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THE PYRROLIZIDINE ALKALOIDS OF *ECHIUM PLANTAGENIUM* L

By

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قلويدات البروليزيدين في نبات الأيشيم بلانتاجنيم

(*Echium Plantagenium* L)

مقدمة من الطالب

حسين السنوسي أسحيم

بإشراف

الأستاذ دكتور فخري عبد الونيس العبار

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‘‘ The Pyrrolizidine alkaloids of *Echium Plantagenium* L’’

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LIST OF ABBREVIATIONS

Ac	Acetyl
Ang	Anoeloyl
Bu	Butyl
CRO	Crotanecine
Ech	Echimidinoyl
Ech-number	Alkaloid-Compound Number
EI	Electron Impact
EM	Electron Multiplier
EtOH	Ethanol
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GLC	Gas Liquid Chromatography
HEL	Heliotridine
HPLC	High performance Liquid Chromatography
Int	Interrinecoyl
Las	Lasiocapoyl
MeOH	Methanol
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OTO	otonecine
PAs	Pyrrolizidine alkaloids
Ph	Phenyl
RET	Retronecine
Rf	Retention Factor
RI	Retention Indice
Rt	Retention time
Sar	Sarrascinoyl

Sen	Senecioyl
SUP	Supinidine
Tig	Tigloyl
TLC	Then Layer Chromatography
Tra	Trachelanthoyl
Vir	viridifloyl

ABSTRACT

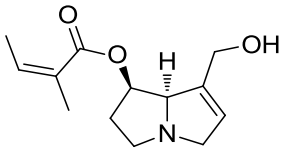
This study includes brief review of previously isolated and identified pyrrolizidine alkaloids from various Boraginaceae plant species by using various isolation and characterization techniques.

The primary aim of this research was isolation and characterization of pyrrolizidine alkaloids from *Echium plantagenium* (Boraginaceae); an uninvestigated Libyan species which grown around El marj city.

Gas Chromatographic Mass Spectrometry technique were used for isolation of pyrrolizidine alkaloids from Methanolic extraction of *Echium plantagenium*. Which result in isolate a twelve compounds. The chemical structures of the isolated compounds have been elucidated by using their Mass spectrometric fragmentation pattern and the value of retention indices; in compare with those of previously identified pyrrolizidine alkaloids as reference standard values.

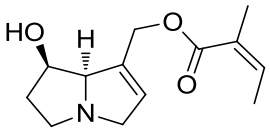
A number of isolated compounds were tentatively identified; while the rest of them is not elucidated accurately, but has been proposed a number of isomers to represent their chemical structure as follow:

Ech-1



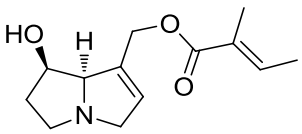
7-angeloylretronecine

Ech-2



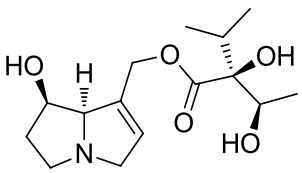
9-Angeloylretronecine

Ech-3



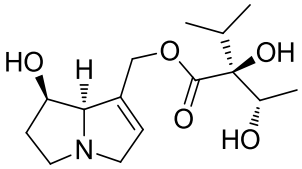
9-Tigeloylretronecine

Ech-4



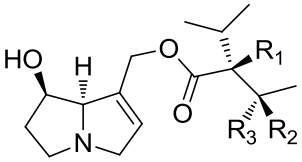
Intermedine

Ech-5



Lycopsamine

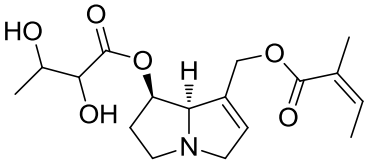
Ech-6



Assumed one of the following isomers

R_1	R_2	R_3	
OH	OAc	H	3'-acetylintermedine
OAc	OH	H	2'-acetylintermedine
OH	H	OAc	3'-acetylycopsamine
OAc	H	OH	2'-acetylycopsamine

Ech-7

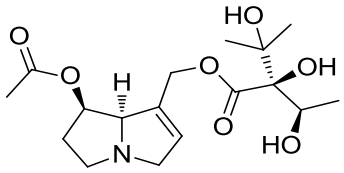


7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine

Ech-8 and Ech-9

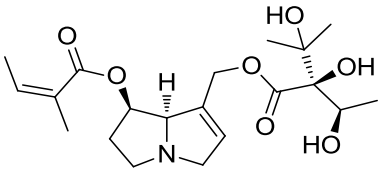
Assumed to be isomer of Ech-7

Ech-10



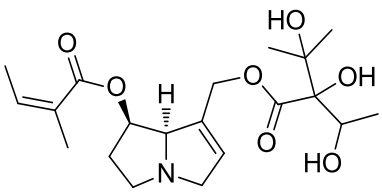
Uplandicine

Ech-11



Echimidine

Ech-12



Echiumine

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I offer the unlimited thanks to ALLAH in making me have the momentum to meet all efforts required during the course of this work.

I would like to express my deep feeling of gratitude, great indebtedness and sincerer appreciation to Prof. Dr. Fakhri A.Elabbar, Chemistry Department, Faculty of science, University of Benghazi for his kind supervision, helpful guidance and continuous encouragement during the course of this work.

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The Auther

CHAPTER ONE

1. INTRODUCTION

1.1. Natural Products

By definition, natural products are compounds present in or produced by nature and not artificial or man-made[1].

Naturally occurring compounds may be divided into three broad categories. Firstly, primary metabolites compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. such as the nucleic acids and the common amino acids and sugars. Secondly, high-molecular-weight polymeric materials such as cellulose, the lignins and the proteins which form the cellular structures. Thirdly, there are the secondary metabolites compounds that are not essential to the growth and development of the producing organism, and characteristic of a limited range of species[2].

The term natural product really refers to any naturally occurring substance but is generally taken to mean a secondary metabolites which Cannot be divided in a clear manner because of the large variation in their structures[3].

Scientists working with natural products with interest for the purpose of and elucidation of their structures and their chemistry or characterization of their biological effects

1.2. Alkaloids

Alkaloids are secondary metabolites compounds with low molecular weight present mainly in many families of the plant kingdom, and they are produced by a large variety of organisms including microbiology organism (e.g bacteria, fungi), plants, and animals[4-6].

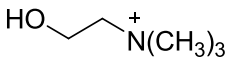
The term alkaloid, coined in 1819 in Halle, Germany, by the pharmacist, Carl Meissner, finds its origin in the Arabic name *al-qali*, the plant from which soda was first isolated[7].

Ladenburg defined alkaloids as naturally occurring plant compounds having a basic character and containing at least one nitrogen in a heterocyclic ring.' With the advent of recent advanced knowledge in the chemistry of various alkaloids two more characteristic features were logically and justifiably added to the definition of alkaloids, namely:(a) Complex

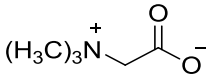
molecular structure, (b) Significant pharmacological activity. Furthermore, it was broadly observed that the basic properties of the alkaloids is solely by virtue of the presence of N-atom embedded into the ring system[8].

Therefore, the alkaloids are now generally defined as, physiologically active basic compounds of plant origin, in which at least one nitrogen atom forms part of a cyclic system.' Even this definition has a few anomalies as stated below[8] :

(i) Cholines and Betaines: These two substances have the N-atom in the side chain and not in the aromatic ring as shown below:



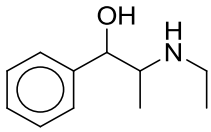
Choline



Betaine

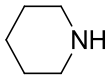
The cholines and betaines are regarded as simple alkylamines and not classified as alkaloids. They are designated by some school of thoughts as 'biological-amines' or 'protoalkaloids'.

(ii) Ephedrine: It has the N-atom only in the side chain and not embedded in the aromatic ring as given below:



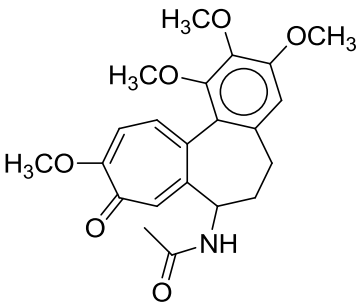
Ephedrine

(iii) Piperidine: It is obtained Piper nigrum (Black Pepper) and does not possess any pharmacological activity, but has a N-atom in a heterocyclic ring as given below:



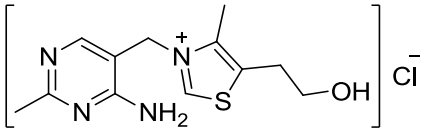
Piperidine

(iv) Colchicine: It is found to be neither basic nor it contains the N-atom in a heterocyclic ring, whereas it is considered as an alkaloid due to the fact it possesses distinct pharmacological activity as shown below:



Cholchcine

(v) Thiamine (Vitamin B1): It confines to the definition of alkaloids but is not regarded as an‘alkaloid’ because of its almost universal distribution in living matter.



Thiamine monochloride

More than 27000 structure of alkaloid has been diagnosed from variety plant species. A single plant species usually contains only a few kinds of alkaloids, but certain plant families may be rich in alkaloids[9].

Alkaloids They includes in their structure one nitrogen atom or more typically in the form of a primary, secondary, or tertiary amines; that's what makes them bases[10, 11]. Many alkaloids which are toxic to other organisms can be purified from crude extracts by acid - base extraction[12].

Many alkaloids have a wide range of pharmacological activities and found use in traditional or modern medicine, or as starting points for drug discovery. Other alkaloids possess psychotropic (e.g. psilocin) and stimulant activities (e.g. cocaine)[13], Alkaloids can be toxic too (e.g. atropine, tubocurarine). Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly evoke a bitter taste[8].

1.2.1. General Characteristics of Alkaloids

1.2.1.1. The basicity of alkaloids

The alkaloids are organic nitrogenous bases found mainly in plants. They have one or more nitrogen atoms present, typically as primary, secondary, or tertiary amines, and this usually confers basicity to the alkaloid, facilitating their isolation and purification since water-soluble salts can be formed in the presence of mineral acids[11].

the degree of basicity varies greatly, depending on the structure of the alkaloid molecule, and the presence and location of other functional groups.45 However, most alkaloids have basic properties with pH value between 6-7[11, 14] . and present in plants in the form of free bases, combined with acids to be salts, or in form of N-oxides. free alkaloids are soluble in organic solvents but mingy water solubility. Alkaloidal salts are soluble in water but insoluble in organic solvents. This difference in solubility provides an excellent way to separate the alkaloids from their natural sources[15].

The alkaloids are usually neutrallized with acids to form salts that may be converted to the corresponding free-base by the cautious addition of selective weak bases, such as, ammonia, calcium hydroxide or sodium carbonate. The usage of either NaOH or KOH solutions must be avoided to prevent the decomposition or destruction of highly sensitive alkaloids[16].

1.2.1.2. The solubility alkaloids

The solubility of different alkaloids and their respective salts usually exhibit considerable variation, which may be attributed from their extremely complex and varied chemical structures. However, it has been observed that the free alkaloid bases as such are invariably found to be fairly soluble in organic solvents, such as: ether, chloroform, relatively non-polar solvents (hexane, benzene, petroleum ether), immiscible solvent, lower alcohols (methanol, ethanol); but they are either practically insoluble soluble in water. Whereas the alkaloidal salts are almost freely soluble in water, relatively less soluble in alcohol and mostly either insoluble or sparingly soluble in organic solvents[8].

1.2.2. Stability of alkaloids

Alkaloid, are not very stable . They normally undergo degradation or decomposition on being exposed to air, light, moisture and heat, besides chemical reagents. For example an aqueous solution of alkaloids undergo rapid decomposition or degradation as compared to their solid forms[16].

During the course of extraction of alkaloids followed by isolation, the solvent is preferably removed effectively by distillation under vacuum (or reduced atmospheric pressure) or by subjecting it to evaporation in a Rotary Thin-Film Evaporator under vacuum so that the desired product is not exposed to excessive heat[17], thus avoiding decomposition.

1.2.3. Testing and precipitating of alkaloids

A good number of alkaloids obtained from various plant sources invariably give a distinct precipitate with certain specific reagents to an extent as small as one microgram. Based on these observations, these alkaloid-precipitating reagents are sometimes employed for either detecting the presence or absence of alkaloids in a crude extracts or plant materials, and for ascertaining whether a specific extraction procedure has exhausted completely the alkaloidal contents or not[8].

Most alkaloids are precipitated from neutral or slightly acid solution by Mayer's reagent (potassio mercuric iodide solution), by Wagner's reagent (solution of iodine in potassium iodide), by solution of tannic acid, by Hager's reagent (a saturated solution of picric acid), or by Dragendorff's reagent (solution of potassium bismuth iodide). These precipitates may be amorphous or crystalline and are of various colours: cream (Mayer's), yellow (Hager's), reddish-brown (Wagner's and Dragendorff's)[15].

The table (1) different qualitative chemical tests can be performed for establishing profile of given extract for its chemical composition.

Name of reagent	Composition
Mayer's Reagent[18] (Potassium-Mercuric Iodide Test Solution)	Mercuric chloride = 1.36 g Potassium Iodide = 5.00 g Distilled water to make = 100.00 ml

Wagner's Reagent[18] (Potassium Triiodide)	Iodine = 1.27 g Potassium = 2.0 g Distilled water to make = 100.00 ml
Dragendorff Reagent[14]	Solution A 55.5 mL of 36% HCl bismuth nitrate = 2 g Distilled water to make = 100.00 ml Solution B potassium iodide = 32 g Dragendorff reagent prepared by the addition of 20 mL of solution (A) and 20 mL of solution (B) then complete to 100 ml with distilled water
Kraut's Reagent [8] (Modified Dragendorff'sReagent)	Bismuth Nitrate = 8.0 g Nitric Acid = 20.0 ml Potassium Iodide = 27.2 g Distilled water to make = 100.00 ml
Marme's Reagent[8] (Potassium-Cadmium Iodide Reagent)	Cadmium Iodide = 10.0 g Potassium Iodide = 20.0 g Distilled water to make = 100.00 ml
Scheibler's Reagent [8] (Phosphotungstic Acid Reagent)	Sodium Tungstate = 20.0 g Disodium Phosphate = 70.0 g Distilled water to make = 100.00 ml Note: Acidify with nitric acid to litmus paper.
Hager's reagent[18]	a saturated solution of picric acid
Sonnenschein's Reagent[8] (Phosphomolybdic Acid	A 1% (w/v) solution of phosphomolybdic acid in ethanol.
Bertrand's Reagent[8] (Silicotungstic Acid)	A 1% (w/v) solution of silicotungstic acid in distilled water.

Reineckate salt solution[8]	Ammonium Reineckate = 1.0 g $NH_4[Cr(NH_3)_2(SCN)_4]$ Hydroxylamine HCl = 0.3 g Ethanol = 100.00 ml Note: Filter and store in a refrigerator.

Table 1: Composition of common reagents used for detection of alkaloids

1.2.4. Classification of Alkaloids

The classification of the alkaloids is complex and may be guided by a set of rules that take into account the structure and other chemical features of the alkaloid molecule, its biological origin, as well as the biogenetic origin where known[19]. some of these rules are describe briefly as below[20]:

Pharmacological classification based on the clinical use or pharmacological activity (e.g Cardioactive alkaloids)

Taxonomic classification based on family or genus. Without reference to the chemical type of alkaloid present (e.g Solanaceous alkaloids)

Biosynthetic classification based on precursors or building block compounds used by plants to synthesize alkaloids (e.g Morphine, papaverine, and colchicines may be listed as phenylanaline and tyrosine derived bases)

Chemical classification based on Chemical structure of basic ring in the alkaloid(e.g Atropine is a tropane alkaloid; quinine is a quinoline alkaloid; papaverine is an isoquinoline; strychnine and ergometrine are indol alkaloids)

According to the fact that all alkaloids must contain nitrogen atom at least, alkaloids may be classified based on the nature of the nitrogen-containing structure as follow[21]:

True alkaloids that contain nitrogen atom in heterocyclic ring, and derived from amino acid.

Proto alkaloids or amino alkaloids that does not have nitrogen heterocyclic ring, and derived from amino acid.

Pseudo alkaloids contain nitrogen atom in heterocyclic ring, but not derived from amino acid.

Based on chemical nature alkaloids are further classified into two groups broad divisions[15]:

I . Non-heterocyclic or atypical alkaloids, called ‘protoalkaloids’ or biological amines.

1. Hordenine or N -methyltyramine
2. Mescaline, related to tryptamine
3. Ephedrine
4. Colchicine (tropolone nucleus with nitrogen in side-chain)
5. Erythromycin (an antibiotic)
6. Jurubin (steroid with 3-amino group)
7. Pachysandrine A (steroid with N -containing C-17 side-chain)
8. Taxol (a modified diterpene pseudo alkaloid)

II . Heterocyclic or typical alkaloids, divided into 14 groups according to their ring structure.

1. Pyrrolidine alkaloids e.g., Hygrine;
2. Piperidine alkaloids e.g., Lobeline;
3. Pyrrolizidine alkaloids e.g., Senecionine;
4. Tropane alkaloids e.g., Atropine;
5. Quinoline alkaloids e.g., Quinine;
6. Isoquinoline alkaloids e.g., Morphine;
7. Aporphine alkaloids e.g., Boldine;
8. Indole alkaloids e.g., Ergometrine;
9. Imidazole alkaloids e.g., Pilocarpine;
10. Diazocin alkaloids e.g., Lupanine;
11. Purine alkaloids e.g., Caffeine;
12. Steroidal alkaloids e.g., Solanidine;
13. Amino alkaloids e.g., Ephedrine;
14. Diterpene alkaloids e.g., Aconitine.

1.3. Pyrrolizidine alkaloids

1.3.1. Occurrence and distribution

The group of compounds known as pyrro1izidine alkaloids comprises over three hundred compounds identified to date, the majority of which occur naturally, although an increasing number are being derived synthetically or semi synthetically[22].

Naturally occurring pyrrolizidines are predominantly of plan origin and have been isolated from over four hundred and fifty species distributed among eighty-seven genera of fourteen plant families[23, 24].The largest number of pyrrolizidine alkaloids occur in the genus Senecio. For this reason, and because the first pyrrolizidine alkaloid was isolated from this genus4l, these alkaloids are often referred to as the Senecio alkaloids[22].

Pyrrolizidine alkaloids (Pyrrolizidine alkaloids) are a well-known class of defense compounds with a wide variety of structures. belong to the plant families Asteraceae, Boraginaceae, Orchidaceae and Fabaceae[25],

More than 660 pyrrolizidine alkaloids and N-oxide derivatives have been identified in over 6000 plants of previous families are considered to be potentially toxic[26]. The main sources of these toxic alkaloids are almost all genera of the family Boraginaceae, the tribes Senecioneae and Eupatorieae of the family Asteraceae and the genus Crotalaria of the family Fabaceae[27]

plants containing pyrrolizidine alkaloids are known to be toxic and have caused widespread livestock losses in many parts of the world[24]. Human poisoning has also been reported to occur through ingestion of pyrrolizidine alkaloids from contaminated foodstuffs and traditional herbal remedies and teas[28]. Some pyrrolizidine alkaloids have also been shown to exhibit anti-tumour activity and as a result are of great interest in medical research fields[28, 29].

Pyrrolizidine alkaloids have also been used as chemotaxonomic markers in various botanical studies[30, 31].

Pyrrolizidine alkaloids have also been isolated from some animal sources. Butterflies and moths of various genera feed on pyrrolizidine-containing plants and the ingested alkaloids are used for defence against predators and as pheromones[32, 33]. For example, the black and white moth Gnophaela latipennis (Arctiidae) has recently been shown to contain pyrrolizidines[34], whilst other workers have demonstrated the uptake of pyrrolizidines from host plants by the parasitic genus Pedicularis. In all these instances the pyrrolizidines were obtained from plant sources in the feeding process[35].

There is a group of naturally occurring Pyrrolizidine alkaloids which are not of plant origin; a number of ant genera have been shown to produce pyrrolizidines as defence compounds[36].

1.3.2. Pyrrolizidine alkaloid Structure

All pyrrolizidine alkaloids contain the basic Pyrrolizidine ring figure (1). The molecules comprises two five-membered rings which share a nitrogen atom at position 4, this structural core is bicyclic ring system named systematically azabicyclo[3,3,0]octane(1).The diagram shows the traditional system for numbering the ring, which has been adopted throughout this work. The orientation of substituents is labelled as shown in figure (1). The fully saturated ring structure is non-planar and adopts an open 'V' shaped configuration. Whether the V opens towards or away from the viewer depends on the orientation of the substituents on C8[37, 38].

Most pyrrolizidine alkaloids identified so far have the basic structure system as shown in figure (1). A few alkaloids possess substituents such as methyl, methylene, carboxaldehyde, Carboxylic acid and nitrogen groups at Cl , while some possess hydroxyl groups at other positions on the ring system, usually at C2 or C6. One or both of the hydroxyl groups (C7 or C9) may be esterified with a variety of acids[39]. The unesterified ring system is usually referred to as the amino alcohol "necine" and the acid portion as the "necic acid"[37, 38].

Pyrrolizidine alkaloids are esters of hydroxylated methyl pyrrolizidines, consisting of a necine base and necic acid moiety see figure (1). The necine base can either be 1,2-unsaturated (e.g Retronecine (8) and Supinidine (12) or saturated (e.g Alexine(3), Australine(4))[24]. The hepatotoxic pyrrolizidine alkaloids are monoesters, diesters or macrocyclicdiesters of unsaturated necine or otonecine bases[40].

Pyrrolizidine alkaloids can be oxygenated at N position to produce N-oxides. The polar nature of the N-oxide bond greatly enhances water solubility upon these alkaloids, relative to the corresponding tertiary bases[14]. Pyrrolizidine alkaloid N-oxides are unstable , they are easily converted into the tertiary amines in the presence of weak reducing agents[41].To date, about 200 structures have been characterized, but many more have yet to be identified[42].

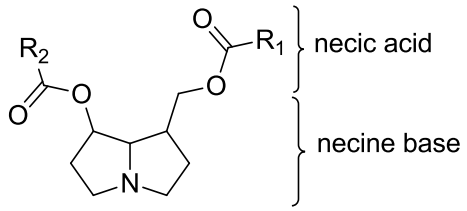


Figure 1: The pyrrolizidine structure

1.3.3. The Necine Bases and Simple alkaloids

There are over forty different necine bases, but only about twenty of these occur commonly[24]. Unesterified bases are rarely extracted from plants, but are obtained by hydrolysis of the alkaloid extract. Simple pyrrolizidine alkaloids are not esterified and are composed of a modified necine base.

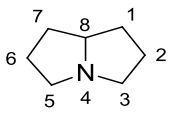
The necine bases are hydroxyl derivatives of 1-methylpyrrolizidine compound[43] , which consisting of two fused five-membered rings with bridgehead nitrogen. The fully saturated ring structure is non-planar and adopts an open 'V' shaped configuration Whether the V opens towards or away from the viewer depends on the orientation of the substituents on Carbon atoms[37, 38]. The necine bases are generally classified into five main types, Retronecine (8)

(RET), Heliotridine (9), crotanecine (10), supinidine(11), otonecine(12) (OTO)[43]. The necine can either be saturated or possess a double bond in the l,2-position. Moreover, they may additionally bear one or two hydroxyl groups at C-2, C-6 or C-7 resulting in the formation of stereoisomers. Dashed and thickened (wedges) lines denote α and ß-orientations of bonds, respectively; α meaning orientation away from the observer, ß toward the observer[39].

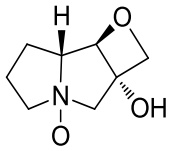
The otonecine (12) is exception to the rest of the necines bases, in that it does not derived from 1-methylpyrrolizidine compound, but derived from an N-methylated azacyclooctan-4-one System. It act as a pyrrolizidine ring System by resonance operation leads to binding between N atom and the CO group[39]. The Pyrrolizidine alkaloids derived from these structures constitute a subgroup of the otonecine (12) alkaloids (O Pyrrolizidine alkaloids).

All known Pyrrolizidine alkaloids found in the plants can form N-oxide derivatives except the otonecine (12) alkaloids. The corresponding esterification of necines containing a double bond in the 1,2- position results in the formation of the toxic alkaloids[44].

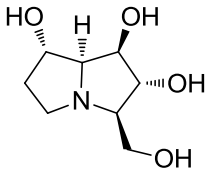
A number of new and simple pyrrolizidine alkaloids compounds have recently been identified[22].



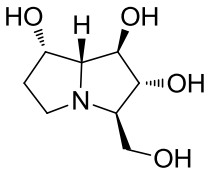
(1)



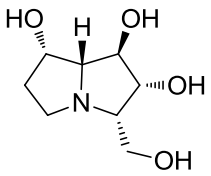
(2) subulacine-4-oxide



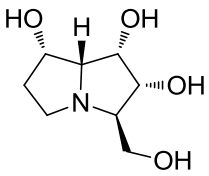
(3) Alexine



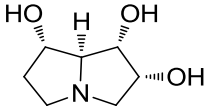
(4) Australine



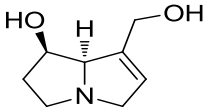
(5) 3-Epiaustraline



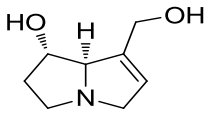
(6) l-epiaustraline



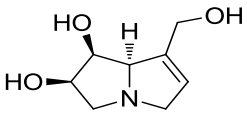
(7) 1,7α-diepialexine



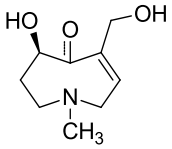
(8) Retronecine



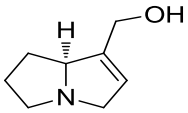
(9) Heliotridine



(10) crotanecine



(11) otonecine



(12) supinidine

Figure 2: The structures of several necine bases and simple Pyrrolizidine alkaloids

1.3.4. Necic acids

The acid Which is esterified with necine base called necic acid. Exception of acetic acid necic acids containing 4 to 10 carbon atoms and differ from each other in their composition.Where they may be mono or di-carboxylic acids with branched carbon chains. And containing substituted groups such as hydroxy, methoxy, epoxy, carboxy, acetoxy or other alkoxy groups[39];and they can also be found in the form of Stereo- and diastereoisomers[44]. Figures (3 and 4) illustrate most important Necic acids that have been detected in alkaloids sofar.

The esterification possibilities are exemplified by several alkaloids. Necines containing one hydroxy group can be esterified with one monocarboxylic acid only as in Amabiline (40). Necines bearing two hydroxy groupssuch as 7,9-necinediols can be esterified with a

monocarboxylic acid either in the 7- or 9-position as demonstrated by7-angeloyl respectively 9-Angeloylretronecine (56). Echimidine (32) is an example of a twofold esterification. With dicarboxylic acids a double esterification takes place exclusively to form macrocyclic diesters (linking C7 with C9) of a necine [14, 45]. Base leading to the formation of alkaloids with 11- to 14-membered ring Systems. The most widely known Pyrrolizidine alkaloids are the11-membered monocrotaline, the 12-membered alkaloids senecionine and senkirkine, the 13-membered doronenine, and the 14-membered parsonsine.

Through combination of necines with necic acids an unimaginably large number of alkaloids may be theoretically obtained In nature.more than about 640 alkaloids were found so far and their structures elucidated[46].

1.3.5. Monoester alkaloids

Monoester alkaloids are composed of a necine base esterified at either C7 or C9. A number of previously known monoester alkaloids have been isolated from new plant sources. Echinatine (50) and Lycopsamine (21)were isolated from *Anchusa arvensis*[47] Heliotrine (53)and Rinderine (47)were isolated from *Arnebia decumbens*[48].

1.3.6. Acyclic diester alkaloids

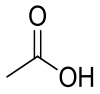
Most new alkaloids of this class have Retronecine (8) as thenecine base.

Hydroxymyoscorpine (30)was isolated from *Llthospermwn erythrorhlzon*, along with Myoscorpine (29)andIntermedine (18)[49]. Doriasenine (49) was isolated from *Senecio doria*[50]

1.3.7. Macrocyclic diester alkaloids

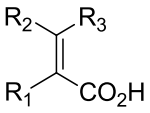
A macrocyclic diester alkaloid is composed of a necine base esterified to a dibasic acid, to produce a macrocyclic structure. Many such alkaloids are known [22].

Necic acids with two carbon atoms



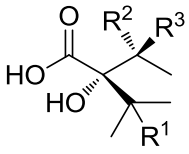
Acetic acid (Ac)

Necic acids with five carbon atoms

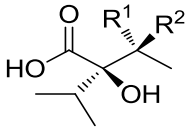


R_1	R_2	R_3	
H	Me	Me	Senecioic acid (Sen)
Me	Me	H	Tiglic acid (Tig)
Me	H	Me	Angelic acid (Ang)
CH_2OH	H	Me	Sarracinic acid (Sar)

Necic acids with seven carbon atoms



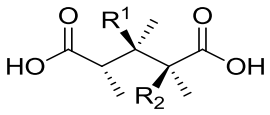
R_1	R_2	R_3	
H	H	OH	(-)-Viridifloric acid
H	OH	H	(+)-Trachelanthic acid
OH	OH	H	Echimidinic acid
OH	OMe	H	Lasiocarpic acid



H	OH	(-)-Trachelanthic acid
OH	H	(+)-Viridifloric acid

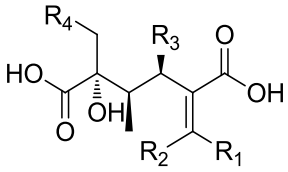
Figure 3: The Most Important Monocarboxylic Necic Acids

Necic acids with eight carbon atoms

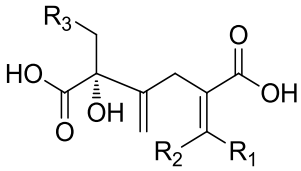


R_1	R_2	
OH	OH	Monocrotalic acid
H	OH	Crotaleschenic acid

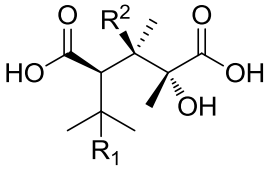
Necic acids with ten carbon atoms



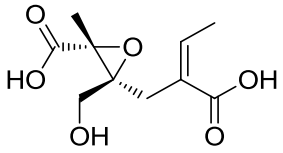
R_1	R_2	R_3	R_4	
Me	H	H	H	Senecinic acid
H	Me	H	H	Integerrinecic acid
H	H	Me	H	Senecivernic acid
Me	H	H	OH	Isatinecic acid
H	Me	H	OH	Retronecic acid



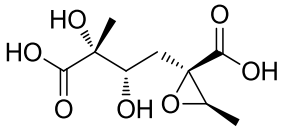
Me	H	H	Seneciphyllic acid
H	Me	H	Spartioidinic acid
Me	H	OH	Riddelliic acid



H	H	Incanic acid
H	OH	Trichodesmic acid
OH	OH	Globiferic acid



Erucifolinecic acid



Petasinecic acid

Figure 4: Most Important Dicarboxylic Necic Acids

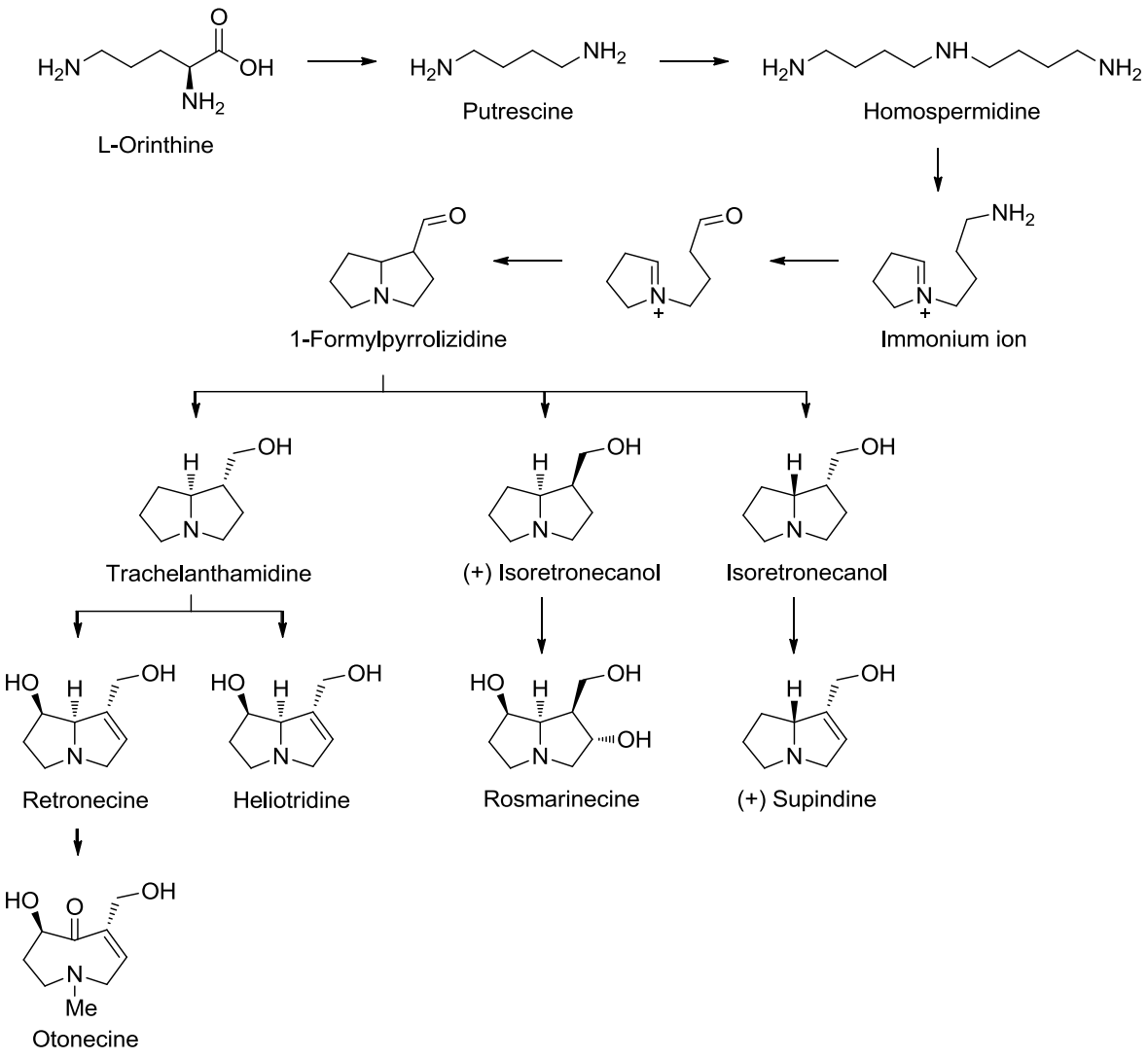
1.3.8. Biosynthesis of Pyrrolizidine Alkaloids

By using radioactive precursors it has been shown that pyrrolizidines are biosynthesized from the amino acid L-omithine via putrescine. Incorporation of two molecules of putrescine into homospermidine has been confirmed by enzymatic experiments. Homospennidine synthase catalyses the formation of homospermidine from two molecules of putrescine[51, 52].

An indication of how homospermidine is converted into necines was provided by the conversion of this triamine into trachelantharnidine using enzymes under physiological conditions [53, 54]. It is likely that oxidation of one terminal amino group of homospermidine produces an immonium ion [55], which can then undergo oxidation of the remaining primary amino group, followed by a non-enzymatic intramolecular cyclization of the aldehyde to give the more stable 1-formylpyrrolizidine. Reduction of this aldehyde then afforded trachelantharnidine. The formation of this base using enzymes suggested that the simple 1-hydroxymethylPyrrolizidine and trachelanthamidine should be tested as intermediates in the biosynthesis of more complex necines such as retronecine and heliotridine[55] as shows in scheme (1).

The biosynthetic pathways outlined in scheme (1) showed that the tracnelanthamidine is an efficient precursor for the retronecine and heliotridine; and the immonium ion is a key intermediate in the biosynthetic pathway of different necine bases including retronecine, heliotridine, rosmarinecine, isoretronecanol and supinidine from homospermidine in the plant species [56].

Otonecine, on the other hand, basic component of the otonecine alkaloids, is produced from retronecine, presumably by further hydroxylation and formation of a ketonic group with simultaneous cleavage of the C-N bond and N-methylation[57].



Scheme 1 : Biosynthesis of different necines bases

1.3.9. Analytical separation techniques

To determine pyrrolizidine alkaloid by analytical methods, the alkaloids must be present as free alkaloids. As pyrrolizidine alkaloid extracted from plants - with the exception of the otonecine alkaloids - generally occur as N-oxides and are thus only poorly soluble in organic solvents but readily soluble in water, the respective mixture should be reduced with zinc dust in the presence of dilute mineral acid[44]

In many cases, plants contain complex mixtures of alkaloids which cannot easily be separated, and the total alkaloid content of the plant is very small. Accordingly, pure components cannot be obtained in sufficient quantity for conventional analysis and other methods have to be used to analyze such mixtures[38] .

Analytical thin-layer chromatography is a very useful technique. Silica coated plates are used most frequently for pyrrolizidine alkaloid work. Alkaloids are detected on chromatograms by a variety of spray reagents. Dragendorff's reagent is commonly used. The iodoplatinate reagent is more specific, but also more expensive. The most convenient and sensitive method for visualising unsaturated pyrrolizidine alkaloids is to spray the chromatogram with a chloranil solution, heating briefly, then spraying with Ehrlich's reagent[58].

Thin-layer chromatography can thus give an indication of the number and type of pyrrolizidines present in the crude alkaloid mixture [59-65]. Tentative identification of alkaloids by comparison of Rf values is possible if standards are available, but many alkaloids have identical Rf values and components are often missed due to incomplete separation.

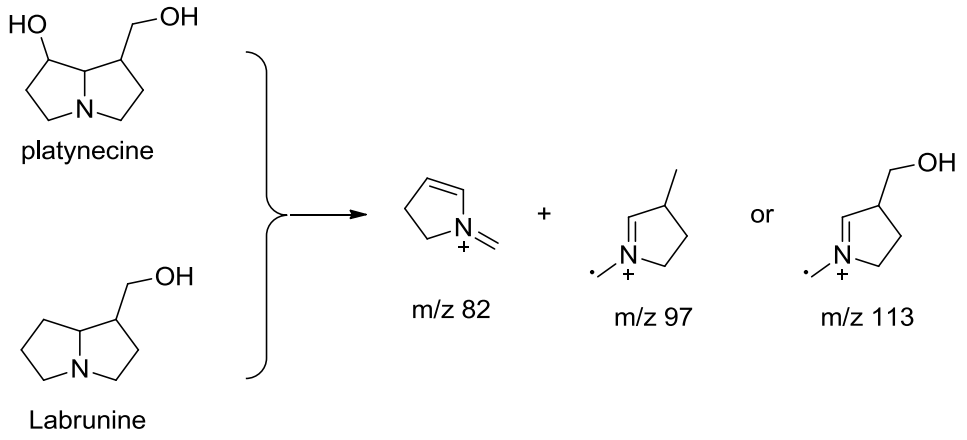
High-performance liquid chromatography (HPLC) may be used in conjunction with mass spectrometry for identification of the components of complex alkaloidal mixtures[24]

A more frequently used technique is tandem gas chromatography mass spectrometry (GC-MS). The main drawback of gas chromatography is that underivatized alkaloids often decompose due to the high temperatures used for the separation. Many workers have analyzed alkaloid samples by derivitazation .The recent development of specially inert columns for polar components has facilitated the GC study of underivatized pyrrolizidines and many workers now use GC-MS for analysis of complex alkaloid mixtures[66]. The alkaloid fraction of *Echium plantagineum* L was analyzed by gas chromatography and mass spectrometry in different ionization modes[67]. Twelve pyrrolizidine alkaloids were characterized and identified by this method. Unfortunately, mass spectrometry is unable to distinguish between isomeric alkaloids, and results obtained can therefore be ambiguous. Reference standards are necessary for comparison of retention times and mass spectra in order to identify compounds unambiguously.

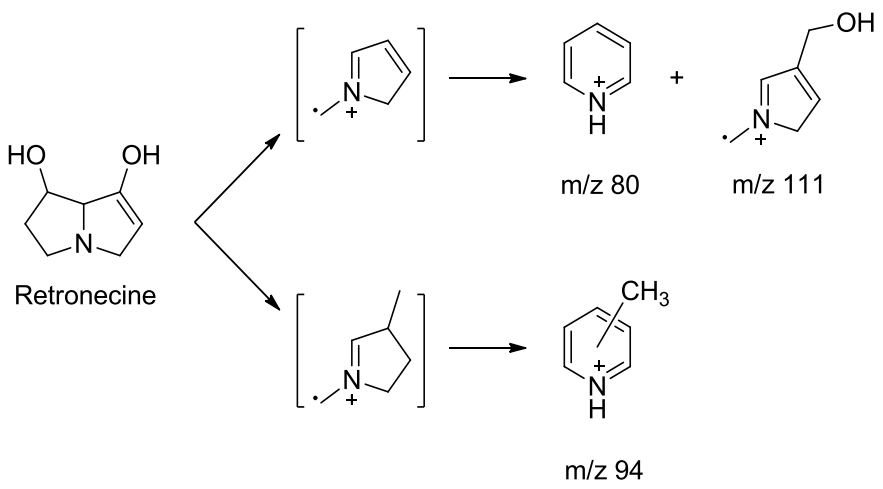
1.3.10. Mass spectrometry of Pyrrolizidine alkaloids

Pyrrolizidine alkaloids exhibit characteristic peaks in their mass spectra. It is possible to obtain a great deal of information about the structure of a Pyrrolizidine alkaloid from electron impact mass spectrometry.

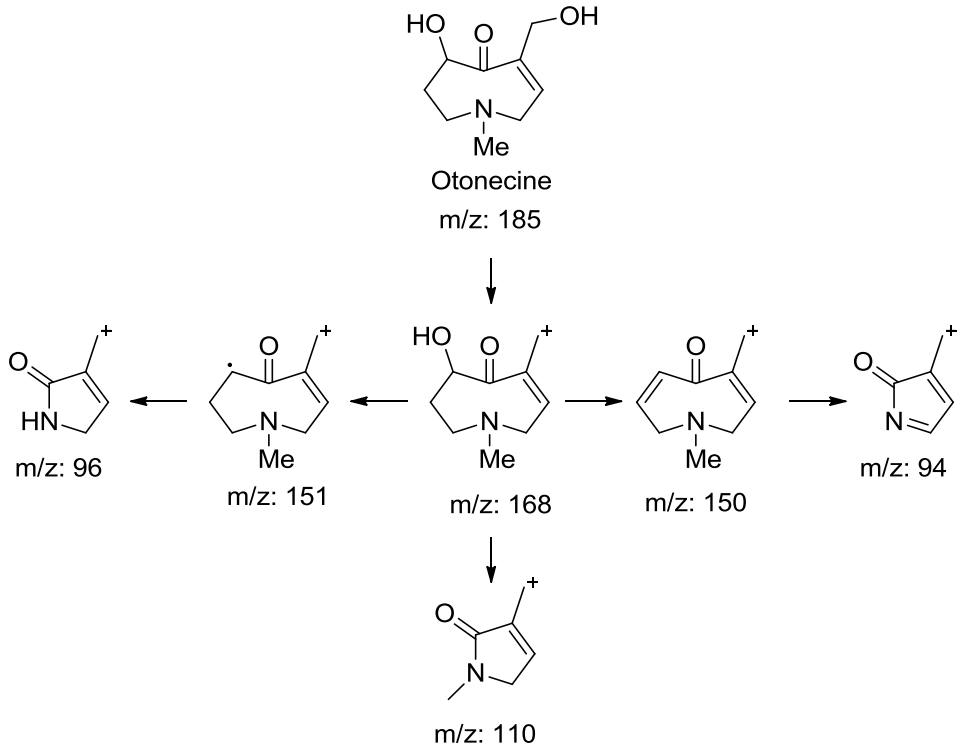
It is possible to identify the type of necine base present using diagnostic peaks. Saturated bases, such as platynecine (124) show intense ions at m/z 82 or m/z 83, together with peaks at m/z 97or m/z 113[24, 68]. The fragmentation pathway giving rise to these peaks is shown in Scheme (2). Unsaturated bases such as Retronecine (8) show intense ions at m/z 80 or 81 and 95 or 111[22, 24]. As shown in Scheme (3). Otonecine (12) type bases exhibit characteristic peaks at m/z94, 96 and 110 [22, 24] Scheme (4).



Scheme 2 : A fragmentation pathway for Saturated necine bases



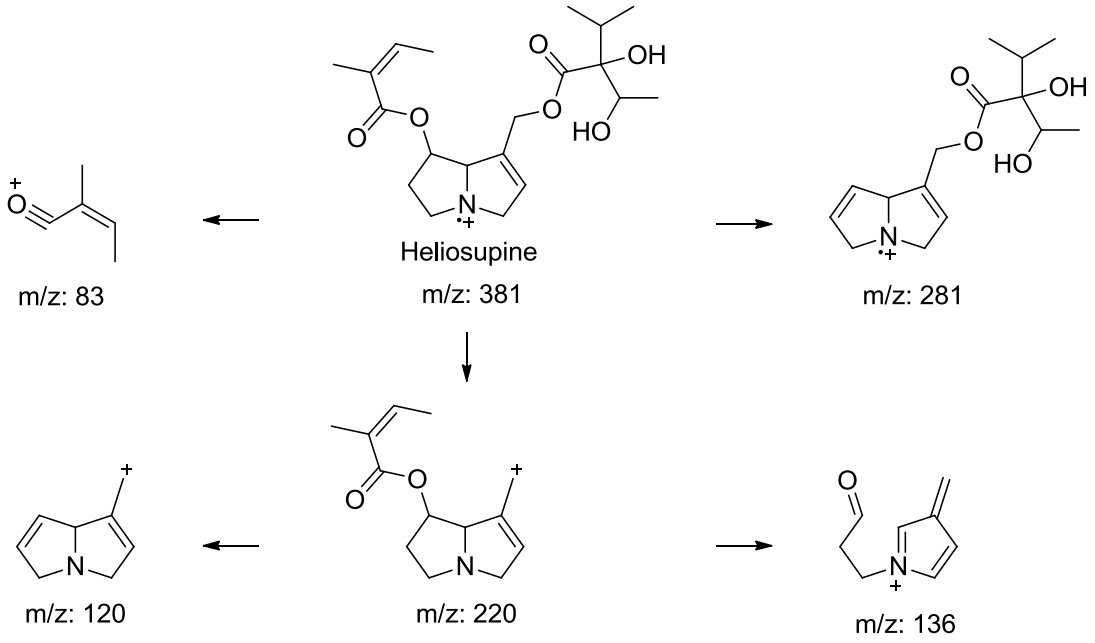
Scheme 3 : A fragmentation pathway for Retronecine



Scheme 4 : Fragmentations pathway for otonecine

The croalbinecine (125) type base is saturated and possesses a hydroxyl group at C2 see page 61. Dehydration occurs readily and the mass spectrum can thus exhibit peaks characteristic

of both a saturated and unsaturated base[69]. It may also be difficult to identify the necine base of an alkaloid when it is esterified, since peak intensities are altered and the esterifying acid can give rise to peaks which are misleading; for example, the presence of angelic acid in Heliosupine (42) gives rise to a peak at m/z 83, as shown in Scheme (5) [22, 69].In esterified necines account must be taken of a range of diagnostic peaks.

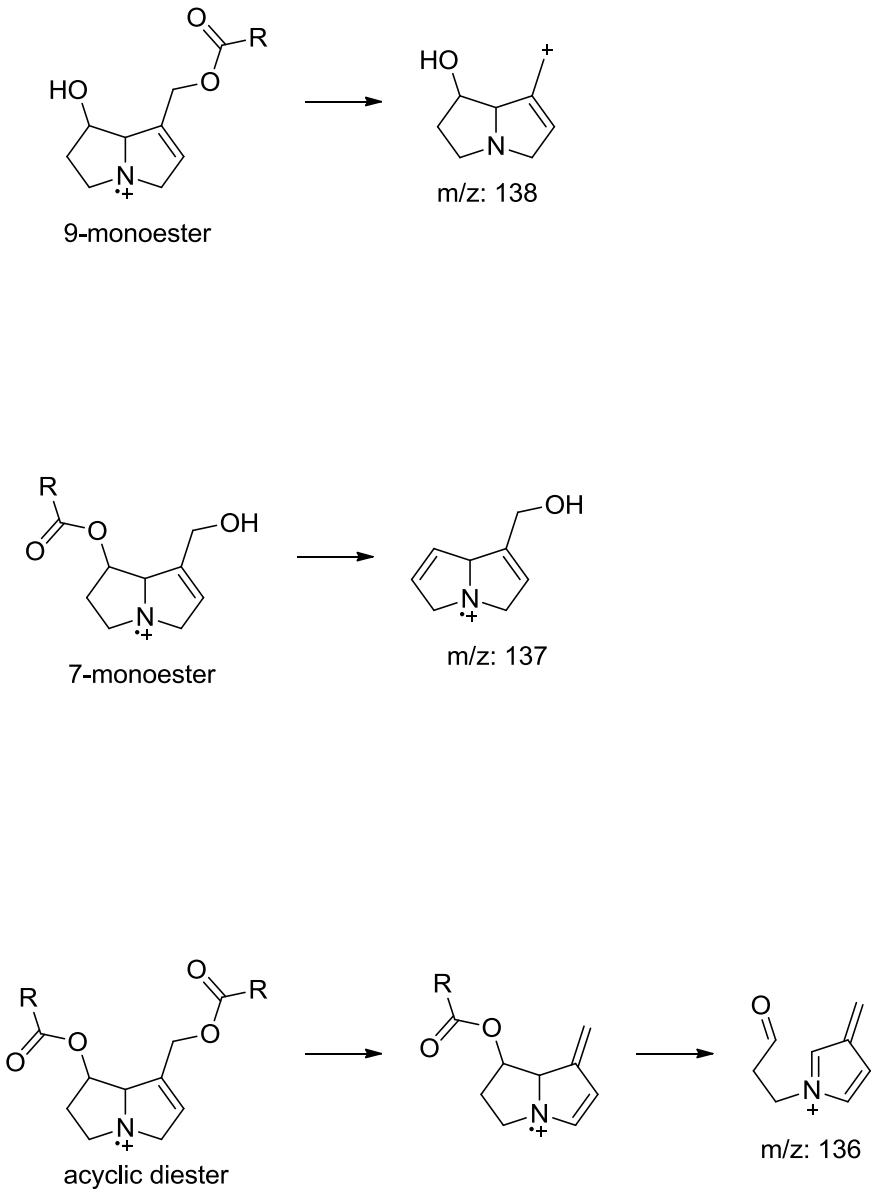


Scheme 5 : Fragmentation pathway for heliosupine

It is possible to ascertain whether the base is monoesterified or diesterified, and whether the diester is acyclic or macrocyclic. C9-monoesters of Retronecine (8) exhibit an intense peak at m/z 138, C7-monoesters at m/z 137 and acyclic diesters at m/z 136 Scheme (6) [70-72]. The corresponding monoesters of platynecine (124) exhibit peaks at m/z two mass units higher; i . e at m/z 138, 139 or 140[22].

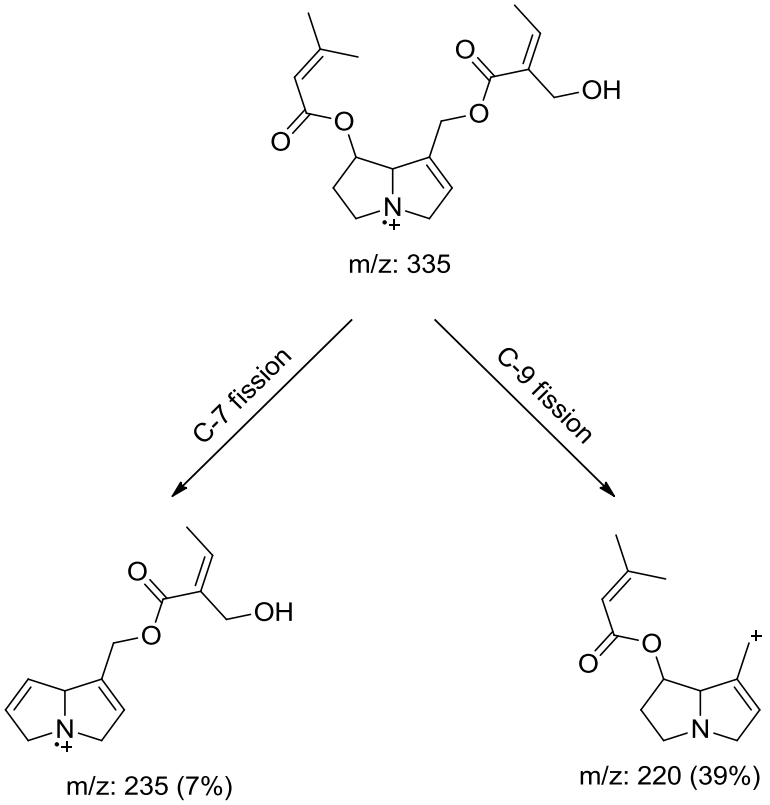
Macrocyclic diesters exhibit the characteristic "triads" of peaks; a group of peaks at m/z 80 and 81 with a second group at m/z 119, 120, 121 and a third at m/z 136, 137, 138indicate an unsaturated base alkaloid[24]."Triads" of peaks atm/z 82 and 83; m/z 121, 122, 123 and m/z

138, 139 and 140 are exhibited by saturated base alkaloids [24]. The triad at m/z 119,120 and 121 (or two mass units higher) also appears in the spectra of acyclic diesters and monoester alkaloids[22].

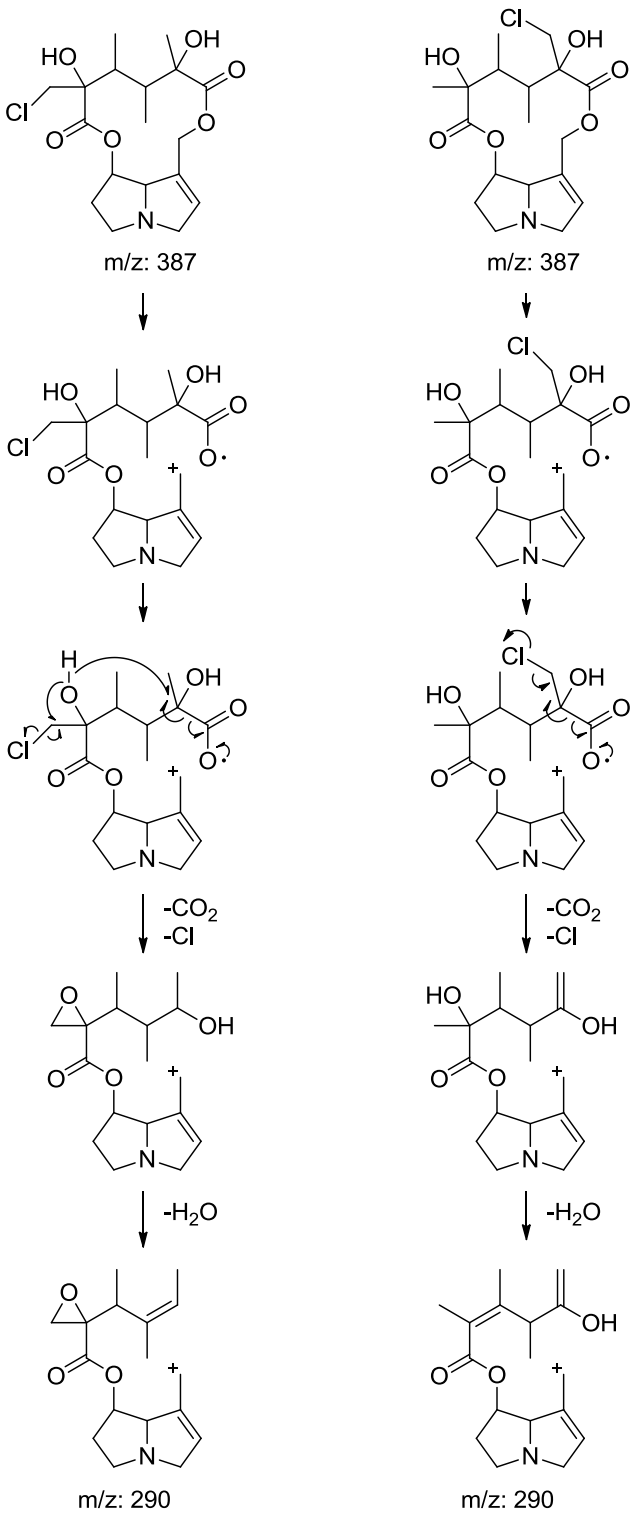


Scheme 6 : Fragmentation of monoesters and acyclic diesters

A great deal of information can be obtained from the relative intensities of peaks in the spectrum. The C9 ester bond is weaker than the C7 bond and will break more easily; hence ions corresponding to this cleavage will be more intense than ions corresponding to cleavage at the C7 linkage Scheme (7). In the case of acyclic diesters this enables one to ascertain the mode of ester attachment. In the case of macrocyclic diesters however, confusion can occur, since the ion corresponding to C9 cleavage can also be formed by cleavage at C7 with subsequent rearrangement[73]. This is shown in Scheme (8).

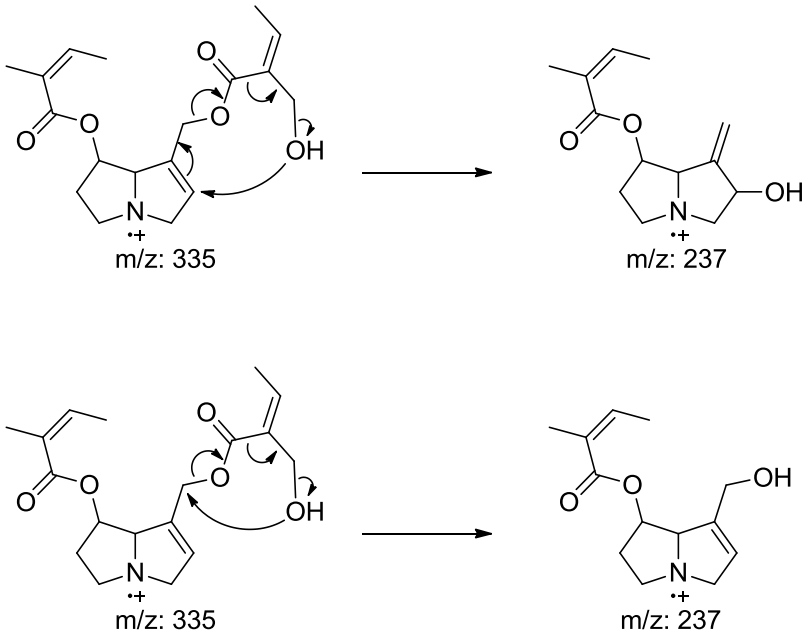


Scheme 7 : The cleavage of C-7 and C-9 linkages of Pyrrolizidine diester



Scheme 8 : Rearrangement of macrocyclic diesters

Rearrangements play a large role in the fragmentation of acyclic diester and monoester alkaloids. Scheme (9) shows two possible mechanisms for such a rearrangement[22].



Scheme 9 : Possible mechanisms for rearrangement in acyclic diesters

These fragmentation pathways apply mainly to the Retronecine (8) and platynecine (124)111 type bases. The less common Pyrrolizidine bases exhibit other characteristic peaks. A base peak at m/z70 is considered by a number of authors to be indicative of a pyrrolizidine in which ring A is unsubstituted Clarynecine (126)[74, 75], as is the combination of a base peak at m/z 83 and a prominent peak at m/z 98[76]. A base peak at m/z 82 occurring with a major peak at m/z 113 is characteristic of a 1-hydroxymethylpyrrolizidine with an OH at C6 or C7 (e.g . 114)[76]. This combination is also considered characteristic of platynecine (124) type pyrrolizidines[77] . A base peak at m/z 83 is typical of the macronecine base (127)[78]. The otonecine base (12) shows characteristic fragments at m/z 168, 150, 122, 110 and 96 [79] ,while a base peak at m/z 124 is typical of 1-methylenepyrrolizidines (128)[80] .

Other typical diagnostic peaks occur. A peak at m/z M-44(loss of CO_2) is typical of all macrocyclic diesters. A peak at m/z M-17 is indicative of an OH group at C12 since this peak

is not observed in the spectra of alkaloids lacking this group[81] (figure (5) shows the numbering). A peak at m/z M-31shows that a methyl ester is present)[78]. A base peak at m/z M-31 is indicative of the loss of an exocyclic CH_3OH group[68].

A strong peak at m/z 180 is typical of a 7-Acetylretronecinederivative [82]. Acyclic diesters of Retronecine (8) in which the ester at C7 is an acyl group show intense fragments at m/z180, 136 and 120[70] . A peak at m/z M-15 indicates the presence of a CH_3 group on the nitrogen atom[81].A peak at m/z M-18 indicates the loss of H_2O, which is considered to occur when there is an OH at C15 [81] (figure (5) shows the numbering). Other workers have found that a peak at M-18 occurred when there was a CH_2OH group present at C12[83]. Acyclic diesters of Retronecine (8) or platynecine (124) which possess a C7-angeloyloxy (or tigloyloxy) group exhibit intense peaks at m/z 220, due to cleavage of the C9 bond[84] The same acid group at C9 leads toan intense peak at m/z 237. A peak at m/z 101 is due to protonated angelic, tiglic or senecioic acid [84].A peak at m/z253 is indicative of a retronecine ester of hydroxysenecioic acid and one at m/z 235 indicates a retronecine ester of hydroxyangelic acid[50].

Although mass spectrometry can provide useful information concerning the overall structure of a pyrrolizidine alkaloid, relative stereochemistry cannot be determined, nor can geometric isomers always be distinguished. It is necessary to make use of reference standards for unambiguous structural assignment. Mass spectrometry is a powerful tool in its own right, but the information it provides can be greatly enhanced by the concurrent use of other spectroscopic techniques, where possible[22].

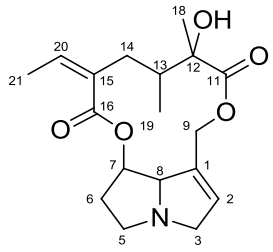


Figure 5: Numbering of macroesters pyrrolizidine alkaloids

1.4. Occurrence of pyrrolizidine alkaloids in Boraginaceae family

plant	pyrrolizidine alkaloids	reference
Alkana trinctoria	Triangularine (76) 7-Angeloylretronecine (55) Dihydroxytriangularine (105)	[45, 85, 86]
A. orientalis	7-Angeloylretronecine (55) 9-Angeloylretronecine (56) 7-Tigloylretronecine (60) 7-Senecioylretronecine (57) 9-Senecioylretronecine (59) 7-Angeloyl-9-(hydroxypropenoyl)retronecine (66) 7-Tigloyl-9-(hydroxypropenoyl) retronecine (68) 7-Angeloyl-9-(2,3-dihydroxypropanoyl) retronecine (62) 7-Tigloyl-9-(2,3-dihydroxypropanoyl) retronecine (67) Triangularine (76) Triangularine (76) Dihydroxytriangularine (105)	[47]
A. tuberculata	7-Angeloylretronecine (55) 7-Tigloylretronecine (60) 9-Tigloylretronecine (61) Trachelanthamine (92) 7-Angeloyl-9-(hydroxypropenoyl)retronecine (66) 7-Tigloyl-9-(hydroxypropenoyl) retronecine (68) 7-Acetyl-9-sarracinoylretronecine (75) 7-Angeloyl-9-(2,3-dihydroxypropanoyl) retronecine (62) 7-Tigloyl-9-(2,3-dihydroxypropanoyl) retronecine (67) Triangularine (76)	[47]

	Triangularine (76) Dihydroxytriangularine (105)	
Anchusa arvensis	9-Acetyltrachelanthamidine (87) 9-Angeloyltrachelanthamidine (88) Supinine (107) Intermedine (18) Lycopsamine (21) 7-Acetylintermedine (19) 7-Acetyllycopsamine (22) 7-diacetylintermedine (20) 7-Diacetyllycopsamine (23)	[47]
A. arvensis	Echinatine (50)	[85]
A. milleri	Retronecine(8) Heliotridine (9) 7-Angeloylretronecine (55) Supinine (107) Viridiflorine (114) Rinderine (47) 9-Curassavoylheliotridine (84) 7-Acetyl-9-curassavoylheliotridine (80)	[47]
A. officinalis	Laburnine (93) Acetyllaburnine (94) Intermedine (18) Lycopsamine (21) 7-Acetyllycopsamine (22) Clivorine (123)	[45, 85, 87-89]
Borago officinalis	Intermedine (18) Lycopsamine (21)	

	7-Acetylintermedine (19) 7-Acetyllycopsamine (22) Amabiline (40) Supinine (107) Thesinine (110) Thesinine- 4'-O-β-D-glucoside (111)	[45, 59-61, 85]
Cordia gilletii.	no Pyrrolizidine alkaloids could be detected in extracts of the root bark and leaves of the Congolese specimen of Cordia gilletii.	[90]
Cynoglossum amabile	Supinine (107) Amabiline (40) Rinderine (47) Echinatine (50) 7-Acetylechinatine (51)	[91]
C. officinale	Heliosupine (42) 12-Acetylheliosupine (44) Echinatine (50) 7-Angeloylheliotridine (79) Viridiflorine (114) 7-Tigloylheliotridine (83) 7-Angeloyl-1-fomryl-6,7-dihydro-5H-pyrrolizdine (121)7-Angeloylrinderine (48) Rinderine (47) 7-Angeloyl-9- (2-methylbutyryl) heliotridine (81) 7-Angeloyl-9- (2.3-dihydroxybutyryl) heliotridine (81) 7-Angeloylechinatine (52) Echinatine (50) Trachelanthamine (92)	[39, 45, 91, 92]

	7-Tigloylheliotridine (83) Rinderine (47) 7-Angeloylrinderine (48) 7-Angeloyl-1-fomryl-6,7-dihydro-5H-pyrrolizdine (121) 7-Angeloyl-9- (2-methylbutyryl) heliotridine (81) 7-Angeloyl-9- (2.3-dihydroxybutyryl) heliotridine (81)	
Echium amoenum	Echimidine (32) 7-Angeloylretronecine (55) 7-Tigloylretronecine (60)	[62, 85]
E. glomeratum	(7S, 8R)-Petranine (115) (7S, 8S)-petranine (116) (7R, 8R)-petranine (117) (7R, 8S)-petranine (118) 7-Angeloylretronecine (55) 9-Angeloylretronecine (56)	[45, 93]
E. Horridum	7-Angeloylretronecine (55) 7-Tigloylretronecine (60) Lycopsamine (21) 7-Angeloyl -9-(2-methylbutyryl)retronecine (63) 7-Tigloyl-9-(2-methylbutyryl) retronecine (69) 7-Acetyllycopsamine (22) Uplandicine (26) 7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine (65) 7-Tigloyl-9-(2,3-dihydroxylbutyryl)retronecine (70) 7-Angeloyllycopsamine (25) 7-Tigloyllycopsamine (24) Echimidine (32)	[94]
E. pininana	Myoscorpine (29)and its N-oxide	[95]

	Hydroxymyoscorpine (30) Echimidine (32 7-Acetylintermedine (19) Echiupinine (58) and its N-oxide	
E. rauwolfii	7-Angeloylretronecine (55) 7-Tigloylretronecine (60) Lycopsamine (21) 7-Angeloyl -9-(2-methylbutyryl)retronecine (63) 7-Tigloyl-9-(2-methylbutyryl) retronecine (69) 7-Acetyllycopsamine (22) Uplandicine (26) 7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine (65) 7-Tigloyl-9-(2,3-dihydroxylbutyryl)retronecine (70) 7-Angeloyllycopsamine (25) 7-Tigloyllycopsamine (24) Echimidine (32)	[94]
E. setosum	Echimidine (32) 7-Angeloylretronecine (55) 9-Angeloylretronecine (56) 7-Tigloylretronecine (60) 9-Tigloylretronecine (61) 7-Angeloyl -9-(2-methylbutyryl)retronecine (63) 7-Tigloyl-9-(2-methylbutyryl) retronecine (69) Uplandicine (26) 7-Angeloyl-9-(2.3-dimelhylbutyryl)retronecine (64) 7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine (65)	[96]
E. vulgare	7-Tigloylretronecine (60) 9-Tigloylretronecine (61)	[85, 96, 97]

	7-Angeloyl -9-(2-methylbutyryl)retronecine (63) 7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine (65) 7-acetylvulgarine (14)and its N-oxide 7-Tigloyl-9-(2-methylbutyryl) retronecine (69) 9-Angeloylretronecine (56) 9-Senecioylretronecine (59) Acetylechimidine (34) and its N-oxide Echimidine (32) and its N-oxide Echimiplatine (17) Echiuplatine (122) 7-Echivulgarine (15) and its N-oxide Hydroxymyoscorpine (30) Leptanthine (16) and its N-oxide Retronecine (8) Uplandicine (26) and its N-oxide Vulgarine (13) and its N-oxide	
E. wildpretti	Echimidine (32) and its N-oxide	[63]
E. angustifolium	Echimidine (32)	[98]
E. diffusum	Heliotridine (9)	[99]
E. humile	Echimidine (32) Echihumiline (35) Lycopsamine (21) 7-Acetyllycopsamine (22)	[100]
E. hypertropicum	Echimidine (32), Echihumiline (35) 7-Senecioylretronecine (57) 9-Angeloylretronecine (56)	[101]

	Lycopsamine (21)	
E. italicum	Echimidine (32)	[99]
E. lycopsis	Echimidine (32) Echiumine (102) Uplandicine (26) Lycopsamine (21) Intermedine (18) Echiuplatine (122) 9-Angeloylretronecine (56), Leptanthine (16).	[97, 102]
E. sericeum	Echimidine (32) Symlandine (27) (26) (or Symphytine (31)).	[103]
E. simplex	7-Angeloylretronecine (55) 9-Angeloylretronecine (56).	[104]
E. stenosiphon Webb subsp. stenosiphon	Echimidine (32)	[101]
E. tuberculatum	Echimidine (32)	[99]
Ehretia aspera	Ehretinine (112)	[99]
Eritrichium rupestre	7-Angeloylretronecine (55)	[99]
H. longituba	9-Angeloylretronecine (56).	[99]
Heliotropium acutifolium	Heliotrine (53)	[105]
H. amplexicaule	Indicine (99)	[99]
H. angiospermum	Retronecine (8) Supinidine (11)	[106]
H. arbainense	Europine (108) Heliotrine (53)	[99, 107]

	Lasiocarpine (45).	
H. arborescens	Indicine (99) Lasiocarpine (45)	[99]
H. arguzioides	Heliotrine (53)	[99]
H. bacciferum	Europine (108) Heliotrine (53) Heleurine (106) Supinine (107)	[99]
H. bovei	Europine (108) 7-Acetyleuropine (109) Lasiocarpine (45) Lasiocarpine (45) N-oxide	[99]
H. bracteatum	Helibractinecine (89) Retronecine (8)	[99]
H. bursiferum	7-Angeloylretronecine (55).	[99]
H. circinatum	Echinatine (50) Europine (108) Heleurine (106) Heliotrine (53) Lasiocarpine (45)	[99]
H. confertifolium	Retronecine (8) Supinidine (11)	[106]
H. crassifolium	Europine (108) and its N-oxide	[99]
H. curassavicum	7-Angeloylheliotridine (79) Retronecine (8) Supinidine (11)	[106, 108]
H. dasycarpum	Heliotrine (53)	[99]

H. digynum (H. luteum)	Europine (108) Heliotrine (53) Lasiocarpine (45)	[99]
H. disciforme	Heliotrine (53) Heliotrine (53) N-oxide Heleurine (106)	[99]
H. dissitiflorum	Heliotrine (53) Heliotrine (53) N-oxide Europine (108).	[99]
H. eichwaldii	Heliotrine (53) 7-angeloylheliotrine Lasiocarpine (45).	[99]
H. esfandiarii	Europine (108) Europine (108) N-oxide.	[109]
H. europaeum	Europine (108) Heleurine (106) Heliotrine (53) Lasiocarpine (45) Heliotrine (53) n-oxide Supinine (107)	[109, 110]
H. foliosisimum	Retronecine (8) Supinidine (11)	[106]
H. fruticosum	Retronecine (8) Supinidine (11)	[106]
H. hirsutissimum	Europine (108) Heliotrine (53) Heleurine (106) Lasiocarpine (45)	[108, 111-113]

	3'-Acetyllasiocarpine (46) Supinine (107)	
H. indicum	Echinatine (50) Helindicine (119) Heliotrine (53) Heleurine (106) Indicine (99) Lasiocarpine (45) Lycopsamine (21) Rinderine (47) Supinine (107) Retronecine (8) Supinidine (11) Trachelanthamine (92)	[108, 111, 112, 114]
H. keralense	Intermedine (18) Retronecine (8)	[115]
H. maris mortui	Europine (108) Lasiocarpine (45).	[107]
H. megalanthum	Lycopsamine (21)	[116]
H. olgae	Heliotrine (53)	[117]
H. ovalifolium	Retronecine (8)	[118]
H. procumbens	Retronecine (8) Supinidine (11)	[106]
H. queretaroanum	Retronecine (8) Supinidine (11)	[106]
H. racemosum	Retronecine (8) Supinidine (11)	[106]

H. rotundifolium	Europine (108) Heliotrine (53) Lasiocarpine (45).	[70, 119]
H. scabrum	Heliscabine (91) Retronecine (8).	[120]
H. sessei	Retronecine (8) Supinidine (11).	[106]
H. spathulatum	Amabiline (40) Retronecine (8) Supinidine (11)	[23, 108]
H. steudneri	Lycopsamine (21)	[24]
H. strigosum	Strigosine (113)	[121]
H. subulatum	Retronecine (8) Heliotrine (53) 7-Angeloylheliotridine (79)	[122]
H. supinum	Echinatine (50) Heliosupine (42) Heliotrine (53) 7-Angeloylheliotridine (79) Lasiocarpine (45) Supinine (107).	[24]
H. transalpinum	Intermedine (18) Indicine (99) Lycopsamine (21) Rinderine (47) Supinine (107).	[123]
H. transoxanum	Heliotrine (53)	[105]

H. wigginsii	Retronecine (8) Supinidine (11)	[106]
Gastrocotyle hispida	Retronecine (8) 7-Angeloylheliotridine (79) Trachelanthamine (92) Supinine (107) Trachelanthamine (92) isomer Intermedine (18) Lycopsamine 7-Acetyllycopsamine (22)	[47]
Heliotropium amplexicaule	Indicine (99)	[85, 124]
H. arborescens	Lasiocarpine (45) Indicine (99) 12-Acetylindicine (101)	[39]
H.hovei	7-Acetyleuropine (109)	[61]
H. indicum L.	Strigosine (113) Supinine (107) Heleurine (106) Heliotrine (53 Indicine (99) 3-Acetylindicine (100) Lycopsamine (21) Echinatine (50) Europine (108) Heliosupine (42) Lasiocarpine (45) Helindicine (119)	[124, 125]

H. rotundifolium	Europine (108) N-oxide	[119]
H. scabrum	Retronecine (8) Helifoline (90) Helibractinecine (89)	[120]
H. arborescens	Indicine (99) 12-Acetylindicine (101)	[45]
H. europaeum	Heliotrine (53) Europine (108) Lasiocarpine (45) Supinine (107) Heleurine (106)	[45]
H. indicum	Indicine (99) eliotrine (53) Supinine (107) Lasiocarpine (45) Echinatine (50) Europine (108) Heleurine (106) Helindicine (119) Heliosupine (42) Lycopsamine (21)	[45]
Lappula intermedia	Lasiocarpine (45)	[125]
L. spinocarpos	7-Acetyl-9-sarracinoylretronecine (75) 7-Angeloylheliotridine (79) 7-Acetylintermedine (19) 7-Acetyllycopsamine (22)	[47]

	Amabiline (40) 7-Angeloylheliotridine (79) Intermedine (18 Lycopsamine (21) Retronecine (8) Supinine (107) Trachelanthamine (92) Viridiflorine (114)	
L. myosotis Moench	Lycopsamine (21) 7-Acetyllycopsamine (22) Intermedine 7-Acetylintermedine (19)	[126]
Lithospermum canescens	Lycopsamine (21) 7-Acetyllycopsamine (22) 7-Acetylintermedine (19) Canescine (95) Canescenine (97) 13-Acetylcanescine (96) 13-acetylcanescenine (98)	[127]
L. erythrorhizon	Intermedine (18)	[128]
L. officinale	Lithosenine (103) 12-Acetyllithosenine (104)	[39, 45, 61, 85]
Maris-Mortui	Europine (108)N-oxide	[119]
Myosotis palustris	Myoscorpine (29) 7-Acetylscorpioidine (39) Symphytine (31)	[85]
M. scorpioides	Myoscorpine (29) Scorpioidine (38)	[39, 45]

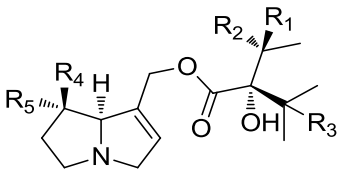
	7-Acetylscorpioidine (39) (38) Symphytine (31)	
Onosma alborosea	Intermedine (18) Lycopsamine (21) 7-Acetylintermedine (19) 7-Acetyllycopsamine (22)	[129]
O. arenaria	5,6-Dihydro-7,9-dimethoxy -7H- pyrrolizine (120) 7-Acetylretronecine (54) 9-(Butyryl-2-ene) supinidine (86) (85) 7-Acetyl-9-(2-melhylbutyryl) retronecine (72) 7-Acetyl-9-(2.3-dimelhylbutyryl)retronecine (71) 7-Acetyl-9-(2-hydroxy-3-methylbutyryl) retronecine (73) 3'-Acetylsupinine (41) 7-Acetyl-9-(2,3-dihydroxybutyryl)retronecine (74) 7-Acetyllycopsamine (22) Uplandicine (26)	[130]
O. leptantha	Echihumiline (35) and its N-oxide Leptanthine (16) 3'-Acetylechihumiline (36)	[45]
O. alboroseum sanguinolentum	9-Angeloylretronecine (56) Echimidine (32) Lycopsamine (21) Intermedine (18) 7-Acetylintermedine (19) 7-Acetyllycopsamine (22).	[131, 132]
O. arenaria	7-Acetyllycopsamine (22) 7-Acetylretronecine (54) 9-(Butyryl-2-ene) supinidine (86) (85)	[130]

	3'-Acetylsupinine (41) Uplandicine (26).	
O. arenaria subsp. pennina	Intermedine (18) Lycopsamine (21) 7-Acetylintermedine (19) 7-Acetyllycopsamine (22).	[131]
O. leptantha	Echihumiline (35) 3’-Acetylechihumiline (36) Leptanthine (16) and their N-oxides.	[133]
O. stellulatum	Echimidine (32) Leptanthine (16) Lycopsamine (21) and heir N-oxides 7-Acetylintermedine (19) Uplandicine (26)	[134]
Paracaryum intermedium	Heliotridine (9) 7-Angeloylrinderine (48) 7-Senecioylheliotridine (82) Supinine (107) Viridiflorine (114) Rinderine (47)	[47]
Paracaryum rugulosum	7-Angeloylheliotridine (79) Viridiflorine (114) Rinderine (47) Echinatine (50) 7-Angeloylrinderine (48) 7-Senecioylrinderine (49) Heliosupine (42)	[47]

Symphytum asperum	Asperumine (78) Echiumine (102) Symlandine (27) Symphytine (31) Myoscorpine (29) Echinatine (50) Echimidine (32)	[85]
Symphytum asperum	Lasiocarpine (45) Intermedine (18) Echimidine (32) Lycopsamine (21) Symlandine (27) Symviridine (28) Heliosupine (42) N-oxide Myoscorpine (29) Echinatine (50) Symphytine (31) Asperumine (78)	[39, 45]
Symphytum caucasicum	Echimidine (32) Asperumine (78) Echinatine (50) Lasiocarpine (45)	[45]
Symphytum caucasicum Bieb	Echimidine (32) Asperumine (78) Echinatine (50) Lasiocarpine (45)	[39]

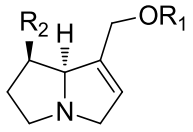
Symphytum officinale	7-Acetylintermedine (19) 7-Acetyllycopsamine (22) Echimidine (32) Echinatine (50) Heliosupine (42) Heliotrine (53) Intermedine (18) Lasiocarpine (45) Lycopsamine (21) Myoscorpine (29) Symlandine (27) Symphytine (31) Symviridine (28) Viridiflorine (114)	[39, 45, 85, 124, 135]
Symphytum sylvaticum	Echimidine (32) and its N-oxide	[136]
Symphytum tuberosum L	Symlandine (27) Echimidine (32) Anadoline(37) 7-Acetyllycopsamine (22)	[39, 45, 85]
Symphytum uplandicum	Intermedine (18) Lycopsamine (21) Symlandine (27) Symphytine (31) Uplandicine (26)	[45]
Symphytum x uplandicum Nyman	Intermedine (18) Lycopsamine (21) 7-Acetylintermedine (19)	[39, 45]

	7-Acetyllycopsamine (22) Uplandicine (26) Symlandine (27) Symviridine (28) Myoscorpine (29) Symphytine (31) Echimidine (32)	
Tournefortia acuminata	Supinine (107)	[124]
Trichodesma africanum	Retronecine (8) Viridiflorine (114) Intermedine (18) Lycopsamine (21)	[47]
Trichodesma zeylanicum	Supinine (107)	[124]



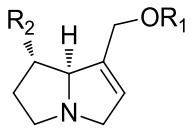
R_1	R_2	R_3	R_4	R_5		
OH	H	OAng	OH	H	Vulgarine	13
OH	H	OAng	OAc	H	7-acetylvulgarine	14
OH	H	OH	OAng	H	Echivulgarine	15
OH	H	OH	OH	H	Leptanthine	16
H	OH	OH	OH	H	Echimiplatine	17
H	OH	H	OH	H	Intermedine	18
H	OH	H	OAc	H	7-Acetylintermedine	19
H	OH	H	OAc	OAc	7-diacetylintermedine	20
OH	H	H	OH	H	Lycopsamine	21
OH	H	H	OAc	H	7-Acetyllycopsamine	22
OH	H	H	OAc	OAc	7-Diacetyllycopsamine	23
OH	H	H	OTig	H	7-Tigloyllycopsamine	24
OH	H	H	OAng	H	7-Angeloyllycopsamine	25
OH	H	OH	OAc	H	Uplandicine	26
OH	H	H	OAng	H	Symlandine	27
OH	H	H	OSen	H	Symviridine	28
H	OH	H	OTig	H	Myoscorpine	29
H	OH	OH	OTig	H	Hydroxymyoscorpine	30
OH	H	H	OTig	H	Symphytine	31
H	OH	OH	OAng	H	Echimidine	32
H	OH	OH	OTig	H	Echimidine (isomer)	33
H	OH	OAc	OAng	H	Acetylechimidine	34

H	OH	OH	OSen	H	Echihumiline	35
H	OAc	OH	OSen	H	3’-Acetylechihumiline	36
H	OTig	H	OH	H	Anadoline	37
OTig	H	H	OH	H	Scorpioidine	38
OTig	H	H	OAc	H	7-Acetylscorpioidine	39
OH	H	H	H	H	Amabiline	40
H	OAc	H	H	H	3' -Acetylsupinine	41
OH	H	OH	H	OAng	Heliosupine	42
OAc	H	OH	H	OAng	3'-Acetylheliosupine	43
OAc	H	OH	H	OAng	12-Acetylheliosupine	44
H	OMe	OH	H	OAng	Lasiocarpine	45
H	OAc	OH	H	OAng	3'-Acetyllasiocarpine	46
H	OH	H	H	OH	Rinderine	47
H	OH	H	H	OAng	7-Angeloylrinderine	48
H	OH	H	H	OSen	7-Senecioylrinderine	49
OH	H	H	H	OH	Echinatine	50
OH	H	H	H	OAc	7-Acetylechinatine	51
OH	H	H	H	OAng	7-Angeloylechinatine	52
H	OMe	H	H	OH	Heliotrine	53

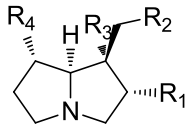


R_1	R_2		
H	OH	Retronecine	8
H	OAc	7-Acetylretronecine	54
H	OAng	7-Angeloylretronecine	55
Ang	OH	9-Angeloylretronecine	56

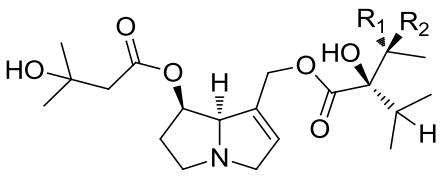
H	OSen	7-Senecioylretronecine	57
(+)-Trac	OSen	Echiupinine	58
Sen	OH	9-Senecioylretronecine	59
H	OTig	7-Tigloylretronecine	60
Tig	OH	9-Tigloylretronecine	61
2,3-dihydroxypropanoyl	OAng	7-Angeloyl-9-(2,3-dihydroxypropanoyl) retronecine	62
2-methylbutyryl	OAng	7-Angeloyl -9-(2-methylbutyryl)retronecine	63
2.3-dimelhylbutyryl	OAng	7-Angeloyl-9-(2.3-dimelhylbutyryl)retronecine	64
2,3-dihydroxybutyryl	OAng	7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine	65
hydroxypropenoyl	OAng	7-Angeloyl-9-(hydroxypropenoyl)retronecine	66
2,3-dihydroxy propanoyl	OTig	7-Tigloyl-9-(2,3-dihydroxypropanoyl) retronecine	67
hydroxypropenoyl	OTig	7-Tigloyl-9-(hydroxypropenoyl) retronecine	68
2-methylbutyryl	OTig	7-Tigloyl-9-(2-methylbutyryl) retronecine	69
2,3-dihydroxy propanoyl	OTig	7-Tigloyl-9-(2,3-dihydroxylbutyryl)retronecine	70
2.3-dimelhylbutyryl	OAc	7-Acetyl-9-(2.3-dimelhylbutyryl)retronecine	71
2-melhylbutyryl	OAc	7-Acetyl-9-(2-melhylbutyryl) retronecine	72
2-hydroxy-3-methylbutyryl	OAc	7-Acetyl-9-(2-hydroxy-3-methylbutyryl) retronecine	73
2,3-dihydroxybutyryl	OAc	7-Acetyl-9-(2,3-dihydroxybutyryl)retronecine	74
sarr	OAc	7-Acetyl-9-sarracinoylretronecine	75
t Sarr	OAng	Triangularine	76
t Sarr	OTig	Triangularicine	77
Tig	OTig	Asperumine	78



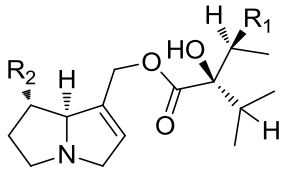
R_1	R_2		
H	OH	Heliotridine	9
H	OAng	7-Angeloylheliotridine	79
Cura	OH	7-Acetyl-9-curassavoylheliotridine	80
2.3-dihydroxybutyryl	OAng	7-Angeloyl-9- (2.3-dihydroxybutyryl) heliotridine	81
2-methylbutyryl	OAng	7-Angeloyl-9- (2-methylbutyryl) heliotridine	82
H	OSen	7-Senecioylheliotridine	83
H	OTig	7-Tigloylheliotridine	84
Cura	OH	9-Curassavoylheliotridine	85
Butyryl-2-ene	H	9-(Butyryl-2-ene) supinidine	86
Ac	H	9-Acetyltrachelanthamidine	87
Ang	H	9-Angeloyltrachelanthamidine	88



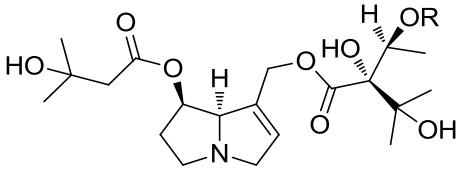
R_1	R_2	R_3	R_4		
H	OH	OH	OH	Helibractinecine	89
OH	OH	H	OH	Helifoline	90
H	OH	OH	OH	Heliscabine	91
H	O(+)Trach	H	H	Trachelanthamine	92
H	H	CH_2OH	H	Laburnine	93
H	H	CH_2OMe	H	Acetyllaburnine	94



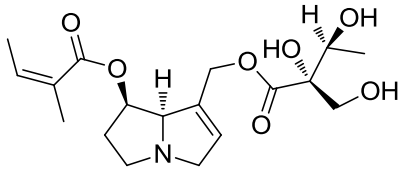
R_1	R_2		
H	OH	Canescine	95
H	OAc	13-Acetylcanescine	96
OH	H	Canescenine	97
OAc	H	13-Acetylcanescenine	98



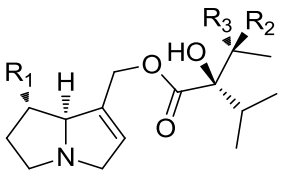
R_1	R_2		
OH	OH	Indicine	99
OH	OAc	3-Acetylindicine	100
OAc	OH	12-Acetylindicine	101
OH	OAng	Echiumine	102

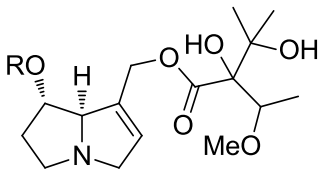


R		
H	Lithosenine	103
OAc	12-Acetyllithosenine	104

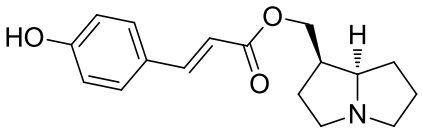


Dihydroxytriangularine 105

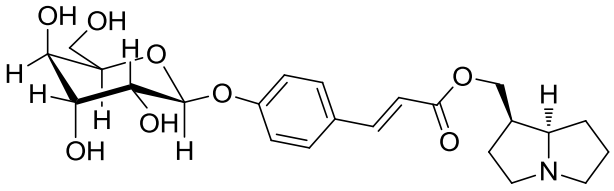




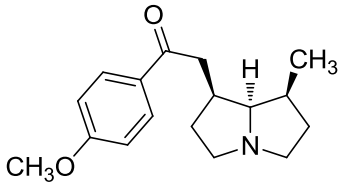
R_1	R_2	R_3			R		
H	H	OMe	Heleurine	106	H	Europine	108
H	H	OH	Supinine	107	Ac	7-Acetyleuropine	109



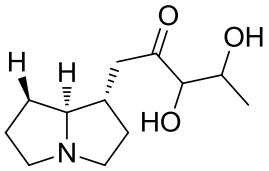
Thesinine 110



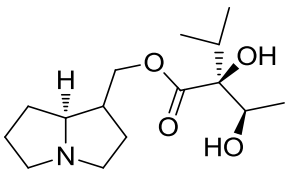
Thesinine- 4’-O-β-D-glucoside 111



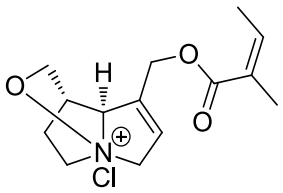
Ehretinine 112



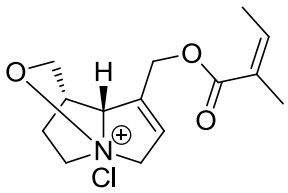
Strigosine 113



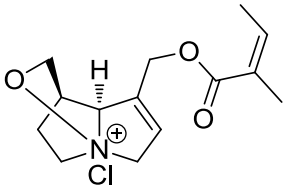
Viridiflorine 114



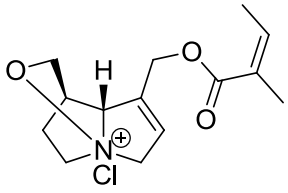
(7S, 8R)-Petranine 115



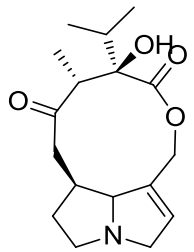
(7S, 8S)-Petranine 116



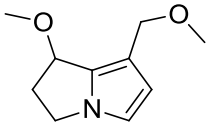
(7R, 8R)-Petranine 117



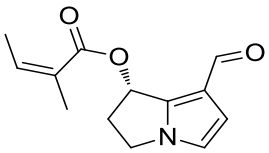
(7R, 8S)-Petranine 118



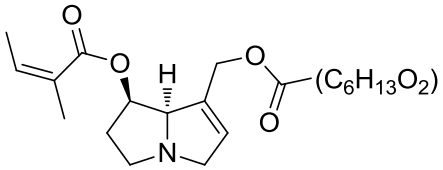
Helindicine 119



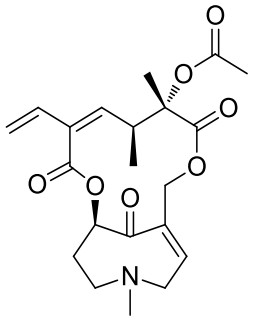
5,6-Dihydro-7,9-dimethoxy -7H- pyrrolizine 120



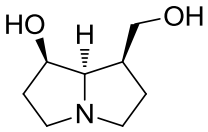
7-Angeloyl-1-fomryl-6,7-dihydro-5H-pyrrolizdine 121



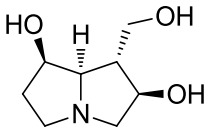
Echiuplatine 122



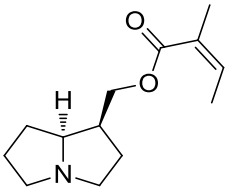
Clivorine 123



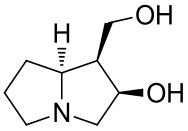
platynecine 124



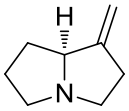
croalbinecine 125



Clarynecine 126



macronecine 127



1-methylenepyrrolizidines 128

1.5. Aim of the study

The present study aimed to isolate and identify the pyrrolizidine alkaloid content of *Echium plantagineum* L which grown in Libya (El-marj) by the Retention index and Gas chromatography Mass Spectroscopy application . while the extraction and isolation is to be performed by acid base extraction technique of reduced N-oxide pyrrolizidine alkaloids and free alkaloids.

Proposal skeletons of isolated compound would be studied by comparison their mass fragmentations and retention indices with those of previously worked pyrrolizidine alkaloids as standard values.

CHAPTER TWO

2. EXPERIMENTAL

2.1. The description of *Echium plantagineum* L.

Echium plantagineum L shown in figure (6) is an erect annual or biennial herb, 20-60 cm tall. With 1-many flowering stems. Covered with soft tubercle-based spreading hairs. Basal leaves usually in a rosette, ± ovate. 40-120 × 12-15 mm, with distinct, lateral nerves, covered with more or less soft appressed setae; cauline leaves smaller, more or less cordate at the base, obLong to lanceolate. Cyme terminal or axillary. Calyx 6-10 mm at anthesis. up to 15 mm in fruit, lobes linearlanceolate. Corolla blue, infundibuliform. 18-35 mm long; glabrous except on the veins and the margins; limb rounded, 11-14 mm in diameter. Only 2 stamens exserted; anthers violet, oblong. c. 1 mm long; style bifid, very sparcely pilose. Style bifid for nearly 1 mm. Nutlets 4, c. 3×3 mm, ovoid, bipyramidal with dorsal and ventral keel, tuberculatc and faintly striate[137].

2.2. Plant Collection

Flowers, leaves, stems of *Echium plantagineum* L plant materials were collected from El-marj, Gabel Akhder at the east Northern part of Libya during April 2012. Plant raw material spread in three square meter shaded area for air-drying at room temperature for about two weeks. After drying, exact weights were recorded was 378 gram. Whole plant grounded to powder using a laboratory hammer mill. Powdered materials were maintained at room temperature (25 ^{o}C), protected from light by storing the powder in dark glass container to avoid the effect of light on pyrrlolizidine alkaloids until required for extraction and analysis.

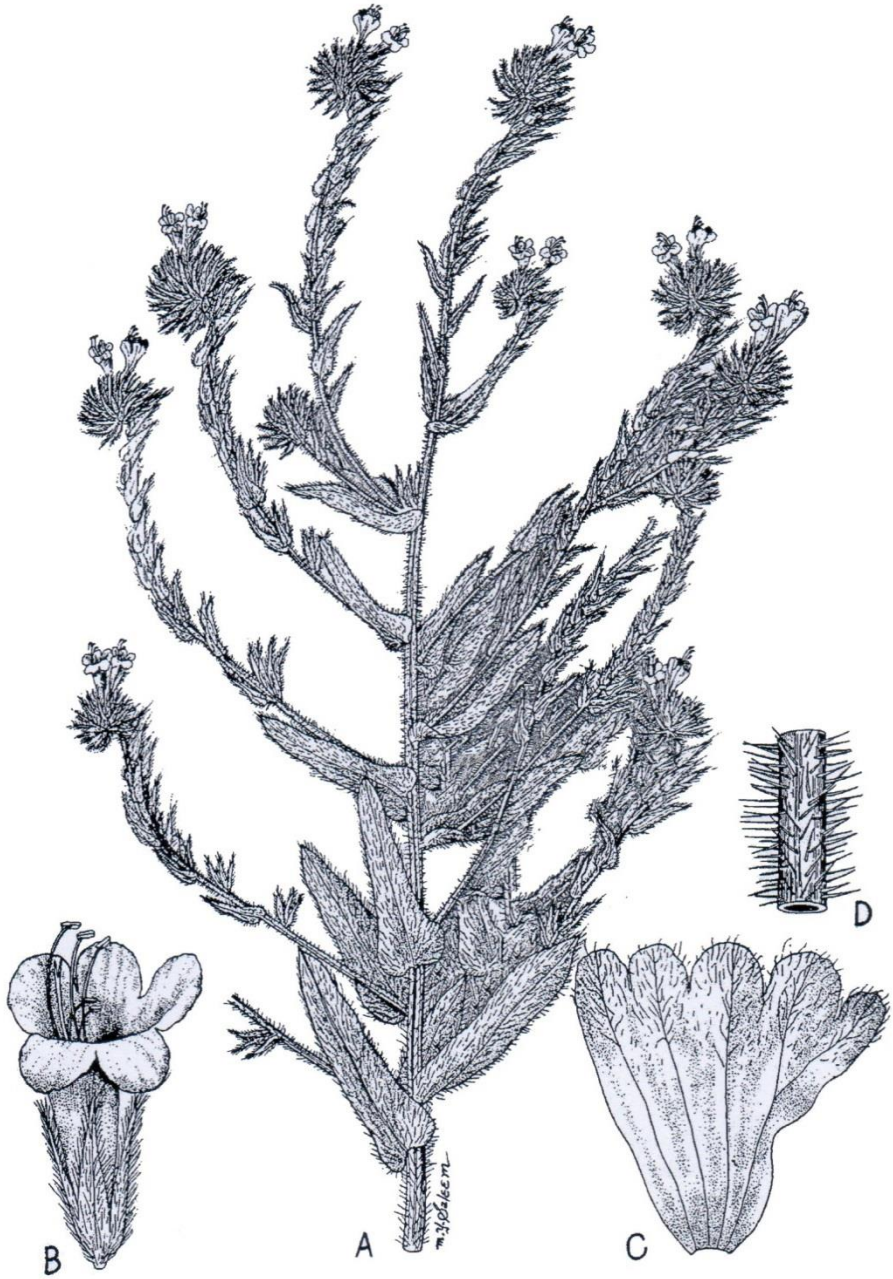


Figure 6: A. apportion of flowering stem×0.5, B.flower×2, C. dissected corolla×2apportion of stem×0.5

2.3. Solvent Extraction of *Echium plantagineum* L

Flowers, leaves, stems of dried plant materials (378 gram) were bowered down and macerate for 24 hours with 2L methanol with intermittent shaking.

The maceration process was then repeated several times for exhaustive extraction. The methanolic extract is then collected and concentrated almost to dryness by using rotary evaporator under vacuum at 40 ºC[14].

2.4. Screening for alkaloids

The alkaloids nucleus could be detected by Dragendorff's reagent. The methanolic extract was dropped into a porcelain basin, dried by blower and added with a few drops of Dragendorff's reagent. A change occurred with red-brown precipitations within several minutes indicated the presence of alkaloid nucleus[138].

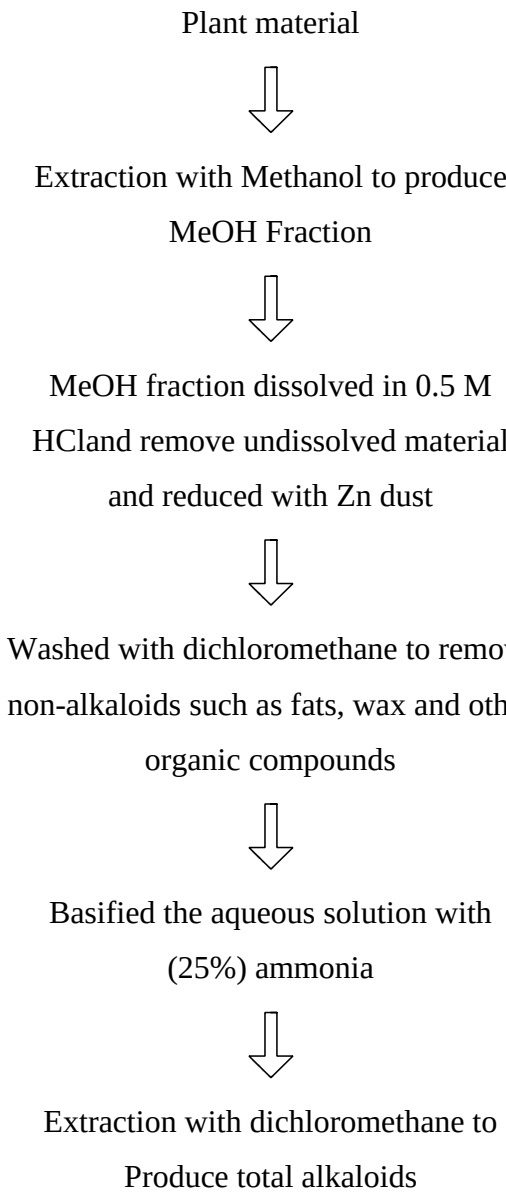
2.5. Alkaloid extraction

All alkaloids contain at least one nitrogen atom, in the majority of cases, those compounds are basic. This means that salt formation can occur in the presence of acid. This fundamental property of alkaloids is used in their extraction and further clean-up as shown in schem (10)[14].

The methanolic extract which gave negative test with Dragendorff's reagent was brought to 2N HCl and reduced with Zn dust with stirring overnight to convert Pyrrolizidine alkaloids N-oxides to the corresponding free bases[93, 139-141].

Excess Zn was removed by filtration. The aqueous acidic solution was washed with CH_2Cl_2, and made alkaline with NH_4OH Using pH meter to make sure that pH in base range, After basification process, the solution was extracted with ethylene chloride, then dried using anhydrous sodium sulfate (Na_2SO_4 50 to 100 g/L organic layer) with stirring for three minutes using glass rod and allowed to stand for 5 minutes then filtered. Organic layer was then combined and concentrated under vacuum at 30 ºC using rotary evaporator to obtain the

total alkaloids (tertiary Pyrrolizidine alkaloids and Pyrrolizidine alkaloids N-oxides) in form of tertiary Pyrrolizidine alkaloids[93, 139-142]. Alkaloid extracts were dissolved in methanol and analyzed by GC-MS.



Scheme 10 : Extraction scheme of Pyrrolizidine alkaloids from Echium plantagineum

2.6. GC-MS analysis

Aglient 6890 chromatograph equipped with aglient mass spectrometric detector, with a direct capillary interface and fused silica capillary column HP-5MS (30 m × 0.32 mm × 0.25 μm film thickness).

Samples were injected under the following conditions:
Helium was used as carrier gas at approximately 1.0 ml/min, pulsed splitless mode. The solvent delay was 3 min. and injection size of sample was 0.001 ml. The mass spectrometric detector was operated in electron impact ionization mode with energy of 70 ev. Scanning from m/z 50 to m/z 500. The ion source temperature was 230 °C. and the quadruple temperate was 150 °C. the electron multiplier voltage (EM voltage) was maintained 1050 v above auto tune. The GC temperature program was started at 70°C then elevated to 300°C at rate 6 °C/min.

2.6.1. Kovats Retention indices (RI)

Kovats retention index RI is a linear scale based upon thermodynamic properties and observation of chromatographic data trends that show a distinct advantage in predicting retention time. The system uses n-alkanes as an index against which retention times of other compounds can be measured. The retention index is information unique to a compound[143].

The temperture programed Kovats retention index RI is defined for given substance (A) as follow[144, 145]

$$RI_A = 100\left(\frac{t_{rA-}t_{rn}}{t_{rN-}t_{rn}} + n\right)$$

trA is retention time of unknown substance (A).

trn is retention time of n-alkane elute before (A).

trN is retention time of n-alkane elute after (A).

n and N refer to the carbon numbers in n-alkane.

Unlike retention time, retention index of compounds has the following advantages[146, 147].

(i) Its dependence on temperature is small and linear.

(ii) It is independence of the column constants and of type of chromatographic apparatus.

(iii)It provide chemical information about the chemical nature of the substances under examination.

2.7. Reagents

1. Petroleum ether, Analytical grade, range of boiling 45-60 °C, Scharlau Chemie S.A. Barcelona, Spain.
2. Dichloromethane, Synthesis grade, range of boiling 39-40 °C. 99.5%. mgchemicals, Belgium. . CAS No. 75-09-2.
3. Ammonia solution. About 25% NH_3. Chemically pure. Frutarom (UK) LTD. CAS No. 1336-21-6.
4. Ethanol, Synthesis grade, 99.9%. Scharlau Chemie S.A. Barcelona, Spain.
5. Methanol, analytical grade, boiling range 64-65.5 °C. 99.5%. BDH England.
6. Acetone, Analytical grade, Laboratory Chemicals, England.
7. Chloroform, pure. Lonover Laboratory Chemicals, England.
8. Anhydrous sodium sulphate, Laboratory reagent. India

2.8. Instrumentation

1. Rotary Evaporator, RE 200, BIBBY, made in U.K. by BIBBY STERILINE LTD.
2. UV lamp, Herolab, made in Germany by GmbH laborgeate K W-254 nm.
3. JENWAY pH meter 3310.

CHAPTER THREE

3. RESULTS AND DISCUSSION

3.1. Structural Elucidation of Isolated Compounds

Gas chromatography combined with mass spectrometry has been increasingly used over last two decades for the convenient analysis of alkaloidal extracts containing pyrrolizidine alkaloids [147-150]. The combination of retention indices (RI), molecular mass [M^+], and mass fragmentation pattern provide sufficient information for unambiguous identification of most pyrrolizidine alkaloids[138, 147].

3.2. GC-MS analysis of the alkaloidal extract of *Echium plantagineum* L

Gas chromatography combined with mass spectrometry has been increasingly used over last two decades for the convenient analysis of alkaloidal extracts containing pyrrolizidine alkaloids [147-150]. This method of choice for the analysis of crude alkaloidal fraction which contain about 10 individual components and enables the identification of even trace or stereoisomers. The combination of retention indices (RI), molecular mass [M^+], and mass fragmentation pattern provide sufficient information for unambiguous identification of most pyrrolizidine alkaloids.

GC analysis of the alkaloidal extract of *Echium plantagineum* L revealed the presence of 12 alkaloids of pyrrolizidine type as illustrated in table (2). Figure (7) shows the total profile obtained by GC-MS of the alkaloidal extract of *Echium plantagineum* L.

Kovats retention indices of isolated pyrrolizidine alkaloids were calculated using co-chromatographed standard n-alkanes (C17- C33). The retention index of data were calculated with the respect to the separately injected standard hydrocarbon illustrated in table (3) and figure (8).

Peak No	Alkaloid	Retention time (min)	Retention index
1	Ech-1	4.53	1787
2	Ech-2	4.7	1797
3	Ech-3	5.46	1843
4	Ech-4	10.75	2133
5	Ech-5	10.94	2145
6	Ech-6	13.01	2255
7	Ech-7	14.21	2315
8	Ech-8	14.38	2325
9	Ech-9	14.51	2533
10	Ech-10	14.58	2337
11	Ech-11	18.72	2560
12	Ech-12	30.24	3178

Table 2: GC data of the alkaloidal extract of Echium plantagineum L

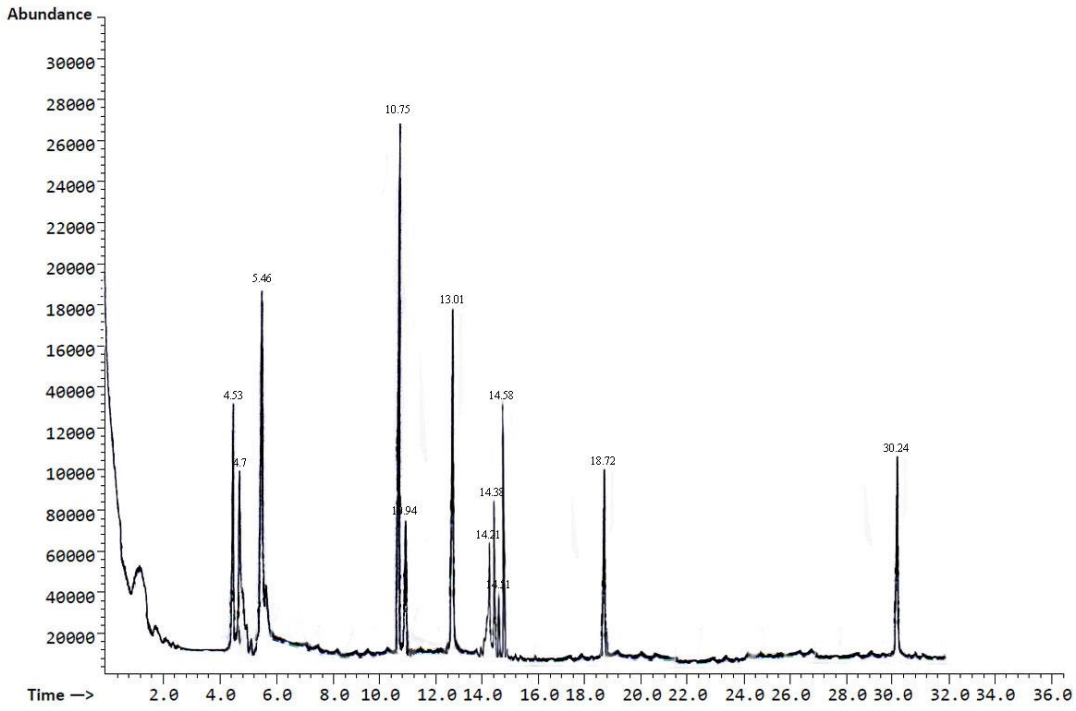


Figure 7: The GC alkaloidal profile of Echium plantagineum L

Peak No	n-Alkane	Retention time (min)
1	C-17	3.05
2	C-18	4.75
3	C-19	6.41
4	C-20	8.15
5	C-21	10.2
6	C-22	11.85
7	C-23	13.95
8	C-24	15.65
9	C-25	17.7
10	C-26	19.4
11	C-27	21.1
12	C-28	23.2
13	C-29	24.8
14	C-30	26.9
15	C-31	28.6
16	C-32	30.7
17	C-33	32.4

Table 3: GC data of the injected standard hydrocarbon

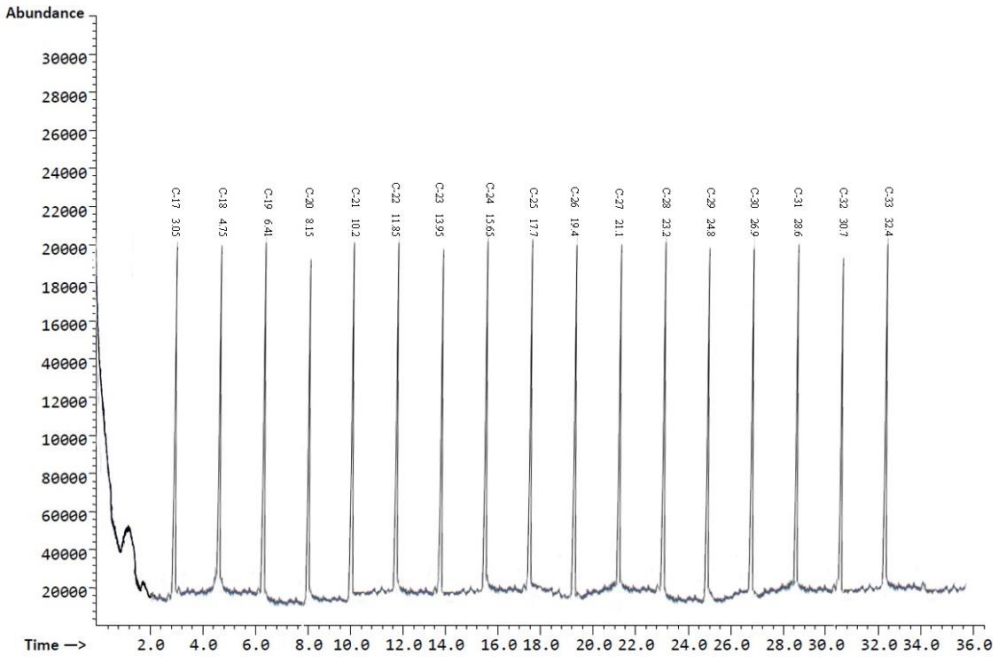


Figure 8: The GC profile of separately injected standard hydrocarbon

The table (4) shows the pyrrolizidine alkaloids that have been identified using GC-MS technique; these compounds has been used as a standards for elucidate and identify the separated compounds in these research.

Alkaloid	**RI**	**Formula**	**M^+**	**Characteristic ions(%)**	**ref**
7-Angeloylretronecine	1787	$C_{13}H_{19}NO_3$	237	237(2), 219 (3), 204(0.5), 191(1), 154(2), 138(5), 137(23), 136(18), 124(23), 111(38), 106(40), 94(20), 93(6), 83(11), 80(100), 55(22).	[151]
9-Angeloylretronecine	1797	$C_{13}H_{19}NO_3$	237	237(1), 219(0.5), 193(3), 154(16), 138(32), 137(25), 136(10), 126(7), 120(2), 108(2), 94(25), 93(100), 83(8), 80(10), 55(13).	[151]
9-Tigloylretronecine	1843	$C_{13}H_{19}NO_3$	237	193(5), 154(15), 138(20), 137(26), 136(13), 126(7), 119(5), 109(4), 94(23), 93(100), 83(10), 80(12), 55(18).	[151]
Intermedine	2133	$C_{15}H_{25}NO_5$	299	299(0.4), 156(9), 139(35), 138(100), 137(13), 136(13), 120(10), 95(15), 94(50), 93(80), 80(14), 67(9), 45(7), 43(18).	[47]
Lycopsamine	2145	$C_{15}H_{25}NO_5$	299	299(0.5), 254(1), 156(8), 139(31), 138 (100), 137(12), 136(12), 120(10), 108(4), 95(15), 94 (55), 93(84), 80(14), 67(10), 45(8), 43(20).	[100]

Acetylintermedine	2255	$C_{17}H_{27}NO_6$	341	341(5), 298 (4), 255 (16), 139 (20), 138(100), 137 (12),136 (12), 94 (30), 93 (71), 80 (10), 43 (21).	[47]
7-Angeloyl-9-(2,3-dihydroxybutyryl) retronecine	2315	$C_{17}H_{25}NO_6$	339	339(1), 239(5), 238(5), 237(5), 221(25), 220(99), 219(15), 141(20), 138(10), 137(11), 136 (100), 121(15), 120(83), 119(34), 106(10), 94(55), 93(95), 83(41), 80(20), 75(2), 57(10), 55(40), 45(10).	[151]
7-Tigloyl-9-(2,3-dihydroxybutyryl) retronecine	2325	$C_{17}H_{25}NO_6$	339	239(5), 238(5), 237(8), 221(22), 220(90), 219 (20), 141(20), 138(10), 137(11), 136(100), 121(15), 120(80), 119(35), 106(10), 94(58), 93(90), 83(46), 80(20), 75(2), 57(9), 55(40), 45(10) .	[151]
7-Angeloyl-9-(2,3-dihydroxybutyryl) heliotridine	2333	$C_{17}H_{25}NO_6$	339	339(1), 324(1), 294(1), 239(6), 222(25), 221(25), 220(65), 219(8), 138 (20), 137(10), 136(81), 121(24), 120(100), 119(85), 106(15), 94(50), 93(85), 83(24), 80(18), 75(2), 57(10), 55(25), 45(10).	[152]

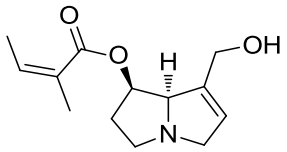
Uplandicine	2337	$C_{17}H_{27}NO_7$	357	357(4), 342(5), 297(23), 281(4), 256(4), 207 (7), 206(52), 181(80), 180 (100), 179 (43), 136(75), 121(49), 120(85), 119 (50), 101(23), 94(55), 93(74), 80(28), 73(74), 59(23), 45(20), 44(69), 43(48).	[153]
Echimidine	2560	$C_{20}H_{31}NO_7$	397	397(0.1), 382 (0.1), 352 (0.1), 297(2), 221(21), 220(100), 219(5), 138(5), 137(6), 136(48), 121(26), 120(75), 119(30), 106(5), 94(30), 93 (61), 83(39), 80(10), 59(10), 55(25), 43(18).	[151]
Echiumine	3178	$C_{20}H_{31}NO_7$	381	381(0.4), 338(1), 337(1), 336(1), 281(2), 255(1), 238(9), 237(2), 221(35), 220(100), 141(16), 138(8), 136(47), 121(32), 120(69), 119(20), 106(7), 94(39), 93(50), 83(28), 80(17), 59(1), 55(37), 53(9).	[154]

Table 4: GC-MS data of the standard Pyrrolizidine alkaloids previously separated

3.3. Characterization of Ech-1 pyrrolizidine alkaloid

The mass spectrum of Ech-1 pyrrolizidine alkaloid figure (9) showed molecular ion peak at m/z 237 for $C_{13}H_{19}NO_3$. The mass spectra data table (5) showed significant ions at m/z 136, m/z 120, m/z 119, m/z 93, and 80 m/z. these fragments are characteristic to unsaturated necine base [22, 24, 138, 155], the intense ion at m/z 137 (M^+-angelic acid) and m/z 106 are characteristic for C-7 monoester; which corresponding to the loss of ester and primary alcohol group respectively [138, 155] as shown in scheme (11) , the ion at m/z 219 is due to the loss of water molecule (M^+-H_2O)

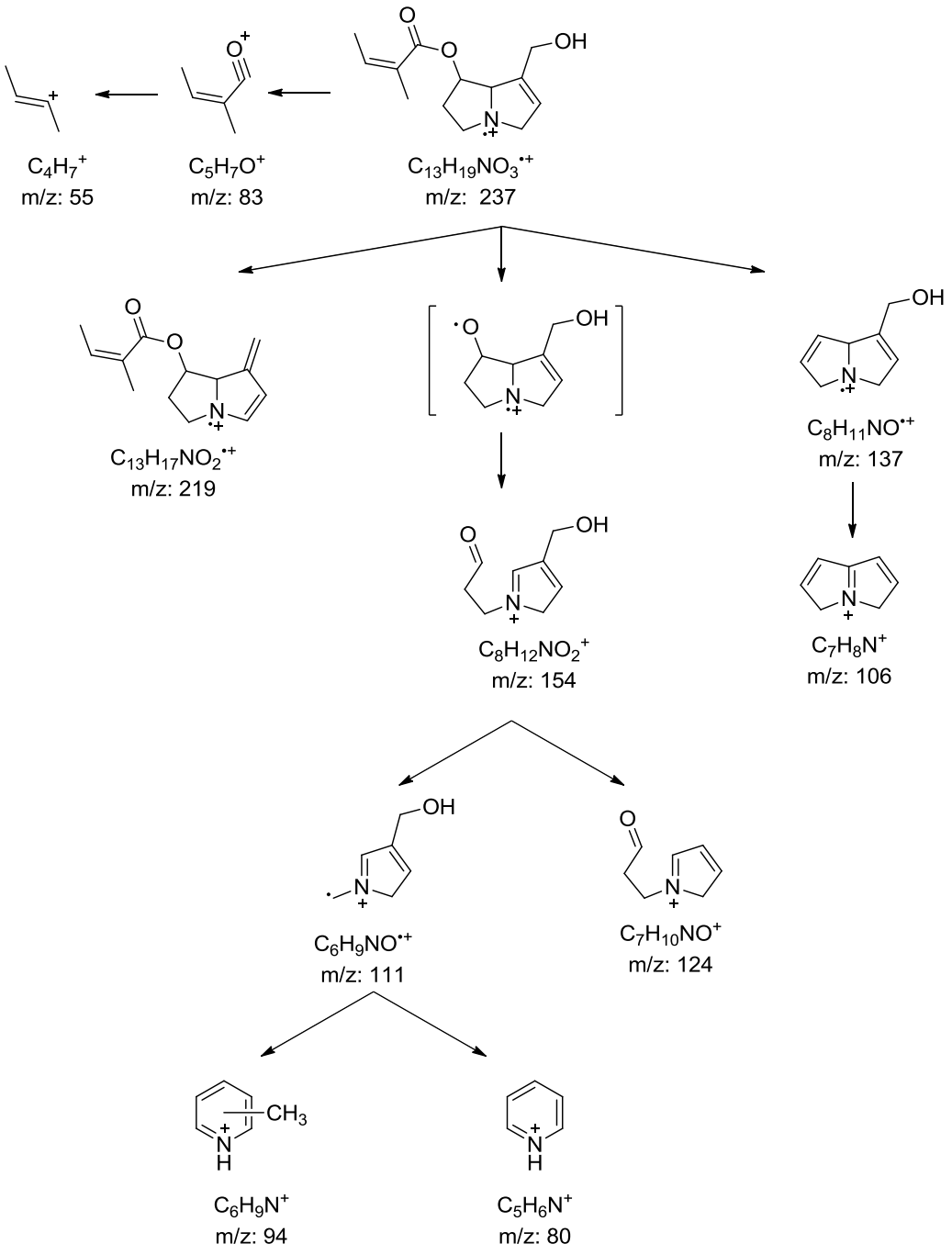
According to data given in literature [99, 151]The alkaloid Ech-1 could be unambiguously identified by its specific retention index and its mass fragmentation pattern as 7-angeloylretronecine



7-angeloylretronecine

m/z	Relative abundance (%)	Expected formula
237	2	$C_{13}H_{19}NO_3$
219	3	$C_{13}H_{17}NO_2$
154	2	$C_8H_{12}NO_2$
137	23	$C_8H_{11}NO$
124	23	$C_7H_{10}NO$
111	38	C_6H_9NO
106	40	C_7H_8N
94	20	C_6H_9N
83	11	C_5H_7O
80	100	C_5H_6N
55	22	C_4H_7

Table 5: the mass spectral data of Ech-1



Scheme 11 : A fragmentation pathway for Ech-1 alkaloid

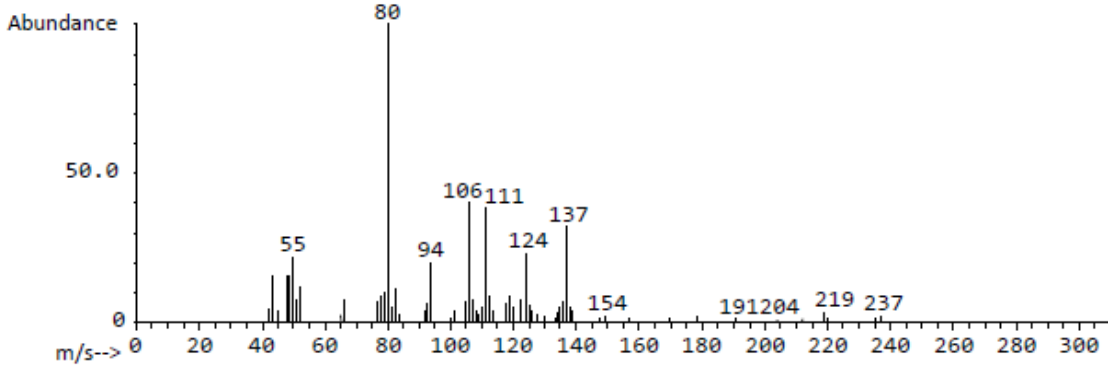


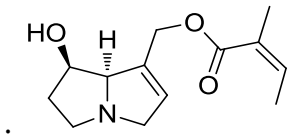
Figure 9: Mass spectrum of Ech-1 alkaloid

3.4. Characterization of Ech-2 and Ech-3 pyrrolizidine alkaloids

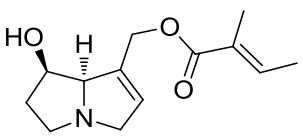
The Ech-2 and Ech-3 alkaloids both exhibit approximately the same fragmentation pattern in mass spectrum figure (10 and 11) only small different occurred in relative intensities of some fragment ions tables (6).

Apparently both Ech-2 and Ech-3 alkaloids have a molecular mass of 237 and observe the characteristic peaks for monoesters of an unsaturated base, viz at m/z 80, 93, 136, 136 and 138[22, 24, 138, 151, 156]. An intense peak at m/z 138 indicates that the compounds are 9-monoesters[22, 70-72] scheme (12). By comparison of retention indices and mass spectra to data given in literature[151], both alkaloids were tentatively identified as 9-angelylretronecine for Ech-2, and 9-tigelylretronecine for Ech-3 alkaloids.

The tigloyl esters are delayed during GC over the angeloyl esters. This is due to trans configuration of the carbonyl group and methyl group on tigloylesters versus the cisconfigurationon the angeloyl esters[147, 157].



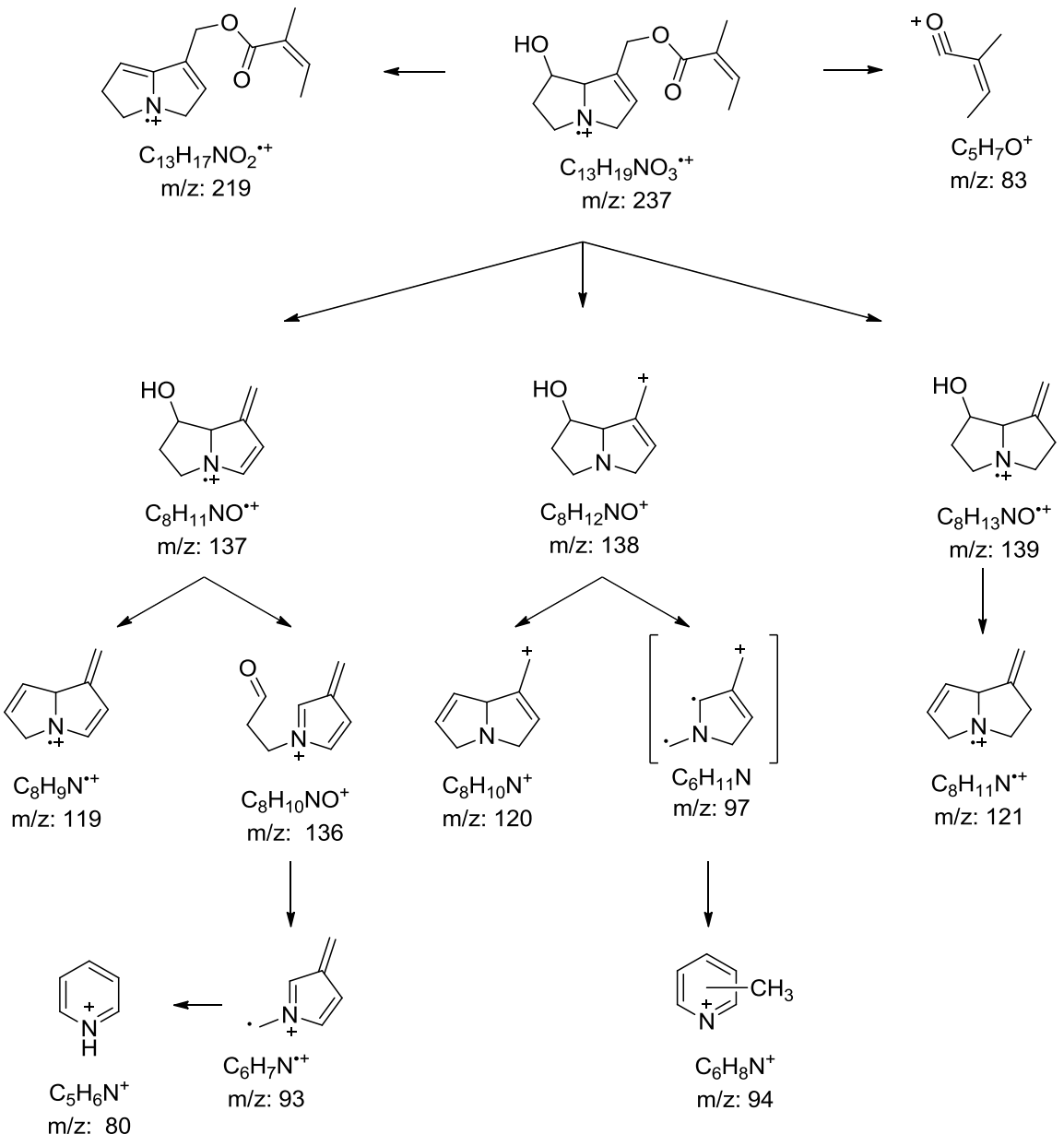
9-Angeloylretronecine



9-Tigeloylretronecine

m/z	Relative abundance (%)		Expected formula
	Ech-2	Ech-3	
237	1	1	$C_{13}H_{19}NO_3$
219	0.5	0.4	$C_{13}H_{17}NO_2$
138	32	20	$C_8H_{12}NO$
137	25	26	$C_8H_{11}NO$
136	10	13	$C_8H_{10}NO$
119	5	5	C_8H_9N
120	2	2	$C_8H_{10}N$
94	25	23	C_6H_8N
93	100	100	C_6H_7N
83	8	10	C_5H_7O
80	10	12	C_5H_6N

Table 6: the mass spectral data of Ech-2 and Ech-3



Scheme 12 : A fragmentation pathway for Ech-2 and Ech-3 alkaloids

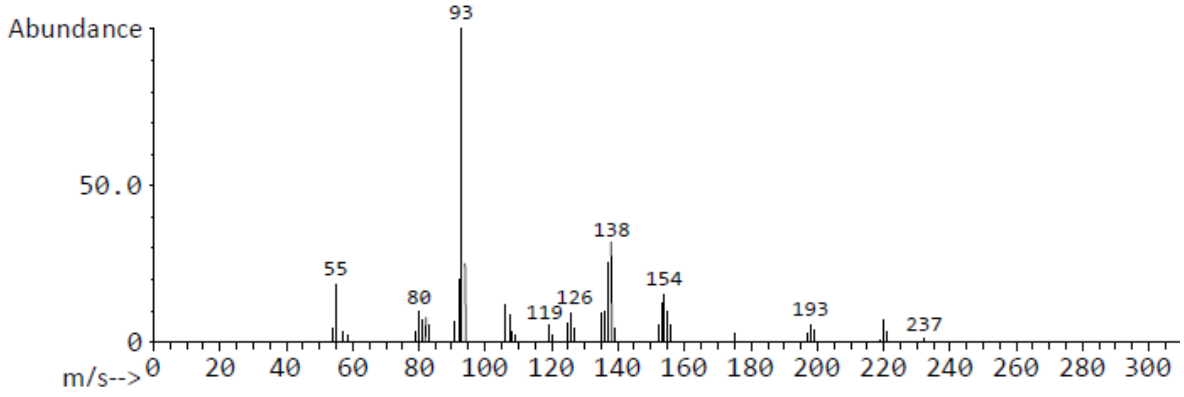


Figure 10: Mass spectrum of Ech-2 alkaloid

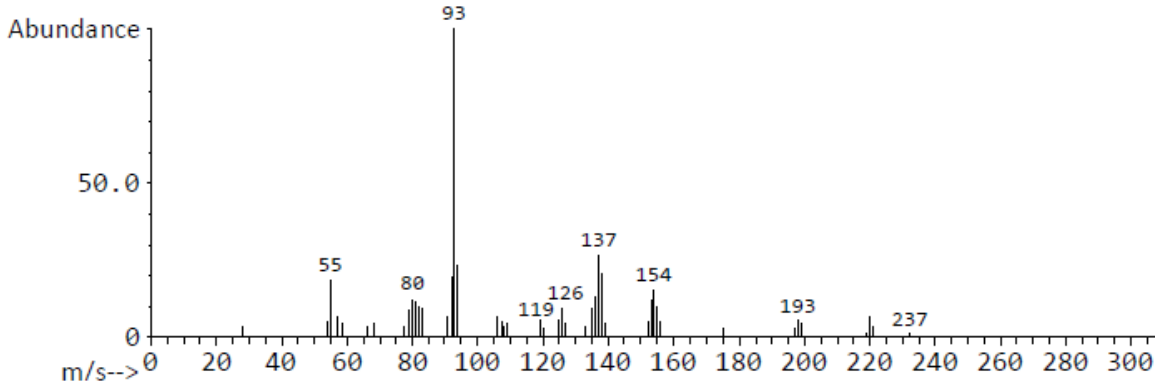


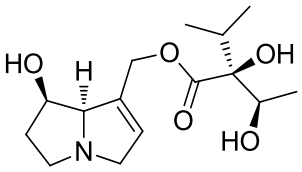
Figure 11: Mass spectrum of Ech-3 alkaloid

3.5. Characterization of Ech-4 and Ech-5 pyrrolizidine alkaloid

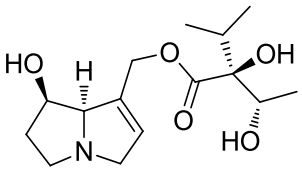
Ech-4 and Ech-5 alkaloids both show a molecular ion peak at m/z 299 figures (12) and (13), which corresponds to the molecular formula $C_{15}H_{25}NO_5$ tables (7). The Pyrrolizidine alkaloidse peak m/z 138 is due to cleavge of the weak allylic ester bond scheme (13) give strong evidence for the presence of free OH group at C-7[22, 70-72, 155].

significant ions m/z 136, m/z 94, m/z 93, and m/z 80 is characteristic to 1,2 unsaturated necine base[22, 24, 47, 99, 138, 155, 158].

Comparison of retention indices and mass fragmentations with those reported in literature[147, 159, 160] show clearly that Ech-4 alkaloid is Intermedineand Ech-5 alkaloid is lycopsamine.



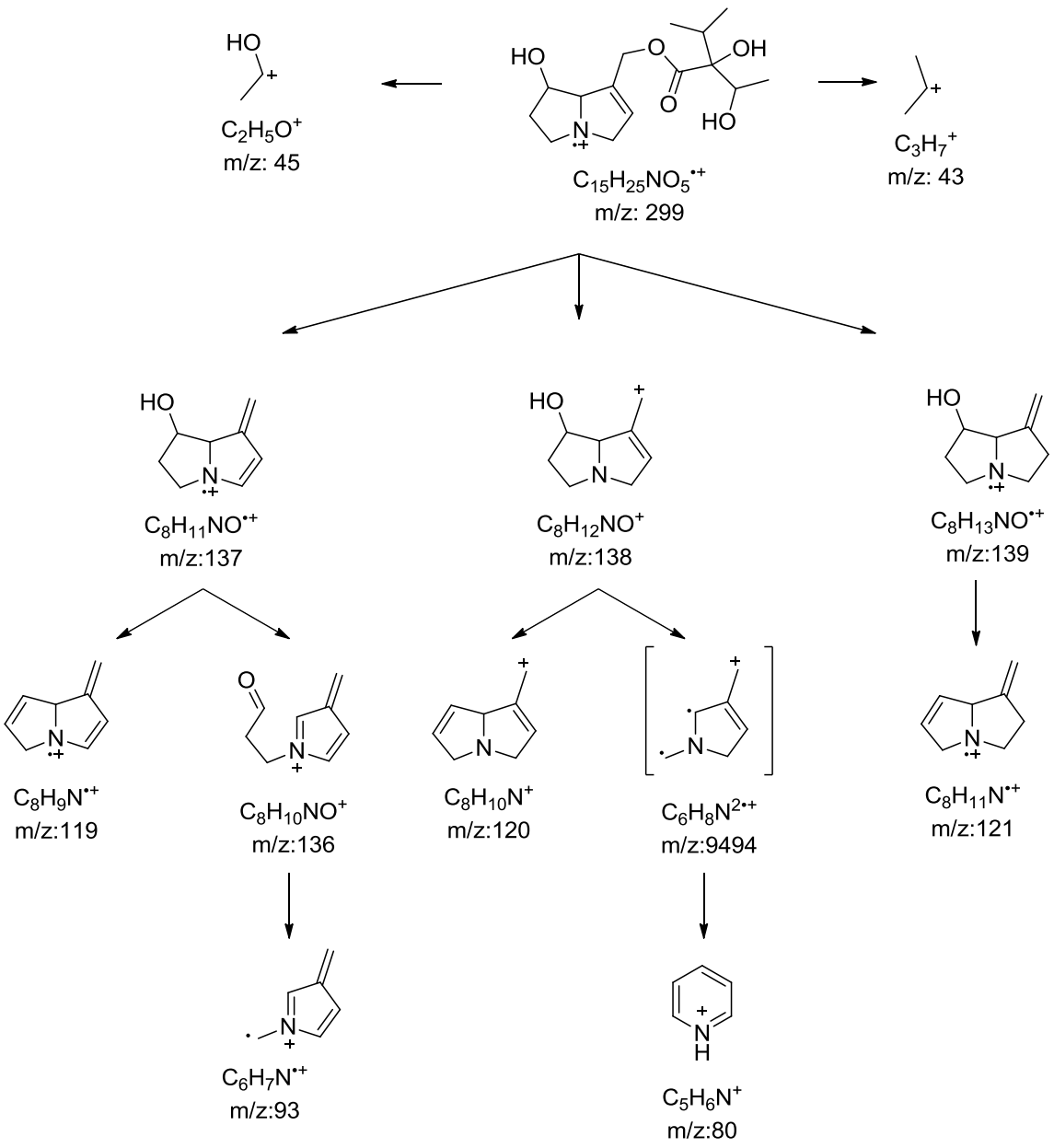
Intermedine



Lycopsamine

m/z	Relative abundance (%)		Expected formula
	Ech-4	Ech-5	
299	0.4	0.5	$C_{15}H_{25}NO_5$
139	35	31	$C_8H_{13}NO$
138	100	100	$C_8H_{12}NO$
137	13	12	$C_8H_{11}NO$
136	13	12	$C_8H_{10}NO$
120	10	10	$C_8H_{10}N$
94	50	55	C_6H_8N
93	80	84	C_6H_7N
80	14	14	C_5H_6N
45	7	8	C_2H_5O
43	43	20	C_3H_7

Table 7: the mass spectral data of Ech-4 and Ech-5



Scheme 13 : A fragmentation pathway for Ech-4 and Ech-5 alkaloids

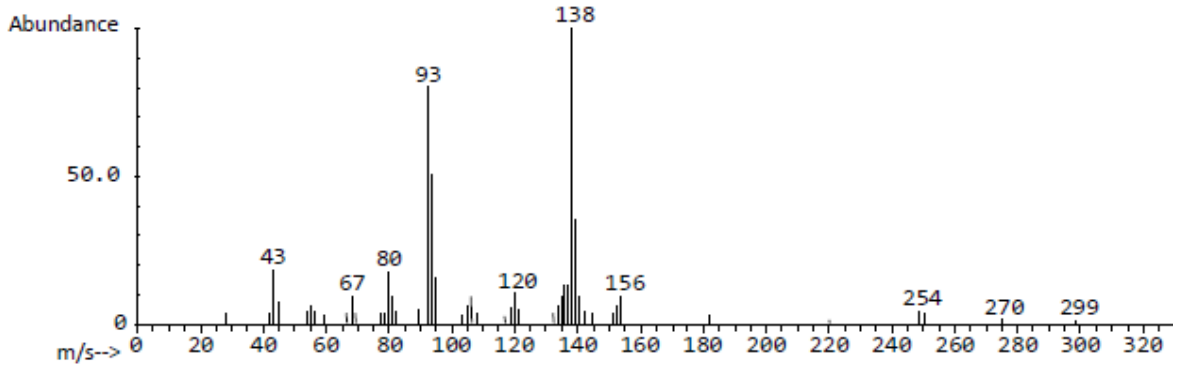


Figure 12: Mass spectrum of Ech-4 alkaloid

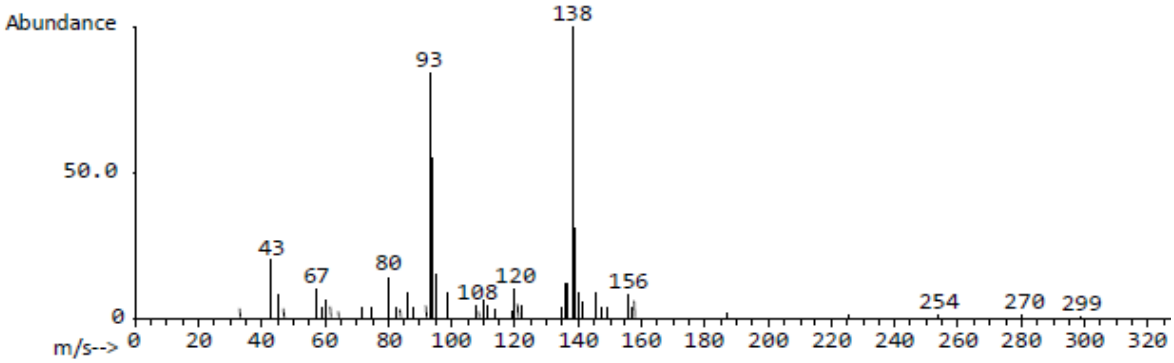


Figure 13: Mass spectrum of Ech-5 alkaloid

3.6. of Ech-6 pyrrolizidine alkaloid

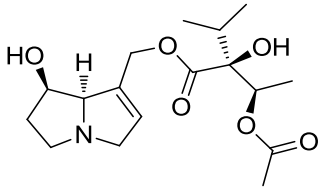
The mass spectrum figure (14) showed M^+ at m/z 341, this parent ion is corresponding to the following molecular formula $C_{17}H_{27}NO_6$ table (8).

The fragment ions m/z 136, m/z 94, m/z 93, and m/z 80 is characteristic to 1,2 unsaturated necine base [22, 24, 47, 99, 138, 155, 158].

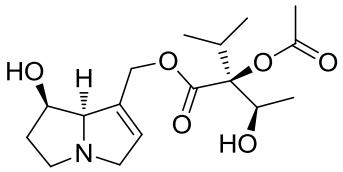
The peak at m/z 138 is formed through C-9 cleavage scheme (14), this peak is characteristic for C9-monoesters [22, 70-72, 155].

A base peak at m/z 138 due to cleavage of the weak allylic ester bond, providing strong evidence for the presence of a free OH at C-7[100, 161]

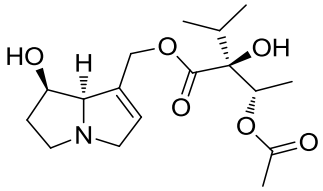
Comparison of retention index and mass fragmentations with those reported in literature [47, 99] show that the Ech-6 alkaloid is is acetylderivative of Intermedineor acetylderivative of lycopsamine. However, it needs to be established by further technique whether the acetyl group is in the 3' or 2' position.



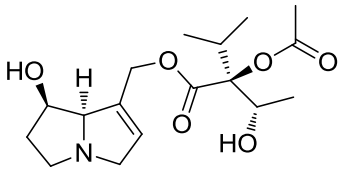
3'-acetylintermedine



2'-acetylintermedine



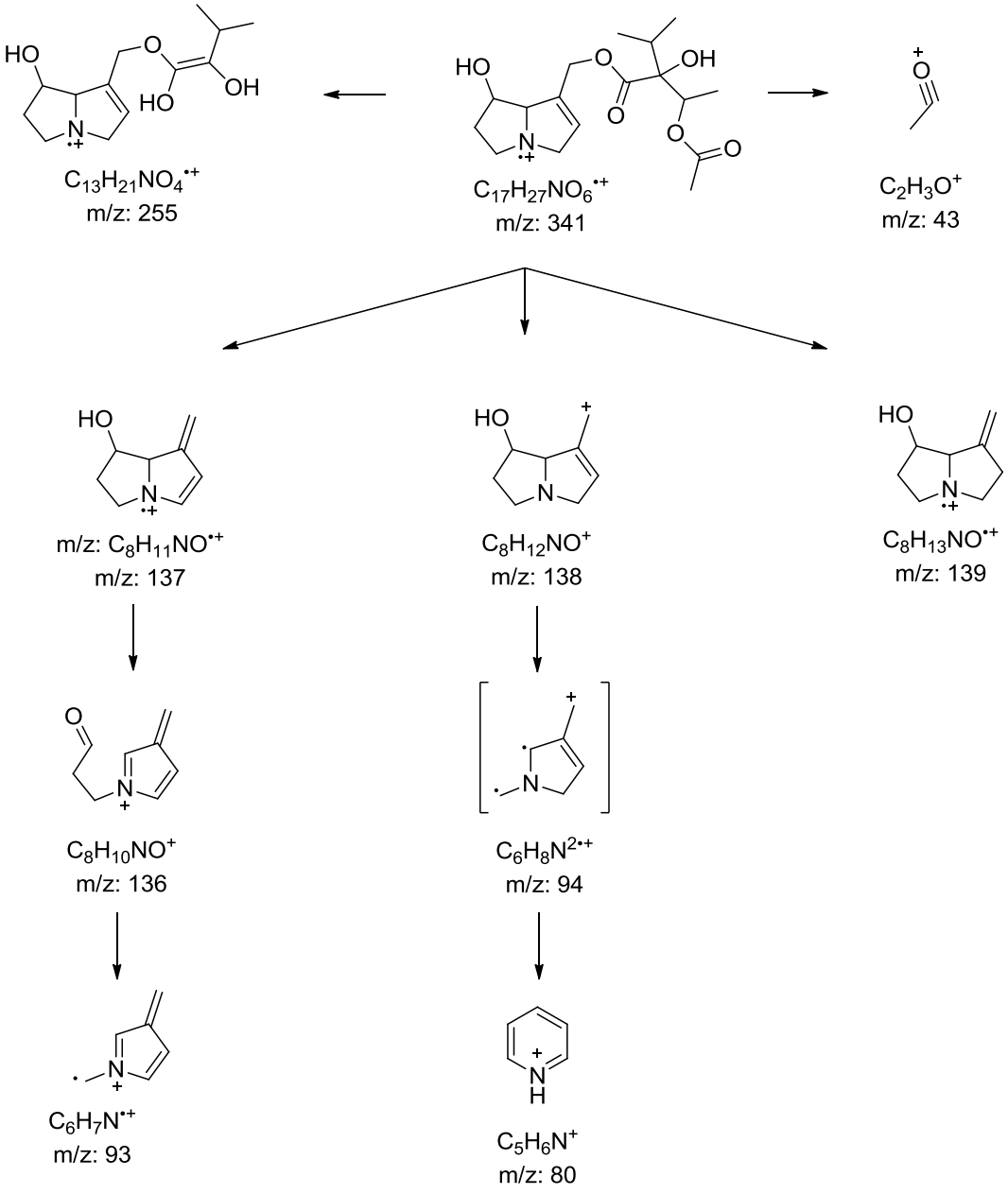
3'-acetylycopsamine



2'-acetylycopsamine

m/z	Relative abundance (%)	Expected formula
341	5	$C_{17}H_{27}NO_6$
138	100	$C_8H_{12}NO$
137	12	$C_8H_{11}NO$
136	12	$C_8H_{10}NO$
94	30	C_6H_8N
93	71	C_6H_7N
80	10	C_5H_6N
43	21	C_2H_3O

Table 8: the mass spectral data of Ech-6



Scheme 14 : A fragmentation pathway for Ech-6 alkaloid

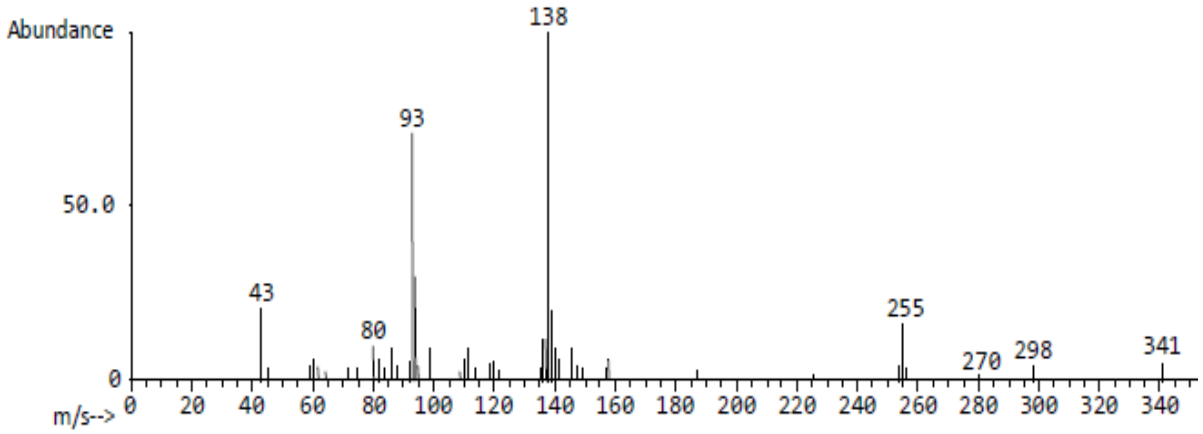


Figure 14: Mass spectrum of Ech-6 alkaloid

3.7. Characterization of Ech-7, Ech-8 and Ech-9 pyrrolizidine alkaloid

Ech-7,Ech-8, and Ech-9 alkaloids all have molecular ion peak at m/z 339 which corresponding to the formula ($C_{17}H_{25}NO_6$) tables (9) respectively. And they show the fragmentation pattern of un saturated diester pyrrolizidine alkaloid which are m/z136, m/z 120, m/z 119, m/z 93, and m/z 80[22, 24, 47, 99, 138, 155, 158].

The strong peak at m/z 220(M^+- $C_4H_7O_4$) is due to the cleavage of the weak allylic bond at C-9.and the ion peak at m/z 239(M^+-angelic acid) is due to the loss of acid attached to C-7 scheme (15).

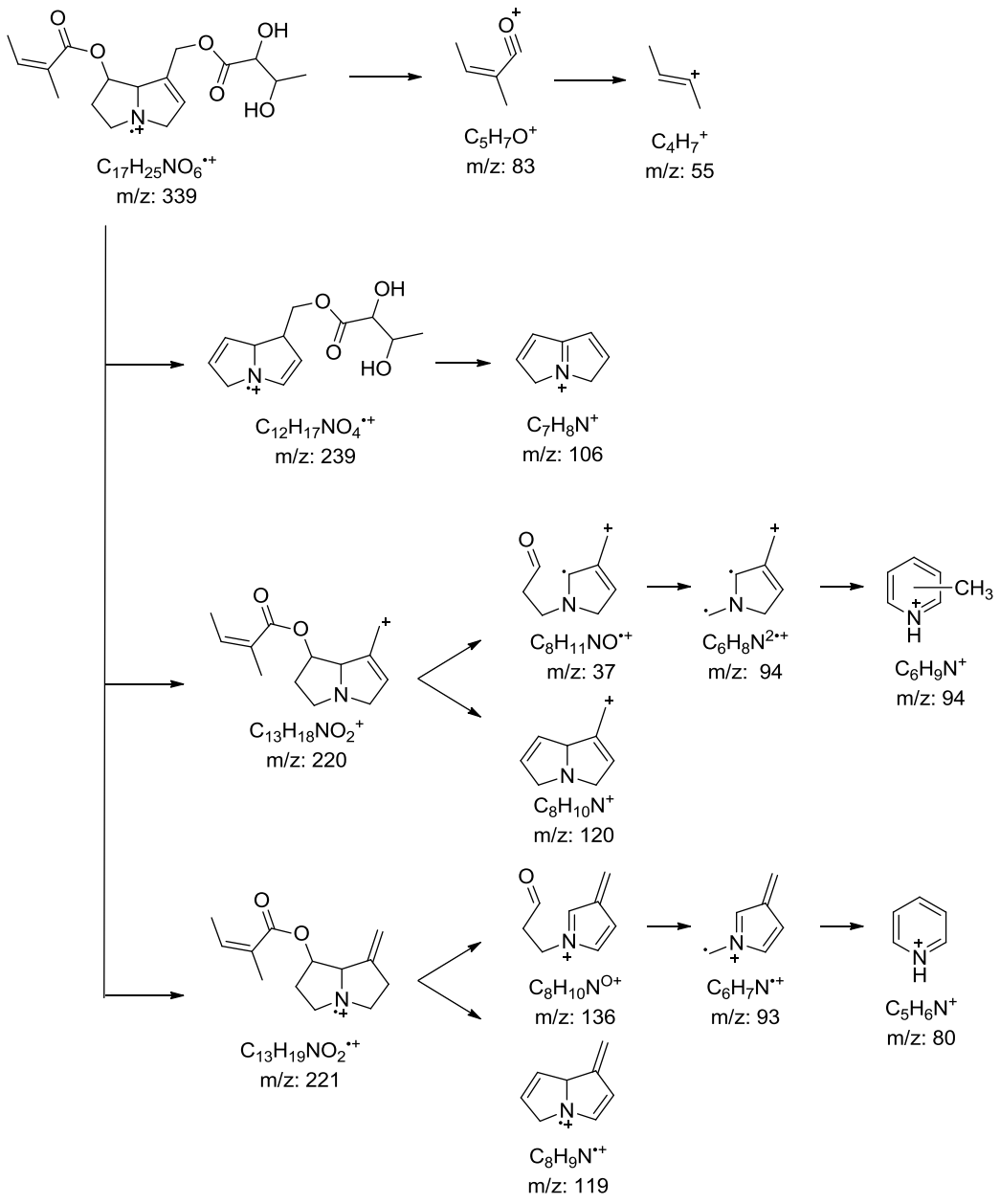
The fragment at m/z 57 (C_3H_5O) is probably derived from m/z 75 ($C_3H_7O_2$) through loss one molecule of water.

Ech-7, Ech-8, and Ech-9 alkaloids show similar fragmentation pattern figures (15), (16), and (17) respectively but they differ in the retention indices and in the relative intensities of some fragment ion.

Ech-7 alkaloid was tentatively identified as 7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine according to literature data [95, 99, 147, 152], and alkaloids Ech-8 and Ech-9 were assumed to be isomers of Ech-7.

m/z	Relative abundance (%)			Expected formula
	Ech-7	Ech-8	Ech-9	
339	1	1	1	$C_{17}H_{25}NO_6$
239	5	5	6	$C_{12}H_{17}NO_4$
221	25	25	25	$C_{13}H_{19}NO_2$
220	99	90	65	$C_{13}H_{18}NO_2$
136	100	100	81	$C_8H_{10}NO$
120	83	80	100	$C_8H_{10}N$
119	34	35	85	C_8H_9N
106	10	10	15	C_7H_8N
94	55	58	50	C_6H_9N
93	95	90	85	C_6H_7N
83	41	46	24	C_5H_7O
55	40	40	25	C_4H_7

Table 9: the mass spectral data of Ech-7, Ech-8, and Ech-9



Scheme 15 : A fragmentation pathway for Ech-7, Ech-8 and Ech-9 alkaloids

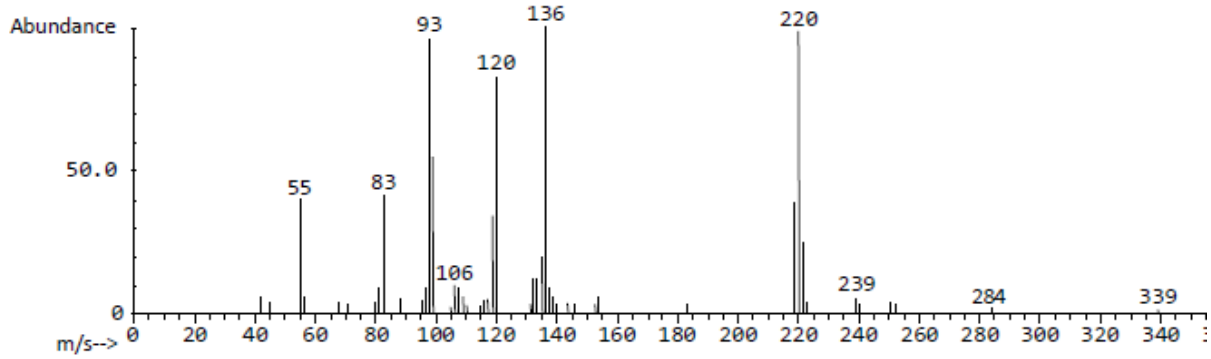


Figure 15: Mass spectrum of Ech-7 alkaloid

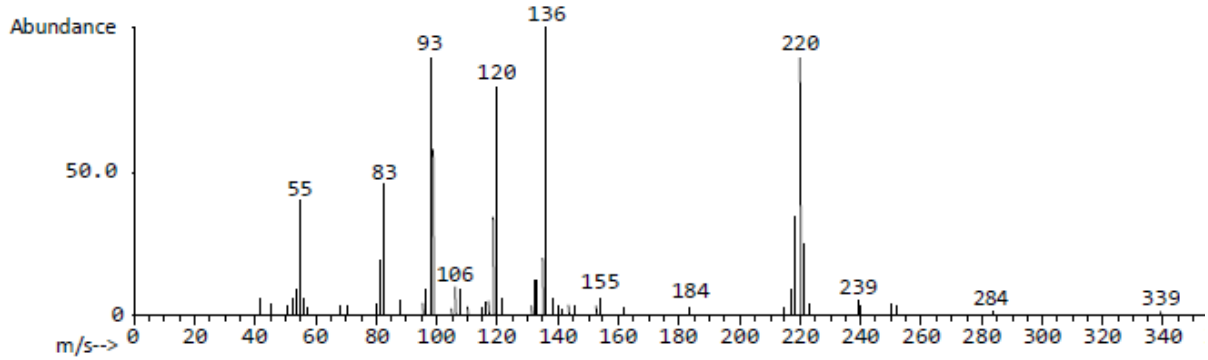


Figure 16: Mass spectrum of Ech-8 alkaloid

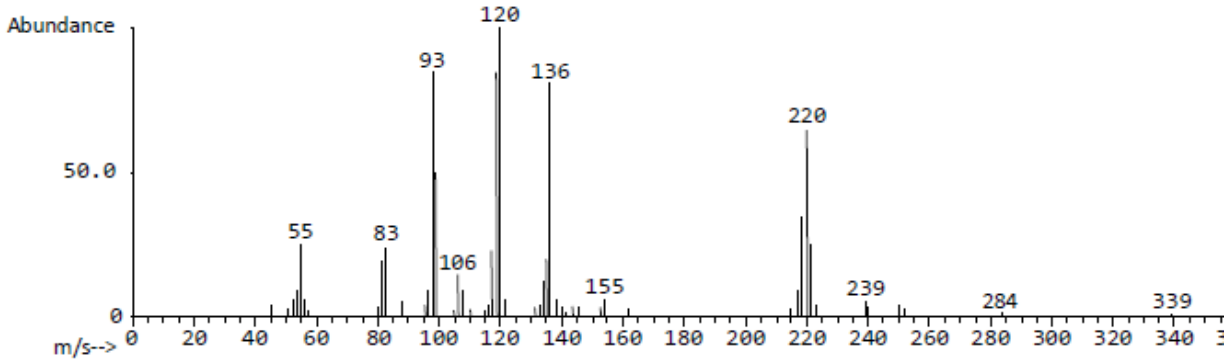


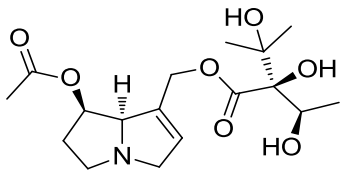
Figure 17: Mass spectrum of Ech-9 alkaloid

3.8. Characterization of Ech-10 pyrrolizidine alkaloid

Figure (18) shows M+ at m/z 357 (corresponding to molecular formula $C_{17}H_{27}NO_7$) table (10). The ion series at m/z 136, 120, 119, 93, and 80 are characteristic of 1.2-unsaturated diester pyrrolizidine alkaloids[22, 24, 47, 99, 138, 155, 158] , and the base peak at m/z 180 (M-$C_7H_{13}O_5$) provides strong evidence for the presence of C-7 acetoxy group[154, 155] .

The low intensity peak at m/z 297 is due to lose of acid attached at C-7 (M-$C_2H_3O_2$) scheme (16).

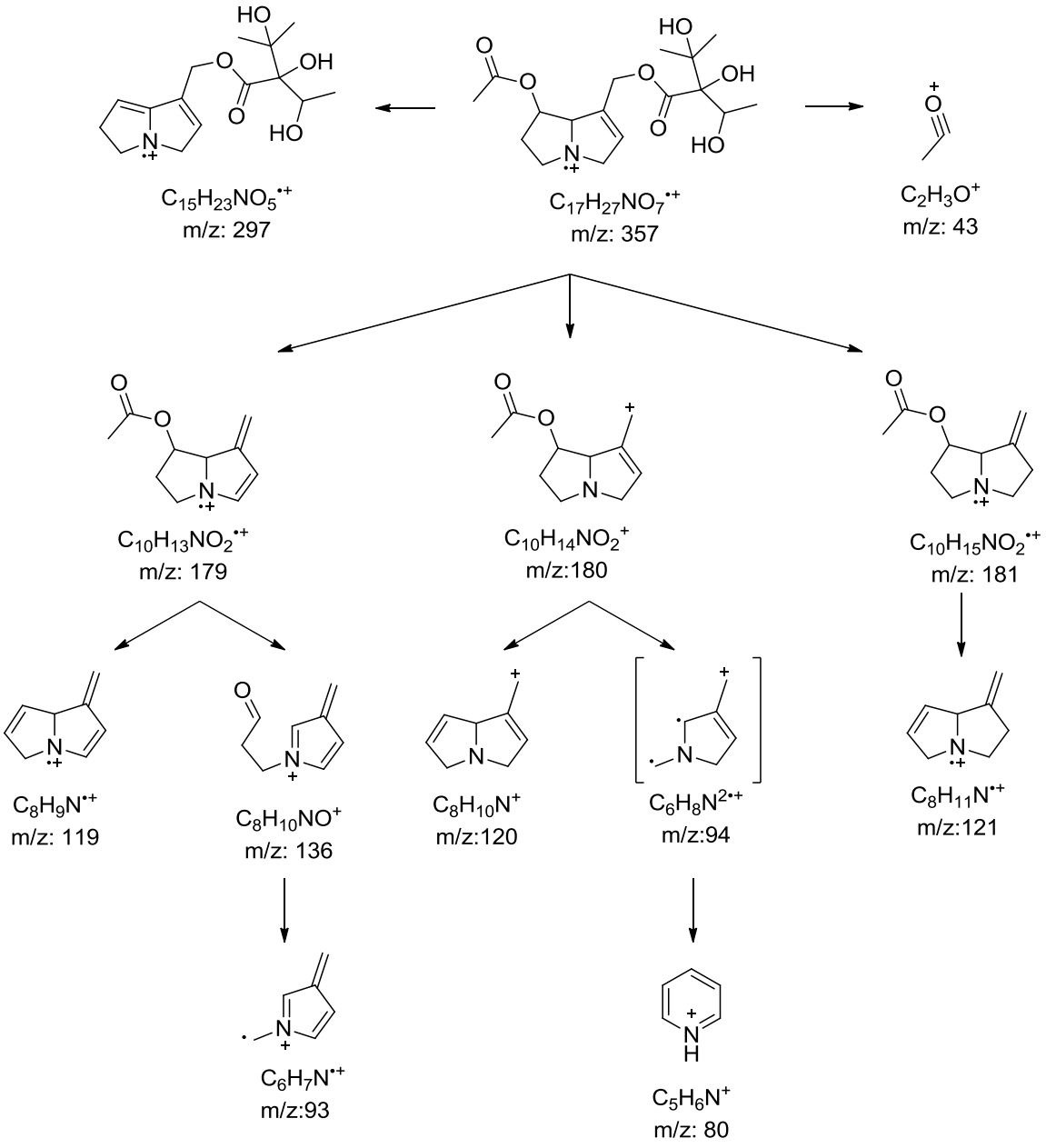
By direct comparison of retention indice of Ech-10 alkaloid (RI) with literature data[152], The acid attached at C-9 has formula of ($C_7H_{13}O_5$) can be identified as 5'-OH (+)-Trach (Echimidinyl), hence the alkaloid Ech-10 with RI 2337was identified as 7-acetyl-9-echimidinylretronecine (uplandicine).



Uplandicine

m/z	Relative abundance (%)	Expected formula
357	4	$C_{17}H_{27}NO_7$
297	23	$C_{15}H_{23}NO_5$
181	80	$C_{10}H_{15}NO_2$
180	100	$C_{10}H_{14}NO_2$
179	43	$C_{10}H_{13}NO_2$
136	75	$C_8H_{10}NO$
121	49	$C_8H_{11}N$
120	85	$C_8H_{10}N$
119	50	C_8H_9N
93	74	C_6H_7N
80	28	C_5H_6N
43	20	C_2H_3O

Table 10: the mass spectral data of Ech-10



Scheme 16 : A fragmentation pathway for Ech-10 alkaloid

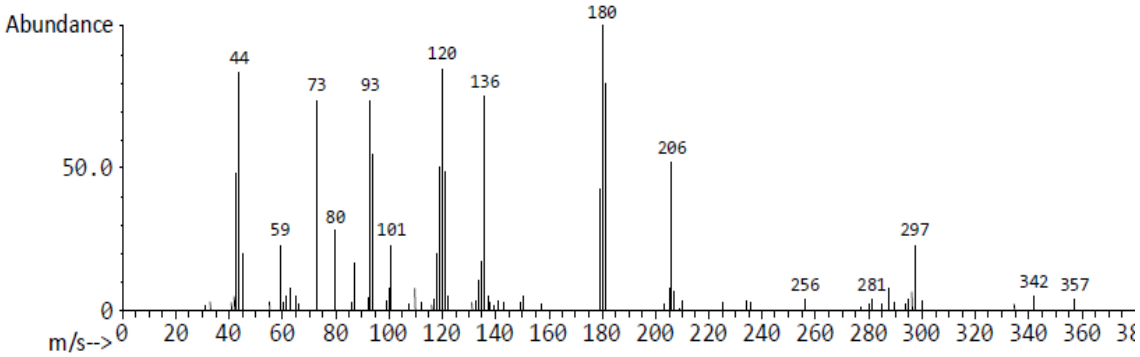
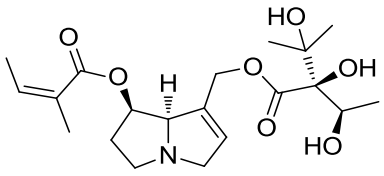


Figure 18: Mass spectrum of Ech-10 alkaloid

3.9. Characterization of Ech-11 pyrrolizidine alkaloid

The MS spectrum of Ech-7 figure (19) showed Molecular ion peak at m/z 397; where the relative abundance is 0.1 %for $C_{20}H_{31}NO_7$.The mass spectral data table (11) Showed significant ions at m/z 136, m/z 120, m/z 119, m/z 93, and m/z 80.These fragments are characteristic to 1,2-unsaturated necine base[22, 24, 138, 151, 156] , the base peak ion at m/z 220 is formed through C-9 cleavage of diesterpyrrolizidine alkaloid bearing angeloxy group.

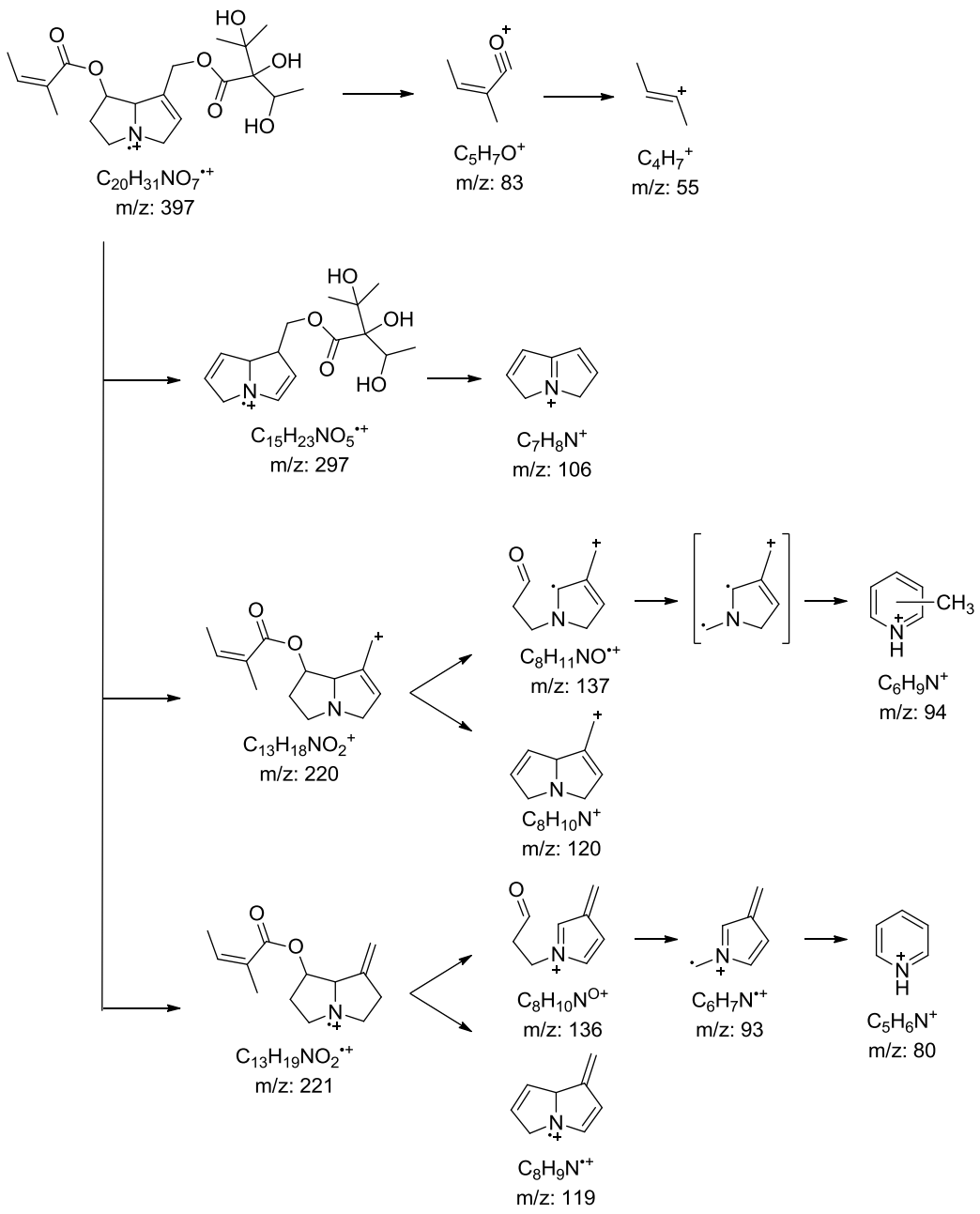
This result was confirmed by the presence of the fragment ion at m/z 297 (M^+-angelic acid) as shown in scheme (17) [138, 162]



Echimidine

m/z	Relative abundance (%)	Expected formula
397	0.1	$C_{20}H_{31}NO_7$
297	2	$C_{15}H_{23}NO_5$
221	21	$C_{13}H_{19}NO_2$
220	100	$C_{13}H_{18}NO_2$
136	48	$C_8H_{10}N_O$
120	75	$C_8H_{10}N$
119	30	C_8H_9N
106	5	C_6H_7N
94	30	C_6H_8N
93	61	C_6H_7N
83	39	C_5H_7O
80	10	C_5H_6N
55	25	C_4H_7

Table 11: the mass spectral data of Ech-11



Scheme 17 : A fragmentation pathway for Ech-11 alkaloid

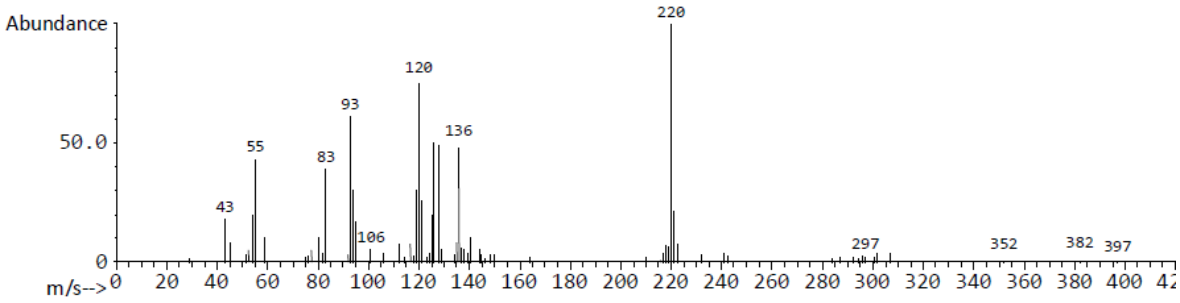
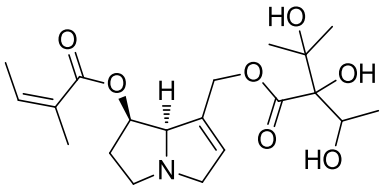


Figure 19: Mass spectrum of Ech-11 alkaloid

3.10. Characterization of Ech-12 pyrrolizidine alkaloid

Ech-12 alkaloids have a molecular mass of 381 figure (20), and exhibit the characteristic peaks of an unsaturated Nicene base (m/z 80, 93, 119, 120, 121 and 136) [22, 24, 138, 151, 156]. Peaks at m/z 83 and 220 indicate the presence of an angelyl type group, while a peak at m/z 281 indicates a(±)- Trachelanthyl group[84]. The peak at m/z 220 is significantly more intense than that at m/z 281, suggesting that the(±)-Trachelanthyl group is attached at C-9 and the angelyl at C-7 [84]. This group of diesters is thus isomeric, with angelyl or tiglyl groups at C-7 and (±)-Trachelanthyl or (±)-Viridifloryl groups at C-9.

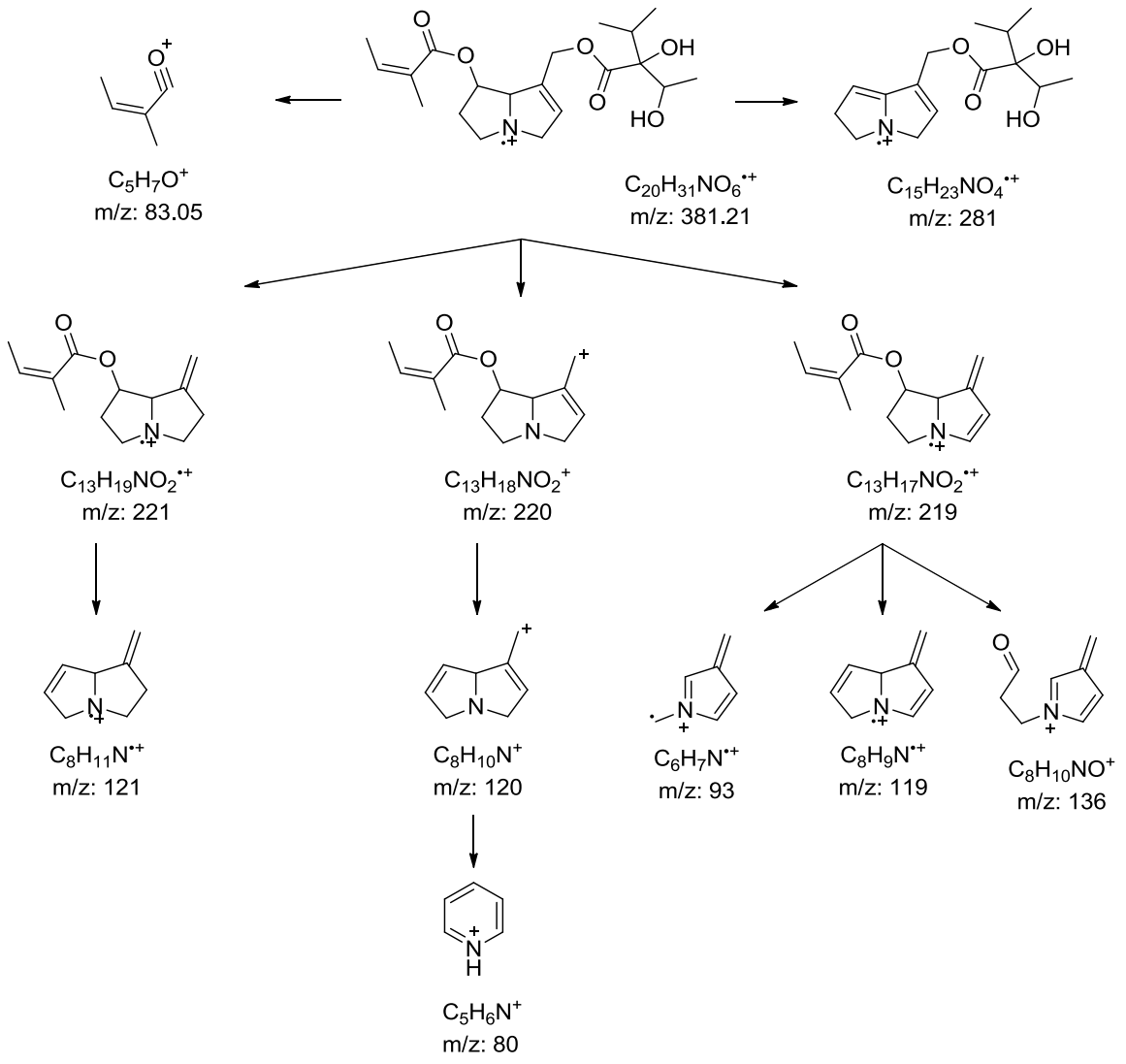
The retention index of Ech-12 is 3179. Comparison this value and mass spectra with those of standard alkaloids identified by GC-MS and other techniques in literature [154, 163] indicate that the Ech-12 combound is Echiumine which is a diester alkaloids and of unsaturated necine base.



Echiumine

m/z	Relative abundance (%)	Expected formula
397	0.4	$C_{20}H_{31}NO_7$
221	35	$C_{13}H_{19}NO_2$
220	100	$C_{13}H_{18}NO_2$
136	47	$C_8H_{10}NO$
120	69	$C_8H_{10}N$
119	20	C_8H_9N
106	7	C_7H_8N
94	39	C_6H_9N
93	50	C_6H_7N
83	28	C_5H_7O
80	17	C_5H_6N
55	37	C_4H_7

Table 12: the mass spectral data of Ech-12



Scheme 18 : A fragmentation pathway for Ech-12 alkaloid

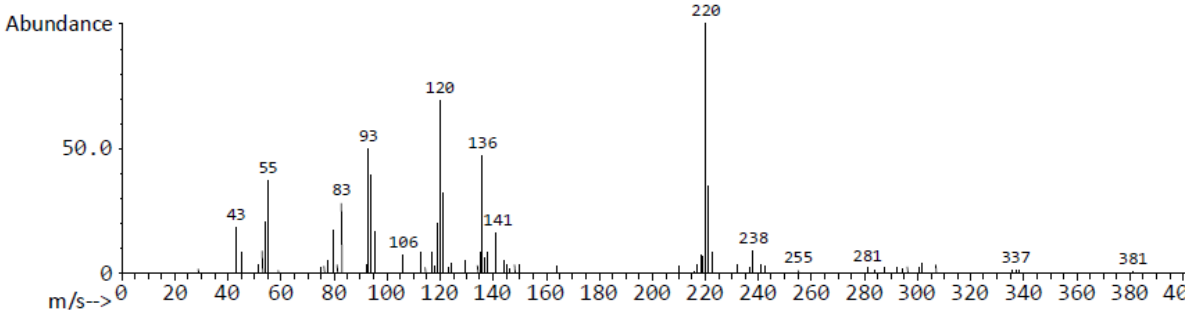


Figure 20: Mass spectrum of Ech-12 alkaloid

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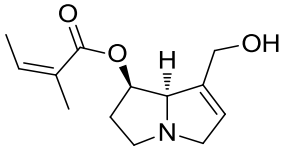
الملخص

هذه الدراسة تتضمن عرض مختصر لقلويدات البيروليزيدين التي تم فصلها سابقا من أصناف متعددة لنباتات من العائلة الشفوية باستخدام تقنيات مختلفة

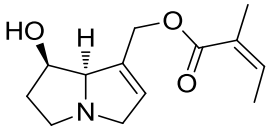
الهدف الأساسي لهذا البحث هو فصل وتحديد تركيب قلويدات البيروليزيدين من مستخلص الميثانولي لنبات *Echium plantagenium* (يعرف محليا باسم المصيص) الذي يعود للعائلة الشفوية والذي ينمو بالقرب من مدينة المرج حيث تم فصل أثني عشر مركبا:

Ech-1



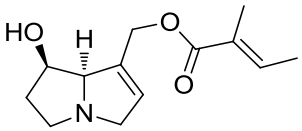
7-angeloylretronecine

Ech-2



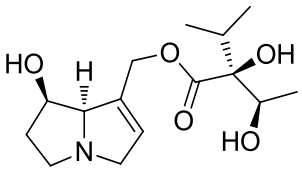
9-Angeloylretronecine

Ech-3



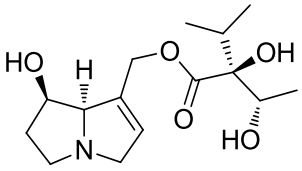
9-Tigeloylretronecine

Ech-4



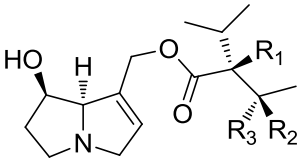
Intermedine

Ech-5



Lycopsamine

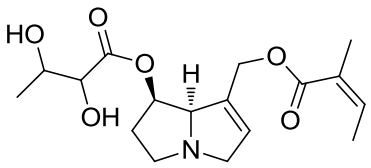
Ech-6



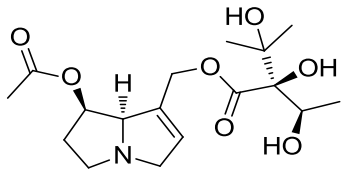
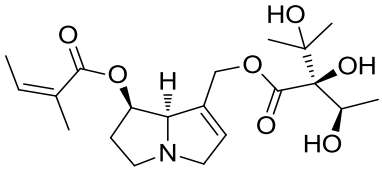
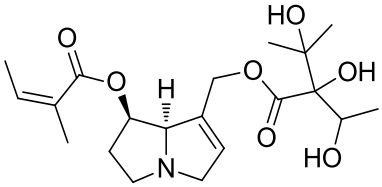
Assumed one of the following isomers

R_1	R_2	R_3	
OH	OAc	H	3'-acetylintermedine
OAc	OH	H	2'-acetylintermedine
OH	H	OAc	3'-acetylycopsamine
OAc	H	OH	2'-acetylycopsamine

Ech-7



7-Angeloyl-9-(2,3-dihydroxybutyryl)retronecine

Ech-8 and Ech-9 Assumed to be isomer of Ech-7	Ech-10  Uplandicine
Ech-11  Echimidine	Ech-12  Echiumine

تم التعرف على المركبات المفصولة باستخدام تقنية Gas Chromatographic Mass Spectrometry و كذلك حساب قيم Retention Indices (RI) حيث تم مقارنة النتائج المتحصل عليها مع نتائج اخرى لمركبات مفصولة سابقا كقيم معايرية.