PHYTOCHEMICAL AND SOME BIOLOGICAL SCREENING OF THE LEAVES OF *CELTIS INTEGRIFOLIA* LAM (ULMACEAE)

BY

USMAN HAMIDU

JANUARY, 2006

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BY

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M.Sc./PHARM SCI/30960/2001-2002

A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (PHARMACEUTICAL CHEMISTRY)

JANUARY, 2006

DECLARATION

I hereby declare that this research work was carried out by me under the joint supervision of Dr. A.K. Haruna and Prof. M. Ilyas. It has not been accepted in any previous publication for a higher degree elsewhere. The works of other researchers and investigators are acknowledged and referred to accordingly.

……
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CERTIFICATION

The thesis entitled "Phytochemical and Some Biological Screening of the Leaves of *Celtis integrifolia* Lam (Ulmaceae)" by USMAN HAMIDU meet the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to my beloved wife – Rabi'atu and our children: Rasheeda (Iya), Fatimah (Mâmo) and Aliyu (Khálifa).

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ABSTRACTS

The plant *Celtis integrifolia* Lam (Ulmaceae) is a tree widely used in West Tropical and Sub-Saharan Africa as a remedy for rheumatic pains, boils, burns and measles. Phytochemical screening of the leaves of this plant revealed the presence of alkaloids, cardenolides, flavonoids, tannins, saponins, steroids and carbohydrates. Palmitic acid and stearic acid were isolated from the petroleum ether extract, identified by spectroscopic technique and comparison with the authentic reference compounds. A flavonols glycoside was isolated from the n-butanol extract and characterised as quercetin 3,7-O-diglucoside by spectroscopic techniques. The n-butanol portion was further subjected pharmacological screening in rodents; investigations were carried out on acetic acid-induced writhing in mice and hind paw oedema in rats. Results showed the extract to possess significant ($P<0.05$) anti-nociceptive activity between 25 and 100 mg/kg body weight intraperitoneal (*i.p.)* in mice and a dose dependent anti-inflammatory activity at 50 and 100 mg/kg body weight *i.p.* in rats comparable to that of piroxicam 20 mg/kg body weight *i.p.* The LD_{50} of the crude ethanolic was found to be 226.3 mg/kg body weight *i.p.* in mice. The *in-vitro* antimicrobial activity of the leaf extracts (ethanolic, n-butanol, aqueous) of *Celtis integrifolia* were assayed using the agar plate diffusion and broth dilution methods using *Bacillus subtilis* (NCTC 8326 B76), *Escherichia coli* (ATCC 11775), *Pseudomonas aeruginosa* (ATCC 10145), *Staphylococcus aureus* (ATCC 021001). *Candida albicans* and *Aspergillus niger* (laboratory isolates) as test organisms. The n-butanol extract inhibited all the test bacteria. The ethanolic extract had activity against the test bacteria except *Pseudomonas aeruginosa.* The aqueous portion inhibited only *Staph. aureus* and *Bacillus subtilis.* All the extracts had no activities on the fungi tested. The ethanolic and n-butanol extracts showed minimum inhibition concentration (MIC) of 6.25 mg/ml against *Staph.*

aureus and *E. coli*. The aqueous extract showed an MIC of 12.5 mg/ml against *Staph. aureus*. The minimum bactericidal concentration (MBC) of ethanolic extract is 6.25 mg/ml against *Staph aureus*. For n-butanol extract, the MBC was 12.5 mg/ml against *E. coli*. The aqueous extract had an MBC of 50 mg/ml against *Staph. aureus* and *B. subtilis*. The n-butanol extract showed the broader antibacterial activity than the ethanolic extract. This study has justified the traditional use of the plant for treating wounds and burns.

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LIST OF ABBREVIATIONS

$AlCl_3$	Aluminium chloride
AQE	Residual Aqueous Extract
NBE	n-Butanol soluble extract
CEE	Crude Ethanolic extract
CPE	Petroleum Ether extract
$CHCl_3$	Chloroform
EtOH	Ethanol
MeOH	Methanol
EtOAc	Ethylacetate
n-Hex	n-Hexane
$FeCl_3$	Ferric chloride
H_2SO_4	Sulphuric acid
CU_2O	Cuprous oxide
MS	Mass Spectrometer
^{1}H-NMR	Proton Magnetic Resonance
TLC	Thin Layer Chromatography
IR	Infra-Red Spectroscopy
UV	Ultra-Violet spectroscopy
NaOAc	Sodium acetate
NaOMe	Sodium methoxide
HCl	Hydrochloric acid
CD_3OD	Deuterated Methanol
CD_3Cl_3	Deuterated chloroform

NaOH	Sodium hydroxide
KOH	Potassium hydroxide
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
Kg	Kilogram
MP	Melting point
Rf	Retention factor
i.p	Intra-peritoneal
mm	Millimetre
mg	Milligram
μg	Microgram
ppm	Parts per million
Hz	Hertz
e.g.	Example
e.t.c.	Et cetera
δ	Delta
λ	Lambda

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The use of plants in healing is as ancient as medicine itself. Until the dawn of this century, natural products have served as the mainstay of all medicine worlds over. Although, the use of herbs as remedy for treatment of disease ailments has declined in the West, it continues to exist throughout the developing world (Rahaman and Choudhary, 1999). Over 80% of the World's population use herbs as their primary source of medication (Farnsworth *et al.,* 1991; Cordell, 2000). It is then evident that "*there is a plant for every disease condition on every continent"* (Cordell, 2000).

The usefulness of certain herbs and other flora as therapeutic agents has been known for thousand of years through the enormous contribution of the primitive man world wide by virtually eating plants in his locality to ascertain their use (Thomson, 1978). Some relieved symptoms of discomfort or sickness, many were not harmful or dangerous, a few nourished him, a very few elevated his mood through hallucinations while quite a number made him ill or even killed him. Therefore, by this gradual method of differentiation, he was able to sort out plants he could eat, those he could not, and those with healing properties; and hence, the knowledge concerning the medicinal properties and usage of such herbs had been passed over from one generation to the next. For this reason, large species of plants are used as medicinal plants today. Hence, plant kingdom have been described as the sleeping giant of drug development (Verpoote *et al.,* 1987) and the whole forest as the "God's own Pharmacy" (Trenben, 1986).

Medicinal plants has been defined in many facets but according to (Sofowora, 1993a) medicinal plants is defined as any plant which in one or more of its organs (parts) contain substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. Medicinal plants consist of many kinds of components and their biological activities are not usually attributable to a single moiety (Cho *et al.,* 2003).

The medicinal property of plant is most usually due to the secondary metabolites; such constituents may include all or some of these substances: phenolic compounds (tannins, flavonoids), terpenes, glycosides (cardiac, flavonoids, steroidal, saponins), anthraquinones, volatile oils, gums and mucilages, coumarins, alkaloids. Phytochemical screenings of these constituents have been made possible by improved methods of extraction, separation, isolation, characterization and structure elucidation (Harborne, 1973a).

Historically, natural products provide the oldest source for new medicines. Natural selection during evolution and competition between species has produced powerful biologically active natural product which can serve as chemical leads to be refined by Chemist to give more specifically biologically active drugs (Ganellin, 1993). One of the major means of identifying the pharmacologically active natural products has been the folkloric remedies which are mainly plant products: alkaloids such as morphine (from *Opium poppy* known in Egypt), atropine and hyoscine (from plants of *Solanaceae* family known to the ancient Greeks) and reserpine (from *Rauwolfia serpentine,* the snakeroot popular in India as a herbal remedy), others are the non-nitrogenous natural products such as Salicylates e.g. Salicin (from the Willow tree-genus *Salix*, botanical sources to Hippocrates; and the glycosides e.g. Digitoxin and Digoxin known for centuries from the foxglove {in folk use in England for centuries} (Ganellin, 1993).

Obviously, a large number of medicinal plants have been known as potential drugs in the treatment of human and animal diseases, many of which the active principle had been isolated and characterised; these include: Quinine (antimalarial, from *Chincona* bark, Digoxin and Digitoxin (cardiotonic activity), morphine (analgesic, from *Papaver somniferum*) (Sofowora, 1993b). Therefore, as a result of some of these potential resources and the widespread use of medicinal plants in the rural areas (Gadhi *et al.,* 2001), a survey carried out by WHO revealed that more than 33% of the modern drugs are medicinal products derived from plants (Ghani, 1986), bearing in mind that humans have derived many benefits from natural plants and compounds (Cho *et al.,* 2003); it is therefore, pertinent to screen phytochemically our medicinal plants used locally by the traditional healer with a view of isolating the active components which will serve as a starting material for the development of clinically useful products.

The plant *Celtis integrifolia* Lam, used locally here in Northern Nigeria to treat a variety of ailments, and that no attempt have been made to isolate and characterise the active components.

1.2 AIMS AND OBJECTIVES OF THE RESEARCH

The aim of this work are to phytochemically screen and isolate the biologically active compounds of the leaves of *Celtis integrifolia* Lam (Ulmaceae), which is used as a medicinal plant and a *pot-herb* in some parts of Northern Nigeria.

The objectives are among other things search for plant's active constituents with view to understanding the rationale for the purported usage in traditional medicine. This includes the isolation, characterization of the constituents and studying their biological responses with a view of obtaining compounds with better potential and low toxicity.

1.3 LITERATURE REVIEW

1.3.1 *Celtis Integrifolia*

KINGDOM: *Plantae*

DIVISION: *Spermatophyta*

SUB-DIVISION: *Angiospermae*

CLASS: *Dicotyledoneae*

SUB-CLASS: *Archiclamydeae*

ORDER: *Urticales*

FAMILY: *Ulmaceae*

GENERA: *Celtis*

SPECIE: *Celtis integrifolia* Lam

COMMON NAMES:

Guouwa; Kamagnan; Kamina Gamiah; Nkaminga.

LOCAL NAMES:

Hausa: *"Dunki, Zuwo"* Kanuri: "*Nguzo"*; Nupe: "*Gimachi"*; Fulani: "*Ganki"*-tree and *"Wanko"*–leaves; Shuwa Arab: *"Abun gatu"*; Yoruba: *"Akpe"* (Keay, 1989). The plant is known in Arabic as *"Mohagria"* (El-Tahir and Gebauer, 2004). The plant is also known as African nettle tree and sometimes African false elm (Dalziel, 1955).

The plant *Celtis integrifolia* Lam is widely distributed mainly in the drier savannah regions, often along rivers and streams – locally abundant (Keay, 1989). Also distributed, but westerly limited in Senegal, easterly in Uganda, northwards to the Sahel zones of Niger Republic. Found also widespread but localized in the Guinea, Sudan and Sahel

zones of Northern Nigeria and extends southwards to the Savannah zone of Southern Nigeria; also present in the Arabian Peninsula (Jackson, 1973).

1.3.2 Morphology

Celtis integrifolia is a tree of up to 24 m high and 5 m girth or more; bole distinct when old, crown small and sharp, not extending far up to the bole. Bark grayish fairly smooth, flaking off in large, thin rather rounded hard plates on old trees; slash alternate bands of cream dark brown. Twigs and young foliage had short whitish-gray hairs (Irvine, 1961; Keay, 1989). The leaves are about 3.5 cm – 9.0 cm long by 2.5 cm – 5.0 cm broad, unsymmetrically ovate, shortly acuminate, very unsymmetrical at the base, upper edge usually being much more rounded than the lower but meeting the stalk at the same level; markedly rough on both surface with short stiff hairs, entire (except on sucker shoots) the basal pair of main nerves running to the upper tip, each having 3 – 5 prominent lateral running out parallel with the margin, midrib with 1 – 2 upper lateral nerves on each side all the nerves are prominent beneath and often have minute tufts of hairs at their base.

Plate I: *Celtis integrifolia* Lam Tree

Plate II. Leaves of *Celtis integrifolia* Lam

The plant flowers (December – April), greenish in slightly branched axillary inflorescences 1.0 – 5.0 cm long, crowded with very small males below and each branch terminating in a much larger female; ovary hairy, ending in a conspicuous twice-branched style about 0.64 cm long. The plant fruits (May – September), appearing ovoid brown up to 1 cm long; usually only one developing in each inflorescence (Keay, 1989).

1.3.3 Uses of The Plant

The leaves of *Celtis integrifolia* with those of *Anthonatha macrophyla* form an ingredient in Congo Basin as a medicine for difficult delivery (Staner and Boutique, 1937). The leaf decoction is used on the Ivory Coast as a beverage for measles (Kerharo and Bouquet, 1950). The leaves are also used in the French Equatorial Africa as a vermifuge and in headache lotions (Walker, 1953). Dalziel, (1955) reported that the root, bark and leaves are used medicinally for rheumatic pains and for other undetermined conditions. The decoction of the leaf from this plant is used in Guinea Conakry to provoke abortion in pregnant women – though the effects described are from multi-component treatment (Vasileva, 1969). Various parts of the tree serve to treat sterility, boils, oedema and asthenia – weakness of the body (Giffard, 1974). The roots are applied to cure mental disorder (Geerling, 1982). In Northern Nigeria, the paste made from the leaves of this plant with those of *Pistia stratiotes* is been used locally to sooth cases of burns and to relieve stomach discomfort (Personal communication).

In various parts of Tropical Africa, the leaves are regarded as a good *pot-herb* and a sustaining food, used in soup; baobab leaves being added in Northern Tropics and Northern Nigeria; sometimes they are even eaten raw. A sort of salad composed of the fresh leaves with groundnuts and condiments is served in Hausa land as *"dinkim"* or

"dinkin". The young shoots are also cut as fodder for domestic animals especially in the dry season. The fruits are also edible (Dalziel, 1955).

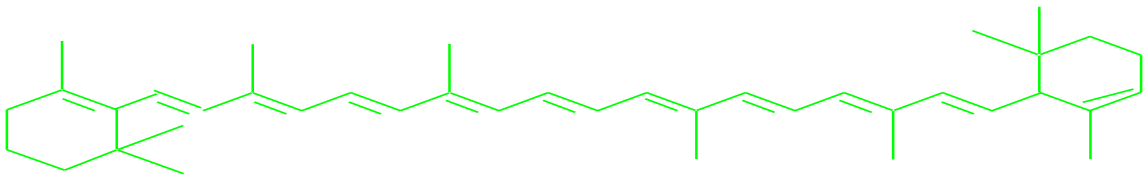
The bast fibres are plain into bands mats and for other purposes. The wood is pale-yellow, soft-grained, easily worked and finishes well; though perishable, the yellowish-white timber is of good quality (Irvine, 1961).

1.3.4 Biological Activities of The Plant

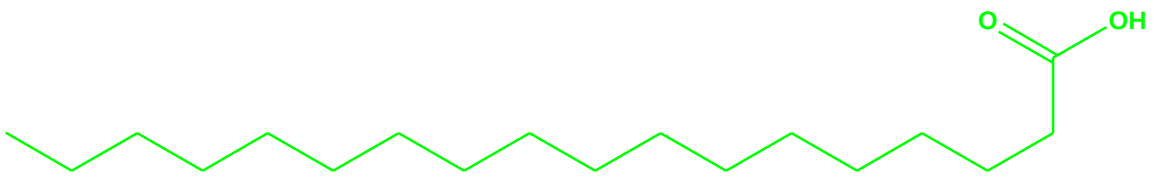
It appears that there has been no documented or reported literature on the biological activities of any extracts from this plant specie.

1.3.5 Phytochemistry and Presence of Compounds

From the literature survey carried out so far, there has not been any report on the presence of the chemical constituents and/or active components of the leaves of *Celtis integrifolia* Lam. However, the only reported work was on the seed, where β-Carotene [$C_{40}H_{56}$] (I) and Stearic acid [$C_{18}H_{36}O_2$] (II) were identified in the seed oil of the plant (Grindley, 1948).



(I)



(II)

However, preliminary phytochemical screening conducted showed the presence of flavonoids, alkaloids, tannins, saponins and fatty acids; it is therefore, of importance to discuss concisely the chemistry of some of these phytochemicals.

1.3.6 Chemistry of Flavonoids

Flavonoid is a term that is used to embrace all compounds whose structure is based on flavone and have been in existence for years (Carlo *et al.,* 1999). They are chemically known as benzo-γ-pyrone (III) derivatives (Finar, 1999). They occur throughout the entire plant kingdom and compose of the largest group of naturally occurring polyphenols present in fruits, vegetables, grains, bark, root, stem, flowers appearing either in free state, as glycosides, methylated derivatives or both; with their glycosides mostly as O-glycosides; though, few occur as C-glycosides (Harborne and Williams, 2000; Viana *et al.,* 2003).

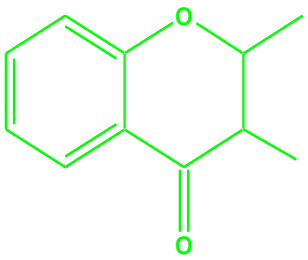
Over 4000 different plant derived flavonoids (bioflavonoids) have been described (Harborne, 1993). More than 2000 of these compounds are known with about 500 being in free state. The flavones and their close relations are often yellow in colour and the extent of colour intensity increases with the number of hydroxyl group. The flavonoids are pigments responsible for the autumnal burst and hues and the many shades of yellow, orange and red flowers (Timberlake and Henry, 1986). They have also been found to play an important role in the growth, development and defense of plants (Cody *et al.,* 1986).

The flavonoids glycosides are generally soluble in water and alcohol, but insoluble in organic solvents. The genins are only soluble in water but insoluble in ether (Trease and Evans, 1997).

1.3.6.1 *Structure and Classification of Flavonoids*

The flavonoid aglycone (IV) consist of a benzene ring (A) condensed with a six-membered ring (C) which in the 2-position carries a phenyl ring (B) as a substituent as shown in structure IV below. The position of the benzenoid substituent divides the flavonoid class into: Flavonoids (2-position) and Isoflavonoids [3-position] (Harsteen, 1983; Rajnarayana *et al.*, 2001).

Flavonoids are often hydroxylated in position 3, 5 and 7; and frequently at positions 3′, 4′ and 5′. Positions 5, 7, and 4′ are generally methylated. When flavonoids occur as glycosides, the glycosidic linkages are normally located in position 3 or 7 and the sugars can be L-Rhamnose, D- Glucose, Gluco-Rhamnose, Galactose or Arabinose (Middleton, 1984; Finar, 1999).



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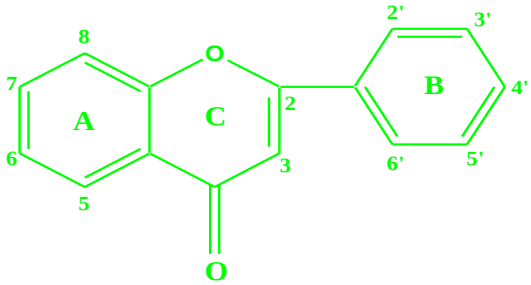


(IV)

Generally, flavonoids containing a double bond at C2-C3 position of ring C and phenyl ring at the C2 are termed flavone as in Apigenin (V); while those without double bond at the C2-C3 position (dihydro derivatives are termed flavonones (Naringenin – [VI]). Flavonoids may also exist as glycosides; typical examples are the baicalin (VII) a flavone glycoside and rutin (VIII)-flavonols glycoside.

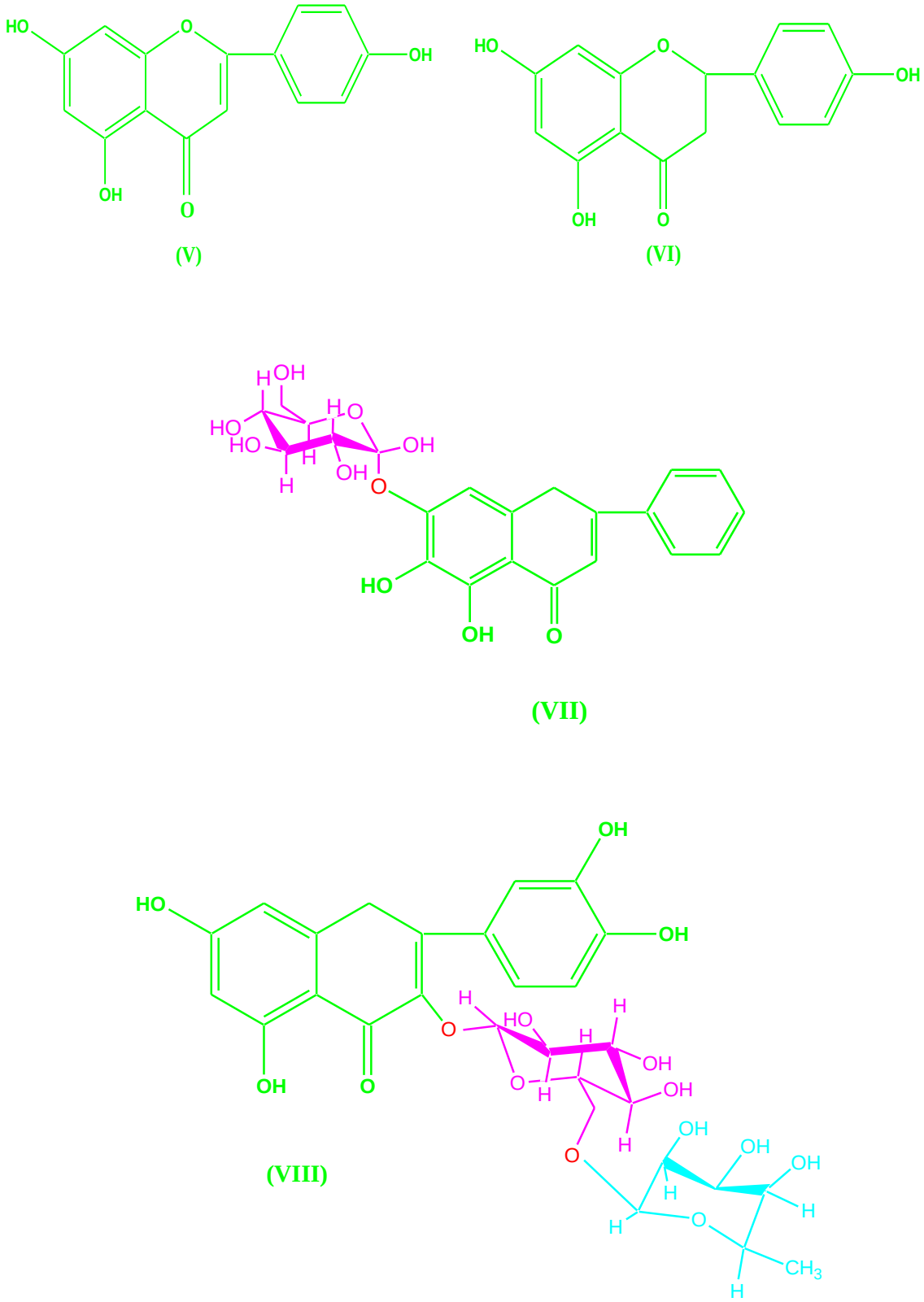
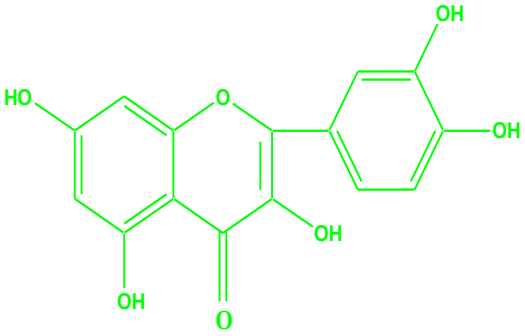


Fig. 1.1 Structures of some flavonoids

Other important flavonoids derivatives are those with C3 – position in the flavone hydroxylated known as flavonols e.g. is the quercetin (IX), isoflavonols-genistein (X), flavanolols e.g. taxifolin (XI) and flavan-3-ols typical example of which is the catechin (XII).

Pigments of relevance to the flavonoids are the derivatives in which the C-ring nucleus is open at the 1-position; these are the anthocyanides e.g. Chalcones (XIII) and the Chalcones example is the 2-hydroxy chalcone (XIV) and aurone (XV).



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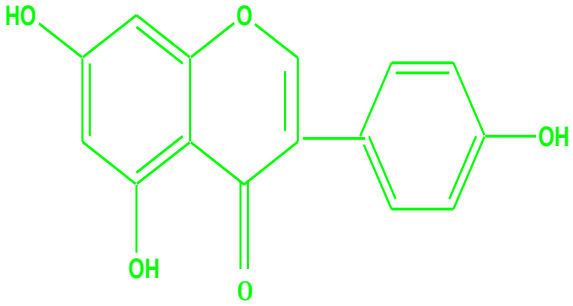

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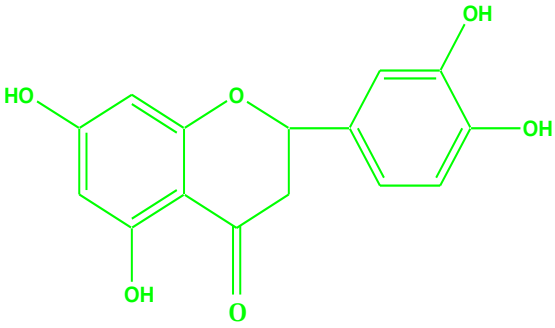

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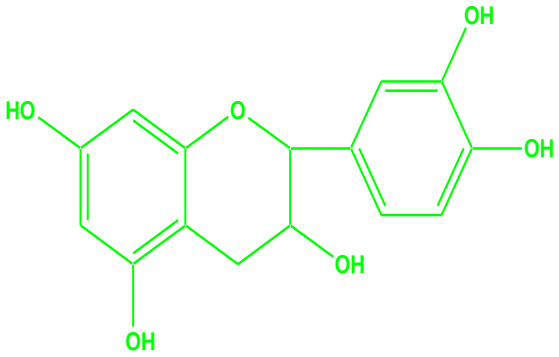

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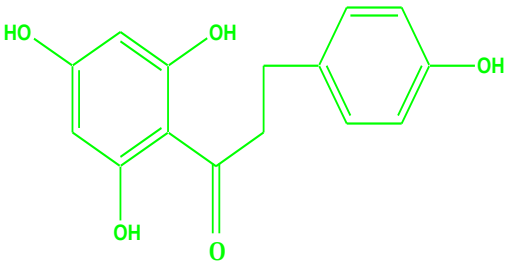

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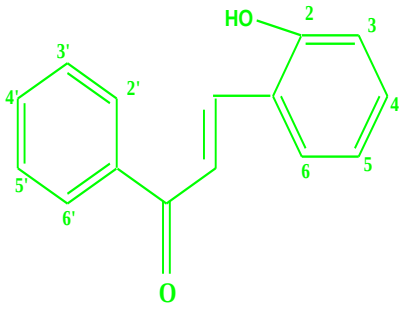

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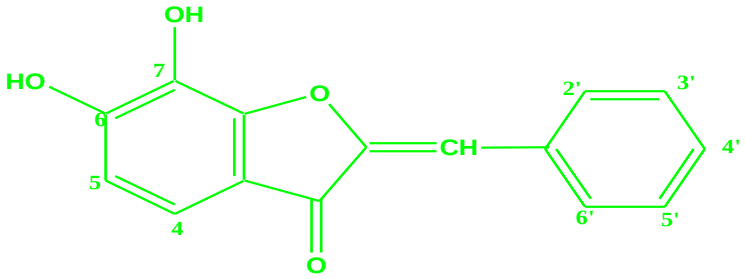
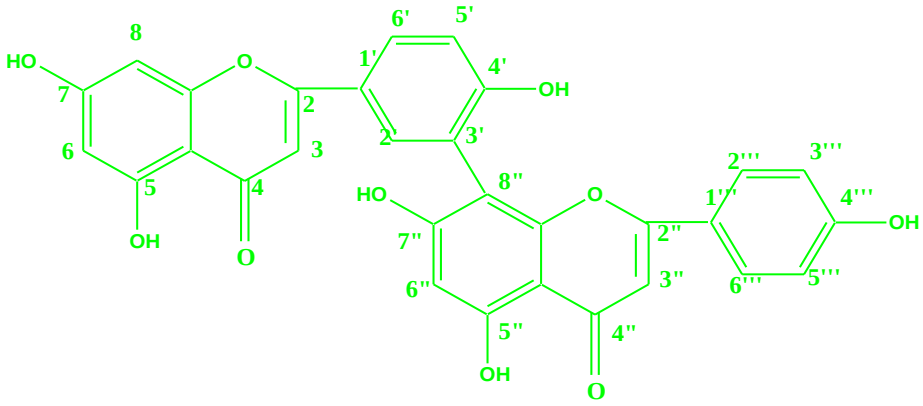


(XV)

Another class of flavonoids of therapeutic importance are the bisflavonoids; in which one flavonoid nucleus fuses with another or more nucleus. Typical example of this group is the biflavonoid known as amentoflavone (XVI)



(XVI)

Fig 1.2 Structures of some flavonoids and related compounds

1.3.6.2 *Characterization of Flavonoids Structure*

The flavonoid glycosides, after isolation is subjected to acid hydrolysis; so as to knock out the flavone aglycone from the sugars by breaking the glycosidic linkages. The usual analytical methods are then applied to determine the number of hydroxyl or methoxyl groups present in the molecule (Finar, 1999).

(i) In order to ascertain the positions of the sugar residues after hydrolysis, the sugar is identified by the usual methods of sugar chemistry; and this includes the use of paper chromatography. If two or more monosaccharide is present, they are first of all examined whether they appear as such or as disaccharides by methylating the flavonoids with suitable enzyme and hence methylated disaccharide may be isolated intact.

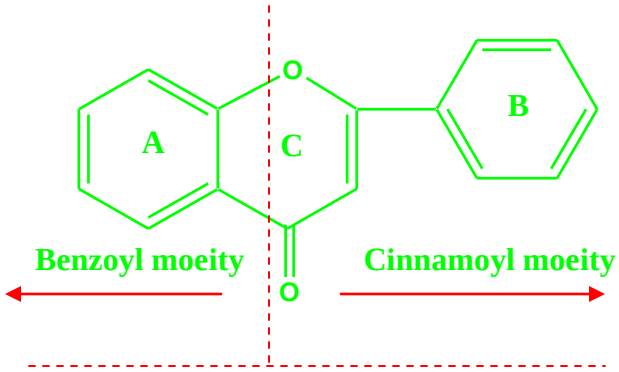
(ii) After acid hydrolysis, the position of free hydroxyl group determines the point of attachment of sugar residues.

(iii) Flavonoids with free hydroxyl group in the 3-position are very readily oxidized by ferric chloride.

Thus, by the above techniques, the flavonoid is fairly assigned to its class by its absorption spectrum.

1.3.6.3 *Ultra-Violet Spectroscopy of Flavonoids*

The methanol spectra of flavones and flavonols exhibit 2 major absorption peaks in the region 240 – 400nm. These 2 peaks are commonly referred to as Band I (usually 300 – 380nm) and Band II {240 – 280nm} (Mabry *et al.,* 1970). Band I is attributed to be due to the B-ring (cinnamoyl moiety) and Band II is related to the absorption due to A-ring (benzoyl system) as shown in structure (XVII).



(XVII)

The UV spectra generally gives information about the level of substitution of flavonoids e.g. the flavones and flavonols oxygenated in the A-ring, but not in the B-ring tend to give spectra in methanol with a pronounced Band II and a weak Band I; in a similar molecules which also possess B-ring oxygenation; Band II is more pronounced and appears at a longer wavelengths. Therefore, the methanol spectrum, particularly, the position of Band I, provides information about the types of flavonoid as well as its oxidation pattern. Thus, Band I of flavones occurs in the range 304 – 350nm whereas Band I of flavonols appeared at a longer wavelength between 352 – 385nm. However, in flavonols with a substituted 3-hydroxyl group (methylated or glycosylated), Band I usually appears at 328 – 357nm which overlaps the region for Band I in flavones, and the general shape of the spectral curves approach those of flavones (Mabry *et al.*, 1970).

Effects of Shift Reagents on UV Spectra of Flavonoids

(i) Effect of Sodium Methoxide (NaOMe)

Sodium methoxide being a strong base ionizes almost all hydroxyl groups on the flavonoid nucleus. So it's difficult to correlate the spectral shift obtained in its addition with the flavonoids hydroxylation. However, the effects of sodium methoxide has been employed in UV spectra of flavones and flavonols for the detection of free 3- and/or 4′-

hydroxyl groups, which usually produces a bathochromic shifts in all absorption bands; with Band I having about 40 – 65nm without a decrease in intensity (diagnostic for the presence of free 4′-hydroxyl group) while those lacking free 4′-hydroxyl group also give 50 – 60nm bathochromic effect in band I with a decrease in intensity of the peak (Mabry *et al.*, 1970).

(ii) Effect of Sodium Acetate (NaOAc)

Sodium acetate (NaOAc) is another shift reagent, though weaker base compared to NaOMe, it ionizes only to the more acidic hydroxyl group in flavones and flavonols i.e. the 3, 7 and 4′-hydroxyl groups. Since ionization of 7-hydroxyl group mainly affects Band II, it's in fact, a very useful reagent for the detection of free 7-hydroxyl groups. Thus, the UV spectra of flavones and flavonols containing free 7-hydroxyl groups with fewer exception exhibit a diagnostic 5 – 10nm bathochromic shift of band II in the presence of this reagent; while those possessing 4′-hydroxyl group and no free 3- or 7 - hydroxyl group usually show a pronounced long wavelength (λ) side of Band I in the presence of NaOAc (not fused) (Mabry *et al.*, 1970).

(iii) Effect of Sodium Acetate (NaOAc)/Boric Acid

Boric acid in the presence of sodium acetate will chelate with ortho-dihydroxyl groups at all the location in flavonoids nucleus, except at the C-5 position. Thus, flavones and flavonols with ortho-dihydroxyl group at the B-ring give a consistent bathochromic shift of 12 – 30nm in band I in the presence of these reagents.

(iv) Effect of $AlCl_3$ and $AlCl_3$/HCl

Acid stable complexes are formed between aluminium chloride and flavones/flavonols which contain the C-3 and C-5 hydroxyl groups; likewise it forms acid labile complexes with flavonoids that exist as ortho-dihydroxy nucleus (Mabry *et al.*,

1970). The complexes formed between $AlCl_3$ and ortho-dihydroxyl group due to ring A and B decompose in the presence of the acid, but in contrast, the C-3 or C-5 hydroxyl group appeared to be stable when $AlCl_3$ in the presence of acid complexes with C-4 keto function.

The $AlCl_3$ and its acidified form can therefore be used to detect the presence of ortho-dihydroxyl group in the B-ring of flavones or flavonols. Usually, a hypsochromic shift of 30 – 40nm is observed in band I or band Ia if one or two peaks spectrum of $AlCl_3$ respectively results; meanwhile the presence of three adjacent hydroxyl group in ring B produces a hypsochromic shift of 20 nm in addition of the acid to the $AlCl_3$ (Mabry *et al.,* 1970).

1.3.6.4 *Infra-Red Spectroscopy of Flavonoids*

This is one of the important techniques for the flavonoids identification. All the peaks in the IR spectra are associated with the specific group present in the molecule that may serve as a lead in the comparison with reference spectrum; so that identification and determination of unknown structure is made possible.

Absorption at frequency 3100 cm^{-1} is due to hydroxyl group (hydrogen bonded) in the C – 3 and C – 5 positions; the free hydroxyl group is observed at 3300 cm^{-1}. Methyl group usually give a strong band at 2930 cm^{-1}. The absorption of carbonyl group of the heterocyclic occurs at 1685 cm^{-1} in flavononones where conjugation is only due to the A ring; the absorption is lowered to 1650 cm^{-1} in 5 – hydroxyl flavononones. In flavones (VII) where the conjugation is due both to A and B rings, absorption due to carbonyl group in 5,7 – dihydroxy derivatives is 1655 cm^{-1} and 1630 cm^{-1} for the 7 – hydroxyl flavones. In the case of flavonols (X), the absorption due to carbonyl is lowered by about 30 cm^{-1} compared with that of homologous flavones (Markham, 1982; Manguro *et al.,* 2005).

The aromatic double bond usually absorbs between 1500 and 1610 cm^{-1}, a peak at 1585 cm^{-1} appeared when the double bond is in conjugation with benzene ring; phenolic hydroxyl group shows a strong absorption band at 1360cm^{-1} with a secondary peak at 1200 cm^{-1}, while absorption at 1165 cm^{-1} is due to one of the meta dihydroxyl substitution {5, 7 – dihydroxyl} (Markham, 1982).

1.3.6.5 *Nuclear Magnetic Resonance Spectroscopy (NMR) of Flavonoids*

Nuclear magnetic resonance is divided into two: Proton NMR (^{1}HNMR) and carbon-13 NMR (^{13}CNMR). Proton signals obtained in the NMR spectra of tetramethyl-sylated flavonoids generally occur in the range 0 – 9 ppm (Mabry *et al.,* 1970).

The A-ring protons

The A-ring protons (C6 & C8) of flavones, flavonols and isoflavones which contain the common 5,7-dihyroxy substituted pattern give rise to two doublets (J=2.5Hz) in the range 6.0 – 6.5 ppm. The H-6 doublet occurs consistently at higher field (6.0 – 6.2 ppm) than the signal for the H-8 (6.3 – 6.5 ppm); but when a sugar is attached to the oxygen at the C-7, the signals for both H-8 & H-6 are shifted downfield (i.e. 6.5 – 6.9 ppm and 6.2 – 6.4 ppm respectively); while for the dihydroxyflavanones, dihydroxyflavonols and their 7-O-glycosides, the signals appeared at higher field in all cases compared to those described earlier (i.e. 5.75 – 5.95 ppm & 5.9 – 6.1 ppm for H-6 & H-8 respectively), their glycoside appeared at 5.9 – 6.1 ppm & 6.1 – 6.4 ppm as H-6 & H-8 signals respectively (Mabry *et al.*, 1970).

Of high interest in the NMR spectra region of flavonoid is the differentiation of signals due to C-6, C-8 & C-3 proton. Thus, as for the C-3 proton, a signal usually appear as a singlet near 6.3 ppm; hence indicating the difference between the 3 protons (Mabry *et al.*, 1970).

The B-Ring protons

The protons of ring B usually appear in the range 6.7 – 7.9 ppm, (downfield from the region where the A-ring proton absorbs. The signal observed for the B-ring protons is characteristics for the substitution pattern of that ring and in addition, suggests the oxidation level of ring C. Therefore, if B-ring is oxygenated at C-4′ a typical four-peak pattern of 2 doublet (each J=8.5Hz) is observed. The doublet for the C-3′ & C-5′ always appears up field from the C-2′ & C-6′ and generally falls in the range 6.65 – 7.10 ppm for all types of flavonoids. The position of the C-2′, C-6′ doublet depends on the oxidation level of C-ring; meanwhile, it appears consistently in the range 7.1 – 8.1 ppm which is a lower field than the C-3′, C-5′ doublet (Mabry *et al.,* 1970).

For the oxygenation of the B-ring of flavonoids at 3′, 4′- position, the protons of interests is C-2′, C-5′ & C-6′. Thus, C-5′ proton of the flavones and flavonols of this type of oxygenation appears as a doublet centered between 6.7 – 7.1 ppm (J=8.5Hz) while C-2′ & C-6′ proton which often overlaps, usually occur between 7.2 – 7.9 ppm (Mabry *et al.,* 1970).

The C-Ring protons

The C-ring proton is usually the C-3 and C-2 as in flavones and isoflavones respectively. The C-3 proton usually appeared as a sharp singlet at 6.3 ppm which often overlaps the signals produced by the A-ring proton. The C-2 proton in Isoflavonoids, which is in a β-position to the C-4 keto function, occurs in the range 7.6 – 7.8 ppm (in CCl_4) a region downfield from where most aromatic proton signals appear. The C-2 protons of flavonones appears as a quartet (2 doublet, J_{cis}=5Hz, J_{trans}=11Hz) near 5.2 ppm as a result of coupling of C-2 with the C-3 protons. The C-3 protons couple with each

other (J=17Hz) thus, giving rise to overlapping quartets near 2.8 ppm. In the dihydroflavonols, the C-2 proton signal occurs as a doublet (J=11Hz) near 5.2 ppm, while the C-3 proton doublet appears further up field at about 4.3 ppm (Mabry *et al.*, 1970).

The Sugar Protons

The chemical shift of the C-1″ proton of a sugar directly attached to the flavonoid hydroxyl group depends both on the nature of the flavonoid and on the position and stereochemistry of the attachment to it. With glucosides and some glycosides, a sugar on the C-3 hydroxyl group can be readily distinguished from that at C-4′, C-5 or C-7. In the 3 types other than the flavonols 3-O-glucosides, the C-1″ proton signal appears near 5.0 ppm while that of the C-3-O-glucoside appears at 5.8 ppm. As glucose commonly forms a β-linkage in flavonoid glycosides and the C-1″ proton of the β-linked sugar has a diaxial coupling with the C-2 proton. Therefore, the C-1″ proton usually appears as a doublet with a coupling constant of about 7 Hz (Mabry *et al.*, 1970).

In flavonoid 7-O-glucosides, however, the glucosyl C-1″ proton does not appear as a sharp doublet rather a complex multiplet, due mainly to the fact that the 7-O-glucosyl rotates with respect to the flavonoid nucleus.

Flavonoid rhamnosides occur naturally as α-L-rhamnosides with C-1″ has an equatorial - equatorial coupling with the C-2″ proton (J= 2Hz appx.). In both 3- and 7-O-rhamnosides the C-1″ proton occurs in the range 5.0 – 5.3 ppm. The signal of methyl group in the flavonoid rhamnosides occur in the range of 0.8 – 1.2 ppm, which is a distinguishing feature with other flavonoid glycosides.

The Methoxyl and Acetoxyl Protons

The protons due to the methoxyl group attached to the flavonoids nucleus appears between 3.7 – 4.1 ppm; those of the acetoxyl group are evident between the ranges of 1.65 – 2.50 ppm.

1.3.6.6 ***Mass Spectroscopy of Flavonoids***

The electron impact mass spectrometry (EI-MS) of flavonoids aglycone and glycosides serve as guide in determining their structures (Harborne *et al.*,1975); by this method molecular ion is produced by electron capture (M^-) or by the loss of electron (M^+) (Markham, 1989). Molecular ion (M^+) normally appears as a major peak in the mass spectra of aglycone, and exact mass measurement of this permits calculation of the elemental composition of the aglycone. The M^+ must be an even number and be related to the molecular weight of the flavonoid nucleus; thus for flavones (222) and flavonols (238) with 16 and 30 mass unit added usually for the hydroxyl and methoxyl group respectively. There is commonly loss of hydrogen (H-1) in flavonoids. 6 or 8 – methoxyl flavones /flavonols lose CH_3 (M^+-15) to generate an intense ion (M-15) which is usually found to be more than the M^+ itself. The 6 or 8 – methoxyl flavonoids are easily distinguishable from one another by their relative intensities of these ions (Markham, 1989). Lower intensities of the M-15 ions originate from flavonoids O-methylated at other site. The loss of water (M-17) is common in O- and C-flavonoid glycoside; while 28(29) mass unit(m.u) is loss due to CO(CHO) respectively from a keto function to form a five-membered ring especially in 3-hydroxy flavones, but the loss of 31 m.u. would indicate the loss of OCH_3 from 2′-methoxy flavonoid. The presence of isopentyl substituent is indicated by the loss of 53 m.u. The loss of 43 m.u. could be as a result of the CH_3 and CO or CH_3CO in methoxylated flavonoids.

1.3.7 Therapeutic Importance of Flavonoids

Flavonoids have certain nutritive effects though they are non-nutritive compounds (Hertog *et al.,* 1997). A considerable research has been directed towards their activity as anti-oxidant and radical scavenging properties (Hostettmann *et al.,* 2001) as well as anti-mutagenic and anti-carcinogenic properties and also their potential in the prevention of coronary heart disease (Meltzer, 1997). The health effects of flavonoids have been epidemiologically studied (Hollman and Katan, 1999). The use of flavonoids in the treatment of diseases is to a large extent much older than the science of chemistry (Rajnarayana *et al.,* 2001). In view of the safe and effective treatment, a study of the role of *Silymarin* in the management of non-B acute viral hepatitis was investigated, which showed significantly earlier recovery from hepatomegaly and enlarged spleen in patients receiving *Silymarin*. Also, treatment with hydroxyl ethyl rutosides significantly improved the sensation of limb swelling "bursting" pain, heaviness, tension and mobility (Rajnarayana *et al.,* 2001).

Many flavonoids have been reported to possess a variety of biological activities including antiallergic, analgesic, anti-inflammatory (e.g. structure (V), (IX)); antiproliferative effects (Shahidi *et al.,* 1998). Antibacterial and antifungal activities had also been reported; all the samples studied were active against the fungal and gram-positive bacterial strains (Wild and Fassel, 1969).

Other flavonoids-containing plants have been known to act as diuretic (e.g. buchu and broom) as well as antispasmodic (e.g. liquorice and pasley) activities (Trease and Evans, 1997). Some flavonoids acts as a strong antioxidant property as well as scavengers of various oxidation specie and have been shown to act as quenchers of singlet oxygen (Harborne and Williams, 2000). They also displayed an antiviral, including anti-HIV

activity. Out of 28 flavonoids tested, flavan-3-ol (XII) was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2 and similar immunodeficiency virus infections (Gerdin and Strensso, 1983).

1.4 CHEMISTRY OF FATTY ACIDS

Fatty acids are group of long chain aliphatic hydrocarbon molecules containing a hydrocarbon length at one end *(Head)* joined to the terminal carboxylic acid moiety at other end *(Tail).* Fatty acids occur mainly in plants in bound form, esterified to glycerol as fats or lipids; they seldom occur free in nature. These lipids comprise up to 7% of the total dry leaves in higher plants and are important as membrane constituents in the chloroplasts and mitochondria (Harborne, 1973b).

Most fatty acids are straight-chain compounds with the most frequently being an even number of carbon atoms. Although, only very few branched-chain, hydroxyl and keto acids, a few odd numbered carbon atoms from C7 – C15 have been detected free or as esters in fatty substances appreciably. Thus, fatty acids chain-lengths generally range from 2 to 80 but commonly from 12 up to 24. Those with a chain length from 2 to 6 (or 4) are called short-chain, from 8 (or 6) to 10 are called medium-chain and 12 up to 24 are called long-chain fatty acids (Leonard, 2004). Their physical and biological properties are related to this partition in 3 classes. The simplest fatty acids are referred to as saturated fatty acids. They have no unsaturated linkages and cannot be altered by hydrogenation or halogenation. When double bonds are present, fatty acids are said to be unsaturated, monounsaturated (MUFA) if only one double bond is present and polyunsaturated fatty acids if multiple double bonds are present in the molecule **(**PUFA). Therefore, the presence of double bond significantly lowers the melting point relative to the saturated

fatty acids. The vast majority of fatty acids has an unbranched carbon chain and differs from one another in chain length and degree of unsaturation. Oleic acid is the most widespread natural fatty acids occurring practically in every naturally fit mixture (Robinson, 1972).

Stearic acid is the nature's most common long chain saturated fatty acids, derived from animal and plant sources; while palmitic acid appeared to be the major saturated fatty acid in the leaf lipids, stearic acid is found less prominent in the leaf but mainly in the seed fats in number of plant families. Other less common fatty acids are linoleum, palmitoleic, and myristic acids (Shorland, 1963).

1.4.1 Saturated Fatty Acids

These are fatty acid with no double bond in the parent nucleus. They are represented by the general molecular formula $R\text{-}(CH_2)_n\text{-}COOH$; in which **n** is mostly an even number. At present, there is evidence that most of the possible saturated fatty acids between C-4 to C-6, occur in plant. However, while the even numbered fatty acids from C-10 to C-18 comprise over 80% of the total fatty acids, the odd numbered ones rarely exceeds 1% of the plant lipids. Saturated fatty acids of less than 8 carbon atoms are liquid, while those greater than this value are solid at room temperature. Normally fatty acids exhibit appreciable solubility in water compared to the corresponding hydrocarbons due to the presence of the polar carboxyl group and much less than other polar organic compounds. The first members of the saturated fatty acid (C-2 to C-4) series are miscible with water in all proportions at room temperature. The higher melting point values of the saturated acids reflect the uniform rod-like shape of their molecules.

Table 1.1 List of most common saturated fatty acids

S/No.	Systematic name	Trivial name	Structural formula	Molecular weight	Ave. Melting point (°C)
1	Butanoic	Butyric	$CH_3(CH_2)_2COOH$	88.1	-7.9
2	Hexanoic	Caproic	$CH_3(CH_2)_4COOH$	116.1	-3.4
3	Octanoic	Caprylic	$CH_3(CH_2)_6COOH$	144.2	16.7
4	Decanoic	Capric	$CH_3(CH_2)_8COOH$	172.3	31.6
5	Dodecanoic	Lauric	$CH_3(CH_2)_{10}COOH$	200.3	44.2
6	Tetradecanoic	Myristic	$CH_3(CH_2)_{12}COOH$	228.4	53.9
7	Hexadecanoic	Palmitic	$CH_3(CH_2)_{14}COOH$	256.4	63.1
8	Heptadecanoic	Margaric	$CH_3(CH_2)_{15}COOH$	270.4	61.3
9	Octadecanoic	Stearic	$CH_3(CH_2)_{16}COOH$	284.4	69.6
10	Eicosanoic	Arachidic	$CH_3(CH_2)_{18}COOH$	412.5	75.3
11	Docosanoic	Behenic	$CH_3(CH_2)_{20}COOH$	340.5	79.9
12	Tetracosanoic	Lignoceric	$CH_3(CH_2)_{22}COOH$	368.6	84.2

The solubility behaviour of the fatty acids in organic solvents is of considerable theoretical and industrial importance.

Table 1.2 Solubility data for the most common saturated fatty acids (in grams acid per litre at 20°C).

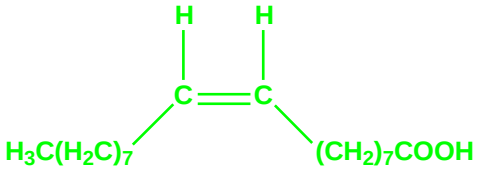
Carbon number	$CHCl_3$	C_6H_6	C-hexane	Acetone	EtOH 95%	Acetic acid	MeOH	Acetonitrile	H_2O
10	3260	3980	3420	4070	4400	5670	5100	660	0.15
12	830	936	680	605	912	818	1200	76	0.055
14	325	292	215	159	189	102	173	18	0.02
16	151	73	65	53.8	49.3	21.4	37	4	0.007
18	60	24.6	24	15.4	11.3	1.2	1	<1	0.003

On the basis of solubility data, it can be concluded that the normal saturated fatty acids are generally more soluble in chloroform and less soluble in water relative to the solvents investigated (St-Onge, 2002).

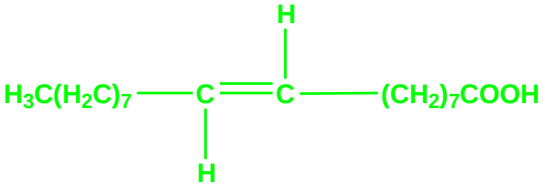
1.4.2 Unsaturated Fatty Acids

These fatty acids comprise a greater percentage of plant derived fatty acids than the saturated derivatives. The predominant unsaturated fatty acids are the C-16 & C-18 acids. The presence of unsaturation centres in fatty acids makes it possible to undergo positional isomerism; the *cis- and trans-* geometry. The numeric designations of fatty acids come from the number of carbon atoms followed by the degree of unsaturation; the site of unsaturation in a fatty acid is indicated by the delta (Δ) symbol. Thus, the predominant unsaturated fatty acids are the *cis-* isomers of the C-16 & C-18 carbon atoms.

The best known examples of *Cis-Trans* double bond isomerism in fatty acids are oleic (XVIII) which has a kink shape and its *Trans* isomer elaidic acid (XVIX) with a linear shape.



(XVIII)



(XIX)

Polyenoic fatty acids are usually liquids or solids at room temperature. The presence of double bond significantly lowers the melting point relative to a saturated fatty acid. Thus, the polyenoic fatty acids are usually of lower melting point compared with the monoenoic derivatives (Jacks, 1973). The position of unsaturation is also a factor in determining the melting point. Therefore, the *trans* isomer generally have higher melting point than the *cis* isomer of olefinic fatty acids. The *cis*-double bond(s) in the unsaturated fatty acids introduce a kink or bend in their shape, which makes it more difficult to pack their molecules together in a stable repeating array or crystalline lattice.

Table 1.3 List of some common unsaturated fatty acids

Systematic name	Trivial name	Structural formula	M. W	Ave. M.P (°C)
Cis-Delta-9-Hexadecenoic acid	Palmitoleic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7$ COOH	254	0
Cis-Delta-9-Octadecenoic acid	Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7$ COOH	282	13
Cis,cis-Delta-9,12-Octadecadienoic acid	Linoleic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7$ COOH	280	-5
Cis,cis,cis-Delta-9,12,15-Octadecatrienoic acid	Linolenic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2$ $CH=CH(CH_2)_7$ COOH	278	-11
Cis,cis,cis,cis-Delta-5,8,11,14-Icosatetraenoic acid	Arachidonic acid	$CH_3(CH_2)_3CH_2CH=CHCH_2CH=CH$ $CH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	304	-49

1.4.3 Characterization of Fatty Acids

Fatty acids can be characterised by the means of chromatography (paper and thin layer) and spectral analytical techniques (MS, GC-MS, ^{1}HNMR, ^{13}CNMR). The conjugated fatty acid has been successfully characterised using Ultra-violet spectroscopy. Thus, unsaturated fatty acids show absorption band within the range 210 – 440 nm. The common fatty acid with conjugated unsaturation is eleaostearic and shows absorption maxima at about 270 nm. Conjugated systems containing acetylenic as well as ethylenic bonds exhibit same absorption maxima with other conjugation types, but they may be recognised by other spectral differences such as intensity and side bonds (Robinson, 1972; Orhan *et al.,* 2003).

Chromatography has been extensively used in characterising fatty acids. Gas liquid chromatography is used for determination of fatty acids. The recent method is the Gas Chromatography with Mass Spectrophotometer (GC-MS) which has a duo property of identifying as well as recording the mass unit of the fatty acids through their fragmentation pattern.

1.4.4 Therapeutic Values of Fatty Acids

Fatty acids saturated or unsaturated have been of great importance in medicine. They are important for a wide array of cell structural components and for many chemical reactions in the body including hormonal and energy activities (Orhan and Sener, 2003). They also play a role in establishing lipid barrier in the skin to block irritants, infections and some of them have beneficial effects on human health such as cardio-protective activity. (Orhan *et al.,* 2003)

Shorter-chained saturated fats have been used therapeutically as conjunctive treatment in liver disease, while the medium-chained fatty acids are directly absorbed into

the bloodstream (Kabar, 2003). Generally, saturated fatty acids can be used to boost the immune system, for weight management, as anti-microbials, as dietary adjuncts in cases of chronic degenerative diseases such as cancer, cardiac and liver diseases (Kabar, 2003). Typical example is as exhibited by coconut (containing mostly saturated fatty acids of C-8 to C-16 nucleus), which has been used traditionally over time, among other things: as skin moisturisers, sunscreen, for the treatment of head lice, for ulcers, wounds, burns, dissolution of kidney stones and in the treatment of cholera, thus, the palm tree is known as *"Tree of Life"*. It is of interest, to note that the composition of fats in coconut oil is comparable to the fats found in human mother's milk (Kabar, 2003).

CHAPTER TWO

2.0 EXPERIMENTAL

2.1 MATERIALS

2.1.1 Chemicals/Reagents

All chemicals and reagents used were of Analar grade. The Silica gel for column chromatography is of mesh size (60–120G, Merck®) and that of thin layer chromatography (TLC) is Kieselgel 60G (Merck®) made to a thickness of 0.25 mm. Sephadex LH-20 for column chromatography (Pharmacia, Biotech).

2.1.2 Analytical Methods

Thin Layer Chromatography (TLC):

Technique: Ascending

Stationary phase: Silica gel (Kieselgel 60G, MERCK Darmstadt) slurry was made by dissolving 40 g of the silica gel in 80 ml of distilled water and then spread onto the plates (20 x 10 cm; 20 x 20 cm) using a spreader and activated at 100 ^{o}C for an hour.

Thickness: 0.25 mm for analytical plates and activated at 110 ^{o}C for 1 hour before use.

Spotting and Development: The spots were applied manually using glass capillary tubes and plates developed at room temperature using a Shandon Chromotank®

Mobile phase: Details in text

Visualisation: The developed chromatograms were dried in a fume cupboard and viewed under:

(i) Ultra-Violet light for any fluorescence spots

(ii) Exposed to iodine vapour in a closed chamber.

(iii) Sprayed with suitable chromogenic reagents.

Column Chromatography

Technique: Gradient elution

Column: Sintered glass column (75 x 1.7 cm; 45 x 1.0 cm) packed by wet slurry method.

Adsorbent: Silica gels (60 – 120G) mesh size and sephadex LH – 20.

Eluting Solvents: Details in text.

Paper Chromatography (PC)

Technique: Descending

Stationary phase: Whatmann No. 1

Spotting and development: The spots were applied manually using glass capillary tubes and paper developed at room temperature.

Visualisation: The developed paper chromatograms were dried in a fume cupboard and sprayed with aniline phthalate to observe the presence of sugars.

Melting point

The melting points of the isolated compounds were determined using Gallenkamp capillary melting point apparatus and the results were uncorrected.

Infra-Red spectroscopy (IR)

The IR spectra of the isolated compounds (AF1a, AF2a, and NBA2b) were recorded as a thin film in Nujol using Genesis series Fourier Transform–Infra-Red spectrophotometer (FT-IR).

Nuclear Magnetic Resonance (NMR)

(a) ^{1}HNMR – AF1a was recorded using Bruker-AC 400MHz (Bruker Instruments Inc. Karlsruhe, Germany), AF2a was recorded on Mercury BB 400MHz and NBA2b at 400MHz.

(b) ^{13}CNMR – AF1a was recorded using Mercury BB 100MHz.

2.2 SPRAY/VISUALISATION REAGENTS

(i) Iodine vapour

(ii) Ferric chloride solution

(iii) Liebermann-Burchard spray reagent

2.3 PHYTOCHEMICAL SCREENING

2.3.1 Preliminary Phytochemical Screening

A little quantity each of CPE, CEE, NBE and AQE were used to test for the presence of the following plant constituents.

2.3.1.1 Test for Carbohydrates

General Test (Molisch's Test)

A few drops of Molisch's reagent were added to each of the extract dissolved in distilled water. This was followed by addition of 1 ml of conc. tetraoxosulphate (VI) acid (H_2SO_4) by the side of the test tube; so that the acid formed a layer beneath the aqueous layer. The mixture was allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet colour at the interphase of the two layers is taken as a positive test (Trease and Evans, 1997).

Test for Monosaccharide (Barfoed's Test)

About 0.2 g of each extract was dissolved in distilled water and filtered. 1 ml of the filtrate was mixed with 1 ml of Barfoed's reagent in a test tube and then heated on a water bath for a period of 2 minutes. A red precipitate of cuprous oxide is taken as a positive test (Brain and Turner, 1975).

Test for Free Reducing Sugars (Fehling's Test)

About 0.2 g of each plant extract was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide (CU_2O) indicates the presence of reducing sugars (Trease and Evans, 1997).

Test for Combined Reducing Sugars

About 0.2 g of each plant extract was hydrolysed by boiling with 5 ml of dilute hydrochloric acid and the resulting solution neutralised with sodium hydroxide solution. Few drops of Fehling's solution was added to it and heated on a water bath for 2 minutes. Formation of a reddish-brown precipitate of cuprous oxide shows the presence of combined reducing sugars (Trease and Evans, 1997).

Standard Test for Ketoses (Salivanoff's Test)

A crystal of resorcinol and 2 ml of hydrochloric acid were added to a small quantity of each extract and the solution boiled for 5 minutes. A red colouration was an indication of the presence of ketoses (Vishnoi, 1979).

Test for Pentoses

To a small quantity of the extract was added 1 ml of hydrochloric acid (HCl) and a little phloroglucinol. The mixture was heated on a low flame and a red colour indicates the presence of pentoses (Vishnoi, 1979).

Test for Soluble Starch

A small quantity of each extract was boiled with 1 ml of 5% potassium hydroxide (KOH), cooled and acidified with H_2SO_4. A yellow colour indicates the presence of starch (Vishnoi, 1979).

2.3.1.2 Test for Tannins

About 0.5 g of each extract was stirred with about 10 ml of distilled water and then filtered. The filtrate was used for the following test:

To 2 ml of the filtrate, few drops of 1% ferric chloride solution was added, occurrence of a blue-black, green or blue-green precipitate was taken as the presence of tannins.

A mixture of equal volume of 10 % lead ethanoate was added to 2 ml of the filtrate. Formation of a white precipitate indicates the presence of tannins.

The filtrate of each extract was boiled with 3 drops of 10% HCl and 1 drop of methanol, a red precipitate was taken as evidence for the presence of tannins (Sofowora, 1993b; Trease and Evans, 1997).

2.3.1.3 Test for Phlobatannins

A small amount of each extract was boiled with distilled water and then filtered. The filtrate was further boiled with 1% aqueous HCl. A red precipitate shows the presence of phlobatannins (Trease and Evans, 1997).

2.3.1.4 Tests for Glycosides

Test for Free Anthraquinones (Borntrager's Test)

About 0.5 g of each plant extract was shaken with 10 ml of benzene and then filtered. 5 ml of the 10% ammonia solution was added to the filtrate. The mixture was then

shaken and appearance of a pink, red or violet colour in the ammoniacal (lower) phase is taken as the presence of free anthraquinones (Trease and Evans, 1997).

Test for Combined Anthraquinones (Borntrager's Test)

About 0.5 g of each plant extract was shaken with 10 ml of aqueous H_2SO_4 and then filtered while hot, the filtrate was shaken with 5 ml of benzene; the benzene layer separated and half its own volume of 10% ammonia solution was then added. The presence of a pink, red or violet colouration in the ammoniacal (lower) phase is an indication of combined anthraquinones (Trease and Evans, 1997).

2.3.1.5 Test for Cardiac Glycosides

A. (i) Salkowski Test

About 0.5 g of the extract was dissolved in 2 ml of chloroform. Tetraoxosulphate (VI) acid was carefully added by the side of the test tube to form a lower layer. Appearance of a reddish-brown colour or yellow at the interphase indicated the presence of a steroidal ring (i.e. aglycone portion of cardiac glycoside) or methylated sterols (Silva *et al.*, 1998).

(ii) Liebermann-Burchard Test

Steroidal Nucleus

About 0.5 g of the extract was dissolved in 2 ml of acetic anhydride and cooled well in ice. Conc. tetraoxosulphate (VI) acid was carefully added. Thus, development of colour from violet to blue or bluish-green indicates the presence of a steroidal ring i.e. aglycone portion of cardiac glycoside (Silva *et al.*, 1998).

Terpenoids

A little of the extract was dissolved in ethanol. To it 1 ml of acetic anhydride was added followed by the addition of conc. H_2SO_4. A colour change from pink to violet was an indication of the presence of terpenoids (Silva *et al.,* 1998).

B. Test for Cardenolides

Legal Test

Solution I: 0.5% of a recently prepared sodium nitroprusside in water and solution II: 0.2N NaOH. About 0.2 g of each extract was dissolved in 3 drops of pyridine, a drop of solution I was added followed by four drops of solution II one at a time. A deep-red colour was taken as the presence of cardenolide aglycone (Silva *et al.,* 1998).

Keller-Killiani Test

About 0.5 g of each extract was dissolved in 2 ml of glacial acetic acid containing a drop of ferric chloride solution. This was underlayed with 1 ml of conc. tetraoxosulphate (VI) acid. Appearance of a brown ring at the interphase indicates the presence of digitoxose sugar characteristic of cardenolide. A violet ring may appear just below the brown ring, while in the acetic acid layer; a greenish ring may form just above the brown ring and gradually spread throughout this layer (Trease and Evans, 1997).

2.3.1.6 Test for Saponins Glycosides

About 1 g of each extract was boiled with 5 ml of distilled water, filtered and the filtrate divided into 2 portions:

To the first portion, about 3 ml of distilled water was added and then shaken for about 5 minutes. Frothing which persist on warming was an evidence for the presence of saponins (Sofowora, 1993b).

To the second portion, 2.5 ml of a mixture of equal volume of Fehling's solution A and B was added. A brick-red precipitate was taken as the presence of saponins glycosides (Vishnoi, 1979).

2.3.1.7 Test for Flavonoids

Shinoda's Test

About 0.5 g of each extract was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips was then added to the filtrate followed by few drops of conc. HCl. A pink, orange, or red to purple colouration was an evidence for the presence of flavonoids (Markham, 1982).

Ferric Chloride Test

Each of the extract was boiled with water and then filtered. To 2 ml of the filtrate, few drops of 10% ferric chloride solution were added. A green-blue, violet colouration indicates the presence of a phenolic hydroxyl group (Trease and Evans, 1997).

Lead Ethanoate Test

A small quantity of the extracts were dissolved in water and filtered. To 5 ml of each of the filtrate, 3 ml of lead ethanoate solution was added. A buff coloured precipitate indicates the presence of flavonoids (Brain and Turner, 1975).

Sodium Hydroxide Test

About 2 ml of the filtered extract was dissolved in 10% aqueous sodium hydroxide to give a yellow colouration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid indicates the presence of flavonoids (Trease and Evans, 1997).

2.3.1.8 Tests for Alkaloids

Preliminary Test for Alkaloids

About 0.5 g of each extract was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 3 ml was taken and divided equally into 3 portions in a test tube. To the first portion, few drops of Dragendorff's reagent was added, occurrence of orange-red precipitate (ppt) was taken as positive. To the second 1 ml, Mayer's reagent was added and appearance of buff coloured ppt. was an indication of alkaloids and to the last 1 ml, few drops of Wagner's reagent was added and a dark-brown ppt. indicates the presence of alkaloids (Brain and Tuner, 1975).

Confirmatory Test for Alkaloids

A confirmatory test designed to eliminate non-alkaloidal compounds capable of eliciting "false positive reaction" was carried out for the extract which gave a preliminary positive test for alkaloids (Sofowora, 1993b). A modified form of thin layer chromatography described by Farnsworth and Euler, (1962) was adopted. 1 g of each extract was treated with 40% calcium hydroxide until the extract was distinctly alkaline with litmus paper, and extracted twice with 10 ml portion of chloroform. The chloroform extracts were then combined and concentrated at reduced pressure to about 5 ml. this was then spotted on TLC plates and developed using four solvents of different polarities. The presence of alkaloids in the developed chromatograms was detected by spraying with freshly prepared Dragendorff's spray reagent. A positive reaction was indicated by the appearance of an orange or darker coloured spots against a pale yellow background on the chromatograms was taken as confirmatory evidence that the extract contain alkaloid.

2.3.1.9 Test for Higher Fatty Acids

The alkaline aqueous solution of the extract was exhaustively extracted with ether and acidified with conc. HCl (pH 3-4). The presence of higher fatty acids is indicated when the acidic solution turned opalescent (UNIDO, 1970).

2.4 EXTRACTION

2.4.1 Collection and Preparation of Plant Material

The leaves of *Celtis integrifolia* Lam were collected from Luvuwa village of Gwoza (Lat. 11^{o} 11′N; Long. 13^{o} 35′E), Borno State, Nigeria; in July 2003. The taxonomic identification and authentication of the plant sample was confirmed by Mr. U. A. Gallah of the Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria; where a herbarium specimen was made and a voucher deposited with number: 1363.

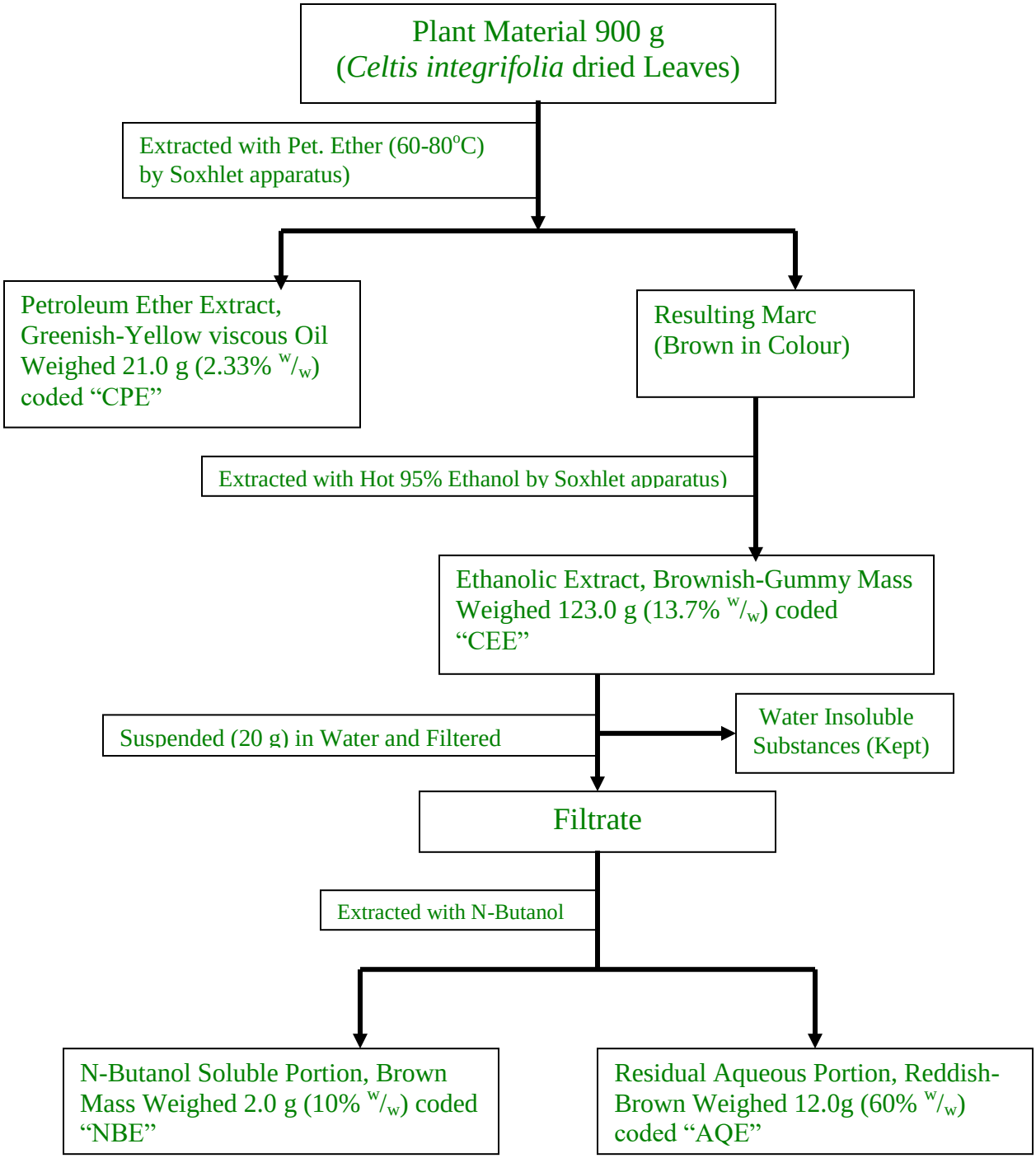
The leaves of *Celtis integrifolia* were air-dried for four days pulverised and coded *"plant material*".

2.4.2 Extraction of Plant Material

The air-dried powdered plant material (900 g) was extracted with petroleum ether (60^{o} - $80\ ^{o}$ C) to exhaustion using soxhlet extraction for 72 hours. The petroleum ether extract was then concentrated *in-vacuo* to obtain a greenish-yellow viscous oil weighing 21.0 g coded "CPE" (2.33% $^{w}/_{w}$).

The defatted marc was further air-dried and subsequently extracted with 95% ethanol (in H_2O) to exhaustion using soxhlet for 72 hours; as described by Lin *et al.*, (1999). The ethanolic extract was concentrated *in-vacuo* to obtain a brownish mass weighing 123.0 g coded "CEE" (13.7% $^{w}/_{w}$). About 20 g of CEE was suspended in distilled water; the resulting suspension was successively partitioned with n-Butanol. The

combined n-butanol extract when concentrated gave a light brown mass weighing 2.0 g (10% $^{w}/_{w}$) coded "NBE" was obtained; while the residual aqueous portion was concentrated *in-vacuo* to yield a reddish-brown gummy mass weighing 12.0 g (60% $^{w}/_{w}$) coded "AQE" as shown in scheme I below.



Scheme I. Extraction Profile

2.5 COLUMN CHROMATOGRAPHIC SEPARATIONS

2.5.1 Separation of Petroleum Ether extract

About 5 g of the CPE was mounted over glass column packed with silica gel (mesh size 60-120 G) of dimension 75 cm by 1.7 cm using the wet packing technique. The column separation was carried out using n-Hexane, n-Hexane: $CHCl_3$, $CHCl_3$: MeOH mixtures successively. 10 mL aliquots of the eluent were collected and the separation monitored by TLC (prepared by making slurry of silica gel powder {Kieselgel 60 G}; where eluent of similar RF values from same solvent systems I, II and III pooled together. The results of elution progresses were summarised in table 3.2a. Elution with n-Hexane (100%) gave a total of 3 fractions (4 – 6) and also a total of 10 fractions (17 – 26) weighed 122.0 and 47.8 mg coded "AF1 and AF2" respectively.

AF1 was further purified by crystallizing over acetone: methanol (2:1) to yield 3 components: coded 'AF1a, AF1b & AF1c. The AF1a was obtained as white crystalline shiny powder (54.1 mg). TLC of 'Af1a' using n-Hexane (100%) and Benzene (100%) showed a single spot. Meanwhile, IR, and M.P. were taken.

AF2 was re-chromatographed over column packed with silica gel (mesh size 60-120 G) of dimension 75 cm by 1.7 cm using the wet packing technique. The column separation was carried out using n-Hexane, n-Hexane: $CHCl_3$, mixtures successively. 10 mL aliquots of the eluent were collected and the separation monitored by TLC (prepared by making slurry of silica gel powder {Kieselgel 60 G}); where fractions of similar RF values from same solvent system pooled together as shown in table 3.2b below. Elution of the AF2 was monitored and a total of 10 fractions (4 – 13) were pooled which appeared as a white crystalline powder; purified by re-crystallization in acetone to afford a white crystalline amorphous powder (14.5 mg) which appeared as a single spot in 4 different

solvent systems (I – IV, as n-Hexane (100%), $CHCl_3$ (100%), n-Hex: $CHCl_3$ (8: 2) and n-Hex: EtOAc (8: 2) respectively, which was then coded AF2a. The M.P., IR, NMR spectroscopy were assayed.

2.5.2 Separation of n– Butanol Extract

About 2 g of the NBE was packed into glass column of dimension 75 cm by 1.7 cm with silica gel (mesh size 60-120 G) using the wet packing technique. The column separation was succeeded by using $CHCl_3$, $CHCl_3$:MeOH and Methanol, mixtures successively. 10 mL aliquots of the eluent were collected and the separation monitored by TLC (prepared by making slurry of silica gel powder {Kieselgel 60 G}); where eluent of similar RF values from same solvent system were pooled together as presented in the table 3.3a.

A total of 42 fractions (48 – 96) were pooled and then crystallized over acetone; the acetone insoluble was coded NBA1 whose TLC gave mixtures. The acetone soluble portion was then concentrated and TLC showed 3 major spots coded NBA2. NBA2 was further purified over sephadex LH – 20 in a column of dimension 45.0 cm by 1.0 cm, using methanol as eluting solvent (shown in Table 3.3b) to give fractions of similar Rf values (8 – 9 and 10 – 17) coded NBA2a and NBA2b respectively. All the isolates were soluble in methanol, ethanol, but insoluble in petroleum ether, benzene, chloroform.

CHAPTER THREE

3.0 RESULTS

3.1 PRELIMINARY PHYTOCHEMICAL SCREENING

The petroleum ether extract contained cardenolides, steroids and terpenoids, while the ethanolic extract and its partitioned portions (n-butanol and residual aqueous) were found to contain alkaloids, carbohydrates, cardenolide aglycons, flavonoids, tannins, saponins in varying amounts (see table 3.1).

3.2 DESCRIPTION OF THE EXTRACTS

(a) Petroleum ether extract (CPE) yielded a greenish-yellow viscous oil weighing 23.0 g (2.33% $^{w}/_{w}$)

(b) 95% ethanolic extract (CEE) yielded a brownish gummy mass weighing 123.0 g (13.7% $^{w}/_{w}$)

(c) n-Butanol soluble portion of ethanolic extract yielded a light brown mass weighing 2.0 g (10% $^{w}/_{w}$ of 20 g CEE)

(d) Residual aqueous portion (AQE) of ethanolic extract yielded a reddish-brown gummy mass weighing 12.0 g (60% $^{w}/_{w}$ of 20 g CEE)

Table 3.1 Phytochemical constituents of the Leaves extracts of *Celtis integrifolia.*

S/No	GROUP CONSTITUENTS	TEST	OBSERVATION	INFERENCE			
				CPE	CEE	NBE	AQE
1	**CARBOHYDRATES**						
	General test	Molisch's	Red or dull violet colour at the interphase	-	+++	+	+++
	Monosaccharide	Barfoed's	Red ppt. of cuprous oxide formed	-	++	-	+++
	Free reducing sugar	Fehling's	Red ppt. of cuprous oxide formed	-	++	+	+++
	Combined reducing sugar	Fehling's	Red ppt. of cuprous oxide formed	-	++	-	+++
	Ketoses	Salivanoff's	No colouration formed	-	-	-	-
	Pentoses		Red colouration formed	-	+	-	+
	Soluble starch		Yellow colour formed	-	++	-	+
2	**ALKALOIDS**	Dragendorff's	Orange-red ppt	-	++	++	+
		Mayer's	Buff coloured ppt	-	++	++	+
		Wagner's	Dark-brown ppt	-	+++	++	+
3	**TANNINS**	$FeCl_3$	Blue-black ppt	-	+++	++	+
		10% lead ethanoate	White ppt	-	++	++	+
		10% HCl	No ppt formed	-	-	-	-
4	**PHLOBATANNINS**		No ppt formed	-	-	-	-
5	**ANTRAQUINONES**						
	Free anthraquinones	Borntrager's	No colour change	-	-	-	-
	Combined anthraquinones		No colour change	-	-	-	-
6	**STEROIDAL NUCLEUS**	Salkowski's	Reddish-brown at the interphase	+++	-	-	-
		Liebermann-Burchard's					
		(i) Steroidal nucleus	Violet to blue-green colour formed	++	-	-	-
		(ii)Terpenoid	Pink – violet colour formed	++	-	-	-

7	**FLAVONOIDS**	Shinoda's	Red – pink colour formed	-	+++	+++	+
		Ferric chloride's	Greenish-black colour formed	-	++	+++	+
		Lead ethanoate's	Buff coloured ppt formed	-	++	+++	+
		Sodium hydroxide's	Colour changes from yellow to colourless on addition of dilute HCl	-	++	++	+
8	**SAPONINS**	Frothing	Frothing persist on warming	-	+++	+	+++
		Saponins glycosides	Brick-red colouration formed	-	++	+	++
9	**CARDENOLIDES**	Legal	Deep-red colour formed	-	++	++	+
		Keller-Killiani	Reddish-brown ring at the interphase	+++	+++	+++	-
10	**FATTY ACIDS**		Opalescent colour at the acidic layer	+++	-	-	-

Key: - = absent, + = faintly present, ++ = moderately present, +++ = highly present, CEE = Crude ethanolic extract, CPE = crude petroleum ether extract, NBE = n-butanol soluble portion, AQE = residual aqueous portion.

3.3 COLUMN CHROMATOGRAPHY

3.3.1 Chromatographic Separation and Isolation of Af1a

Comprehensive column chromatographic separations of AF1a as summarised in Table 3.2a revealed the melting point of AF1a as 63 – 65^{o}C. The compound AF1a was obtained as white crystalline bright powder (54.1 mg); TLC of AF1a and co-TLC with authentic palmitic acid using n-Hexane (100%) and Benzene (100%) showed a similar single spot which was soluble in Pet. ether, benzene, and chloroform but insoluble in methanol. The results for the separation of the petroleum ether extract are as shown in table 3.2a while the Rf values of Af1a in 2 different solvent is summarised in table 3.2a.i below. AF1a gave positive test for fatty acid, which was then subjected to spectral analysis: IR, and ^{1}HNMR.

Table 3.2a Column Chromatographic separation of Petroleum ether extract.

S/No	Fractions	Eluting solvent	No. of spots
1	1 – 3	n-Hex (100%)	--
2	**4 – 6**	**n-Hex (100%)**	**3**
3	7 – 12	n-Hex (100%)	3
4	13 – 16	n-Hex (100%)	2
5	**17 – 26**	**n-Hex (100%)**	**2**
6	27 – 33	n-Hex (100%)	2
7	34 – 93	n-Hex (100%)	3
8	94 – 119	n-Hex:$CHCl_3$ (19 : 1	2
9	120 – 128	n-Hex:$CHCl_3$ (19 : 1)	2
10	129 – 132	n-Hex:$CHCl_3$ (19 : 1)	1
11	133 – 156	n-Hex:$CHCl_3$ (9 : 1)	1
12	157 – 162	n-Hex:$CHCl_3$ (9 : 1)	3
13	163 – 177	n-Hex:$CHCl_3$ (8 : 2)	4
14	178 – 188	n-Hex:$CHCl_3$ (8 : 2)	3
15	189 – 215	n-Hex:$CHCl_3$ (8 : 2)	3
16	216 – 227	n-Hex:$CHCl_3$ (8 : 2)	2
17	228 – 231	n-Hex:$CHCl_3$ (7 : 3)	3
18	232 – 251	n-Hex:$CHCl_3$ (7 : 3)	1
19	252 – 260	n-Hex:$CHCl_3$ (6 : 4)	4
20	261 – 272	n-Hex:$CHCl_3$ (6 : 4)	3
21	273 – 288	n-Hex:$CHCl_3$ (5 : 5)	4
22	289 – 300	n-Hex:$CHCl_3$ (4 : 6)	2
23	301 – 320	n-Hex:$CHCl_3$ (3 : 7)	4
24	321 – 330	n-Hex:$CHCl_3$ (2 : 8)	2
25	331 – 350	n-Hex:$CHCl_3$ (1 : 9)	3

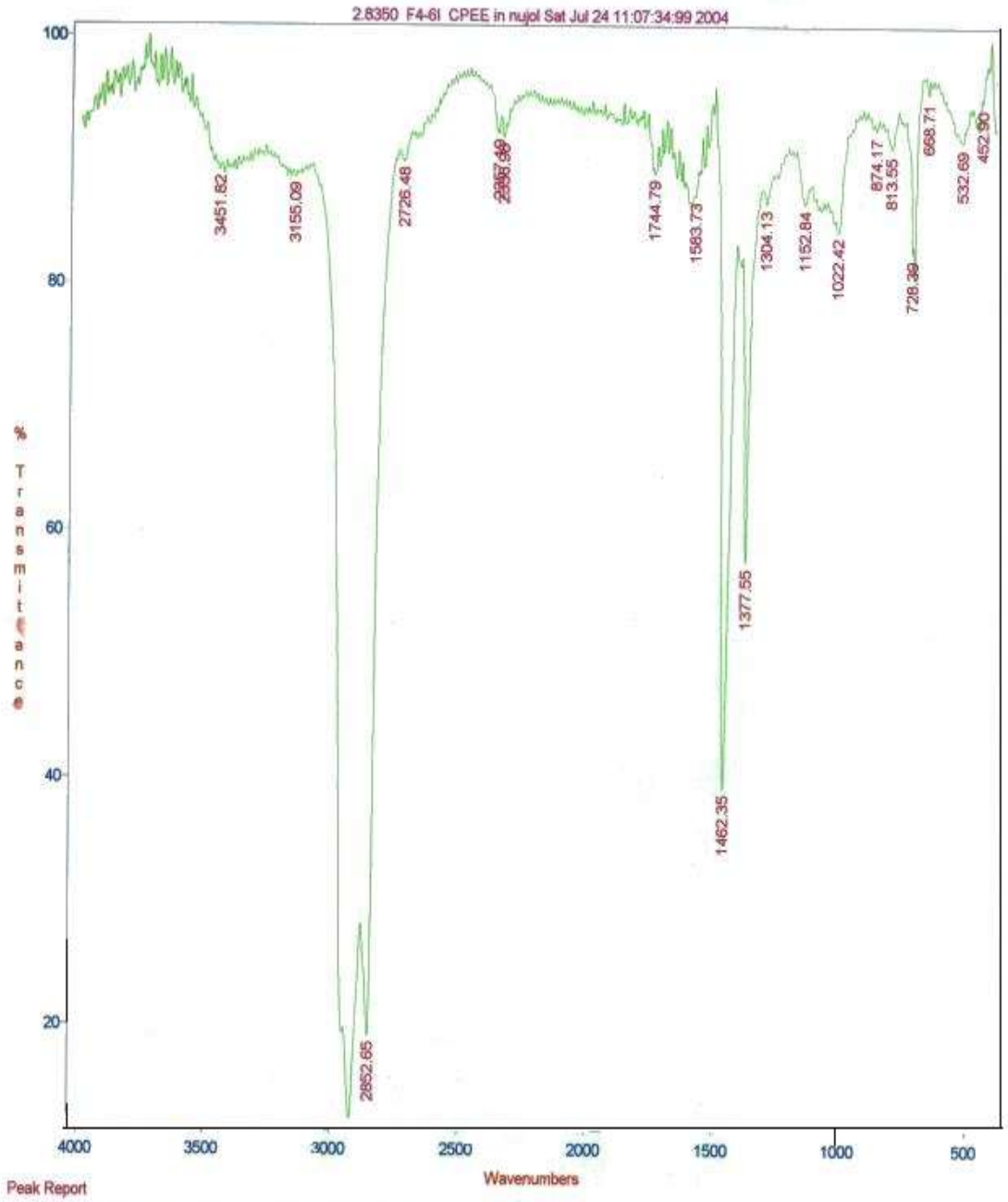
TLC solvent system used:
(I) n-Hexane (100%) (II) $CHCl_3$ (100%)
(III) n-Hex: $CHCl_3$ (8: 2) (IV) n-Hex: EtOAc (8: 2)

Table 3.2a.i TLC and co- TLC profile of AF1a and authentic Palmitic acid

Solvent Systems	Rf values		Colour in Iodine
	AF1a	Palmitic acid	
Petroleum ether (100%)	0.92	0.90	Brown
Benzene (100%)	0.96	0.95	Brown

Table 3.2a.ii Proton NMR of AF1a in $CDCl_3$

H-Type	δ_H-Value (ppm)
TMS (standard)	0.08
$-CH_3$(terminal)	0.88 – 0.91
$-CH_2$	1.27 – 1.33
$-CH_2$	1.54
$-CH_2$-C-O	2.18
$CDCl_3$ (solvent)	7.27



Peak Report
File: c:\first\data\tmp
Title: 2.8350 F4-6I CPEE in nujol Sat Jul 24 11:07:34:99 2004
Filter: Three Point Center of Gravity

cm-1	%T	cm-1	%T	cm-1	%T
2922.68	12.4236	2852.65	19.0940	3451.82	89.0925
3155.09	88.5664	2726.48	89.8498	2357.19	92.1333
2336.98	91.8356	1744.79	88.9078	1583.73	87.1166
1462.35	39.1594	1377.55	57.4342	1152.84	86.5075
1022.42	84.2837	1304.13	86.5194	813.55	91.0659
874.17	92.5793	728.39	82.6806	532.69	91.6798
452.90	94.9272	668.71	95.4923		

Fig. 3.1 Infra-red Spectra of compound AF1a

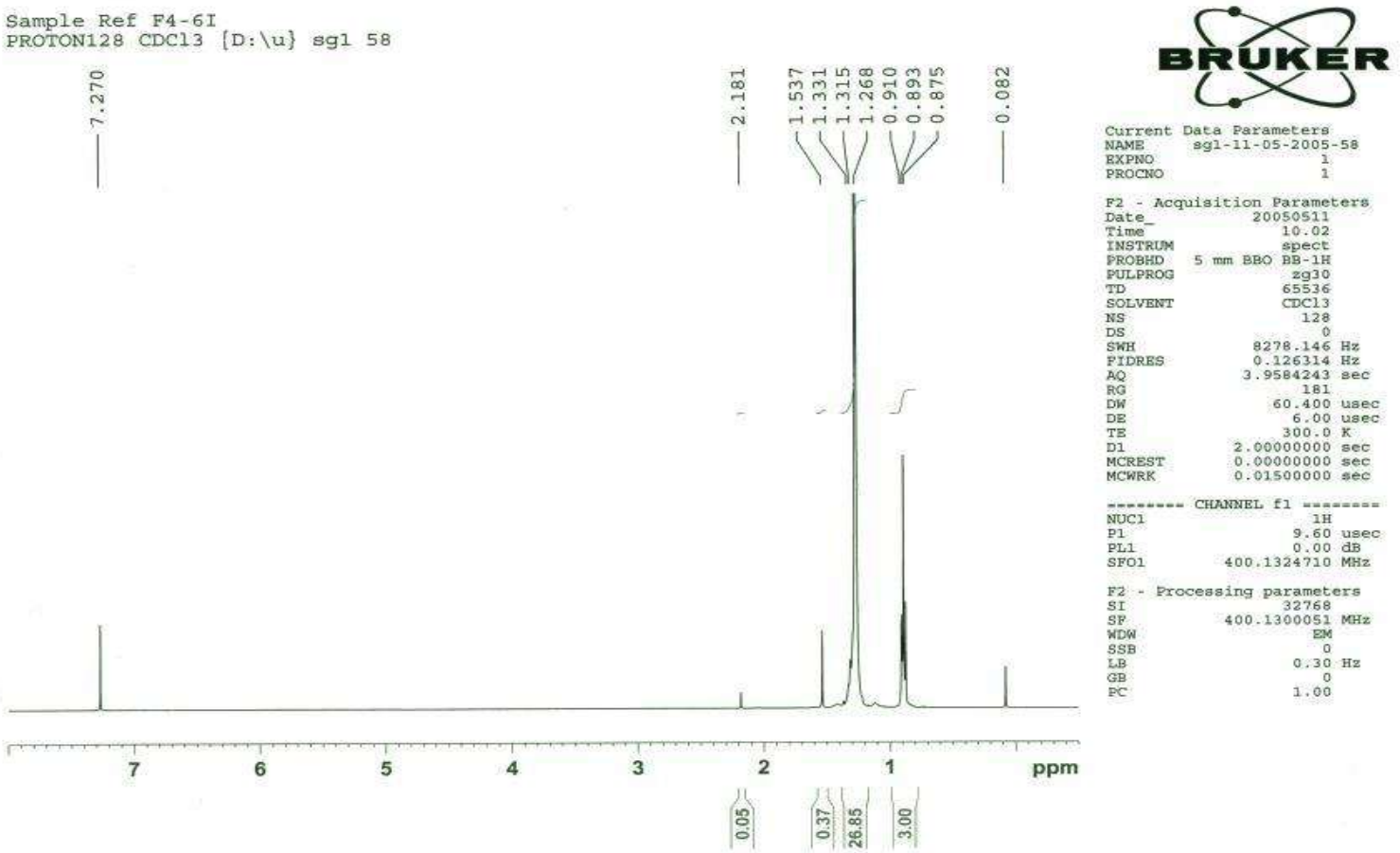


Fig. 3.1a Proton NMR of compound AF1a

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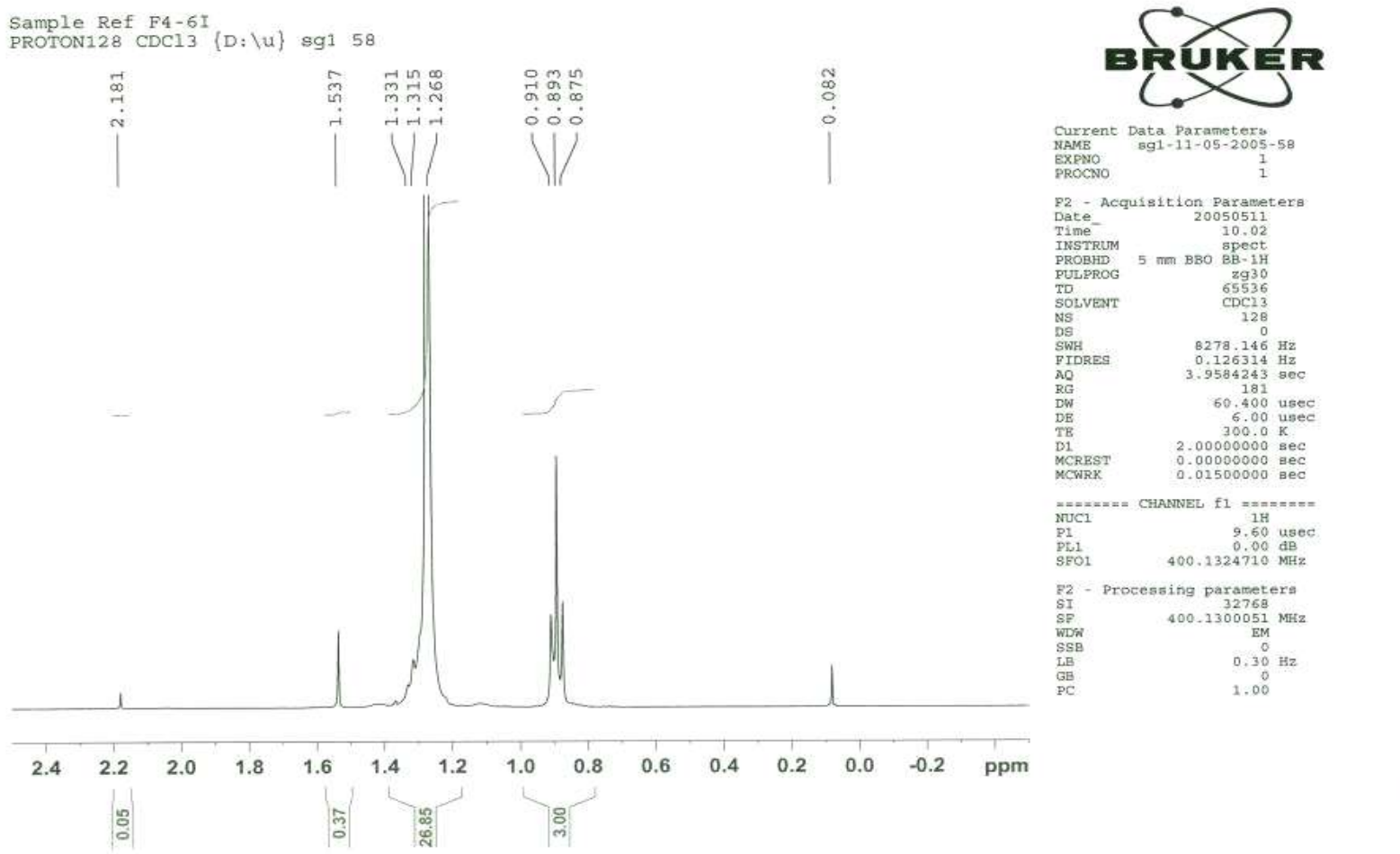

Fig. 3.1b Proton NMR of compound AF1a

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3.3.2 Chromatographic Separation and Isolation of Af2a

Sample AF2a was obtained as a white crystalline amorphous powder (14.5 mg) as shown in 3.2b. The melting point was obtained between 67 – 69^{o}C. The TLC of AF2a and co-TLC with authentic stearic acid using n-Hexane (100%) and Benzene (100%) showed a similar single spot; which was soluble in Pet. ether, benzene, and chloroform but insoluble in methanol. The Rf values of Af2a in 2 different solvent is summarised in table 3.2b.i below. The compound AF2a was positive to test for fatty acid, which was then subjected to spectral analysis: IR and proton NMR.

Table 3.2b Column Chromatographic separation of fraction AF2 on Silica Gel

S/No	Fractions	Eluting Solvent	No. of Spots
1	1 – 3	n-Hexane (100%)	1
2	**4 – 13**	n-Hexane (100%)	**2(1major,1 minor)**
3	14 – 18	n-Hexane : $CHCl_3$ (19 : 1)	3
4	19 – 28	n-Hexane : $CHCl_3$ (19 : 1)	2
5	29 – 31	n-Hexane : $CHCl_3$ (19 : 1)	--
6	32 – 48	n-Hexane : $CHCl_3$ (9 : 1)	1
7	49 – 53	n-Hexane : $CHCl_3$ (8 : 2)	3(1major,2 minor)
8	54 - 60	n-Hexane : $CHCl_3$ (8 : 2)	2

TLC solvent system used:
(I) n-Hexane (100%) $CHCl_3$ (100%)
(III) n-Hex : $CHCl_3$ (8 : 2) (IV) n-Hex : EtOAc (8 : 2)

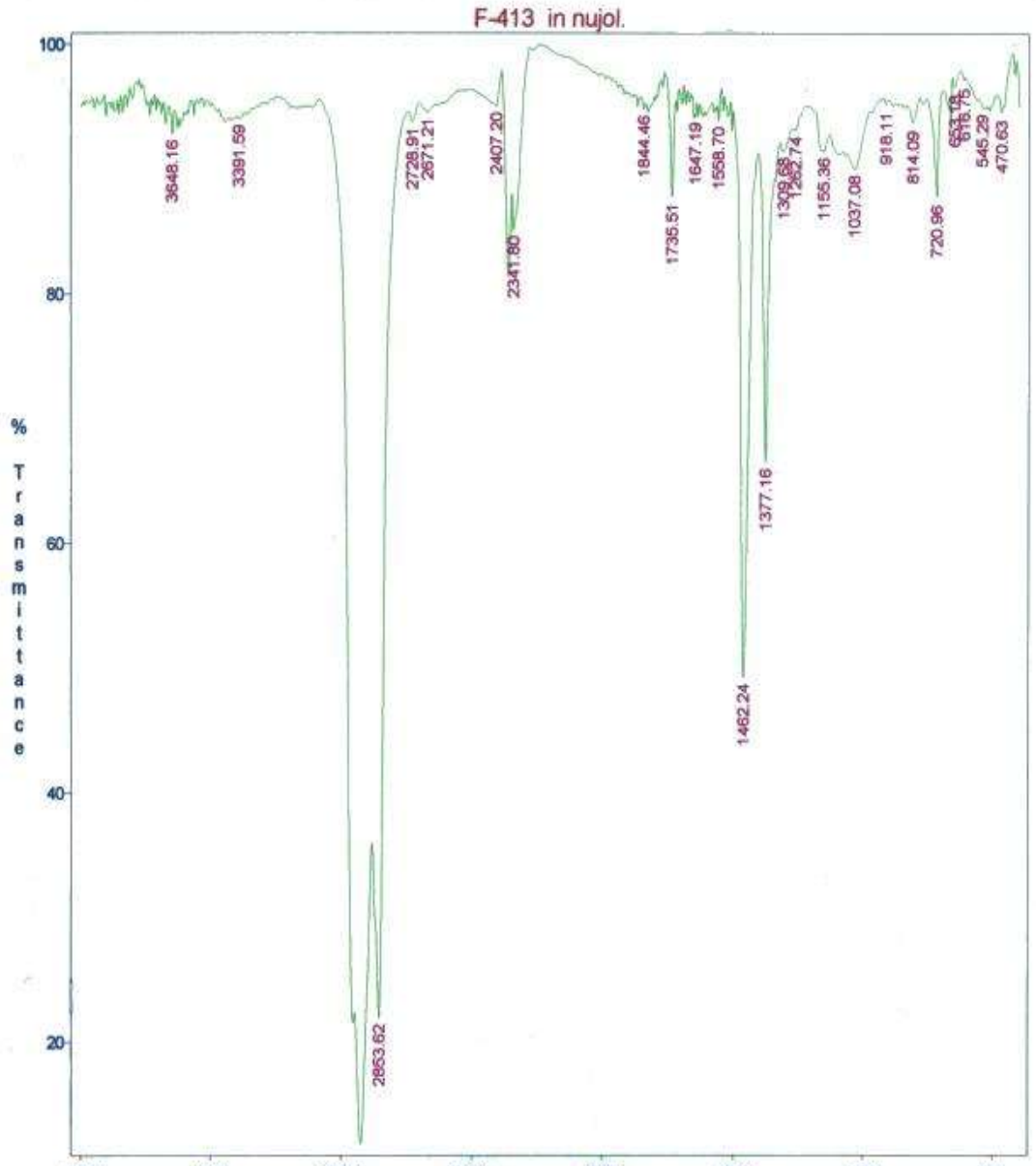
Table 3.2b.i TLC and co- TLC profile of AF2a and authentic Stearic acid

Solvent Systems	**Rf values**		**Colour in Iodine**
	AF2a	Stearic acid	
a	0.90	0.89	Brown
b	0.85	0.86	Brown
c	0.70	0.70	Brown

a = Chloroform (100%) b = n-Hexane: Chloroform (4:6)
c = n-Hexane: Chloroform (6:4)

Table 3.2b.ii Proton NMR and ^{13}CNMR data of AF2a in$CDCl_3$

Proton NMR		**Carbon-13 NMR**	
H-type	**δ_H-Value (ppm)**	**C-type**	**δ_H-Value (ppm)**
TMS (standard)	0.07	TMS (standard)	1.01
-CH_3(terminal)	0.80 – 0.95	-CH_3(terminal)	14.0
-CH_2	1.25	-CH_2	22.6 – 29.7
-CH_2	1.61	-CH_2	31.9
-CH_2-C-O	2.29	-CH_2	34.4
$CDCl_3$ (solvent)	7.26	$CDCl_3$(solvent)	77.0
		-C=O	174.0



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2671.21	94.5688	2853.62	21.9270	2923.77	11.8630
1735.51	87.7791	1844.46	94.7692	1462.25	49.2036
1377.16	66.4349	1037.08	90.0117	1155.36	91.3998
1309.68	91.4389	1262.74	93.0746	814.09	93.7152
720.96	87.8029	653.18	96.6548	470.63	94.5966
545.29	95.0518	616.75	97.1414	1558.70	93.8452
1647.19	94.2177	3648.16	92.7844	3391.59	94.0271
918.11	95.2761				

Fig. 3.2 Infra-red Spectra of compound AF2a

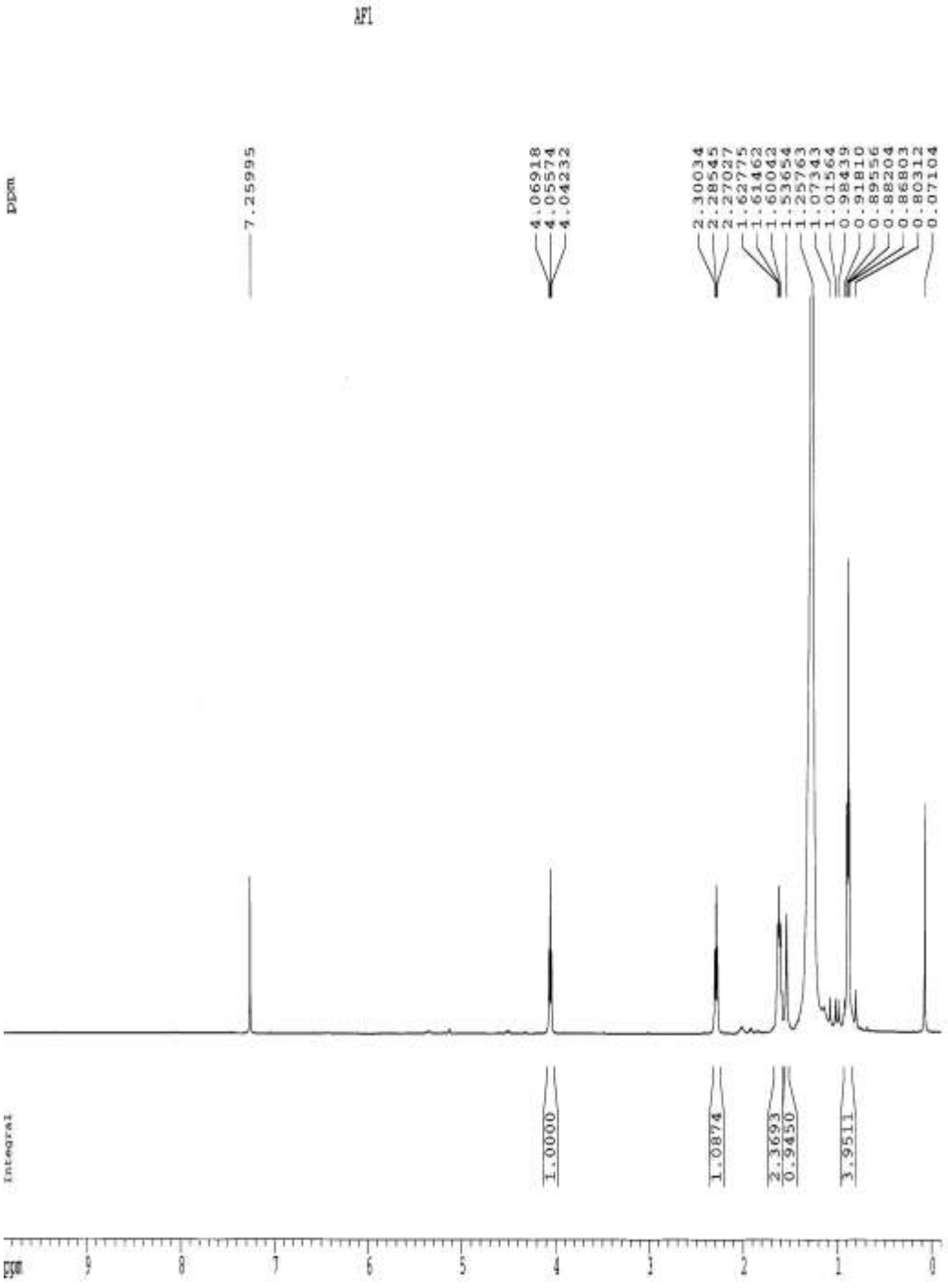


Fig. 3.2a Proton NMR of compound AF2a

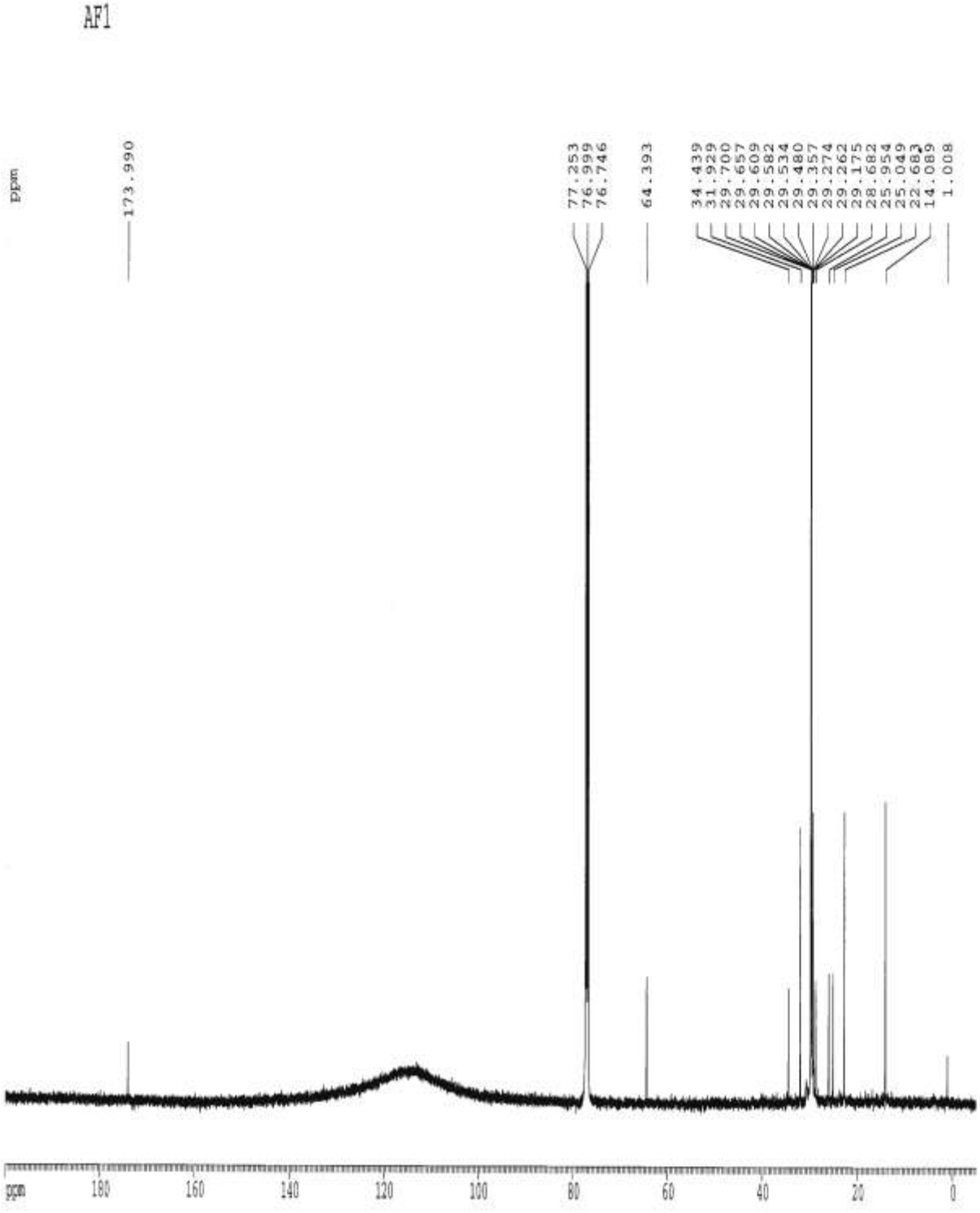
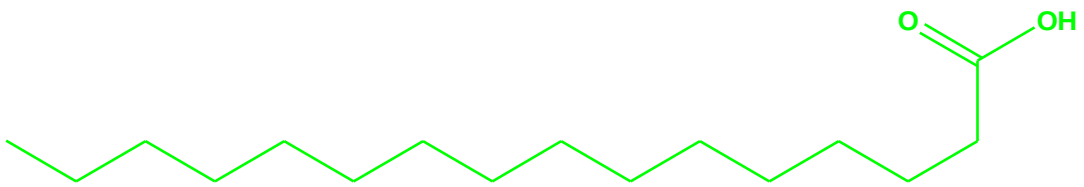
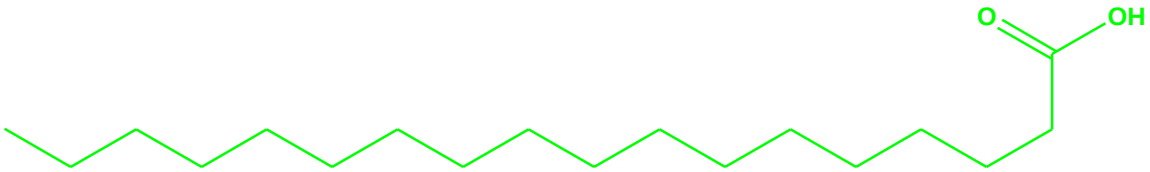


Fig. 3.2b Carbon-13NMR of compound AF2a



Compound AF1a: Palmitic acid (Hexadecanoic acid) {$C_{16}H_{32}O_2$}



Compound AF2a: Stearic acid (Octadecanoic acid) {$C_{18}H_{36}O_2$)

3.3.3 Chromatographic Separation of the n-Butanol Extract

Extensive chromatographic studies of n-butanol portion as summarised in table 3.3a and 3.3b afford 3 components; coded NBA1 – yellow crystalline powder (40.0 mg) which gave mixtures, the acetone soluble portion was then concentrated and TLC showed 3 major spots coded NBA2 (24.4 mg). NBA2 was further purified over sephadex LH – 20 (as presented in Table 3.3b) to give fractions of similar Rf values (8 – 9 and 10 – 17) coded "NBA2a - yellow amorphous powder (4.3 mg)" and "NBA2b - brownish-yellow amorphous powder (12.0 mg)." Chromogenic tests and other chemical tests conducted (UV in NH_3 solution gave yellow fluorescence, Shinoda's test gave a pink colour, $FeCl_3$ test was blue-black) strongly suggests that the compound 'NBA2b' is a flavonol. Due to the quantity obtained only NBA2b was subjected to further studies, the M.P. was found to be 205-208^{o}C (uncorrected), the compound was also subjected to spectroscopic analyses: IR and ^{1}HNMR. The separation profile is as presented in table 3.3a and 3.3b. All the compounds were soluble in methanol and ethanol, but insoluble in petroleum ether, benzene and chloroform.

Table 3.3a. Column Chromatographic separation of n-Butanol portion on Silica Gel

S/No	Fractions	Eluting solvent	No. of spots
1	1 – 3	$CHCl_3$ (100%)	--
2	4 – 6	$CHCl_3$ (100%)	2
3	7 – 12	$CHCl_3$ (100%)	--
4	13 – 19	$CHCl_3$:MeOH (19:1)	3
5	20 – 33	$CHCl_3$:MeOH (19:1)	4
6	34 – 48	$CHCl_3$:MeOH (9:1)	2
7	**49 – 96**	**$CHCl_3$:MeOH (8:2)**	**3**
8	97 – 128	$CHCl_3$:MeOH (8:2)	1
9	129 – 159	$CHCl_3$:MeOH (7:3)	2
10	160 – 178	$CHCl_3$:MeOH (6:4)	1
11	179 – 196	$CHCl_3$:MeOH (5:5)	3
12	197 – 211	$CHCl_3$:MeOH (9:1)	1

TLC solvent system used:
(I). Ethylacetate:Methanol: Water (100:16.5:13.5) (II). Chloroform: Methanol: Water (3:3:1)
(III). Ethylacetate:Formic acid: Water (10:1:2)

Table 3.3b Column Chromatographic separation of NBA2 on Sephadex LH-20

S/No	Fractions	Eluting solvent	No. of spots
1	1 – 3	Methanol (100%)	--
2	4 –5	Methanol (100%)	2
3	6 – 7	Methanol (100%)	Mixtures
4	**8 – 9**	**Methanol (100%)**	**1**
5	**10 – 17**	**Methanol (100%)**	**1**
6	18 – 21	Methanol (100%)	--
7	22 – 24	Methanol (100%)	--

TLC solvent system used:
(I). Ethylacetate:Methanol:Water(100:16.5:13.5) (II).Chloroform:Methanol:Water (3:3:1)
(III). Ethylacetate:Formic acid: Water (10:1:2)

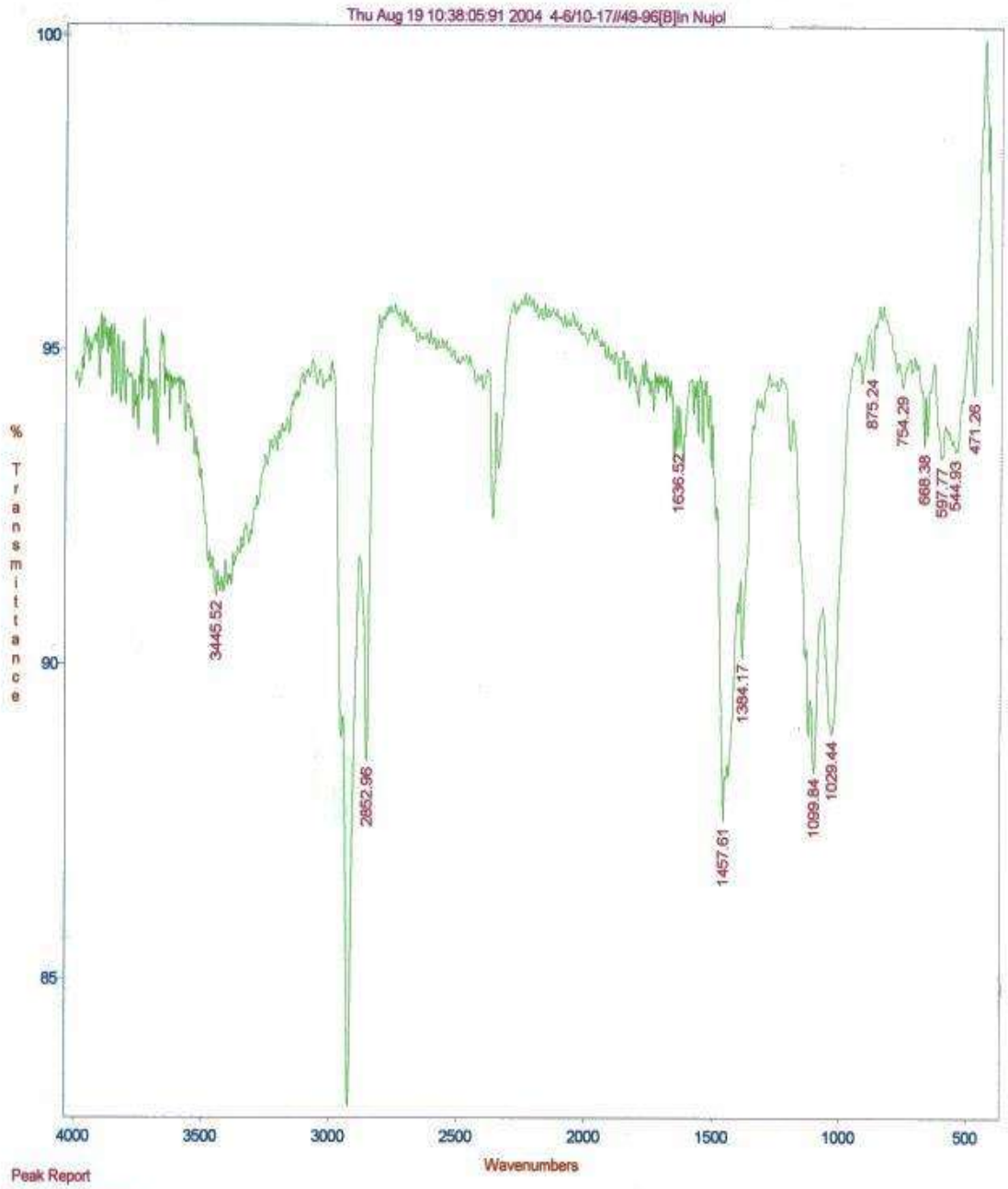
Table 3.3b.i TLC profile of NBA2b

Solvent Systems	Rf	Colour		
	NBA2b	In iodine	UV/NH_3	Under sunlight
a	0.75	Yellow	Yellow fluorescence	Yellow
b	0.47	Yellow	Yellow fluorescence	Yellow
c	0.48	Yellow	Yellow fluorescence	Yellow

a = Chloroform: Methanol: Water (3:3:1)
b = Ethylacetate:Formic acid: Water (10:1:2)
c = Ethylacetate:Methanol:Water (100:16.5:13.5)

Table 3.3b.ii Proton NMR data of NBA2b in CD_3OH

H-Position	**δ_H-Value (ppm)**
6	6.49 (1H, d, J=12.4Hz)
8	6.56 (1H, d, J=10.4Hz)
5′	6.95 (1H, d, J=8.4Hz)
6′	7.53 (2H, d, J=8.4Hz)
2′	7.98 (2H, d, J=8.8Hz)
C-1″, C-1‴	6.24 (1H, d, J=6.8Hz); 5.04 (1H, d, J=10.0Hz)
$C\text{-}6''_B$, $C\text{-}6''_A$	4.25 (m), 4.09 (m)
$C\text{-}6'''_B$, $C\text{-}6'''_A$	3.93 (m), 3.84 (m)
C-5″, C-5‴	3.80 (m), 3.79 (m)
C-3″, C-3‴	3.69 (m), 3.44 (m)
C-4″, C-4‴	3.19 (m), 3.15 (m)
C-2″, C-2‴	3.13 (m), 3.07 (m)



Peak Report
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Filter: Three Point Center of Gravity

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471.26	94.3084	544.93	93.4011	597.77	93.2961
668.38	93.4949	754.29	94.4271	875.24	94.6987
1029.44	88.8871	1099.84	88.2947	1457.61	87.5106
1384.17	90.1179	1636.52	93.4560	2852.96	88.4479
2924.28	82.9725	3445.52	91.0796		

Fig. 3.3 Infra-red Spectra of compound NBA2b

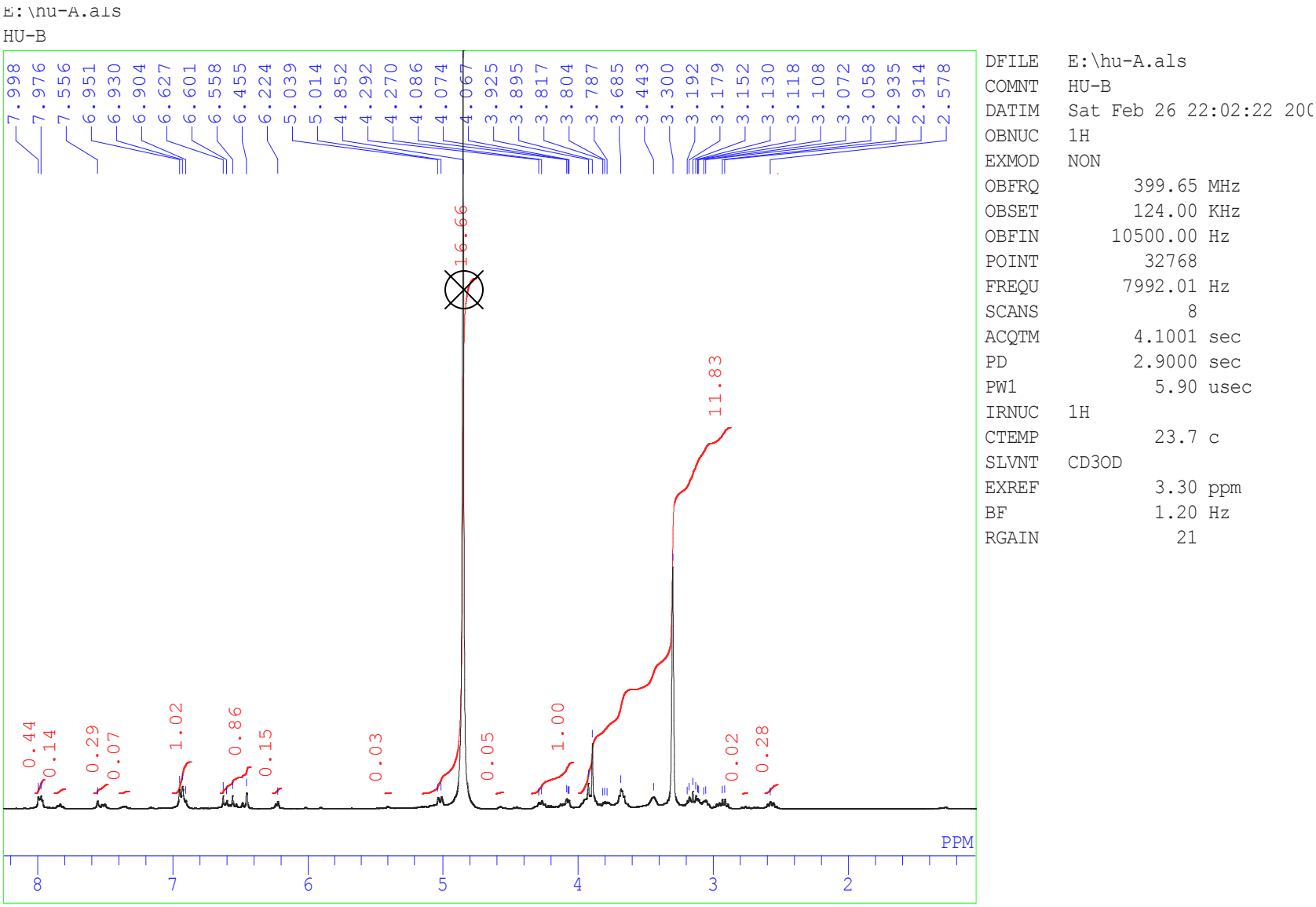


Fig. 3.3a Proton NMR of compound NBA2b

65

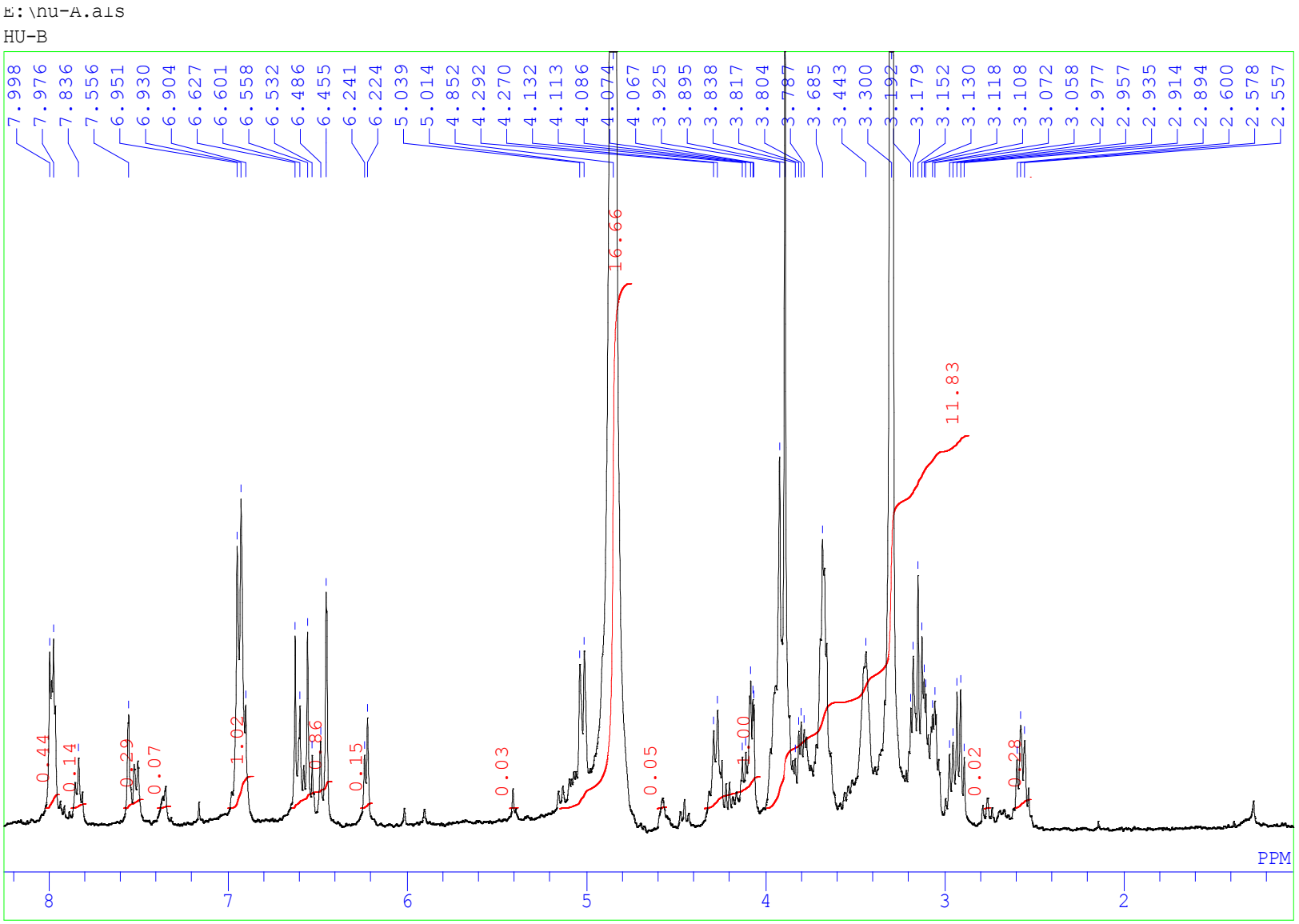


Fig. 3.3b Proton NMR of compound NBA2b

66

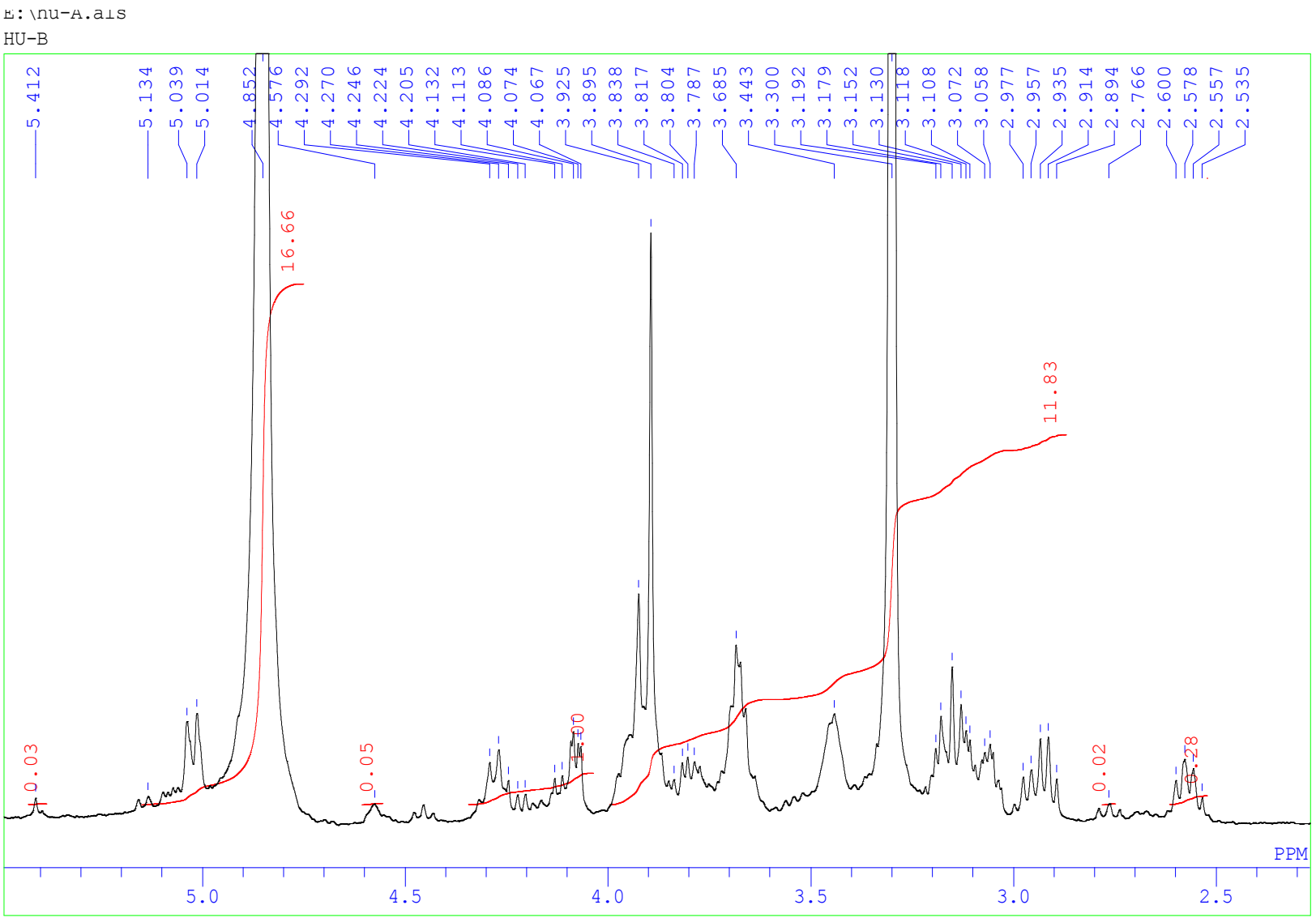


Fig. 3.3c Proton NMR of compound NBA2b

67

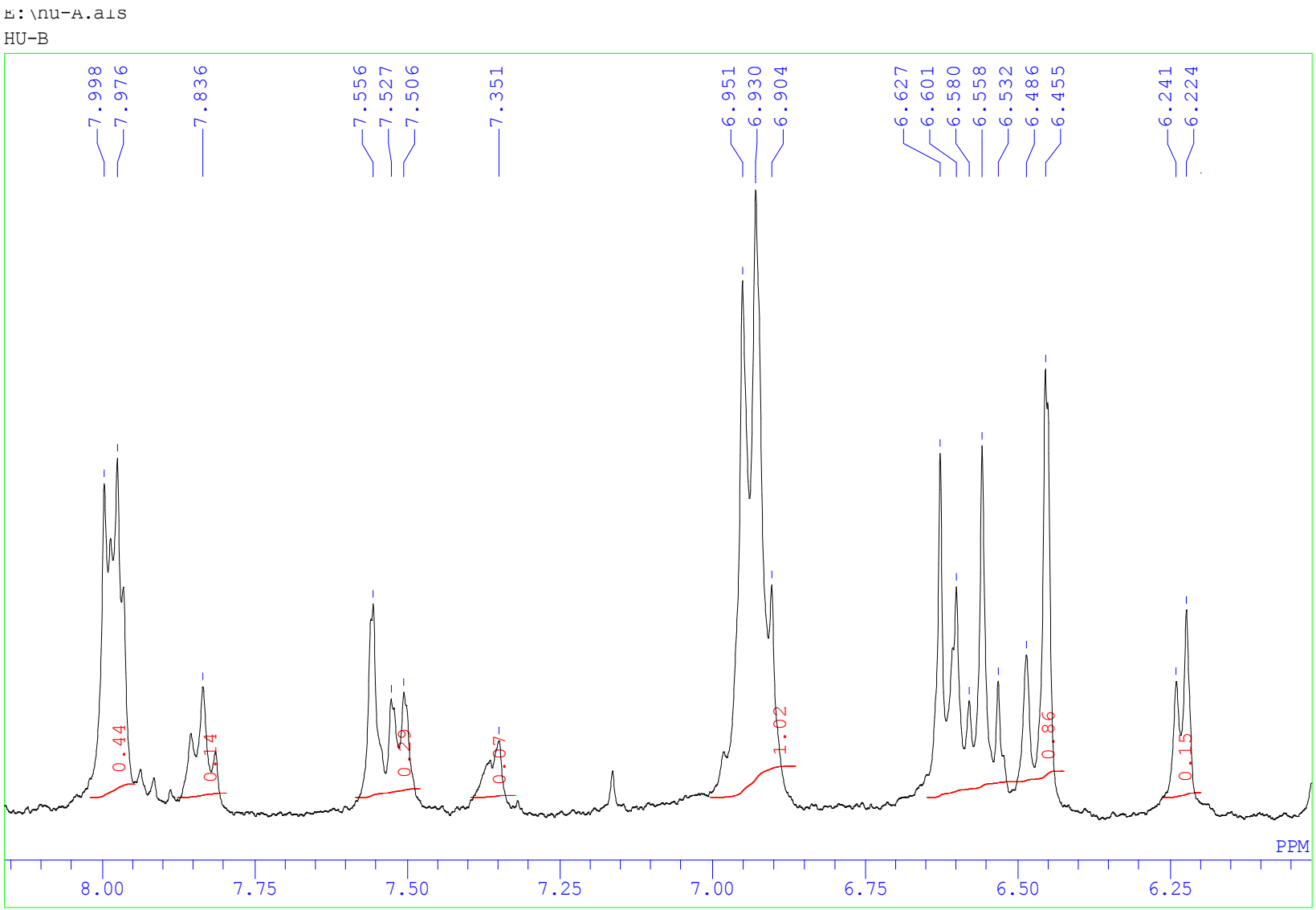
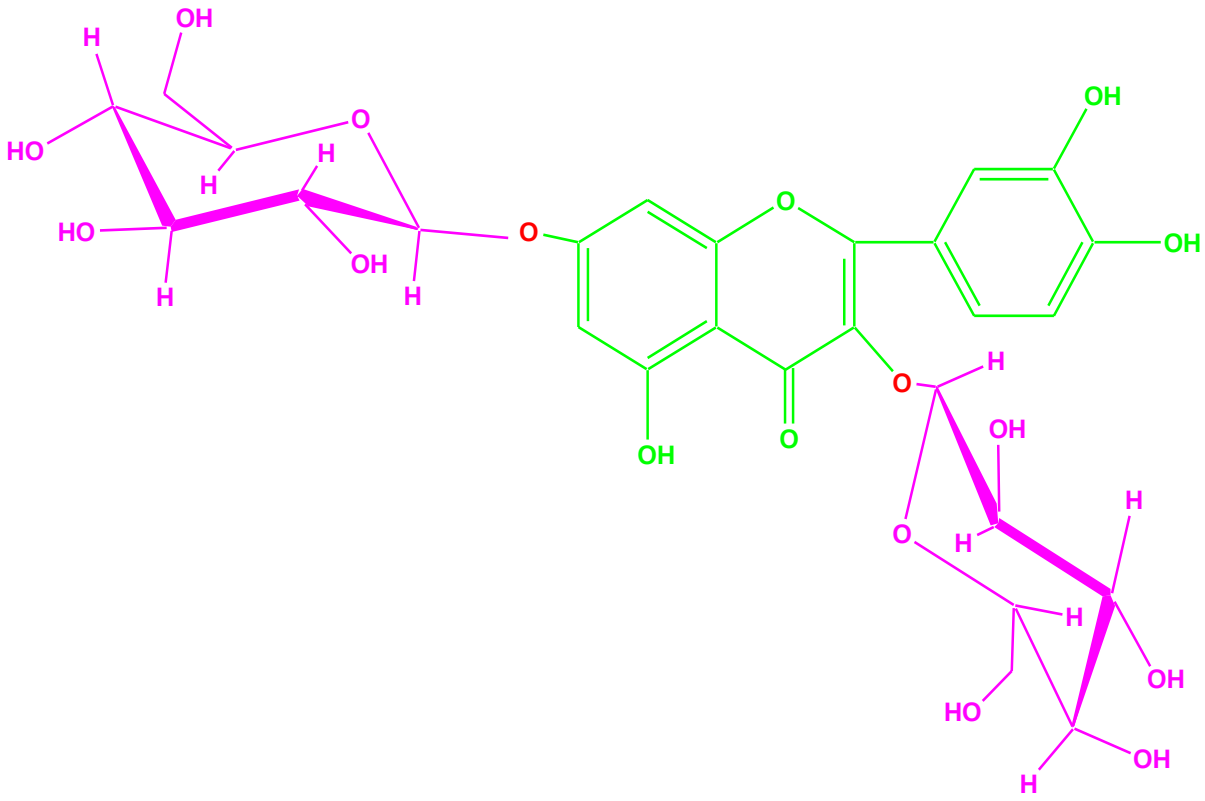


Fig. 3.3d Proton NMR of compound NBA2b

68



Compound NBA2b: Quercetin-3,7-*O*-diglucoside (5,3′,4′,-trihydroxyflavone-3-*O*-β-glucosyl-7-*O*-β- glucoside). MF: $C_{27}H_{30}O_{17}$; MW: 626.

CHAPTER FOUR

4.0 DISCUSSIONS

From the preliminary phytochemical tests carried out on the petroleum ether, ethanol, n-butanol, and residual aqueous extracts respectively, the leaves of *Celtis integrifolia* was found to contain among other constituents, alkaloids, flavonoids, saponins, steroids and fatty acids.

The petroleum ether extract after extensive separation afforded two compounds coded AF1a and AF2a, while the n-butanol soluble portion of ethanol extract following extensive chromatographic separations affords NBA2b.

Compound Af1a gave positive to higher fatty acids and negative test for steroids. The IR spectrum of compound AF1a showed frequency of 1744.8 cm^{-1} diagnostic of α, β-unsaturated carbonyl functions, other frequencies are 3451.8, 2852.7, and 1462.3, 1377.5 1152.8 cm^{-1} due to –OH, -CH_2, and -CH respectively (Kemp, 1991). ^{1}HNMR signals was observed at δ0.91 (CH_3), 1.33, (CH_2) 1.54, (CH_2) 2.18 (CH_2-C-O) and δ7.27 ($CDCl_3$). Though, only IR and ^{1}HNMR was conducted for AF1a, but an insight was sought from that of the AF2a by looking at their ^{1}HNMR where similar bulky nature of -CH_2 was observed; other signals such as due to carbonyl would have been observed if the ^{13}CNMR was conducted. Thus, co-TLC and mixed-melting point with that of AF1a showed close resemblance to palmitic acid which was further confirmed from the literature report (Orhan and Stener, 2003). Thus, AF1a has a molecular formula of $C_{16}H_{32}O_2$ (Hexadecanoic acid) and MW: 256.

Similarly, compound Af2a gave positive to higher fatty acids but negative test for steroids. The IR absorption peaks for compound AF2a were observed at 3391.6, 2853.6,

and 1462.2, 1377.2 cm^{-1}, which are due to -OH, $-CH_2$, and -CH respectively and absorption at 1735.8 cm^{-1} is diagnostic of α, β-unsaturated carbonyl functions (Kemp, 1991). The proton and ^{13}Carbon NMR revealed that the structure of the compound could be a long chain alkanes typical of long chain fatty acids as was evident from the big clusters of carbon in about δ29 in the ^{13}CNMR spectrum (Silverstein *et al.*, 1989). Eighteen carbon signals were observed in the ^{13}CNMR spectrum. This is confirmed absolutely from the presence of big signals due to methylene group ($-CH_2$) at δ1.25 in ^{1}HNMR spectrum. Other signals at δ14.0 and δ34.4 in Carbon-13 NMR correlates to the signals at δ0.8-0.9 and that centred at δ2.28 in HNMR spectrum which are due to terminal CH_3 and CH_2C-O respectively. The carbonyl group is shown by the absorption at δ174.0 in ^{13}CNMR spectrum, this is typical of the carbonyl of the –COOH group which absorbs between δ66-181 (Kemp, 1991). All the spectral data revealed the likelihood of the compound AF2a to be long chain fatty acid; this was later confirmed by the spectral comparison and by conducting mixed-melting point. Co-TLC with authentic stearic acid with that of AF2a showed similarity in all respect and no depression was observed in their MP values; hence suggest AF2a as a Stearic acid, with molecular formula $C_{18}H_{36}O_2$ and MW of 284.

The compound NBA2b gave pink colour to Shinoda's test, yellow fluorescence in UV/NH_3 and bright-yellow under sunlight; which are characteristics of flavonols compounds. The IR spectrum of NBA2b showed a broad peak at 3445.5 cm^{-1}, a diagnostic absorption for hydroxyl (-OH) group. The presence of a signal at 1636.5 cm^{-1} was attributed to chelated keto group, hence confirming the presence of 5-OH group (Mekonen and Gebreyesus, 2000). Peak at frequency 1457.6 cm^{-1} was due to aromatic nucleus

(C=C), the broad band at 1099.8 – 1029.4 cm^{-1} are indicative of the glycosidic character of the compound (Tang *et al.,* 2003).

The proton NMR of NBA2b shown in Table 3.3bii and Figs. 3.3a-d indicated signals at δ6.49 (1H,d,J=12.4 Hz) and δ6.56 (1H,d,J=10.4 Hz) which are due respectively to *meta* related protons assigned to H-6 and H-8 of a 5,7-dihydroxy substitution in ring A of flavonoid nucleus (Mabry *et al.,* 1970); but due to little unresolved mixture (δ4.85) in the compound, the splitting constant values was higher compared to the literature value. The signals observed at δ6.95 (1H,d,J=8.4Hz) was assigned to H-5′ of ring B, whereas a broad singlet overlapping a doublet at δ7.53 (2H,d, J=8.4Hz) and δ7.98 (2H,d, J=8.8Hz) assigned to H-6′ and H-2′ respectively (Mekonen and Gebreyesus, 2000). The presence of many peaks in the region of δ3.50 – 3.06 is an indication that the compound contained a sugar moiety in its nucleus, while the absence of singlet at δ6.4-7.2 is an indication that the C-3 is substituted (Mekonen and Gebreyesus, 2000). Most anomeric proton appears at δ5.0 ppm in the C-4′ C-5, or C-7 flavonoids which can easily be distinguished from that of flavonol 3-*O*-glucoside, where the anomeric signal appears downfield at about δ5.8 (Mabry *et al.,* 1970). Therefore, the anomeric proton due to C-3-*O*-glucosyl was deshielded due to keto group at C-4 thus and appeared at δ6.24 (1H,d,J=6.8Hz) assigned to H-1″; the 7-*O*-glucose appeared up field at δ5.04 (1H,d,J=10.0Hz) due to H-1‴. The appearance of multiplet in the δ3.50 – 3.06 region integrates for 12 unresolved protons are due to 2 sugar moieties. Other sugar protons are δ4.25 (m, H-6″$_B$), 4.09 (m, H-6″$_A$); 3.93 (m, H-6‴$_B$), 3.84 (m, H-6‴$_A$); 3.80 (m, H-5″), 3.78 (m, H-5‴); 3.69 (m, H-3″), 3.44 (m, H-5‴); 3.19 (m, H-4″), 3.15 (m, H-4‴); 3.13 (m, H-2″), 3.07 (m, H-2‴) for 3-*O*-glucoside(″) and 7-*O*-glucoside(‴) respectively. No singlet was observed at δ3.7–3.9 indicative of

absence of methoxyl group, similarly no peak was noticed between δ0.8-1.2 characteristics of Rhamnose sugar (Mabry *et al.,* 1970; Manguro *et al,* 2005) and hence the absence of these molecules. The compound NBA2b was characterised as Quercetin 3,7-*O*-diglucoside owing to their spectral data characteristics.

CHAPTER FIVE

5.0 BIOLOGICAL SCREENING

The rationale for this studies is to scientifically ascertain the purported traditional claim of some of the uses of this plant in the treatment of rheumatic pains, oedema/inflammation; curing of boils and wound resulting from burns. The efficacy of the extract (NBE) was screened for analgesic and anti-inflammatory activity; the antimicrobial studies was conducted on the crude ethanolic extract (CEE) of the leaves of this plant and its partitioned extracts (NBE and AQE) with the view to finding which of the extracts had the capacity to inhibit microbial growth *in-vitro.*

5.1 PHARMACOLOGICAL STUDIES

The activity of n-butanol soluble portion was tested against the acetic acid-induced writhes and hind paw oedema were determined in mice and rats respectively.

Experimental Animals

Adult male Wistar rats weighing 160 – 250 g and adult male Swiss albino mice weighing 18 – 27 g were obtained from Animal house, Department of Pharmacology and Clinical Pharmacy, kept under well-ventilated conditions, fed on standard feeds (Excel feeds Plc) and allowed water *ad libitum*.

5.1.1 Acute Toxicity Studies (LD_{50})

LD_{50} determination was conducted using the method of Lorke, (1983). In the initial phase mice were divided into 3 groups of three mice each and treated with ethanol extract at doses of 10, 100 and 1000 mg/kg body weight *i.p* and observed for 24 hour. In the final phase, 4 mice were divided into 4 groups of one mouse each and ethanol extract

administered at doses of 80, 160, 320, and 640 mg/kg body weight *i.p* and the final LD_{50} value calculated.

5.1.2 Anti-nociceptive Studies in Mice

This test was conducted employing the method described by Koster *et al.*, (1959). Swiss albino mice were divided into 5 groups of 6 mice each. The first group served as control, (3% gum acacia, 1 ml/100 g body weight) while groups 2, 3 and 4 received the extract at doses of 25, 50 and 100 mg/kg body weight *i.p* and the 5^{th} group was given piroxicam at a dose of 20 mg/kg *i.p.* 30 minutes later, all the groups were treated with acetic acid (0.06%, l ml/100 g *i.p.*). Mice were placed in an individual cage. The number of abdominal constrictions was counted 5 minutes after acetic acid injection for a period of 10 minutes. Percentage inhibition of writhing was obtained using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Mean Number of writhing (control)} - \text{Mean No. of writhing (test)}}{\text{Mean Number of writhing (control)}} \times 100$$

5.1.3 Anti-inflammatory Studies in Rats

A modification of the method of Winter *et al.*, (1963) was used. Male rats were divided into four groups (n = 6) and treated as follows, 3% gum acacia solution (control) 1 ml/100 g; 50 and 100 mg/kg body weight of the extract *i.p.* and piroxicam 20 mg/kg body weight *i.p*. After 30 minutes each group was administered 0.1 ml raw egg albumin sub planter to the left hind-paw. Measurements of the foot volume were done using a digital plethysmometre (Ugo basile LE 7150) at 20 minutes interval for 120 minutes after albumin treatment.

5.1.4 Statistical Analysis

The results of the experiment were expressed as Mean $\pm$ SEM. Statistical analysis was carried out using the Students t – test with level of significance set between $P< 0.05$ and $P< 0.001$ (Duncan *et al.*, 1977).

5.2 ANTIMICROBIAL STUDIES

Many African plants are used in traditional medicine as antimicrobial agents but only few are documented (Lewis and Elvin-Lewis, 1977, Bellomaria and Kacou, 1995). The use of plants is as old as mankind and in the coming years, the market will see new products containing natural products (Dweck, 1996). Since over 80% of the world's population use plant as their primary source of medication (Farnsworth *et al.;* 1985; Cordell, 2000) and in view of the fact that antibiotics are sometimes associated with adverse side effects to the host including hypersensitivity, immuno-suppressive and allergic reactions (Ahmad *et al.*, 1998), it is of interest to develop alternative antimicrobial drugs such as medicinal plants for the treatment of infectious diseases (Clark, 1996, Cordell, 2000) and thus the purpose for this study.

5.2.1 Bacterial and Fungal strains

The Gram – positive organisms used in this study are: *Bacillus subtilis* (NCTC 8326 B76) and *Staphylococcus aureus* (ATCC 021001), while Gram – negative organisms are: *Escherichia coli* (ATCC 11775) and *Pseudomonas euroginosa* (ATCC 10145). The fungal strains are *Candida albicans* and *Aspergillus niger* – which are laboratory isolates. All the organisms were obtained from the Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria – Nigeria.

5.2.2 Antimicrobial Susceptibility Studies

A preliminary antimicrobial activity of the 3 extracts (CEE, NBE and AQE) were determined using disc diffusion technique as described by Chung *et al.,* 1990, by preparing a stock extracts concentration of 100 mg/ml prepared by dissolving l g of each extract into 10 ml of sterilized distilled water. The micro organisms were maintained on agar slants. The inocula were prepared by inoculating the test organisms in nutrient broth and incubated for 24 hours at 37^{o}C. After incubation, the broth cultures were diluted to 1:1000 for Gram-positive bacteria and 1:5000 for the Gram-negative bacteria. One millilitre of the diluted cultures was inoculated into 19 ml sterile molten nutrient agar (48^{o}C) and poured into sterile Petri dishes. These were gently swirled and allowed to solidify. Afterwards, wells were bored into the solidified and inoculated nutrient agar plates using number IV cork borer. All the wells were filled with equal volumes of 0.1 ml (10 mg/hole) of each extract. Ciprofloxacin and ofloxacin standard disc (5 μg each) was placed on the agar plate. About 2 hours was allowed for the extract to diffuse into the agar. Plates were then incubated overnight at 35 ^{o}C and 37 ^{o}C for fungi and bacterial tests respectively.

At the end of the incubation period, inhibition zones were recorded in millimetres as the diameter of growth free zones around the bored holes using a transparent metre rule. Each extract and standard antibiotics were independently tested in duplicate. Diameters of zones of inhibition $\geq$ 10 mm were considered active (Zwadyk, 1972).

Minimum Inhibitory Concentration (MIC)

MIC was determined using the broth dilution technique (Sidney *et al.,* 1978; Vollekovà *et al.,* 2001). The minimal inhibitory concentration value was determined for the microorganisms that were sensitive to the extracts under study (CEE, NBE and AQE). The microorganisms were prepared as described earlier. Each extract was first diluted to the highest concentration (100 mg/ml) in sterile distilled water, and then two-fold serial dilution of each extracts were made to a concentration ranging from 0.098 - 50 mg/ml using nutrient broth. The extracts were inoculated with 0.2 ml suspension of the organisms. MIC is defined as the lowest concentration where no visible turbidity was observed in the test tubes.

Minimum Bactericidal Concentration (MBC)

MBC were determined by using the broth dilution technique previously described by Vollekovà *et al.,* (2001) by assaying the test tubes resulting from MIC determinations. A 1oopful of the content of each test tube was inoculated by streaking on a solidified nutrient agar plate and then incubated at 37^{o}C for 24 hours and observed for bacterial growth. The lowest concentration of the subculture with no growth was considered the minimum bactericidal concentration.

5.3 RESULTS

Table 5.1: Effects of n-butanol soluble portion on acetic acid-induced writhing in mice

Treatment	Dose (mg/kg body weight)	Number of abdominal constrictions	% inhibition	P value
3% gum acacia solution (*i.p.*)	-	[b] 23.4±3.69	-	
Extract (*i.p.*)	25	[a] 3.60±1.61	84.6	P<0.001
Extract (*i.p.*)	50	[a] 5.00±1.34	78.6	P<0.01
Extract (*i.p.*)	100	[a] 6.25±1.70	73.3	P<0.01
Piroxicam (*i.p.*)	20	[a] 4.83±0.70	79.4	P<0.001

Table 5.2a: Effects of the n-butanol soluble portion and controls on egg albumin-induced oedema in rats.

Treatment	Paw volume (ml) at various time (minutes)							
	Dose (mg/kg body weight)	**0**	**20**	**40**	**60**	**80**	**100**	**120**
3% gum acacia solution (negative control)	-	0.310± 0.016	0.440 ± 0.031	0.380 ± 0.037	0.450 ± 0.075	0.390 ± 0.060	0.390 ± 0.035	0.360 ± 0.033
Extract	50	^{c}0.190 ± 0.027	^{d}0.190 ± 0.034	^{c}0.210 ± 0.031	^{d}0.210 ± 0.031	^{c}0.200 ± 0.026	^{c}0.180 ± 0.032	^{b}0.160 ± 0.050
Extract	100	^{c}0.190 ± 0.027	^{c}0.240 ± 0.026	^{b}0.260 ±0.028	^{d}0.210 ± 0.028	^{d}0.160 ± 0.029	^{d}0.130 ± 0.022	^{d}0.130 ± 0.007
Piroxicam (positive control)	20	^{c}0.150 ± 0.034	^{c}0.220 ± 0.016	^{b}0.200 ± 0.015	^{d}0.160 ± 0.015	^{d}0.130 ± 0.019	^{d}0.120 ± 0.007	^{d}0.140 ± 0.045

b = P< 0.05, c = P< 0.01, d = P<0.001

80

Table 5.2b: Percentage inhibition expressed by n-butanol soluble portion and piroxicam on egg albumin-induced oedema in rats.

		% Inhibition at various time (minutes)							Mean inhibition (%)
Treatment	**Dose (mg/kg body weight)**	**0**	**20**	**40**	**60**	**80**	**100**	**120**	
Extract	50	38.7	56.8	44.7	53.3	60.8	53.8	55.6	51.96 ± 2.88
Extract	100	38.7	45.5	31.6	53.3	68.6	66.7	63.9	52.61 ± 5.50
Piroxicam	20	51.6	50.0	47.4	64.4	74.5	69.2	61.1	59.74 ± 3.92

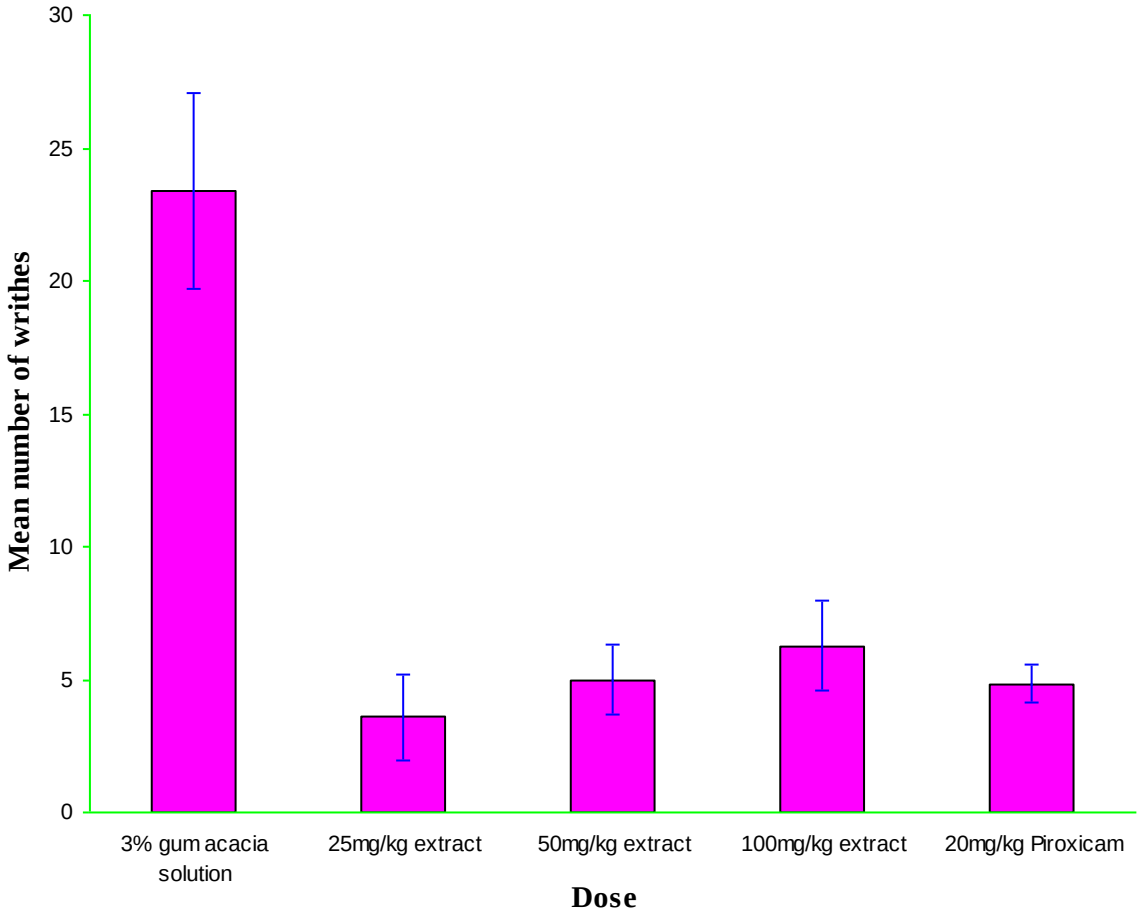


Fig 5.1a. Effects of n-butanol portion on acetic acid-induced writhing in mice

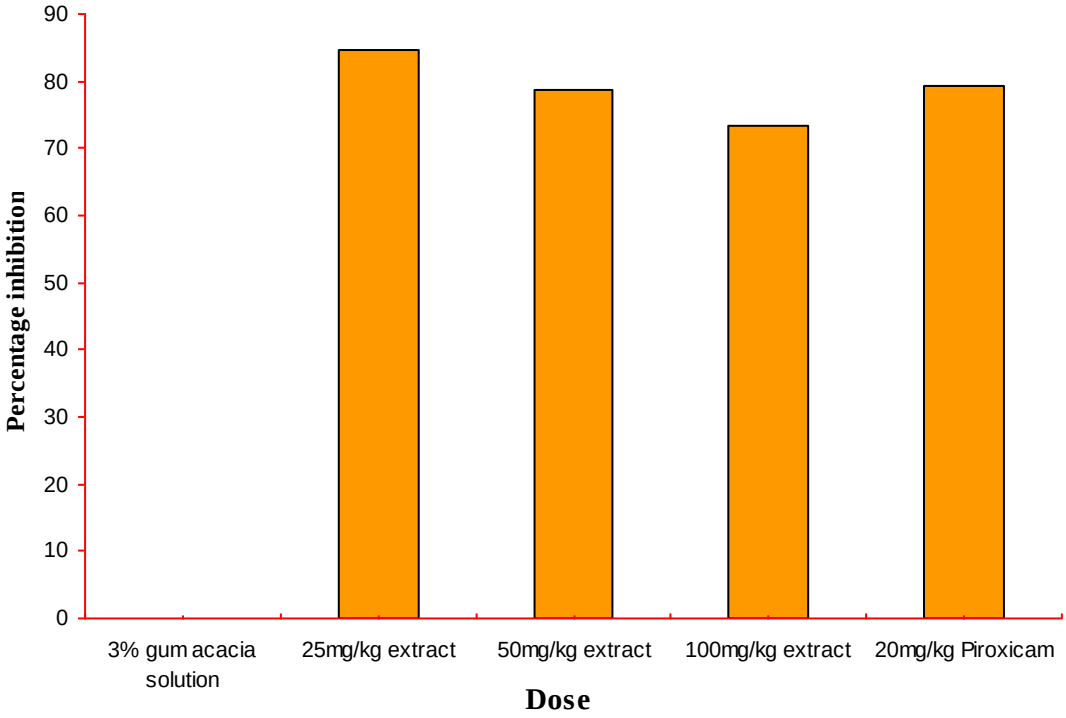


Fig. 5.1b. Percentage inhibition of acetic acid-induced writhing produced by *i.p* . pre-tretment with n-butanol portion

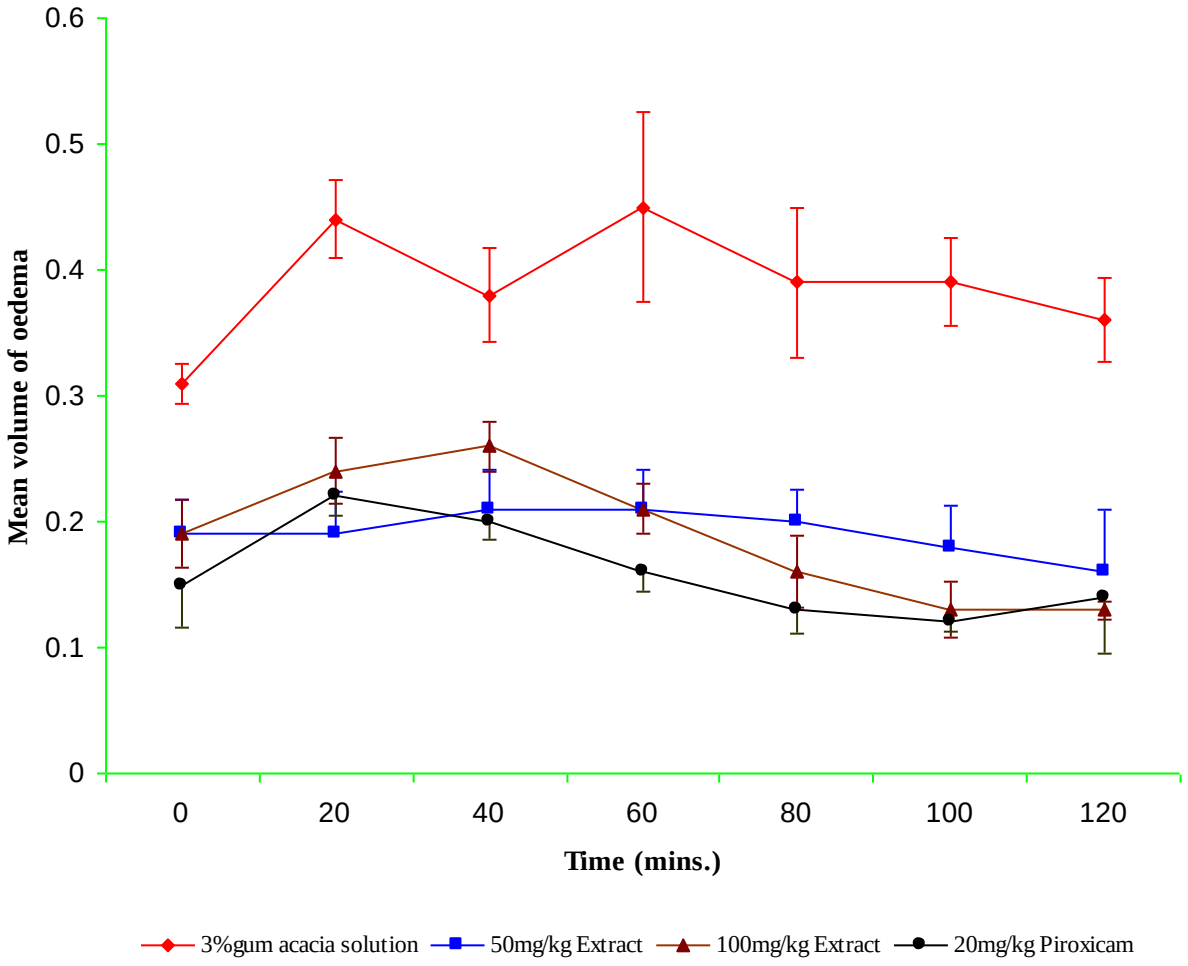


Fig. 5.2a. Effects of n-butanol portion on egg albumin-induced paw oedema in rats

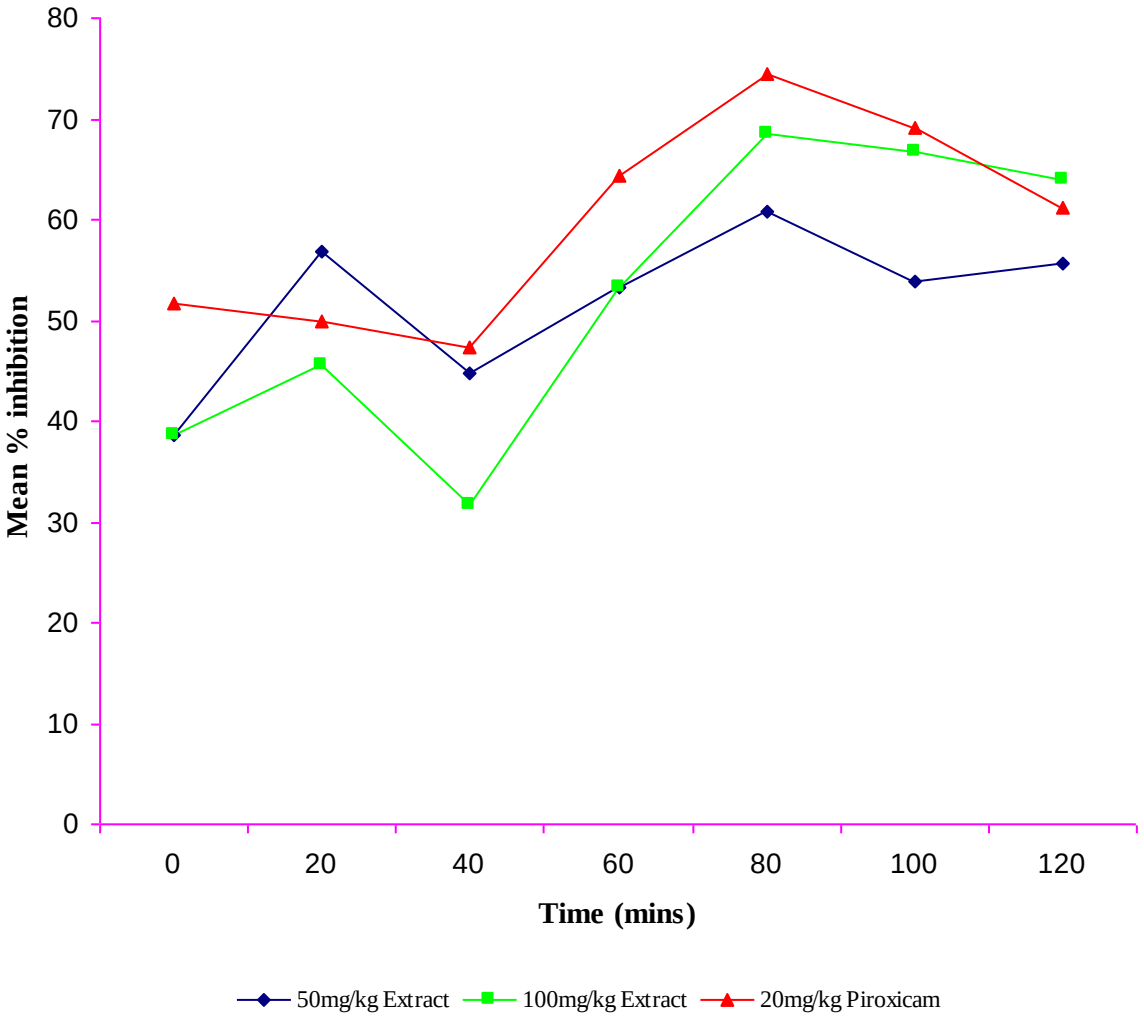


Fig. 5.2b. Effect of n-butanol soluble portion and Piroxicam on egg albumin-induced oedema expressed as percentage

Table 5.3: Antibacterial and Antifungal effects of the extracts

		Organisms zone of inhibition (mm)					
Extracts / Standards	**Conc. (mg/hole)**	**S.a**	**B.s**	**E.c**	**P.e**	**C.a**	**A.n**
CEE	10	11.0 ± 0.0	12.0 ± 0.0	11.5 ± 0.5	∗	∗	∗
NBE	10	11.5 ± 0.5	13.0 ± 0.0	11.0 ± 0.0	12.0 ± 0.0	∗	∗
AQE	10	11.0 ± 0.0	12.0 ± 0.0	∗	∗	∗	∗
Ofloxacin	5µg/disc	15.0 ± 0.0	24.0 ± 0.0	25.0 ± 0.0	35.0 ± 0.0	⊗	⊗
Ciprofloxacin	5µg/disc	21.0 ± 1.0	26.0 ± 0.0	29.5 ± 0.0	20.0 ± 0.0	⊗	⊗

Key: S.a = *Staphylococcus aureus*, B.s = *Bacillus subtilis*, E.c = Escherichia coli, P.e = *Pseudomonas euroginosa*, C.a = *Candida albicans*, A.n = *Aspergillus niger*, CEE = crude ethanolic extract, NBE = n-butanol portion, AQE = residual aqueous portion, ⊗= not tested, ∗ = no inhibition.

Table 5.4 Minimum inhibitory concentrations expressed by the extracts

Extracts	Organisms	Concentration (mg/ml)									
		0.098	**0.195**	**0.390**	**0.780**	**1.563**	**3.125**	**6.250**	**12.500**	**25.000**	**50.000**
NBE	P.e	+	+	+	+	+	+	+	α	α	α
NBE	E.c	+	+	+	+	+	+	α	α	α	α
CEE	E.c	+	+	+	+	+	+	+	α	α	α
NBE	S.a	+	+	+	+	+	+	+	+	α	α
CEE	S.a	+	+	+	+	+	+	α	α	α	α
AQE	S.a	+	+	+	+	+	+	+	α	α	α
NBE	B.s	+	+	+	+	+	+	+	+	α	α
CEE	B.s	+	+	+	+	+	+	+	+	α	α
AQE	B.s	+	+	+	+	+	+	+	+	α	α

Key: S.a = *Staphylococcus aureus*, B.s = *Bacillus subtilis*, E.c = Escherichia coli, P.e = *Pseudomonas euroginosa*, C.a = *Candida albicans*, A.n = *Aspergillus niger*, CEE = crude ethanolic extract, NBE = n-butanol portion, AQE = residual aqueous portion, + = visible growth, α = no visible growth.

Table 5.5 Minimum bactericidal concentrations expressed by the extracts

Extracts	Organisms	Concentration (mg/ml)									
		0.098	**0.195**	**0.390**	**0.780**	**1.563**	**3.125**	**6.250**	**12.500**	**25.000**	**50.000**
NBE	P.e	+	+	+	+	+	+	+	+	ϕ	ϕ
NBE	E.c	+	+	+	+	+	+	+	ϕ	ϕ	ϕ
CEE	E.c	+	+	+	+	+	+	+	+	ϕ	ϕ
NBE	S.a	+	+	+	+	+	+	+	+	+	ϕ
CEE	S.a	+	+	+	+	+	+	ϕ	ϕ	ϕ	ϕ
AQE	S.a	+	+	+	+	+	+	+	+	+	ϕ
NBE	B.s	+	+	+	+	+	+	+	+	+	ϕ
CEE	B.s	+	+	+	+	+	+	+	+	+	ϕ
AQE	B.s	+	+	+	+	+	+	+	+	+	ϕ

Key: S.a = *Staphylococcus aureus*, B.s = *Bacillus subtilis*, E.c = Escherichia coli, P.e = *Pseudomonas euroginosa*, C.a = *Candida albicans*, A.n = *Aspergillus niger*, CEE = crude ethanolic extract, NBE = n-butanol portion, AQE = residual aqueous portion, + = growth, ϕ= no growth.

5.4 DISCUSSIONS

Acute toxicity studies (LD_{50}) of the ethanol extract was found to be 226.3 mg/kg body weight in mice *i.p.* The extract at doses between 25 and 100 mg/kg body weight significantly decreased the number of acetic acid-induced writhes. All values were significant ($P< 0.001$) compared with negative control as shown in Table 5.1 and Figs. 5.1a, b. Thus, activity resides more at lower doses of the NBE extract (25 mg/kg body weight), highest percentage inhibition was observed (84.6%) at a dose of extract 25 mg/kg body weight numerically above that of piroxicam 20 mg/kg body weight (79.4%). The method employed in this study also called abdominal constriction response, is very sensitive and able to detect anti-nociceptive effects of compound(s) at dose level that may be inactive in other methods like tail-flick test (Collier *et al.,* 1968; Bentley *et al.,* 1981). The abdominal constriction response is postulated to partly involve local peritoneal receptors (Bentley *et al.,* 1983). Since the extract under study contains Quercetin 3,7-O-diglucoside and owing to the fact that analgesic and/or anti-inflammatory effects has been reported with flavonoids as well as tannins (Ahmadiani *et al.,* 1998, 2000). It is therefore, pertinent to say that the extract may be a good source of analgesic, since quercetin which has been earlier reported to have analgesic and anti-inflammatory activities (Shahidi *et al.,* 1998). It is then possible that the active component of the NBE could be quercetin 3,7-O-diglucoside; since it is a derivative of quercetin.

The extract also possesses a significant anti-inflammatory effect comparable to that of piroxicam 20 mg/kg body weight. Results shown in Table 5.2a and b, and Figs. 5.2a, b revealed that the extract caused a dose dependent inhibition of albumin-induced oedema over a period of 120 minutes. Peak inhibitory effects of the extract (68.6%) was observed at a dose of 100 mg/kg body weight, 80 minutes after treatment; however,

highest percentage inhibition was observed with piroxicam 20 mg/kg body weight and all results were tested significant ($P<0.001$) compared to negative control. The extract under study, as shown in Table 3.1 was flavonoid-rich portion as quercetin 3,7-O-diglucoside (Flavonol) was isolated; it is therefore, pertinent to say that results agreed with the findings of Duke, (1992) that, flavonoids isolated from medicinal plants have been proven to possess anti-nociceptive and/or anti-inflammatory effects. Other flavonoids (Manthey, 2000) potently inhibit prostaglandins, a group of powerful pro-inflammatory signalling, molecules. Manthey *et al.,* (2001) reported that flavonoids also inhibit phosphodiesterases involved in cell activation, much of whose effect is upon the biosynthesis of protein cytokines that mediate adhesion of circulating leucocytes to sites of injury. Protein kinases are another class of regulatory enzymes affected by flavonoids. Thus, the inhibition of these key enzymes provides the possible mechanism by which the extract (NBE) acts on inflammation, hence these activities exhibited by the extract could possibly be related to quercetin 3,7-O-diglucoside.

The antibacterial and antifungal activities of the individual extracts are summarized in table 5.3. The NBE showed broad-spectrum activity against the test bacteria, while AQE had activity only on *Staphylococcus aureus* and *Bacillus subtilis.* CEE also had a broad activity but was not able to inhibit the growth of *Pseudomonas aeruginosa*. None of the extracts showed any antifungal effects on the test organisms *(C. albicans and A. niger).* The two antibiotics disc used in this study inhibited the growth of the test bacteria. The zones of inhibition produced by the antibiotic disc were higher than those produced by the three extracts. The antibacterial activity exhibited by the extracts could not be unrelated to the presence of carbohydrates, alkaloids, flavonoids, saponins, tannins in the leaves extracts of this plant. These classes of compounds are known to

show curative activity against several pathogens (Hassan *et al.*, 2004) and therefore could explain its use traditionally for the treatment of wide array of illnesses.

The results in Table 5.4 showed that, NBE had MIC of 6.25 mg/ml against *E. coli.* CEE and AQE showed MIC of 6.25 mg/ml and 12.5 mg/ml respectively against *S. aureus.* All the extracts exhibited equal concentration of 25 mg/ml as the MIC on *B. subtilis.* The minimum bactericidal concentrations of 12.5 mg/ml against *E. coli* and 50 mg/ml against both *S. aureus* and *B. subtilis* was shown by NBE, while CEE showed a bactericidal concentration at 6.25 mg/ml towards *S. aureus* and a 50 mg/ml against *B. subtilis;* AQE showed an MBC of 50 mg/ml against both *B. subtilis* and *S. aureus* as presented in Table 5.4.

From the results of the MIC and MBC, it can be surmised that the n-butanol soluble portion (NBE) expressed the broadest activity, as it cut across both Grams positive and negative organisms utilized in this work. The NBE had a very good inhibition effects on the Gram negative organisms *(E. coli and P. aeruginosa)* with 6.25 mg/ml, 12.5 mg/ml and 12.5 mg/ml; 25 mg/ml, as MIC and MBC respectively. The CEE, on the other hand exhibit strong inhibitory effects against the Gram-positive bacteria *S. aureus* (having a concentration of 6.25 mg/ml as MIC and MBC), a pyogenic bacteria known to play a significant role in invasive skin diseases including superficial and deep follicular lesion (Srinivasan *et al.,* 2001). AQE displayed only little inhibitory effects against Gram-positive organism with none on Gram-negative bacteria.

The broad-spectrum antibacterial activity exhibited by the NBE and CEE could not be unrelated with the high intensities of flavonoids and tannins in these extracts. In line with these findings, early studies had revealed that flavonoids may posses multi-targeting antibacterial properties (Okurelu and Ani, 2001; Sato *et al.,* 2002), it could therefore,

surmised that the presence of quercetin 3,7-O-diglucoside and possibly other flavonoids compounds in the extract aided these activities. Trease and Evans (1978), reported that tannins had been widely used as an application to sprains, bruises and superficial wounds. The inability of AQE to inhibit the growth of Gram-negative organism could be probably due to high level of glycosides (saponins and carbohydrates); as sugars are being utilized by the bacteria for their cellular and metabolic activities.

CONCLUSIONS

Extensive phytochemical studies were carried out on the leaves extracts of *Celtis integrifolia*. Two compounds were isolated from the petroleum ether extract identified as Palmitic and Stearic acid; consequently, flavonol diglucoside identified as quercetin 3,7-O-diglucoside was isolated from the n-butanol soluble portion of ethanolic extract. Thus, the presence of this flavonoid and some other classes of secondary metabolites in the extracts studied could be the possible constituents responsible for its wide array of medicinal values.

The pharmacological screening revealed that the extract from the leaves of this plant possesses significant anti-nociceptive and a moderate anti-inflammatory effects, which revealed by the n-butanol extract. Thus the n-butanol portion of the ethanol extract could be a strong analgesic and/or anti-inflammatory agent(s), as the plant is used locally as a remedy for rheumatic pains and burns.

It can be concluded from the antimicrobial studies that the extracts could moderately inhibit microbial growth. This finding corroborates to some other uses of this plant in traditional medicine as a remedy for the treatment of wounds, burns and stomach discomfort among others that are caused by most of the organisms used in the study. From these findings, the extract from the leaves of this plant could not be use as a remedy for wounds or other skin infections/lesions as a result of fungal infestation.

Further work needed to be done in order to authenticate the basis for the traditional claim of this plant.

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APPENDICES

Appendix I. Lethal dose (LD_{50}) determinations

First Phase

S/No.	Group	Weight of mice (g)	Dose (mg/kg)	No. of Death
1	1	25	1000	1
2	1	25	1000	1
3	1	23	1000	1
4	2	24	100	0
5	2	25	100	1
6	2	24	100	1
7	3	24	10	0
8	3	16	10	0
9	3	21	10	0

Second Phase

S/No.	Group	Weight of mice (g)	Dose (mg/kg)	No. of Death
1	I	26	80	0
2	II	28	160	0
3	III	28	320	1
4	IV	25	640	1

Appendix II. Anti-nociceptive studies

Group 1. Negative control: 3% Gum acacia solution 1ml/100kg body weight.

S/No.	Weight of mice (g)	Number of abdominal constrictions
1	20	0
2	20	11
3	19	24
4	17	28
5	19	33
6	18	21

Group 2. Extract 25 mg/kg body weight.

S/No.	Weight of mice (g)	Number of abdominal constrictions
1	17	2
2	21	5
3	20	5
4	22	3
5	18	3
6	22	0

Group 3. Extract 50 mg/kg body weight.

S/No.	Weight of mice (g)	Number of abdominal constrictions
1	20	0
2	20	4
3	24	2
4	17	10
5	20	5
6	20	4

Group 4. Extract 100 mg/kg body weight.

S/No.	Weight of mice (g)	Number of abdominal constrictions
1	23	0
2	17	3
3	17	0
4	19	6
5	24	11
6	25	5

Group 5. Positive control: Piroxicam 20 mg/kg body weight

S/No.	Weight of mice (g)	Number of abdominal constrictions
1	17	5
2	23	7
3	17	4
4	24	6
5	26	2
6	22	5

Appendix III. Anti-inflammatory Screening

Group 1. Positive control: Piroxicam 20 mg/kg body weight.

S/No.	Weight of rats (g)	Paw volume (ml) at various time (minutes)						
		0	**20**	**40**	**60**	**80**	**100**	**120**
1	115	0.21	0.20	0.33	0.15	0.21	0.13	0.11
2	130	0.05	0.18	0.12	0.18	0.09	0.12	0.12
3	140	0.13	0.27	0.28	0.21	0.16	0.15	0.16
4	150	0.28	0.26	0.24	0.17	0.13	0.10	0.22
5	138	0.16	0.18	0.09	0.17	0.09	0.11	0.12
6	135	0.09	0.21	0.11	0.10	0.10	0.11	0.08

Group 2. Negative control: 3% Gum acacia solution 1ml/100kg body weight.

S/No.	Weight of rats (g)	Paw volume (ml) at various time (minutes)						
		0	**20**	**40**	**60**	**80**	**100**	**120**
1	223	0.32	0.35	0.43	0.53	0.35	0.40	0.44
2	210	0.36	0.53	0.52	0.52	0.37	0.30	0.41
3	209	0.35	0.52	0.25	0.72	0.67	0.38	0.38
4	211	0.31	0.47	0.37	0.21	0.63	0.52	0.21
5	195	0.25	0.38	0.36	0.40	0.63	0.45	0.35
6	210	0.29	0.39	0.35	0.30	0.43	0.30	0.34

Group 3. Extract 50 mg/kg body weight.

S/No.	Weight of rats (g)	Paw volume (ml) at various time (minutes)						
		0	20	40	60	80	100	120
1	171	0.18	0.31	0.27	0.24	0.12	0.30	0.38
2	150	0.25	0.27	0.12	0.18	0.30	0.10	0.06
3	152	0.21	0.21	0.29	0.13	0.15	0.11	0.11
4	145	0.06	0.11	0.23	0.30	0.18	0.13	0.07
5	185	0.18	0.14	0.21	0.11	0.23	0.21	0.11
6	155	0.23	0.12	0.11	0.28	0.22	0.21	0.21

Group 4. Extract 100 mg/kg body weight.

S/No.	Weight of rats (g)	Paw volume (ml) at various time (minutes)						
		0	20	40	60	80	100	120
1	160	0.25	0.28	0.20	0.16	0.11	0.11	0.15
2	180	0.23	0.28	0.33	0.13	0.09	0.19	0.13
3	167	0.23	0.13	0.19	0.15	0.08	0.07	0.10
4	165	0.09	0.19	0.36	0.28	0.21	0.21	0.12
5	147	0.19	0.28	0.23	0.26	0.24	0.10	0.12
6	162	0.12	0.26	0.26	0.27	0.21	0.12	0.13

Appendix IV. Antimicrobial Susceptibility Test

Antibacterial and Antifungal Activities

		Organisms zone of inhibition (mm)					
Extracts / Standards	**Conc. (mg/hole)**	**S.a**	**B.s**	**E.c**	**P.e**	**C.a**	**A.n**
CEE	10	11.0	12.0	11.0	-	-	-
		11.0	12.0	12.0	-	-	-
NBE	10	11.0	13.0	11.0	12.0	-	-
		12.0	13.0	11.0	12.0	-	-
AQE	10	11.0	12.0	-	-	-	-
		11.0	12.0	-	-	-	-
Ofloxacin	5µg/disc	15.0	24.0	25.0	35.0	NT	NT
		15.0	24.0	25.0	35.0	NT	NT
Ciprofloxacin	5µg/disc	21.0	26.0	29.0	20.0	NT	NT
		21.0	26.0	30.0	20.0	NT	NT