SPECTRAL PROPERTIES AND CHROMOPHORIC CHARACTERIZATION OF *Lannea fulva* AND *Terminalia brownii* DYE EXTRACTS

BY

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DECLARATION

Declaration by the candidate

This thesis is my original work and has not been presented for any course in any other University.

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KEY WORDS AND DEFINITIONS

Auxochromes - groups of atoms that modify ability of chromophore to absorb light

Chromophore - groups in a molecule which consist of alternating single and double bonds (conjugation) and absorb light in visible region

Chromophoric - adjective of any chemical groups that produce colour

Lannea fulva - deciduous shrub of family Anacardiaceae

Spectra - physics of the set of colours into which beam of light can be separated

Terminalia brownii - tree of Combretaceae family which is found in many parts of Africa and it is 15 -18m long.

ABBREVIATIONS

API - Atmospheric Pressure Ionization

CID - Collision induced dissociation

ESI - Electrospray Ionization

FTIR - Fourier Transform Infrared

HOMO - Highly Occupied Molecular Orbital

HPLC - High Performance Liquid Chromatography

JKUAT - Jomo Kenyatta University of Agriculture and Technology

LC - Liquid Chromatography

LC-MS - Liquid Chromatography Mass Spectrophotometer

LC-DAD-ESI-M - Liquid Chromatography Diode Array Detector Electrospray Ionization Mass

LUMO - Lowest Unoccupied Molecular Orbital

m/z - Mass to charge ratio

MS - Mass Spectrophotometer

MSD - Mass Selective Detector

PAD - Photo Array Detector

PDAD - Photo Diode Array Detector

RP-HPLC - Reverse Phase High Performance Liquid Chromatography

TIC - Total Ion Current

UPF - Ultraviolet Protection Factor

UV-VIS -Ultraviolet Visible

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ABSTRACT

Lannea fulva, and *Terminalia brownii,* have been used for decades not only in herbal medicine, but also as a major source of dye for traditional handwork, especially by Marakwet community in Kenya. However, as a source of dye, there exists wide gap in knowledge on the specific chromophores and spectral properties such as: molecular mass, molecular structure and dye disintegration pattern of individual dyestuff compounds responsible for dyeing capability. Similarly, the lack of individual dye components identification has hindered the exploitation of the dyes in modern textile industries in the face of the huge challenges posed by synthetic dyes such as their carcinogenicity to human health and toxicity to environment, more so when applied in textile and printing industries. The main purpose of this study was to assess the dyeing properties and to characterize the compounds responsible for the property in *L. fulva* and *T. brownii* dye extracts. Maceration and soxhlet method were used to extract dyes using both organic and aqueous solvents. Qualitative analysis of the extracts was done using UV-VIS, FT-IR, and LC-MS spectroscopic methods. UV-VIS analysis identified delocalization of different types of functional groups like carbon-carbon double bonds (C=C), carbon-oxygen double bonds (C=O) lone pairs electron on oxygen and benzene rings as the main chromophores responsible for colour. These were confirmed by FT-IR with characteristic absorption spectra displayed at 1598.31 cm^{-1}, attributed to C=C conjugated system common in benzene rings, absorption band at 1401.66 cm^{-1} due to C=O double bond of carboxylic acid and C-OH stretch from polysaccharides shown at frequency 1069.61cm^{-1} to 605.67 cm^{-1} confirmed by LC-MS to be water soluble sugars. *L. fulva* glycosides identified for the first time include: Rubiadin primeveroside, ruberythric acid, lucidin primeveroside. Rubiadin, alizarin, lucidin and xanthopurpurin were also identified in *L. fulva* as aglycones. In *T. brownii* for example, the chromophoric compounds identified include: Catechin, epiChatechin, genipin and baccatins. Gentianose, geniposide and genipin were reported for the first time to be present in *T. brownii.* The identification on *T. brownii* compounds were based on two peaks at UV-VIS λ_{max} 258.5nm which was identified as C=C owing to delocalized electron from fluorescent aromatic compounds (benzene rings) confirmed by FT-IR by absorption band occurring at 1597.61 cm^{-1} and 1400.77 cm^{-1} which is attributed to stretching of C=C of aromatic/benzene rings. The second peak with absorbance at λ_{max} 361 nm was attributed to C=O due to lone pairs electron on the oxygen atom causing absorbance to occurs at longer wavelength. This was further confirmed by FT-IR at frequency 3131.94 cm^{-1}due to stretching vibration of OH from carboxylic acid [COOH] and C-H stretch or CHO spectra displayed at frequency 2935.48 cm^{-1} vibration due to carbohydrates present in geniposide and gentianose which confirmed their decarboxylation in LC-MS. Maceration method of extraction was better in extracting natural dyes compounds from *L. fulva* and *T. brownii* dye extracts. The identification of chromophores and other functional groups was valuable in unlocking full chemical information embedded in the dye structure. The spectral properties, in particular degradation pattern, have bearing in determination of nature of dye dyestuff.

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CHAPTER ONE

1.0 Introduction

1.1 Background of the study

For decades plants remain to be treasured natural products throughout the world. They have profound cultural, educational and social-economic values attached to them (Teklemedhin, 2018). Research by Saravanan, Chandramohan, Saivaraj, & Deepa (2013), noted that natural dyes from plant origin have been part of human life from the ancient times. A Number of communities have been using and applying natural dyes in colouration of natural fibre, leather and wool. This is due to wide range of colours and shades that they possess (Haddar, Ticha, Meksi & Guesmi, 2016). Amongst Marakwet community in Kenya, *L. fulva*, common names: Marakwet and Tugen: Lolotwo; Luo: Logologo and *T. brownii,* common name: Marakwet, Pokot and Tugen: Koloswo; Swahili: Mbarao; Luo: Ong'ang'; Luhya: Shirah; Kamba: Muuku and Embu: Mururuku, have been used for decades not only in herbal medicine, but also as a major source of dye for traditional handwork.

Literature has further shown that organic dyes derived from plant origin are most preferred by green minded consumers due to their medicinal value (Venkatalakshmi, Vadivel, & Brindha, 2016).

At the moment, the call for these natural dyes is snowballing worldwide due to toxicity of synthetic colours. Interestingly, recent research by Malinauskiene (2012) noted that the usage of artificial dyestuff in colouring and printing industry has been vilified owing to introduction of contaminants and noxious waste into the environment. Most of these

synthetic dyes are disperse dyes which are the greatest causative agents of allergens amongst textile dyes. They pose health hazards when they are discharged into rivers, lakes and oceans (Krizova, 2018). In addition, they also release toxic amines and reductive cleavage of azo-linkages. Nowadays, human is drifting towards the application of eco-friendly and biodegradable dyestuff component in textile and printing industry. This increase appeal for natural dyes is driven by strong believe that they are safe unlike man-made dyes that have been proven to be noxious to human and detrimental to the environment (Ali, Hussain, & Nawaz, 2009). It is for this reason that research by Chaitanya (2014) showed scientists in field of natural dyes to be continuously working towards improvement of natural dye quality so as to minimize or eliminate batch to batch variation that has been the main problem associated with natural dyes. Majority of natural dyes show soft, soothe shade and colours when applied on a fabric. However, Lee, Kang, Lee, & Lee, (2013) noted that the effectiveness of natural dye has been weighed down by batch to batch variation due to complexity of dye chromophores and co-existence of dye chromophores with other contaminants.

Consequently, research by Saldanha, Vilegas, & Dokkedal (2013) showed that natural dyes efficacy, safety, quality and repeatability can be enhanced by analyzing and characterizing all chromophoric compounds present in crude extract so as to identify and separate specific dye chromophores that cause batch-to-batch variation. This has net effect of improving dye colour and quality. Essentially, natural dye colour is a measure of quality of product by appealing better to consumers which in turn influence them to buy the product (Kundal, Singh, & Purahit, 2016).

In determination well-known natural dye colour, research has shown that when chromophoric compound (molecule or part of molecule) is subjected to UV-VIS (Zainuri, Razak, & Arshad, 2018), or high quantized energy (exhibiting both wave and particle like properties), it absorbs higher frequency photons in form of ultraviolet or visible of wavelength equivalent to the difference between antbonding orbital (highest occupied molecular orbital) and bonding orbital (lowest unoccupied molecular orbital) commonly called HOMO-LUMO energy gap (Rahmani, Boukabcha, Chouaih, Hamzaoui, & Goumri-Said, 2018). The wavelength and energy-photon absorbed by the chromophore is used to excite and promote electrons in bonding or non-bonding orbital into one of the empty anti-bonding orbitals ($\pi \rightarrow \pi^*$ and/or $n \rightarrow \pi^*$) (Zainuri et al., 2018).

However, electrons at higher energy state are usually unstable and they will quickly fall back to the lower energy state. Generally, this fall emit photon with energy equal to the difference in energy levels. This is measured as absorbed wavelength (El Kouari, Migalska-Zalas, Arof, & Sahraoui, 2015). Ordinarily, electrons transition and absorbed wavelength are characteristic of certain functional groups within the fluorescent or aromatic conjugated system. The absorbed wavelength is unique to the chromophoric compound and acts like the fingerprint of each functional group or chromophores present (Khalid et al., 2017).

Such properties of electron transition in UV-VIS can be employed to tentatively classify the chromophoric compounds or functional groups present in the dye molecule which can be confirmed further using FT-IR, which convert light absorption for each mirror into light absorption of each wavelength (actual spectrum) due to specific rotational or vibrational energy (Salah, 2015; Arjunan, Rani, Mythili, & Mohan, 2011).

The specific molecule or chemical structure will produce unique fingerprint depending on the structure and functional groups present. The individual compound can additionally be identified by comparing the absorption peak with literature or to data bank of spectra.

According to Vlase, Muntean, Cobzac, & Lorena (2014), chromophoric compounds in dyes can be separated into individual dyestuff components by RP-HPLC due to difference in affinity of mobile and stationary phase. The stationery phase in RP-HPLC is non-polar or slightly polar whereas the mobile is always polar such as methanol, acetonitrile, tetrahydrofuran or water. However, previous research by Nowik (2000) had suggested that hyphenated LC-DAD-ESI-MS method of analysis provides definitive structural and molecular mass information for confirmation of each dye component, especially when atmospheric pressure ionization (API) and mass selective detector (MSD) are used in line with photo diode array detectors (PDAD). These two detectors will allow pre-selective of coloured compounds prior to analysis.

Additionally, research by Singh and Srivastava, (2015) recognized the analysis of spectral and chromophoric properties of natural dyes has green area in field of textile research due to overemphasis on environmental protection as a consequence of huge challenges posed by utilization of chemicals from synthetic dyes during coloration. Generally, spectral and chromophoric evaluation of any natural dye producing plants will reveal the exact chromophoric compounds responsible for dyeing capabilities and improve the dye quality and safety.

This natural dye quality has become extremely important in international markets which focus on protecting consumers from lethal and undesirable effects of artificial dyes which have been proven to pose risk to both human health and environment. Recent research by

Shabbir et al. (2016) has shown that these natural dyes are safe and have medicinal properties. They have been used in textile functional treatment such as de-odorizing, antimicrobial, anti-allergic, anti-feedant, fluorescence and UV protection factor. Historically, Marakwet sub-tribe of the Kalenjin community in Kenya have been using *L. fulva* (Anacardiaceae) family and *T. brownii* (Combretaceae*)* family as ethno botanical medicinal plants (Kioko, Baker, Shannon, & Kiffner, 2015; Kigen et al., 2014). The two ethno medicinal plants are also highly treasured by Marakwet craftsmen/women because they also double up as dyeing plants. Over the years, plants have been used in dyeing of sisal fibre for making traditional baskets, locally known as Kiyondo. There is however, limited research carried out on the analysis of spectral chromophoric compounds responsible for dyeing properties. This research attempts to bridge the gap in knowledge which exists by revealing the spectral and chromophoric properties responsible for dyeing capabilities of these two plants. Further, this research aims to evaluate the spectral properties and characterize chromophoric compounds responsible for dyeing capabilities in *L. fulva* and *T. brownii* dye extract.

1.2 Problem Statement

At present, there is general concern about toxicity of synthetic dyes applied in textile and printing industries because they have been proven to be toxic to human and hazardous to environment (Ali et al., 2009). The chromophoric compounds and spectral properties of *L. fulva* and *T. brownii* used by traditional dyers and home craft in Marakwet community have not been well evaluated and documented despite their widespread use. Though, these plants have been used in dyeing of sisal fibre for making traditional baskets, locally known as

"Kiyondo", there is little or no research that has been carried out on the analysis of spectral chromophoric compounds responsible for dyeing properties.

The absence of chromophoric and spectral properties analysis in most of natural dyeing plants has resulted to batch-to-batch variation during dyeing using natural dyes. Recent research eludes this as major challenge hampering utilization of dyes from natural origin. Research by Thiyagarajan, Balakrishnan, & Tamilarasi (2015), attributed this challenge to the existence of unknown chromophores with other degradants. Majority of chromophoric compounds and degradants have different degradation pattern resulting to colour variation. Similar research done by Prashanth, Kumar, Madhu, & Sagar (2011) noted that improper identification of functional groups responsible for dyeing properties has greatly affected the efficacy, quality and utilization of natural dye. So far, the functional groups responsible for dyeing capabilities in *L. fulva* and *T. brownii* crude extract have not been identified.

1.3 Objective

1.3.1 General objective

The main objective of this study was to qualitatively analyze the natural dye compounds present in *L. fulva* and *T. brownii* dye extracts.

1.3.2 Specific objectives

The specific objectives of this study were to:

(i) Evaluate the extraction methods and solvents that gives high dyes yield from *L. fulva* and *T. brownii* plant material

(ii) Identify the functional groups responsible for dyeing properties

(iii) Determine the spectral properties of the dye extracts

(iv) Characterize the chromophoric compounds present in the dye extracts

1.4 Justification

The use of natural dyeing plants by traditional dyers and home craft users in both developed and third world countries is on increase in the demand. This is driven by the belief that they are harmless, possess medicinal properties and biodegradable (Budeanu, Curteza, & Radu, 2014). *L. fulva* of the family of Anacardiaceae and *T. brownii* of Combretaceae family are examples of plants that have been used by Marakwet community in Kenya as herbal medicine and dye for home craft (Kigen, et al., 2014). The analysis and identification of individual chromophoric compounds and spectral properties responsible for dyeing properties is important for minimizing or eliminating the batch to batch variation commonly associated with the use of natural dyes. This will enhance uniformity of colour and shade. Unlike toxicological effect and environmental pollution resulting from the use of synthetic dyes, this research will attempt to bridge the gap in knowledge which exists in *L. fulva* and *T. brownii* dye extract by revealing the spectral and chromophoric properties responsible for dyeing properties. Therefore, spectral and chromophoric characterization of natural dyes aids in the identification and separation of specific chromophore responsible for colour variation.

L. fulva and *T. brownii* have been used as ethno medicinal plant by Marakwet community since time immemorial (Kigen, et al., 2014). Recent research by Shabbir et al. (2016) noted that natural dyes can as well be used in textile functional treatment such as de-odorizing, anti-microbial, anti-allergic, anti-feedant, fluorescence and UV protection factor. In achieving that, Mena et al. (2012) noted that comprehensive identification of phenolic compounds from plants is central in assessing chromophores or in analyzing and separation of chromophore from other degradants which aid in determination of fastness properties, especially wash and light fastness.

In essence, individual dye chromophore in natural dye complex differs from one another only on the position of functional group (auxochromes). These functional groups or auxochromes act as centers for mordant (metallic ion) which helps in improving fastness to fabric, resulting to dramatic change in the colour of the natural dye extract (Upadhyay & Choudhary, 2012). Therefore, by identifying the functional groups responsible for dyeing properties in *L. fulva* and *T. brownii* dye extract, colour variation and shade can easily be achieved. Besides, such identification will be significant in improving efficacy and quality of the dyes. Recent research by Ford, Rayner,& Blackburn (2015) noted that functional groups such as sugar moiety in natural dyes often provides ample potential for binding to fibre or metal ion (mordant) during dyeing. The analysis of the dye extract from these two plants will enable the incorporation of metal ion to the functional groups that will improve dye quality, efficacy and enhance colour fastness (Kundal et al., 2016).

1.5 Hypothesis of the study

For objective one (i) of the study to be achieved and satisfied, two hypothesis were formulated and tested at significant level of 0.05.

Null hypothesis 1 (H_{01}), the amount of dye extracts does not vary with method of extraction.

Null hypothesis 2 (H_{02}), the extracts does not vary with solvent used during extraction.

CHAPTER TWO

2.0 Literature Review

2.1 Dye

A dye is defined as any synthetic or natural substance that chemically bonds to substrate to which it is being applied to. It also adds colour or changes the colour of something. Depending on the origin, some dyes may require mordant or polyvalent metal ion (Kundal et al., 2016) to form complexes between dye and the fabric after reaction (Packianathan & Karumbayararn, 2010).

2.2 Types of Dyes

2.2.1 Synthetic dyes

These are dyes which are petrochemical in nature and have dominated both the dyeing and textile industries for quite some time (Ali, Shakra, Youssef, & Aysha, 2012). Recent research by Malinauskiene, (2012), has confirmed that synthetic dye is made up of chemical compounds that can pose a health risk to persons handling it as well as eliciting multiple side effects such as: skin irritation, erythema, urticaria, and even alopecia (Mbwambo, Erasto, Nondo, Innocent, & Kidukuli, 2011).

This has catalyzed what is referred to as the "return to nature" where most scientists are shifting back to doing research on natural dyes with medicinal properties as viable alternatives to toxic and strictly controlled synthetic dyes (Vlase et al., 2014). Such focus minimizes the use and application of harmful chemicals and creates dyes that are environmentally friendly whose effluent is biodegradable, hence leading to reduction in

water pollution and display matchless appearance on dyed textile or fabric (Sivathasan, 2007).

Previous research by Nilani and Karumbayaram (2010) has noted that the application and use of synthetic dyes have been cited as highly contaminant to the environment because it contains heavy metals like lead, mercury and chromium. Therefore, they have to overcome innumerable regulatory barriers in advance, before it reaches its destination. This has resulted to paradigm shift towards the use of natural dyes (Yusuf, Shabbir, & Mohammad, 2017), which not only offers a rich and diverse source of dyestuff, but also, offers serious prospects of an income, through sustainable yield and contracts from these dyeing plants (Saravanan, et al., 2013).

2.2.2 Natural dyes

These are colours resulting from plants, insects or animals with or without chemical processing (Nilani et al., 2008). The current increase in call for natural dyes is due to consumer awareness about the effect of synthetic dyes. This has made it necessary to search for t naturally derived dyes which are believed not to be harmful. Most of them are non-toxic, non-carcinogenic and biodegradable when compared to the synthetic ones. They have been shown to be eco-friendly and possess health-promoting properties. These unique characteristics in apparel have made it prudent to check out on the use of natural dyes (Anderson, 2009).

Literature has also shown that some of natural dyes containing fragrances which retain freshness to dyed fabric (Singh & Srivastava, 2015). Previous studies have shown that characterization of any natural dye improves the aesthetic value of a dyed fabric, dye quality

and increases financial worth in the market (Chaitanya, 2014). Besides, it imparts unique characteristic of uncommon soothing soft shades, playing indispensable role to finishing process to textile material (Farhan, 2012; & Khan, 2012).

Currently, the demand for natural dyes with known chromophoric and the spectral properties is on increase. This surge is occasioned by non-toxicity, eco-friendliness and medicinal property accompanying natural dyeing (Ali et al., 2009). Flavonoids which are the major coloring factors of most yellow dyes (Islam & Yousif, 2013) are known to be antioxidant just like red anthraquinones alizarin, purpurin and rubiadin which are anti-proliferative. When used as dye they are greatly favoured by environment conscious consumers and have a niche in the market (Saxena & Raja, 2014).

2.3 Extraction and purification of natural dye extracts by HPLC

Majority of natural dyes are glycosylated compounds. Consequently, the choice of solvent and method of analysis is paramount (Ford et al., 2015). Interestingly, research has shown that water and ethanol give higher yield during extractions of glycosylated compounds from plant origin although it has the net effect of hydrolyzing glycosides under high temperatures. In such cases, hexane can be used as a solvent of choice. Further research has revealed that ethanol is known to cause lucidin-ethyl ether by LC-MS when used as solvent at reflux temperature (Henderson, Rayner, & Blackburn, 2013).

Recent research by Ford (2017) showed that hydrolytic enzymes convert the glycosides to respective aglycones as a result of cleavage of glycosidic bond. This makes majority of yellow flavonoid and red anthraquinones to be polar and soluble in nature. Such explains polarity of the dye extract. Usually, chemical reaction between dye and the fabric during

dyeing occurs as a result of numerous hydroxyl groups and sugar moiety in natural dyes. These also functional groups depict their polarity and serve as centers for mordant (metal ions) during dyeing.

2.4 Dye plants

2.4.1 *Lannea fulva*

The *L. fulva* from Anacardiaceae family is found in the lowland areas, mainly with rocky hills and sloping ground with shallow soils. It is mainly used as fruit and anti-venom (Wigrup, 2005). For decades, Marakwet community has been employing the bark of the plant as a source of red or brown dye, anti-inflammatory drugs as well as to remove bitter taste of the guard or calabash before using it for the fermentation of soar milk, locally known as *Mursik*.

Figure. 2.1: Aerial part of *Lannea fulva* (from Oinobmoi in Baringo County)

2.4.1.1 Dyeing property of *Lannea fulva* dye extracts

In Marakwet community, *L. fulva* is locally called *Lolotwo* (Kipkore, Wanjohi, Rono, & Kigen, 2014). The plant is chiefly employed as a basis of red or brown colour for dyeing sisal fibre used in making traditional basket locally known as *Kiyondo*. The colour can be varied from red to dark brown depending on duration of fermentation. Generally, the dye extract produces a very uncommon red colour accompanied by soothing or soft shades which display permanent conspicuous brown-red colour. The degree of the colour depends mainly on duration of fermentation, temperature of the surrounding and/or concentration of the natural dye extract. However, the chromophore responsible for this red colour has not been identified.

2.4.2. *Terminalia brownii*

T. brownii belongs to the family of Combretaceae. In Kenya, for instance, the plant is locally known by different names depending on the local dialects to which it is found. For instance, in Samburu, it is known as *Lbukoi*, in Arabic its known by the name (*subaraya*, *subagh or darot*), in Swahili (*mwahambe*, *Mbarao*), in Pokot, Tugen, Keiyo and Marakwet (*Koloswo*), in Borana (*baressa*), and in Luganda (*Nkalati*) (Duduku, Nithayanandam, & Sarbartly, 2012).

The plant mainly grows in areas such as: woodlands, bushland, grassland and riverine forests with altitudes ranging from 700-2000m above the sea level. The tree is mainly used as firewood, charcoal, timber and for its medicinal purposes. In Samburu, for instance, the stem or bark is used in preparation of hot concoctions for managing yellow fever, gastro intestinal tract and in managing Trypasonomiasis (Divya & Ravi., 2013).

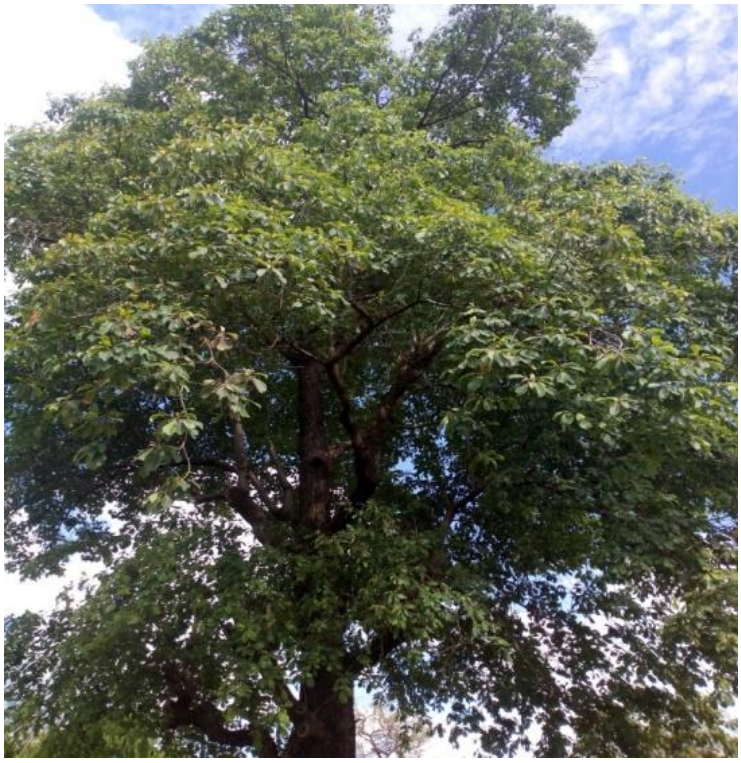

Figure 2.2: Stem and aerial parts of *Terminalia brownii*

(from Samar village in Elgeiyo Marakwet county).

2.4.2.1. Dyeing property of *Terminalia brownii* dye extract

Traditionally, *T. brownii* (Combretaceae) family has been exploited as a source of yellow dye by the Marakwet people. The yellow dye has been used to colour sisal fibre in making traditional baskets, locally known as *kiyondos*. The bark produces a yellow tinge, hence its local name *Koloswo,* meaning yellow. This colour is used to dye sisal fibres for making the traditional basket mentioned earlier. Despite this colouration, there is little knowledge on the specific chromophoric compound responsible for dyeing ability in this plant.

2.5 Analytical methods for spectral properties of natural dye

2.5.1 LC-MS Analysis

Mass spectrometer has been described as a perfect instrument for analyzing dye complex. It is often used to measure mass to charge ratio (*m/z*) of a dye stuff components. The product ions are isolated and fragmented several times to generate molecule-specific fingerprints. The recognized peak is known by comparing retention time and mass spectrum with known standard. In cases where there is no reference standard, the peak will be classified by comparing its retention time and mass spectrum with literature available (tentative determination) (Abdel-Hameed, Bazaid, & Salman, 2013).

ESI-MS has been regarded as accurate and rapid method developed for identification of natural dyes (Tateo, Bononi, & Gallone, 2010). However, UV-VIS and FT-IR, serves as complementary for dye structural identification and functional groups characterization. They require minimum sample because it is not destructive method, hence suitable for empirical investigation of organic colourants (Lee, Kang, Lee, & Lee, (2013). The magnificent characteristics of chromophore manifested by natural dye extract are dependent on the type of the compounds present. This unique phenomenon of dyes depends on the number of the constituents therein. Typically, natural organic dye is well suited for analysis by ESI- MS which is most sensitive for molecular analysis capable of measuring the *m/z* of molecules and produce data on molecular weight as well as structure of the analyte (Abdel-Hameed et al., 2013).

L. fulva is a well-known dyeing plant by the Marakwet people. It contains substantial red dye that has been used in dyeing materials. Furthermore, the extracts have been used medically as anti-venom and anti-inflammatory herbs. To date, the information on the main

dye chromophores responsible for dyeing capability of this plant remain scanty. Usually, the interpretation of spectral property of dye molecule involves focusing on specific information on specific functional groups for structural characterization. This hinges on the structure of the chromophores which display the overabundance of colours (Chengaiah, Rao, Kumar, Alagusundaram, & Chetty, 2010; Lee et al., 2013).

Other research conducted in the recent past have shown that proper understanding of chromophoric compounds responsible for dyeing capabilities entails comprehensive evaluation of MS spectral properties such as fragmentation pattern. This spectral property is central in the assessment of bioactive constituents present like molecular mass, chemical structure, disintegration configuration, dyeing properties, quality, effectiveness and safety. This bio-analysis of chromophoric and spectral properties is vital in guaranteeing the reliability and reproducibility of natural dye for quality control and textile application (Kang, 2012).

Natural dyes from plants origin are generally, complex mixtures. Prashanth et al. (2011) noted that the complexity in such dye mixture creates pseudo-molecular ions in LC-PDA, ESI-MS. These pseudo-molecular ions provide definitive molecular mass for confirming the structures of the chromophoric compound present.

Similarly, study by Adawiyah, Lioe, & Anggraeni, (2012) revealed that molecular weight of the major dyestuff component is shown by highest peak in ESI-MS spectra. Such spectrum discloses the aglycones identical to flavone (yellow flavonoids dye) or alizarin, rubiadin, purpurin, and xanthopurpurin (red anthraquinones) from their glycoside. Essentially, when evaluating natural dye extract, relative abundance of mass spectrum can be used to analyze and identify specific chromophores present. Alternatively, the distinctive spectral masses of

individual dye chromophore can be evaluated by retention times, molecular ion mass and fragmentation pattern. These have been used to confirm the known compounds and identify the unknown dye chromophore, especially in the field of material science (Osman, Zidan, & Kamal, 2014). Berthelette, Swann, & Fountain (2015) noted that LC-UV-MS or LC-PDA-ESI-MS, gives complimentary analysis in the production, separation and identification of charged species. The detectors operate entirely on different principles, yielding single superior orthogonal valuable information with minimum efforts. It also adds rich information to spectrum without compromising the existing data (Naegele, 2011).

Other studies have noted that unknown dyestuffs (*L. fulva* and *T. brownii),* are best identified by mass spectra analysis. This will involve monitoring of relevant molecular weight or masses of the target chromophoric compounds by measuring the *m/z* of chromophores (Silver, Cammarata, & Ronald, 2013). In majority of research where there are no reference standards available, the specific information on dye chromophores (molecular weight and structure) can be constructed entirely based on specific fragmentation pattern or by comparing with MS entries in libraries. Similarly, exact masses of the chromophoric compounds are extracted using total ion current (TIC) with narrow window (Hird, Lau, Schuhmacher, & Krska, 2014).

On the other hand, chromophoric determination and spectral characterization of natural dye such as *T. brownii* and *L. fulva* dye requires LC-MS equipped with Photo diode array detectors (PDA) and mass spectrometer. These two detectors offer complete separation of chromophoric compounds which substantially, minimizes unevenly dyeing of fibre or batch to batch variation. Furthermore, it also provides (semi) quantification data or spectral data

for specific application of dye/ chromophore in the industry (Karapanagiotis & Karadag 2015).

Furthermore, Photo array detector (PAD) monitored at specific wavelength normally identify polyphenolic compounds in dye extract or gives spectral properties or absorbance criterion which is magnificent indicator of many applications in textile industry such as determination of possible fading of the dye, absorbance behavior under UV-light/ sun-light and act as crucial factor for determining UV protection on dyed fabric, often called Ultraviolet protection factor (UPF) (Hussein & Elhassaneen, 2013).

Ideally, natural colours chromophore can be known using Electrospray Ionization mass spectrometer (ESI-MS). This generates characteristic absorption spectra that are dye-discriminate but differ chemically. This is useful in finding a specific chromophore that substitutes synthetic dyes that have been suspected or proven to be hazardous (Carey, Rodewijk, Xu, & van der Weerd, 2013). It equally identifies different dye stuff component such as chemical bond and other functional groups (chromophores/auxochromes) responsible for dyeing capabilities. The spectral data such as characteristic peak values are indicative of specific functional groups in the natural dye complex (Ashokkumar & Ramaswamy, 2014).

Literature further has pointed out that unknown natural dye complexes like *T. brownii* and *L. fulva* are best analyzed using R-PHPLC or LC in tandem with Mass Spectrometer (LC-MS) operating in both positive mode and negative ionization mode with femtogram-to-pecogram sensitivity. This delivers both qualitative and quantitative results (Edgar, 2012).

The positive mode ionization involves the protonation of the dye molecules $[M+H]^+$, especially the decomposition of glycosides like red lucidin primeveroside in *Rubia* species.

In most cases, the negative mode ionization involves the deprotonating $(M-H)^{n-}$, causing ionization of aglycones such as purpurin, alizarin, rubiadin (red natural dyes) or flavone and flavonones in yellow natural dyes, making it compatible with wide range of natural dye. The characteristics fragmentation pattern of product ions yields sequential losses which can be identified by comparing with the reference standard or reported data (Mena et al., 2012; Abdel-Hameed et al., 2013).

These two ionization mode in MS detects and characterizes polyphenolic structures and other chromophoric compounds with weak or no Ultra Violet-Visible (UV-Vis) absorbance (Silver et al, 2013). Most importantly, when analyzing natural dyes using Atmospheric pressure ionization (API) in addition to electro spray analyzer, the isolated chromophoric compounds are fragmented to generate molecule-specific ions that enhance tentative identification of the chromophoric compound present (Saleh, Abd-El-Hady, & El-Badry, 2013; Naegele, 2011). This allows determination of natural dye chemical structure both known and unknown, within relatively short analysis time, thus playing an instrumental role in screening of flavonoids and other phenolic (Saldanha, Vilegas, & Dokkedal, 2013).

Dyestuff components from *Rubia* spp. have been used as alternative to heterocyclic amines or azo compounds accompanying the usage of synthetic dyes that are known to introduce aromatic amines causing allergy to skin, carcinogenic effect and eventually posing health hazards to human and environment (Tateo et al., 2010).

2.5.1.1 Spectral properties of natural red dye

Lannea fulva dye extract is red in colour. In most red dyeing plants or anthraquinones dyes, pseudo -purpurin can be found alongside lucidin primeveroside and its aglycones for instance lucidin, xanthopurpurin and Nordamnacanthal which is converted by air to

munjistin. Literature has shown that the detection and identification of characteristic red-brown anthraquinones dye is by employing photo-array detector (PAD) and mass spectrometer (MS). This offers robust sensitivity with minute sample within the shortest time, making it suitable for screening anthraquinones and other polyphenolic compounds (Saldanha, Vilegas, & Dokkedal, 2013; Morgan, 2015).

Research by Vlase, et al. (2014) showed that red anthraquinones dyes are detected, separated and characterized on the basis of their structure, molecular masses and functional groups present. The generation of chromatogram or characteristic absorption spectra in LC-MS, enables separation, isolation and identification of chromophoric compounds in natural dye complex (Lynn, Kinks, & Muddinman, 2013).

Remarkably, research by Divya and Siri (2014) established that aglycones such as alizarin and purpurin which are derivatives of red natural dyes such as *Rubia tinctorium* L. hold medicinal properties, specifically anti-proliferative, anti-carcinogenic and anti-mutagenicity. In another research on *Rubia cardiofolia,* the bioactive compounds responsible for the red colour are mainly hydroxyanthraquinones and their glucosides (xylose/ribose, primeveroses) such as lucidin primeveroses, Ruberythric acid and methyl Geniposide (iridoid). Recent study has linked the presence of sugar moiety in galiosin to the protection of carboxylic acid [COOH] from decarboxylation. According to Henderson et al. (2013) the decomposition of pseudo purpurin glucoside can cause pseudo purpurin to be lost entirely from the red dye extract. It further showed that spectra data at *m/z*=277 and *m/z*= 255 are distinctive characteristic of purpurin. Glycosidic compound such as ruberythric acid m/z=533, rubiadin primeveroside m/z =548 and lucidin primeveroside m/z=564 are major compounds.

Literature (Lajkó et al.2015) on qualitative analysis of aqueous extract of red anthraquinones dye from *Rubia tinctorium* L. showed that munjistin with molecular mass of 284a.m. u, to be the main dye chromophore with minor red anthraquinones identified to be pseudo purpurin, Rubiadin, Nordamnacanthal and alizarin.

In the spectral characterization of natural red chromophores for example, pseudo purpurin, purpurin, and alizarin, researchers have relied heavily on unique spectral capabilities, like fragmentation pattern which generate specific fingerprint of individual dye molecule (Bruker, 2010). This spectral characterization, assists in structural determination of degradation products by measuring mass to charge ratios (m/z). The core chromophoric information generated by the spectral data is based on available standard or literature (Gevrenova, 2010). Essentially, red/ brown dyes are mainly anthraquinones. Recep, Emine, & Gokhan (2014), have proven that alizarin -containing plants, belong to the important groups of red dye anthraquinones such as madder. In such red anthraquinones dye plants, alizarin m/z =239 and purpurin m/z= 255 and m/z=277 are the main dyestuff components. The analysis and characterization of such dyestuff component is achieved by evaluating major peaks, retention time and the molecular mass (MS), which is anticipated to retain much of primary structure of the natural dye (Adawiyah., et al., 2012; Deveoĝlu, Torgan, & Karadağ, 2012).

2.5.2 UV-VIS analysis

UV-spectroscopy is valuable in semi qualitative analysis of organic compounds with conjugated system in which every bond is double bond (Martelo-Vidal & Vazquez, 2014). In essence, UV absorption spectrum shows electron transition from pi bonding orbital or non-bonding orbital to pi*/ anti-bonding orbital (Birdwell and Engel, 2010).

Since majority of chromophoric compounds are conjugated system, there is correlation between structure of organic molecule and colour (Priyanka, Prabaharan, Hilal, & Islam, 2018: Tomaszewska et al., 2013). For example, when part of a molecule suspected to be chromophoric compound is subjected to UV light of high frequency or high energy (exhibiting both wave and particle or photon) of wavelength equivalent to HOMO-LUMO energy gap. The energy absorbed in form of wavelength will be used to excite and promote electrons from non-bonding or bonding orbital into one of the empty anti-bonding orbitals either $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ (Daengngern, Camacho, Kungwan, & Irle, 2018; Shapley, 2012) as shown by **Fig. 2.3** and **Fig. 2.4**

Such photon or light energy absorbed is given by equation $E = hv = hc/\lambda$ (where= energy, h= plank's constant, v= frequency, c=speed of light and λ, =wavelength). Chromophoric compounds or molecule exhibiting such characteristic absorbs energy (light) in the visible region making the molecule appear coloured (Ren, Li, Zhang, Wang & Wang, 2016). The delocalization of different types of functional groups such as fluorescent aromatic compounds or carbon-carbon double bonds, carbon-oxygen double bonds or lone pairs on oxygen and nitrogen in conjugated system causes absorption spectra to appear at longer wavelength region (bathochromic shift i.e. region of longer wavelength or low energy) than benzene which absorbs at 255nm. However, such absorption does not exceed 400nm (Fritsch, Lavayen, & Merlo, 2018). Likewise, Li, et al. (2013) noted that the size of organic compound is influenced strongly by conjugated system or increase in size of conjugated system and the presence of functional groups which shift the wavelength to the right or favour the side with less energy.

2.5.2.1 Single and double peak in UV Spectrum in identification of chromophores

Commonly, a molecule or chromophoric compound producing single peak in UV spectrum is due to presence of delocalized electrons or double bond ($\pi \rightarrow \pi^*$ electron transition only) with no non-bonding electrons (Schnaiter, et al., 2003), whereas those chromophoric compounds that produce two peaks have pi-electrons as a part of the double bond ($\pi \rightarrow \pi^*$) and unpaired electron on the oxygen atom ($n \rightarrow \pi^*$). These two chromophores are common in carbon oxygen double bond (C=O) and carbon -carbon double bond (C=C) involving in the absorption of light energy for electrons jump from a pi bonding to a pi anti-bonding orbital (mostly first peak) and second peak occurs at higher wavelength due to excitation of lone pair electron on an oxygen which is a non-bonding orbital to pi anti bonding molecular orbitals) (Amendola &Menenghetti, 2009).The non-bonding orbital has a lower energy than a pi bonding orbital consequently absorbs energy (light) of a lower frequency, lower energy and higher wavelength. In effect, broad absorption peak shows that the chromophoric dyestuff is in fact absorbing light energy over a whole range of wavelengths by a whole range of energy jumps (Thakre, Kaswan, Shukla, & Kumar, 2017).

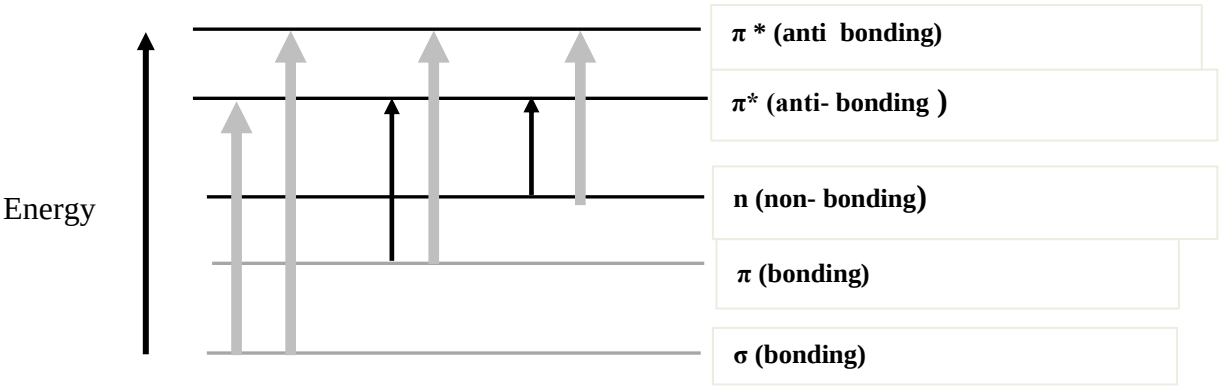

Figure 2.3: Electronic transition in UV-Visible absorption spectra

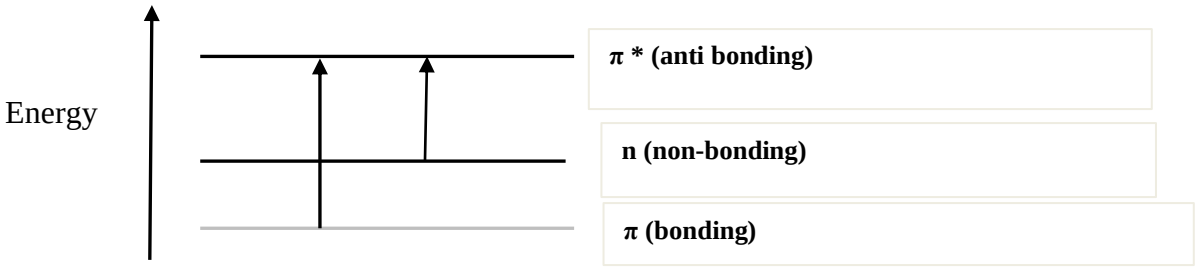

Figure. 2.4: Bonding and non-bonding in UV-Visible, absorption spectra

2.5.3 FT-IR Analysis

Once a molecule or compound is exposed to FT-IR or infrared radiation, energy is absorbed in form of IR radiation. The energy absorbed excite electron from ground state into a higher vibrational state. The wavelength of light absorbed by a specific molecule is a function of the energy difference between the lower energy level and higher vibrational level (Taha, Hassan, Essa, & Tartor, 2013). In actual fact, the energy absorbed is measured in wavelengths and it is characteristic of the sample or the compound molecular structure. Typically, FT-IR spectra are commonly presented as graph of intensity/absorbance versus wavenumber with cm^{-1} as SI unit. (Wave number is the reciprocal of the wavelength) whereas the intensity is plotted as absorbance at each wavenumber (Simona, Carla, Giulia, & Elisabetta, 2017).

The Fourier transform infrared absorption bands identify molecular components such as functional groups, chromophores and molecular structures based on the vibrational excitation of molecular bonds (Liang et al., 2013). Usually, the totality of vibrational spectra for chromophoric compound produces IR absorption spectrum that is identical to molecular "fingerprint" for the molecule or the sample in question. The spectral properties or the spectrum can be used in identification of functional groups and other chromophores present.

However, according to Erwanto, Muttaqien, Sugiyono, Sismindari,& Rohman, (2016) FT-IR spectrum of any unidentified molecule can be gauged using the reference spectra and library containing an identical or a very similar spectrum. This is because FT-IR is more informative when polar functional groups are under study. It categorizes chemical bonds in a molecule by producing an infrared absorption spectrum which is distinct and serves as molecular fingerprint for detecting functional groups and characterizing covalent bonding (Baran, Fieldler, Schultz, & Baranska, 2010).

The spectrum further analyses phenolic compounds with characteristic moiety groups and shed some light on the individual dye components (chemical functionality and bonds) present. The identification is by absorption band within fingerprint region, making it an irrefutable means of confirming chemical composition of a material with spectral specific to functional groups or phenolic groups (Espinosa-Morales, Reyes, Hermosín, & Azamar-Barrios, 2012).

FT-IR employs principles of IR in identifying the structure of a compound based on interaction of an atom /molecule with IR radiation. This has been employed to determine the possible functional groups of chemical compounds present. The molecular vibration is localized within a functional group and can be identified as absorption band. Strikingly, a band within fingerprint region arises from functional groups present, making it irrefutable means of identifying chemical repetition composition of a material. The spectral is specific to functional groups present in dye molecule (Yuen, Ku, Choi, Kan, & Tsang, 2005).

FT-IR is mainly for identification of natural dyes which are organic in nature (Peter,.2009). Functional groups are known to create electrostatic force of attraction or ionic bonding between themselves in the fabric and dye chromophore. This understanding is significant in

the area of natural dyes (Nilani et al., 2008) stem from flora and fauna are believed to be safe, none toxic, none carcinogenic and biodegradable in nature (Ali et al., 2009).

According to Lyon (2010), a dye molecule must satisfy the following requirements: must have a chromophore, the chromophore must be part of the conjugated system in which single bond is alternating with double bond. Additionally, the auxochromes as functional groups must be ring substituents, thereby providing the desired colour and enhancing the solubility of the dye.

Islam and Yousif (2013) noted that the auxochromes (colour helpers) for instance, hydroxyl groups (OH), sulfonic (SO_3H) or carboxylic acid (COOH) shift the colour and influence the solubility of the dye, especially if many polar functional groups such alcohol and/or hydroxyl(OH) or carboxylic acid (COOH) are directly attached to the aromatic ring.

In another research by Ford et al. (2015), it was noted that polar functional groups in dye chromophore act as binding centers for polyvalent metal ions known as mordant. Mordant is used to provide various shades of colours. Furthermore, the presence of functional groups such as sugar moiety provides ample potential for binding to fibre or metal ion (mordant) during dyeing. Individual dye molecule in natural dye complex differs from one another only on the position of functional groups (auxochromes). It is also of importance to note that auxochromes such as OH or COOH act as centres for mordant (metallic ion) which help in improving fastness to fabric with overall effect is dramatic change in the colour of the natural dye extract (Upadhyay & Choudhary, 2012).

2.5.3.1 Identification of Functional groups in red natural dyes extract

L. fulva dye extract is normally red/brown colour. Though there is no study that has been carried out to reveal its functional groups. It is generally expected that it will display functional groups characteristic of anthraquinones structure. Recent research by Lajkó, et al. (2015) noted that in red dye plants for instance *Rubia tinctorium* L, the functional groups (chromophore) is what controls the red colour while the auxochromes functional groups such as hydroxyl groups (OH)) attached to anthraquinones structure modify the colour. Fundamentally, the presence of hydroxyl functional groups in dye chromophore indicates that the dye molecule undergoes oxidation or dehydration. The hydroxyl molecule will be eliminated as water. In another study by Henderson et al., (2013) the presence of carboxylic acid (COOH) as a functional group is clear indication that the dye/chromophore will undergo decarboxylation upon elimination of carbon (IV) oxide during fermentation, drying in the sun or under MS condition.

Equally, study by Daniels, Devièse, Hacke, & Higgitt (2014) on red natural dyes, suggested that the presence of primeveroside in some of the chromophoric compounds protect the carboxylic acid functional groups from decarboxylation. Shabbir et al. (2016) stated that some natural yellow dyes are tannin based with good substantivity towards textile during dyeing. This is because of presence of auxochromes functional groups such as hydroxyl groups (OH) and carboxylic acid (COOH).

Principally, the characterization of chemical compounds responsible for dyeing properties entails the detection of the characteristic peak patterns of the individual functional groups (chromophore and auxochromes) in the natural dye compound. The study by Thilagavath &

Rathinamoorthy, (2014), using FT-IR on *T. chebula* dye extract, reveals more of carboxylic groups [COOH] owing to presence of active metabolites such as Ascorbic acid in the extract.

Similarly, Shabbir et al. (2016), while studying on *T. chebula* dye extracts, noted that the characteristic moiety groups such as carboxyl (-COOH), Amino ($-NH_2$), and Hydroxyl groups (-OH). He further stated that *T. chebula* dye extract have functional groups identified in FT-IR bands at 3240cm^{-1} which is due to -OH groups, 2980cm^{-1} attributed to aromatic (C-H) stretching vibration whereas absorption band at 1710cm^{-1} is due to C=O functional groups. The signal at 1595cm^{-1} attributed C=C stretching vibration and absorption at 1201-1040 cm^{-1} is due to C=O functional groups. Other study by Shabbir et al., (2016), noted that these functional groups in dye extracts, serves as centres of dye mordant (metal ions) causing coordination complex.

The mordant attaches firmly to the fabric, ensuring the fixing of the colour during dyeing. Packianathan & Karumbayaram, (2010), noted that polyvalent metal (mordant) forms coordination complexes with specific dyes or agent that facilitate attachment between dye and the fabric.

2.6 Chromophoric characterization of natural dye extracts

2.6.1 Red anthraquinones dye extracts

The exploitation of *L. fulva* as source of red/brown dye has been interesting, especially by the aforementioned. The bark is extremely rich in red colour akin to that of madder which belongs to the chemical family of anthraquinones (Ford 2017). Although the individual chromophores responsible for dyeing capabilities of *L. fulva* have not been identified, a study by Derksen,,van Beek, de Groot, & Capelle (1998), stated that chromophoric

evaluation of natural red dyed plants showed anthraquinones complex to be chromophoric compound responsible for red colour in majority of natural red dyeing plants. Therefore, the red/orange dye extract from *L. fulva* may be empirically assumed to be in the chemical class of anthraquinones and also expected to display similar chromophoric and spectral properties in LC-MS method of analysis. Possibly, the dazzling physical characteristics manifested by natural red anthraquinones dye like madder is dependent on the type of the polyphenolic compound. The hydroxyl-anthraquinones structure is responsible for its dyeing property and is best identified using chromatographic and spectrometric method due to sensitivity to chromophores (Samanta & Agarwal, 2009).

There are three traditional dyeing processes that have been employed by the Marakwet community to enhance hue and colour of *L. fulva* dye. These methods include steaming, crushed bark, fermenting the extract under the sun for some days or adding some Soda. Though mechanism of action for such process was not explained, recent study by Ford et al. (2015), however, linked the variation of colour and hue to induction of hydrolytic enzymes that hydrolyze glycosides to corresponding aglycones. These aglycones augment brightness and colour depth. Similarly, Cai, Sun, Xing, & Corke (2004) point out that majority of such aglycones such as alizarin, purpurin and lucidin are hydroxyanthraquinones and they are predominantly antioxidant and glycosylated.

Usually, the glycoside moiety holds the key to the dyeing chromophores. Glycosides as a rule provides enough binding site with metal ion (Espinosa-Morales et al., 2012). Often, glycosides or glycosidic bond defines polar chromophore, which calls for polar solvent such as alcohol or water during extraction. Water is necessary in hydrolyzing glycosidic bond. Previous studies have explained various conditions leading to formation of corresponding

aglycones from glycosides such as glycosidic bond being hydrolyzed enzymatically or by analytical conditions like by MS. Also, fermentation under the sun has been noted to induce hydrolytic enzymes leading to the formation of aglycones such as alizarin from decomposition of Ruberythric acid and Rubiadin from decomposition of *Rubiadin primeveroside* (Derksen, van Beek, de Groot, & Capelle, 1998).

Recent research by Daniels et al. (2014), noted that aglycones determine the types of chromophores present in natural dye and is what controls the colour in majority of natural dyes. For example, in red dyeing plants for example madder, the most important anthraquinones responsible for red/brown colour is pseudo purpurin and alizarin. Pseudo purpurin is as a result of the decomposition of unstable galiosin while alizarin results from the decomposition of ruberythric acid. For this reason, chromophoric analysis of compound responsible for dyeing property is key to identification of specific anthraquinones or aglycones responsible for red colour in plants. According to Manhita, Ferreira, Candeias, & Dias (2011), typical natural anthraquinones dyes **(Fig. 2.3)** have conjugation system/ double bond which allow the delocalization of electrons in the whole chromophore, resulting to creation of color.

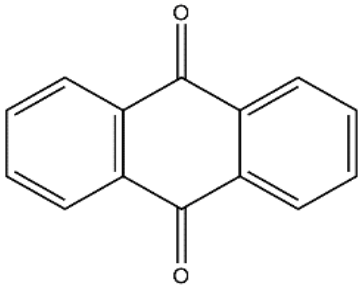

Figure 2.5: Chemical structure of Anthraquinone

2.6.2 Yellow natural dyes extracts

Deveoĝlu, et al. (2011) noted that chromophoric characterization of natural yellow dyes is very important to scientists because it enable them to identify the predominant flavonoid responsible for yellow colour. Yellow colour is perhaps the most plentiful colour in nature. It corresponds to wide array of chromophoric compounds such as flavonols, flavonones, flavones and chalcones. However, Blanc et al. (2006) noted that sometimes natural dye chromophore display colour after various chemical reactions such as complexation with metal ion, hydrolysis or oxidation by air. Largely, yellow yielding dye plants outnumber those yielding other dye components. Petroviciu, Medvedovici, Albu, Creţu,& Berghe, (2012) noted that determination of the exact chromophoric compounds responsible for yellow colour in natural dye plants has been challenging to scientists for quite some time. This is largely due to wide spectrum of chromophores and the complexity in chemical compositions as a result of co-existence of chromophores with other degradants, making the separation and identification of such chromophoric compound difficult.

However, Taujenis & Vilma (2012) stated that the separation, identification and determination of yellow dye chromophores is by evaluating related chemical structures and unique spectral properties. Intrinsically, bulk of yellow chromophoric compounds in dye plants exists as glycoside. The glycosylated forms aid in the solubility of the dye component and often dictate the method of extraction and evaluation. Yellow dye chromophores are determined by analysis using "soft" ionization method. Acids such as hydrochloric acid (HCl) or extreme condition often cause hydrolysis and/or destruction of O-glycosidic bond

in the yellow or flavonoid dye (Nowak, 2000; Manhita, et al., 2011), resulting to loss of glycosylation and subsequent loss of chromophores/colour.

Recent experimental analysis on yellow dye plants has shown that flavonoids are the main dye components in majority of yellow dyeing plants. Unlike flavone whose yellow colour is stable to photochemical degradation, flavonols dye is unstable and quickly fades, resulting to losses colour. Therefore, mordant (metallic ion) is necessary fixing the dye into fabric, stabilize the dyes and enhance wash and colour fastness. Hence, identifying specific dye chromophore is critical to colour fastness during dyeing (Karapanagiotis & Karadag, 2015). More often, flavonoid yellow dyes are good antioxidant. This intrinsic medicinal property has made most of natural dyeing plants to be popular and to be considered as alternatives to toxic, mutagenic and allergic synthetic dyes. Yellow dye plant, like Weld, luteolin is the main dye component while Golden rod, Quercetin is major dye component or glycoside. The colour property is linked to long conjugate double bonds (Samanta & Konar, 2011) degrade.

In majority of yellow dye yielding plants, these chromophores are stable to photo degradation because they are flavones. Yellow dye plants are biocolour because they contain many polyphenol compounds. Ordinarily, these polyphenolic chromophores give stable yellow dye (flavones). Conversely, extreme light and oxygen (photo oxidation) activation may cause yellow natural dye like Catechin to degrade to hydroxybenzoic acid or experience most intensely fading and subsequent loss of colour (Manhita et al., 2011).

CHAPTER THREE

3.0 Materials and Method

3.1 Materials, Reagents and Equipment

Hexane, methanol and acetone for extraction were of regular grade, manufactured by Merck Company, and were sourced and supplied by gel sap laboratories Nairobi. Hexane, Acetic acid, formic acid and ethanol were of LC-MS purity was purchased from Merck limited (Brussels, Belgium). The water (distilled) were obtained from Milli-Q apparatus (Billerica, MA, USA). Water bath, Analytical balance, Rota evaporator (BÜCHI Rota vapor R-114), Thermometer and PTFE membrane filters (0.45mm) were locally sourced. Data collection was done by UV-VIS (Shimadzu model UV-1800 (Japan) and FT-IR spectrometer was Brucker alpha model, (Germany) and LC-MS was LCQ/Surveyor/LC-Q/LC-Q model (USA).

3.2 Methods

3.2.1 Plant collection and processing

The sample of mature barks of *L. fulva* was collected from Oinobmoi in Baringo County and of *T. brownii* was collected from Samar village in Elgeyo Marakwet County. Baringo and Elgeyo Marakwet are among the 47 counties in Kenya being their natural habitat. After sample collection, the plants under study were carried to Moi University, Botany Department for taxonomical identification, voucher number allocation and processing. Thereafter, plant samples were air-dried under shade till constant weights were attained. Then they were chopped into small pieces and grounded into course powder using a Willy

Mill. The sample powders obtained from the grinding were stored in airtight container ready for use.

3.2.2 Extraction of dyestuff components

To determine the most effective method of extraction that gives high yield of individual dye components during extraction, soxhlet method and maceration methods were compared using two solvents of different polarity then the respective percentage yield tabulated.

3.2.2.1 Soxhlet extraction

About 30 grams of each dye powder were weight and kept sequentially in soxhlet extractor. Then 200 ml of each dye sample solvent was serially extracted in order of increasing polarity starting with hexane followed by water. The choice of these two solvent is extreme polarity. Then the solvent containing each extractive was collected and rota-vaporized and the yields were tabulated.

3.2.2.2 Maceration technique

About 30 grams of L. fulva and T. brownii dye powder was weighed and soaked separately using 200ml of hexane and water each, at 60ºC for 60 minutes with gentle stirring. The solution was then filtered using whatman number 54 and the solvent was removed by rota-evaporator and the resultant solid yield from each solvent was weight and tabulated.

3.2.2.3 Determination of solvent of choice for extracting the dyestuff compounds.

The most desirable solvent for extracting the dyestuff components were evaluated by calculating the mass difference between the measured mass of the clean empty bottles used

from the mass of bottle, plus the content of each extractive solvent (hexane and water) using the following formula.

$$\text{Total \% yield } = \frac{\text{Mass of extract}}{\text{Mass of sample}} \times 100\%$$

3.2.2.5 The individual extraction for HPLC separation

The procedure by Recep et al., (2014) was followed in which the crude powder from both *L. fulva* and *T. brownii* were extracted using 300 µL mixture of 37% hydrochloric acid, water and methanol (3/1/1: v/v/v) in a conical glass tube and heated gently for 10 min in water bath at 90ºC. The dye mixture was then rapid cooled under running cold water and the solution evaporated to dryness in a water bath at temperature of 50ºC under a gentle stream of nitrogen. The dry dye residues were then re-dissolved in a 300µl mixture of methanol/water (3:1; v/v) filtered using polypropylene syringe filters (0.2µm) and 15 µL of the supernatant was injected into the HPLC apparatus.

3.2.3 Identification of chromophores in *Lannea fulva* and *Terminalia brownii* dye extracts by UV-VIS spectroscopy

The procedure entailed preparing 10% concentration of each dye sample by weighing 0.5mg of each dye sample (*L. fulva* and *T. brownii*) and diluted in 50mL of pure distilled water. Then the aliquot was introduced into a quartz cell (1cm pathway) and analyzed in Shimadzu model UV-1800 series (spectrophotometer) and a scan of 200-800nm was performed in order to generate the characteristic absorption spectra of each dye chromophore. Thereafter, qualitative identification of each dye chromophore was confirmed by matching the absorption spectrum with literature or library available.

3.2.4 Identification of functional groups in *Lannea fulva* and *Terminalia brownii* dye extracts by FT-IR Analysis

The procedure involved weighing 4mg of each of the dye sample (*L. fulva* and *T. brownii*) powder, and then mixed thoroughly with 180 mg of potassium bromide (KBr) until homogenized in a mortar. The mixture was placed into the sample compartment for thin film of translucent sample disc to be formed in Bruker FT-IR spectrometer (Model Alpha) and the spectra were recorded over an absorption band of 4000-500 cm^{-1}.

3.2.5 Characterization of chromophoric compounds in *Terminalia brownii* and *Lannea fulva* dye extracts

3.2.5.1 HPLC separation and analysis

The chromatographic profiles of dyestuff compounds were obtained by performing reverse HPLC (RP-HPL) and the separation of the chromophoric compounds were achieved by multiwavelength detectors (MWD). The eluted peaks were monitored at 214nm, 280nm, 310nm and 330nm. Analytical and guard column was maintained at 30 ^{0}C. The HPLC chromatographic separation of the hydrolyzed sample was performed using a gradient elution program that utilizes the two solvents A (acetonitrile) and solvent B (water acidified with 0.1% formic acid).

3.2.5.2 LC-DAD-MS Analysis.

The LC-DAD-MS analysis of spectral properties (molecular structure, molecular mass and disintegration pattern) was achieved using Manhital et al., (2011) in which an LCQ Advantage Thermo Finnigan spectrometer equipped with an ESI source using anion trap mass analyser and a PDA detector (San Jose, CA, USA). The conditions of the MS were

capillary temperature of 300ºC, source voltage of 5.0 kV, source current of 100.0µA, and capillary voltage of -20.0 V in negative ion mode and 22.0V in positive ion mode. The dye analytes was detected in full MS mode with mass to charge ratio (m/z) 100-1500). In negative ionization mode, two segments were used, 30% CID running from 0 to 3 min and 60% CID from 3-6 min, however, in positive ionization mode 60% CID was employed from 0 to 6 min. The two dye extracts were injected in negative and positive ion mode in which the column temperatures were set at 30ºC and tray temperature was set at 24ºC. The PDA detectors were set at 214nm, 280nm, 310nm and 330nm. The MS and PDA equipment was coupled with LC system with auto sampler Surveyor Thermo Finnigan in which the analytical column was a reversed phase Fortis C-18(Fortis Technologies; C_{18}, particle size3.0 µm, 150*2.1mm). The mobile phase consists of acetonitrile (A) and water acidified with 0.1% formic acid (B). The gradient used will be 0 – 100%A from 0-3 min, then 100% A from 3 – 6 min. In between the injections 0-75% B was injected to re-equilibrate. The injection volume was set at 15µL.

3.3 LC-MS identification of chromophoric compounds in *Lannea fulva* and *Terminalia brownii* dye extracts

The characteristic spectral properties such as retention time, molecular mass, molecular structure, disintegration pattern and mass to charge ratio (m/z) were generated by hyphenated LC-MS as suggested by (Hird et al., 2014; Silver et al., 2013). The characteristic absorption spectra in mass spectra (highest peak in ESI-MS mass chromatogram and unique fragmentation pattern, resulting from sequential losses of compound) was used to discriminate chromophores or dyestuff compounds that are similar or have equal colour but have different chemical structure. The mass chromatogram and unique disintegration pattern

was compared with previous literature to confirm the known chromophores and identify unknown dye chromophore.

3.4. Data analysis and presentation

3.4.1 Data analysis

The extraction was done in triplicates in which the average mean value was calculated and recorded. Thereafter the UV-Vis data was analyzed by measuring the HOMO-LUMO energy gap of electron transition in bonding or non-bonding orbital jumping into one of the empty anti-bonding orbitals ($\pi \rightarrow \pi^*$ and/or $n \rightarrow \pi^*$).

The electrons at higher energy state (π^*) are unstable and they will quickly fall into ground state, emitting photon with energy equal to the difference in energy levels. This quantized-energy absorbed by the chromophore was measured as absorbed wavelength and usually correlated to the type of chemical bond which is characteristic of chromophore present in the dye molecule.

Equally, FT-IR analysis, of the individual functional group was determined by evaluated the energy absorbed by electron from ground state to an excited vibrational state. The wavelength of light absorbed by a specific molecule is a function of the energy difference between the lower energy level and the higher vibrational level. Such energy absorbed is shown by a wavelength which is characteristic of the sample chemical bond, chromophore or functional groups existing in the sample.

In LC-DAD-MS analysis, the eluted chromophoric dyestuffs were identified using MS and their unique fragmentation pattern or m/z of molecules resulting from sequential losses of compound (Hird, Lau, Schuhmacher, & Krska, 2014). Often, mass spectrometer software

makes a choice in real time about the selection of ions to be fragmented based on the intensity of each peak with threshold set above 1500 absolute count. The total ion current (TIC) spectra and the exact masses of individual dye chromophores were analysed using Hystar software 3.2 and compared with existing mass library and previous literature to confirm the known chromophores and identify unknown dye chromophore. The characteristic absorption spectra in mass spectra was used to discriminate chromophores or dyestuff compounds that are similar or have equal colour but have different chemical structure.

3.4.2 Data presentation

Microsoft excel 2016 (computer software) was used to evaluate the data and presented in form of tables and graphs of absorbance verses wavelength, retention time, absorbance versus wavenumber in cm^{-1} and relative intensity verses mass.

CHAPTER FOUR

4.0 Results and Discussion

4.1. Determination of method of extraction and solvent of choice for the dyestuff Compound.

The most preferable method of extraction and the solvent of choice that gives high yield of the dyestuff components were determined by calculating the mass difference between the mass extract and the sample using the following formula below. The results were tabulated as shown in table 4.1.

$$\text{Total \% yield } = \frac{\text{Mass of extract}}{\text{Mass of sample}} \times 100\%$$

Table 4.1: Percentage yield of the individual dye sample with respect to method variation and the solvent

Sample code	Yield in grams	%
THS	2.4	08.0
THM	2.4	08.0
TAS	3.4	11.3
TAM	4.6	15.3
LHS	3.2	10.7
LHM	4.3	14.3
LAS	3.6	12.0
LAM	4.4	14.7

Key:

THS –*Terminalia brownii* when hexane was used as solvent and the method was soxhlet

THM –*Terminalia brownii* when hexane was used as solvent and the method was maceration

TAS –*Terminalia brownii* when aqueous was used as solvent and the method was soxhlet

TAM- *Terminalia brownii* when aqueous was used as solvent and the method was maceration

LHS –*Lannea fulva* when hexane was used as solvent and the method was soxhlet

LHM- *Lannea fulva* when hexane was used as solvent and the method was maceration

LAS – *Lannea fulva* when aqueous was used as solvent and the method was soxhlet

LAM- *Lannea fulva* when aqueous was used as solvent and the method was maceration.

The results showed that method and solvent plays a fundamental role in determination of percentage yields of individual dye extracts. For all practical purpose, maceration method was efficient on the account of high percentage yield of the dye extracts when compared with soxhlet method of extraction.

Figure 4.1: *L. fulva* dye extracts

Figure 4.2: *L. fulva* dye powder

Figure 4.3: *T. brownii* extracts

Figure 4.4: *T. brownii* dye powder

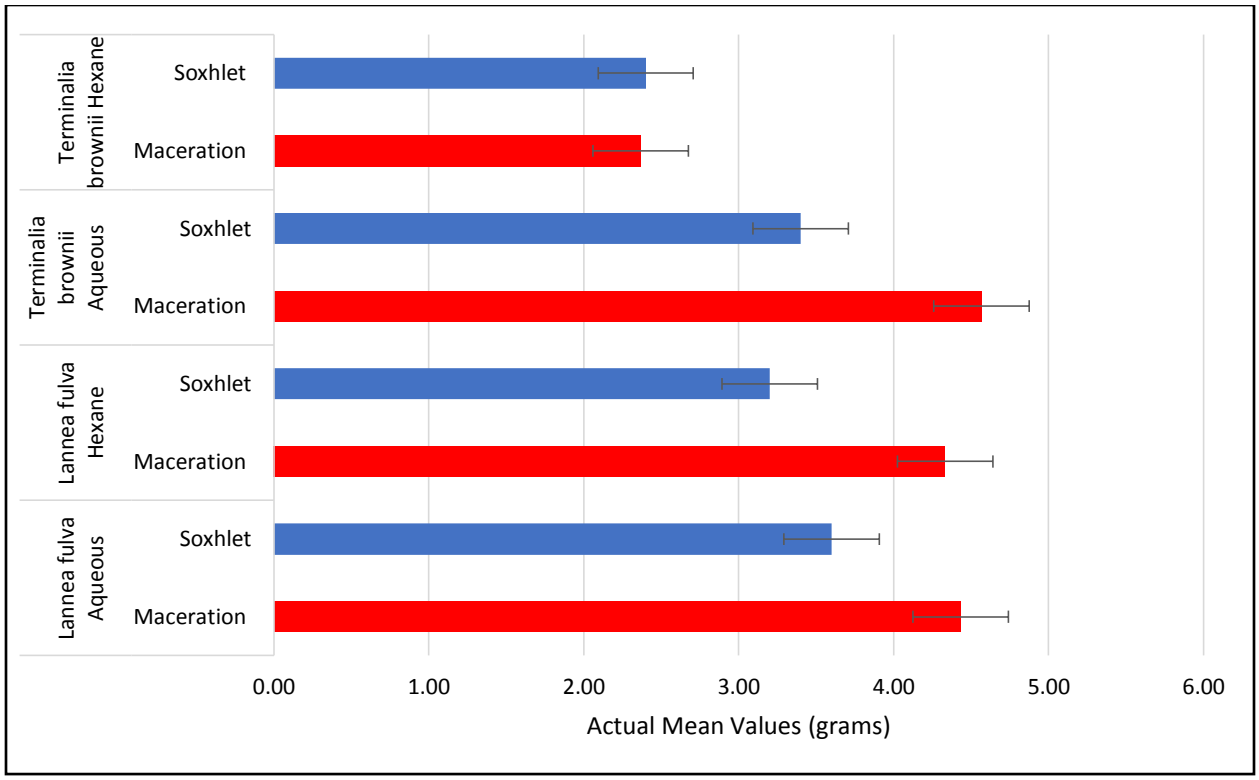

Figure 4.5: Comparison of Soxhlet and Maceration method on the two solvents hexane and water.

This study findings showed that amount of extracts vary with method. The difference in extractive yields was statistically significant, (P< 0.05) as shown in figure 4.5. This is further indicated by 95% CI error bars that do not overlap. On the adopted maceration method, hexane as a solvent gave to low yield (2.4 mg which is 8%) unlike water extractive that yielded high (4.6 mg which is 15.3%). The difference was statistically significant, (*P=0.0145*), which is less than 0.05 with error bars not overlapping. On the other hand, *L. fulva*, aqueous and hexane gave almost similar amount. The difference was statistically insignificant, (*P< 0.05*) as shown in figure 4.5 with error bars overlapping. In such case, hexane was however preferred for subsequent experiments

because it less ionized and unlikely to interfere with peak spectra under UV-VIS, FT-IR and LC-MS during subsequent experiment. The observation was consistent with Ford, et al. (2015).

4.2 Identification of chromophores present in *Lannea fulva* and *Terminalia brownie* dye extracts by UV-VIS spectroscopy

4.2.1 *Lannea fulva* dye extracts

When *L. fulva* dye extracts was subjected to UV-VIS, there was absorption of electromagnetic radiations causing electron transition between HOMO and LUMO. This resulted to a spectrum of single peak with absorbance λ_{max} 275.5 nm as shown in figure 4.6. Such peak is characteristic of conjugated system or chromophore resulting from delocalized electrons or carbon carb on double bond (C=C) with no non-bonding electron. This chromophore in the dye molecule (*L. fulva*) is responsible for absorption of light by creates electronegativity which changes the size of the energy in the delocalized system, causing electron transition from the π-π^* in the process controls the colour of dyestuff.

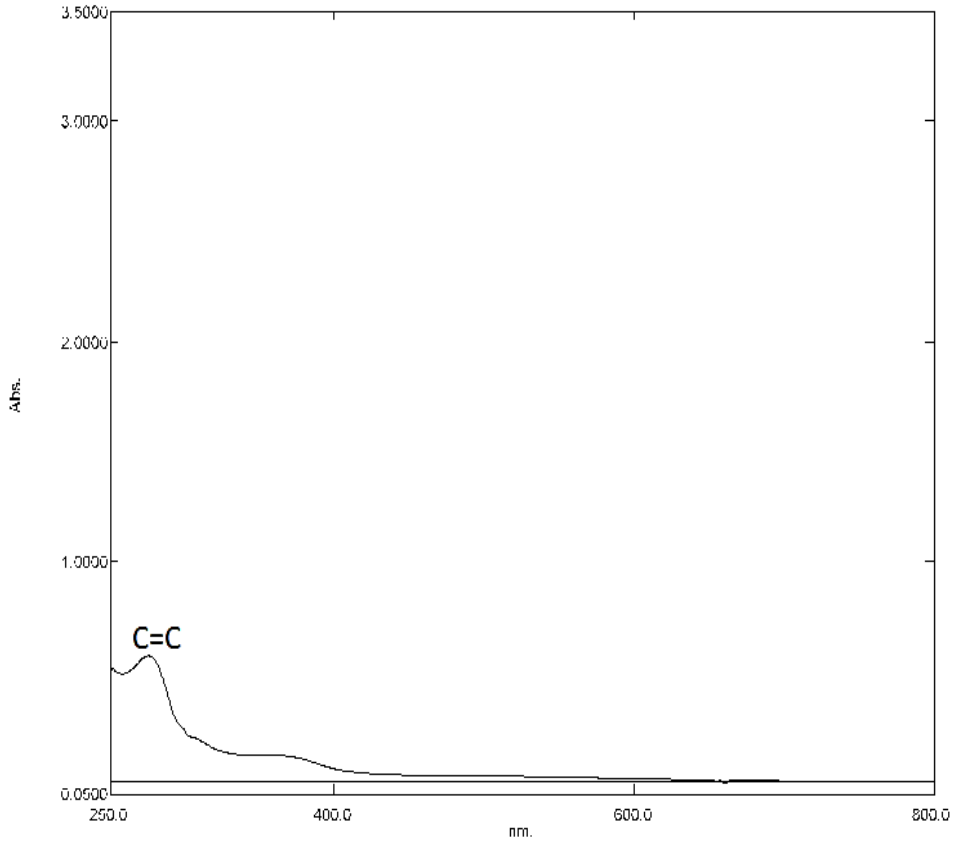

Figure 4.6: UV-VIS spectrum of *L. fulva* dye extracts

From the figure 4.6 above the wavelength at which the absorbance occurs at highest was at λ_{max} 275.5nm, which is slightly higher than that of benzene λ_{max} 255, implying that the chromophore C=C responsible for absorption spectra was due to delocalization of different types of functional group as attached to fluorescent aromatic compounds (benzene rings) or carbon-carbon double bonds in the conjugated system. This observation compares well with Thakre et al., (2017). Furthermore, the presence of such functional group/chromophore attached to benzene rings causes absorption spectra to appear at longer wavelength region or low energy (bathochromic

shift) than benzene which is absorbed at 255nm which is consistent Schnaiter et al., (2003). Such C=C conjugated system is common in benzene rings.

4.2.2 *Terminalia brownii* dye extracts

Similarly, when *T. brownii* dye extracts was exposed to UV-VIS light, there was absorption of light energy resulting to electron excitation in pi bonding orbital or non-bonding orbital to pi* anti-bonding orbital resulting to spectrum with two peaks as shown by figure 4.7.

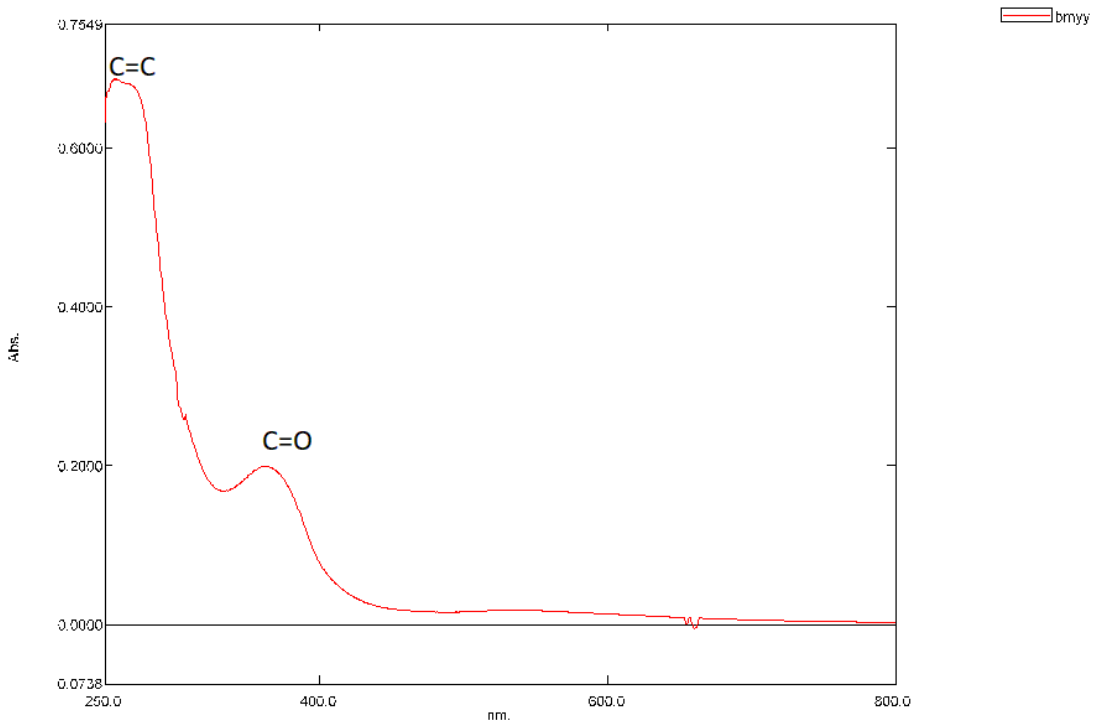

Figure 4.7:*T. brownii* dye extracts Under UV-VIS

The first peak was absorbed at λ_{max} 258.5nm which was attributed to presence of conjugated system having delocalized electrons or double bond resulting from jumping of electrons from highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO). This electron jump is also expressed as π–π^*

and the chromophore responsible for absorption of light resulting to the colour is C=C conjugated system. This observation is supported by high energy gap and short wavelength which is characteristic of C=C chromophore. The second peak UV-VIS spectra (Fig. 4.7) absorbed at,λ_{max} 361 nm, was attributed to non-bonding electron or lone pairs electron on the oxygen atom (carbon oxygen double bond or C=O),which is comparable with Shapley (2012). The chromophore C=O represented by λ_{max} 361 nm absorb Ultra Violet-Visible light of a lower frequency, lower energy and higher wavelength attributed non-bonding electron jump and it is in agreement with Naskar et al., (2017).

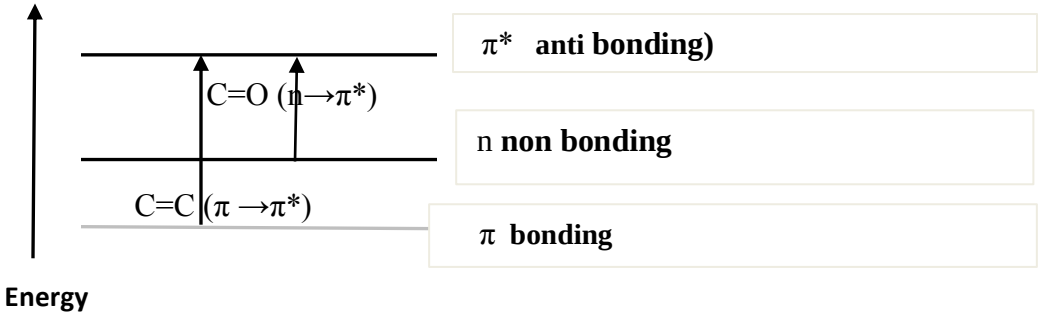

Figure 4.8: Allowed Electronic transition in UV-Vis absorption spectra

4.3 Identification of functional groups in *Lannea fulva* and *Terminalia brownii* dye extracts by FT-IR analysis

4.3.1 *Lannea fulva* dye extracts

To understand the chemical functionality, chemical bonds and confirm the chromophores present in *L. fulva* dye extracts, FT-IR was used to give the spectrum of functional groups present in the dye molecule as shown in figure. 4.9.

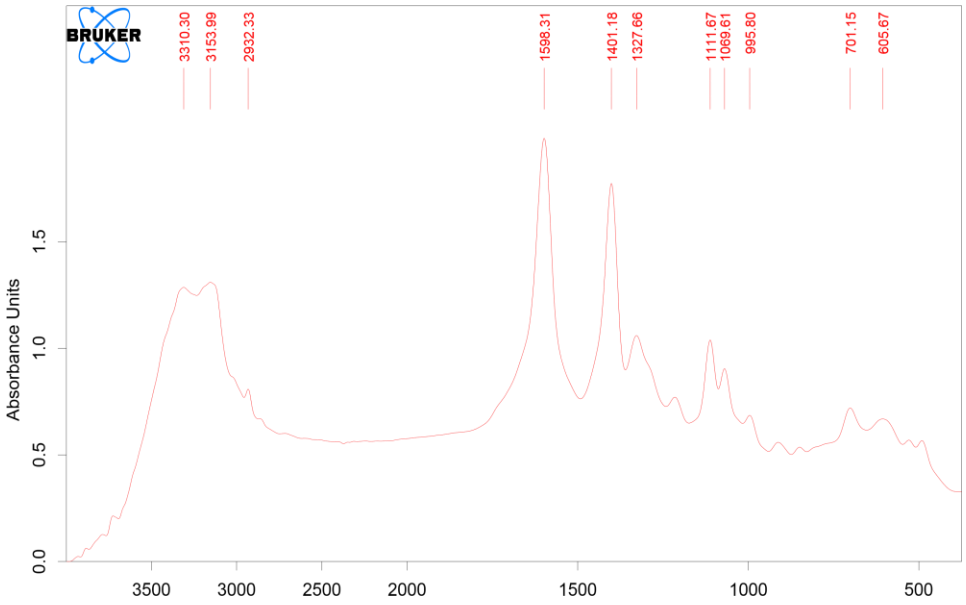

Figure 4.9: FT-IR spectra of *Lannea fulva* dye extract

The absorption band at 3310.30 cm^{-1} was attributed to O-H attached to phenolic group. Absorption band at 3153.99 cm^{-1}, was attributed to C=C which is aromatic structure. This is observation was confirmed by absorption band occurring in the region of 1615-1580 cm^{-1} showing aromatic stretch. The absorption spectrum at 2932.33 cm-1,was attributed to C-H stretch of sugar moiety or polysaccharides and they are comparable to Amir et al., (2013).

The characteristic absorption spectra displayed at 1598.31 cm^{-1} was assigned to C=C conjugated system common in benzene rings. Similarly, the absorption band at 1401.66 cm^{-1}, is due to C=O double bond of carboxylic acid. This is typical characteristic of anthraquinones structure in dyestuff like Ruberythric acid. The absorption spectra observed at frequency 1327.66 cm^{-1}, denote primary alcohol O-H or phenolic

compounds. The absorption spectra observed at frequency at 1111.67 cm^{-1},was attributed to C-O-C, whereas the absorption band at 1069.61cm^{-1} to 605.67 cm^{-1} shows C-OH stretch or C-H of monosaccharide's and they are in agreement with Kedzierska-Matysek et al., (2018). Essentially, the spectrum at these regions confirms the presence of low molecular weight carbohydrates or monosaccharide's like glucose or xylose. Such chromophoric component of a plants dye is what controls the dye colour. Additionally, auxochromes which are functional groups like OH, COOH attached to anthraquinones structure modify the dye colour (Cai *et al*., 2004). Recent research by Mbonyiryvuza *et al*. (2015), suggest that in identifying functional groups by FT-IR is instrument, specific fragmentation pattern by MS is essential.

4.3.2 *Terminalia brownii* natural dyes

The vibrational chemical bonds characteristic of certain functional groups, present in *Terminalia brownii* dye extracts, was revealed by the absorption of radiation in mid IR region between 4000- 400cm^{-1} and the functional groups present were revealed by the spectrum displayed in figure. 4.10.

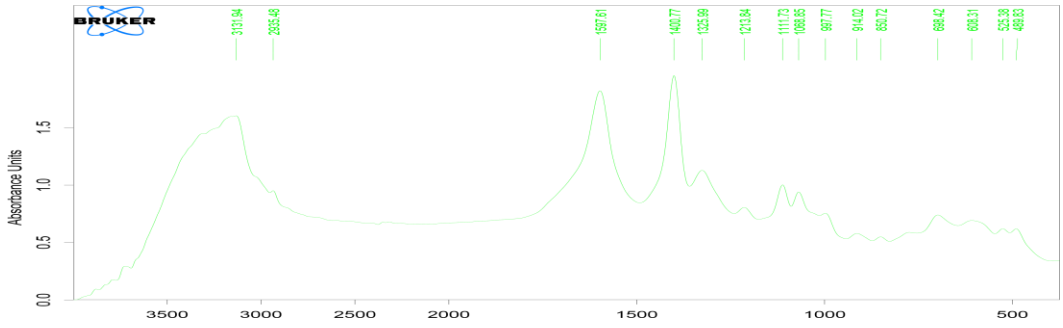

Figure 4.10: FT-IR spectra of *Terminalia brownii* dye extracts

The Figure 4.10 revealed spectra displayed at frequency 3131.94 cm^{-1} was attributed to stretching vibration of OH from carboxylic acid [COOH]. The spectra displayed at frequency 2935.48 cm^{-1}vibrations was assigned to CHO of carbohydrates which is in agreement with Wei et al., (2015). The absorption band occurring at 1597.61 cm^{-1} and 1400.77 cm^{-1} was attributed to stretching of C=C of aromatic/benzene rings. The absorption spectra displayed at frequency 1325.99 cm^{-1} was assigned to O-H of phenolic group or carboxylic group. The absorption band displayed by 1213.87 cm^{-1} is due to C-O-C, confirming Geniposide structure. This functional group can be observed in other structures like Catechin and Epicatechin. The absorption band occurring at 1111.73 cm^{-1} is due to C-O-C whereas, the absorption band between 1065.85 cm^{-1} to 608.31 cm^{-1} was assigned to C-OH phenolic compound out of plane deformation which compares well with Wang et al (2010).

4.4 Characterization of chromophoric compounds in *Terminalia brownii* and *Lannea fulva* dye extracts by LC-MS spectral analysis

4.4.1 Lannea fulva

4.4.1.1 Mass spectra of chromophoric compounds detected in positive ionization mod

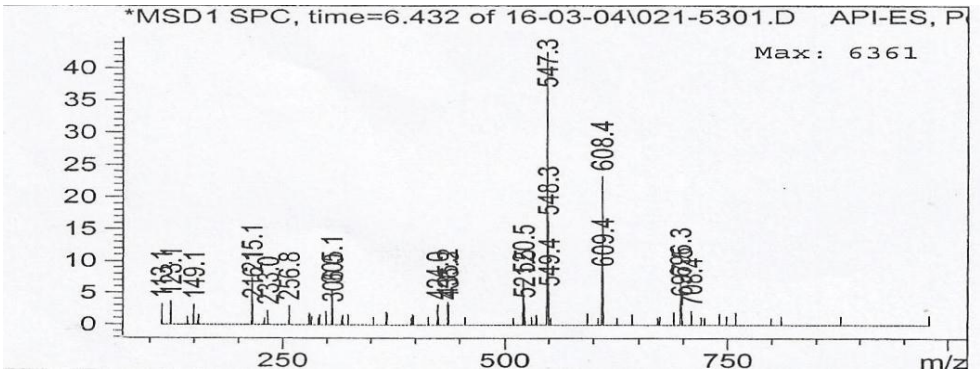

Figure 4.11 : Product Ion of Rubiadin primeveroside

The chromophoric compound displayed by the mass spectra m/z 549.4 was positively identified as Rubiadin primeveroside, based on cleavage of primeveroside (Xylose + Glucose) with mass 294amu. The decarboxylation of this compound result to formation of Rubiadin $[M+H]^+$ m/z=256 which compares well with Ford et al., (2015).

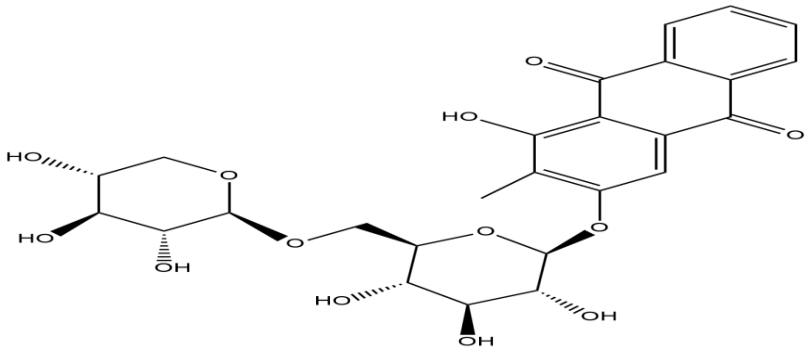

Figure 4.12: chemical structure Rubiadin primeveroside

The decarboxylation of Rubiadin primeveroside result to formation of Rubiadin $[M+H]^+$ m/z=256, which agrees with the reported data (Ford, 2017). The

decarboxylation of primeveroside (Xylose + Glucose) confirms the absorption spectra at 2932.33 cm^{-1}, due to C-H stretch of sugar moiety or polysaccharides and absorption band at 1069.61cm^{-1} to 605.67 cm^{-1} shows C-OH stretch or C-H of monosaccharaides (Xylose + Glucose) which is comparable to Kedzierska-Matysek et al., (2018).

4.4.1.2 Mass spectra chromophoric compounds detected in negative ionization mode

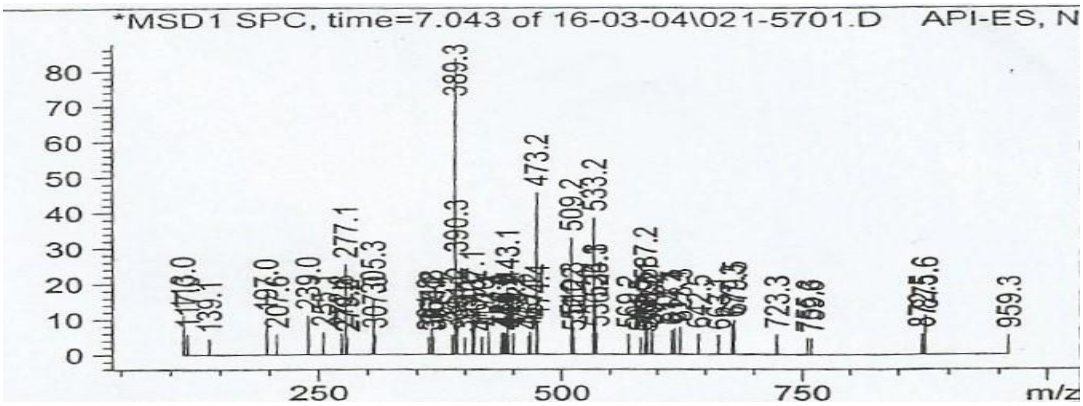

Figure 4.13: Product Ion of Ruberythric acid

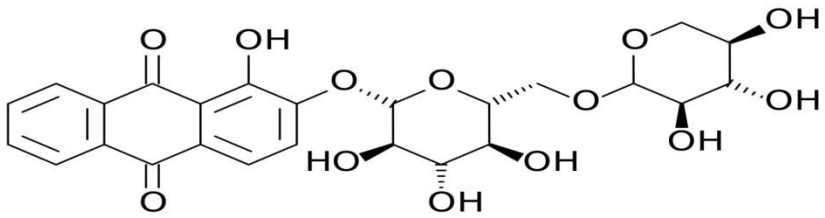

Figure 4.14: Chemical structure Ruberythric acids

The chromophoric compound with mass spectra of m/z 533 as displayed in figure 4.14 was positively identified to be Ruberythric acid also called alizarin primeveroside. This chromophoric compound has molecular weight of m/z 533.The decarboxylation

of Ruberythric acid ([M-294]$^{+}$ resulted to alizarin with molecular weight of m/z=239. This observation was comparable to Rocep et al (2014). This analogy was supported by cleavage of primeveroside with molecular weight of 294 (xylose molecular weight of 132 and glucose molecular weight of 162) which is consistent with Henderson, et al., (2013).

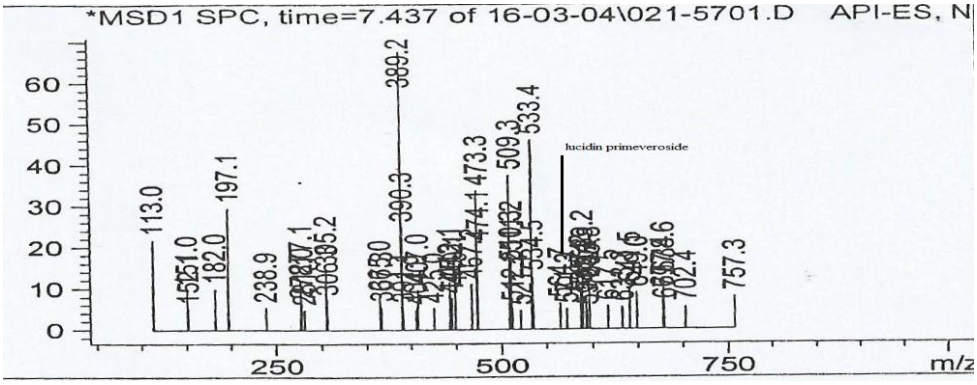

Figure 4.15: Product Ion of Lucidin primeveroside

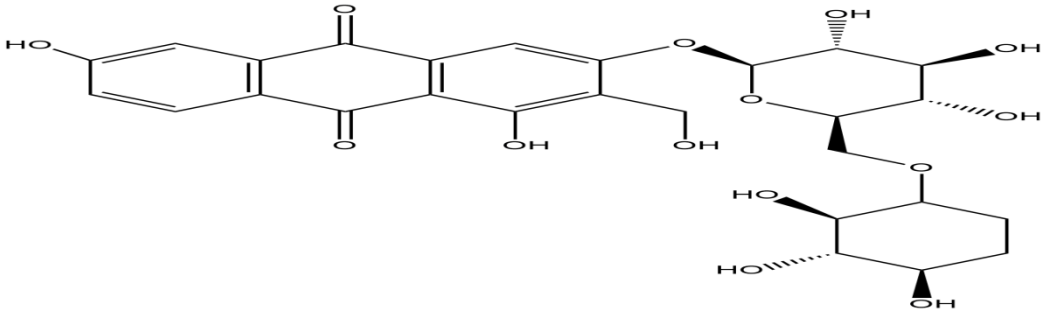

Figure 4.16: Lucidin primeveroside

The dyestuff compound characterized by mass spectra m/z 564 as displayed by figure 4.15 was positively identified as Lucidin primeveroside. This follows characteristic fragmentation of Lucidin primeveroside m/z 564 to Lucidin which is unstable

compounds and quickly gain stability by cleavage of CHO forming Xanthopurpurin with m/z=239. This observation was consistent with Cai et al (2004). The hydrolytic cleavage of primeveroside [M-H-Xyl + O-Glu] equivalent to loss of total mass of 294 amu, result to lucidin m/z=270, which is easily oxidized by air to Nordamnacanthal $[M-2H]^-$, m/z=268 (Daniels *et al*., 2014). The cleavage of CHO led to formation of xanthopurpurin whose spectral mass $[M-CHO]^-$, m/z=239. This analogy was in good agreement with the literature Lajko et al., (2015).

4.4.1.3 *Terminalia brownii*

Mass spectra chromophoric compounds detected in positive ionization mode

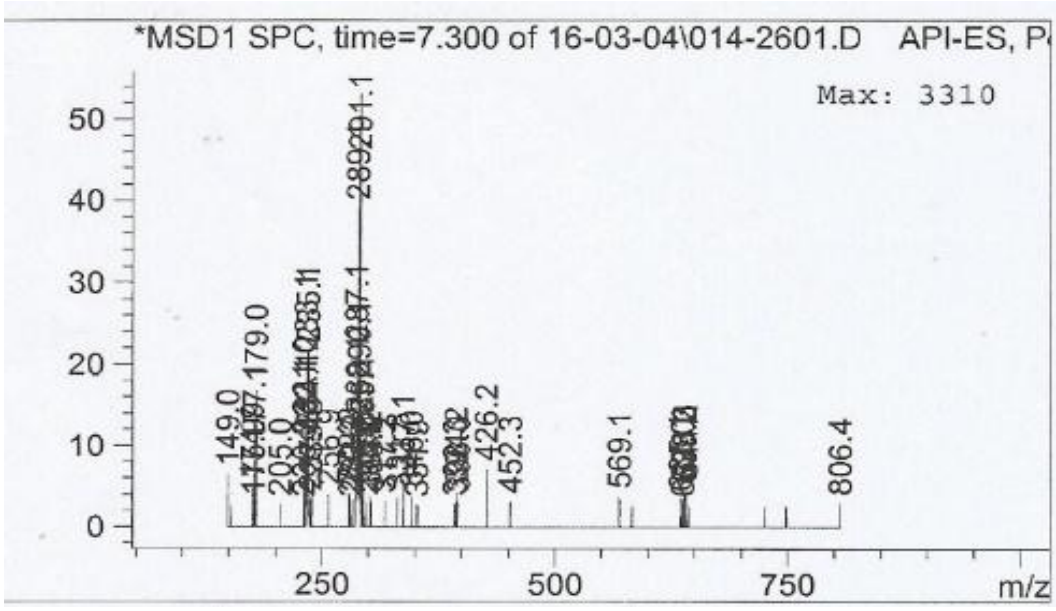

Figure 4.17: Product Ion of Catechin m/z 289.2

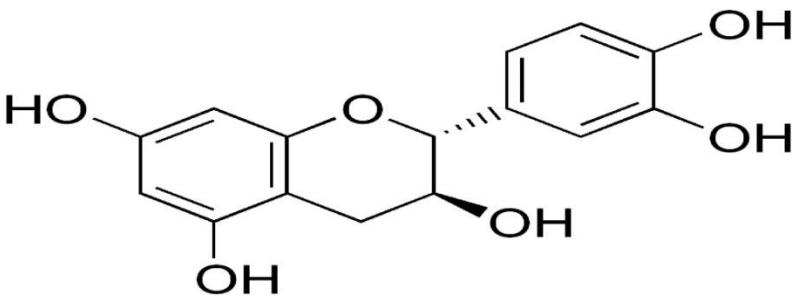

Figure 4. 18: Chemical structure of Catechin

The chromophoric compound displayed by retention time =7.3 was positively identified Catechin, based on mass spectra m/z 289.2. The chromophoric compound is common in most of yellow dye producing plants. This was consistent with compound previously identified in *T. catappa* (Sirdaarta *et al*., 2015).

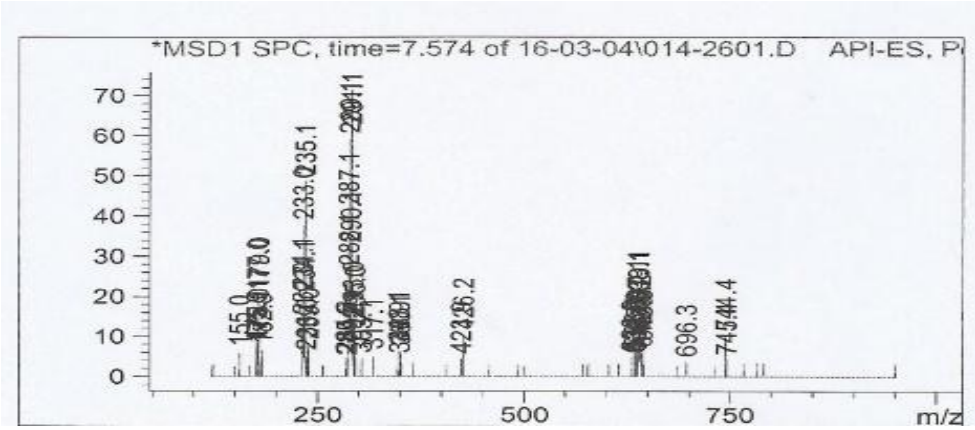

Figure 4.19: Product Ion of Epicatechin m/z 290.1

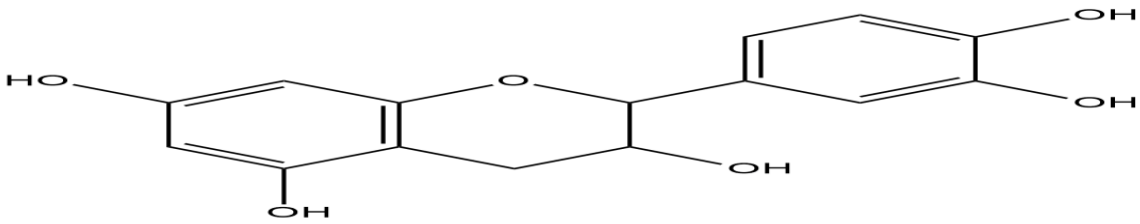

Figure 4. 20: Chemical structure of Epicatechin

The chromophoric compound displayed by retention time =7.57 with mass spectrum $[M+H]^+$, m/z = 290.1 was tentatively identified as Epicatechin, based on their mass spectrum, fragmentation pattern and the literature (Abdel-Hameed et al., 2013). The chromophoric compound is common in most of yellow dyeing plants. Catechin and Epicatechin are two isomeric compounds which are ionization dependent and can only be chemically distinguished by retention time or elution order.

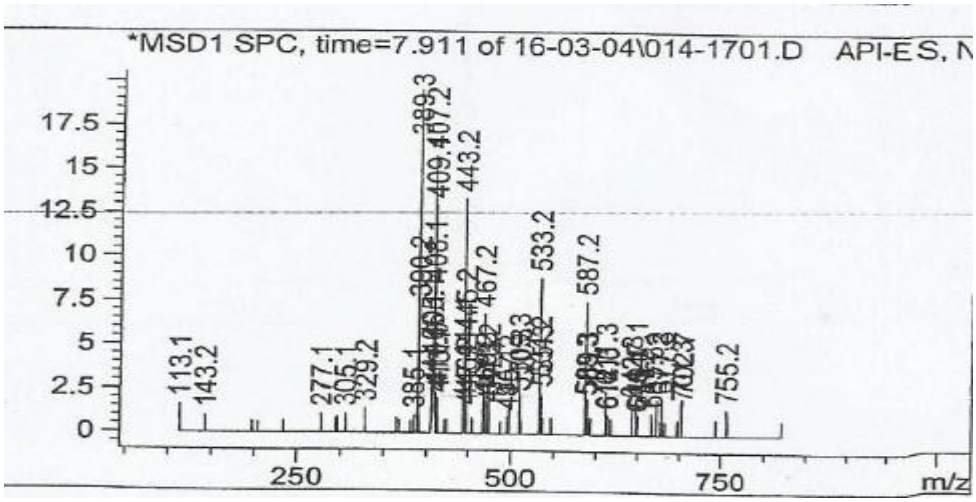

Figure 4.21: Product Ion of Baccatin m/z 587.2

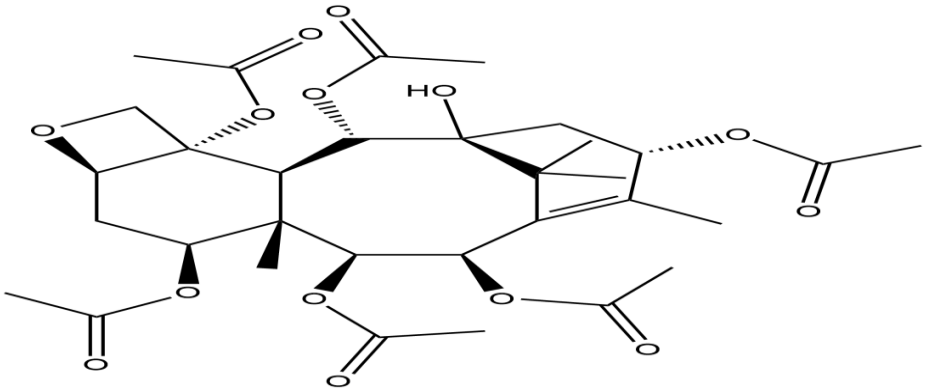

Figure 4.22: Chemical structure of Baccatin

In the same way, the protonated chromophore with peak occurring at retention time 8.0min (Figure 4.21) shows product ion of $[M+H]^+$, m/z = 588. This is the same compound in negative ionization mode which elute at retention time 7.9min with deprotonated product ion $[M-H]^-$, m/z = 587. This yellow Chromophoric compound was tentatively identified as Baccatin which is consistent with previously identified compound in *T. catappa* L. (Venkatalakshmi et al., 2016).

4.4.3.2 Mass spectra chromophoric compounds detected in negative ionization mode

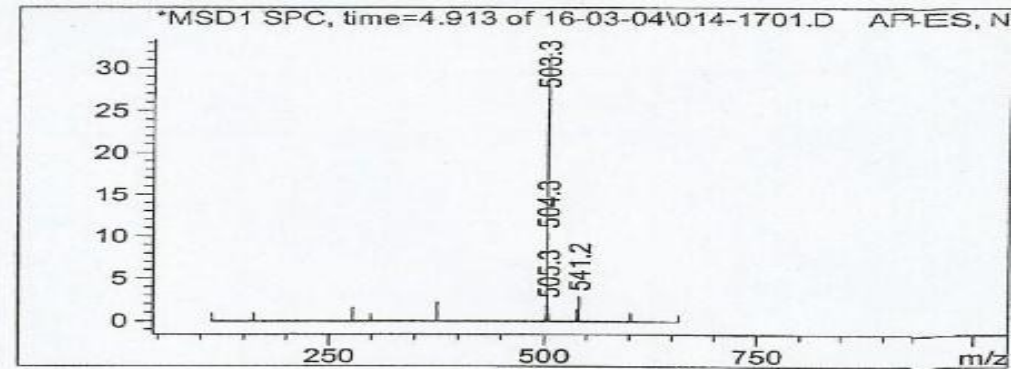

Figure. 4.23: Product Ion of Gentianose trisaccharide m/z 504

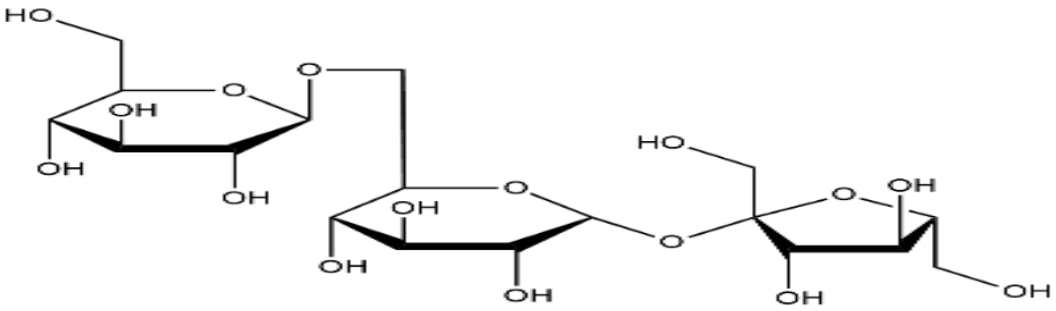

Figure 4.24: Chemical structure of Gentianose trisaccharide

The dyestuff compound characterized by mass spectra m/z 504 as displayed by Fig 4.23 was identified as Gentianose trisaccharide which agrees with Takahashi et al 2014; Peng et al 2018).This compound is composed of beta –D glucopyaranose, Alpha-D glucopyranose and beta –D fructofuran [M-2Glu-Fru]; =0, where M= 504 m/z of Gentianose, Glu =162 and Fru=180

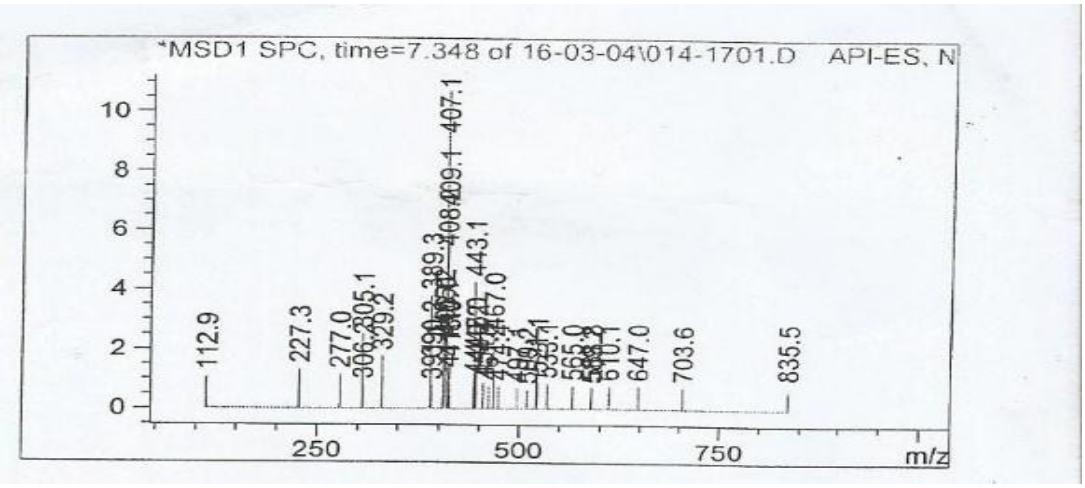

Figure 4.25: Product Ion of Geniposide m/z 389.3

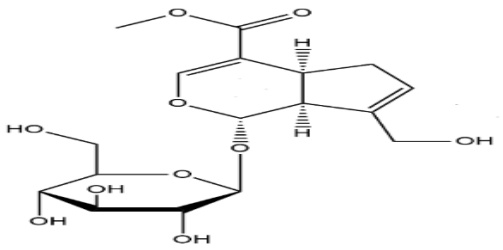

Figure 4.26: Structure of Geniposide

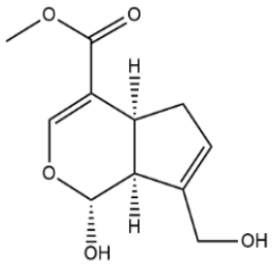

Figure 4.27: Structure of Genipin

The yellow chromophore presented by mass chromatogram $[M-H]^-$, m/z=389, extracted at retention time 7.3min (figure.4.24) was tentatively identified as

Geniposide. The unique fragmentation pattern revealed the formation of another yellow chromophore with molecular ion [M-H-162], m/z=227 which is consistent with Priya & Siril, (2014).This observation prompted us to positively identify the new yellow Chromophoric compound Genipin with respect to cleavage of O-hexose, having 162 mass units. This was previously detected and identified in *T.catappa* L. (Venkatalakshmi et al., 2016).

CHAPTER FIVE

5.0 Conclusion and Recommendation

5.1 Conclusion

Maceration method of extraction was better in extracting natural dyes compounds from *L. fulva* and *T. brownii* dye extracts. This identification of chromophores and other functional groups is valuable in unlocking full chemical information embedded in the dye structure as they serve as centers for metal ions (mordant) when dyeing fabric. These spectral properties, in particular degradation pattern, have bearing in determination of natural dye properties.

5.2 Recommendation

There is need to consider other solvent besides water and hexane to test their effect on the percentage yield on the two methods (maceration and soxhlet).

Isolation of individual dyestuff compounds identified to enhance uniformity of colour and shade during dyeing and minimize batch to batch variation of fabric during dyeing.

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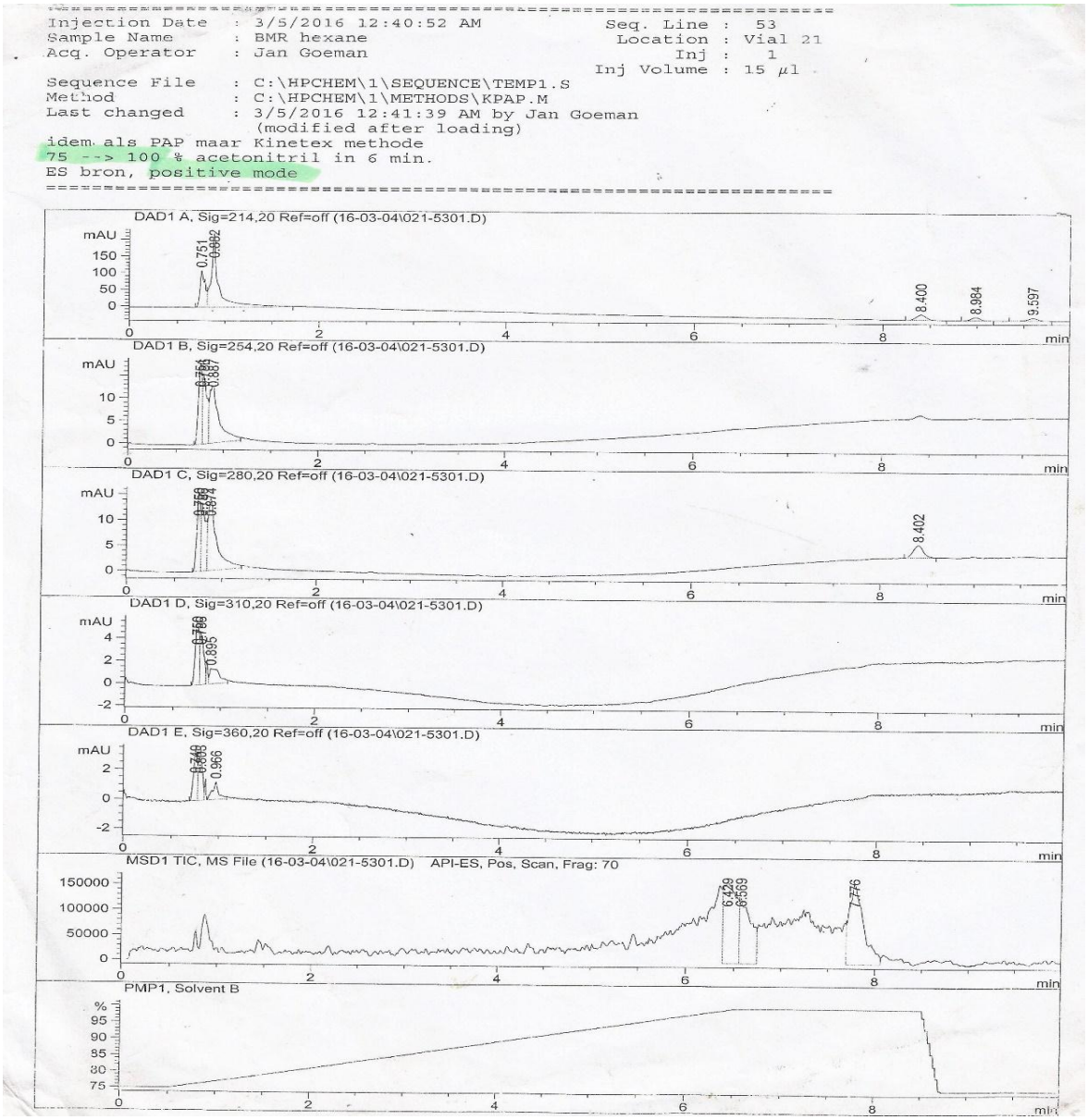
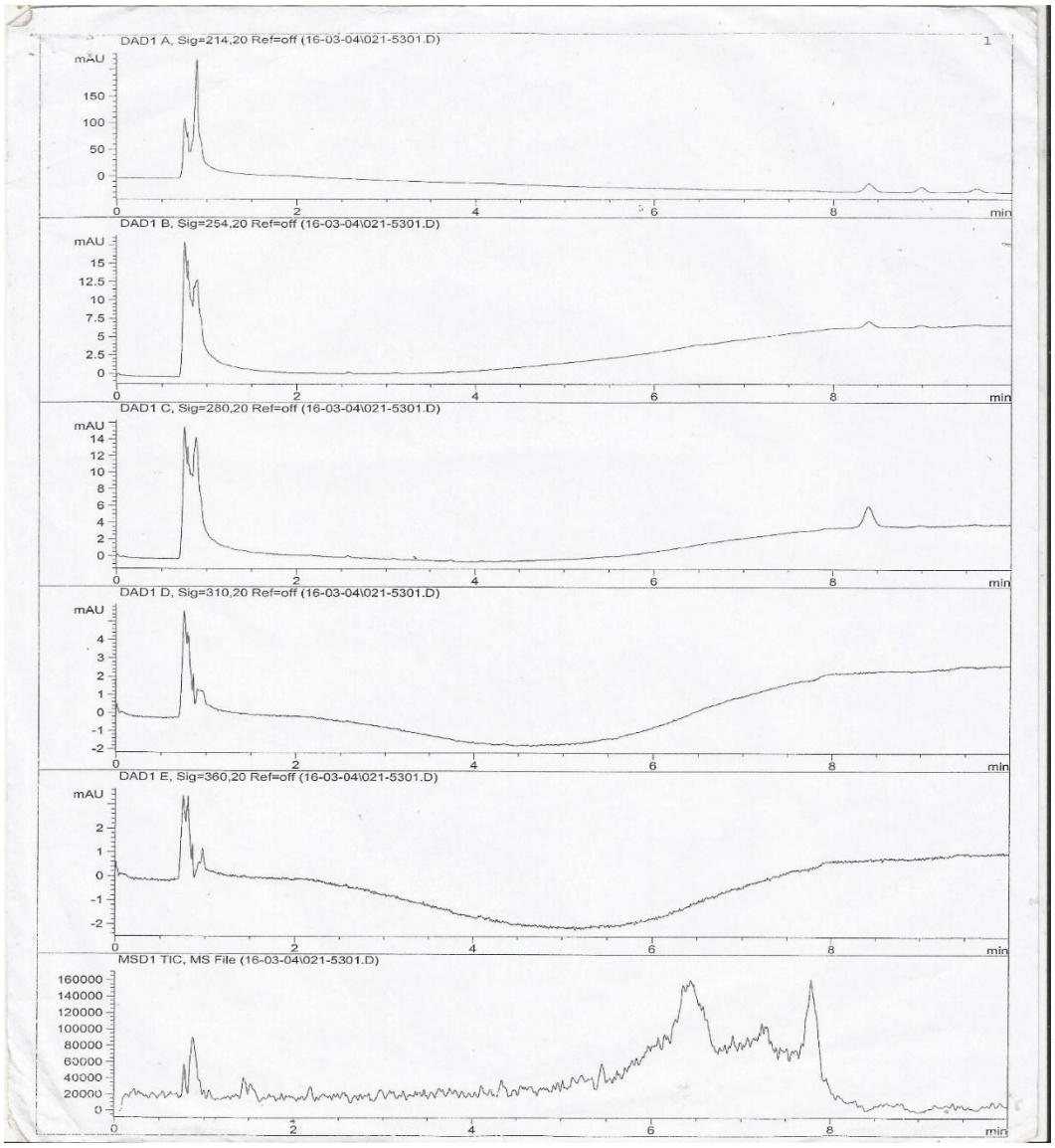
APPENDICES

APPENDIX I: Chromatograms of *Lannea fulva* dye extract in positive mode ionization

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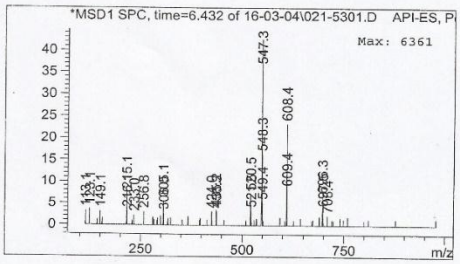
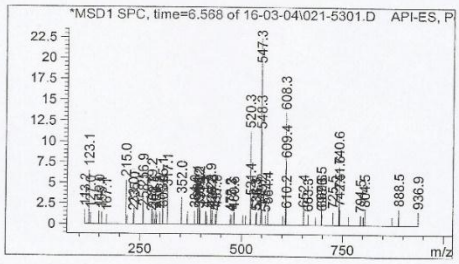
mAU
150
100
50
0
0
2
4
6
8
min
DAD1 B, Sig=254,20 Ref=off (16-03-04\021-5301.D)
mAU
15
12.5
10
7.5
5
2.5
0
0
2
4
6
8
min
DAD1 C, Sig=280,20 Ref=off (16-03-04\021-5301.D)
mAU
14
12
10
8
6
4
2
0
0
2
4
6
8
min
DAD1 D, Sig=310,20 Ref=off (16-03-04\021-5301.D)
mAU
4
3
2
1
0
-1
-2
0
2
4
6
8
min
DAD1 E, Sig=360,20 Ref=off (16-03-04\021-5301.D)
mAU
2
1
0
-1
-2
0
2
4
6
8
min
MSD1 TIC, MS File (16-03-04\021-5301.D)
160000
140000
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
min

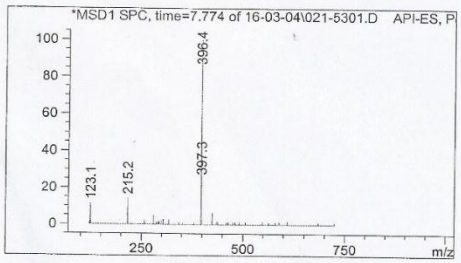
```
Injection Date  : 3/5/2016 12:40:52 AM          Seq. Line :  53
Sample Name     : BMR hexane                    Location : Vial 21
Acq. Operator   : Jan Goeman                        Inj :  1
                                                Inj Volume : 15 µl
Sequence File   : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method          : C:\HPCHEM\1\METHODS\KPAP.M
Last changed    : 3/5/2016 12:41:39 AM by Jan Goeman
                  (modified after loading)
idem als PAP maar Kinetex methode
75 --> 100 % acetonitril in 6 min.
ES bron, positive mode
```

MS Signal: MSD1 TIC, MS File, API-ES, Pos, Scan, Frag: 70
Spectra from peak tops.
Noise Cutoff: 1000 counts.
Reportable Ion Abundance: > 25%.

Retention Time (MS)	MS Area	Mol. Weight or Ion
6.429	1528882	608.40 I
		548.30 I
		547.30 I
6.569	1083652	740.60 I
		609.40 I
		608.30 I
		548.30 I
		547.30 I
		520.30 I
7.776	1753456	397.30 I
		396.40 I

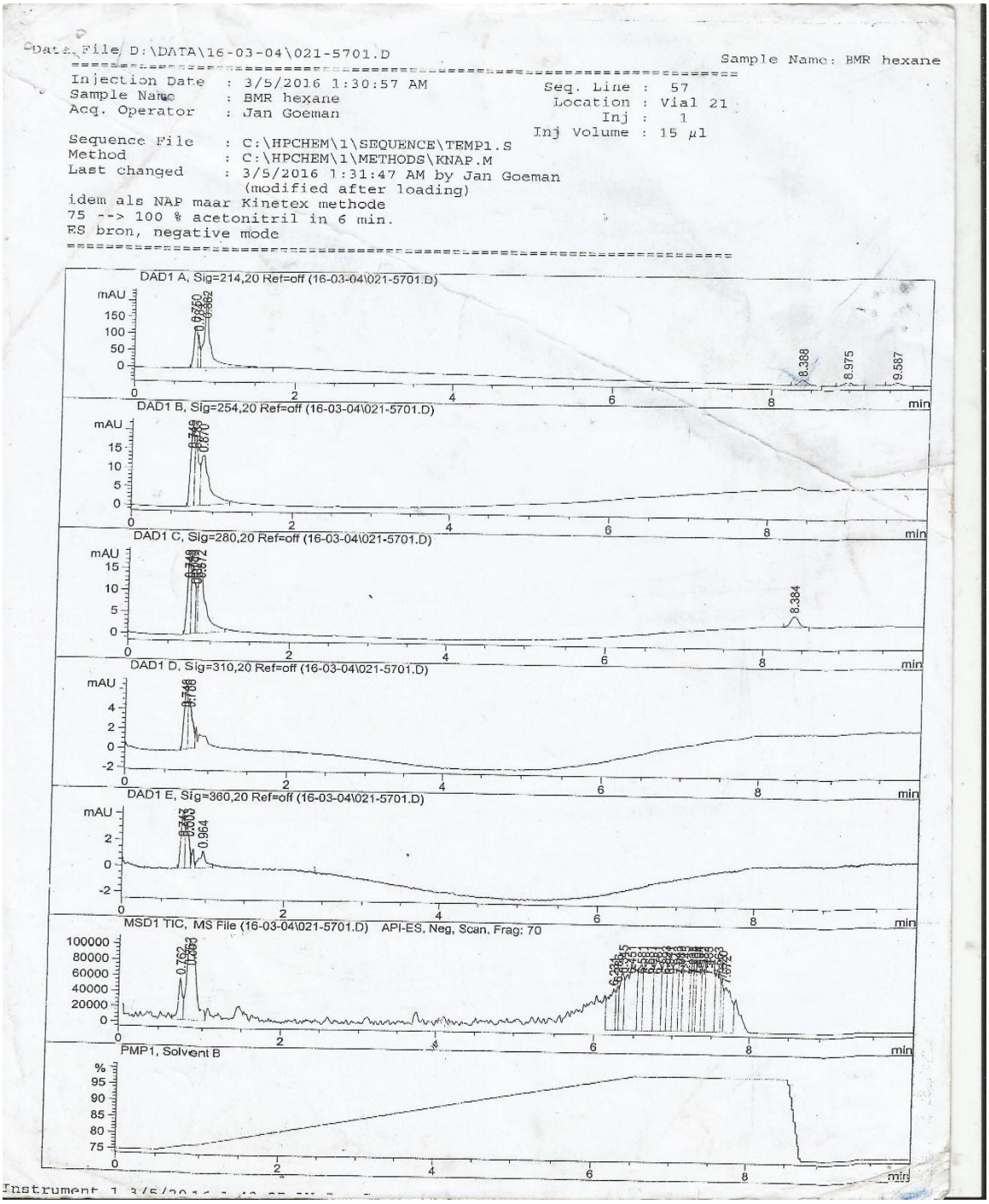


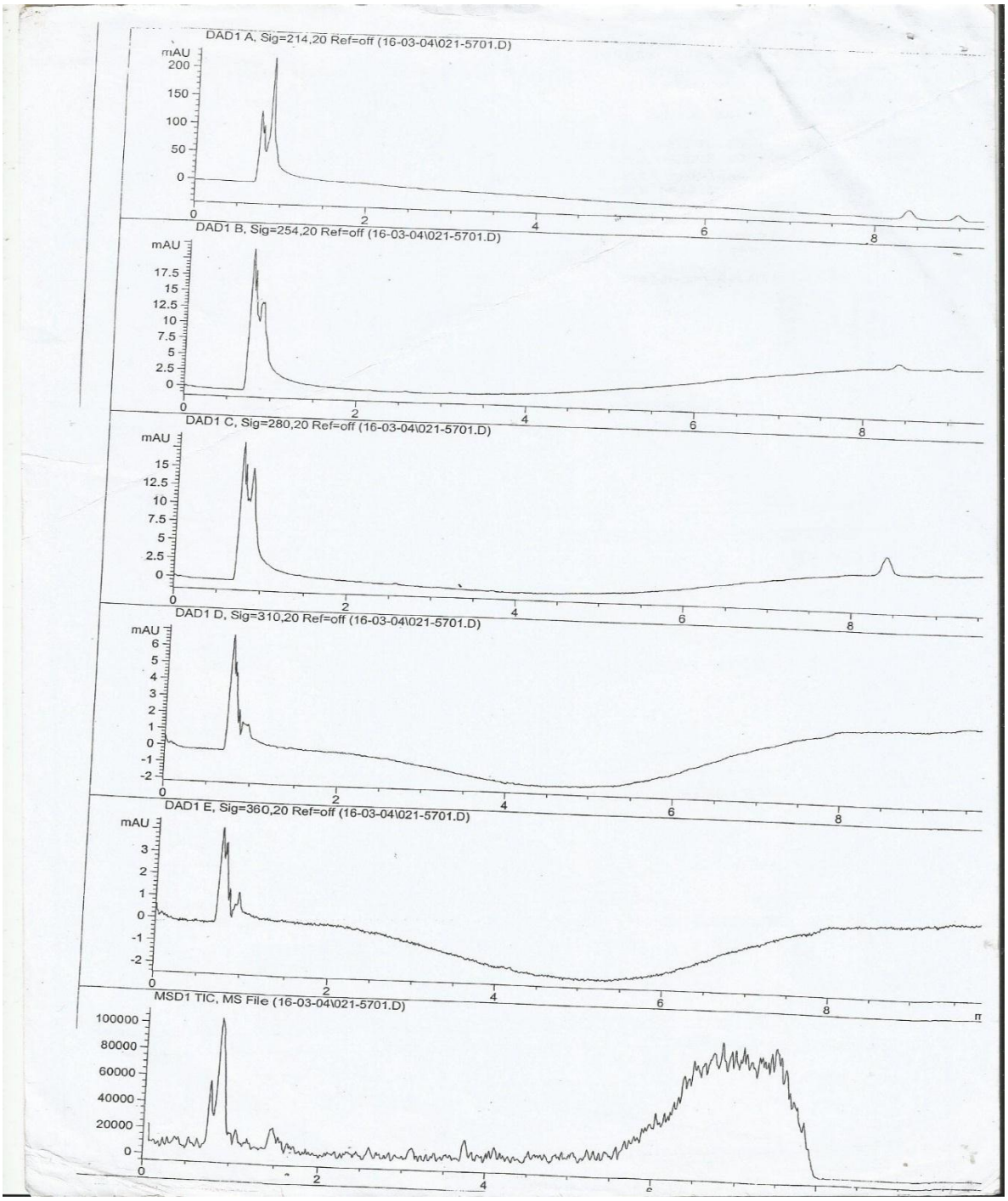




*** End of Report ***

APPENDIX II: Chromatogram of *Lannea fulva* dye extract in negative ionization mode.




DAD1 A, Sig=214,20 Ref=off (16-03-04\021-5701.D)
mAU
DAD1 B, Sig=254,20 Ref=off (16-03-04\021-5701.D)
mAU
DAD1 C, Sig=280,20 Ref=off (16-03-04\021-5701.D)
mAU
DAD1 D, Sig=310,20 Ref=off (16-03-04\021-5701.D)
mAU
DAD1 E, Sig=360,20 Ref=off (16-03-04\021-5701.D)
mAU
MSD1 TIC, MS File (16-03-04\021-5701.D)

Data File D:\DATA\16-03-04\021-5701.D
Sample Name: BMR he

Injection Date : 3/5/2016 1:30:57 AM
Sample Name : BMR hexane
Acq. Operator : Jan Goeman
Seq. Line : 57
Location : Vial 21
Inj : 1
Inj Volume : 15 µl

Sequence File : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method : C:\HPCHEM\1\METHODS\KNAP.M
Last changed : 3/5/2016 1:31:47 AM by Jan Goeman
(modified after loading)
idem als NAP maar Kinetex methode
75 --> 100 % acetonitril in 6 min.
ES bron, negative mode

MS Signal: MSD1 TIC, MS File, API-ES, Neg, Scan, Frag: 70
Spectra from peak tops.
Noise Cutoff: 1000 counts.
Reportable Ion Abundance: > 25%.

Retention Time (MS)	MS Area	Mol. Weight or Ion
0.762	177176	394.90 I
		321.10 I
0.863	614041	577.00 I
		575.00 I
		451.00 I
6.231	321315	389.30 I
6.286	134496	533.30 I
		390.20 I
		389.20 I
6.345	273292	389.20 I
		353.30 I
6.451	745113	389.20 I
		353.30 I
6.581	330003	390.30 I
		389.30 I
		353.40 I
6.681	684559	389.30 I
		353.30 I
6.781	560738	533.30 I
		399.30 I
		390.20 I
		389.20 I
		277.10 I
6.893	373857	473.30 I
		389.30 I
6.941	371561	533.30 I
		473.30 I
		389.30 I
7.043	416038	533.20 I
		509.20 I
		473.20 I
		389.30 I
7.083	255069	533.30 I
		509.20 I
		473.20 I
		389.20 I
7.145	476647	533.30 I
		389.20 I

$(N_3CH_2CH_2$

Instrument 1 3/5/2016 1:43:27 AM Jan Goeman

Data File D:\DATA\16-03-04\021-5701.D

Sample Name: BMR hexar

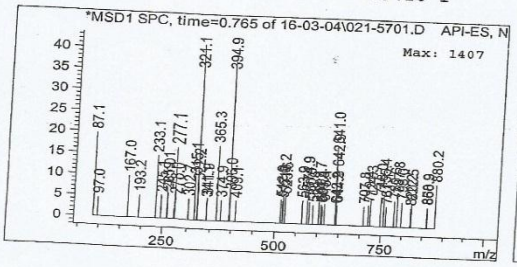
Injection Date : 3/5/2016 1:30:57 AM
Sample Name : BMR hexane
Acq. Operator : Jan Goeman

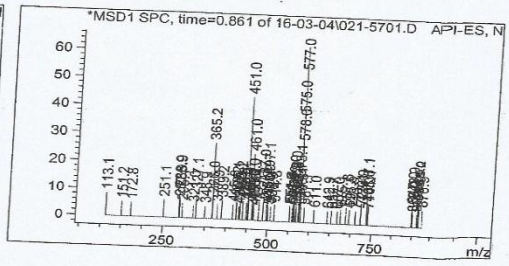
Seq. Line : 57
Location : Vial 21
Inj : 1
Inj Volume : 15 µl

Sequence File : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method : C:\HPCHEM\1\METHODS\KNAP.M
Last changed : 3/5/2016 1:31:47 AM by Jan Goeman
(modified after loading)

idem als NAP maar Kinetex methode
75 --> 100 % acetonitril in 6 min.
ES bron, negative mode

7.238	211118	534.20 I 533.30 I 473.30 I 389.20 I
7.268	176933	533.20 I 509.20 I 473.30 I 389.20 I
7.304	362982	533.30 I 509.30 I 389.20 I
7.382	242763	533.30 I 509.20 I 473.30 I 389.20 I
7.433	672019	533.40 I 509.30 I 473:30 I 389.20 I 197.10 I
7.563	333991	533.20 I 509.20 I 473.40 I 389.30 I
7.620	149443	533.40 I 473.40 I 389.20 I
7.672	402683	509.20 I 389.20 I



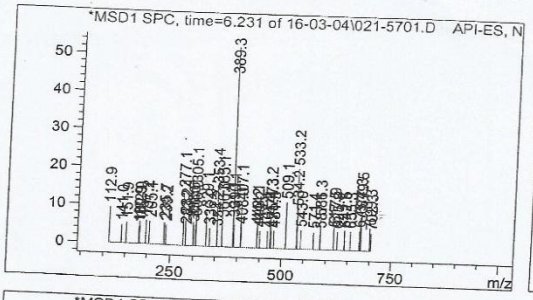


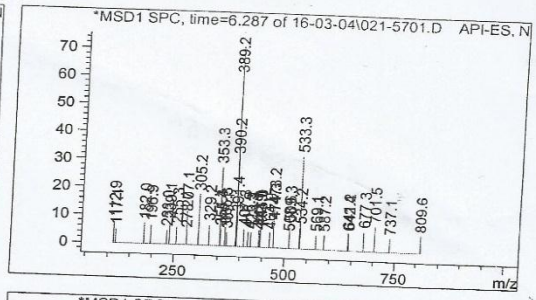
Data File D:\DATA\16-03-04\021-5701.D

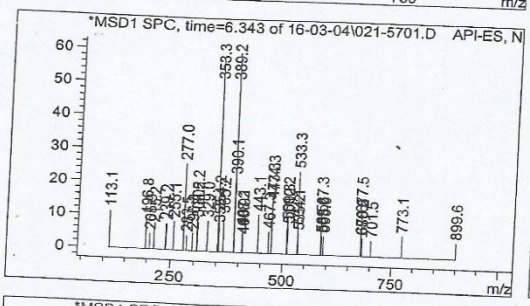
Sample Name: BMR hex

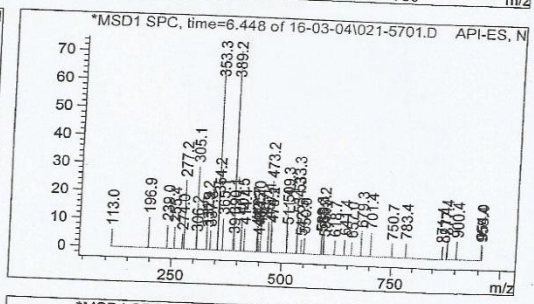
Injection Date	: 3/5/2016 1:30:57 AM	Seq. Line	: 57
Sample Name	: BMR hexane	Location	: Vial 21
Acq. Operator	: Jan Goeman	Inj	: 1
		Inj Volume	: 15 µl
Sequence File	: C:\HPCHEM\1\SEQUENCE\TEMP1.S		
Method	: C:\HPCHEM\1\METHODS\KNAP.M		
Last changed	: 3/5/2016 1:31:47 AM by Jan Goeman (modified after loading)		

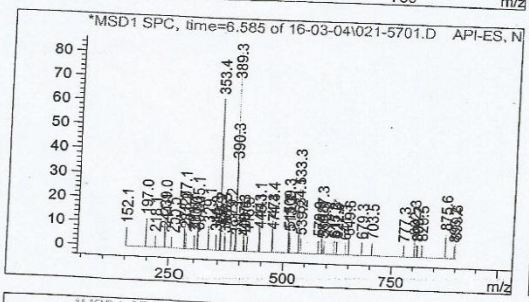
idem als NAP maar Kinetex methode
75 --> 100 % acetonitril in 6 min.
ES bron, negative mode

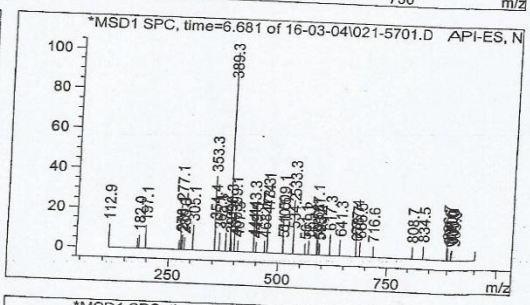


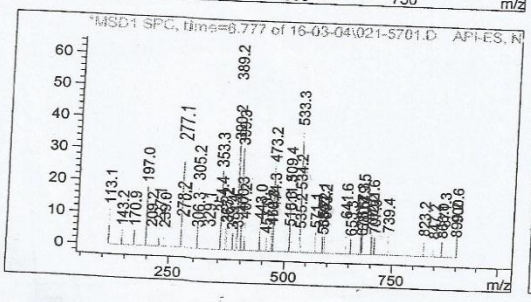


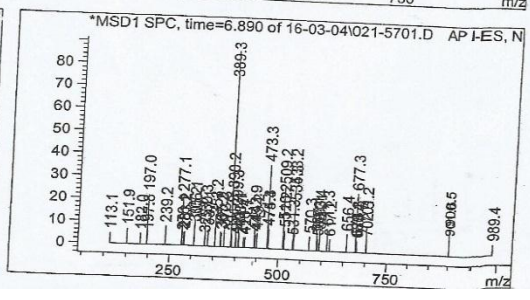


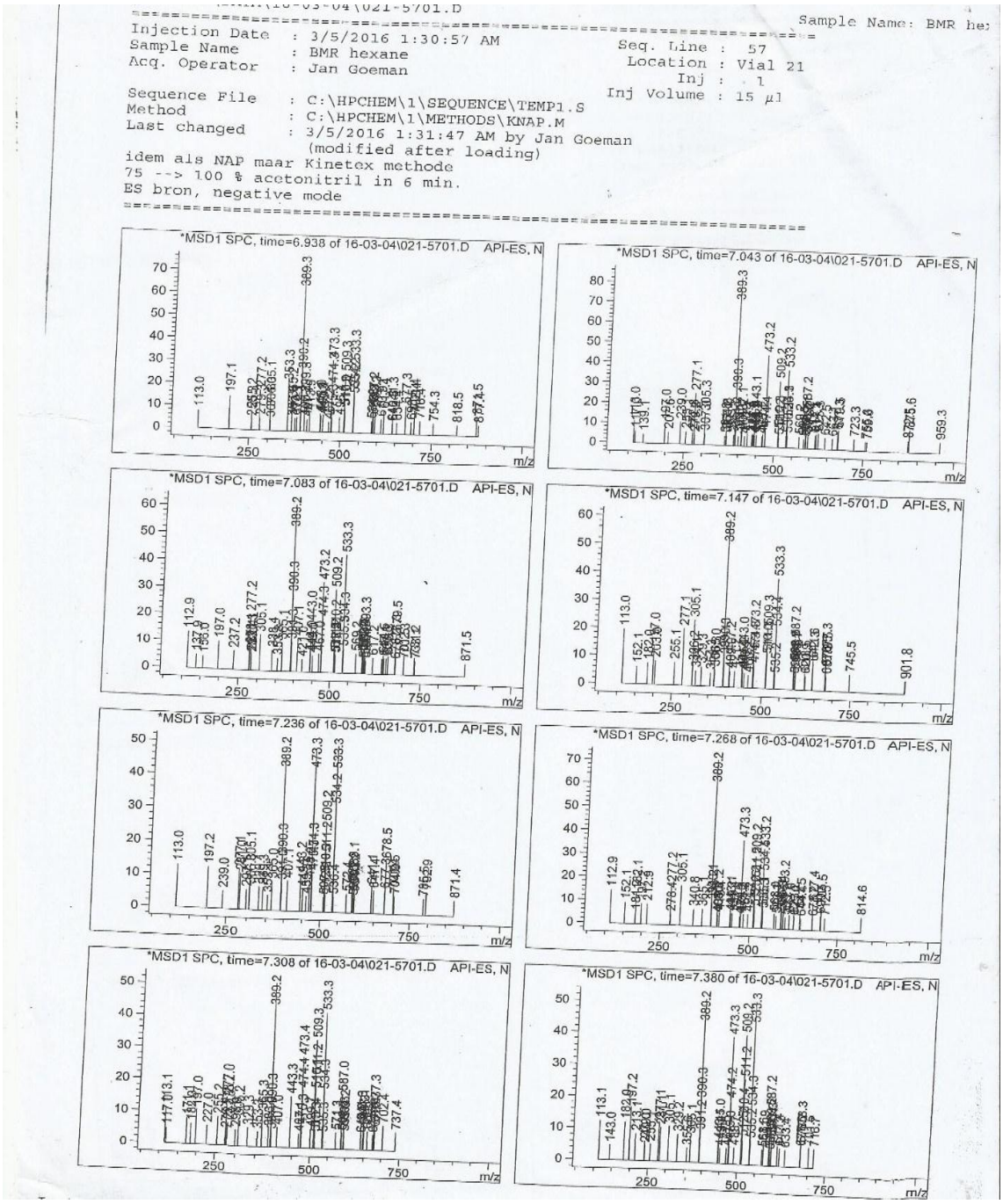












...\16-03-04\021-5701.D Sample Name: BMR he;

Injection Date : 3/5/2016 1:30:57 AM Seq. Line : 57
Sample Name : BMR hexane Location : Vial 21
Acq. Operator : Jan Goeman Inj : 1
Inj Volume : 15 µl

Sequence File : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method : C:\HPCHEM\1\METHODS\KNAP.M
Last changed : 3/5/2016 1:31:47 AM by Jan Goeman
(modified after loading)

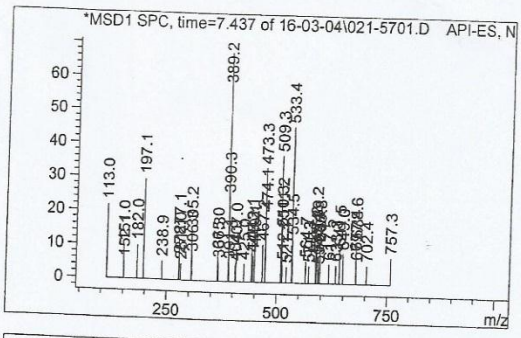
idem als NAP maar Kinetex methode
75 --> 100 % acetonitril in 6 min.
ES bron, negative mode

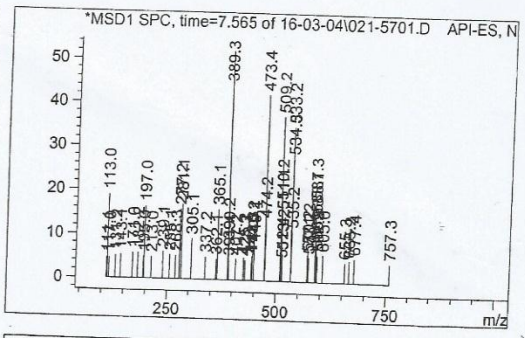
...\021-5701.D Sample Name: BMR hexa

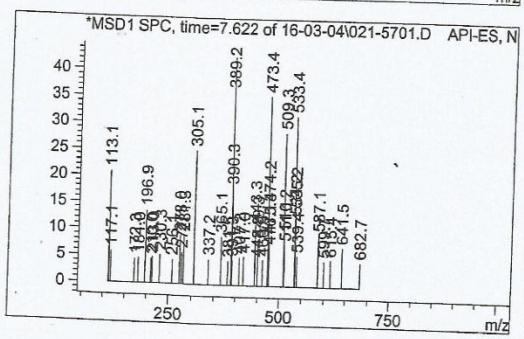
Injection Date : 3/5/2016 1:30:57 AM Seq. Line : 57
Sample Name : BMR hexane Location : Vial 21
Acq. Operator : Jan Goeman Inj : 1
Inj Volume : 15 µl

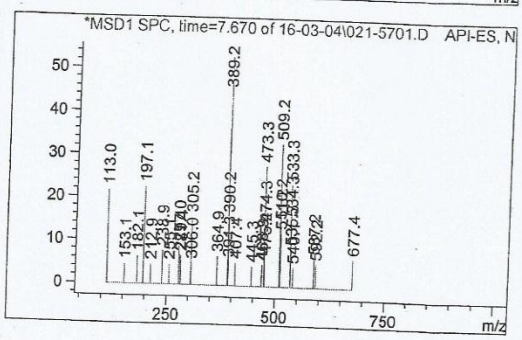
Sequence File : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method : C:\HPCHEM\1\METHODS\KNAP.M
Last changed : 3/5/2016 1:31:47 AM by Jan Goeman
(modified after loading)

idem als NAP maar Kinetex methode
75 --> 100 % acetonitril in 6 min.
ES bron, negative mode



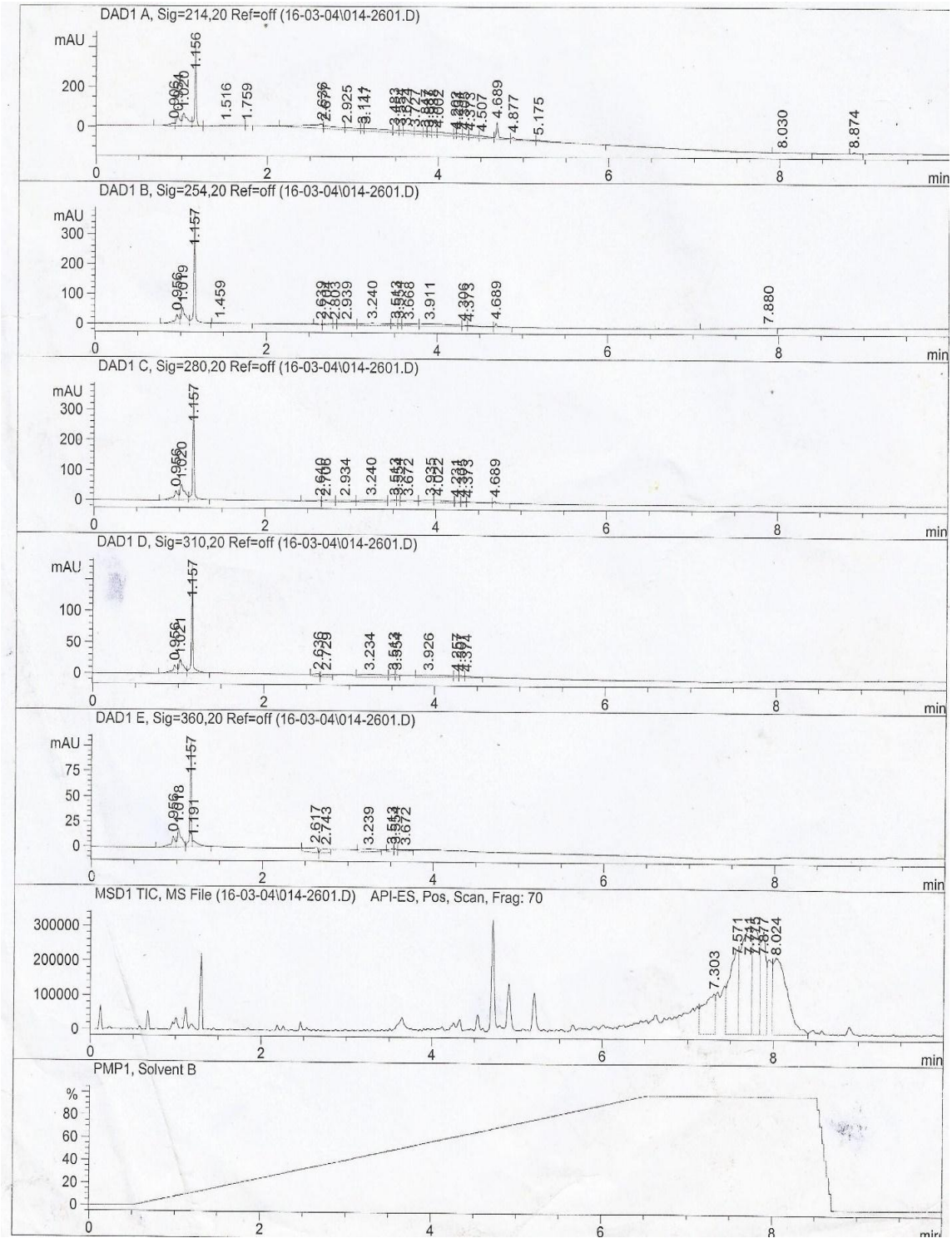


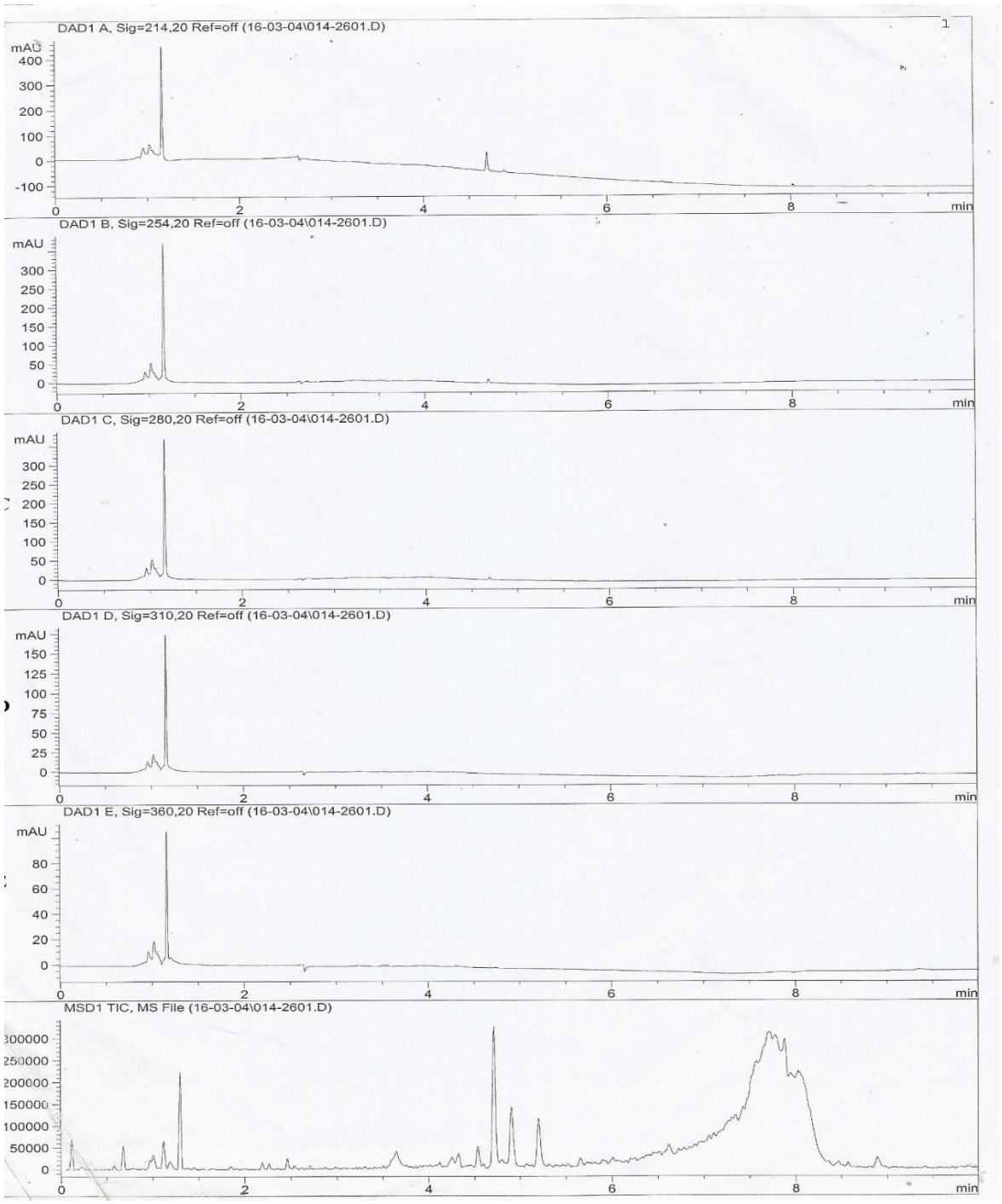




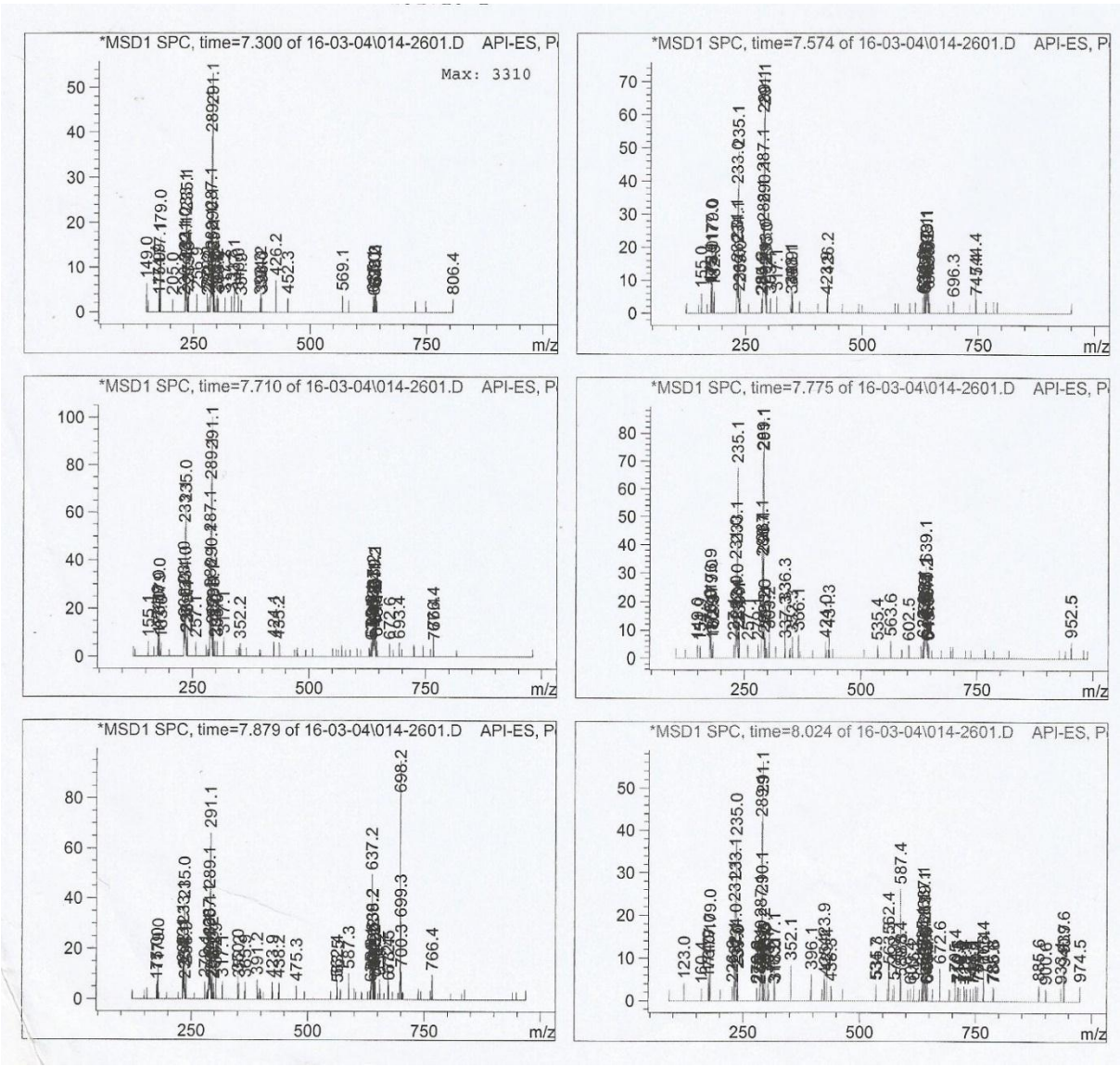
*** End of Report ***

APPENDIX III: Chromatogram of *Terminalia brownii* dye extract in positive mode ionization

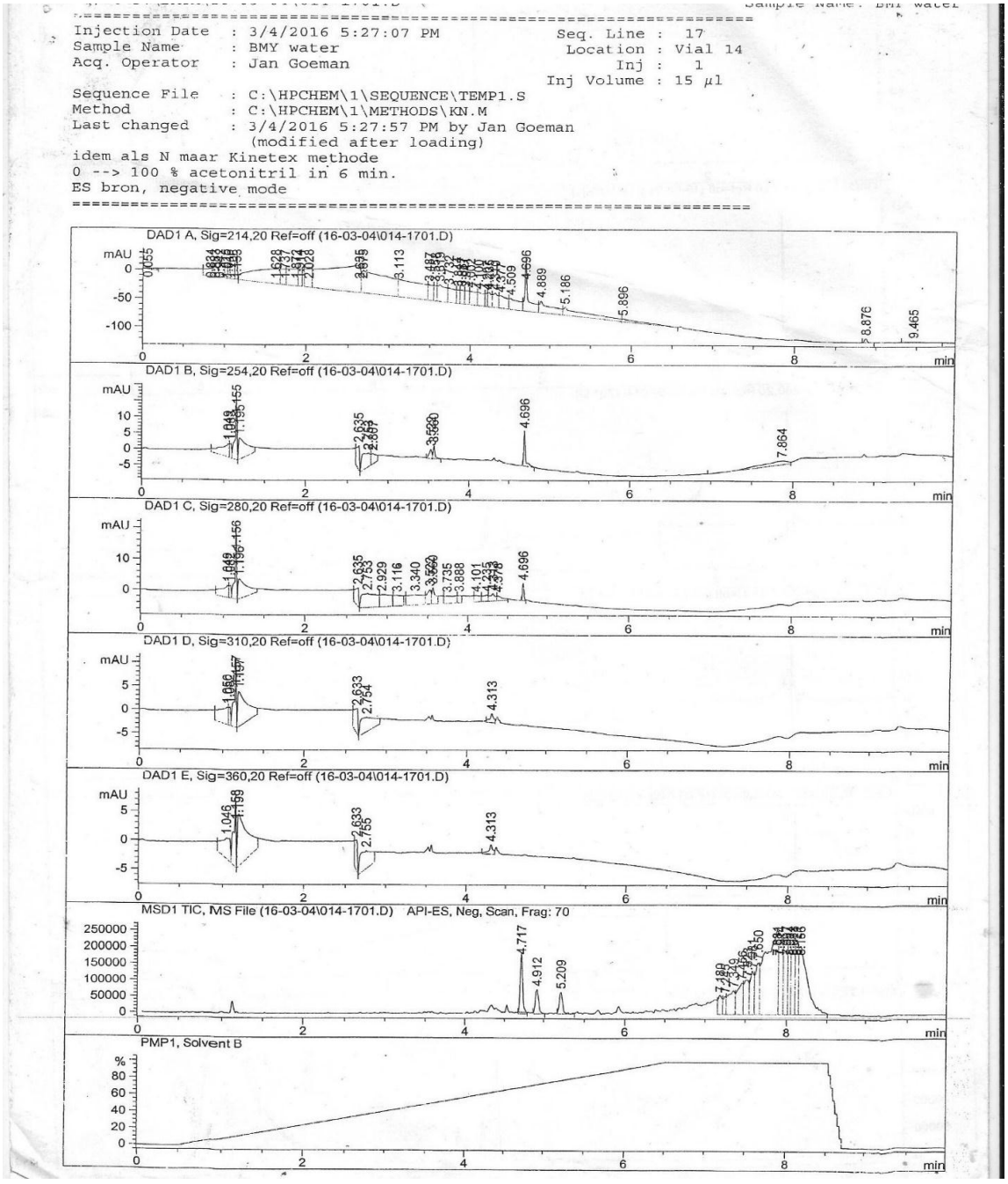


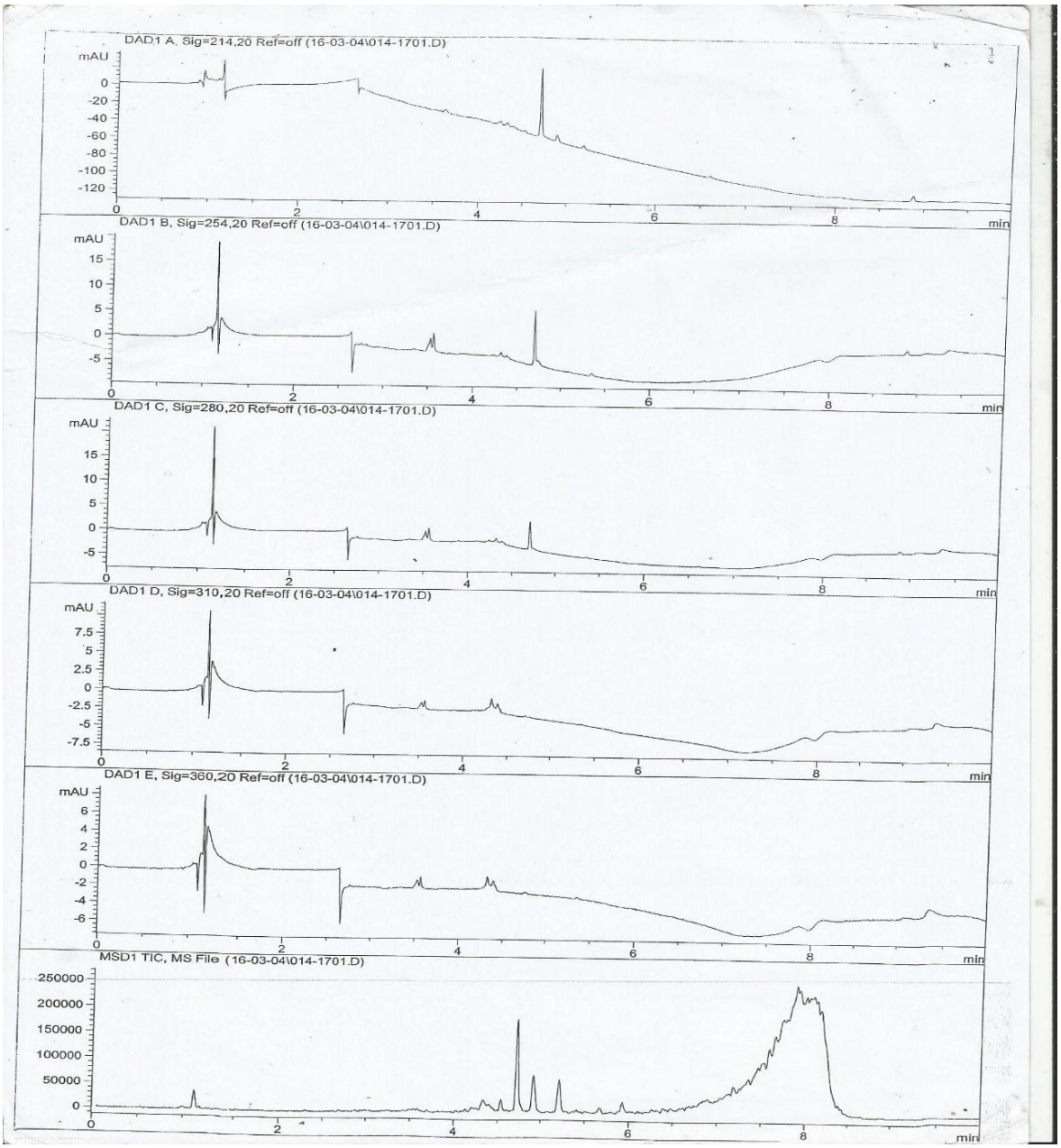

DAD1 A, Sig=214,20 Ref=off (16-03-04\014-2601.D)
mAU
400
300
200
100
0
-100
0
2
4
6
8
min
DAD1 B, Sig=254,20 Ref=off (16-03-04\014-2601.D)
mAU
300
250
200
150
100
50
0
DAD1 C, Sig=280,20 Ref=off (16-03-04\014-2601.D)
DAD1 D, Sig=310,20 Ref=off (16-03-04\014-2601.D)
150
125
75
25
DAD1 E, Sig=360,20 Ref=off (16-03-04\014-2601.D)
80
60
40
20
MSD1 TIC, MS File (16-03-04\014-2601.D)
300000
250000
200000
150000
100000
50000

```
==========================================================================
Injection Date  : 3/4/2016 7:16:02 PM            Seq. Line :  26
Sample Name     : BMY water                       Location : Vial 14
Acq. Operator   : Jan Goeman                         Inj :   1
                                                 Inj Volume : 15 µl
Sequence File   : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method          : C:\HPCHEM\1\METHODS\KP.M
Last changed    : 3/4/2016 7:16:53 PM by Jan Goeman
                  (modified after loading)
idem als P maar Kinetex methode
0 --> 100 % acetonitril in 6 min.
ES bron, positive mode
==========================================================================

MS Signal: MSD1 TIC, MS File,  API-ES, Pos, Scan, Frag: 70
    Spectra from peak tops.
    Noise Cutoff: 1000 counts.
    Reportable Ion Abundance: > 25%.


Retention                   Mol. Weight
Time (MS)      MS Area      or Ion

   7.303       1110082       291.10 I
                             290.10 I
                             289.10 I
                             287.10 I
                             235.10 I
                             233.10 I
                             179.00 I

   7.571       1755935       291.10 I
                             290.10 I
                             289.10 I
                             288.10 I
                             287.10 I
                             235.10 I
                             234.10 I
                             233.00 I
                             231.10 I
                             179.00 I
                             177.00 I

   7.711       2633729       641.20 I
                             639.10 I
                             291.10 I
                             290.10 I
                             289.10 I
                             288.10 I
                             287.10 I
                             235.00 I
                             234.00 I
                             233.10 I
                             231.00 I

   7.775       1668171       639.10 I
                             291.10 I
                             290.10 I
                             289.10 I
                             288.10 I
                             287.10 I
                             235.10 I
                             233.10 I
                             231.00 I
                             176.90 I

   7.877       1256730       699.30 I
                             698.20 I
                             639.20 I
                             638.20 I
                             637.20 I
                             291.10 I
                             289.10 I
                             287.10 I
```


*MSD1 SPC, time=7.300 of 16-03-04\014-2601.D API-ES, P
Max: 3310
*MSD1 SPC, time=7.574 of 16-03-04\014-2601.D API-ES, P
*MSD1 SPC, time=7.710 of 16-03-04\014-2601.D API-ES, P
*MSD1 SPC, time=7.775 of 16-03-04\014-2601.D API-ES, P
*MSD1 SPC, time=7.879 of 16-03-04\014-2601.D API-ES, P
*MSD1 SPC, time=8.024 of 16-03-04\014-2601.D API-ES, P
m/z

APPENDIX IV: Chromatograms of *Terminalia brownii* dye extract in negative mode ionization




DAD1 A, Sig=214,20 Ref=off (16-03-04\014-1701.D)
mAU
DAD1 B, Sig=254,20 Ref=off (16-03-04\014-1701.D)
mAU
DAD1 C, Sig=280,20 Ref=off (16-03-04\014-1701.D)
mAU
DAD1 D, Sig=310,20 Ref=off (16-03-04\014-1701.D)
mAU
DAD1 E, Sig=360,20 Ref=off (16-03-04\014-1701.D)
mAU
MSD1 TIC, MS File (16-03-04\014-1701.D)
min

```
==========================================================================
Injection Date  : 3/4/2016 5:27:07 PM            Seq. Line :  17
Sample Name     : BMY water                       Location : Vial 14
Acq. Operator   : Jan Goeman                         Inj :   1
                                                 Inj Volume : 15 µl
Sequence File   : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method          : C:\HPCHEM\1\METHODS\KN.M
Last changed    : 3/4/2016 5:27:57 PM by Jan Goeman
                  (modified after loading)
idem als N maar Kinetex methode
0 --> 100 % acetonitril in 6 min.
ES bron, negative mode
==========================================================================

MS Signal: MSD1 TIC, MS File,  API-ES, Neg, Scan, Frag: 70
    Spectra from peak tops.
    Noise Cutoff: 1000 counts.
    Reportable Ion Abundance: > 25%.


Retention                    Mol. Weight
Time (MS)      MS Area       or Ion

   4.717        415486       277.10 I

   4.912        222520       504.30 I
                             503.30 I

   5.209        187222       504.30 I
                             503.20 I

   7.180        187689       409.10 I
                             407.10 I

   7.245        136338       409.10 I
                             407.20 I
                             305.20 I

   7.349        424212       409.10 I
                             408.20 I
                             407.10 I

   7.456        509057       443.20 I
                             409.00 I
                             408.10 I
                             407.10 I
                             389.30 I

   7.523        431609       443.10 I
                             409.00 I
                             408.10 I
                             407.10 I
                             405.00 I
                             389.20 I

   7.581        437970       742.30 I
                             467.20 I
                             443.10 I
                             409.10 I
                             408.00 I
                             407.20 I
                             389.20 I
                             305.20 I

   7.650        554823       467.20 I
                             443.20 I
                             409.10 I
                             408.10 I
                             407.10 I
                             406.00 I
                             405.00 I
                             389.20 I

   7.881       2723828       533.30 I
```

Injection Date : 3/4/2016 5:27:07 PM Seq. Line : 17
Sample Name : BMY water Location : Vial 14
Acq. Operator : Jan Goeman Inj : 1
Inj Volume : 15 µl

Sequence File : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method : C:\HPCHEM\1\METHODS\KN.M
Last changed : 3/4/2016 5:27:57 PM by Jan Goeman
(modified after loading)

idem als N maar Kinetex methode
0 --> 100 % acetonitril in 6 min.
ES bron, negative mode

		467.20 I
		445.10 I
		443.20 I
		409.10 I
		408.10 I
		407.20 I
		405.10 I
		390.10 I
		389.30 I
7.914	851152	587.20 I
		533.20 I
		467.20 I
		445.20 I
		443.20 I
		409.10 I
		408.10 I
		407.20 I
		405.10 I
		390.20 I
		389.30 I
7.997	753587	587.20 I
		533.30 I
		473.30 I
		467.20 I
		445.20 I
		443.10 I
		409.10 I
		408.10 I
		407.10 I
		406.10 I
		405.10 I
		390.20 I
		389.30 I
8.034	568267	587.10 I
		533.30 I
		473.20 I
		467.30 I
		443.00 I
		409.10 I
		408.10 I
		407.10 I
		405.00 I
		390.20 I
		389.30 I
8.078	705795	534.30 I
		533.20 I
		473.30 I
		467.10 I
		445.10 I
		444.10 I
		443.10 I
		409.10 I
		408.20 I
		407.10 I
		406.00 I
		405.10 I

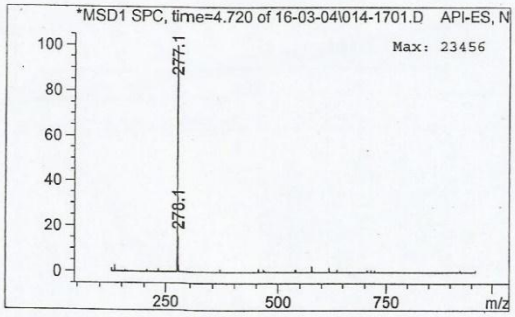
Data File D:\DATA\16-03-04\014-1701.D Sample Name: BMY water

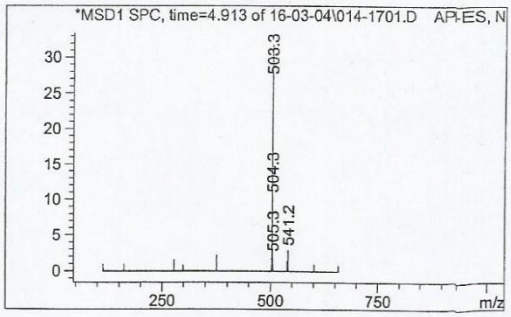
Injection Date : 3/4/2016 5:27:07 PM Seq. Line : 17
Sample Name : BMY water Location : Vial 14
Acq. Operator : Jan Goeman Inj : 1
Inj Volume : 15 µl

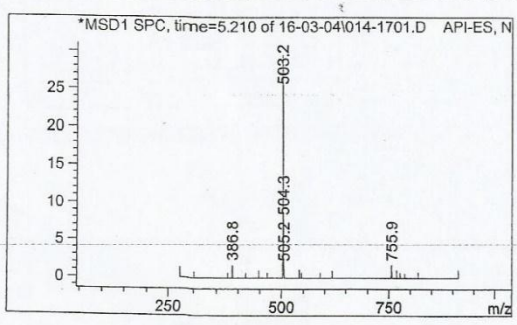
Sequence File : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method : C:\HPCHEM\1\METHODS\KN.M
Last changed : 3/4/2016 5:27:57 PM by Jan Goeman
(modified after loading)

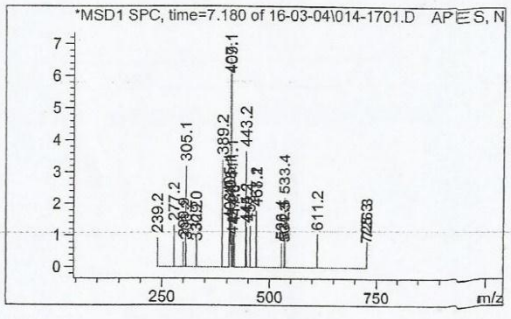
idem als N maar Kinetex methode
0 --> 100 % acetonitril in 6 min.
ES bron, negative mode

		390.30 I
		389.30 I
8.121	552277	587.20 I
		534.20 I
		533.30 I
		473.30 I
		445.00 I
		443.20 I
		409.10 I
		408.10 I
		407.20 I
		405.00 I
		390.30 I
		389.20 I
8.166	1541935	587.20 I
		533.30 I
		443.20 I
		409.10 I
		408.20 I
		407.10 I
		390.20 I
		389.20 I

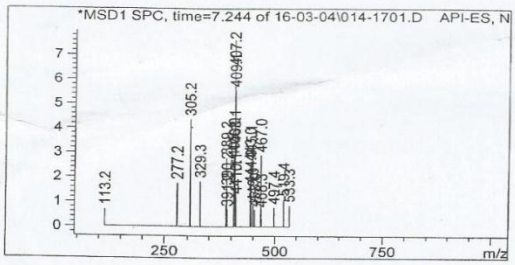


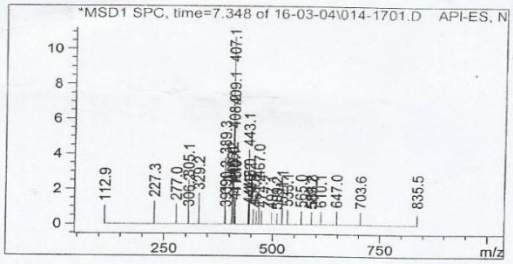


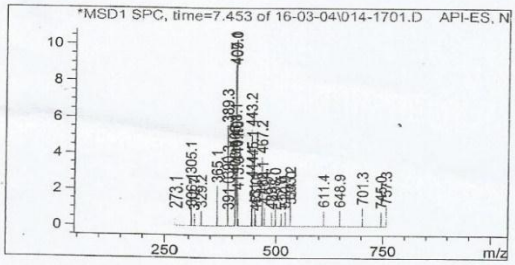


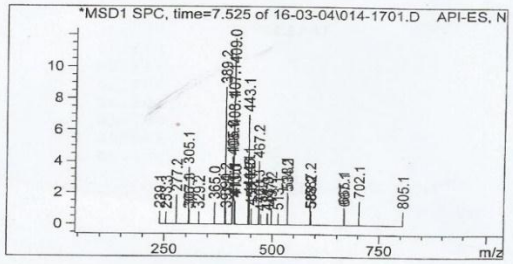


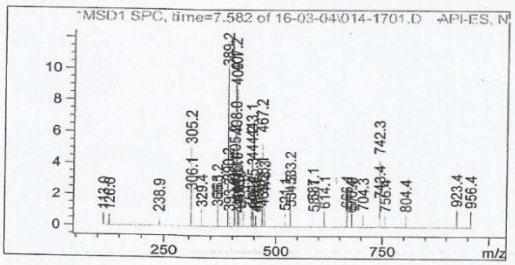
```
Injection Date   : 3/4/2016 5:27:07 PM              Seq. Line :  17
Sample Name      : BMY water                        Location : Vial 14
Acq. Operator    : Jan Goeman                           Inj :  1
                                                  Inj Volume : 15 µl
Sequence File    : C:\HPCHEM\1\SEQUENCE\TEMP1.S
Method           : C:\HPCHEM\1\METHODS\KN.M
Last changed     : 3/4/2016 5:27:57 PM by Jan Goeman
                   (modified after loading)
idem als N maar Kinetex methode
0 --> 100 % acetonitril in 6 min.
ES bron, negative mode
```

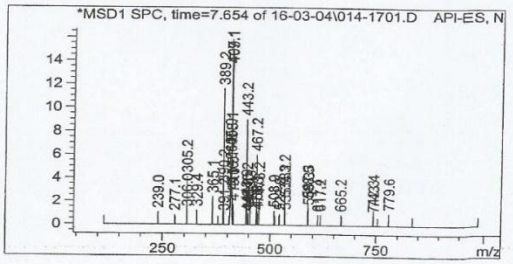


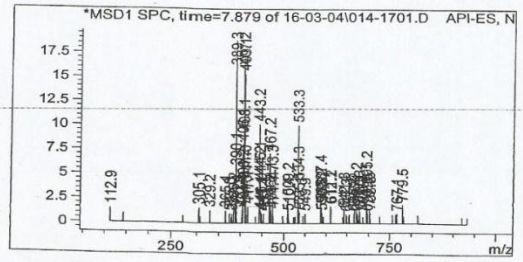












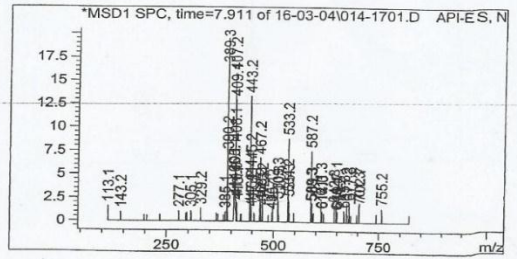


Table 4.2 Multiple comparisons of the two solvents (hexane and water) using the two extraction methods (soxhlet and maceration) on the two dye plants.

(I) Type of Plant & Extraction	(J) Type of Plant & Extraction	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Soxhlet *L.fulva* Hexane	Soxhlet *Lannea fulva* Aqueous	-.4000*	.1453	.014	-.708	-.092
	Soxhlet *Terminalia brownii* Hexane	.8000*	.1453	.000	.492	1.108
	Soxhlet *Terminalia brownii* Aqueous	-.2000	.1453	.188	-.508	.108
	Maceration *Lannea fulva* Hexane	-1.1333*	.1453	.000	-1.441	-.825
	Maceration *Lannea fulva* Aqueous	-1.2333*	.1453	.000	-1.541	-.925
	Maceration *Terminalia brownii* Hexane	.8333*	.1453	.000	.525	1.141
	Maceration *Terminalia brownii* Aqueous	-1.3667*	.1453	.000	-1.675	-1.059
Soxhlet *Lannea fulva* Aqueous	Soxhlet *Lannea fulva* Hexane	.4000*	.1453	.014	.092	.708
	Soxhlet *Terminalia brownii* Hexane	1.2000*	.1453	.000	.892	1.508
	Soxhlet *Terminalia brownii* Aqueous	.2000	.1453	.188	-.108	.508
	Maceration *Lannea fulva* Hexane	-.7333*	.1453	.000	-1.041	-.425
	Maceration *Lannea fulva* Aqueous	-.8333*	.1453	.000	-1.141	-.525
	Maceration *Terminalia brownii* Hexane	1.2333*	.1453	.000	.925	1.541
	Maceration *Terminalia brownii* Aqueous	-.9667*	.1453	.000	-1.275	-.659
Soxhlet *Terminalia brownii* Hexane	Soxhlet *Lannea fulva* Hexane	-.8000*	.1453	.000	-1.108	-.492
	Soxhlet *Lannea fulva* Aqueous	-1.2000*	.1453	.000	-1.508	-.892

	Soxhlet *Terminalia brownii* Aqueous	-1.0000*	.1453	.000	-1.308	-.692
	Maceration *Lannea fulva* Hexane	-1.9333*	.1453	.000	-2.241	-1.625
	Maceration *Lannea fulva* Aqueous	-2.0333*	.1453	.000	-2.341	-1.725
	Maceration *Terminalia brownii* Hexane	.0333	.1453	.821	-.275	.341
	Maceration *Terminalia brownii* Aqueous	-2.1667*	.1453	.000	-2.475	-1.859
Soxhlet *Terminalia brownii* Aqueous	Soxhlet *Lannea fulva* Hexane	.2000	.1453	.188	-.108	.508
	Soxhlet *Lannea fulva* Aqueous	-.2000	.1453	.188	-.508	.108
	Soxhlet *Terminalia brownii* Hexane	1.0000*	.1453	.000	.692	1.308
	Maceration *Lannea fulva* Hexane	-.9333*	.1453	.000	-1.241	-.625
	Maceration *Lannea fulva* Aqueous	-1.0333*	.1453	.000	-1.341	-.725
	Maceration *Terminalia brownii* Hexane	1.0333*	.1453	.000	.725	1.341
	Maceration *Terminalia brownii* Aqueous	-1.1667*	.1453	.000	-1.475	-.859