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SYNTHESIS AND CHARACTERIZATION OF XYLOPIC ACID DERIVATIVES WITH POTENTIAL ANTIMICROBIAL ACTIVITY

BY

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DECLARATION

I hereby declare that this thesis report is my own work and that, to the best of my knowledge, it has not been submitted for the award of any other degree by this university or any other university, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to the Almighty God who has granted me the strength, knowledge and motivation to carry it out.

ACKNOWLEDGEMENTS

....God of our weary years, God of our silent tears, thou who hast brought us thus far on the way ;… (James Weldon Johnson)

I thank the Almighty God for his constant grace and love through the calm and storms alike.

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Finally, I am very grateful to all my friends and colleagues especially Mawunyo, for their moral support and company.

ABSTRACT

Xylopic acid was isolated from the dried fruits of *Xylopia aethiopica,* crystalized and characterized. The isolate was used to synthesize five novel derivatives of xylopic acid and these have been sufficiently characterized. The structure of derivatives was related to the observed trend in terms of antimicrobial activity. Base catalyzed ester formation was employed in the synthesis of the esters while direct coupling with HBTU was employed in the synthesis of the amide derivative. Deacetylation of xylopic acid was achieved by refluxing xylopic acid with 10% methanolic KOH. The structures of the derivatives were confirmed using ^{1}H NMR, mass spectrometry and IR spectroscopy. The broth dilution method was employed in the antimicrobial assay. All the synthesized derivatives were more active than xylopic acid against the microorganisms tested (*Staphylococcus aureus, Streptococcus pyrogenes, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*). The ester derivatives were most active with MICs of up to 160µg/mL. The benzyl amide and the ester of deacetyl xylopic acid generally exhibited a lower antimicrobial activity with MICs of up to 320µg/mL.

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LIST OF ABBREVIATIONS

ABTS	2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid
DBP	Diastolic Blood Pressure
DCC	Dicyclohexylcarbodiimide
DMSO	Dimethyl Sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDC	1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride
GC-FID	Gas Chromatography-Flame Ionization Detector
GLC	Gas Liquid Chromatography
HATU	2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate, Methanaminium]
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOBt	1-Hydroxybenzotriazole
ICR	Institute of Cancer Research
LPS	Lipopolysaccharide
MBC	Minimal Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
NMR	Nuclear Magnetic resonance
PCA	Passive Cutaneous Anaphylaxis,
SBP	Systolic Blood Pressure
TMS	Tetra Methyl Silane

CHAPTER ONE

INTRODUCTION

1. 1 GENERAL INTRODUCTION

Apart from serving as a source of food, plants invariably remain a major source of medicine in most parts of the world. This is particularly true about the people of Africa especially those in the tropical regions of the continent probably due to the poor access to orthodox healthcare, or the fact that the tropical and subtropical regions of Africa alone contain about 45,000 species of plants with potential medicinal value. Despite the overabundance, only about 5,000 of these plant species have been exploited for medicinal use so far and even much less have been investigated for corroboration of the said therapeutic use and safety. For instance, Boakye-Yiadom *et al* showed that xylopic acid, a compound isolated from the fruits of *Xylopia aethiopica* possesses antimicrobial activity against gram positive and gram negative bacteria and fungi (Boakye-Yiadom *et al*., 1976) and quite recently, Fetse *et al* also showed that total alkaloidal extract of *Alstonia boonei* root bark possess a good wound healing and antimicrobial activity (Fetse *et al*., 2014).

Xylopia aethiopica or Ethiopian pepper as it is usually called, is an angiosperm belonging to the family Annonaceae and is among the species that thrive in the evergreen rain forests of tropical and subtropical Africa. *Xylopia aethiopica* matures as a slim, tall tree of approximately 60 cm in diameter and up to 30 m high, usually has a straight stem, and has a slightly stripped bark. It bears odoriferous fruits, in the form of slender pods slightly curved (fig. 1.1) with

about 15 carpels and are arranged in capitula to form bouquets of 12-20 bacciferous-like capsules (Tairu *et al*., 1999).

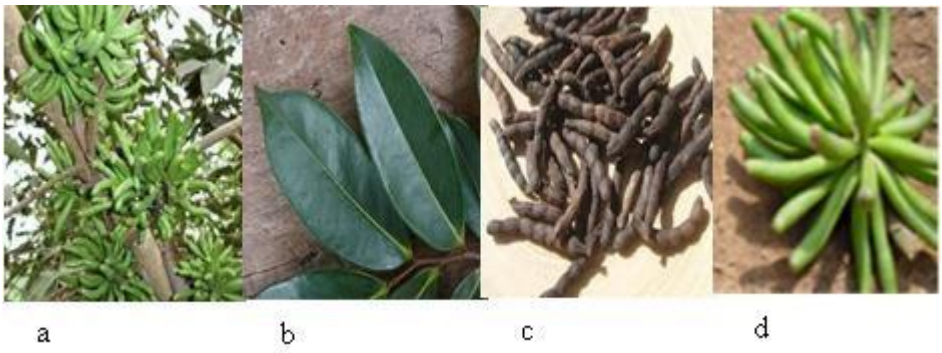


Fig. 1.1 (a) fruits of Xylopia aethiopica still attached to the tree (b) leaves of Xylopia aethiopica (c) dried fruits of Xylopia aethiopica and (d) a cluster of Xylopia aethiopica fruits.

Xylopia is compressed from the Greek words "*xylon pikron"* which mean "bitter wood". The second part of the plant's binomial name, *aethiopica*, refers to its origin, Ethiopia; however, currently it grows most prominently as a crop in Ghana (Orwa, 2010). The plant has several local names, in Ghana it is known as *'hwenteaa'* in Akan, *'etso'* in Ewe, *'so*' in Ga and '*samaamdabile'* by the Waala people in the Upper West Region. This plant has played a key role in African traditional medicine for several centuries owing to its wide array therapeutic indications. *Xylopia aethiopica* is used in the treatment of cough, biliousness, bronchitis, rheumatism, dysentery, malaria, uterine fibroid, amenorrhea (Burkill, 1995), boils, sores, wounds and cuts among others (Busia, 2007).

1.2 JUSTIFICATION

Xylopic acid, a kaurene diterpene is among the major constituents in the fruits of *Xylopia aethiopica*. Various *in vitro* studies conducted on this compound

have revealed that it possesses a wide array of biological and pharmacological properties (e.g. antimicrobial, anticancer, analgesic and anti-inflammatory etc.). Over the years, this compound has been on the cutting edge of various studies conducted particularly at the Faculty of Pharmacy, KNUST, yielding groundbreaking findings. For instance, in a recent study, Biney *et al* investigated various neuropharmacological effects of xylopic acid in an *in vivo* experiment using mice (Biney *et al*., 2014). Again, Woode *et al* also showed that xylopic acid possess very good analgesic activity (Woode *et al*., 2012b). In a much earlier study, Boakye-Yiadom *et al* showed that xylopic acid possesses antimicrobial activity against gram positive and gram negative bacteria and fungi (Boakye-Yiadom *et al*., 1976). Elsewhere, Davino *et al* also reported that xylopic acid and its epimer, acetylgrandifloric acid exhibited significant antimicrobial against the tested microorganisms with MIC greater than or equal to 250µg/mL (Davino *et al*., 1988). In spite of all these interesting findings, very little has been done in terms of forming simple derivatives of xylopic acid and investigating how these derivatives compare with xylopic acid for most of the established biological activities of the latter. Nonetheless, it is important to state that some work has been done in this regard. For instance, Soh *et al* reported that epoxide derivatives of xylopic acid have shown to possess good trypanocidal activity against *Trypanosoma brucei* with no detected cytotoxicity. In the same study, 15-oxo-ent-kaur-16-en-19-oic acid, obtained by oxidation of xylopic acid exhibited significant cytotoxic effects on MRC-5 fibroblast suggesting its potential to be used as an anticancer agent (Soh *et al*., 2013). It is against this background that we intend to form semi-synthetic derivatives of xylopic acid and compare their antimicrobial activities with that of xylopic acid

and time permitting, we hope to investigate further, the analgesic effects of the derivatives.

1.3 RESEARCH OBJECTIVES

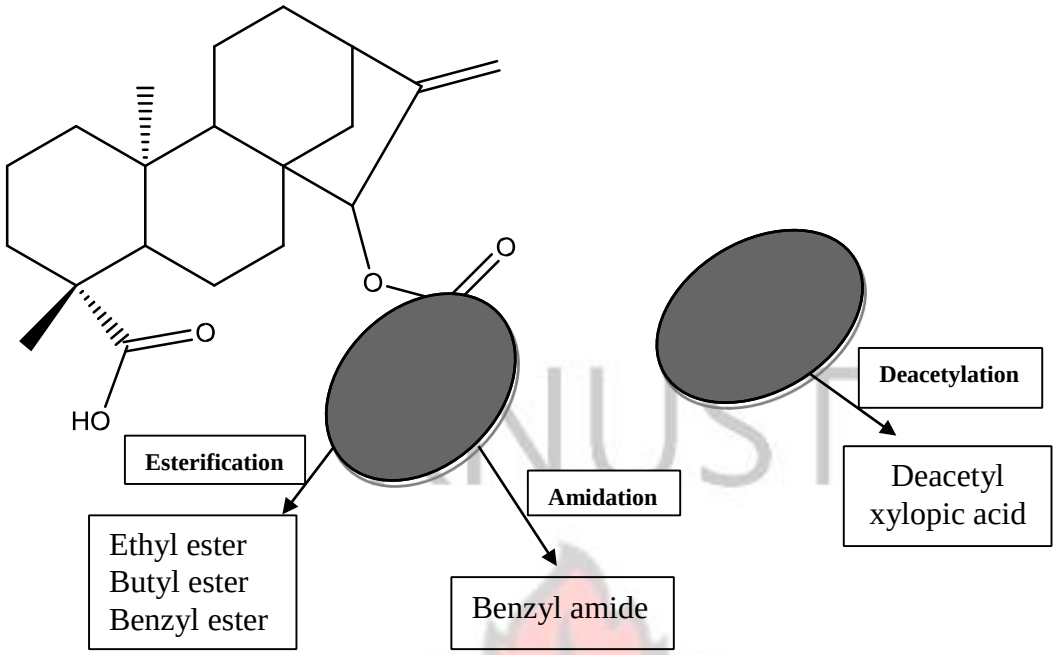
1.3.1 General objective

The purpose of this research is to synthesize and characterize various derivatives of xylopic acid with potential antimicrobial activity. The antimicrobial activities of the formed derivatives will be compared with that of xylopic acid. The structures of derivatives will then be related to the observed trend in terms of antimicrobial activity. In addition, the analgesic activity of these derivatives would also be investigated, the findings of which may be published later.

1.3.2 Specific objectives

To isolate, purify and characterize xylopic acid obtained from the dried fruits of *Xylopia aethiopica.*

1. To form ester (ethyl, butyl and benzyl), amide (benzyl) and deacetylated derivatives of xylopic acid (scheme 1.1).
2. To characterize the formed derivatives using IR spectroscopy, ^{1}H NMR spectroscopy and mass spectrometry.
3. To investigate the antimicrobial activities of the formed derivatives by determining their MICs.
4. To compare observed antimicrobial activities of the formed derivatives with that of xylopic acid.
5. To relate structure of derivative to observed trend in antimicrobial activity.



Scheme 1.1 A scheme showing the various modifications to be effected on xylopic acid.

CHAPTER TWO

LITERATURE REVIEW

2.1 TAXONOMY

The genus *Xylopia* consists of about 150 species which occur in tropical and subtropical Africa (Irvine, 1961). *Xylopia aethiopica*, also known as Negro pepper, is an angiosperm belonging to the custard apple family, Annonaceae (Choumessi *et al*., 2012).

2.2 ETHNOBOTANICAL USES

Xylopia aethiopica possesses great nutritional and medicinal values in traditional medicine (Oloyede and Aduramigba-Modupe, 2011). Almost all parts of *Xylopia aethiopica* are very useful medicinally, but the fruits are most

commonly used for therapeutic purposes. Extracts of the fruits are used in the treatment of cough, biliousness, bronchitis, rheumatism, dysentery, malaria, uterine fibroid and amenorrhea [(Soh *et al*., 2013), (Burkill, 1995), (Woode *et al*., 2012a)]. The fruits can also be crushed and mixed with Shea butter and used as body creams, cosmetic products or perfumes (Ayedoun *et al*., 1996). It has also been shown that the essential oil from the seeds of *Xylopia aethiopica* can be used in the formulation of shampoos due to its high saponification value (Ajiwe *et al*., 1998). In Benin, the dried fruits are commonly used as a constituent of extracts for bathing, and as a potion administered to new-borns (Ayedoun *et al*., 1996). The seeds are crushed and applied topically on the forehead to treat headache and neuralgia. It can also be taken as a decoction, concoction or even chewed and swallowed for the management of various aches and pains (Igwe *et al*., 2003). It has also been shown experimentally, that the seeds possess good anthelminthic activity against *Nippostrongylus brasiliensis* and as such its use in man as an anthelminthic may be investigated (Suleiman *et al*., 2005). Various extracts of *Xylopia aethiopica* have also demonstrated some promise in its employment as an adjunct therapy in the management of sickle cell disease (Uwakwe, 2013). An oily extract of the seeds is used as a lotion for boils and eruptions, and as a liniment for lumbago. Traditional medical practitioners and birth attendants use a decoction of the seeds to induce placental discharge postpartum due to its abortificient effect [(Burkill, 1995), (Woode *et al*., 2012a)].

The roots of *Xylopia aethiopica* are used in tinctures, administered orally as an anthelminthic, or in teeth-rinsing and mouth-wash extracts against toothache

(Ayedoun *et al*., 1996). They are also used as an antihemorrhagic agent. Aqueous concoction of the root is administered after child birth as an antiinfective agent (Nwangwa, 2012). The powdered root is also employed as a dressing and in the local treatment of cancer (Oloyede and AduramigbaModupe, 2011).

The leaves and bark are used in traditional medicine to manage boils, sores, wounds and cuts (Busia, 2007). A decoction of the leaves is used as an antiemetic. Powdered leaves are also taken as snuff for the treatment of headaches (Yapi *et al*., 2012). In some parts of Congo, the plant is used to manage asthmatic attacks, stomach aches and rheumatism. In the Ivory Coast, it is recommended as a postpartum tonic and also taken to promote fertility and ease of childbirth [(Burkill, 1995), (Iwu, 2014)]. *Xylopia aethiopica* is also used locally as carminative, stimulant and adjunct to other remedies for the treatment of skin infections (Konning *et al*., 2004).

2.3 CHEMICAL COMPOSITION OF *XYLOPIA AETHIOPICA*.

Xylopia aethiopica is known to have myriad chemical constituents with diverse therapeutic and pharmacological properties. These compounds, most of which have been isolated and characterized include saponins, sterols, carbohydrates, glycosides, mucilage, acidic compounds, tannins, balsams, cardiac glycosides, volatile aromatic oils, phenols[(Esekhiagbe *et al*., 2009), (Ezekwesili *et al*., 2010), (John-Dewole *et al*., 2012)], alkaloids, rutin and fixed oils [(Nwaichi and Igbinobaro, 2012), (Asekun and Kunle, 2004)]. The plant also contains vitamins A, B, C, D, and E, and proteins together with high amounts of minerals like copper, manganese and zinc [(John-Dewole *et al*., 2012), (Nwaichi and Igbinobaro, 2012)].

2.3.1 Essential oils

Among the commonest groups of chemical compounds conspicuously present in the various parts of *Xylopia aethiopica* are the essential oils. Different studies conducted on these essential oils have shown the presence of a wide diversity of chemical compounds. In one of the early studies conducted, Ogan (1970) identified for the first time, an aromatic aldehyde specifically, cuminal (*p*isopropyl-benzaldehyde) as a component of the essential oils obtained from the fruits of *Xylopia aethiopica* (Ogan, 1971). After almost a decade later, Karawya *et al* also analysed the essential oil from the dried fruits of *Xylopia aethiopica* and the only aldehyde identified was cuminic aldehyde at a concentration of

6.5%, corroborating Ogan's work. Other compounds were also identified; namely β-pinene a monoterpene hydrocarbon, bisabolene a sesquiterpene hydrocarbon, terpinene-4-ol which is an alcohol and the oxide 1, 8-cineole among others (Karawya *et al*., 1979).

Ogunwande *et al* also reported for the first time, the presence of Zerumbone, a known sesquiterpene ketone as a constituent of *Xylopia aethiopica (Ogunwande et al., 2005)*. Elsewhere, Ayedoun *et al* identified about 60 components in the essential oil obtained from the fruits and leaves of *Xylopia aethiopica* sourced from Benin of which about 45 have previously been identified. The components that occurred in relatively high quantities are α-pinene (4-16%), sabinene (3-35%), β-pinene (12-42%), 1,8-cineole (trace-15%) and (Z)-β-ocimene (trace18%). The leaf oil additionally contained 14.9% of elemol (Ayedoun *et al*.,

1996). In another research to investigate the composition of the essential oil of *Xylopia aethiopica* dried fruits from Benin, Poitou *et al* identified and characterized forty-one compounds representing 82.3% of the oil. Two major fractions were identified, namely oxygenated (28.8%) fraction and the hydrocarbon fraction which comprised mainly of monoterpenes (>50% of the whole oil), with sabinene (36.0%) as the main component. 1, 8-cineole (12.8%), linalool (3.9%) and terpinen-4-ol (7.0%) were the major oxygenated monoterpene constituents identified. The major sesquiterpenoid components identified were β-elemene (0.81%) and β-eudesmol (1.9%) (Poitou *et al*., 1996). These results are in agreement with the findings of Ayedoun *et al* except that αpinene, β-pinene and a few other compounds occur in much lower quantities in the samples analysed by Poitou *et al*. Again, Tomi *et al* identified the component of essential oil obtained from the seeds of *Xylopia aethiopica* sourced from Guinea. It was observed that monoterpenes were the most predominant (81.4-

84.1%); prominent among them were hydrocarbons; namely β-pinene (37.040.5%) and α-pinene (13.6-18.4%) which are the major constituents, sabinene (7.1-7.6%) and 1, 8-cineole (6.5-8.4%). Also present in some of the analysed plant samples were α-phellandrene (7.9%) and germacrene D (6.5%), which are sesquiterpenes (Tomi *et al*., 1996). Yapi *et al* analysed the chemical composition of 48 essential oil samples isolated from the leaves of *Xylopia aethiopica* harvested in six Ivoirian forests. The analysis was carried out using GC-FID and ^{13}C-NMR. The findings were not so different from what has been reported in previous research; about 23 components were identified accounting for 82.5–96.1% of the oil composition. Here again, the monoterpene

hydrocarbons β-pinene (up to 61.1%) and α-pinene (up to 18.6%) were the most dominant together with the sesquiterpene hydrocarbon germacrene D (up to 28.7%) (Yapi *et al*., 2012). The essential oil of *Xylopia aethiopica* dried fruits from Mali have been analysed using combined GC and GC/MS. The principal constituents identified were β-pinene, γ-terpinene, trans-pinocarveol, *p*cymene, α-cadinol, α-pinene and 1,8-cineole. Among these, β-pinene was the most prominent (Keita *et al*., 2003). Lamaty *et al* analysed the essential oil of *Xylopia aethiopica* fruits from Cameroon by GLC and GC-MS. It was reported that monoterpene hydrocarbons (66.6%) were the most conspicuous among which sabinene is the most abundant (23.9%), oxygenated compounds particularly terpinen-4-ol and α-terpineol made up 25.1% of the oil and 8.3% of the oil was sesquiterpenene hydrocarbons with α-muurolene making up 4.3% of the latter (Lamaty *et al*., 1987).

Yet again, Koba *et al* evaluated essential oil extracted from air-dried fruits of *Xylopia aethiopica* harvested in Togo and the chemical composition examined by GC, GC/MS. Thirty-five compounds were identified, and these represented 89.9% of total oil. The major constituents identified were ß-pinene (23.6%), αpinene (11%), sabinene (9.8%), germacrene D (8.3%) and 1, 8 cineole (8.2%) (Koba *et al*., 2008). The general trend of results obtained in all these studies, particularly the various compounds identified in the essential oil of *Xylopia aethiopica* is illustrative of the fact that similar species of plants will usually have identical chemical composition. On the contrary, the variations observed in composition and/or concentration of some components, especially those sourced from different countries could possibly be a reflection of the influence

of the geographical origin on the chemical composition of plants belonging to the same species.

2.3.2 Diterpenes

Most of the acidic compounds isolated from *Xylopia aethiopica* are the various kaurane, kolavane and trachylobane diterpenes, which are reportedly present in the stem bark and fruit of the plant. Quite an extensive research has been conducted on most of these diterpenes leading to the elucidation of their structures. Typically, xylopic acid (fig. 2.1), a kaurene diterpene has been taken through extensive research to the extent that some derivatives of it have been synthesized. Ekong and Ogan isolated xylopic acid from the dried powdered fruits of *Xylopia aethiopica* by extracting the latter with light petroleum (b.p.6080°C). The extract was subsequently concentrated and crystallised from ethyl acetate to obtain xylopic acid, its melting point was determined to be 259-260°C (Ekong and Ogan, 1968). Again, Soh *et al* also extracted xylopic acid using hexane where the extract was chromatographed over silica gel using hexaneethyl acetate (95:5) mixtures to obtain xylopic acid as a white powder with melting point of 230- 232^{o}C (Soh *et al*., 2013). Although the two groups of researchers reported to have isolated xylopic acid, there was a vast difference in the melting point of the crystals obtained. This could possibly be attributed to the fact that a particular group isolated xylopic acid of low purity and as such, the impurities might have altered the actual melting point of the compound. Better still, it could be that one group isolated a compound closely related to xylopic acid but not xylopic acid itself. Apart from the difference in melting point, Ekong and Ogan elucidated the structure of the compound isolated as

15β-acetoxy-(-)-kaur-16-en-l9-oic acid while Soh *et al* determined theirs to be 15α-acetoxy-ent-kaur-16-en-19-oic acid. Fiagbe *et al* also solved the structure of xylopic acid as 15β-acetoxy-(-)-kaur-16-en-l9-oic acid by use of crystallography (Fiagbe *et al*., 1979). Elsewhere, Adosraku and Oppong Kyekyeku isolated xylopic acid from the dried fruits of *Xylopia aethiopica* using petroleum ether (40-60°C) and recrystallized the former with distilled alcohol. The melting point obtained for these crystals was 261-262°C (Adosraku and Oppong Kyekyeku, 2011). In another research, Fahim *et al* determined the melting point of isolated xylopic acid as 265 – 266°C (Fahim *et al*., 1953). The melting points obtained in these two separate studies for xylopic acid are in much agreement with that obtained by Ekong and Ogan (259-261°C) as compared with the melting point obtained by Soh *et al* (230-232°C). A major issue that requires further research is whether only one of these stereochemical forms of xylopic acid actually exists, or whether both do exist concurrently in the same plant or separately depending on the geographical location of the plant. Finally, the effects of the stereochemical difference if any on the melting point of the compound should be investigated.

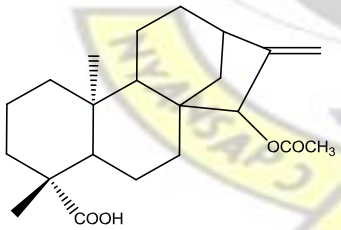


Fig. 2.1 Chemical structure of xylopic acid.

Other kaurane diterpenes isolated from *Xylopia aethiopica* (structures of which are shown in figure 2.2) include xylopioxyde, which has its nomenclature as 16, 17-epoxy-15-oxo-ent-kauran-19-oic acid, 15-oxo-ent-kaur-16-en-19-oic acid,

ent-kaur-16-en-19-oic acid and (-) kaur-16-en-15-hydroxy-19-oic acid (melting point 204-206ºC) among others. Xylopioxyde is obtained by column chromatographic separation (eluent: hexane-ethyl acetate 85:15, $^{v}/_{v}$) of hexane extract of *Xylopia aethiopica* fruits and exists as a white powder with melting point of 190-192ºC. 15-oxo-ent-kaur-16-en-19-oic acid could also be obtained upon elution of hexane extract of *Xylopia aethiopica* fruits (eluent: hexane-ethyl acetate 90:10, $^{v}/_{v}$) and occurs as a white powder with melting point 192-194ºC. Ent-kaur-16-en-19-oic acid is also obtained as a white powder having a melting point of 172-174ºC upon column chromatographic separation of the hexane extract of *Xylopia aethiopica* fruits on a silica gel column (eluent: hexane-ethyl acetate 80:20, $^{v}/_{v}$) [(Soh *et al*., 2013), (Ekong *et al*., 1969)]. Trachyloban-19-oic acid, 7β-hydroxytrachyloban-19-oic acid (Diderot *et al*., 2005), 7αhydroxytrachyloban-19-oic acid (Ngouela *et al*., 1998), 15-oxo-(-)-trachyloban19-oic acid and 15β-hydroxy-(-)-trachyloban-19-oic acid (Harrigan et al., 1994) are among the trachylobane diterpenes while kolavenic acid (Diderot *et al*., 2005) and 2-oxo-kolavenic acid (Hasan *et al*., 1982) are typical kolavane diterpenes also present in *Xylopia aethiopica.*

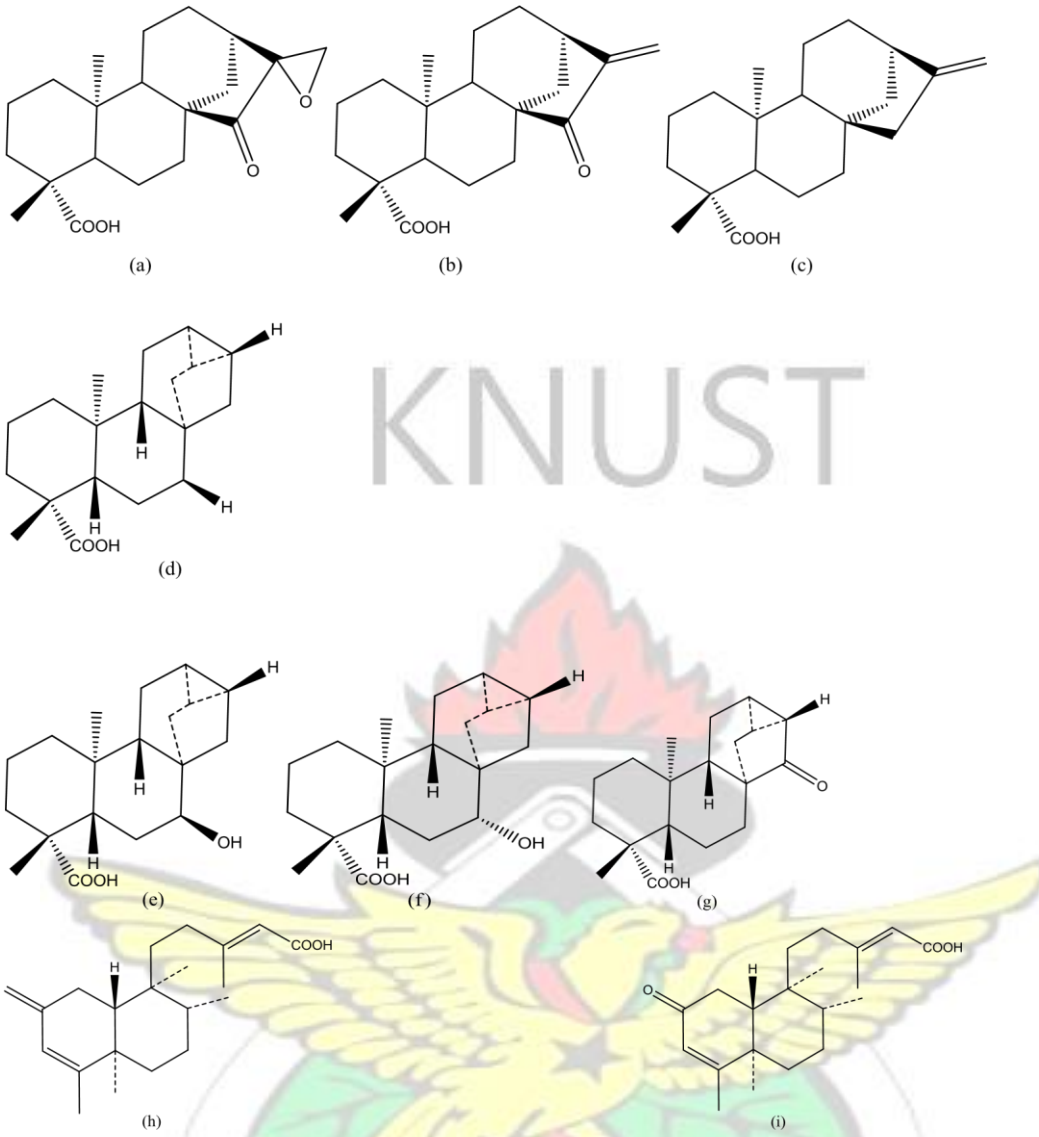


Fig. 2.2 Chemical structures of (a) xylopioxyde (16, 17-epoxy-15-oxo-entkauran-19-oic acid), (b) 15-oxo-ent-kaur-16-en-19-oic acid, (c) ent-kaur-16en-19-oic acid, (d) Trachyloban-19-oic acid, (e) 7β-hydroxytrachyloban-19oic acid, (f) 7α-hydroxytrachyloban-19-oic acid, (g) 15-oxo-(-)-trachyloban-19-oic acid, (h) kolavenic acid and (i) 2-oxo-kolavenic acid

2.4 PHARMACOLOGICAL PROPERTIES OF *XYLOPIA AETHIOPICA*

2.4.1 Analgesic, anti-inflammatory, anti-allergic and CNS effects

An ethanolic extract of the fruits of *Xylopia aethiopica* showed significant analgesic activity in rats and mice upon oral administration of the extract.

Xylopic acid isolated from the dried fruits of *Xylopia aethiopica* also showed comparable results (Woode *et al*., 2012b). The aqueous ethanolic fruit extract of *Xylopia aethiopica* at concentrations of 100, 300 and 600mg/kg all exhibited good anti-arthritic effect in Sprague-Dawley rats. The anti-arthritic effect was achieved because of the suppression of both inflammation and the destruction of the joint in adjuvant arthritic rats (Obiri *et al*., 2014). Obiri and Osafo have investigated the anti-anaphylactic and anti-inflammatory effects of the aqueous ethanolic fruit extract of *Xylopia aethiopica*. In this study, it was reported that the aqueous ethanolic fruit extract when administered orally at doses of 30–1000mg/kg 1 hour before administering compound 48/80 (an anaphylactic reaction inducer), there was significant anti-anaphylactic effects. This antianaphylactic effect was dose-dependent, in that increasing the dose of the extract led to a resultant increase in the median survival of the mice used for the study. Similar results were observed when the extract was investigated for its effect on Lipopolysaccharide (LPS)-induced allergy, pinnal inflammation (passive cutaneous anaphylaxis, PCA), clonidine-induced catalepsy and carrageenan-induced paw oedema (Obiri and Osafo, 2013). Ameyaw and others also showed that an ethanolic extract and xylopic acid from the dried fruits of *Xylopia aethiopica* were able to improve vincristine- induced tactile and cold allodynia, as well as mechanical hyperalgesia. This suggests that the ethanolic fruit extract of *Xylopia aethiopica* and its major kuarane diterpene xylopic acid have anti- allodynic and anti- hyperalgesic properties in vincristine- induced neuropathic pain (Ameyaw *et al*., 2014).

2.4.2 Cytotoxic effects

Elsewhere, the anticancer activity of *Xylopia aethiopica* methanolic fruit extract was studied using human cancer cell lines C_33A (cervical), KB (oral), MCF _7 (breast), A549 (lung) and mouse embryo fibroblast (NIH3T3). In order to investigate the antitumor activity of the extract, the antiproliferative activity of the extract against the various cell lines was first studied. It was noticed that the C_33A cell was the most sensitive to the extract _induced growth inhibition. In addition, the extract showed an inhibition of the proliferation of C_33A cancer cells *via* cell cycle arrest at sub_G0/G1 and G2/M phases. This was corroborated by an increased level of p21 and p53 gene transcripts in extract treated cells (Adaramoye *et al*., 2011).

2.4.3 Antioxidant effect

The free radical scavenging effects, antioxidant and ion toxicity preventive effect of ethanolic extracts of *Xylopia aethiopica* stem bark was investigated by Moukette *et al*. From the results of this research, it was observed that vitamin C used as standard had a significantly ($p < 0.05$) lower value of IC_{50} on nitric oxide (NO), hydroxyl (OH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) radicals as compared to the extracts. The extracts also showed a protective effect against lipid peroxidation. It may therefore be inferred from this study that *Xylopia aethiopica* has a good antioxidant and protective potential against ion-mediated oxidative damage and may be considered as a potential drug against metal mediated toxicity (Moukette Moukette *et al*., 2015). Phenol-rich fruit extracts of *Xylopia aethiopica* exhibited very good free radical scavenging ability in a concentration dependent fashion (0.08-0.53mg/mL). Again in assessing the

ferric reducing antioxidant property of the extract, the study showed that the extract possesses good reducing potentials (Adefegha and Oboh, 2012).

2.4.4 Hypoglycaemic, cardiovascular and other pharmacological effects

The anti-diabetic effect of *Xylopia aethiopica* has also been studied. A chloroformic extract of the dried fruits of *Xylopia aethiopica* administered orally at a dose of 250mg/kg showed an 82% reduction in the blood glucose concentration of alloxan monohydrate induced diabetic Wistar albino rats while diabetic non treated rats and glibenclamide treated rats showed a 6.9% and 74% reduction respectively. It was however, reported that, a lower reduction of 23% was observed when the *Xylopia aethiopica* extract was administered at a dose of 100mg/kg. This suggests that the hypoglycaemic effect of *Xylopia aethiopica* fruit extract is dose dependent (Okpashi *et al*., 2014). In another study, an aqueous extract obtained from the dried fruits of *Xylopia aethiopica* administered at a concentration of 0.26% $^{w}/_{v}$ exhibited some influence on the biochemical profile in Wistar Albino rats. For instance, it was revealed that the extract caused a marked reduction in the plasma cholesterol, triglyceride and sodium levels in the treated rats. These findings pre-suggest that the extract has some beneficial effects of reducing cardiovascular risk factors that are not genetic. Also, the extract was shown to induce hypokalaemia (Nwaichi and Igbinobaro, 2012).

An ethanolic extract of the dried fruits of *Xylopia aethiopica* was shown to cause a significant ($P<0.05$) dose related reduction in sperm count and motility in male albino rats, but does not affect the morphology significantly. The testicular histology also showed a disorientation of the basal layer and histoarchitecture

of the seminiferous tubules. The rats that received higher doses were affected much more than those with lesser doses, suggesting the dose dependency of this effect (Nwangwa, 2012).

Also, the cardiovascular and diuretic activities of kaurene derivatives of *Xylopia aethiopica* in Wistar rats were investigated by Somova *et al*. It was observed that xylopic acid, a major compound in the dried fruits of *Xylopia aethiopica*, produced more pronounced and significant blood pressure lowering effect on both systolic blood pressure (SBP) and diastolic blood pressure (DBP). These observations were made at 20, 30 and 60 mins after an intraperitoneal administration of the former at a dose of 20 mg/kg body weight. There was also a significant gradual decrease in heart rate (HR) by 14, 15 and 20%, respectively.

2.5 ANTIMICROBIAL ACTIVITY OF XYLOPIA AETHIOPICA AND XYLOPIC ACID

2.5.1 Antibacterial and antifungal activities

Xylopia aethiopica has gained wide application in traditional medicine partly because of its usefulness as an effective antimicrobial agent. Extracts and isolates from various parts of the plant through *in vitro* studies have confirmed its anti-microbial activity. For instance, Konning *et al*, reported that a 3%$^{w}/_{v}$ methanol extract of *Xylopia aethiopica* (dried fruits) showed some antimicrobial activity against gram negative and gram positive bacteria and the fungi investigated (Konning *et al*., 2004). The ethanol extract of the dried fruits of *Xylopia aethiopica* was also investigated for antimicrobial activity. In this study, the agar diffusion method was used to determine both the zones of inhibition and the minimum inhibitory concentration (MIC). The extract

exhibited activity against *E. coli, S. typhi, Candida albicans, B. aurium* with 15mg/mL MIC. The extract however, did not show any activity against *S. aureus* and *B. subtilis*. This study has therefore shown to some extent that *Xylopia aethiopica* ethanol extract contain compounds that could be further investigated as potential source of broad spectrum antibacterial agents.(Oloyede and Aduramigba-Modupe, 2011).The essential oil of *Xylopia aethiopica* has been shown to possess antibacterial as well as antifungal activity. In a study, the disk diffusion method was employed for antimicrobial assay with the minimum and maximum zones of growth inhibition as 18mm (against *Bacillus subtilis*) and 32mm (against E. *coli*) respectively (Tatsadjieu *et al*., 2003). In another study, essential oil from various parts of *Xylopia aethiopica* showed varying degrees of activity against gram positive and gram negative bacteria and the fungi used in the study. Interestingly, none of the tested essential oils showed activity against *Escherichia coli* (Fleischer *et al.*, 2008). Once more, Asekun and Adeniyi also showed that the essential oil of *Xylopia aethiopica* fruits possess good antifungal activity but showed no activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* at a concentration of 5 mg/mL (Asekun and Adeniyi, 2004). These findings are mostly in agreement with the results reported by Fleischer *et al* except that the latter reported that *Staphylococcus aureus* was most sensitive among the microorganisms tested. Again, the findings somewhat contradict the results of the study conducted by Tatsadjieu *et al* as the results of the study conducted by the latter showed that both *Escherichia coli* and *Staphylococcus aureus* were susceptible to the essential oil from *Xylopia aethiopica* fruits. In any case, all these findings point out one thing, that there may be specific compounds in these extracts and

essential oils giving rise to the said antimicrobial activities and that the composition of the essential oil varies based on the geographical location of the plant. It is therefore necessary that further research be conducted to identify specific compounds in these extracts that are responsible for the various antimicrobial activities. The identified compound could further be modified chemically for a possible optimization of the activity it possesses.

2.5.2 Anti-protozoan effects

Boampong *et al* showed that xylopic acid, a pure compound isolated from the dried fruits of *Xylopia aethiopica* possess prophylactic antimalarial activities comparable to that of Sulphadoxine/pyrimethamine and a curative antimalarial activity similar to Artemether/lumefantrine. In this study, each male ICR mice (25–30 g) was infected with 1×10^6 *Plasmodium berghei* (NK65) and after three days the animals were treated once daily with three doses of xylopic acid (10, 30, and 100mg/kg p.o.) (Groups 1–3), 4mg/kg p.o. of artemether/lumefantrine (A/L) (standard drug: group 4), and 10 mL/kg p.o. normal saline (group 5) for 5 days. On the fourth and fifth days of treatment, it was observed that mice in group 3 (100mg/kg of xylopic acid) and group 4 (4mg/kg A/L) showed percentage reduction in parasitaemia of 92.8% and 99.6%, and 91.7% and 99.6% respectively. Although the percentage reduction in parasitaemia of the mice in groups 1 and 2 were much less, there was however, no significant difference in percentage chemosuppression caused by 4mg/kg of A/L as compared to the percentage chemosuppression produced by the various doses of xylopic acid on these days. Xylopic acid again showed to possess good prophylactic activity against *Plasmodium berghei* at the various doses investigated, this was observed as reduction in parasite count compared to the

vehicle-treated group. In the same study, xylopic acid (30 and 100 mg/kg) exhibited a significant reduction ($P < 0.05$) in lipopolysaccharide-induced fever in rats. Prednisolone, used as positive control also significantly reduced ($P < 0.05$) lipopolysaccharide-induced fever in the rats (Boampong *et al*., 2013). Essential oil from *Xylopia aethiopica* stem bark have also shown to possess antimalarial properties. In a study conducted by Boyom *et al*, when tested against the W2 strain of *Plasmodium falciparum,* the essential oil demonstrated anti-plasmodial activity with an IC_{50} of 17.8µg/mL (Boyom *et al*., 2003). In spite of these ground breaking findings, further studies like forming and testing semi-synthetic derivatives of xylopic acid and other bioactive compounds from *Xylopia aethiopica* on the malaria parasites should be considered as this may reveal further findings.

Still on the investigation of the anti-protozoan activity of *Xylopia aethiopica,* Soh *et al* showed that two epoxide derivatives obtained by oxidation of xylopic acid, 15α-acetoxy-16,17α-ent-epoxy-kauran-19-oic and 15α-acetoxy-16,17βepoxy-ent-kauran-19-oic acid possess good trypanocidal activity against *Trypanosoma brucei* (ED_{50} 52 and 127 µM, respectively) with no detected cytotoxicity on MRC-5 fibroblast. However, ent-kaur-16-en-19-oic acid and 15oxo-ent-kaur-16-en-19-oic acid displayed cytotoxic effects on MRC-5 fibroblast and this calls for further studies into their potential to be used as an anti-cancer agent (Soh *et al*., 2013). In another research, kaurenoic acid and its derivatives have been investigated for activity against trypomastigote forms of *Trypanosoma cruzi*. In this *in vitro* assay, kaurenoic acid, kaurenol, acutifloric acid and stemodin (with ED_{100} 1.363, 1.386, 1.599 and 1.390µg/mL respectively) all demonstrated a complete clearance of the parasites from the

blood of previously inoculated male Swiss albino mice (18-20 g) (Takahashi *et al*., 2002).

2.6 TOXICOLOGY

In a test for acute toxicity using the brine shrimp (*Artemia salina*) bioassay, it was observed that the hexane extract of *Xylopia aethiopica* dried fruits had low toxicity with LC_{50} of 0.30ng/mL whilst xylopic acid and its deacetyl derivative both gave an LC_{50} of 0.50ng/mL. Again, in qualitative/semi quantitative test for toxicity, the Hippocratic test in rats was employed in a 5-day follow-up period following a single intraperitoneal injection. Here, the hexane extract, xylopic acid and deacetyl xylopic acid all showed no toxicity at a dose of 20mg/kg body weight (Somova *et al*., 2001). In another research, the essential oil from the fruits of *Xylopia aethiopica* was showed to be toxic to *Artemia salina* at concentrations ranging from 10 to 1000 µg/mL (Asekun and Adeniyi, 2004). Koba *et al* investigated the *in vitro* cytotoxicity of essential oil from *Xylopia aethiopica* fruits. The cytotoxicity was evaluated using the human epidermal cell line HaCaT. Here, it was observed that at concentrations in the range of 50 - 1500 µg/mL, the tested essential oil did not show any cytotoxicity but rather induced a significant increase in cell viability (up to 130 %), suggesting their potential as cytoprotectors or antioxidants. At higher concentrations ranging from 1600 to 3000 µg/mL, the same type of toxicity profile was recorded (Koba *et al*., 2008).

An ethanolic extract of a combination of equal quantities of *Alstonia congensis* bark and *Xylopia aethiopica* fruits have been investigated for acute and subacute toxicity. In the acute toxicity study, there were no observable changes in the

behaviour and sensory nervous system responses. In addition, no adverse gastrointestinal effects were observed in male and female mice used in the experiment. At a 20.0 g/kg dose, all the mice that received the extract survived beyond the 24 hours of observation. It was therefore inferred that the median acute toxicity value (LD_{50}) of the extract must be above 20.0 g/kg body weight. Although not entirely representative, these findings to some extent show that ethanolic extract of *Xylopia aethiopica* fruits is relatively safe (Ogbonnia *et al*., 2008). However, it is necessary that further research be conducted to determine what doses are safe and effective when administered to humans.

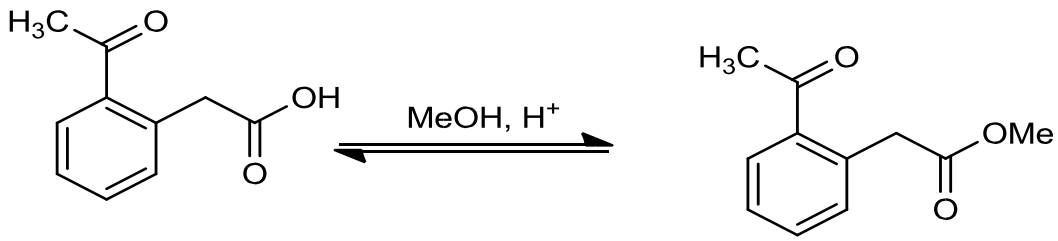
2.7 ORGANIC SYNTHESIS

In organic synthesis, one or more chemical reactions are employed in the preparation of a particular compound of interest. In organic synthesis, it is imperative of the synthetic chemist to always consider a method that require readily available starting materials and one which is environmentally friendly. Typical examples of chemical reactions that could be employed in organic synthesis include esterification, hydrolysis and amidation.

2.7.1 Esterification of carboxylic acids

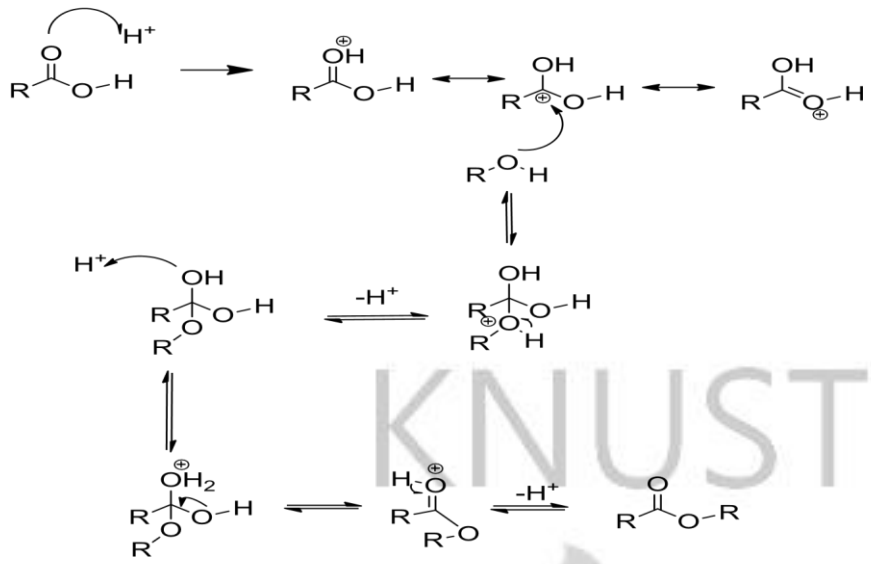
Formations of esters from carboxylic acids generally require the use of a suitable catalyst. This may either be in the form of a strong base or a concentrated acid solution. Acid catalysed ester formation (Fischer esterification)

In this process, a carboxylic acid is condensed with an alcohol in the presence of an acid catalyst with the formation of water molecules.



Scheme 2.1 Reaction Scheme of Fischer Esterification.

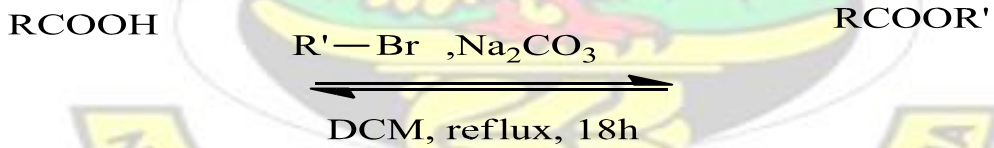
This reaction is reversible and the equilibrium position lies slightly to the side of products when the reactants are simple alcohols and carboxylic acids. Again, the equilibrium position can be shifted to the side of products when either the alcohol or carboxylic acid is used in excess. Furthermore, removing water from the reaction mixture can also shift the equilibrium position to favour the ester formation. Although this method of ester formation is relatively simple and straightforward, it may not be a good choice when the starting material has groups likely to be affected by the concentrated acid catalyst (e.g. xylopic acid). In such situations, the base-catalysed method may be a better choice (Caron, 2011). From the mechanism shown below, there is an initial attack of the protons (H^+) by the carbonyl ($C=O^*$) oxygen atom in the carboxylic acid. The reaction however ends with the loss of a proton from the protonated ester suggesting that the reaction medium remains acidic even at the end of the synthesis.



Scheme 2.2 Reaction mechanism for fischer esterification.

Base catalysed ester formation

The preparation of esters from carboxylic acids may be achieved *via* treatment with an alkyl halide in the presence of a suitable base, which more or less serves as the catalyst. A typical example of such reaction is as shown in the scheme below;



Scheme 2.3 Reaction Scheme for Base Catalysed Ester Formation.

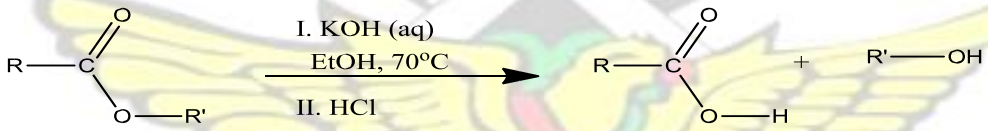
From scheme 2.3, the rate of ester formation can be accelerated by increasing the reaction temperature. However, this is achieved more commonly by employing a more reactive alkyl halide. Considering scheme 2.3, addition of potassium iodide (KI) will convert the alkyl bromide (R'-Br) to the more reactive alkyl iodide (R'-I) *in situ.* This conversion is known as the Finkelstein reaction (Caron, 2011).

2.7.2 Hydrolysis of esters

The ester functional group is commonly found in natural products. In various multi-step syntheses, the ester functionality is employed in protective groups to "mask" the carboxylic acid moiety. One of the very commonly used methods for converting esters to carboxylic acids is *via* hydrolysis. Generally, ester hydrolysis may occur either under acidic or basic conditions.

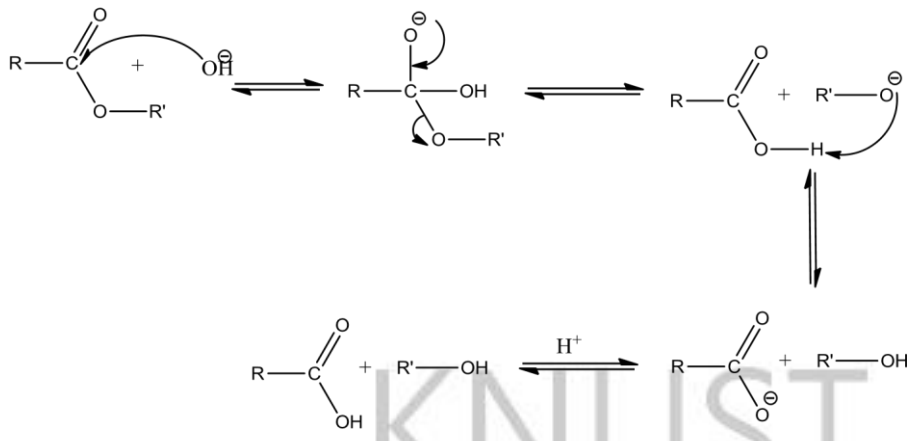
Base catalysed ester hydrolysis

The product of base-catalysed hydrolysis of esters is the carboxylate salt, which can be converted to the corresponding carboxylic acid *via* an acidic workup. Generally, alkali metal hydroxides are the preferred bases for effecting this reaction (Deussen *et al*., 2004).



Scheme 2.4 Reaction equation for base catalysed ester hydrolysis.

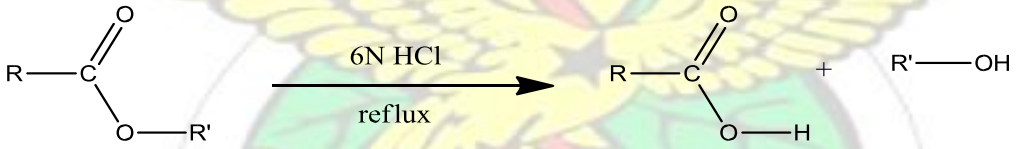
It has been reported that the rates of hydrolysis of esters is usually pH dependent (Chan *et al*., 2008). Generally, carboxylate esters undergo slow hydrolysis at lower pH (pH 5–6) and the rate at which they are hydrolysed increases substantially with increasing pH of the reaction system. From the mechanism shown in scheme 2.5, the hydrolysis is initiated with an attack of the nucleophilic hydroxide ions (-OH) on the carbonyl carbon (*C=O) of the ester. There is rearrangement to form a carboxylate ion and an alcohol.



Scheme 2.5 Reaction Mechanism for Base Catalysed Ester Hydrolysis

Acid catalysed ester hydrolysis

In general, esters that are capable of forming stabilized carbocations (benzhydryl esters) or that readily eliminate to form olefins (t-butyl esters) are cleaved under acidic conditions. Often, dilute acid solutions are used for such hydrolysis. This reaction is reversible as excess water forces the hydrolysis of the ester, whereas removal of water forces the formation of ester.



Scheme 2.6 Reaction equation for acid catalysed ester hydrolysis

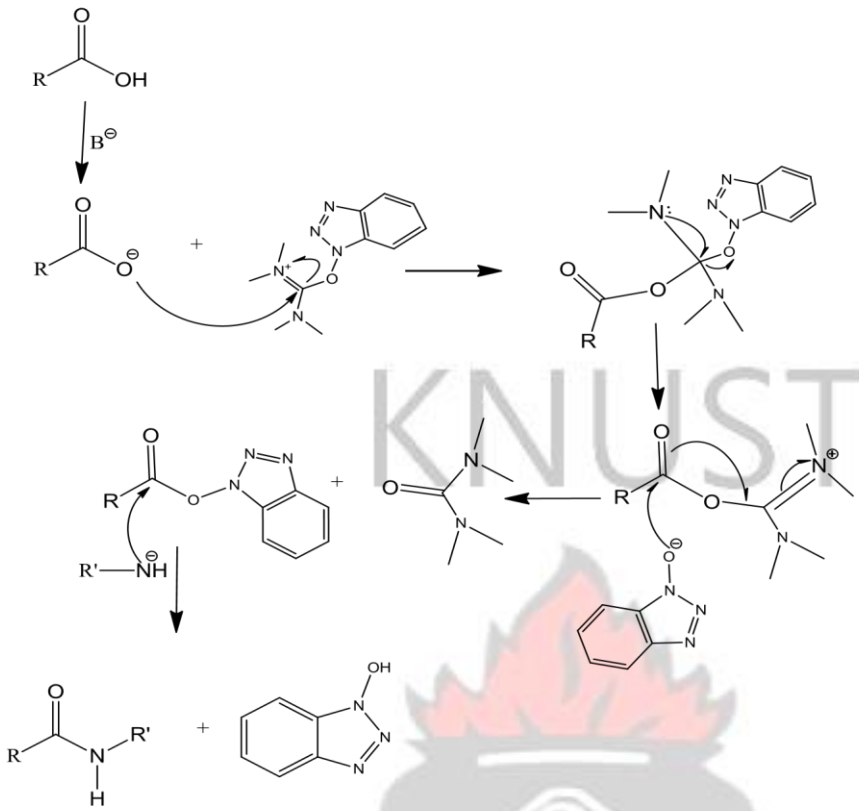
2.7.3 Amide synthesis

Amide formation may be achieved either by direct coupling of carboxylic acids (by activation *in situ*) with amines, or in a two-step process with an initial activation of the carboxylic acid followed by reaction of an amine with the activated intermediate. The latter is most commonly employed in the synthesis of polypeptides.

Direct coupling of carboxylic acids with amines

Carbodiimides such as DCC (dicyclohexylcarbodiimide) and EDC (1-[3(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride) are the most commonly used class of reagents for the direct coupling of carboxylic acids and amines and are often used with various additive (Caron, 2011, Sheehan and Hess, 1955). For instance, HOBt (1-Hydroxybenzotriazole) being one of the most efficient additives, is employed in reactions involving amino acids mainly to reduce isomerization to acceptable levels as carbodiimide activation of amino acid derivatives often causes a partial racemization of the amino acid (Caron, 2011).

Over the years, other coupling reagents such as HBTU [(2-(1H-benzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)] and HATU [2-(1H-7Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate, Methanaminium] have also gained some prominence in synthetic chemistry. One major problem associated with the use of most coupling reagents is the formation of urea by-products hence making chromatography the most appropriate means of purifying the formed amide. This challenge may however be alleviated by the use of EDC as the urea by-product produced with the use of EDC is water soluble and can be conveniently removed through extractive workup or by washing with water (Ormerod *et al*., 2005). The mechanism of amide formation with HBTU as coupling reagent (Dourtoglou *et al*., 1978) is as shown in the scheme below. It could be observed that there is an initial deprotonation of the carboxylic acid. It could also be observed that the attack of the carbonyl carbon (*C=O) by the nucleophilic N-atom of the amine is preceded by an activation of the carboxylic acid with HBTU.



Scheme 2.7 Reaction mechanism for amide formation with HBTU as coupling reagent.

2.8 TOOLS FOR CHARACTERIZATION OF ORGANIC COMPOUNDS

The synthetic chemist relies on various tools to characterize and elucidate the structures of synthesized organic compounds. Typically, tools like infrared spectroscopy, mass spectrometry and NMR spectroscopy all play a vital role structure elucidation.

Infrared spectroscopy

An infrared spectrum may generally be divided into two main regions, namely- the functional group and fingerprint region. The functional group region is the region within wavenumber range of 4000-1500cm^{-1} while the fingerprint region spans from 1500-400cm^{-1} (Cairns, 2012).

Mass spectrometry

Mass spectrometry is a vital tool that enables the chemist to ascertain the elemental composition and some aspects of the molecular structure of an analyte. Some unique features of mass spectrometry include its ability to directly determine the nominal mass (and in some cases, the molar mass) of an analyte, and to produce and detect fragments of the molecule that correspond to specific groups of atoms of different elements that reveal structural features. Mass spectrometry is able to generate more structural information per unit quantity of an analyte than can be determined by any other analytical technique (Watson and Sparkman, 2007). Samples analysed by mass spectrometry must be converted to various (positive or negative) ion fragments before reaching the detector as mass spectrometry measures the mass to charge ratios of the various ion fragments. Ionization techniques can be grouped under two general classes, namely- 'Hard' and 'Soft' ionization. With the hard ionization method, a beam of energetic electrons of approximately 70 eV are used to ionize the sample molecules and as such produces a substantial proportion of ionized molecules with such high internal energy that they fragment before leaving the ion source. It is therefore unlikely to obtain a molecular ion peak on a mass spectrum when hard ionization is employed. Soft ionization on the other hand minimises such further fragmentation hence there is a great chance of obtaining a molecular ion peak on the spectrum. Several soft ionization techniques exist, examples include; Chemical Ionization (CI), Fast Atom Bombardment (FAB), MatrixAssisted Laser Desorption/Ionization (MALDI) and Electrospray Ionization (ESI) among others (McLafferty and Tureček, 1993).

NMR spectroscopy

Aside X-ray crystallography, which is capable of uncovering the complete molecular structure of some pure crystalline materials, NMR spectroscopy is the chemist's most direct and general tool for identifying the structure of both pure compounds and mixtures as either solids or liquids. The process often involves performing several NMR experiments to deduce the molecular structure from the magnetic properties of the atomic nuclei and the surrounding electrons. The assignment of structure on the basis of NMR spectra generally requires knowledge of the relationship between chemical shifts and functional groups (Lambert and Mazzola, 2004).

2.9 ANTIMICROBIAL ASSAY

The current trend in antimicrobial assay particularly in determining the minimum inhibitory concentration (MIC) of an antimicrobial is the use of the broth dilution method rather than the agar well diffusion. One major advantage of the broth dilution over the agar well diffusion is that unlike the latter, broth dilution does not have any challenges with diffusion (Betsy and Keogh, 2005).

Broth dilution method

The broth dilution method is employed in the determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of an antimicrobial agent. The minimal bactericidal concentration is the lowest concentration of an antimicrobial agent needed to kill a given microorganism. The broth dilution test requires that a broth containing the drug be placed in wells of a plastic tray (e.g. a microtitre plate) in a sequence of decreasing concentrations of the antimicrobial agent. Each well is inoculated with the microorganism (bacteria or fungi). After an incubation period, each

well is examined to determine the effectiveness of the concentration of the antimicrobial agent. The well that shows no growth of microorganisms with the lowest concentration of the antimicrobial agent signifies the MIC and MBC that should be used to treat diseases caused by the microorganism (Betsy and Keogh, 2005)

CHAPTER THREE

EXPERIMENTAL

3.1 MATERIALS AND EQUIPMENT

Table 3.1 Materials or reagents used and their respective sources.

Materials/Reagents	Source
Petroleum ether 40/60	BDH
Ethyl acetate	BDH
Dried powdered fruits of Xylopia aethiopica.	Kejetia market, Kumasi.
Anhydrous Dimethyl sulphoxide (DMSO)	Fissons
Dichloromethane (DCM)	BDH
Sodium carbonate	BDH
Potassium hydroxide (KOH)	BDH
Alkyl halides (ethyl iodide, butyl iodide, benzyl chloride)	BDH
Benzyl amine	BDH
HBTU (N,N,N′,N′-Tetramethyl-O-(1Hbenzotriazol-1-yl) uronium hexafluorophosphate)	Aldrich
Triethylamine	Fisher Scientific
Sodium thiosulphate penta hydrate	BDH
Anhydrous sodium sulphate	BDH
Ceric sulphate	BDH
Sulphuric acid	BDH
Various glassware	Supertek Scientific
Precoated thin layer chromatography (TLC) plates	Alugram Sil G
Ethanol (96%)	BDH

Table 3.2 Equipment or apparatus used for the experiment and their source

Equipment/Apparatus	Source
Hot plate with magnetic stirrer	(Model IKA C-MAG HS 7)
Reflux system with recirculator chiller	Buchi
Water bath	Buchi
Melting point apparatus	(Stuart digital, SMP10)
Stuart Scientific Flask shaker	SF1
Analytical Balance	Sartorius (LE623P)

Column chromatography was performed on silica gel 60 (70–230 mesh). FTIR spectra were obtained using a Perkin Elmer FTIR spectrum 2ATR spectrophotometer and Perkin Elmer Spectrum Version 10.03.09 program. 1H NMR (400 and 500MHz) spectra were recorded on a Bruker 400 and "Bruker 500" spectrometers using TMS as an internal standard in DMSO. Data is reported for each compound as follows: chemical shift in ppm (δ), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s= broad singlet), coupling constant (Hz), chemical group bearing specified protons. Mass Spectra were obtained using "Waters-Micromass ZQ2000" LCMS instrument. Melting point determinations were performed with a Stuart digital, SMP10 melting point apparatus and were uncorrected. A magnetic stirrer with heating and ceramic heating plate model "IKA C-MAG HS 7" was used to provide the source of heat for the synthesis. Reagents and solvents used are of commercial grade and were used as supplied without any further purification, except when specified in the experimental procedure.

3.2 METHODS

3.2.1 Isolation of xylopic acid

Dried fruits of *Xylopia aethiopica* were obtained from the Kejetia market, Kumasi and authenticated by the department of Pharmacognosy, KNUST, Kumasi with a voucher number of FP/09/76. The dried fruits (1.025kg) were powdered and extracted with 2 litres of petroleum ether (b. p. 40-60^{o}C) for 72 hours. The extract was concentrated with a rotavapour at 60^{o}C. 500mL of ethyl acetate was added to the concentrated mass and allowed to stand for 48 hours to allow formation of xylopic acid crystals. The concentrate was decanted after the period to separate the deposited crystals from the upper oily mass.

The crude xylopic acid crystals (2.130g) were recrystallized using freshly distilled ethanol and the formed crystals were washed with cold pet ether 40/60 and dried to give xylopic acid as white crystals. The weight of the crystals was 1.318g (yield 0.13%$^{w}/_{w}$).

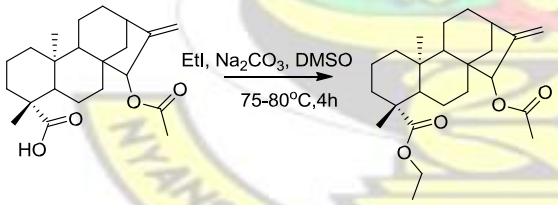
$V_{max}cm^{-1}$; 3280.5, 2927.21, 1722.99, 1704.89, 1271.13, 807.17.

δ_H, (400 MHz, DMSO) 12.10 (1H, br s, COOH), 5.08 (1H, s, C=C-H), 4.97 (1H, s, CH), 4.86 (1H, s, C=C-H), 2.67 (1H, s, CH), 2.17 (3H, s, CH_3), 1.16 (3H, s, CH_3), 0.97 (3H, s, CH_3) 0.96-0.80 (3H, m, CH,CH_2), 1.15-0.98 (6H, m, CH, CH_2), 2.12 -1.17 (9H, m, CH, CH_2), m/z (ES) 359.1 (M^+-H, 100%), 360.1 (M^+-H+1, 30%), 378.1 (M^++ H_2O), 379.1 (M^++H_2O+1), 301.0 (M^+ -$C_2H_3O_2$)

HPLC: Mobile phase: MeOH:H_2O (90:10); flow rate: 0.8mL/min; UV detector (206nm); C-18 column (4.6×250mm); retention time 4.8mins; purity 98.73%.

3.2.2 Synthesis of novel derivatives

Ethyl ester formation



Scheme 3.1 Synthesis of ethyl ester of xylopic acid

A mixture of xylopic acid (0.336g, 0.93207mmol) and sodium carbonate (0.300g, 2.8302mmol) were placed in a reaction vessel and DMSO (5mL) was added. The mixture was stirred at room temperature for 30minutes. Ethyl iodide (0.5mL, 6.2512mmol) was then added and the mixture was heated at 75-80°C while stirring continuously for (using TLC to monitor progress of the reaction)

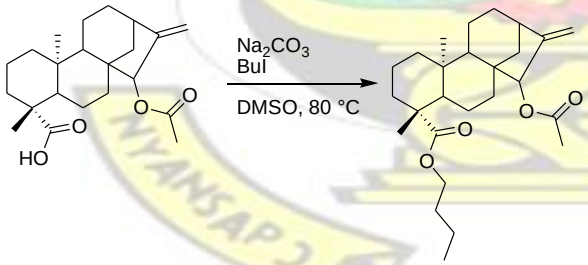
4 hours. The resulting mixture (which occurs as a reddish brown solution) was then cooled to room temperature and the content transferred into a separating funnel. 1 M sodium thiosulphate solution (20mL) was added to the content of the separating funnel before extracting with ethyl acetate (4×10mL). The organic fraction was dried with anhydrous Na_2SO_4, filtered and evaporated to dryness to give the crude sample which was purified by chromatography (silica gel) [eluent – Pet: Et_2O- 100:0- 30:70] to yield the product (322mg, 89.04%) as off-white solid crystals.

V_{max}cm-1; 2923.82, 2854.55, 1737.48, 1712.78, 1456.32, 1225.37, 889.78.

δ_H, (400 MHz, DMSO); 5.08 (1H, s, C=C-H), 4.90 (1H, s, CH), 4.80 (1H, s, C=C-H), 4.05 (2H, q *J*=7.5Hz, CH_2) 2.647 (1H, s, CH), 2.130 (3H, s, CH_3), 1.20 (3H, t *J*=7.5Hz, CH_3), 1.14 (3H, s, CH_3), 0.85 (3H, s, CH_3) 2.35-2.14 (3H, m, CH, CH_2), 2.10-1.3 (9H, m, CH, CH_2), 1.00-0.86 (6H, m, CH, CH_2)

m/z (ES) 302.0 $(M-C_4H_6O_2)^+$, 195.0 $(M+2H)^{2+}$, 329.2 $(M-C_2H_3O_2)^-$

Butyl ester formation



Scheme 3.2 Synthesis of butyl ester of xylopic acid

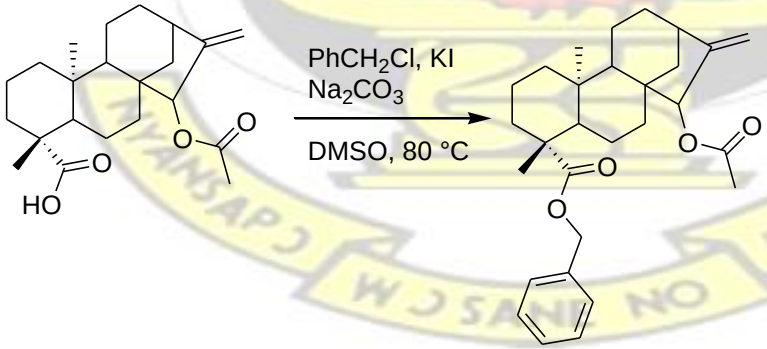
A mixture of xylopic acid (0.353g, 0.9792mmol) and sodium carbonate (0.279g, 2.6321mmol) were placed in a reaction vessel and DMSO (5mL) was added. The mixture was stirred at room temperature for 30minutes.

Butyl iodide (0.5mL, 4.4017mmol) was then added and the mixture was heated at 75-80^{o}C while stirring continuously (using TLC to monitor progress of the reaction) for 6 hours. The resulting mixture (which occurs as a reddish solution) was then cooled to room temperature and the content transferred into a separating funnel. 1 M sodium thiosulphate solution (25mL) was added to the content of the separating funnel before extracting with ethyl acetate (5×10mL). The organic fraction was dried with anhydrous Na_2SO_4, filtered and evaporated to dryness to give the crude sample which was purified by chromatography (silica gel) [eluent – Pet: Et_2O- 100:0- 30:70] to yield the product (388mg, 93.37%) as a pale yellow gum.

V_{max}cm-1; 2934.01, 2873.24, 1739.81, 1720.15, 1663.27, 1229.54, 888.23.

δ_H, (400 MHz, DMSO); 5.08 (1H, s, C=C-H), 4.98 (1H, s, CH), 4.86 (1H, s, C=C-H), 4.04 (2H, q *J*=7.0Hz, CH_2), 2.69 (1H, s, CH), 2.17 (3H, s, CH_3), 1.16 (3H, s, CH_3), 0.95 (3H, t *J*=7.0Hz, CH_3), 0.87 (3H, s, CH_3), 2.12-1.05, (23H, m, CH, CH_2).

Benzyl ester formation



Scheme 3.3 Synthesis of benzyl ester of xylopic acid

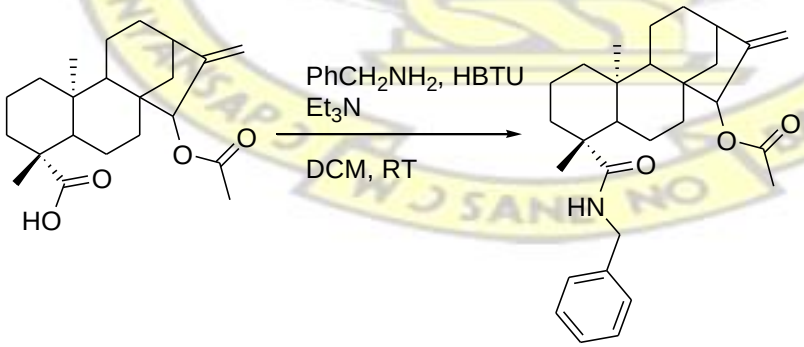
A mixture of xylopic acid (0.347g, 0.9626mmol) and sodium carbonate (0.279g, 2.8396mmol) were placed in a reaction vessel and DMSO (5mL) was added.

The mixture was stirred at room temperature for 30minutes. Benzyl chloride (0.8mL, 6.9521mmol) and potassium iodide (0.896g, 5.3976mmol) were then added and the mixture was heated at 75-80^{o}C while stirring continuously (using TLC to monitor progress of the reaction) for 6 hours. The resulting mixture (which occurs as a reddish brown solution) was then cooled to room temperature and the content transferred into a separating funnel. 1 M sodium thiosulphate solution (25mL) was added to the content of the separating funnel before extracting with ethyl acetate (5×10mL). The organic fraction was dried (with Na_2SO_4) and evaporated to dryness to give the crude sample which was purified by chromatography (silica gel) [eluent – Pet: Et_2O- 100:0- 30:70] to yield the product (224mg, 51.73%) as a white crystalline solid.

δ_H, (400 MHz, DMSO); 7.46-7.37 (5H, m, ArH), 5.06 -5.17 (2H, m, CH_2), 4.97 (1H, s, C=C-H), 4.96 (1H, s, CH), 4.85 (1H, s, C=C-H), 2.67 (1H, s, CH), 2.17 (3H, s, CH_3), 2.14-1.34 (12H, m, CH, CH_2), 1.19 (3H, s, CH_3), 1.17-0.86 (6H, m, CH, CH_2), 0.80 (3H, s, CH_3).

m/z (ES) 435.3 $(M\text{-}CH_3)^+$, 359.2 $(M\text{-}C_7H_7)^-$

Benzyl amide formation



Scheme 3.4 Synthesis of benzyl amide of xylopic acid

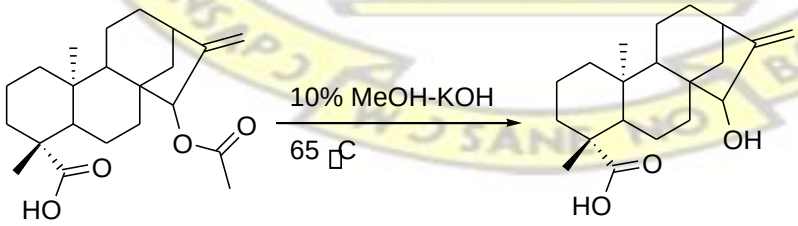
A mixture of xylopic acid (0.100g, 0.2774mmol), triethylamine (0.1mL, 0.7170mmol) and HBTU (0.210g, 0.5537mmol) were placed in a reaction vessel

and DCM (2mL) was added. The mixture was stirred at room temperature for 1.5 h. Benzyl amine (0.15mL, 1.3732mmol) was then added and the mixture stirred continuously (using TLC to monitor progress of the reaction) for 18hours. The resulting mixture was transferred into a separating funnel. The reaction vessel was rinsed with ethyl acetate (5×10mL) and transferred into the separating funnel. The solution in the separating funnel was washed with distilled water (5×10mL) followed by brine (5×10mL). The organic fraction was dried with anhydrous Na_2SO_4, filtered and evaporated to dryness to give the crude sample which was purified by chromatography (silica gel) [eluent – Pet: Et_2O- 100:0- 30:70] to yield the product (58.7mg, 47.11%) as a yellowish gum which solidifies upon standing.

V_{max}cm-1; 2923.72, 2853.36, 1725.62, 1645.55, 1457.74, 961.93, 746.62, 696.75.

δ_H, (500 MHz, DMSO); 7.80-7.20 (5H, m, ArH), 5.03 (1H, s, C=C-H), 4.92 (1H, s, C=C-H), 4.81 (1H, s, CH), 4.77 (1H, s, NH), 4.24 -3.94 (2H, app m, CH_2), 2.64 (1H, s, CH), 2.14-1.34 (12H, m, CH, CH_2), 2.12 (3H, s, CH_3), 1.24 (3H, s, CH_3), 1.11 (3H, s, CH_3) 1.17-0.86 (6H, m, CH, CH_2)

Deacetyl xylopic acid



Scheme 3.5 Synthesis of deacetyl xylopic acid

A mixture of xylopic acid (0.400g, 1.1096mmol) and 10%$^{w}/_{v}$ methanolic KOH (5mL, 8.9118mmol) were placed in a reaction vessel and refluxed while stirring continuously (using TLC to monitor progress of the reaction) for 3hours. The

resulting mixture was transferred into a separating funnel. 10% $^{v}/_{v}$ sulphuric acid solution (35 mL) was added and a blue litmus paper was used to monitor the pH to ensure that it is sufficiently acidified. The cloudy solution in the separating funnel was then extracted with ethyl acetate (5×10mL). The organic fraction was dried with anhydrous Na_2SO_4, filtered and evaporated to dryness to give the crude sample which was purified by chromatography (silica gel) [eluent – Pet: Et_2O- 100:0- 30:70] to yield the product (330mg, 93.52%) as a white crystalline solid.

δ_H, (400 MHz, DMSO); 12.0 (1H, br s, COOH) 5.10 (1H, s, C=C-H), 5.01 (1H, s, C=C-H), 4.81 (1H, s, OH), 2.60 (1H, s, CH), 1.16 (3H, s, CH_3), 0.99 (3H, s, CH_3), 2.42-1.21 (12H, m, CH, CH_2), 0.98-0.80 (7H, m, CH, CH_2) m/z (ES) 317.1 (M^+-H, 100%), 318.1 (M^+-H+1 30%), 336.0 (M^++ H_2O)

TLC was carried out on silica gel 60 F254 pre-coated plates and detection was achieved by staining with a saturated solution of ceric sulphate in 10% sulphuric acid followed by heating.

3.2.3 Antimicrobial assay

The microorganisms used for the experiment [*Staphylococcus aureus (ATCC 25923), Streptococcus pyrogenes (clinical strain), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 4853) and Candida albicans (clinical strain)*] were sub-cultured in sterile nutrient broth and incubated at 37^{o}C for 1824 hours. All the microorganisms were sourced from Komfo Anokye Teaching Hospital (KATH), Kumasi.

Eight concentrations of the test compounds (1000, 800, 500, 400, 250, 200, 100 and 10µg/mL) were prepared as aqueous solutions (methanol: water, 50:50) by serial dilution.

100µL of sterile broth was dispensed into each previously labelled well of the micro titre plate by means of a micropipette. 80µL of each dilution of the test compounds was dispensed into the appropriate well in each case. Finally, 20µL of each test organism was again dispensed into the appropriately labelled wells and the plates were covered, labelled and incubated at 37^{o}C for 20 hours. The resultant concentrations of the compounds obtained after inoculation were 400, 320, 200, 160, 100, 80, 40 and 4µg/mL. The results were recorded and the Minimum Inhibitory Concentration (MIC) determined in each case.

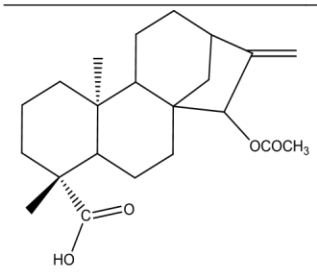
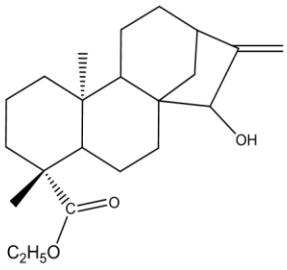
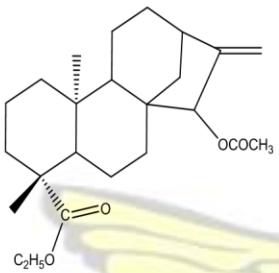
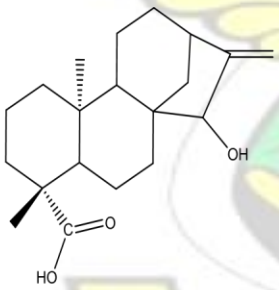
CHAPTER FOUR

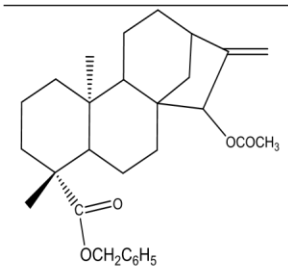
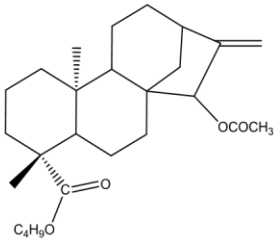
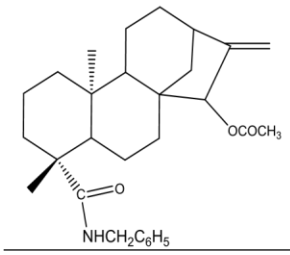
RESULTS

4.1 PHYSICAL DATA FOR COMPOUNDS

Table 4.1 Physical data of various compounds.

Compound	Cod	Mol.	% Yield	Melting	Rf

	e	Weight (g/mol)	(%$^{w}/_{w}$)	pt. (^{o}C)	
	JP 000	360.49	0.13	264-265	0.51 Pet: EtOAc
	JP 002	346.50	32.36		0.48 Pet: EtOAc
	JP 005	388.54	89.04	134-135	0.75 Pet: Et_2O
	JP 006 A	318.45	93.52	204-206	0.29 Pet: Et_2O

	JP 007	450.61	51.73	114-115	0.81 Pet: Et_2O
	JP 010	416.21	93.37		0.77 Pet: Et_2O
	JP 015	449.62	47.11		0.68 Pet: Et_2O

4.2 ANTIMICROBIAL ASSAY

Table 4.2 Minimal inhibitory concentrations (MIC) of xylopic acid and the formed derivatives.

MIC (µg/mL)

Organism	JP 000	JP 002	JP 005	JP 006A	JP 007	JP 010	JP 015	Cefuroxime axetil
Staphylococcus aureus	320	160	100	100	100	100	200	100
Streptococcus pyrogenes	320	100	100	100	100	100	200	100
Escherichia coli	200	100	100	100	100	100	200	100
Pseudomonas aeruginosa	200	160	100	100	100	160	200	80
Candida albicans	320	200	160	160	160	160	200	100

NB: JP 000- xylopic acid, JP 002- deacetyl ester; JP 005- ethyl ester; JP 006Adeacetyl xylopic acid; JP 007- benzyl ester; JP 010-butyl ester; JP 015- benzyl amide.

Xylopic acid exhibited the highest antimicrobial activity against the gram negative microorganisms (*Escherichia coli* and *Pseudomonas aeruginosa*) tested. The ester derivatives of xylopic acid did not show any significant difference in antimicrobial activity as observed from the MIC values obtained. The deacetylated derivatives of xylopic acid also possess activity comparable to that of the ester derivatives. The amide derivative is the least active among all the synthesized compounds.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Isolation and characterization of xylopic acid

Highly purified xylopic acid crystals (purity 98.73%) at a yield of 0.13%$^{w}/_{w}$ were obtained from the isolation procedure. This could be considered as a relatively high yield when compared with a yield of 0.05%$^{w}/_{w}$ obtained by Adosraku and Oppong Kyekyeku who used a similar method for the extraction and isolation of the crystals (Adosraku and Oppong Kyekyeku, 2011). Considering an earlier research conducted by Ekong and Ogan, xylopic acid was isolated at a yield of 1.30% which is ten times more than what was obtained in this experiment (Ekong and Ogan, 1968). In all these experiments, petroleum ether was used in the extraction of xylopic acid, however, Ekong and Ogan used ethyl acetate to recrystallize the extracted xylopic acid. This method of recrystallization was attempted at one point in this research with the hope of increasing the yield of xylopic acid but the extracted xylopic acid was very poorly soluble in relatively large volumes of ethyl acetate even at temperatures as high as 75^{o}C. Hence, ethyl acetate could not be used for recrystallization of xylopic acid. Again, the possibility of using an alternate solvent for extracting the plant material in order to increase the yield of xylopic acid was explored. Here, chloroform was considered, as a minimum volume is able to dissolve relatively large quantities of xylopic acid even at room temperature. The use of this solvent was however abandoned when it was realised that a solution of xylopic acid in chloroform at room temperature begins to show multiple spot on TLC on standing for 24hours but maintains one spot when refrigerated. This observation suggested that the chloroform interacted with xylopic acid in a

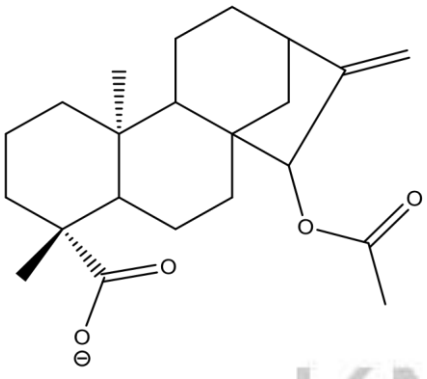
manner that for example led to the degradation of the latter. Having said this, the possibility of employing chloroform as solvent in extracting very pure xylopic acid in relatively high yields cannot be written off, as the option of extraction at a low temperature has not been investigated.

The xylopic acid crystals obtained were first identified by melting point determination. The crystals had a melting point of 264-265ºC. This is comparable with the results obtain by Fahim *et al* who determined the melting point of isolated xylopic acid as 265 – 266°C (Fahim *et al*., 1953). Again, this value is more or less consistent with that obtained by Ekong and Ogan and Adosraku and Oppong Kyekyeku who determined the melting point of the crystals as 259-260ºC and 260-261ºC respectively. The slight differences observed may be due to the purity of crystals obtained in each case and perhaps the type of melting point apparatus used.

Looking at the infrared spectrum of the xylopic acid obtained (appendix A2), some major absorption bands could be identified in the functional group region. For instance, the peak at 3280.5 cm^{-1} signifies the presence of a hydroxyl group (-OH) whilst a relatively broad peak from around 3100-2800cm^{-1} is generally suggestive of a carboxylic acid (-COOH) functional group. Within this same wavenumber range is where the various sp^3 hybridized C-H stretch vibrations occur. These peaks are not diagnostically relevant, as almost all organic compounds possess the C-H bond. Peaks at 1722.99 and 1704.89 cm^{-1} confirm the presence of two carbonyl functional groups. The peak at 1722.99 cm^{-1} is most likely due to the vibration of the carbonyl (C=O) present in the acetyl group while that at 1704.89cm-1 may be due to carbonyl (C=O) in the

carboxylic acid. Generally, carbonyls in esters vibrate at a higher frequency as compared to those in carboxylic acids. This is because in carboxylic acids, the hydrogen atom alternate between the two oxygen atoms thus introducing a partial single bond character in the C=O. In addition, there is an increased tendency of intramolecular hydrogen bonding between the carbonyl and the carboxylic acid –OH. These will consequently decrease the strength of the C=O and decreasing bond strength means a reduction in vibrational frequency (Pavia *et al*., 2008). It is important to indicate that the infrared spectrum obtained in this experiment compares very much with that obtain by Adosraku and Oppong Kyekyeku.

With the mass spectrometric analysis, the ionization method employed in this experiment is the ESI. Considering the mass spectrum of xylopic acid (appendix A3) it could be observed that generally, the compound is better ionized in the negative mode than in the positive mode, as the signal intensity for the negative ionization (MS ES- :359, 4.4×10^{7}) is about a thousand times greater than that of the positive ionization (MS ES+:361, 3.3×10^{4}). In the negative mode, a very intense peak was observed at mass to charge ratio (m/z) of 359.1. This peak corresponded to an $[M-H]^{-}$ peak, as the predicted molecular weight of the compound is 360 thus loss of a proton will lead to a fragment with a molar mass of approximately 359. The product of negative ionization of xylopic acid is shown in fig. 5.1.



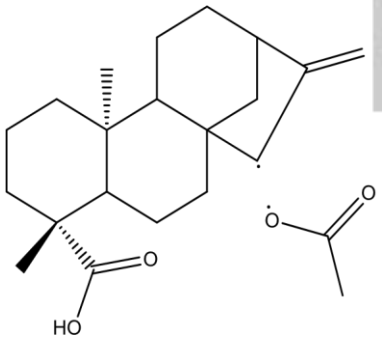
Chemical Formula: $C_{22}H_{31}O_4^-$
Exact Mass: 359.22
Molecular Weight: 359.48
m/z: 359.22 (100.0%), 360.23 (24.3%), 361.23 (3.6%)
Elemental Analysis: C, 73.50; H, 8.69; O, 17.80

Fig. 5.1 Negative ionization of xylopic acid

Again, a peak with m/z of 360.1 and having an intensity of approximately 24% could also be observed. This peak is probably not the molecular ion peak but rather may be due to the presence of elements with only two naturally occurring stable isotopes (e.g. H,C and N), and the mass of the more abundant isotope is one mass unit less than the other [(X+1) elements]. Here, the peak at 359.1 represents the X peak while that at 360.1 represents the (X+1) peak. The (X+1) peak in this case is most likely to be due to the presence ^{13}C rather than ^{2}H because the relative abundance of ^{13}C in nature is 1.09%. Therefore the probability that the $[M-H]^-$ ion contains a ^{13}C isotope [(1.09/98.91)×100 ×22 carbon atoms] is 24.24. This figure mostly corresponds to the intensity of the peak on the mass spectrum (Watson and Sparkman, 2007), (McLafferty and Tureček, 1993).

Considering the mass spectrum for ionization in the positive mode, an $[M+H]^+$ peak with m/z 361 is not observed as expected, however, peaks at 378.1 and

379.1 are observed instead. It could be deduced that the peak at m/z of 378.1 is due an $[M+H_2O]^+$ ion whilst that at m/z of 379.1 represent an (X+1) peak of the latter. There is also another peak at m/z of 301.0, which most probably represents an $[M-OCOCH_3]^+$ ion i.e. a loss of an acetate ion from the molecular ion (as shown in fig. 5.2).



Chemical Formula: $C_{20}H_{29}O_2^{\bullet}$
Exact Mass: 301.22

Chemical Formula: $C_2H_3O_2^{\bullet}$
Exact Mass: 59.01

Fig. 5.2 Loss of acetate ion from molecular ion

From the NMR spectrum of xylopic acid in appendix A1, a broad singlet peak with integration corresponding to 1H could be observed at a chemical shift of 12.05. This signifies the presence of a carboxylic acid proton thus confirming the presence of a carboxylic acid functional group in xylopic acid. Again, the three singlet peaks at 5.08-4.86 could be integrated as 3protons (3H). It could be suggested that the signal at chemical shift 4.98 (i.e. middle peak) corresponds to the proton on C-15 while the two other peaks represents the two diastereotopic olefinic protons which exhibit geminal anisochrony. This may arise from the fact that one of the olefinic protons interacts more with the deshielding acetyl functional group than the other as a result of the rigidity of the C=C double bond. The proton on C-7 is assigned the signal at chemical shift 2.69 due to its closeness to the strongly deshielding carboxylic acid functional

group. The three intense singlets at 2.17, 1.16 and 0.92 are most likely to be due to the methyl protons on C-27, C-20 and C-19 respectively. This assignment stems from the fact that the protons on C-19 are the least deshielded whilst the protons on C-27 are most deshielded due to their increased proximity to the deshielding oxygen atoms.

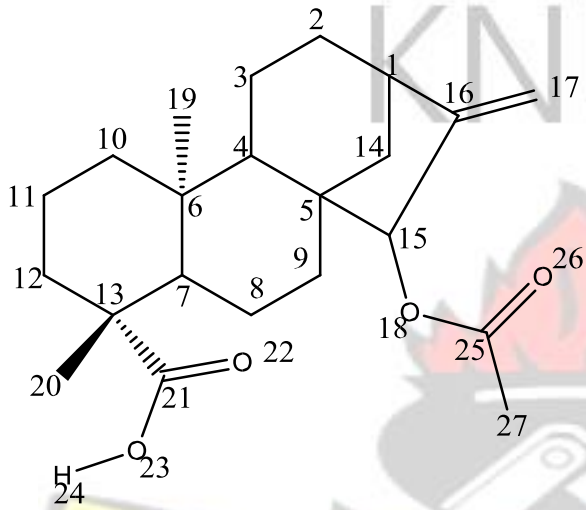


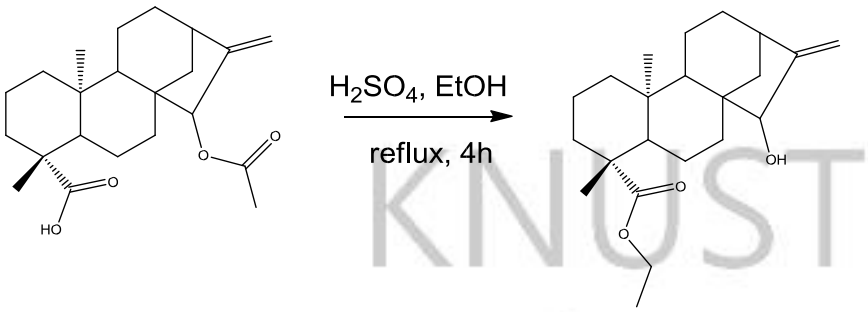
Fig. 5.3 Structure of xylopic acid showing numbering of various atoms

5.1.2 Synthesis and characterization of novel derivatives

Ethyl ester

Acid catalysed ester formation was first attempted in this experiment, but it turned out that the concentrated sulphuric acid (catalyst) interacts with the xylopic acid upon adding the former. This was characterized by a discoloration of the xylopic acid solution upon addition of the catalyst. An attempt to form an ethyl ester of xylopic acid *via* acid catalysis yielded a product, which appeared to be more polar than the xylopic acid starting material, as evidenced by a lower RF than xylopic acid on TLC. It was therefore assumed that there could be a possible cleavage of the acetyl group of xylopic acid upon addition of the acid catalyst followed by the esterification of the deacetylated xylopic acid, leading

to the formation of an ester of deacetyl xylopic acid (compound JP 001) instead of an ester of xylopic acid (see scheme 5.1). This method of ester formation was therefore abandoned.



Scheme 5.1 Suggested reaction for acid catalysed ethyl ester (of xylopic acid) formation.

An infrared spectrum of this compound (appendix B2) showed no peak at 3280 cm^{-1} as in the case of xylopic acid, depicting the disappearance of the carboxylic acid –OH group. However, considering the suggested structure (scheme 5.1), there is an –OH group at C-15 and thus a peak between 3500 and 3200 cm^{-1} was generally expected, on the contrary, this did not occur. Looking at the ^{1}H NMR spectrum obtained for the compound (appendix B1), a quartet was observed at δ 4.05 and a triple was observed at δ 1.15. It could be suggested that, these two groups of protons (depicting the ethyl group of the ester) couple with each other as the two signals have the same coupling constant (*J*=7.5Hz). Again, a singlet at δ 3.15, which is integrated as 1H, is probably due to the –OH group in the deacetylated compound confirming the structure suggested earlier.

Considering the spectral data (especially the NMR spectrum) obtained for the product of the base catalysed ethyl ester formation (appendix C1), it was obvious that the synthesized compound was indeed an ethyl ester derivative of xylopic acid. From the ^{1}H NMR spectrum, it could be observed that the broad

singlet at chemical shift of 12.05 (in the case of xylopic acid) is conspicuously missing. This is indicative of the fact that the carboxylic acid functional group in xylopic acid was lost most likely through ester formation. Again, the three singlet peaks at 5.04-4.82 were integrated to give three protons (3H), the second signal represents the proton on C-15 while the other two represent the two chemically non-equivalent olefinic protons on C-17. In addition, there was also a quartet at δ 4.07-4.01 (*J*=7.5Hz) with an integral of two protons (2H) thus it is most likely to be the protons on C-24. Yet still, the proton on C-7 (just like the case of xylopic acid) is assigned the signal at chemical shift 2.65 due to its closeness to the strongly deshielding carboxylate group. Once more, it is apparent that the three intense singlets at 2.13, 1.14 and 0.85 are most likely to be due to the methyl protons on C-27, C-20 and C-19 respectively. Finally, a triplet could be observed at δ1.20 (*J*=7.5Hz) which was also integrated to three protons (3H). This signal is suspected to be due to the protons on C-25 as its coupling constant (*J*=7.5Hz) is similar to that of the methylene protons on C-24 (*J*=7.5Hz). Generally, protons that couple have similar coupling constants.

In spite of the fact that the NMR spectrum almost completely solved the structure of the compound, an infrared spectrum was also obtained (appendix C2) and it further confirmed the structure elucidated with the NMR spectrum. For instance, the disappearance of an –OH stretch at 3280 cm^{-1} together with the absence of a broad peak between 2700-3100cm^{-1} was strongly indicative of the fact that the carboxylic acid group originally present in xylopic acid was converted to another functional group, typically an ester.

Furthermore, from the mass spectrum of the same compound (appendix C3), some important deductions were made. For example, on the spectrum resulting from positive ionization, an m/z of 302.0 and 195.0 could be attributed to the fragments shown in fig 5.4 while that at 303.1 is most likely due to an (X+1) peak.

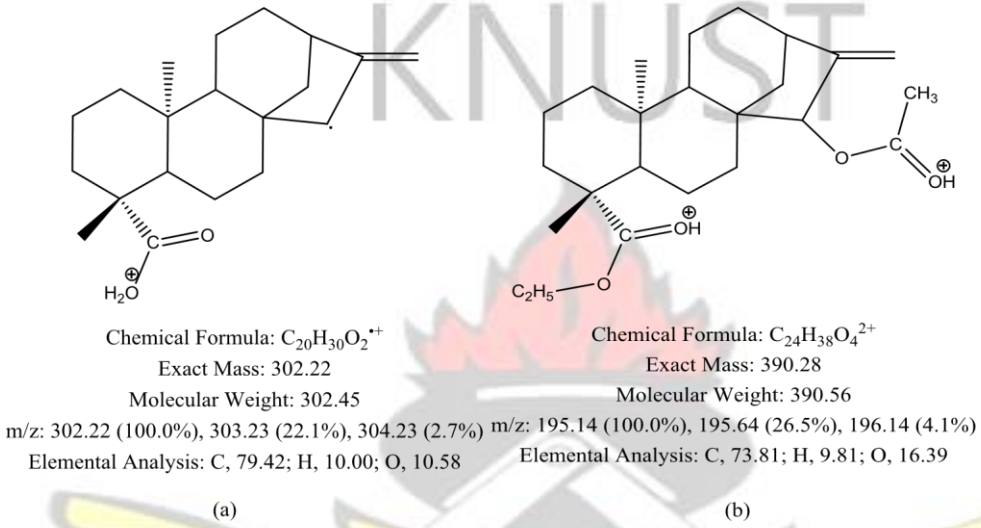


Fig. 5.4 (a) Fragment (ion) with m/z of 302.0 and (b) fragment with m/z of 195.0.

Again, from the spectrum resulting from the negative ionization of the compound, a peak at m/z of 329.2 may be due to the fragment shown in fig 5.5 and that at 330.2 is perhaps another (X+1) peak.

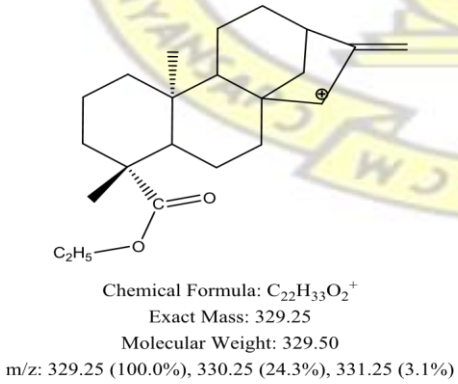


Fig. 5.5 Fragment (ion) with m/z 329.2.

The melting point of the compound which was determined as 134-135^{o}C does not provide any crucial information as there is no such data on the compound. On the other hand, this may be valuable information that could be relied on for further research conducted on the compound. Although the product was obtained in very good yield, (89%), this could be further optimised.

Butyl ester

The structure of the butyl ester was virtually solved with just the ^{1}H NMR data. From the spectrum shown in appendix D1, like the spectrum for the ethyl ester, clearly the broad singlet at δ12.05 has disappeared signifying a loss of the carboxylic acid functional group most probably to a butyl ester formation. Other similarities between this spectrum and that of the ethyl ester could also be identified; for example the three singlet peaks at δ 5.09-4.86 (3H), the singlet at 2.69 for the proton on C-7 and the singlets at δ 2.17, 1.15 and 0.87 (3H) corresponding to the protons on C-26, C-20 and C-19 respectively.

Additionally, signals that were not typically present in the spectrum for the ethyl esters were also observed. For instance, the multiplet at δ 4.07-3.99, which is integrated as two protons (2H), is most likely due to the methylene protons on C-30 and the triplet at δ 0.96 integrated as three protons (3H) is suggested to be due to the methyl protons on C-27.

The infrared spectrum (appendix D2) again confirmed a loss of the carboxylic acid functional group with the disappearance of a peak at 3280 cm^{-1} and the loss of the broad peak at 3100-2800cm^{-1}.

No peaks (ion fragments) were observed for both positive and negative ionization of the butyl ester. This could probably mean that the compound did not get charged both under positive and negative ionization.

As the compound occurs as a semi-solid, melting point determination was not performed. It was however obtain in a very high yield of 93.37%.

Benzyl ester

Furthermore, the benzyl ester, which occurred as a white crystalline solid, was also taken through various spectral analyses i.e. ^{1}H NMR and MS. Like the other compounds, most of the very meaningful spectral information was obtained from the NMR data.

Considering the NMR spectrum for this compound (appendix E1), it could be observed that there is no signal at δ 12.05 indicating the absence of a carboxylic acid. There is however, a multiplet at δ 7.46-7.37 with an integral of five protons (5H). A signal at δ 7-8 is strongly indicative of an aromatic ring and an integral of 5H means that the benzene ring is mono substituted. This is very much in agreement with the proposed structure of the benzyl ester. Again, aside the three singlet peaks at δ 5.05-4.85, there is a multiplet at δ 5.17-5.06, which is suggested to be due to the methyl protons on C-24 due to their closeness to the electron withdrawing oxygen atoms. The other aspects of the spectrum are very similar to that of xylopic acid.

With the aid of some MS interpretation tools (e.g. chemdraw ultra 12.0), some possible ion fragments were assigned to a few peaks on the mass spectrum for the benzyl ester. For instance, looking at the spectrum representing positive

ionization of the compound, a stick at m/z of 435.3 could be due to the ion fragment shown in fig. 5.6 (a) while (b) represents the stick at m/z of 359.2 on the spectrum for negative ionization of the compound.

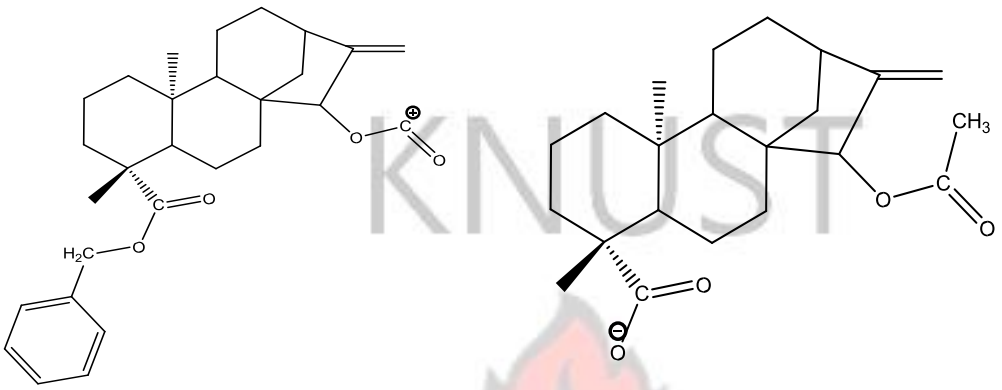


Fig. 5.6 (a) ion fragment for m/z of 435.3 (b) ion fragment for m/z of 359.2

Benzyl amide

The yield of the benzyl amide (of xylopic acid) was the lowest (47.11%) among all the compounds synthesized. This may be because the coupling agent used (HBTU) could not efficiently activate the carboxylic acid for subsequent reaction with the amine to form the desired amide. To confirm the structure of the synthesized compound, infrared and ^{1}H NMR spectra was obtained for the compound. From the IR spectrum (appendix F2), it is obvious that the carboxylic acid functional group in xylopic acid has been converted to another functional group (most probably the amide). This is evident from the fact that the –OH stretch at 3280cm^{-1} and the broad peak between 2800 and 3100cm^{-1} which indicate the presence of a carboxylic acid functional group have both disappeared. Again, an –NH stretch at 3500-3200cm^{-1} is expected because of the presence of the –NH group in the compound. A weak signal near 3470cm^1 could most likely be due to the –NH stretch as these vibrations are usually of

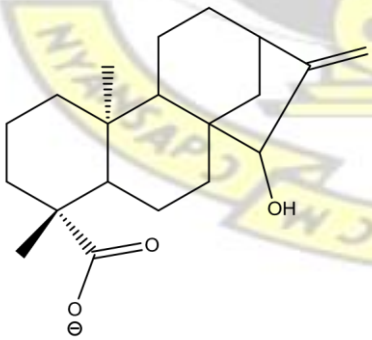
low to medium intensities. Yet again, in the fingerprint region, the presence of a monosubstituted benzene ring is usually characterized by a strong absorption near 690cm^{-1} and another near 750cm^{-1} (Pavia *et al*., 2008). From the spectrum obtained, the occurrence of two strong absorption bands at 696.75 and 746.62cm^{-1} more or less confirms the presence of a monosubstituted aromatic system, which is in this case the benzyl group of the amide synthesized. From the NMR spectrum obtained (appendix F1) disappearance of a signal at δ 12.05 suggests that the carboxylic acid functional group has been lost. In addition, a multiplet is observed at δ 7.8-7.2, integrated as five protons (5H) indicate the presence of a monosubstituted benzene ring. Aside the signal due to the olefinic protons near δ 5.0, the presence of another singlet at δ 4.77 that was integrated as 1H was most probably due to the –NH group in the amide. Finally, the multiplet at δ 4.24-3.94 (2H) is probably due to the CH_2 protons in the benzyl group.

Deacetyl xylopic acid

Base catalysed ester hydrolysis was employed in the deacetylation of xylopic acid. It was observed that when reaction was allowed to proceed for 4hrs, TLC monitoring showed two spots that had very close RF values. However, when the reaction was allowed to proceed for just 1 h, only one spot was observed.

The formation of epimers (α-OH and β-OH) was suspected in the case where two spots were observed on TLC. These two compounds were successfully separated by column chromatography and the yield was in a ratio of 25:1 for the higher and lower RF compounds respectively. The original plan was to synthesize ether derivatives of deacetyl xylopic acid *via* the Williamson's ether synthesis (using potassium hydroxide as alkali) but this was unsuccessful after

several attempts. The melting point of the compound which occurred in a larger quantity was determined as 204-206^{o}C. Ekong and Ogan (1968) obtained the same value (204-206^{o}C) for a compound identified as (-) kaur-16-en-15βhydroxy-19-oic acid (i.e. the β-OH epimer). Because of the very low recovery of compound JP 006B (lower RF compound), its melting point could not be determined. However, both compounds were taken through mass spectrometric analyses. The mass spectra of the two compounds (JP 006A and B) are as shown in appendices G2 and H1 respectively. Like xylopic acid, the two compounds (JP006A and B) appear to be better ionized in the negative mode than the positive mode. This is not far-fetched because the major functional group in the compound i.e. the carboxylic acid functional group will preferentially be deprotonated rather than being protonated. In the negative mode, a very intense peak was observed at mass to charge ratio (m/z) of 317.1. This peak corresponded to an $[M-H]^-$ peak, as the predicted molecular weight of the compound is 318 thus loss of a proton will lead to a fragment with a molar mass of about 317. The product of negative ionization of compounds JP 006A and B is shown in fig. 5.7.



Chemical Formula: $C_{20}H_{29}O_3^-$
Exact Mass: 317.21
Molecular Weight: 317.44
m/z: 317.21 (100.0%), 318.22 (22.1%), 319.22 (2.9%)
Elemental Analysis: C, 75.67; H, 9.21; O, 15.12

Fig. 5.7 Loss of a proton from the molecular ion

Here too, the presence of an (X+1) peak is observed at m/z of 318.1 for both compounds with a peak intensity of 22%. Due to its very small yield, no further analysis was successfully carried out on compound JP 006B but the mass spectrometric data alone gives a strong indication that it may be an epimer of deacetyl xylopic acid. For compound JP 006A, positive ionization is very similar to that of xylopic acid in that there is a peak at m/z of 336.0 which may be due to an $[M+H_2O]^+$ ion and another peak at 302.0 possibly due to a loss of a hydroxyl group from the molecular ion as shown in figure 5.8.

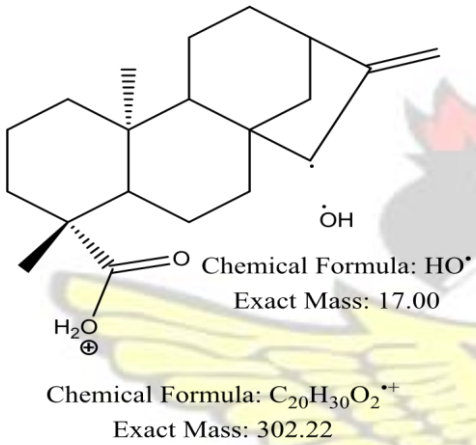


Fig. 5.8 Loss of hydroxyl group from molecular ion

The NMR spectrum of compound JP 006A (appendix G1) showed a broad singlet at chemical shift of 12.0 confirming the presence of a carboxylic acid functional group. Again, like xylopic acid, there are three singlet peaks at 5.104.88 integrated as 3protons (3H). Nevertheless, quite differently, it could be suggested that the signal at chemical shift 4.88 corresponds to the hydroxyl proton (25) while the two other peaks represented the two diastereotopic olefinic protons (i.e. protons on C-17) which exhibit geminal anisochrony. Again, the proton on C-15 is represented by the signal at chemical shift 3.60 rather than the 4.98 observed in xylopic acid. The difference could be a result of the loss of the

acetyl group which consequently results in the loss of one oxygen atom which would otherwise further increase the deshielding (electron withdrawing effect) of the proton on C-15. The proton on C-7 is assigned the signal at chemical shift 2.60 due to its closeness to the strongly deshielding carboxylic acid functional group. The two intense singlets at 1.16 and 0.92 are most likely due to the methyl protons on C-20 and C-19 respectively. This assignment stems from the fact that the protons on C-19 are deshielded to a lesser extent whilst the protons on C-20 are deshielded to a greater extent due to their increased proximity with the deshielding oxygen atoms of the carboxylic acid functional group. Interestingly, the singlet due to C-27 occurring at 2.17 in the spectrum for xylopic acid has disappeared. This therefore confirms the fact that the hydrolysis of xylopic acid led to the loss of the acetyl group bearing the methyl protons on C-27.

5.1.3 Antimicrobial assay

The broth dilution method of MIC determination was employed in this research. Sterility and growth (negative) controls as well as positive control experiments were performed. Again, a duplicate experiment was performed in order to ensure the accuracy of results obtained. The microorganisms used for the antimicrobial assay were two gram positive (*Staphylococcus aureus* and *Streptococcus pyrogenes*) and gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria and a fungus (*Candida albicans*). These microorganisms were selected particularly because they will together help to predict the spectrum of activity of the novel derivatives synthesized. From the results obtained (Table 4.2), it could be observed that clearly, all the derivatives synthesized were generally more active than xylopic acid. Again, the benzyl

amide derivative appeared to be the least active among the various derivatives formed hence the benzyl ester is more active than the corresponding benzyl amide. It will however be inappropriate to assume that ester derivatives of xylopic acid will be more active than their corresponding amide derivatives unless more amides are synthesized and their antimicrobial activities investigated and compared with those of the esters. Deacetylation obviously resulted in a more active compound. Also, esterification of deacetyl xylopic acid resulted in a compound which is less active than deacetyl xylopic acid itself. However, both compounds tended to have a lower antifungal activity as compared with their antibacterial activity. For the ester derivatives, increasing the number of carbon atoms did not significantly affect their antimicrobial activity. In addition, the ester derivatives generally exhibited a greater antibacterial activity as compared with their antifungal activity. To explain the observed trends in antimicrobial activity, it could be suggested that the ester and amide derivatives are more active than xylopic acid because derivatization resulted in a more lipophilic compound, which is more easily transported across the cell wall of the microorganisms. Upon entering the cell, hydrolytic enzymes hydrolyse the compound to produce xylopic acid again, which then interact with the organelles of the microorganism in a way that destroys the latter. Now, the benzyl ester was probably, more active than the corresponding benzyl amide because amide bonds are generally more stable than ester linkages. As a result, hydrolysis occurred more readily in the case of the benzyl ester thus toxic levels of xylopic acid were attained within the cell of the microorganism even with lower concentrations of the ester compared with the amide.

On the contrary, comparing xylopic acid with its deacetylated derivative, the issue of lipophilicity does not hold as xylopic acid is the less polar compound and yet it is less active as compared with its deacetyl derivative. A better explanation to this observation could be based on molecular weight; deacetyl xylopic acid has a molecular weight that is about 42g/mol less than that of xylopic acid. This difference in molecular weight probably makes it possible for deacetyl xylopic acid to be more rapidly transported across the cells of the microorganism compared with xylopic acid. This means that toxic levels of deacetyl xylopic acid are quickly attained within the cells of the microorganisms leading to their death. A similar theory may also be used to explain why deacetyl xylopic acid is generally more active than the deacetyl ester. Finally, the fact that most of the synthesized compounds compare largely in terms of antimicrobial activity with the control drug (cefuroxime axetil) which is a very potent antibiotic on the international market suggests that these compounds possess much promise in their potential to be used as effective antimicrobials. That notwithstanding, it is important to emphasis the fact that other *in vivo* studies such as toxicity and efficacy need to be investigated before any claims can be made.

5.2 CONCLUSION

Xylopic acid was isolated and crystallised from the dried fruits of *Xylopia aethiopica* and characterized. The crystals were used to synthesize novel derivatives of xylopic acid and these were sufficiently characterized using IR, NMR and mass spectrometry. All the synthesized compounds were more active than xylopic acid, which generally exhibited a broad spectrum of activity but had greater gram-negative activity. Again, the ester derivatives generally had

greater antibacterial activity as compared with their antifungal activity. Generally, increasing the carbon chain of the esters did not affect their antimicrobial activity. Deacetylation also resulted in a more active compound. Finally, the benzyl amide derivative had lower antimicrobial activity as compared with the corresponding benzyl ester but this did not suggest that all amide derivatives of xylopic acid would be less active than their corresponding ester derivatives.

5.3 RECOMMENDATIONS

More amide derivatives of xylopic acid should be synthesized and investigated for antimicrobial activity, and the results compared with that of their corresponding esters. Other derivatives of xylopic acid (e.g. epoxides, ethers) should be synthesized, characterized and screened for various biological activities (e.g. antimalarial, analgesic).

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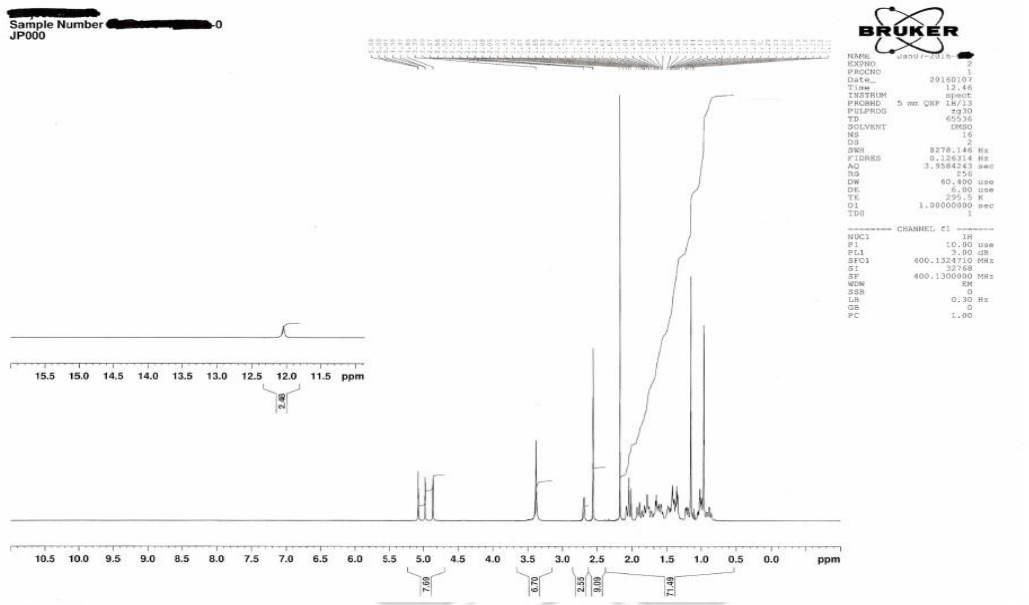
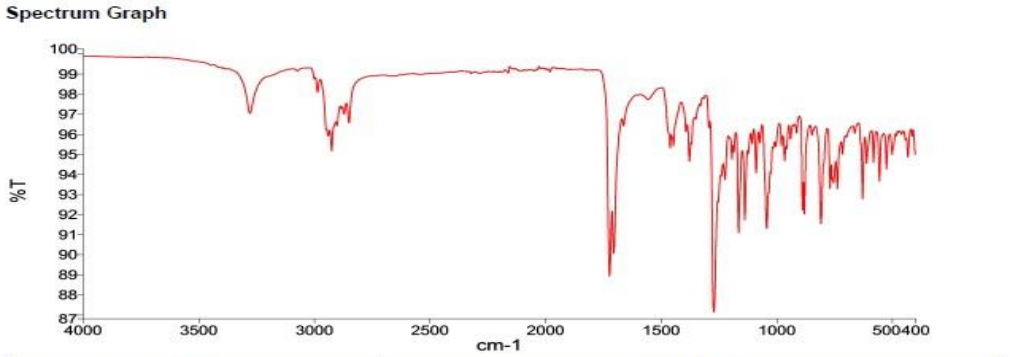
APPENDICES

Appendix A; Analytical data for of xylopic acid.

1H NMR spectrum



IR Spectrum

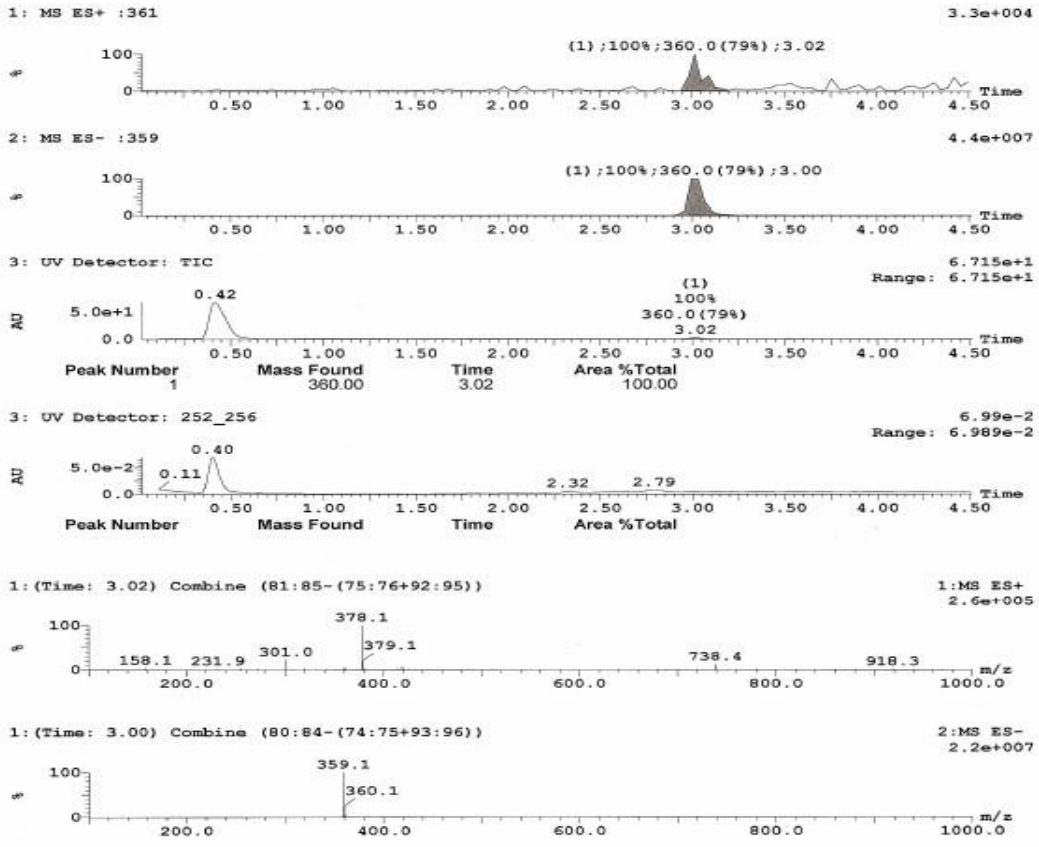


Name	Description
John Fetse1	Xylopic acid

Peak Table

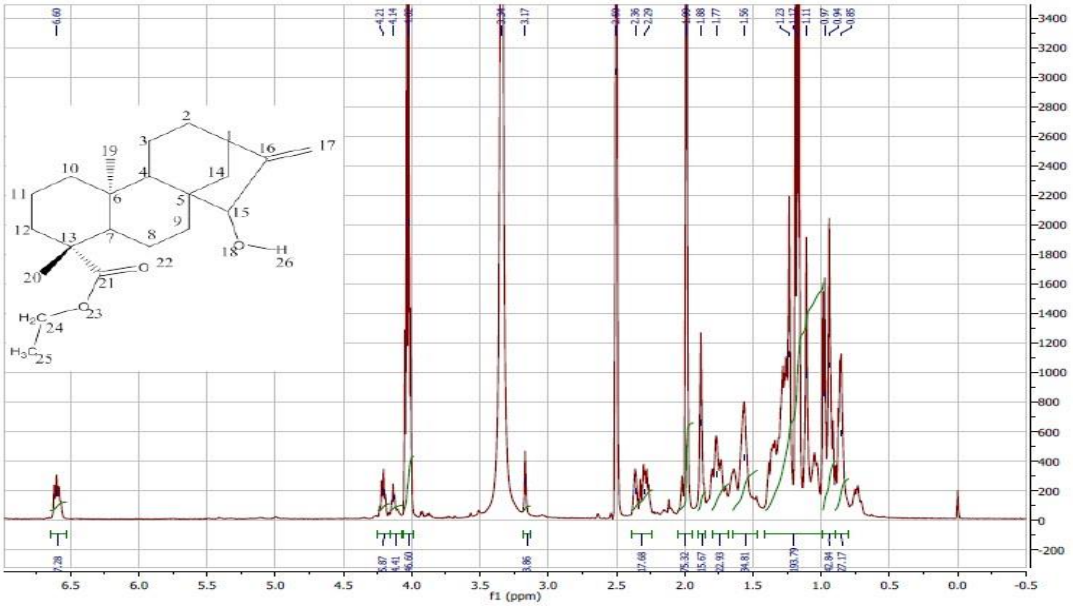
Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)
1	3280.5	97.07	2	2927.21	95.2	3	1722.99	88.92	4	1704.89	90.05
5	1460.92	95.33	6	1377.09	94.67	7	1271.13	87.13	8	1164.7	91.08
9	1137.36	91.74	10	1088.4	94.1	11	1042.76	91.3	12	879.62	92.03
13	807.17	91.55	14	736.82	93.29	15	626.95	92.79	16	554.44	93.68

Mass spectrum



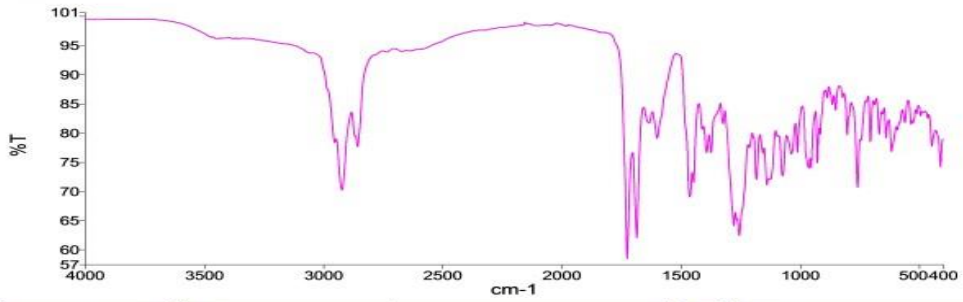
Appendix B; Analytical data for ethyl ester of deacetyl xylopic acid.

^{1}H NMR spectrum



IR Spectrum

Spectrum Graph



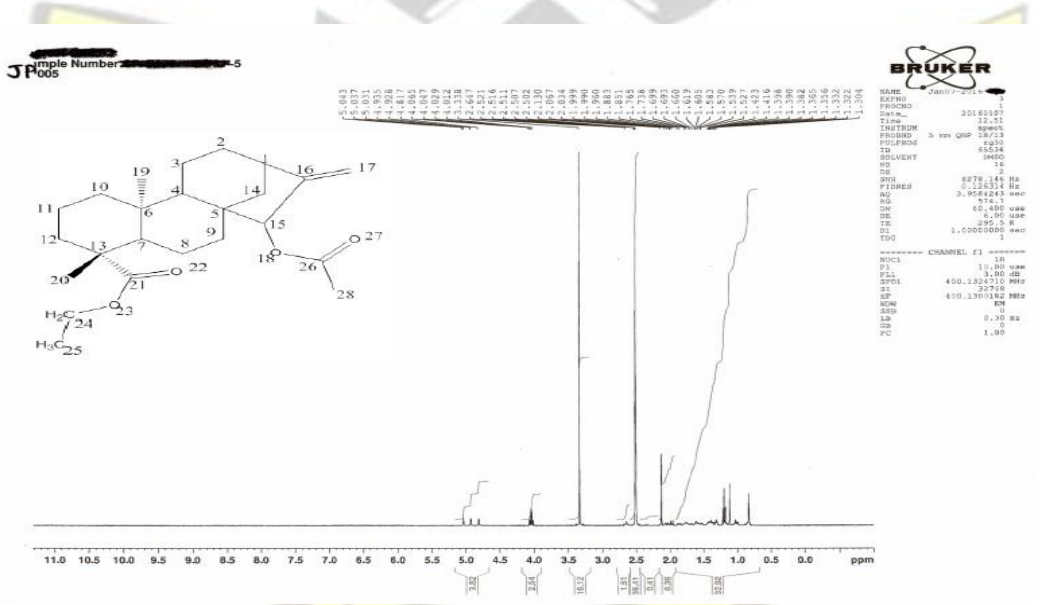
Name	Description
John Fetse 1	Ethyl xylophate

Peak Table

Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)
1	2925.62	70.28	2	2858.99	77.7	3	1726.5	58.48	4	1686.57	62.07
5	1636.05	81.82	6	1600.07	79.15	7	1463.2	69.06	8	1447.17	71.52
9	1392.64	76.69	10	1373.75	76.68	11	1323.62	81.63	12	1278.4	64.1
13	1255.4	62.39	14	1183.98	72.07	15	1134.53	72.05	16	1073.47	72.68
17	1036.55	76.46	18	1011.18	76.77	19	962.51	74.02	20	953.77	74.09
21	927.15	74.93	22	850.84	83.97	23	801.85	79.71	24	758.55	70.73
25	704.78	78.53	26	667.39	79.84	27	638.4	79.23	28	615.41	76.88

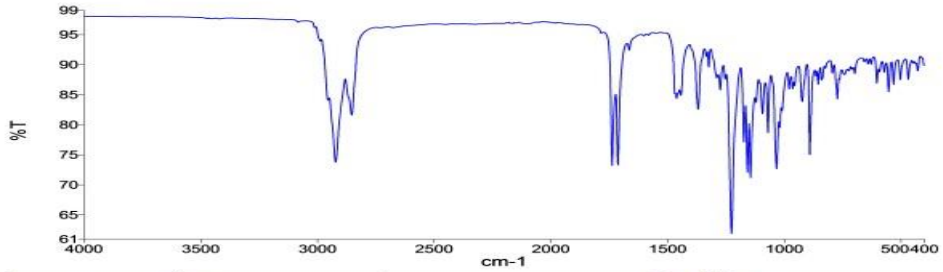
Appendix C; Analytical data for ethyl ester of xylopic acid.

^{1}H NMR spectrum



IR spectrum

Spectrum Graph

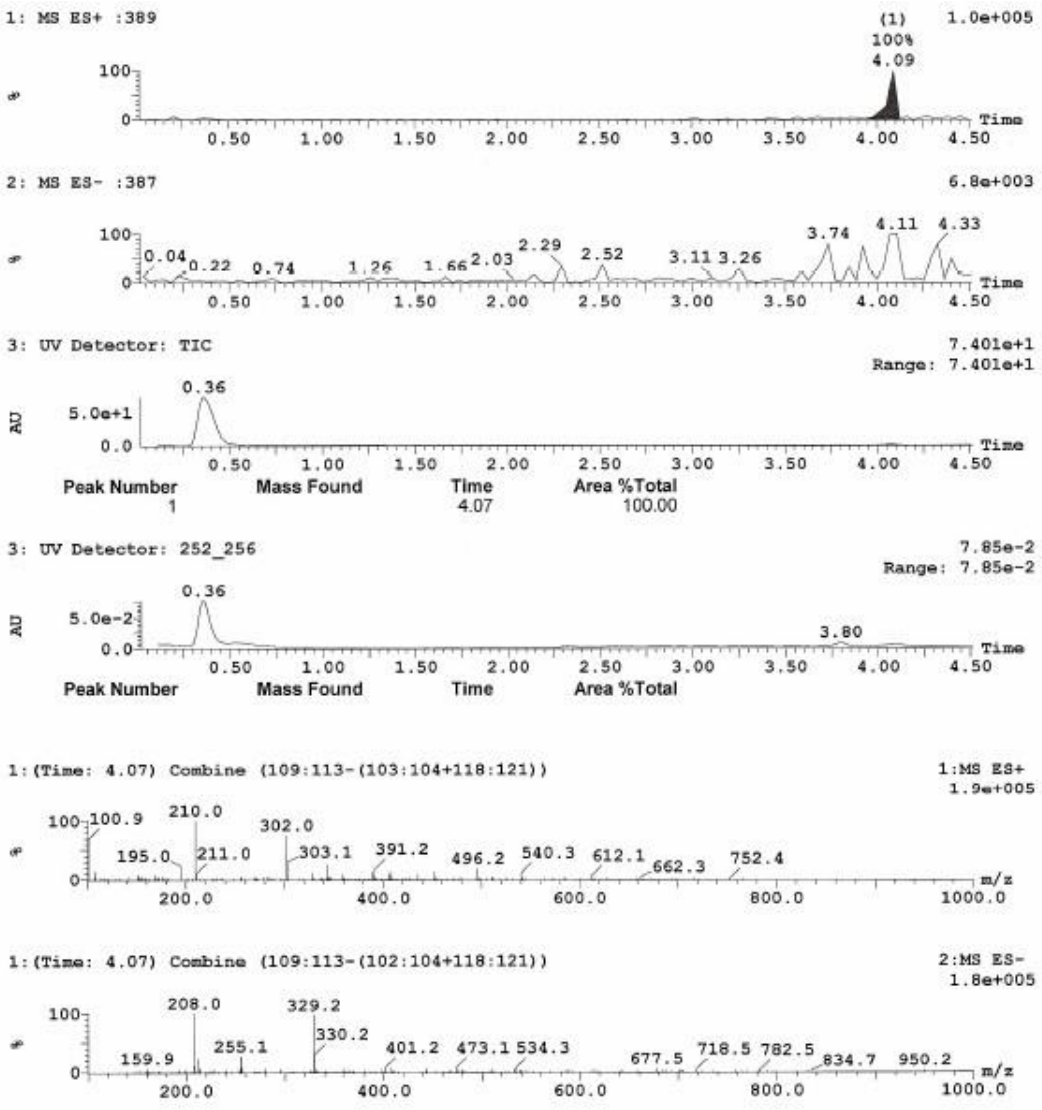


Name	Description
Judith Foli	Xylopic acid

Peak Table

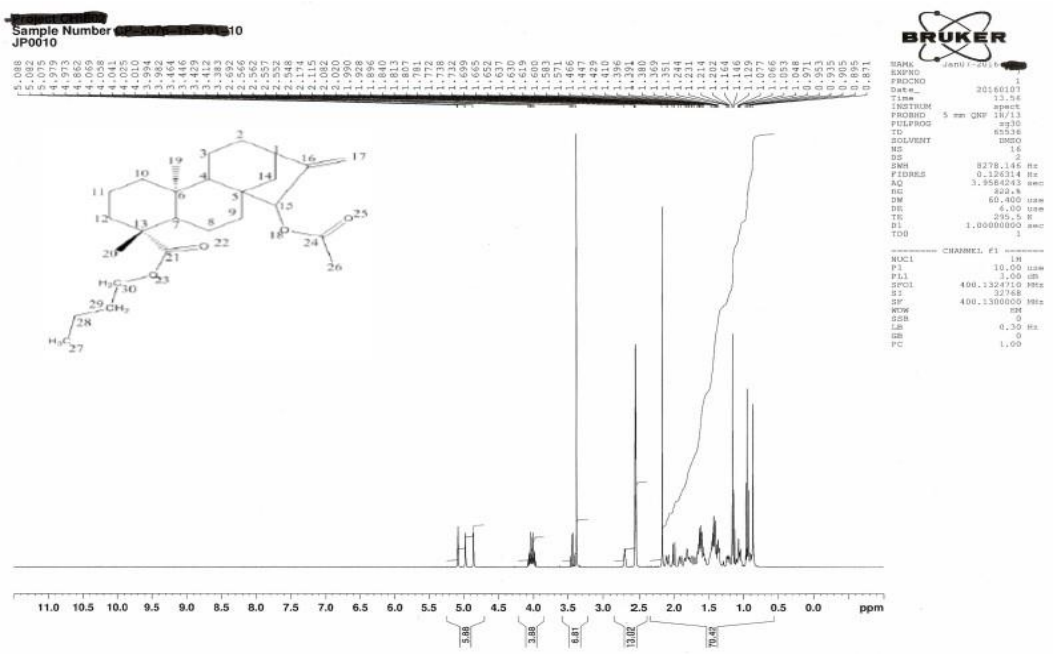
Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)
1	2923.82	73.77	2	2854.55	81.65	3	1737.48	73.22	4	1712.78	73.3
5	1456.32	85.26	6	1368.83	82.63	7	1323.76	89.67	8	1273.49	85.84
9	1225.37	61.88	10	1172.87	77.15	11	1157.52	72.09	12	1143.99	71.17
13	1092.33	81.89	14	1069	78.68	15	1032.53	72.72	16	978.24	85.96
17	962.58	86.01	18	922.09	83.94	19	889.78	75.05	20	854.11	86.81
21	771.23	84.36	22	647.01	91.04	23	601.69	86.98	24	551.54	85.54
25	530.37	86.77	26	501.07	87.56	27	466.76	87.63			

MASS SPECTRUM

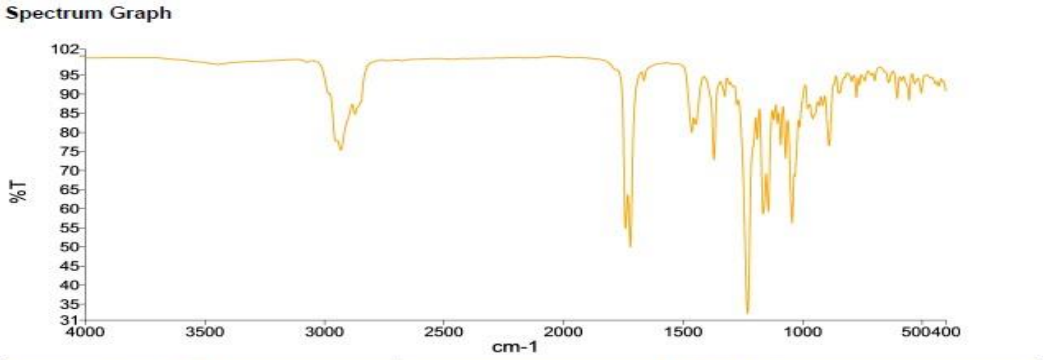


Appendix D; Analytical data for butyl ester of xylopic acid

^{1}H NMR SPECTRUM



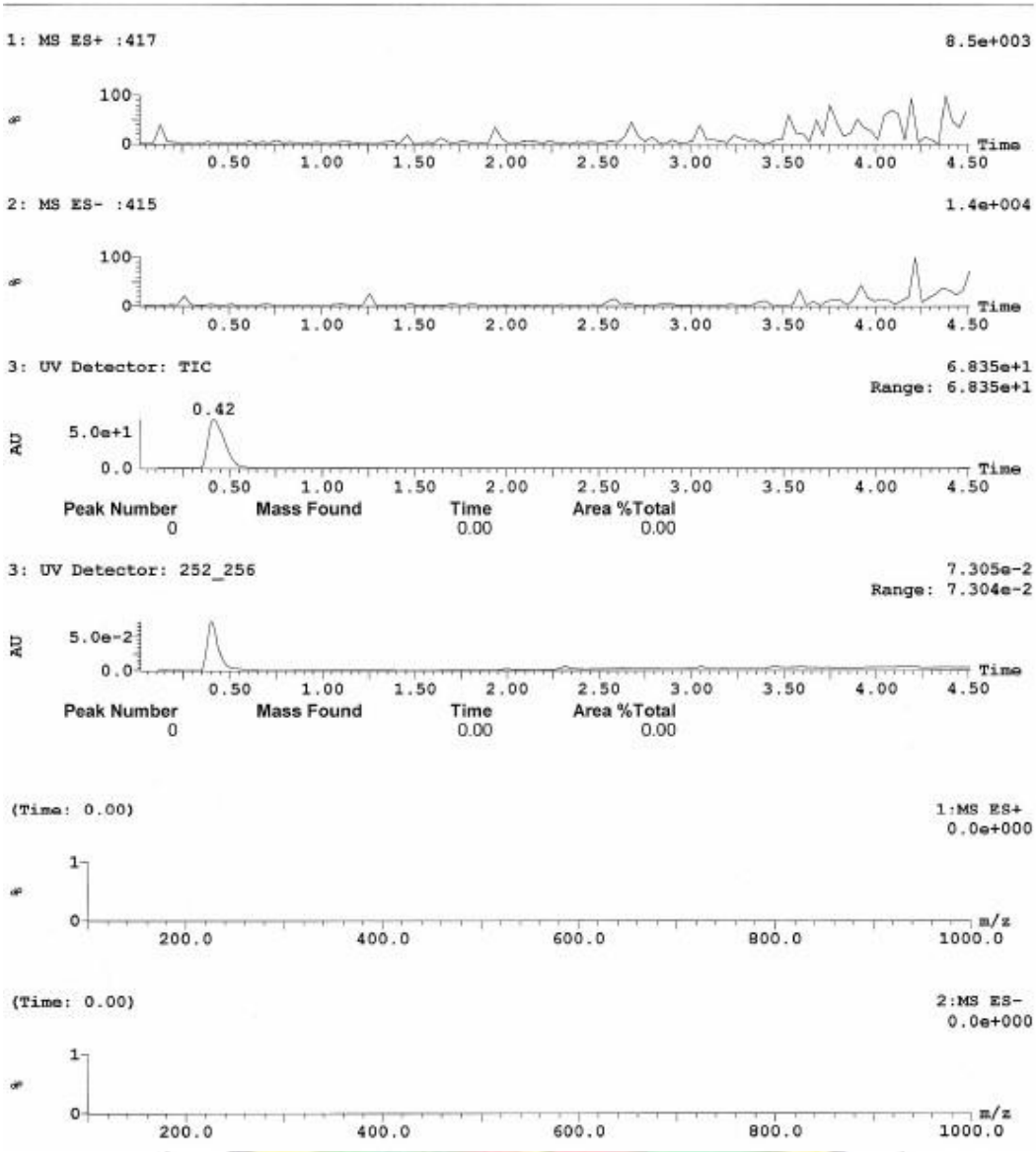
IR spectrum



Peak Table

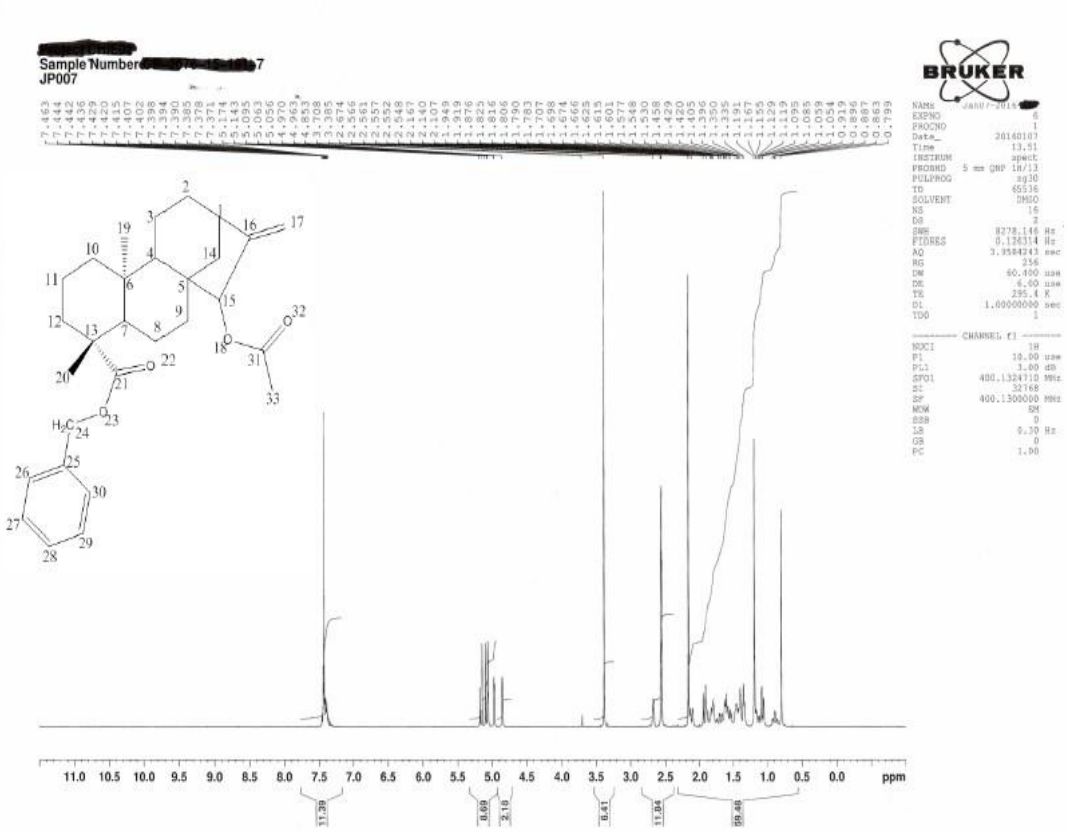
Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)
1	2934.01	75.41	2	2873.24	84.86	3	1739.81	54.89	4	1720.15	49.98
5	1663.27	93.67	6	1462.52	80.03	7	1445.79	82.23	8	1370.35	73
9	1325.43	89.43	10	1229.54	32.51	11	1188.63	78.39	12	1163.72	58.63
13	1142.29	59.3	14	1119.17	83.48	15	1103.82	82.41	16	1090.42	76.74
17	1070.48	73.32	18	1043.18	56.3	19	955.59	83.71	20	914.41	87.17
21	888.23	76.58	22	845.87	90.63	23	773.22	89.27	24	760.76	92.47
25	697.2	93.68	26	637.95	93.2	27	602.96	88.98	28	553.16	88.46

Mass spectrum

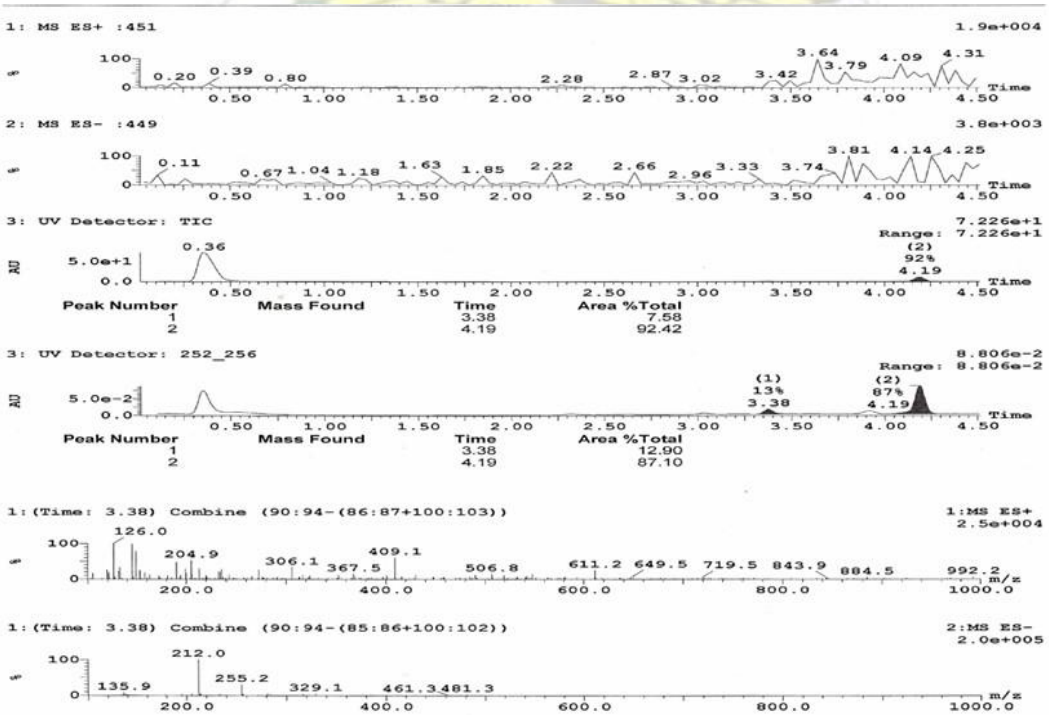


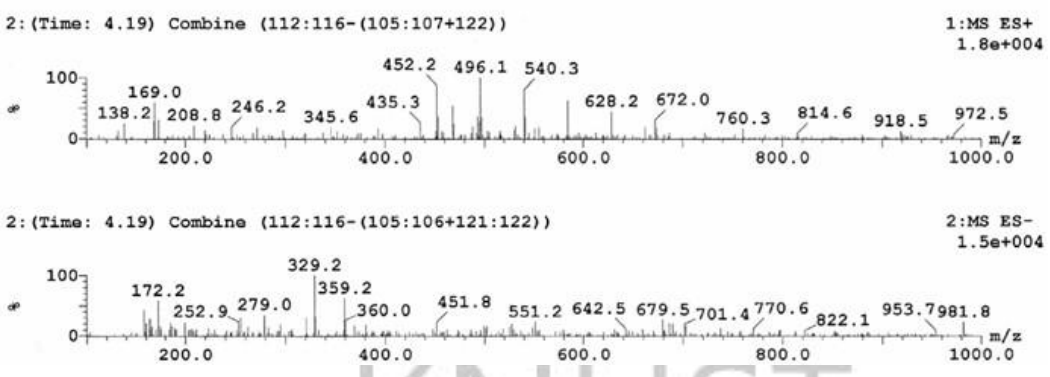
Appendix E; Analytical data for benzyl ester of xylopic acid

^{1}H NMR spectrum



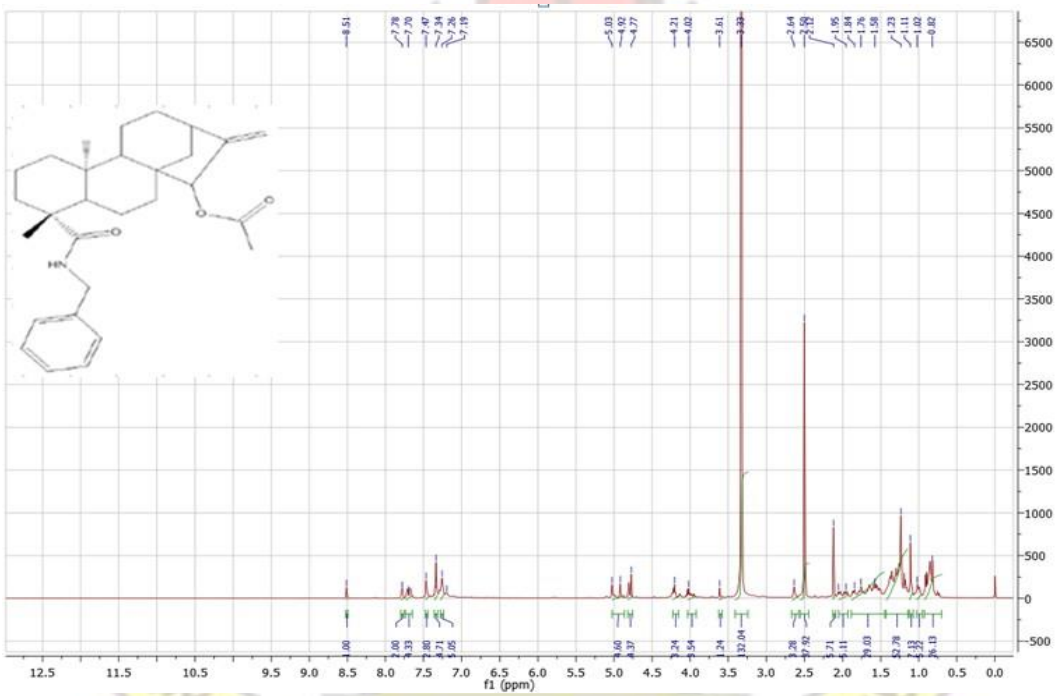
Mass spectrum





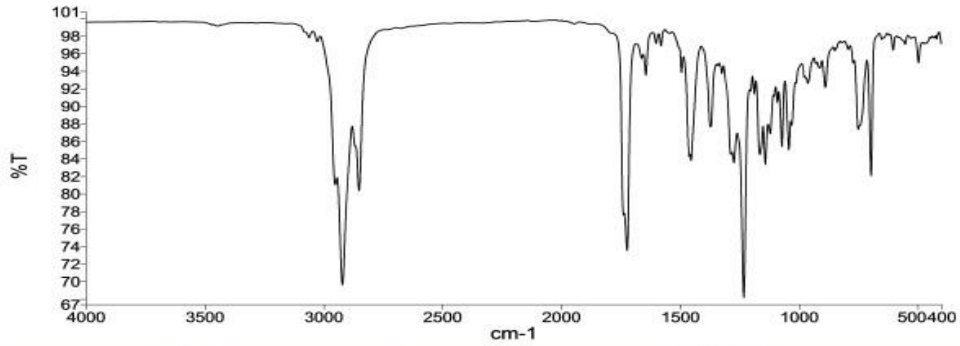
Appendix F; Analytical data for benzyl amide of xylopic acid

^{1}H NMR spectrum



IR spectrum

Spectrum Graph



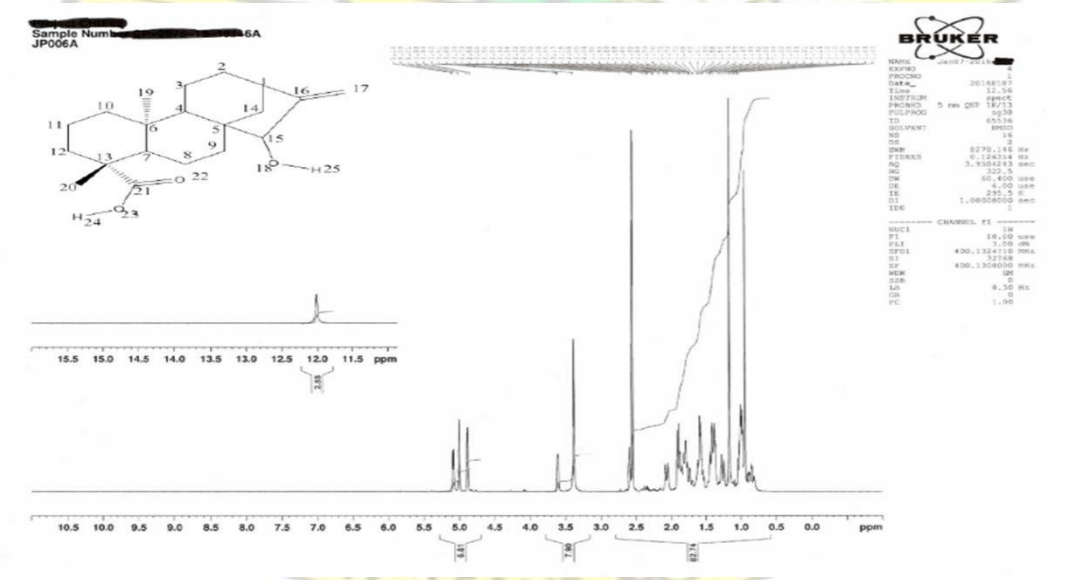
Name	Description
John Fetse	Benzyl amide

Peak Table

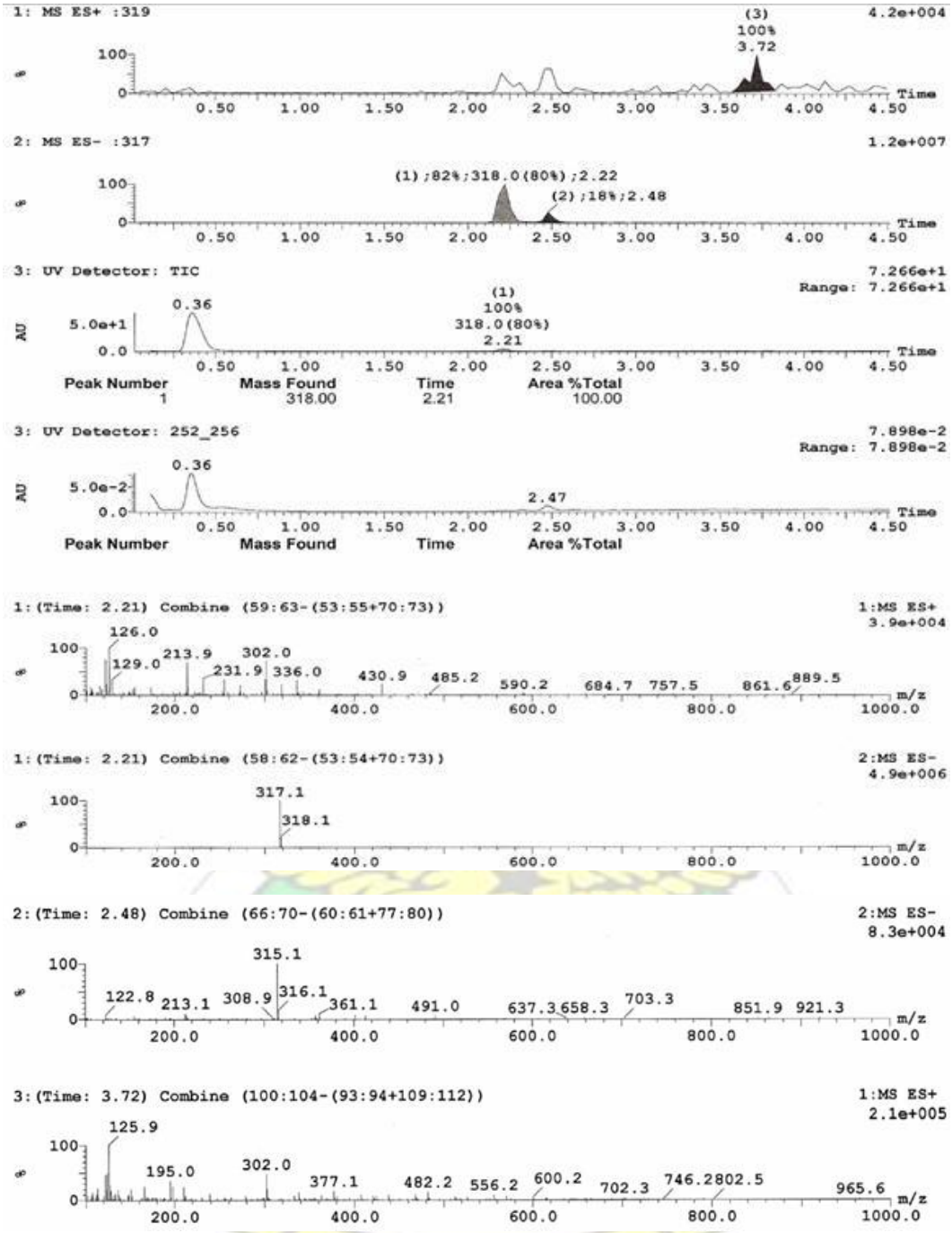
Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)	Peak	X (cm-1)	Y (%T)
1	2923.72	69.6	2	2853.36	80.39	3	1725.62	73.8	4	1645.55	93.62
5	1457.74	84.52	6	1372.72	87.7	7	1274.15	83.6	8	1232.64	68.18
9	1165.16	84.53	10	1141.87	83.38	11	1071.8	85.47	12	1042.84	85.07
13	961.93	92.69	14	889.02	92.21	15	746.62	87.8	16	696.75	82.1
17	496.11	95.02									

Appendix G; Analytical data for deacetyl xylopic acid (β-OH epimer)

^{1}H NMR spectrum



Mass spectrum



Appendix H; Analytical data for deacetyl xylopic acid (α-OH epimer) Mass spectrum