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Extraction and Analysis of Spectral Properties and ChroMophoric Characterization of Natural Dye Extract from Barks of *Terminalia brownii* Fresen (*Combretaceae*)

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Abstract: *Terminalia brownii* is known for its medicinal properties has been used to treatment and management of malaria, ulcers, diarrhea, coughs, hepatitis etc. In Elgeyo-Marakwet County, and amongst the Marakwet community of Kenya, the plant has been known for its dyeing properties and has been used historically for the dyeing of traditional handbags locally known as "kiondos". Literature evaluation indicates little is known concerning the dyeing properties of this plant, hence there was need for such work to be done. This coupled with the fact that there has been an attempted shift to natural dyes from their synthetic counterparts which are considered to be poisonous, allergens and in some cases carcinogenic. In this work, two extraction methods namely soxhlet and maceration were compared for their percentage yields with maceration being chosen as a preferred method. The extracts were then separated and identified via UV-VIS, FT-IR and LC-MS. Preliminarily the UV-Vis was used for identification of chromophores present in the dye extracts at maximum absorption (λ_{max}) due to electron transitions from HOMO-LUMO whereas the FT-IR was used to identify the specific vibrational chemical bonds characteristic of the individual functional groups present in the dye extracts. LC-MS in tandem with collision induced dissociation (CID) was used to accurately identify and characterize the chromophoric compounds based on their structures, molecular (product ion) and fragmentation pattern which were then compared with LC-MS data, library and the literature. Several compounds with dyeing properties such as Catechin, Epi-Catechin, Baccatin, Gentianose, Geniposide and Genipin were identified. Gentianose, geniposide baccatin and genipin are being reported for the first time as chromophoric compounds in *Terminalia brownie.*

Keywords: Chromophores, FT-IR, LC-MS, *Terminalia Brownie*, UV-Vis

1. Introduction

The *Terminalia brownii* Fresen. (*Combretaceae*) is well distributed plant within tropical regions of the world, more so in Savannah region of Eastern African countries such as Kenya, Uganda, Tanzania, Eritrea, Sudan and Somalia [1, 2]. For decades, the plant has been used in different parts of the world in management of various kinds of diseases such as jaundice, malaria, sexually transmitted diseases (gonorrhea and syphilis), diarrhea, ulcers, stubborn coughs, hepatitis and yellow fever. This has been proven with extracts of different parts of the plant showing antibacterial, antimicrobial and anti-plasmodial activities [3].

In Kenya, the plant has not only been useful traditionally for the healing of various ailments but also, the barks of the

plant have been used for the dyeing of tradition handbags commonly referred to as "Kiondos" among the Marakwet community of Kenya. Given the common environmental problems that are associated with synthetic dyes such as carcinogenicity, allergic reaction to the skin and harm to the aquatic environment due to their persistence, natural dyes have often been suggested as alternative to this menace [4] In view of this, extracts from *Terminalia brownii* are often presented as viable alternatives. However, the absence of documented chromophoric and spectral properties has been cited as major challenge hampering utilization of dyes from natural origin. Additionally, batch-to-batch variation during dyeing has been a great challenge due to large number of unknown chromophores co-existing with other degradants each has different fragmentation pattern resulting to colour variation often referred as batch to batch variation.

Therefore, this work aimed at identifying some chromophores and spectral properties such as: molecular mass, molecular structure and dye disintegration pattern of individual dyestuff assumed to be responsible for the dyeing process. This entailed the use of several analytical equipment such as UV-Vis, FT-IR, and LC-DAD-ESI-MS. It has been noted that natural dye chromophores can be identified well by Electrospray Ionization mass spectrometer (ESI-MS) which identifies specific and dye discriminate spectra [5]. Hence together with a UV-VIS and an FT-IR, it is possible to identify among other things functional groups and predictable auxochromes and chromophores [6].

2. Materials and Methods

2.1. Reagent and Chemicals

All chemicals used for LC were of HPLC grade and those used for extraction were of reagent grade. They includedmethanol, acetonitrile, formic acid, acetone, acetic acid and ethanol. All were manufactured by Sigma Aldrich Company and supplied by Kobian Laboratory Nairobi Kenya. PTFE membrane filters (0.45mm) was sourced from Gelsap laboratories. Distilled water was distilled thrice using a Milli-Q apparatus (Billerica, MA, USA).

2.2. Instrument and Apparatus

UV- 1800 Shimadzu, blender (ramtons), rotary evaporator (BÜCHI Rota vapor R-114), FT-IR (Bruker Alpha series) spectrophotometers and LC-DAD-ESI-MS.

2.3. Plant Collection and Processing

Terminalia brownii samples were harvested from their natural habitat in Samar village in Elgeyo Marakwet County (0.9486° N, 35.5085°E). They were later deposited at the Moi University Department of Botany for taxonomical verification. Thereafter, the plants barks were harvested and air dried till a constant weight was obtained.

The barks were later reduced into size by a hammer mill and further reduced to size using a ramtons blender. They were then stored in air tight containers awaiting future use.

2.4. Extraction of Dyestuff Components

2.4.1. Soxhlet Extraction

15 grams of each of the ground powder was weighted and kept in soxhlet extractor and 100 ml of each solvent i.e hexane and water used for extraction.

2.4.2. Maceration Extraction

15 grams of each of the ground powder was weighted and soaked in 100 ml of solvent i.e. hexane and water for approximately 48 hours with slight agitations at room temperatures. For both the percentage yields were calculated as follows.

$$\text{Total \% yield} = \frac{\text{Mass Of extract}}{\text{Mass of sample}} \times 100 \quad (1)$$

2.5. Preparation of HPLC

This followed Recep et al., (2014) with slight modification. The dye extracts (powder) from *Terminalia 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 90°C. The dye mixture was then rapidly cooled under running cold water and the solution evaporated to dryness in a water bath at temperature of 50°C 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.

2.6. Identification of Chromophores in Terminalia Brownii Dye Extracts by Uv-Vis Spectroscopy

The procedure entailed preparation of 10% concentration of the dye sample by weighing 0.5mg of the dye sample (*Terminalia brownii*) and diluted in 50m1 of pure distilled water. Then the aliquot was introduced into a quartz cell (1cm pathway) and analyzed on a Shimadzu model UV-1800 series (spectrophotometer). Scanning of between 200-800nm was performed in order to generate the characteristic absorption spectra of each dye chromophore. The qualitative identification of each dye chromophore was confirmed by matching the absorption spectrum with those of literature or library available.

2.7. Identification of Functional Groups Via FT-IR

The procedure involved weighing 4mg of the dye sample (*Terminalia brownii*) powder, 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 FT-IR spectrometer (Bruker-Alpha) and the spectra was recorded over an absorption band of 4000-500 cm^{-1}.

2.8. 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° C. The HPLC gradient elution was performed using solvent A (acetonitrile and B (water acidified with 0.1% formic acid). The chromatographic separation of the hydrolyzed sample was performed using an isocratic elution program that utilizes the two solvents A (acetonitrile) and solvent B (water acidified with 0.1% formic acid).

2.9. LC-DAD-MS Analysis

The LC-ESI-MS analysis of spectral properties (molecular structure, molecular mass and disintegration pattern) was achieved by using the procedure by Manhital et al., (2011) in which an LCQ Advantage Thermo Finnigan spectrometer equipped with an ESI source used anion trap mass analyser and a PDA detector (San Jose, CA, USA). The conditions of the MS were capillary temperature of 300°C, 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). The collision induced dissociation (CID-MSn) was applied to induce the fragmentation of the molecular ion and the fragments were analyzed using tandem MS spectrometry. 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 dye extracts were injected in negative and positive ion mode in which the column temperatures were set at 30°C and tray temperature was set at 24°C. 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 size 3.0 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. Results and Discussion

3.1. Solvent Extraction

Both methods of extraction i.e. soxhlet and maceration were assessed for extraction with maceration showing to be more efficient hence it was chosen for subsequent extractions. The Percentage yields were as shown below in table 1.

Table 1. *Percentage yields.*

Column1	SOLVENT	YIELD (GM)	% YIELD
MACERATION	HEXANE	2.40	08
	WATER	4.60	15.3
SOXHLET	HEXANE	2.40	08
	WATER	3.40	11.30

The extracted dye components were as follows in figure 1 below.

Figure 1. *(a) Ground powder of the bark and (b) extracted dye.*

3.2. UV Analysis of the Dye Extracts

The yellow dye extracts of *Terminalia brownii* absorbed electromagnetic radiation (light energy) as visible light causing transition of electron from bonding or non-bonding orbital into one of the empty anti-bonding orbitals ($\pi-\pi^*$ or $n-\pi^*$). The chromophores present in the dye extracts were revealed by spectra of two peaks as shown below.

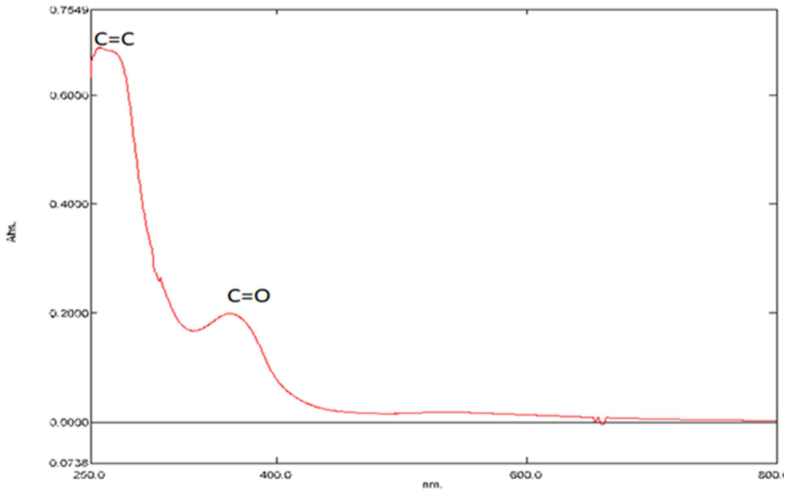

Figure 2. *UV-VIS Spectrum of Terminalia brownii dye extracts.*

The chromophore displayed by the first peak at λ_{max} 258.5nm can be identified as C=C due to delocalized electron from fluorescent aromatic compounds (benzene rings). The transition of electrons from highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO) is commonly expressed as $\pi-\pi^*$. This can be explained by high energy gap and short wavelength which is characteristic of C=C chromophore. Closer look at the absorbance reveals that it occurs at slightly higher wavelength than benzene with λ_{max} 255 nm, meaning that the chromophore is a functional group attached to benzene ring is responsible for bathochromic shift as shown by the λ_{max} 258. 5nm. Such chromophore is absorbed in FT-IR as functional group represented by absorption band occurring at 1597.61 cm^{-1} and 1400.77 cm^{-1} which is characteristic of C=C stretching of aromatic/benzene rings causing the absorbance spectra. This observation is comparable to [7]. The second peak absorbed at λ_{max} 361 nm was due to non-bonding electron or lone pairs electron on the oxygen atom (carbon oxygen double bond or C=O) which is characterized by absorption of UV-VIS light at a lower frequency, lower energy and higher wavelength as a consequence of electron jump from non-bonding orbital to anti bonding orbital commonly referred as $n\rightarrow\pi^*$ electron jump which is in agreement with [8]. This chromophore (C=O) is the same functional group which is absorbed in FT-IR as absorption band displayed by 1213.87 cm^{-1} and the absorption band occurring at 1111.73 cm^{-1} as both confirmatory. This chromophore has been identified in chromophoric compound like Geniposide and Gentianose which is in agreement with [9].

3.3. Identification of Functional Groups in T. Brownii Dye Extracts Using FT-IR Analysis

Most dye extracts absorb in the mid IR region of 4000-400 cm^{-1} to show functional groups mainly linked to long conjugate double bonds [10]. The spectrum of the FTIR was as below in figure 3.

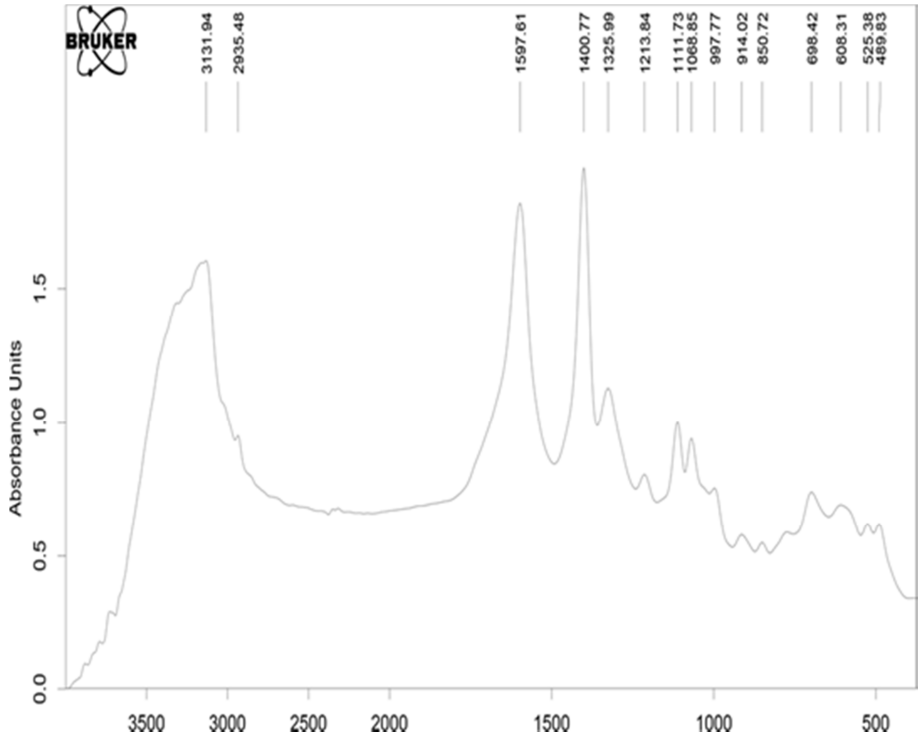

Figure 3. *FTIR Spectrum.*

The bands were displayed at different frequencies with that at 3131.94 cm^{-1} being due to the presence of the stretching vibrations of OH group seen in terminal carboxylic acid [COOH] group. The bands displayed at frequency 2935.48 cm^{-1} vibrations is due to C-H stretch or CHO of carbohydrates which is in agreement with [15]. Those witnessed at 1597.61 cm^{-1} and 1400.77 cm^{-1} are due to C=C stretches of benzene rings. The absorption band at frequency 1325.99 cm^{-1} is as a result of the O-H of either a phenolic or carboxylic. The 1213.87 cm^{-1} absorption band is due to C-O-C. The absorption band between 1065.85 cm^{-1} to 608.31 cm^{-1} is due to C-OH of phenolic compound that is out of plane deformation which is agreeing with [11]

3.4. Characterization of Chromophoric Compounds in Terminalia Brownii Dye Extracts by LC-MS

In positive ionization mode, four dyestuff compounds were tentatively identified as Catechin, Epicatechin, Baccatin and hydroxytiaprofen. The catechin and epicatechin are two isomeric compounds which are ionization dependent and can only be chemically distinguished by retention time or elution order. The third and fourth dyestuff component (Baccatin and hydroxytiaprofen) were tentatively identified after evaluating it in negative mode ionization since it was not ionization

dependent. Therefore, the chromatographic peak occurring at retention time 7.3min with molecular ion $[M+2H]^+$; m/z = 291. Similarly, the chromatographic peak occurring at retention time7.5 min with mass spectrum $[M+2H]^+$, m/z = 291 was tentatively identified as Epicatechin, based on their mass spectrum, fragmentation pattern and the literature. In the same way, the protonated chromophore with peak occurring at retention time 7.9min shows product ions of $[M+H]^+$, m/z = 588 and $[M+H]^+$, m/z = 277. These two chromophoric compounds are also appears in negative ionization mode eluting at retention time 7.9min with deprotonated product ion $[M-H]^-$, m/z = 587 for Baccatin and $[M-H]^-$, m/z = 277 for hydroxytiaprofenic acid. This yellow chromophoric compound was consistent with previously identified compound in *Terminalia catappa* L. [12, 13].

Interestingly, in negative ionization mode, the dyestuff components deprotonate by displaying molecular product ion in form of $[M-H]^-$. The mass chromatogram of dyestuff component eluting at retention time of 4.9 min was identified as Gentianose trisaccharide, based on unique fragmentation resulting to the loss of entire trisaccharide. To the best of our knowledge, this chromophoric compound (Gentianose) is reported for the first time in Terminalia brownii and it is one of the dyestuff compounds responsible for yellow colouration. Additionally, the four yellow chromophoric compounds displayed by mass chromatogram eluting at retention time 7.3 were identified as follows; the yellow chromophoric compound with molecular mass and molecular ions $[M-H]^-$ m/z = 587 was identified as Baccatin, whereas the chromophoric compound represented by $[M-H]^-$; m/z = 389 was identified as Geniposide. This kind of mass spectral fragmentation pattern is typical of Genipin by cleavage of O-hexose molecule [M-H-162], m/z=227. This was previously detected and identified in Terminalia catappa L. [12]. However, to the best of our knowledge these chromophoric compounds (Baccatin, Geniposide and genipin) are reported for the first time in Terminalia brownii and are responsible for yellow colouration. The last compound detected was hydroxytiaprofenic acid with $[M-H]^-$ m/z = 277. This was previously identified in positive ionization mode in a chromatogram peak eluting at retention time 7.9 represented by $[M+H]^+$, m/z = 277 and was consistent with compound reported in Terminalia catappa [13].

These yellow dyestuff components identified in our results justify the traditional application of Terminalia brownii dye extracts more so the maceration method using water extracts in dyeing by Marakwet community in Kenya.

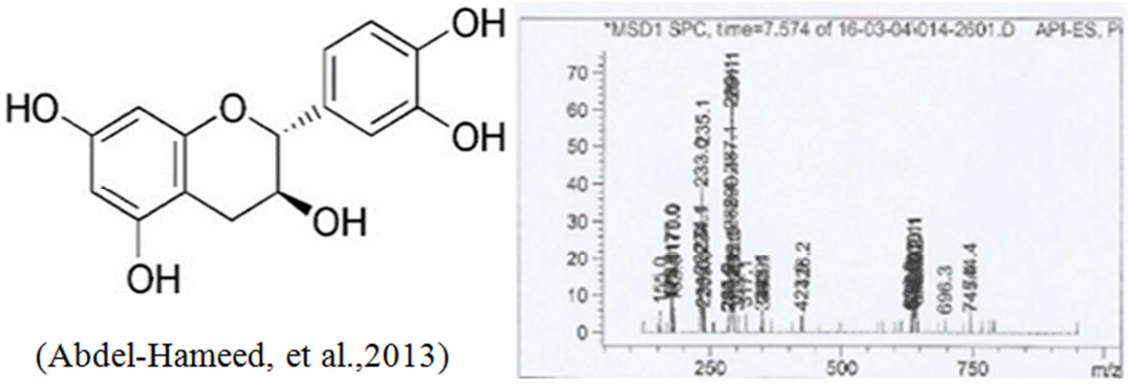

Figure 4. *MS spectrum of Catechin (m/z = 289).*

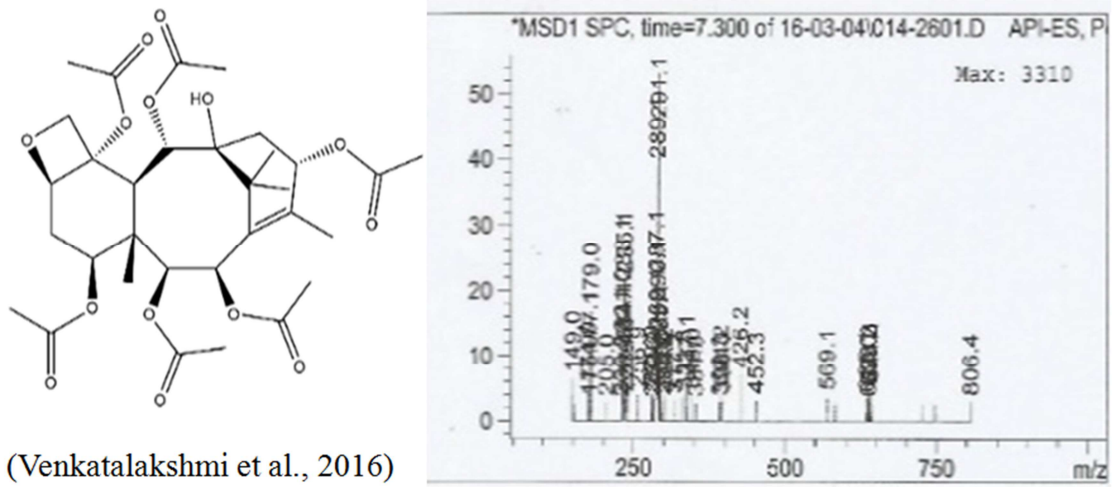

Figure 5. *MS Spectrum of epicatechin (m/z= 291).*

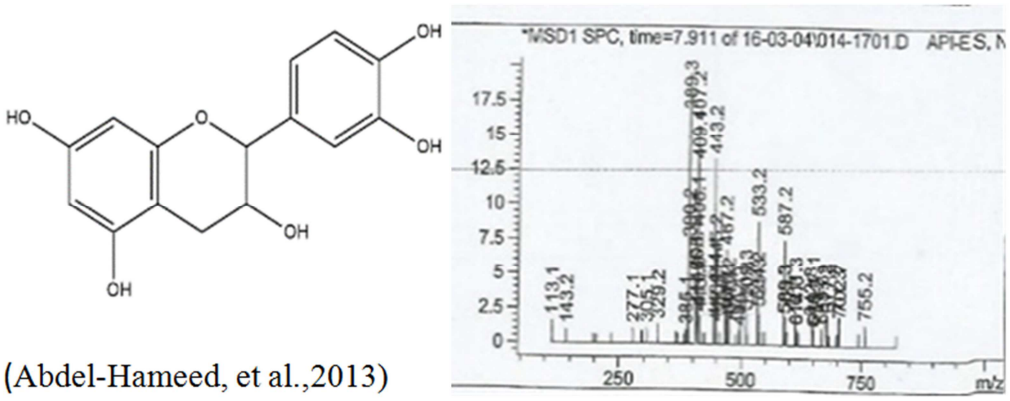

Figure 6. *MS spectrum of Baccatin (m/z=587).*

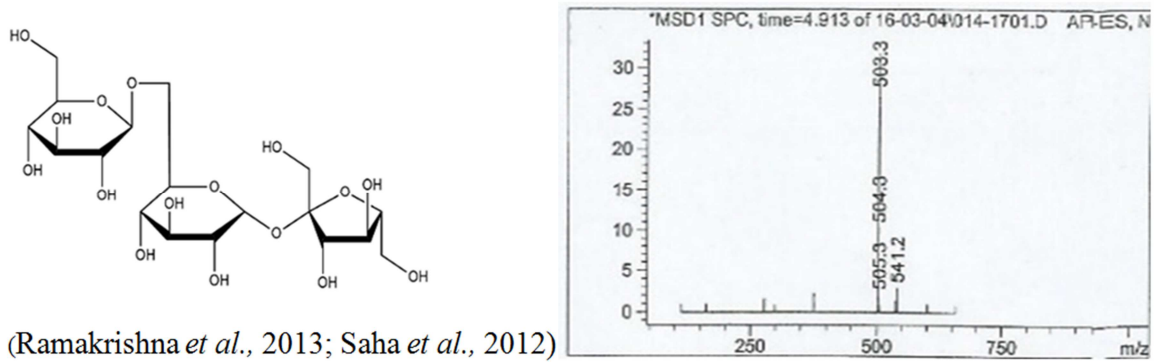

Figure 7. *MS spectrum of gentianose (m/z = 503).*

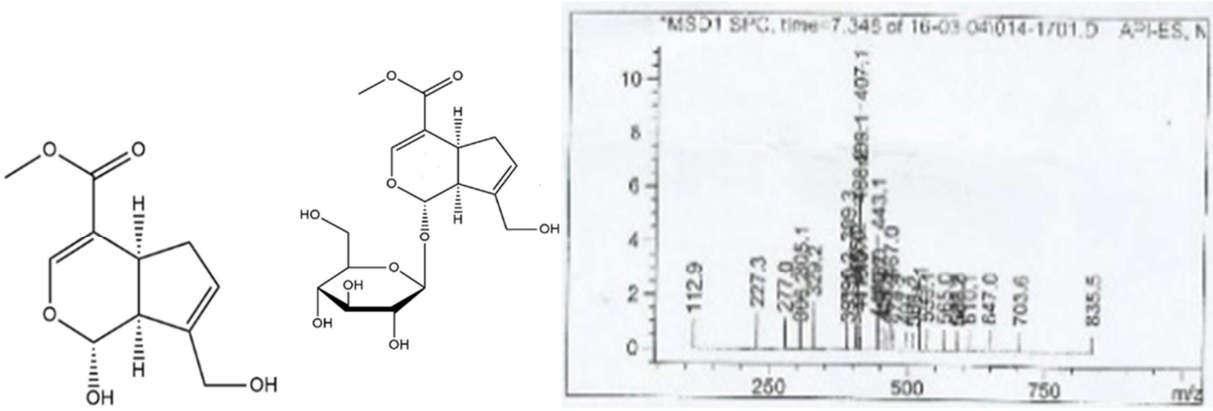

Figure 8. *Genipin (m/z 227) and Geniposide (m/z=389) respectively.*

4. Conclusion

Several compounds from extracts from *T. brownii* that have been previously used for dyeing in traditional settings have been tentatively identified by the use of various instruments. The data obtained on the chromophoric and spectral properties can be extrapolated for the utilization at industrial scale of natural dyes as substitutes of synthetic dyes that have been found to be toxic to humans and pollutants to the environment.

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