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Plant Steroids to Phytoecdysteroids; a new chemical entity over viewed with extraction, purification, and analysis

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ABSTRACT: The phytochemical investigation of ecdysteroids involves the following: extraction of the plant material; separation and isolation of the constituents of interest; characterization of the isolated compounds and quantitative evaluations. Emphasis is placed more on chromatographic procedures and structural elucidations.

KEYWORDS: Ecdysteroids, Extraction, Column chromatography, HPTLC, HPLC, NMR, MS

INTRODUCTION

Plants elaborate a very wide array of steroidal compounds, party as endogenous hormones (in low amounts) or as allelopathic defence compounds (in much higher concentrations). The distributions of the various classes of defence steroids vary between plant families, between species within the family, within the species (ecotypes) and within the organs of the plants, which contain them. It is clear that phytosteroids possess many interesting medicinal, pharmaceutical and agrochemical activities. For this reason alone, further studies are warranted on these compounds to identify lead compounds. Further, Plants are good source of larger numbers of varied analogues suitable for structural-activity studies. Modification of the levels or profiles of phytosteroids by genetically modified (GM) or non-GM means in crop species may result in improved growth and yields (brassinosteroids) or better allelopathic effects (deterrence of vertebrate or invertebrate predators, greater resistance to microbial attacks, etc: bufadienolides, cardenolides, cucurbitacins, ecdysteroids, steroidal alkaloids, sapogenins, vertebrate-type steroids and withanolides) (1).

Plant produces steroid molecules, which can be divided into three groups based on their biological relevance –

(I) Substances, which have physiological roles in the plant itself, as hormones or pheromones. Thus, brassinosteroids (**1**) are growth-promoting phytohormone, whereas antheridiol (**2**) and oogoniol (**3**) are pheromones in an aquatic filamentous fungus.

(II) Allelochemical substances related to animal hormones: ecdysteroids (**4**) are analogues of insect moulting hormones, whereas androgens, oestrogens, progestagens (**5**), corticosteroids and cholecalciferols (**6**) are related to vertebrate hormones.

(III) Plant-specific allelochemical substances, which often display protective (repellent, antifeedant, toxic) action towards phytophagous animals or parasitic fungi: these are, e.g., cucurbitacins (**7**), cardenolides (**8**), bufadienolides (**9**), sapogenins (**10**), withanolides (**11**) and steroidal alkaloids (**12**).

Ecdysone is a prohormone of the major insect-moulting hormone 20-hydroxyecdysone. Insect moulting hormones (ecdysone and its homologues) are generally called “ecdysteroids”. Ecdysteroids act as moulting hormones of arthropods but also occur in other invertebrates where they can play different role. Ecdysteroids also appear in many plants mostly as a protection agent (toxins or antifeedants) against herbivore insects. Phytoecdysteroids deter some insects (2-25 ppm), while other insects are resistant to very high concentrations (400-1000ppm). It appears ecdysteroids concentrations are highest in tissues which are most important for the survival of the plant and that these levels change throughout plant development (2). The most common phytoecdysteroids found in plants is 20-hydroxyecdysone (3-5). While most plant families have at least some species that accumulate ecdysteroids, less than 2% of the world flora and 6% plant species have been investigated for the presence of ecdysteroids (6).

Ecdysteroids and Health Improvement Preparations

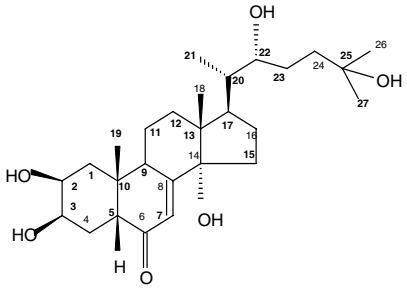
It is much easier and cost effective to isolate ecdysones from plants rather than arthropods because the concentrations of ecdysones are very much higher in plants. Many plants belonging to the various families are reported to be containing ecdysones, often as complex mixtures. These ergogenic supplements are believed to produce anabolic and growth-promoting

effects, and are primarily marketed to weight lifters and sports enthusiasts as pseudo-steroidal muscle enhancers. Plants that contain high amounts of phytoecdysteoids are cultivated in Europe as a source of these compounds for the dietary supplement market (7).

Biological activity

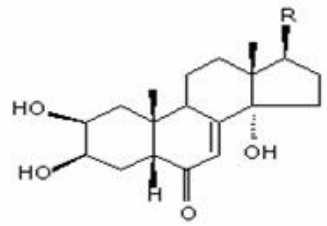
Experimental findings on ecdysteroids have indicated their anabolic and cholesterol level reducing effects. In mammals, ecdysteroids have beneficial effects on induced hypoglycemia and they also potentiate the effect of insulin. Ecdysteroid- containing preparations are recommended in asthenic and astheno-depressive states, somatic and infectious diseases, neurasthenia, neurosis, hypotension, fatigue, for stress, stimulation of functions of the central nerveous system and for increasing appetite and digestion (8)

The chemical structure



Ecdysteroids comprise a class of natural steroids (cyclopentano-perhydro-phenanthrene). Their chemical structure is closely related to sterine and brassinolids. The biosynthetic pathway of ecdysteroids, which originates from isoprene units, leads through cholesterine and its C24 homologues. During biosynthesis the compounds retain the side chain of sterine, the intermediate precursor. It is through this route that C27-C29 ecdysteroids are formed. The C19, C21, and C24 ecdysteroids are produced by cleavage of the side chain. Therefore, ecdysteroids are characterized as C19 steroids with a side chain containing from 2-10 carbons attached to the beta position at C17. In some cases, the side chain closes to form either a lactone ring or cyclic ether. Ecdysteroids are members of the 5ß-androstane series of ecdysteroids. Their A/B ring has the *cis*-fused junction. The C2 position is generally hydroxylated. A steric hinderance between 2ß-OH and 19-Me favors the 5ß-forms. In addition to the ecdysteroids, only three additional natural products have such a ring junction. These are the cardenolides (γ lactone), the bufadienolides (δ lactone) and the bile acids. There is a 7-ene-6-one conjugation in the B-ring (which is responsible for UV absorption), and the possession of a 14α-OH group and several other OH group. Ecdysteroids are highly hydroxylated, with 3 to 9 hydroxyl groups in various positions such as – but not exclusively- at C1, C2, C3, C4, C5, C11, C20, C22, C24, C25, C 26/27, C28, and C29. Other substituents are also possible. In addition to the 6-one, other keto-or carboxylic groups may be present. e.g. a carbonyl at the 3,12,17,20 or 22 positions, a carboxyl group at position 26 (which sometimes occurs as a lactone) or at the 29 positions, in the lactone form. Glucoside, acetate, benzoate, phosphate and various other ester forms are also found. The majority of ecdysteroids contain vicinal 2-3 and 20-22 dihydroxyl groups. The major structural modifications of ecdysteroids include the number and position of the hydroxyl substituents. Other structural changes involve oxidation of the molecule and the number of double bonds (9,10).

Types of phytoecdysteroids *(11)*



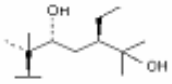
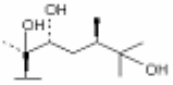
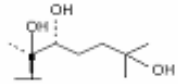
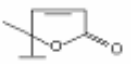
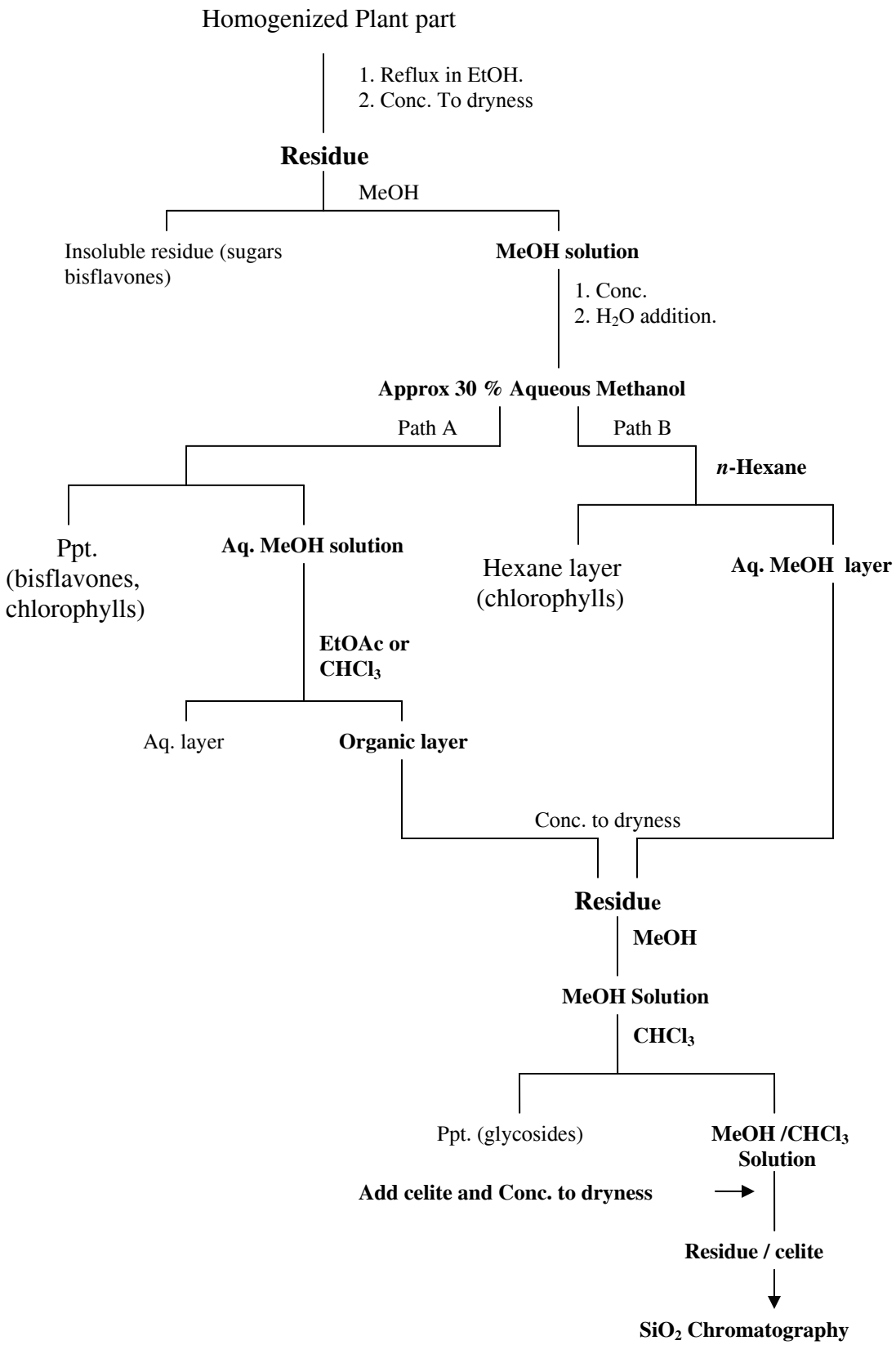
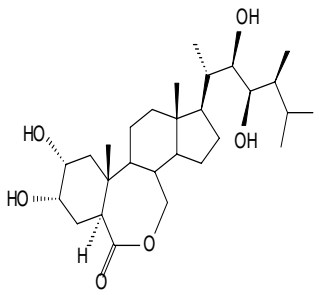
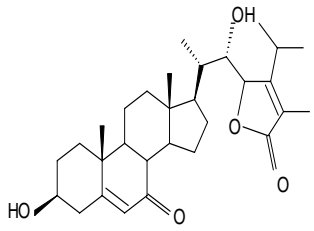
Number of C atoms in the skeleton	R	Example
29		makisterone C
28		makisterone A
27		20-hydroxyecdysone
24		Sidisterone
21		Posterone
19	OH	Dihydrorubrosterone

Fig 1

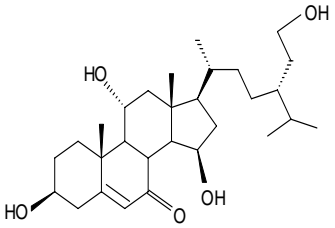

Homogenized Plant part
1. Reflux in EtOH.
2. Conc. To dryness
Residue
MeOH
Insoluble residue (sugars bisflavones)
MeOH solution
1. Conc.
2. H2O addition.
Approx 30 % Aqueous Methanol
Path A
Path B
n-Hexane
Ppt. (bisflavones, chlorophylls)
Aq. MeOH solution
Hexane layer (chlorophylls)
Aq. MeOH layer
EtOAc or CHCl3
Aq. layer
Organic layer
Conc. to dryness
Residue
MeOH
MeOH Solution
CHCl3
Ppt. (glycosides)
MeOH /CHCl3 Solution
Add celite and Conc. to dryness
Residue / celite
SiO2 Chromatography



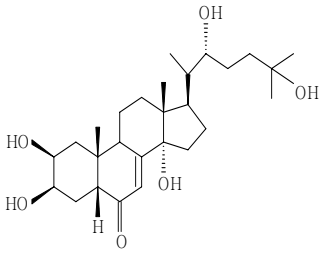
Brassinolide (**1**)



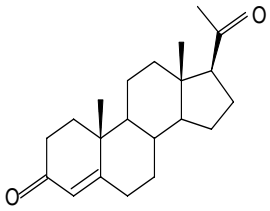
Antheridiol (**2**)



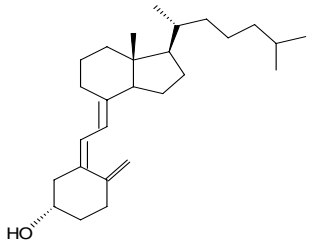
Oogoniol (**3**)



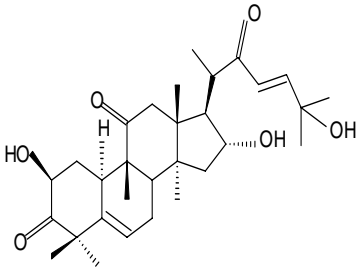
Ecdysone (**4**)



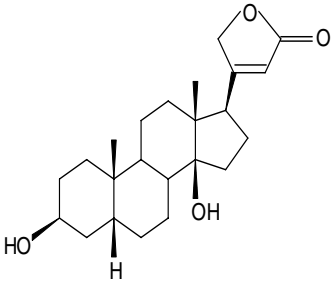
Progesterone (**5**)



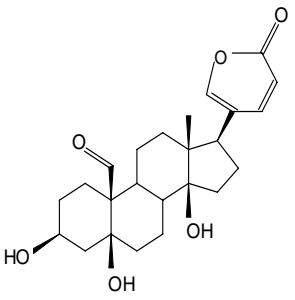
Vitamin D3 (**6**)



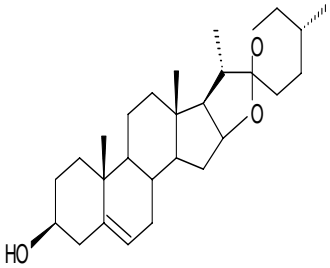
Cucurbitacin D (**7**)



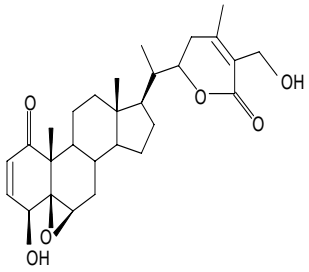
Digitoxigenin (**8**)



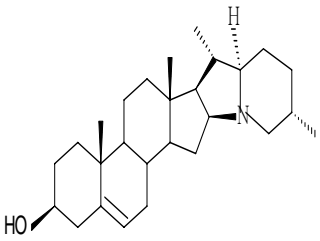
Hellebrigenin (**9**)



Diosgenin (**10**)



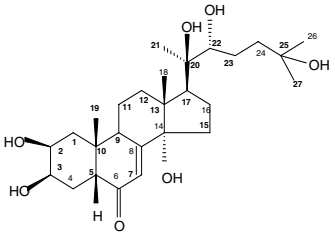
Withaferin A (**11**)



Solanidine (**12**)

The principle ecdysteroid in Invertebrates and Plants: 20-hydroxyecdysone

In invertebrates and in plants, 20-hydroxyecdysone is the most frequently found and widely distributed ecdysteroids. It has been possible to demonstrate some concentration of 20-hydroxyecdysone in almost all plant species tested. Since the discovery of 20-hydroxyecdysone was made by various scientists in different parts of the world at or near the same time, it received an assortment of trivial names such as beta-ecdysone, crustecdysone, ecdysone, polypodine-A, isoinokosterone and 20-hydroxyecdysone. In order to standardize the nomenclature, the IUPAC assigned the name 20-hydroxyecdysone to the compound: 2ß, 3ß, 14α, 20R, 22R, 25-hexahydroxy-5ß-cholest-7-ene-6-one, due to the accumulation of data from studies on it and its many practical applications, 20-hydroxyecdysone has gained remarkable significance among the ecdysteroids. Plants continue to be an optimal source for 20-hydroxyecdysone. That is, they supply the majority of raw materials required to conduct research on insect physiology, pre-clinical pharmacology and receptor binding (12).



20-hydroxyecdysone

Table1. Variations on the 20-hydroxyecdysone molecules (13)

Type	Positions
Hydroxyl groups	
Additional –OH	1,5,11,16,18,19,23,24,26
Missing –OH	2,20,22,25
Oxidation	
(>CHOH → >C = O	3,22
(–CH_2OH→ –COOH)	26
(→ epoxide)	22-23
Epimerization	3α/3β, 5α/5β
Alkyl substitution	24 (methyl, methylene, ethyl, etc.)
Esterification	
Acetates	2,3,22,25
Fatty acyls	22
Benzoates	20,22,25
Cinnamates	2
Coumarates	3
Phosphates	2,22,26
Sulfates	22
Lactone ring formation	concerns mainly C-28 or C-29 ecdysteroids
Etherification	
Intramolecular	between C-22 and C-25
Methoxy ether	25
Ketal acetal formation	
Acetonides	2-3,20-22,
Benzylidene acetals	20-22
Glycosylation	
Galactosides	3,22
Glucosides	3,22,25,26
Dehydration	9(11), 14(15), 24(25), 25(26)
Side-chain cleavage	C-20/C-22, C-17/C-20

Methods of extraction and isolation : ecdysteroids

From plant sample to pure compounds, the purification strategy always comprises a multi-step procedure including extraction, prepurification and the one or several chromatographic steps. Thus, the fresh or dry sample will be cut up or ground to a powder form before (i) extraction with organic solvents (perhaps in a sequence of increasing polarity), water or supercritical CO_2, (ii) solvent partitions to remove less polar and /or more polar compounds, (iii) initial chromatographic steps (flash chromatography, counter-current chromatography or low-pressure column chromatography on silica or alumina) and (iv) final purification by thin layer chromatography (TLC) and /or high -performance liquid chromatography(HPLC). This general strategy is similar

to that used for other classes of plant molecules, such as polypeptides (14).

Extraction (of milled, dry material) can be performed using a large range of solvents (with ca. 10% v/w), among which alcohols are the most widely used. For polar steroids it is possible to first extract with apolar solvents (e.g., a hydrocarbon), which will extract non-polar compounds, and then with a more polar one (alcohol). After concentration, a second step usually involves a partition between two non-miscible solvents. The purpose of such a step is that it can be used whatever the sample size, and it can be very efficient in removing both more poar and less polar contaminants, if two complementary partition steps are being used. More over, the recovery of compounds of interest in nearly quantitative. For example, ecdysteroids can be purified using isobutyl acetate-water (they remain in the water phase), then n-butanol-water (they go into the butanol phase). The choice of partition system relies on the polarity of the compounds of interest, which can roughly be estimated from the number of –OH groups, although it also depends of course on their position on the molecule (1,15,16). A general extraction route of polyhydroxy steroids suggested by by D.H.S.Honn (17) shown in fig 1. As the level of phytoecdysteroids covered a wide range (such as from 3.2% down to the trace) a unified method was elaborated to exploit ecdysteroids from plant (7,18-21). The methods start with sample preparation and draw the end towards isolation.

Homogenization and/or sample preparation is a crucial phase of both any enrichment and isolation work and they contained three essential steps, such as (i) Pulverization (size reductions) and carried out extraction with solvent (simple or multiple process), centrifugation, or filtration (ii) Concentration, fractionated precipitation and/or solvent-solvent partition. (iii) Solid phase extraction. Some out line are drawn below.

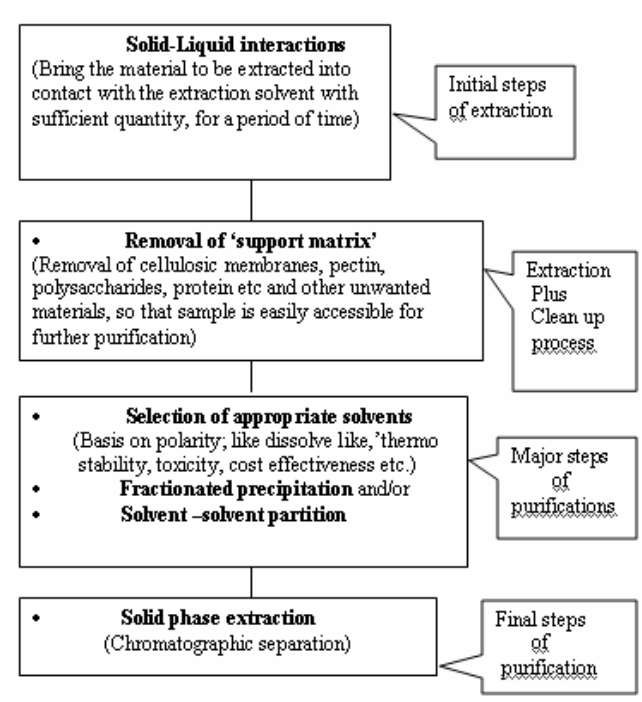


Fig. (2) The common process of the ecdysteroid extraction and purification

Preparative adsorption chromatography for isolation of phytoecdysteroids

Because of their strong polar nature, poor crystallization properties and validations in structures, ecdysones pose many complicated problems in isolation and purification. The separation of closely related ecdysones in pure form has been achieved employing complex processes. However, some general extraction processes for ecdysones have been developed and they can be adopted with suitable modifications. One process is based on extraction with solvents of different polarities followed by adsorption chromatography. In this process, the alcohol extract of

plant sources was fractionated into sugars, *bis*-flavones, chlorophylls, glycoside portions by change of solvents. The remaining solids were column chromatography over different stationary phases. Columns are filled with either normal (polar) phase (silica or alumina) eluted with organic solvents, or non-polar phases (Amberlite XAD-2, polyamide or sephadex LH20) eluted with aqueous mixtures. Ion exchange phases (e.g. DEAE-sephadex) eluted with buffers can be used for polar anionic ecdysteroids. The mobile phase may be a chlorinated hydrocarbon or an alcohol. Dichloromethane - ethanol mixtures with a small amount of water (0.1-1%) are generally used. The stationary phase is generally 4-25 times more than the sample, i.e. for 100 g of sample kilograms of stationary phase. 10-100 liters of the mobile phases and 100 ml of fractions are usual with stepwise gradient elution. Adsorption chromatography generally in 5-20 folds purification, with 75 % recovery.

Stationary phase	Solvent system
Normal phases	
Silica (silica gel, silicic acid or celite)	$CHCl_3$ / MeOH (100:3; 95:5; 80:20; or SG
	$CHCl_3$ / EtOH (19:1)
	CH_2Cl_2 / EtOH (SG)
	EtOAc / MeOH (SG)
Alumina	$CHCl_3$ / MeOH (2:1; or SG)
	$CHCl_3$ / EtOH (SG)
	CH_2Cl_2 / EtOH (9:1; SG)
	EtOAc / MeOH (1:1)
	EtOAc / EtOH (2:1;
	(1:1; or SG)
	Me_2CO / CH_2Cl_2 / H_2O
	(62.5: 15: 10)
Sephadex LH20	$CHCl_3$ / EtOH (88:12)
	CH_2Cl_2 / MeOH (SG)
	CH_2Cl_2 / MeOH (SG)
	CH_2Cl_2 / Me_2CO
Reverse-phases	
Amberlite XAD-2	H_2O / MeOH (SG)
	H_2O, then EtOH
Amberlite XAD-16	H_2O, then EtOH
Sephadex LH20	EtOH / H_2O (7:3)
	MeOH
Polyamide	H_2O
Ion-exchange	
DEAE-Sephadex	Step-gradient of NaCl in H_2O

The separation on alumina is generally better than on silica but irreversible adsorption. Some decomposition, and adjacent group transformation may occur. Planar development may also be used for isolation of ecdysteroids. Silica plates are generally used with dichloromethane and ethanol (96%) (11,22).

Analysis of ecdysteroids

The uniformity of ecdysones was controlled by various analytical procedures that included TLC and HPLC. Purity control was accomplished using both straight-phase thin layer chromatography (NP-TLC) and reverse-phase thin layer chromatography (RP-TLC). The stationary phase was either silica gel with a fluorescence indicator (TLC silica F_{254}) for NP-TLC or octadecyl silica with a fluorescent indicator (RP-TLC C 18 silica F_{254}) for RP-TLC. The selection of an optimal mobile phase was done by a systematic way of trial and error. To ensure the proper selectivity these following mobile phases could be checked for NP-TLC:

A: Dichloromethane –Ethanol 8:2, v/v

B: Ethyl acetate- Methanol- Ammonia (25 % solution), 85:10:5, v/v/v

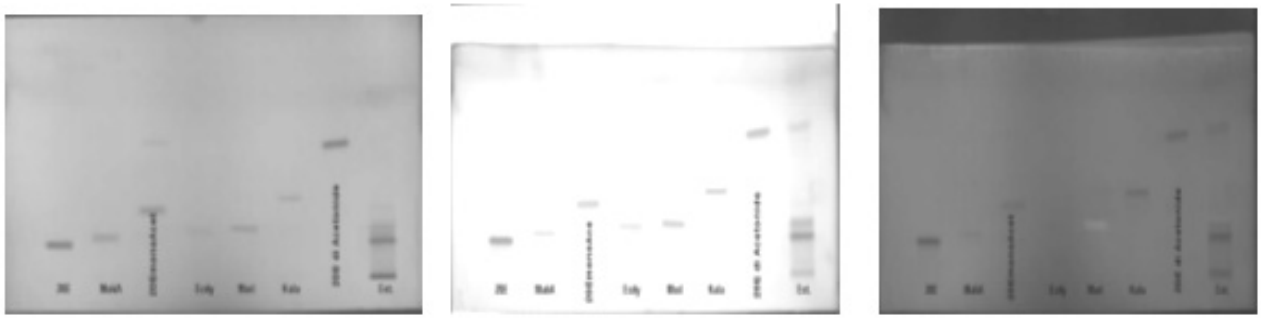
C: Toluene-Acetone-Ethanol (96%)-Ammonia solution, 100:140:32:9 v/v/v/v

D: Chloroform- Methanol- Benzene 25:5:3 v/v/v

E: Ethyl acetate-Ethanol (96%)- Water, 16:2:1 v/v/v

Triple detections were employed to locate the dark spot that was visible under UV light at 254 nm during NP-TLC analysis. After spraying the plate with a vanillin/sulfuric acid or anisaldehyde /sulfuric acid reagent and observed the plates (without any heating) both, on day light also, even fluorescence under 366 nm. (A typical example of several ecdysteroids was identified in NP-TLC plate by triple detection shown on page 24).

TLC- densitometry offers the removal of abundant impurities and reliable analysis of the ecdysteroid. The method is fast, inexpensive, and gives direct comparisons of the series of samples situated on the same TLC plate. The potential decomposition on alumina sorbents is more in case of ecdysteroids; therefore this stationary phase may be substituted by silica. The recent introduction of spherical silica particles to planar chromatography will further improve the potency of TLC. Moreover, the employment of reversed-phase TLC leads to the avoidance of irreversible adsorption, decomposition, and structural alteration of ecdysteroids (7,11).



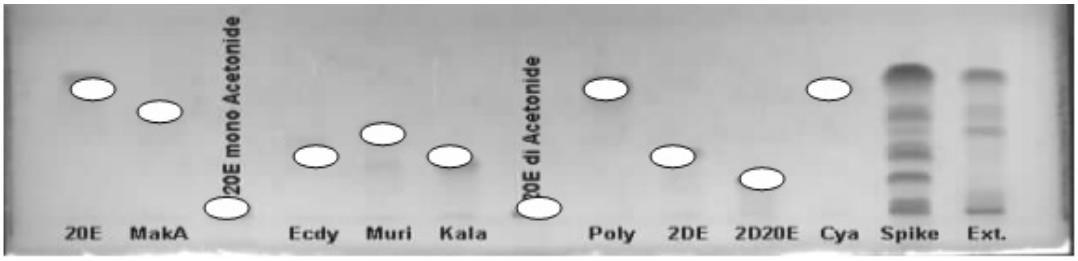


Fig.3: Stationary phase: RP-C18 silica F_{254} plate, mobile phase: methanol: water (50:50), spots 20E(20-hydroxyecdysone), MakA (makisterone-A), 20E monoacetonide (20-hydroxy20, 22 monoacetonide), Ecdy (ecdysone), Muri (muristerone-A), Kala (kaladasterone), 20Edi acetonide (20-hydroxyecdysone 2,3; 20,22 diacetonide), Poly (polypodine-B), 2DE(2-Deoxyecdysone), 2D20E(2-Deoxy20-hydroxyecdysone), Spike of all compounds, kaladana extract

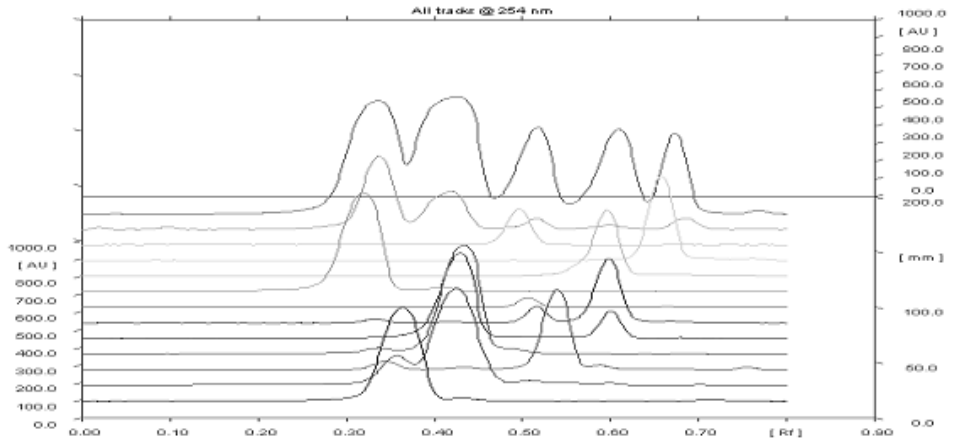


Fig. 4 3D image of the plate

Quantification of ecdysteroids

Both seperation and detection methods were used to determine the ecdysteroids content of the plant extracts. Chromatographic and related seperation methods served to differentiate the ecdysteroids from each other and also from the other compounds. The seperation generated either distinct peaks (at various column techniques) or distinct spots (TLC/HPTLC), however, the detection methods were used to locate of peaks (and spots) belonging to the individual ecdysteroids. When the seperation was monitored using UV detection, it offered certain specificity and also the quantitation. The most popular method for ecdysteroids analysis was HPLC combined with UV detection at 254 nm (HPLC/UV $_{254}$). The detectable signal was the consequence of 7-en-6-one conjugation. As the maximum of ultraviolaet absorbance was really at 240 through 245 nm, the specificity could be slightly increased to performed detection somewhere at that wavelength (23).

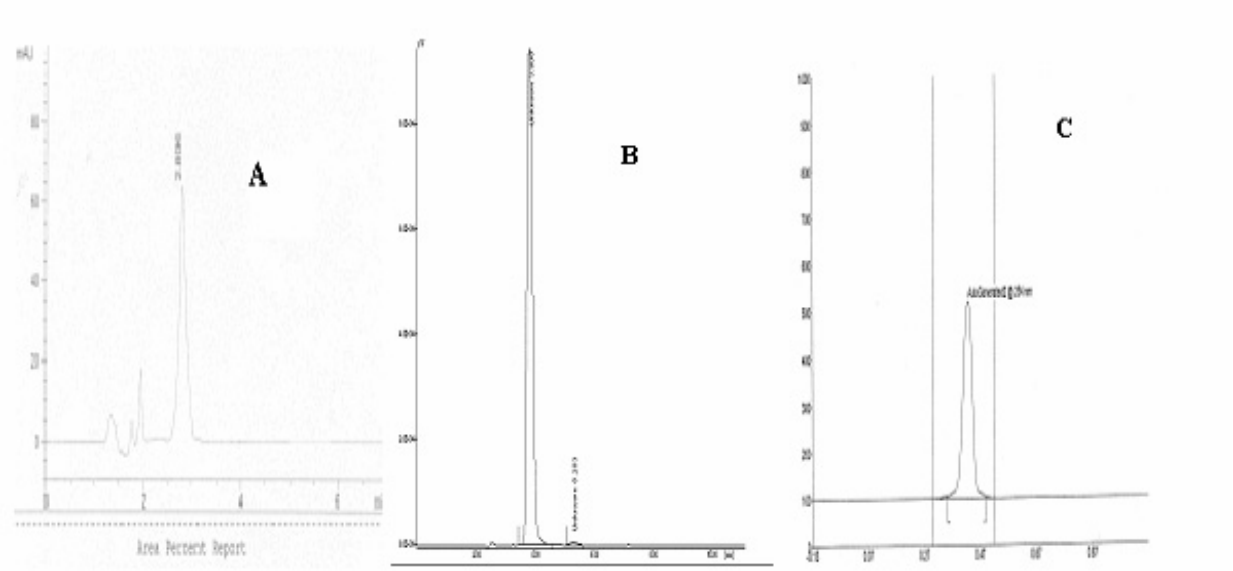


Fig.5 UV detection was performed for 20-hydroxyecdysone, in LC-MS at 246 nm [**A**], in HPLC at 246 nm & 254 nm [**B**] and in HPTLC at 254 nm[**C**].

Structural Elucidation

The 14α- hydroxy-7-en-6-one moiety: UV, IR, NMR, and MS have been utilized to identify the ecdysteroids wherein MS and NMR provided the basic information on the structure. UV and IR rendered this characteristic information on the 7- ene-6-one chromophore: the UV extinction of the 242 nm band is useful for gaining information on sample purity because ecdysones have poor crystalline properties and in many cases the amount is too minute for analytical purification. The IR band at 1650 cm^{-1} is low for a conjugated enone (in KBr disc), which is presumably due to intermolecular hydrogen -bonding with a hydroxyl group in the solid phase, a wide band at 3400 cm^{-1} verifies the presence of hydroxyl groups. The NMR spectra of ecdysones are complex, but the signals of the methyl groups of the ecdysones generally appeared as sharp peaks in the spectra of these compounds and provided valuable clues to their structure. It has been observed that the proton resonance signals are not resolved well enough, but the signals of angular (C-18 and C-19) and the side-chain (C-21, C-26 and C-27) methyl groups are adequately sharp to provide valuable support for the structural evaluations. The methyl signals appear for all ecdysones at about δ 0.6-1.5 ppm. Their chemical shift is the consequence of substitutions on the attached ring as well as the side chain, which gives information on the structure. Signals from hydroxyl, ester and ether groups, as well as olefin protons, appear at higher δ values. A particular characteristic of all the ecdysteroids is the 7-ene-6-one conjugation that is evidently same by a signal at δ 5.75-6.0.

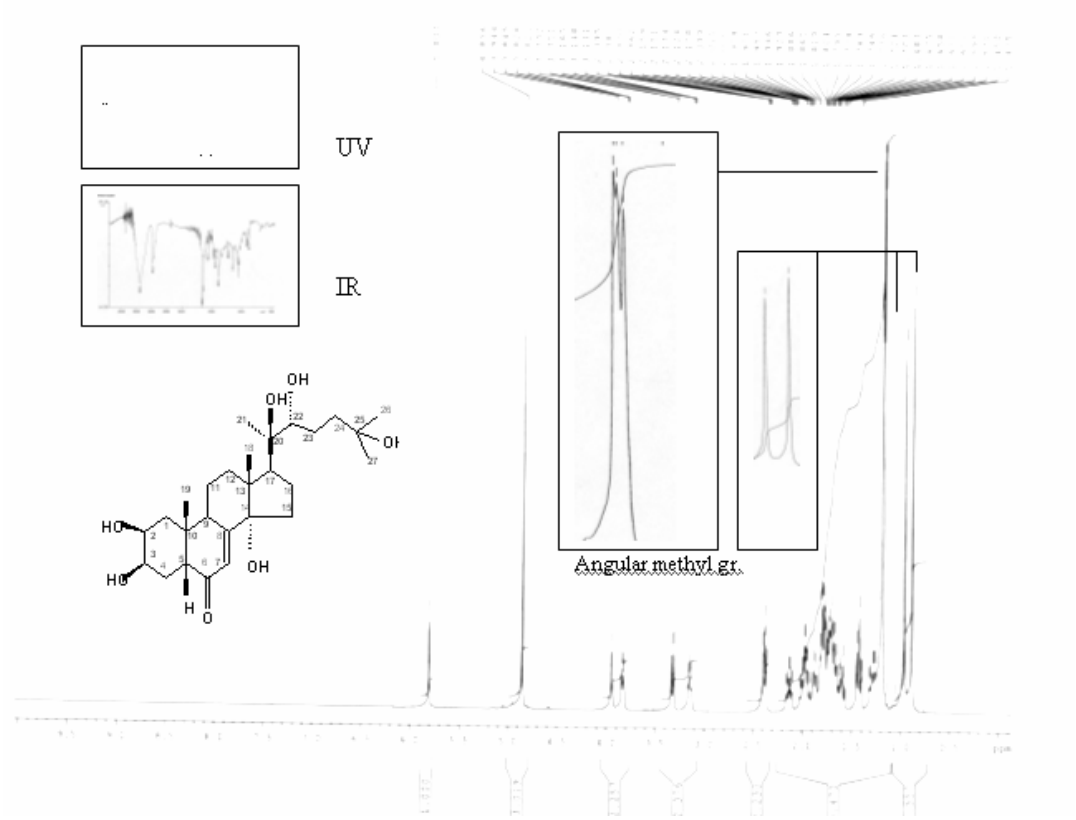
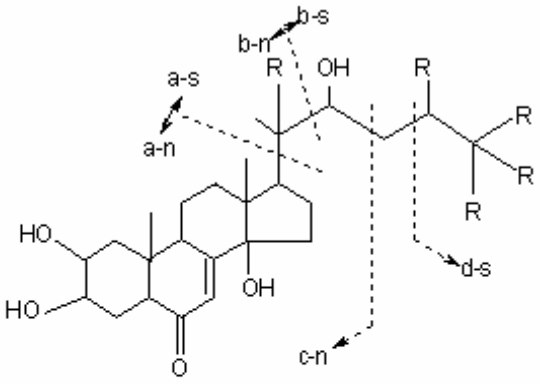


Fig.6 UV, IR, ^{1}H -NMR spectra of 20-hydroxyecdysone



[I] nucleus

a-n, b-n: presence/absence of additional groups

[II] sidechain

d-s: branching at C-24

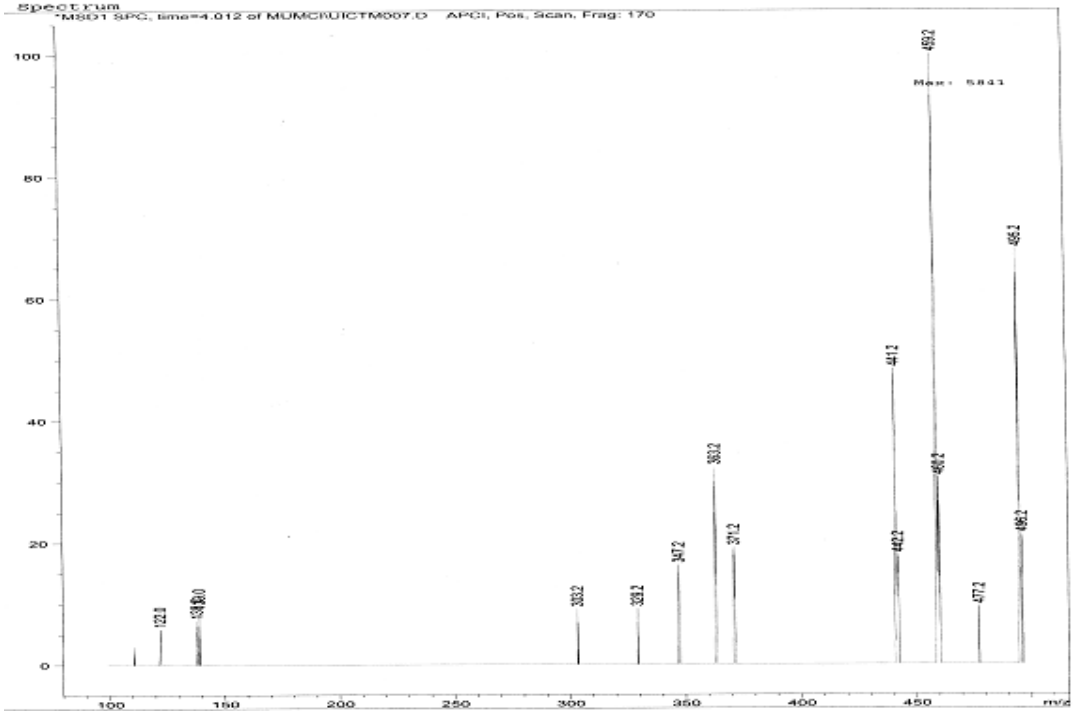


Fig.7 LC-APCI-MS spectra of makisterone-A in positive ESI mode

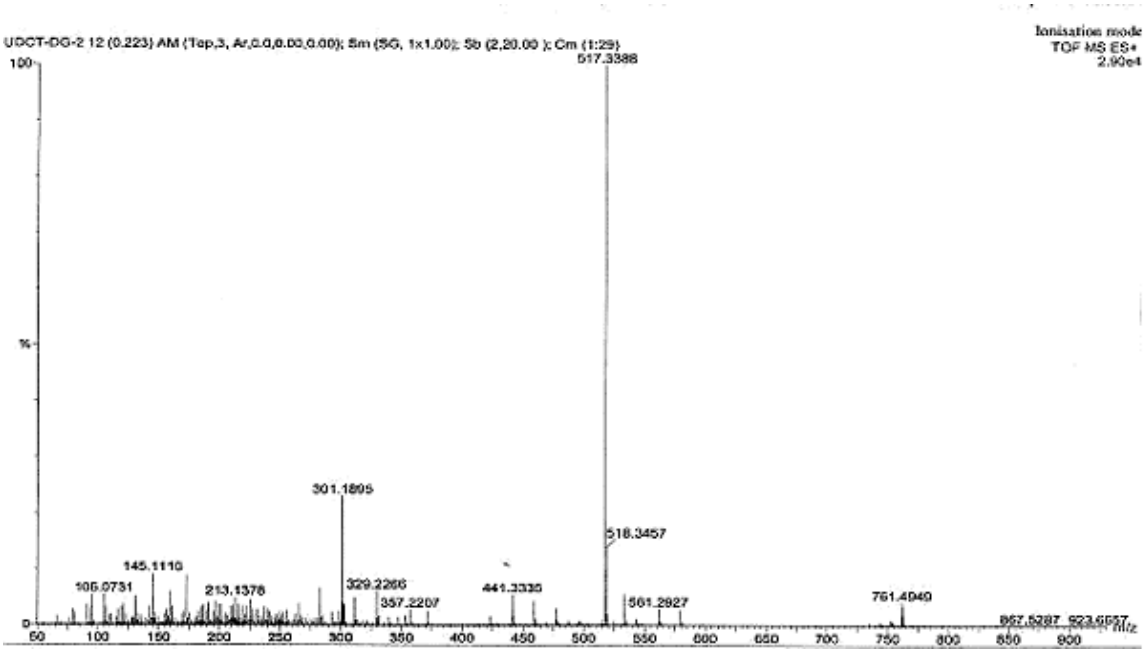


Fig.8 Q-TOF Micromass spectra of makisterone-A

Mass spectra (generalization): The main fragmentation occur at C-17/20 (fission a) and, to a greater extent, at 20/22 (fission b). Both fissions give rise to two series of peaks designated 'n' (nuclear) and 's' (sidechain). Fission c between 22/23 is minor. An additional fission d occurs if branching is present at C-24. Similar fragmentation processes take place with the acetates as well. The 2,3,20,22-diacetonide and 20,22-mono acetonide spectra are characterized by

strong peaks above m/e 300, and many metastable peaks, the main fission occurring at 17/20 (24,25).

Loss of water from the ecdysteroids is facile and can be easily documented. Ions arising from the loss of a molecule of water in makisterone-A shows 477($M+1-H_2O$)$^+$, 459($M+1-2H_2O$)$^+$, 441($M+1-3H_2O$)$^+$, 301, but the major differences was found in Quadruple Time of Flight (Q-Tof) mass spectra where moleculat ion peak is absent but pseudomolecular ion peak or adduct ion peak is prominent $(M+Na)^+$ but in CI, the molecular ion peak $(M+1)^+$or $(M)^+$ is prominent (see above fig. 5 & 6).

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