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Leishmanicidal activity of the Root Bark of *Erythrophleum Ivorense* (Fabaceae) and Identification of some of its compounds by Ultra-Performance Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry (UPLC-QTOF-MS/MS)

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ABSTRACT

Ethnopharmacological relevance: Leishmaniasis is one of the neglected tropical disease caused by a protozoan of the genus Leishmania transmitted by sandflies. High cost and lack of oral formulation of existing drugs, rapid developments of resistance by the parasite coupled with serious side effects require new treatments to augment or replace currently available therapies. The major merits of herbal medicine seem to demonstrate perceived efficacy, low incidence of serious adverse effects and low cost. Erythrophleum plants possess beneficial biological properties and, as such, characterization of the bioactive components of these plants is imperative. Previous work has shown an overwhelming presence of cassaine alkaloids in these plants. However, amongst these plants, the African based specie (Erythrophleum ivorense) is the least studied.

Objective: In the current study, the *in vitro* anti-leishmanial activity of the crude extract, its fractions and isolated compounds were evaluated using direct counting assay of promastigotes of Leishmania donovani using amphotericin B as positive control.

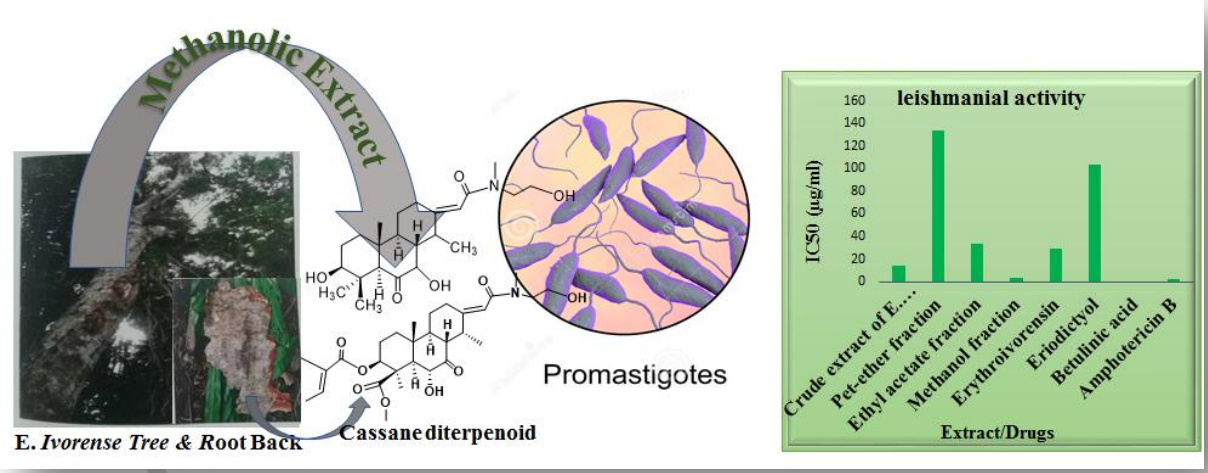
Materials and methods: The anti-leishmanial activity of *E. ivorense* extract was evaluated *in vitro* against the promastigote forms of *Leishmania Donovani* using a direct counting assay based on growth inhibition. Different crude extracts from ethyl acetate, pet-ether, and methanol as well as pure isolated compounds of E. ivorense: Erythroivorensin, Eriodictyol and Betulinic acid were

screened. To know the possible components of the active methanolic extract, attempt was made to elucidate the extract using ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS/MS).

Results: This afforded a weak pet-ether fraction, a moderately active ethyl acetate fraction and a significantly active methanol fraction (IC_{50} = 2.97 µg/mL) compared to Amphotericin B (IC_{50} = 2.40±0.67µg/mL). The novel diterpene erythroivorensin, betulinic acid and the flavanone Eriodictyol, from the ethyl acetate fraction, showed weak activity. UPLC-QTOF-MS/MS was used to identify the cassaine diterpenoids from the active methanol fraction. Here, 10 compounds of this type were putatively identified from the ethanol crude extract.

Conclusion: The fragmentation mechanism of these metabolites is also proposed and are expected to serve as reference template for identification of these and related compounds in future. The presence of these compounds is an indication that they are an inherited and evolutionary component of plants belonging to the Erythrophleum genus. Our results further present another dimension where these compounds and their relative abundances can be used as chemo-taxonomical bio-markers of the genus. The present study also successfully demonstrated/re-affirmed the use of UPLC-QTOF-MS/MS as a robust technique for the characterization of natural products.

Graphical Abstract



Keywords: *Erythrophleum ivorense*, UPLC-QTOF-MS, Cassaine-type diterpenoid, chemo-taxonomy, Leishmaniasis.

1.0 Introduction

Leishmaniasis is a vector-borne disease caused by protozoa and is endemic in large areas of the tropics, subtropics and the Mediterranean basin (Chappuis et al., 2007). This disease is caused by more than 20 leishmanial species and is transmitted to humans by 30 different species of phlebotomine sandflies (Pearson and Sousa, 1996). It can also affect both domestic and wild animals (Camargo and langoni, 2006).

Leishmaniasis is one of the world's most neglected diseases affecting largely the poorest of the poor, mainly in developing countries (Dawit and Shishey, 2014). In Africa, it is endemic to countries mostly in the North, Central, East and the Horn of Africa. The disease is also endemic in West Africa (Sheik-Mohammed and Velema, 1999) although it appears to be one of the less recognized or under-reported parasitic infections in this region (Desjeux et al., 1981). Leishmaniasis has not been reported in Ghana until recently, when some chronic ulcers diagnosed as cutaneous leishmaniasis were observed in the Ho District of the Volta Region (Boakye et al., 2005). Kwakye-Nuako et al, (2015) reported for the first time the characterization of a new member of the *Leishmania enriettii* complex, a new subgenus of Leishmania parasites, isolated from the Volta region of Ghana, through DNA sequencuing and phylogenetic analysis.

Treatments for Leishmaniasis include the use of pentavalent antimony (sodium stiboghconate and meglumineantimoniate), Amphotericin B, pentamidine and paramomyan (Chappuis et al., 2007). These drugs have the disadvantages of high cost, lack of oral formulation or serious side effects including pain, swelling and irritation at injection site (amphotericin B), fever, nausea, vomiting, diarrhea, headache, shortness of breath, muscle or joint aches and tingly feeling that require close monitoring of the patient. Also, rapid developments of resistance by the parasite has been reported (Ephros et al., 1999). Therefore new treatments are needed to augment or replace currently available therapies.

A large number of people in Ghana use herbal medicines for their primary health care needs. The government, recognizing the contribution of herbal medicine to the healthcare needs of the populace, has established herbal clinics in most public hospitals across the country where herbal medicine is practiced alongside allopathic medicine. Thus, natural remedies contribute

significantly to healthcare delivery in Ghana (Amponsah et al., 2014). It is therefore important to investigate the efficacy of plants used in folklore medicine for the treatment of parasitic diseases so that they can be integrated safely into main stream primary health care.

Erythrophluem ivorense, known in Ghana as 'Protrodom' (Akans), is used mainly to treat small pox, convulsion, inflammation and parasitic diseases (Oliver-Bever, 1986). Cassaine-type alkaloids were reported in the 1960s and the 1970s from *Erythrophleum ivorense*; 3(3-methylcrotonyl), Ivorine, Nor-cassaide, Nor-cassamidide, dehydro-nor-erythrosuamide and norerythrosuamide. Recently, Armah *et al.,* (2015) reported the isolation of Erythroivorensin: A novel anti-inflammatory diterpene from the root-bark of *Erythrophleum ivorense*. Elsewhere, the presence of these compounds has been correlated with the various biological activities of the source plants including those from *Erythrophleum* genus (Maurya et al., 2012). The cytotoxicity of the *Erythrophluem ivorense* was reported by Adu-Amoah and co-workers 2014.

In this study, the anti-leishmanial potential of the crude extract, pet-ether, ethyl acetate and methanol fractions and their compounds were investigated. We also report the systematic investigation and the fragmentation behaviors of ten cassaine-type diterpenoid amides from the methanol fraction *via* UPLC-qTOF-ESI-MS/MS as an analytical method for structural identification of new trace natural products. The results of which could add another dimension were these compounds and their relative abundances can be used as chemo-taxonomical bio-markers of the *Erythrophleum* genus.

2.0 MATERIALS AND METHODS

2.1 Plant material

The root-bark of *E. ivorense* (Fabaceae) was harvested from Adukrom, a village in Nzema East Metropolis of Ghana, in October 2014 and was identified by curators of the University of Cape Coast Herbarium (Ghana). A voucher specimen (BHM/Eryth/017R/2014) has also been identified and deposited at the Herbarium of the Department of Herbal Medicine, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Ghana.

2.2 Extraction procedure

Powdered air-dried root bark of *E. ivorense* (1.2 kg) was cold macerated with 70% ethanol for 72h. The resulting extract was then filtered and concentrated under reduced pressure (40 °C) to give the crude extract in a yield of 8.7% (w/w). A portion this extract (100 g) was successively partitioned with petroleum ether (5 L), ethyl acetate (5 L) and methanol (5 L) to afford fractions in the yield of 15 g, 36.3 g and 41.2 g respectively. The methanol extract (0.1 g) was reconstituted in 1mL highly pure methanol (Romil) and filtered through a 0.22 µm nylon syringe filter and stored at -20°C prior to UHPLC-qTOF-MS analysis.

2.2.1 Parasite cultivation

Promastigote strain of *Leishmania donovani* (LV-90) was obtained from the Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, U.K. They were cultivated at 25 °C in medium 199 (Gibco, Invitrogen) supplemented with 5% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS), 10 mM adenine (pH 7.5), and 5 MmL-glutamine. Using a pipette, 500 µL of parasite (containing $14.4x10^6$ parasites) were picked and added to 15 mL of M199 media in a culturing flask. The mixture was incubated at 25°C for five days. The number of parasites was counted daily using a hemocytometer (Z359629 SIGMA) according to the method described by Umakant et al., (2011).

2.3 IN VITRO ANTI-LEISHMANIA ASSAY

2.3.1 Extracts and compounds of E. ivorense

The anti-leishmanial activity of *E. ivorense* extract in comparison to Amphotericin B was evaluated *in vitro* against the promastigote forms of *Leishmania Donovani* using a direct counting assay based on growth inhibition (Callahan et al., 1996). A stock solution of the total crude methanol extract of the plant (1000 µg/mL) was prepared in DMSO. The different concentrations of the extract were prepared by two fold dilution of the stock as follows: 1 ml of the stock solution was dispensed into the first well of the 24 well micro titre plate. It was topped up with 1 mL of M199 media mixing thoroughly. 1 ml of this solution was again dispensed into the second cell and

procedure repeated in subsequent cells. The promastigotes were seeded at an initial concentration of 95.5×10^6 cells/mL, equivalent to that of early log phase. Each cell was then topped with 20 µl of *Leishmania donovani* culture containing of the promastigotes. Finally, each well was topped up with a suitable amount of the medium (M199) to make a final volume of 2 mL thereby achieving concentrations of 15.6, 31.2, 125, 250 and 500 µg/mL. The total concentration of DMSO was thus reduced to $0.5\%^{V}/_{V}$, a concentration which has negligible effect on parasite growth rate and morphology (Khan *et al*., 2012). Total solution contained M199, DMSO, plant extract and 20 µl promastigotes of *L. donovani*. Similarly, the positive control amphotericin B, the pet-ether, ethyl acetate and methanol fractions of *E. ivorense* were tested at the same concentrations. The negative control consisted of the medium M199, DMSO and promastigotes of *L. donovani* mixed evenly in a cell without the extract.

The parasites were incubated up to 72 h at 25°C. However, the numbers of viable parasites were counted with a hemocytometer after 6, 12, 24, 48 and 72 hours. All the *in-vitro* experiments were run in triplicate. Time course curves (% viable cells at 6, 12, 24, 48 and 72 hours) were plotted for each extract/compound. The % inhibition of parasites numbers (calculated from the AUC's) was then plotted against log of concentration. The concentration required for 50% inhibition *in-vitro* (IC_{50}) was determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

$$Y= \frac{a+(b-a)}{\left(1+10^{(LogEC50-X)}\right)}$$

Where, *X* is the logarithm of concentration and *Y* is the response. *Y* starts at *a* (the bottom) and goes to *b* (the top) with a sigmoid shape (Miller, 2003; Motulsky, 2003). Graph Pad Prism for Windows version 5.0 (Graph Pad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

Previous studies in our lab, on the bioactive ethyl acetate fraction of *E. ivorense* resulted in the isolation of the novel cassane diterpene Erythroivorensin, betulinic acid and eriodictyol (Armah et al., 2015). These compounds were similarly tested for anti-leishmanialactivity as described above using the same concentrations**.**

2.4 Ultra High-Performance Liquid Chromatography (UHPLC)

For metabolite separation, 3 µL of the methanol extracts on a UHPLC equipped with an Waters Acquity HSS T3 C18 column (150 mm × 2.1 mm with particle size of 1.8 µm) (Waters Corporation, Manchester, UK). The mobile phase composition was a binary solvent system consisting of solvent A (0.1% formic acid in deionized water), and solvent B (0.1% formic acid in acetonitrile). The column was eluted with a linear gradient starting from 2% B to 60% B at 24 min, ramped to 95% B at 25 min, held constant at 95% B over 25.0 – 27.0 min, and a re-equilibration at 5% B over 27 – 30 min. The flow rate was 0.4 mL/min at a column temperature of 60°C. Chromatographic elution was monitored by a photo-diode array (PDA) detector scanning between 200 - 500 nm.

2.4.1 Mass Spectrometry Acquisition Parameters

In tandem with the PDA detector, MS data was also acquired in both positive and negative polarity electrospray ionization (ESI) modes for *m/z* range: 100 – 1000 Da, scan time 0.2 sec, inter-scan delay: 0.02 sec with leucine encephalin (556.3 µg/mL) as a lock mass standard at a constant of flow rate: 0.1 mL/min to enable acquisition of data within mass accuracy window of 0.5 mDa. Optimized MS settings presented elsewhere [19] were used. Briefly, collision energy of 3 eV, capillary voltage of 2.5 kV, sample cone voltage of 30 V, detector voltage of 1650 V in positive mode and 1600 V in negative mode, source temperature at 120 °C, cone gas flow at 50 (L/h), and desolvation gas flow at 550 (L/h) were used to acquire standard MS data. However, for metabolite fragmentation, data were collected at alternating collision energy levels as previously described elsewhere [Madala et al., 2012]. For tandem MS (MS/MS), pseudo-molecular *m/z* which were believed to represent cassaine-type diterpenoid were selectively trapped on the quadrupole compartment of the MS instrument and ion-specific fragmentation was achieved by increasing both the cone voltage and collision energies until efficient fragmentation was achieved.

3.0 RESULTS & DISCUSSION

Erythrophleum ivorense, commonly known as ‘portrodum’ (Akan) in Ghana, is applied topically to treat parasitic skin diseases. In the communities around the Ankobra river in the Nzema East district of Ghana where the plant material was harvested, the root bark is topically applied to small skin nodules (pox) suspected to be sandflies transmitting the Leishmania parasite (Oliver-

Bever, 1986). This agrees with report of the use of the plant in the topical treatment of small pox in the Ivory Coast (Aubréville, 1959). In this research, the possible anti-leishmanial activity of extracts and compounds of *E. ivorense*, as suggested by folklore medicine, was evaluated.

The extracts, fractions and compounds of *E. ivorense* showed considerable anti-leishmanial activity expressed as the IC_{50} (Table 1). Fractionation of the active *E. ivorense* extract afforded methanol fraction whose activity (IC_{50} = 2.97 µg/mL) was comparable to that of amphotericin B (IC_{50} = 2.4 µg/mL), used as the reference drug (Table 1). This was followed by the ethyl acetate (IC_{50} = 33.07 µg/mL) and pet ether fractions (IC_{50} = 133.6 µg/mL). The novel cassane diterpene erythroivorensin, the flavanone eriodictyol and the triterpene betulinic acid from the bioactive ethyl acetate fraction showed weak leishmanicidal activity (Table 1).

Table 1 Anti leishmanial activity of extracts and compounds of *E. ivorense*

Extract/Drug	IC_{50} (µg/ml)
Crude extract of *E. ivorense*	14.08±1.33
Pet-ether fraction	133.6±0.87
Ethyl acetate fraction	33.07±0.13
Methanol fraction	2.97±0.67
Erythroivorensin	29.10±0.13
Eriodictyol	103.80±0.33
Betulinic acid	No activity
Amphotericin B	2.4± 0.67

A number of diterpenes have been reported to show marked leishmanicidal activity. Fokialakis *et al*., (2006), reported the anti-leishmanial activity of eleven clerodane and seven labdane type diterpenes from *Cistus monspeliensis* and *Cistus creticus* (Cistaceae) with IC_{50} values in the range 3.3 - 3.5 µg/mL for the potent compounds. Habtemariam, (2003) also reported the anti-leishmanial activity of diterpene acids with an even greater activity. In a similar report, cassane diterpene acids from *Caesalpina echinata*, with structures similar to erythroivorensin, were found to show relatively high leishmanicidal activities (Cota et al., 2011). They were also non-toxic to human peripheral blood mononuclear cells *in vitro*. The higher

methanolic extract activity is attributed to relatively numerous forms of diterpenes identified in this extract.

The isolation of the compounds in the active methanol fraction was hampered by their availability in trace amounts. Although ongoing research has focused on harvesting the roots in large quantities to isolate them, for conservation purposes Ultra High-Performance Liquid Chromatography (UHPLC-QTOF-MS/MS) was used to identify ten cassane diterpenoid amides in the very active methanol fraction. This could initiate synthesis of these compounds for bioassays to possibly unveil leads for the discovery of novel anti-leishmanial agents.

3.1 Metabolite identification of active methanol fraction

Using the single ion monitoring (SIM) chromatograms generated using accurate *m/z* representing casseine ditepenoids alkaloids published elsewhere [Hunga et al., 2014; Kablan et al., 2014; Ngounou et al., 2005], respective MS spectra of these molecules were generated and the molecular formulae were computed on the basis that the isotopic fit ratios (iFit) was close to zero as possible and more importantly that the overall MS accuracy was within 5 mDa. (Table 1).

Fragmentations of these molecules by acquiring data with alternating collision energies (CE) were also attempted. However, the more efficient and sensitive corresponding MS/MS spectra were generated (Figure 1). The molecular formulae of these respective bio-markers were selected on the basis of a 5 mDa mass accuracy range. The identities of the perceived compounds were further searched and confirmed using the Dictionary of Natural Products online database (dnp.chemnetbase.com/) and comparison with other published data [Du et al., 2010; Hunga et al., 2014; Kblan et al., 2014; Ngounou et al., 2005]. From the MS fragmentation patterns, several casseine ditepenoid alkaloid derivatives were identified (Figure 2 & 3). Accordingly, molecules **1**– **4**, **5** -**8**, **9** and **10** (Figure 2-4, Figure S2, Table 1) were tentatively identified to be the isomeric molecules of norerythrosuamide, nor-cassamide and nor-cassaide respectively. The presence of these molecules in several *Erythrophleum* species has been reported [Du et al., 2010; Hunga et al., 2014; Kblan et al., 2014; Ngounou et al., 2005; Dade et al., 2015] and their MS fragmentation presented elsewhere [Tsao et al., 2008; Wu et al., 2015; Manfouo et al., 2005]. Based on the individual molecular fragmentation patterns (Figure 1-3), the molecules were positively identified.

Three types of fragmentation rules can be summarized as follows: firstly, ions assigned as loss of H_2O (-18 Da), CH_2OH (-31 Da), methyl formate (-60 Da) and amide side chain (-75 Da) were observed in ESI-MS/MS spectra of casseine ditepenoids alkaloids (Table 2). For example, molecule **1** at retention time (Rt) of 6.83 min (Figure 2) produced a precursor ion at *m/z* 480.2878 [M+H]−($C_{25}H_{37}NO_8$) and fragmentation of this molecule (Figure 2, Scheme 1) generated product ions at *m/z* 405.2289, derived from the loss of amide side chain (-75 Da) after a possible 1,5 methyl rearrangement of demethyl derivative of norerythrosuamide to a more stable N-methyl derivative of norerythrosuamide (Scheme 1) [Du et al., 2010]. Products ions at *m/z* 345.2069 and 285.1793 due to the neutral loss of two methyl formate (-120 Da) and at *m/z* 239.1766 (loss of carbonyl and water molecules) were also observed. Based on these data, molecule **1** was tentatively identified as 3β-acetoxydinorerythrosuamide (**1**). As an example, TOF-ESI-MS/MS spectra and proposed fragmentation pathways of **1-4** are also shown in Figure 2 - 4 and scheme 1-3. McLafferty rearrangement of molecules **1-4** was observed for these molecules, due to methyl formate substituent generating additional ions by loss of tigloyl and acetyl groups respectively (Scheme 1b). Similar rearrangements have been reported elsewhere [Qu et al., 2007]. Interestingly, compounds **2-4** at retention times: 6.37, 8.22 and 9.8 min also showed the same fragmentation pattern (Figure 2 & 3, Scheme 1 & 2) as compound **1,** and as such, these four molecules were identified as either geometrical or regional isomers of norerythrosuamide as 3β-Acetoxynorerythrosuamide (**2**), 3β-Tigloyloxydinorerythrosuamide (**3**) and 3β-Tigloyloxynorerythrosuamide (**4**), respectively.

Table 1. Accurate masses of $[M+H]^+$ ions and formulas of the constituents **1–10** by HPLC/HRMS in positive ion ESI mode.

Constituents	Formulae	Measured (*m/z*) $[M+H]^+$	Calculated (*m/z*) $[M+H]^+$	Error mDa
1	$C_{25}H_{37}NO_8$	480.2878	480.2553	3.3
2	$C_{26}H_{39}NO_8$	494.2657	494.2676	-1.9
3	$C_{28}H_{41}NO_8$	520.3109	520.2832	2.8
4	$C_{29}H_{43}NO_8$	534.2955	534.2989	-3.4
5	$C_{23}H_{35}NO_6$	422.2484	422.2464	2.0

6	$C_{24}H_{37}NO_7$	452.2610	452.2570	3.0
7	$C_{26}H_{39}NO_7$	478.2734	478.2727	0.7
8	$C_{24}H_{37}NO_6$	436.2544	436.2654	-11.0
9	$C_{23}H_{37}NO_4$	392.2737	392.2756	-1.9
10	$C_{23}H_{37}NO_5$	408.3082	408.2705	3.8

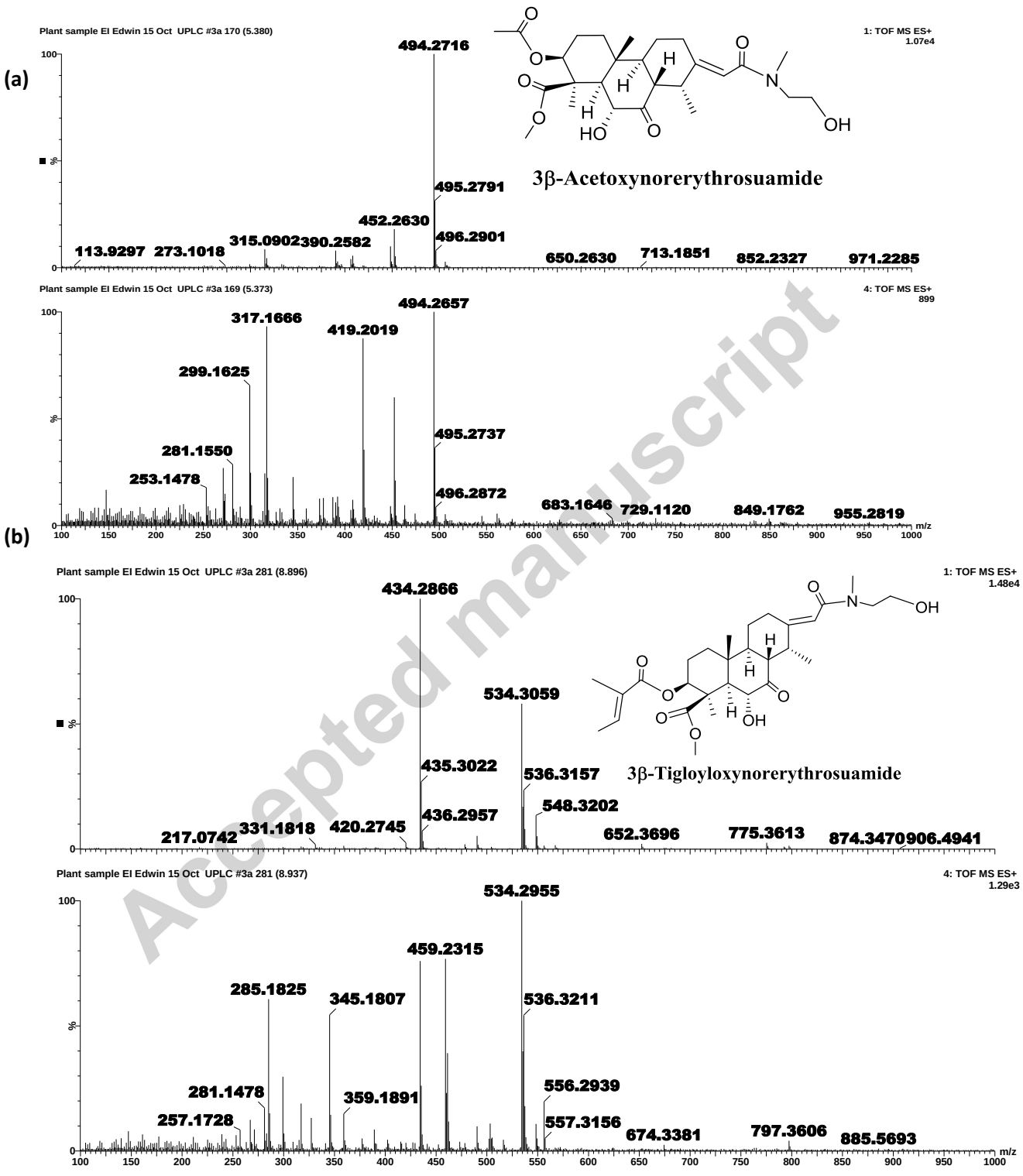


Figure 1: Mass spectrum of compounds (a) 3β-Acetoxynorerythrosuamide and (b) 3β-Tigloyloxynorerythrosuamide

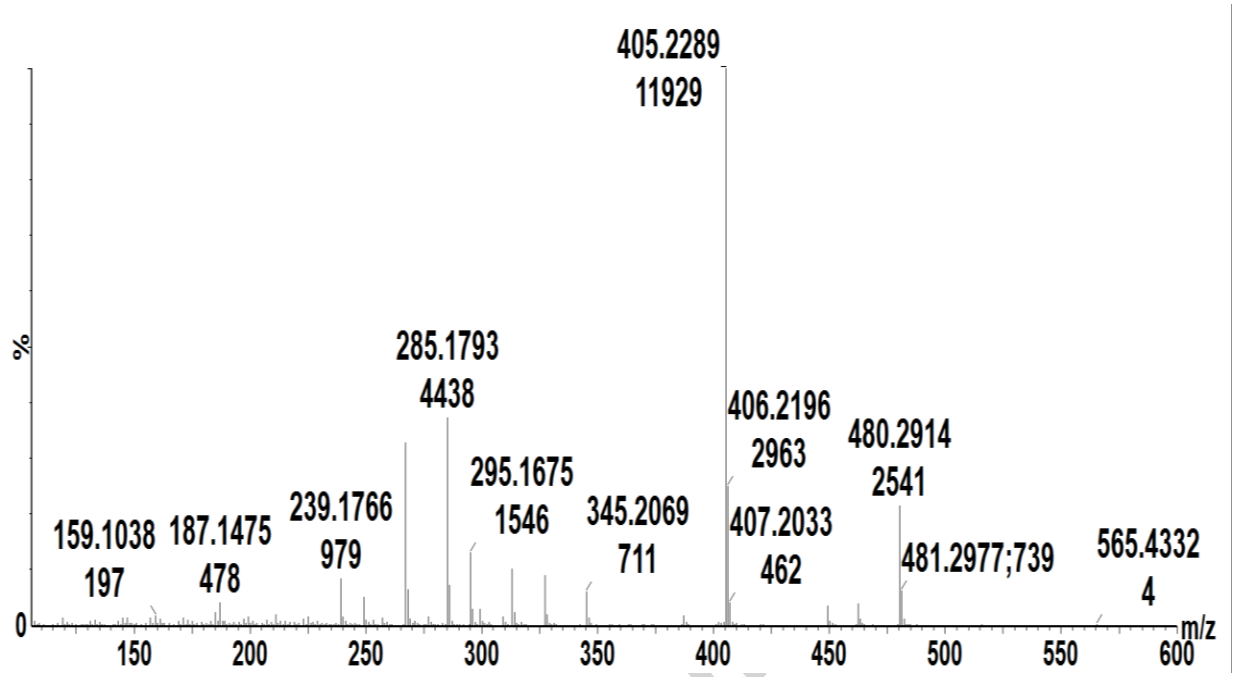
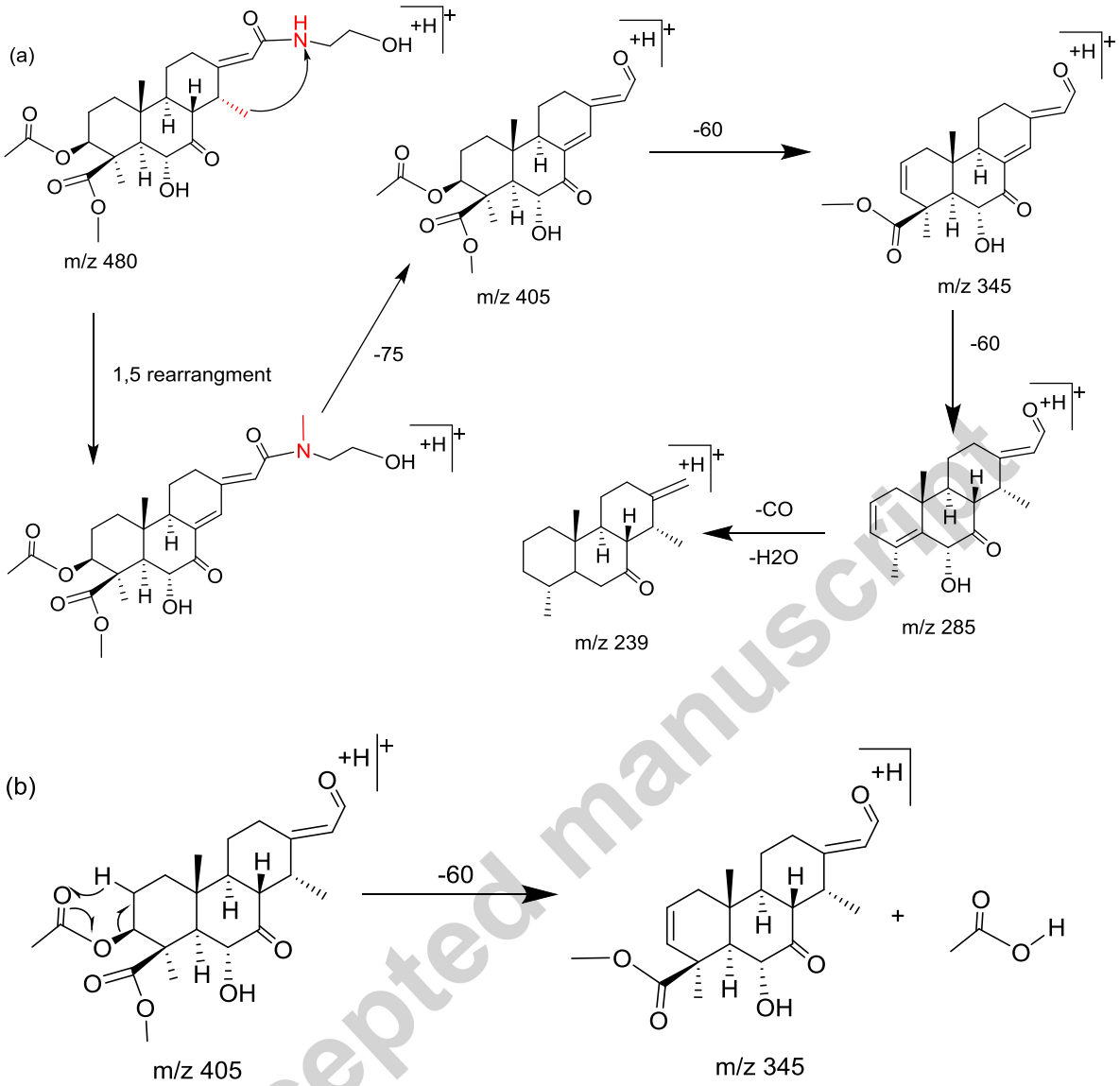


Figure 2: Typical mass spectra of the fragmentation patterns of 3β-acetoxydinorerythrosuamide **1**



Scheme 1: (a) Proposed fragmentation pathways of 3β-acetoxydinorerythrosuamide **1** (b) McLafferty rearrangement of C-3 substituents for 3β-acetoxydinorerythrosuamide

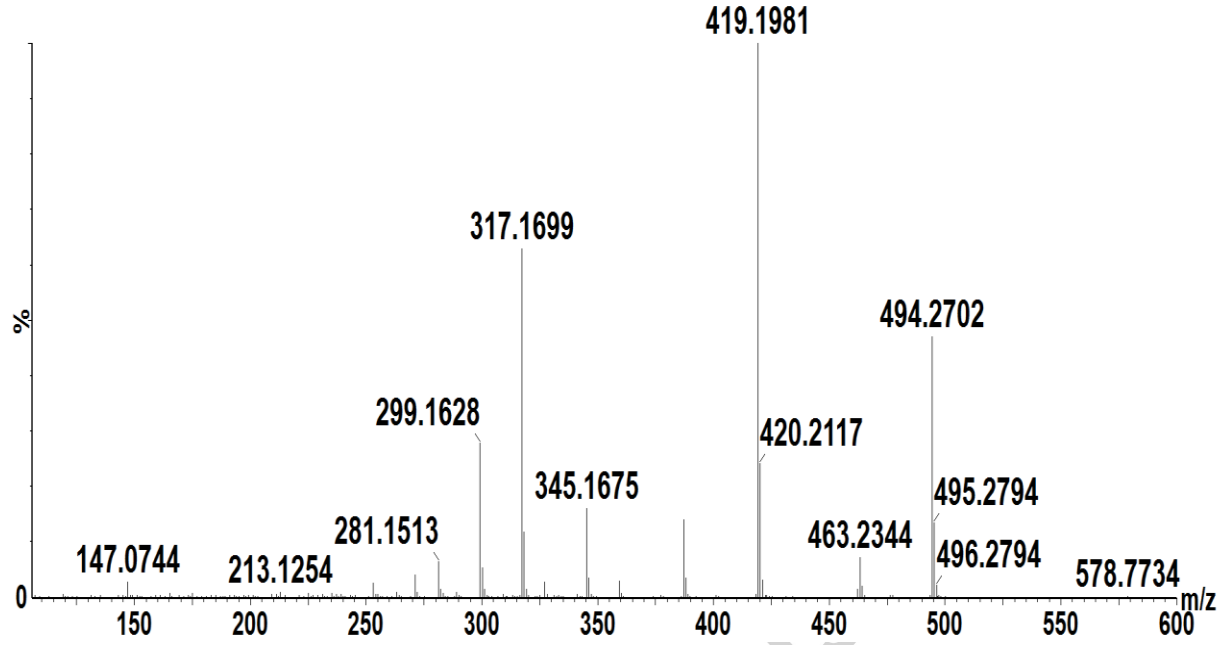
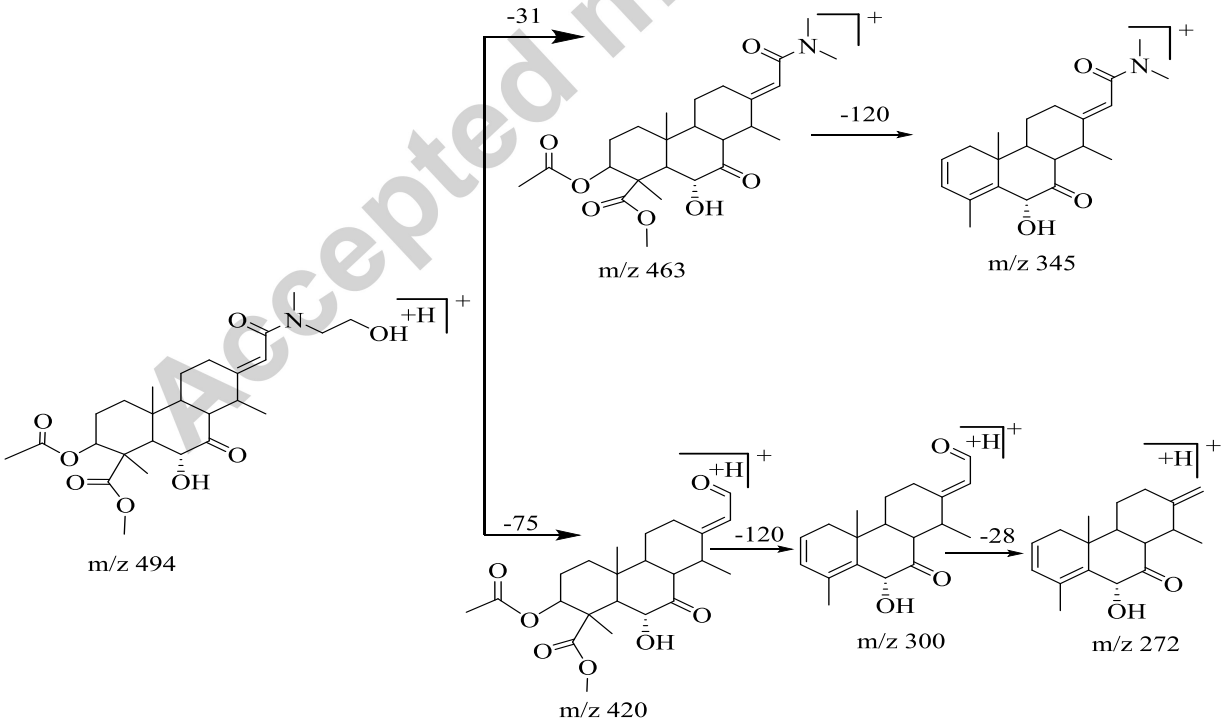


Figure 3. Typical mass spectra of the fragmentation patterns of 3β-acetoxynorerythrosuamide **2**



Scheme 2. Proposed fragmentation pathways of 3β-acetoxynorerythrosuamide **2**

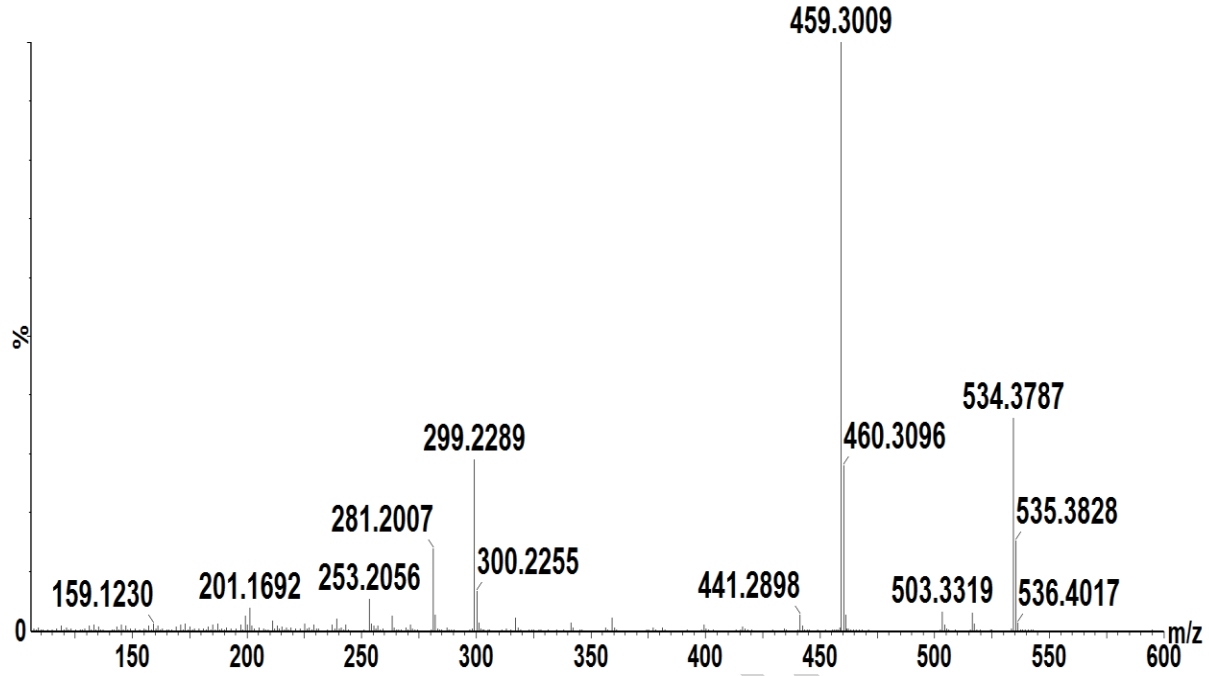
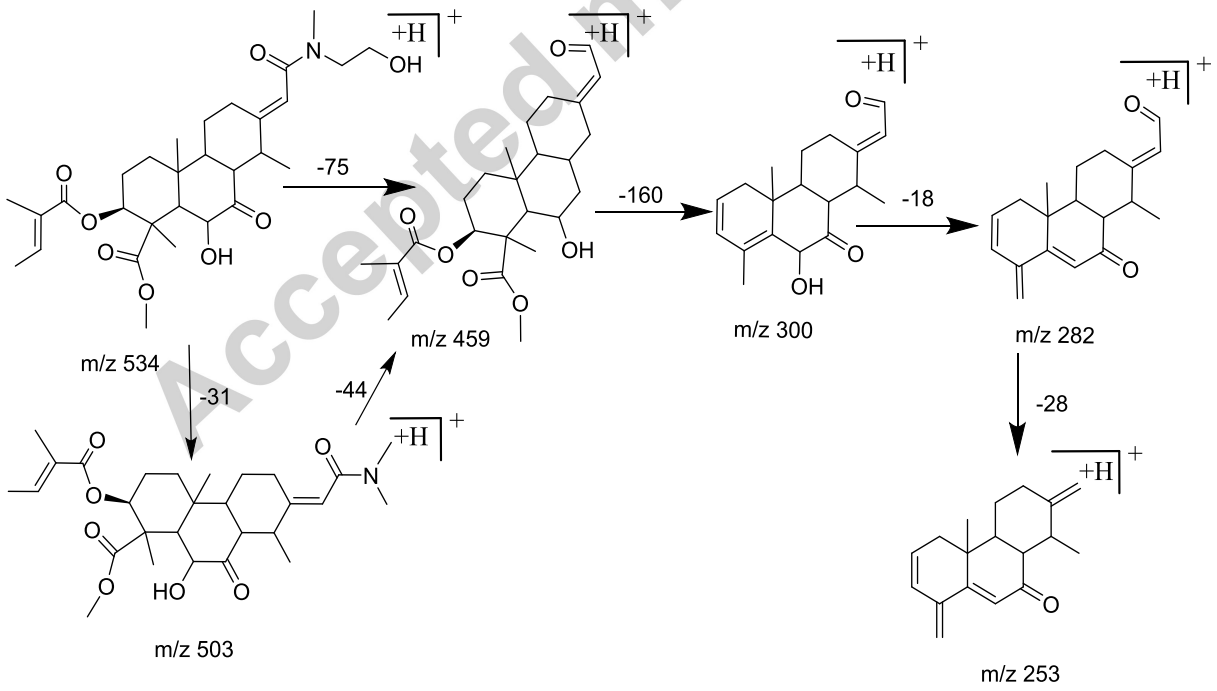


Figure 4. Typical mass spectrum of the fragmentation pattern of 3β-Tigloyloxynorerythrosuamide **4**



Scheme 3. Proposed fragmentation pathways of 3β-Tigloyloxynorerythrosuamide **4**

A fifth and a second form of compound at retention time of 5.27 min (Figure 5) showed a precursor ion of *m/z* 422.3082 [M+H] - ($C_{23}H_{35}NO_6$) and at higher collision energy, several product ions were observed. The most prominent ions were at *m/z* 347.2348 due to the loss of an amide side chain (-75 Da), at *m/z* 288.2205 as well as *m/z* 269.2031 and 241.2069 derived from the loss of methyl formate, water and carbonyl respectively. Similar 1, 5 methyl rearrangement observed in molecule 1 was also evident in molecule **5**. Molecule **5** was identified as 6α-Hydroxydinorcassamide (**5**). Similar cassamide molecules **6**, **7** and **8** (erythrophlamide) were also identified with similar fragmentation pattern (Figure 5, Scheme 4).

A comparison of the fragmentation pathways data of **8** (nor-erythrophlamide) with those of norcassamide **5-7** suggested they share the same skeleton. Compounds **6-8** were elucidated by positive-ion mode accurate HR-MS and upon further fragmentation (Figure 5&6) at higher collision energy, these molecule showed product ions. The molecular formula of **6** was established as $C_{24}H_{37}NO_7$, which showed a pseudo-molecular ion peak at *m/z* 452.2610 (calcd. 452.2604) $[M+H]^+$ at retention time of 4.84 min. Fragmentation pattern showed product ions at *m/z* 452.2892 and 378.2196 due to the loss of an amide side chain (-75 Da), at *m/z* 317.1914 as well as *m/z* 299.1806 and *m/z* 271.1820 derived from the loss of methyl formate, water and carbonyl respectively. Similarly, compound 7 produced molecular ion of *m/z* 478.2764 at retention time of 7.51 min with molecular formulae $C_{26}H_{39}NO_7$. Compound **8** was also identified as $C_{24}H_{37}NO_6$ with *m/z* 436.2685 at retention time of 8.58 min. Analogous fragmentation pattern of these molecules to that of **5** and **6** was also realized (Figure 6, Scheme 5). Based on this data and previous literature reports (Hung et al 2014, Qu et al 2007, Du, et al. 2010), molecule **6 - 8** were identified as 6α-hydroxy-nor-erythrophlamide (**6**), 22-acetoxy-6α-hydroxy-nor-cassamide (**7**) and 6α-hydroxy-nor-cassamine (**8**).

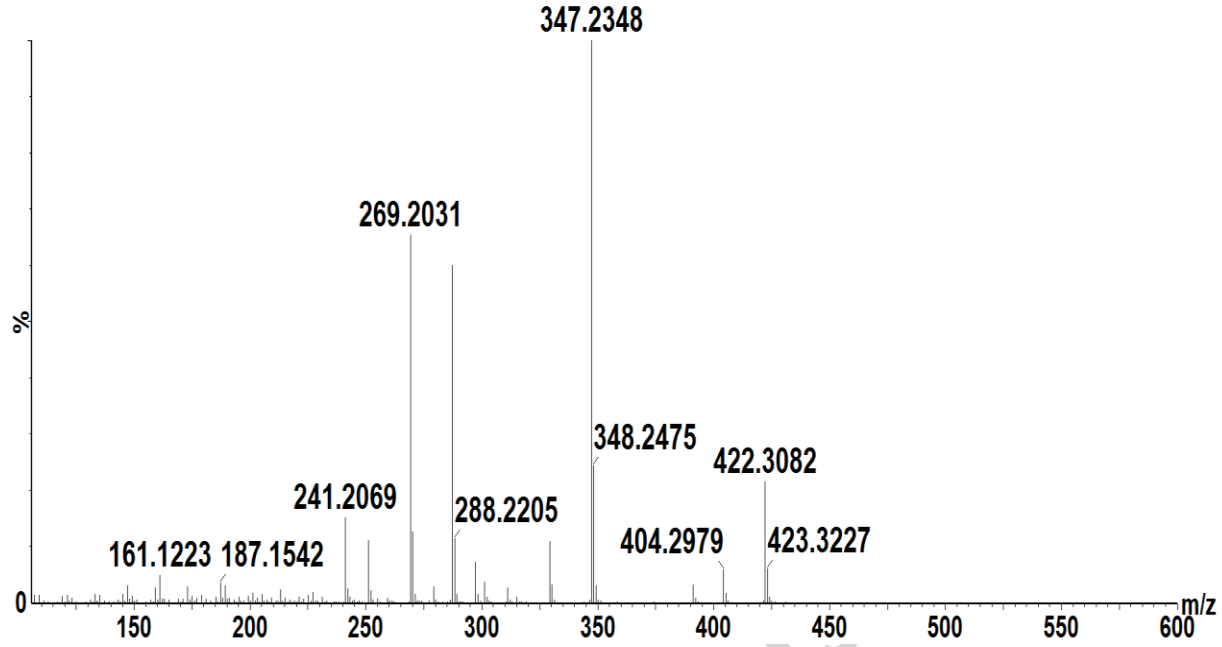
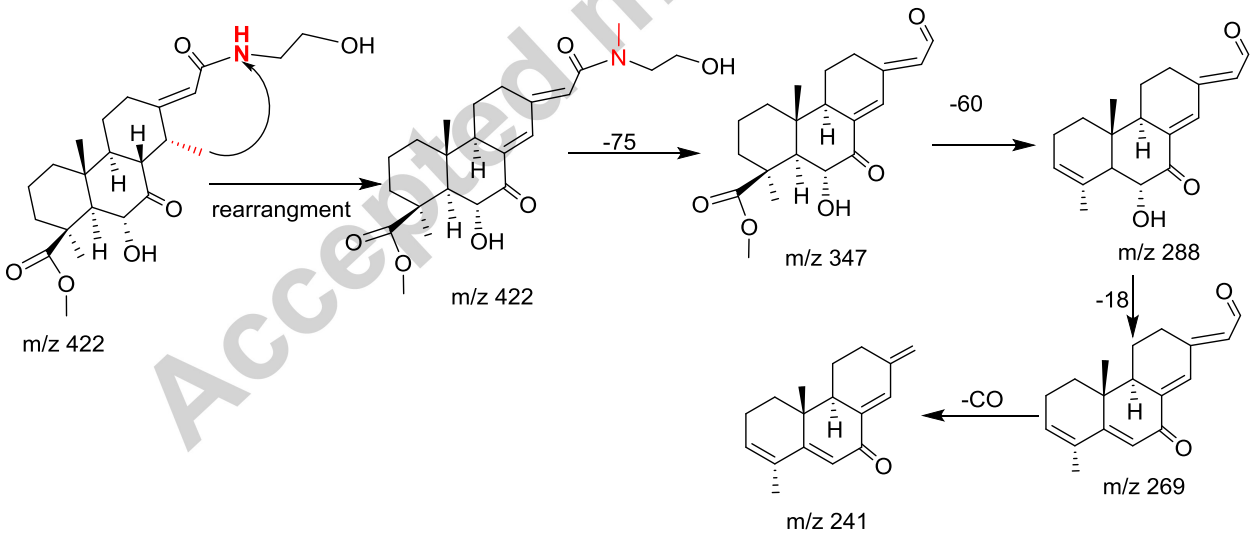


Figure 5. Typical mass spectrum of the fragmentation pattern of 6α-Hydroxydinorcassamide **5**



Scheme 4. Proposed fragmentation pathways of 6α-Hydroxydinorcassamide **5**

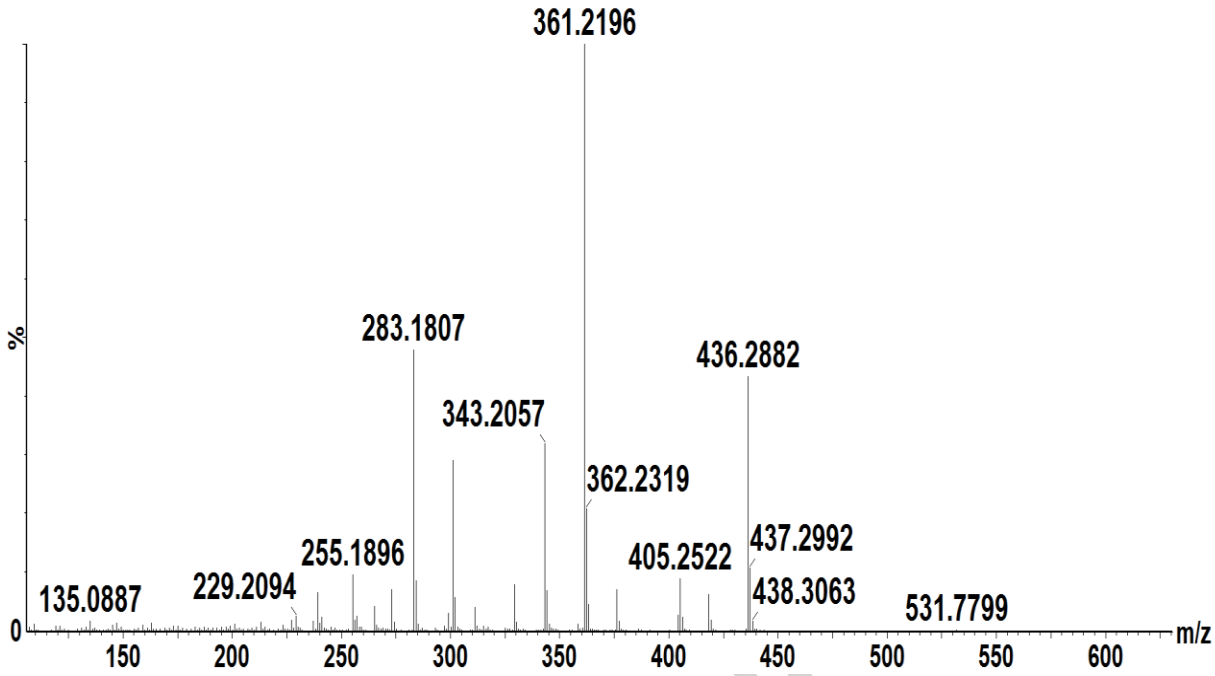
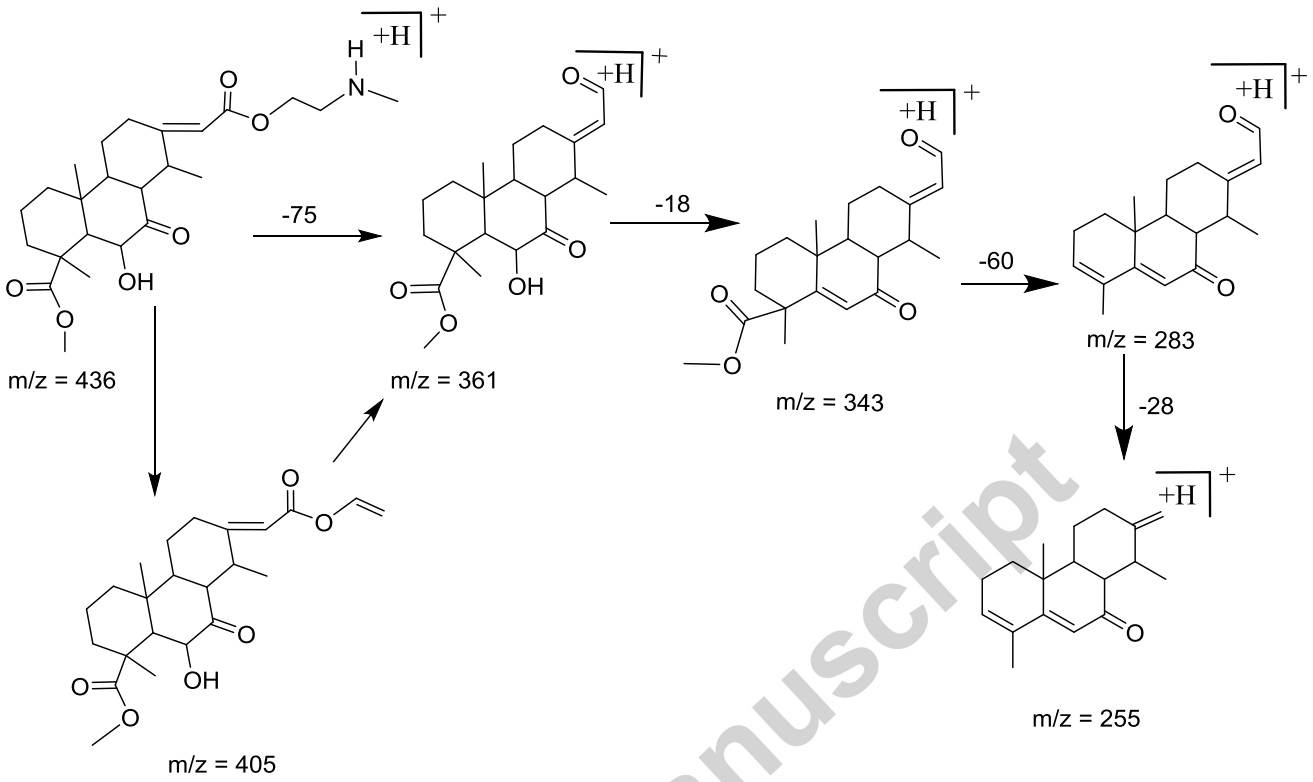


Figure 6. Typical mass spectrum of the fragmentation pattern of 6α-hydroxy-nor-cassamine **8**



Scheme 5. Proposed fragmentation pathways of 6α-Hydroxy-nor-cassamine **8**

Finally, molecules **9** and **10** were identified at rt 5.84 and 5.72 min respectively, (Figure 7) producing a precursor ion at *m/z* 392.2737 [M+H] - ($C_{23}H_{37}NO_4$) and *m/z* 408.3082 [M+H] - ($C_{23}H_{37}NO_5$) respectively. Fragmentation of this molecule at a higher CE level produced product ions at *m/z* 317.2310 resulting from the loss of an ethanol moiety (45 Da), at *m/z* 299.2142 and 271.2207 resulting from a further loss of a water (18 Da) and carbonyl moiety respectively. Looking at their fragmentation patterns (Figure 7 & 8, schemes 6 & 7), they were identified as Norcassaide **9** and 7b-hydroxy-7-deoxo-6-oxonorcassaide **10** based on the work of Ngounou, et al., 2005, Lorder et al., 1972 and Crönlund et al., 1971.

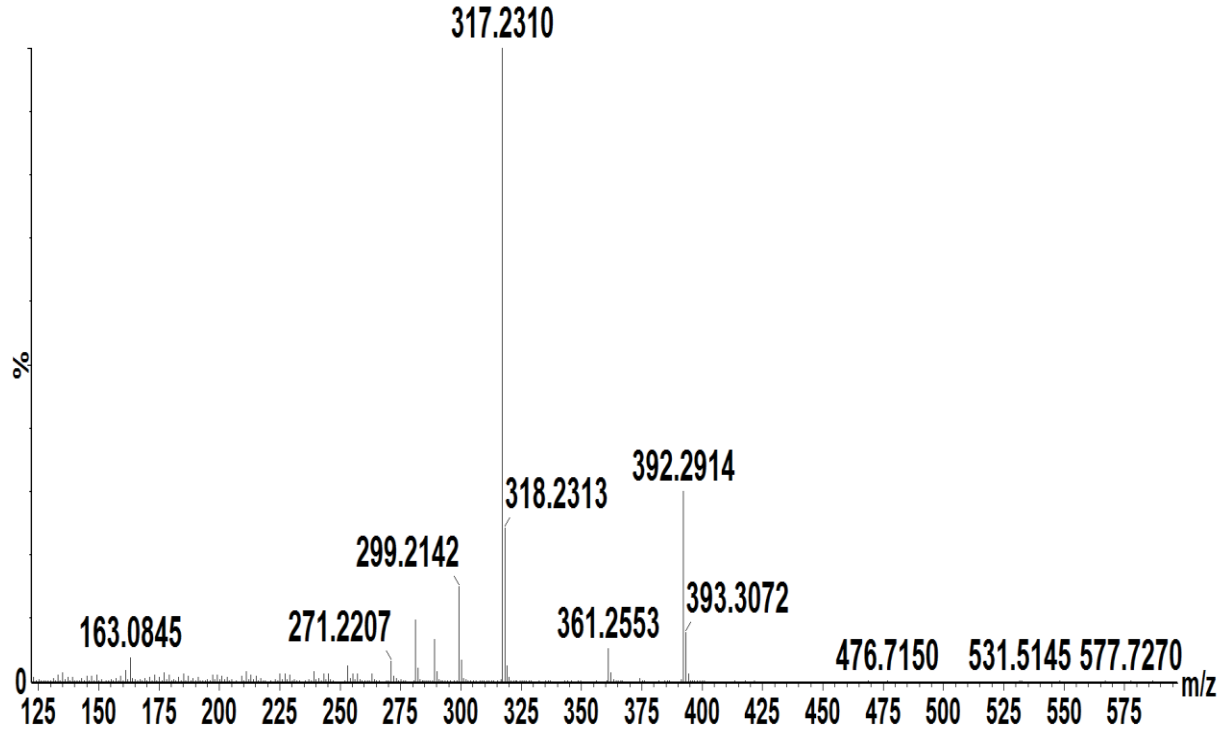
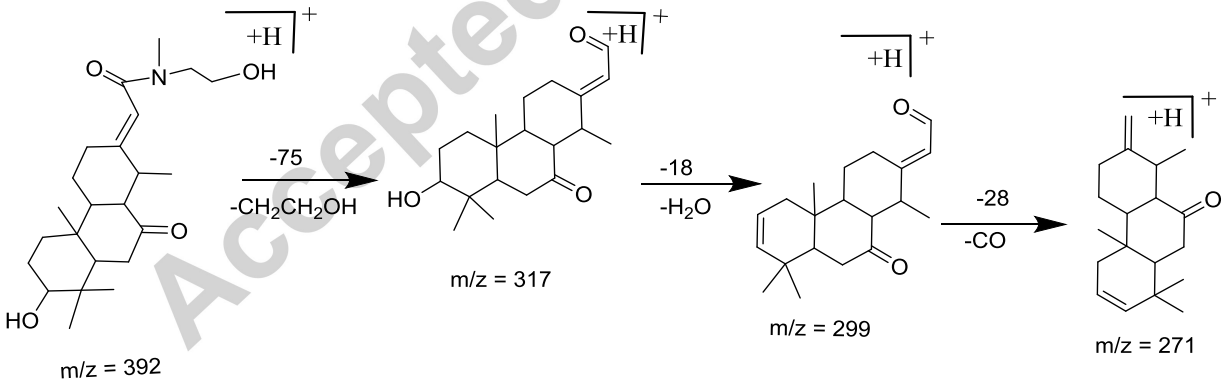


Figure 7. Typical mass spectrum of the fragmentation pattern of Norcassaide **9**



Scheme 6. Proposed fragmentation pathways of Norcassaide **9**

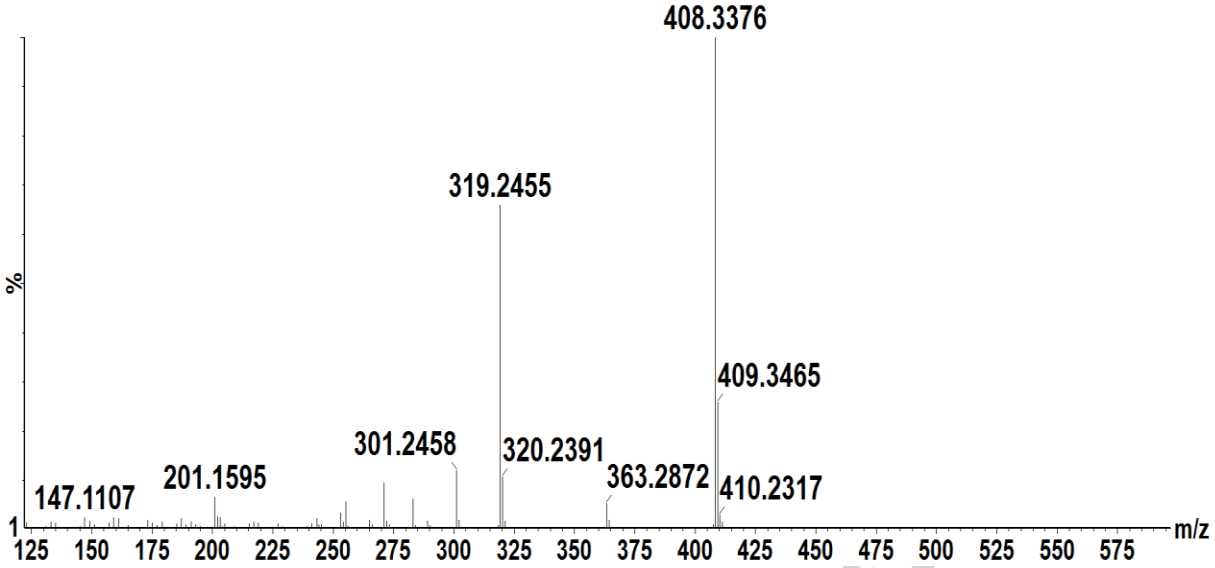
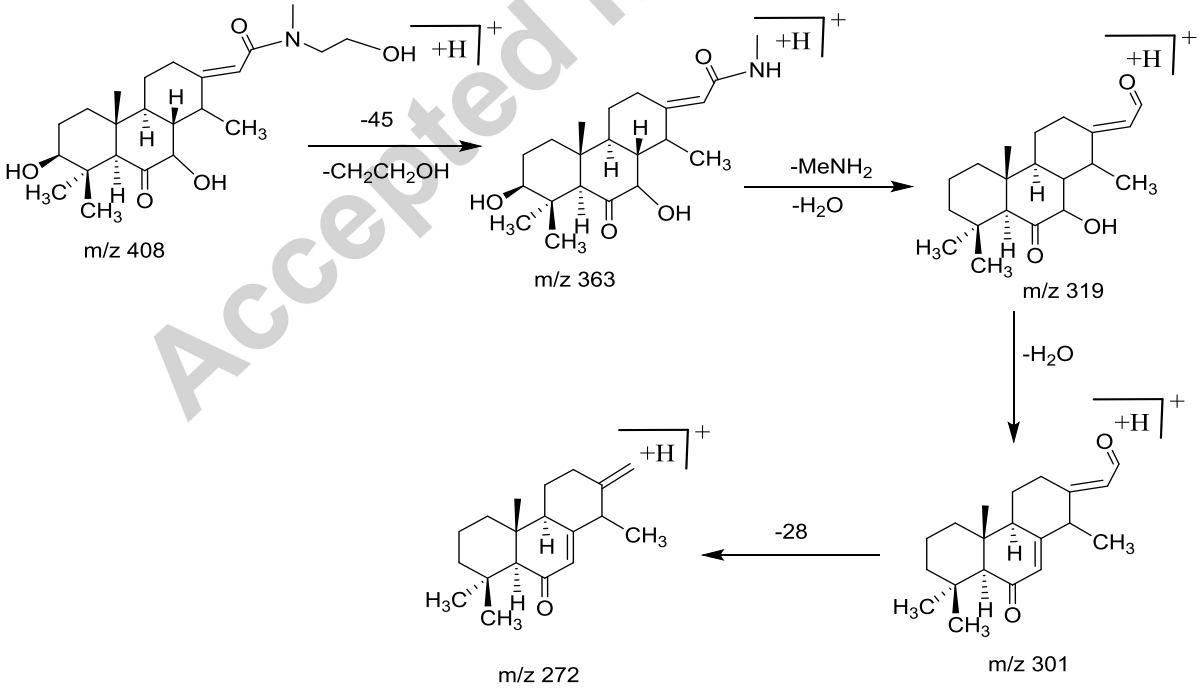


Figure 7. Typical mass spectrum of the fragmentation pattern of 7b-hydroxy-7-deoxo-6-oxonorcassaide **10**



Scheme 7. Proposed fragmentation pathways of 7b-hydroxy-7-deoxo-6-oxonorcassaide **10**

4.0 Conclusion

The methanol extract of *E. ivorense* may contain promising leishmanicidal compounds. Investigation of the active methanol extract using UHPLC-Q-TOF-MS/MS fingerprinting method, based on the in-source collision induced dissociation (ISCID) approach revealed ten cassaine diterpenoid compounds confirmed by their typical fragmentation pattern. The presence of these compounds is an indication that they are an inherited and evolutionary component of the plants from *Erythrophleum* genus. This results further present another dimension were these compounds and their relative abundances can be used as chemo-taxonomical bio-markers of the *Erythrophleum* genus.

Authors' contributions

FAA, AAY and RAD conceptualized the study and FAA reviewed the research proposal. FAA conducted the ethnobotanical survey and analyzed the field data. CKA, FAA, NEM and IKA drafted the manuscript. PAS and NEM conducted the Metabolite identification of active methanol fraction using UHPLC-Q-TOF-MS/MS fingerprinting method, while CKA and NEM interpreted and elucidated the compounds. All the authors Participated in writing and giving feedback on the manuscript. All authors have read and approved the final manuscript.

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LIST OF ABBREVIATION

UPLC-QTOF-MS/MS - Ultra-Performance Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry

LV-90 - *Leishmania donovani*

DMSO - Dimethyl sulfoxide

IC_{50} - The concentration required for 50% inhibition

UHPLC - Ultra High-Performance Liquid Chromatography

SIM - Single ion monitoring

ISCID - In-source collision induced dissociation

FBS - Fetal bovine serum

PDA - Photo-diode array detector

CSIR - Council for Scientific and Industrial Research

NRF - National Research foundation

List of Compounds Studied

1. 3β-acetoxydinorerythrosuamide (**1**)
2. 3β-Acetoxynorerythrosuamide (**2**),
3. 3β-Tigloyloxydinorerythrosuamide (**3**)
4. 3β-Tigloyloxynorerythrosuamide (**4**)
5. 6α-Hydroxydinorcassamide (5).
6. 6α-hydroxy-nor-erythrophlamide (**6**),
7. 22-acetoxy-6α-hydroxy-nor-cassamide (**7**)
8. 6α-hydroxy-nor-cassamine (**8**).
9. Norcassaide (**9)**
10. 7b-hydroxy-7-deoxo-6-oxonorcassaide (**10)**
11. Erythroivorensin
12. Eriodictyol
13. Betulinic acid
14. Amphotericin B