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Original Article

High-performance liquid chromatography and tandem mass spectrometric analysis of beta ecdysone from *Achyranthes aspera* extract: An antimalarial drug

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ABSTRACT

The objective of the paper is to isolate beta ecdysone, an insect molting hormone in *Achyranthes aspera* seed extract by high-performance liquid chromatography (HPLC) and tandem mass spectrometry analysis. Refined extract of *A. aspera* is obtained from Green chem herbal extracts and formulations, Bengaluru. Beta ecdysone is purchased from Sigma. HPLC analysis is performed by Shimadzu LC-20AD prominence gradient system and mass spectra is detected by Shimadzu LC-MS 8030 triple quadrupole mass analyzer. The result reveals that content of beta ecdysone in *A. aspera* extract was 0.05% w/w. Spectra generated for beta ecdysone standard in positive selective ion monitoring gave protonated molecule $[M+H]^+$ 481 and in negative selective ion monitoring gave deprotonated molecule $[M-H]^-$ 479. Mass value (m/z) of beta ecdysone is 480.65. Same mass of $[M+H]^+$ 481 and $[M-H]^-$ 479 in spectra generated for *A. aspera* extract eluted at retention time 12.5 min, confirms the presence of beta ecdysone. Beta ecdysone is found to be richly present in *A. aspera* seed extract further *in vivo* studies should be done to prove its anti-malarial activity to combat the vector borne diseases.

Keywords: *Achyranthes aspera,* beta ecdysone, insect molting hormone, vector borne disease

Introduction

"Vector-borne disease" is the term commonly used to describe an illness caused by an infectious microbe that is transmitted to people by blood-sucking arthropods. Mosquitoes, sand flies, ticks, and other biting bugs can cause some of the most devastating diseases such as malaria, chicken gunia, dengue fever, Japanese encephalitis, and yellow fever.[1] These arthropods cause a particular problem for native populations. Mosquitoes are attracted to people by skin odors and the carbon dioxide from breath.[2]

The use of insect repellents makes a person unattractive for feeding and therefore repels the mosquito. Advantages of using insect repellents are that they are non-invasive, safe and can be easily utilized for application.[3,4] Commonly used insect repellents are N,N-diethyl benzamide and N, N-diethyl-3-methylbenzamide, picardin, pyrethroids, and essential oils.[5,6]

Ecdysone is a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone, which is secreted from the prothoracic glands. Insect molting hormones (ecdysone and its homologues) are generally called ecdysteroids.[7] Induction of molting in *Drosophila* coincides with release from the ring gland of 20-hydroxyecdysone, also known as ecydsone. Ecdysone receptor (EcR) is induced at the beginning of the gene activation hierarchy.[8,9] EcR is induced directly by ecdysone, and provides an auto regulatory loop that increases the level of receptor protein in response to the hormone ligand. EcR exists in three isoforms, each one having an different biological function. The steroid hormone ecdysone is the central regulator of insect developmental transitions.

Ecdysteroids are a class of compounds (polyhydroxylated ketosteroids, with various tails) that are structurally similar to androgens. They are well studied as plant and insect growth factors, and derived their name (ecdy-) from the process of molting in insects, called ecdysis. Ecdysteroid is a category, and popular ecdysteroids include "ecdysone," "ecdysterone," "turkesterone" and "20-hydroxyecdysone."[10]

They have some biological effects in mammals when orally ingested, and have been called by some researchers as behaving similar to anabolic steroids putatively without the androgenic effect. Due to

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the lack of androgenicity, their safety profiles are much greater than anabolic androgenic steroids.

In addition, they seem to have a wide variety of side effects that are deemed as healthy.[11]

Ecdysteroids can lower cholesterol and blood glucose, are seen as healthy for the liver and intestines by increasing protein synthesis rates, and may have protective effects on neural tissue. 20-EC has been shown to dramatically increase prostaglandin synthesis in mammals. Very high concentrations of intracellular arachidonic acid were noted following administration of 20-EC with Vitamin D3.

Achyranthes aspera is an annual, stiff-erect herb found commonly as a weed throughout India.[12,13]The leaf ethyl acetate extract showed high larvicidal activity on the tick larvae of *Rhipicephalus*. It possess medicinal properties and used in treatment of cough, bronchitis and rheumatism, malarial fever, dysentery, asthma, hypertension, and diabetes in Indian folklore.[14,15] The chloroform and ethanol root extracts of the *A. aspera* are reported to have anti-implantation and abortifacient activity. The ethanol extract of the root possess spermicidal activity. Leaf extracts were reported to possess thyroid stimulating and antiperoxidative properties.[16,17]

It strengthen the liver and kidneys, strengthening muscles, tendons and bones, anti-inflammatory, anti-toxin, urine laxative, normalize menstruation, hemostatic, and ease childbirth. Chemical ingredients: Akirantin, glokosa, galactose, reilosa, ramnosa, alkaloids. Hentriakontan, sapogenin, betaine, ecdysterone, triterpenoid saponins.[18,19]

The nomadic Maasai people of East Africa used *A. aspera* to cure malaria. The alcoholic extract of this plant showed lowering the levels of total serum cholesterol and phospholipid, triglyceride (TG) and total lipid in triton-induced hyperlipidemic rats.[20-22]The methanolic leaf extract at showed significant lowering of serum lipids such as total cholesterol, TG, high density lipoprotein, and low density lipoprotein. The whole plant of *A. aspera* found to have cytotoxic compounds.

The objective of the study is to isolate the beta ecdysone from *A. aspera* extract by high-performance liquid chromatography (HPLC) and tandem mass spectrometry analysis.

Materials and Methods

Plant material

A. aspera extract is obtained as a gift sample from Green Chem Herbal Extracts and Formulations, Bengaluru.

Chemicals and reagents

Beta ecdysone is purchased from Sigma, acetonitrile HPLC grade solvents; all analytical grade solvents obtained from E-Merck Ltd., Mumbai, India.

Estimation of beta ecdysone in *Achyranthes* extract by HPLC method

The Shimadzu class LC-20AD HPLC, prominence gradient system, column used was Phenomenex C-18, Luna, - SS column 150 mm × 4.6 mm, 5 µ particle size. The output was monitored and processed using LC solution version 1.21 SPI software on a Pentium computer (Hewlett Packard).

Mobile phase: (A) Water, (B) acetonitrile (gradient system)
Detection: 254 nm
Flow rate: 1.0 mL/min

Gradient system

Time (min)	B concentration (%)
0.01	0
1.5	1.2
5.0	15.0
7.5	25.0
25.0	60.0

Standard solution

10 mg of beta ecdysone reference standard (Chromadex) is accurately weighed and dissolved in 25 ml of 40% methanol. It is heated on water bath for 15-20 min. The volume is made up to 25 ml, with 40% methanol, filtered and the solution is injected.

Sample solution

100 mg of *Achyranthes* extract is weighed accurately and dissolved in 25 ml of 40% methanol. It is heated on water bath for 15-20 min. The volume is made up to 25 ml, with 40% methanol, filtered, and the solution is injected.

20 ul of sample and standard is injected separately. Calculate the area of beta ecdysone in standard as compared to sample and the assay is calculated.

HPLC analysis

Equation 1:

Calculation of assay:

$$\frac{\text{Standard weight}}{\text{Sample weight}} \times \frac{\text{Sample area}}{\text{Standard area}} \times \%\,\text{Assay}$$

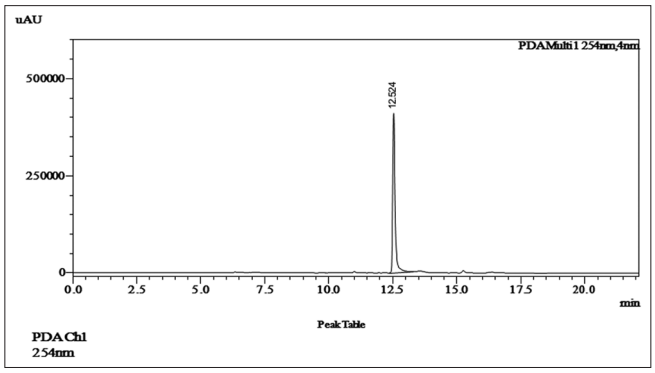
Calculation of assay of beta ecdysone in *A. aspera*:
Standard: Beta ecdysone/reference standard (Chromadex) (00005015-052)
Standard weight: 12.5 mg in 25 ml of 40% methanol
Standard area: 2,616,412
Standard purity: 99%
Sample weight: 100.8 mg in 25 ml of 40% methanol
Sample area: 12,731

Assay of beta ecdysone in test sample:
(12.5/100.8) × (12,731/2,616,412) × 99% = 0.05% w/w

PDA Ch1 254 nm

Peak#	Retention time	Area	Height	Area %	Height %
1	12.524	2616412	410225	100	100
Total		2616412	410225	100	100

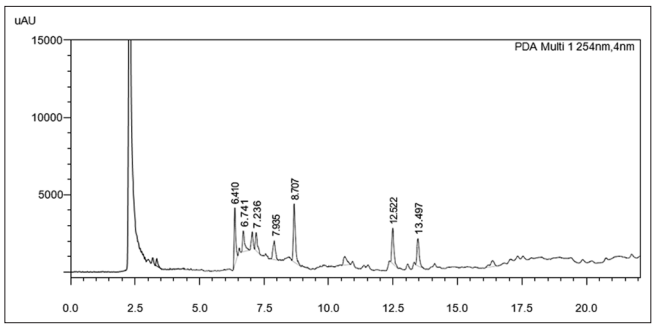
Chromatogram



Sample name: *Achyranthes aspera*
Data filename: Beta ecdysone 04.lcd
PDA Ch1 254 nm

Peak#	Retention time	Area	Height	Area%	Height%
1	3.333	2525	516	2.725	3.106
2	6.410	15,487	3733	16.709	22.451
3	6.741	7792	1339	8.407	8.054
4	7.236	6080	1150	6.560	6.918
5	7.935	7906	1220	8.530	7.336
6	8.707	20,518	3719	22.138	22.370
7	10.671	4950	534	5.341	3.209
8	12.522	12,731	2279	13.736	13.709
9	13.497	10,486	1747	11.314	10.510
10	16.391	4207	388	4.540	2.337
Total		92,682	16,627	100.000	100.000

Chromatogram



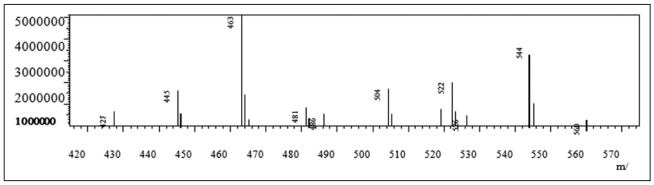
Liquid chromatography–tandem mass spectrometry (LC-MS/MS) scanning for beta ecdysone in *Achyranthes* extract

Shimadzu LC-MS 8030 triple quadruple mass analyzer was run in scanning mode and selective ion monitoring mode. Attached chromatograms show photodiode array and full mass spectra from LC-MS injection of samples beta ecdysone and achyranthes extract. Spectra generated for achyranthes extract in positive ion mode gave protonated molecule $[M+H]^+$ 481 for beta ecdysone similarly spectra generated for achyranthes extract in positive selective ion monitoring gave protonated molecule $[M+H]^+$ 481 for beta ecdysone spectra generated for achyranthes extract in negative ion mode gave deprotonated molecule $[M-H]^-$ 479 for beta ecdysone. Similarly, spectra generated for achyranthes extract in negative selective ion monitoring gave deprotonated molecule [M−H]− 479 for beta ecdysone. Spectra generated for beta ecdysone reference standard in positive selective ion monitoring gave protonated molecule $[M+H]^+$ 481 and in negative selective ion monitoring gave deprotonated molecule $[M-H]^-$ 479.

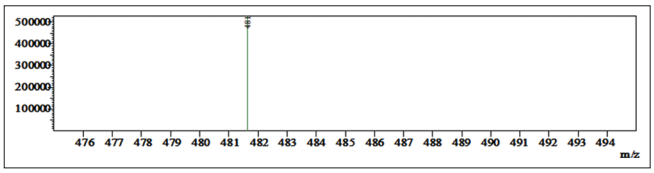
Mass value of beta ecdysone is 480.65.

MS spectrum

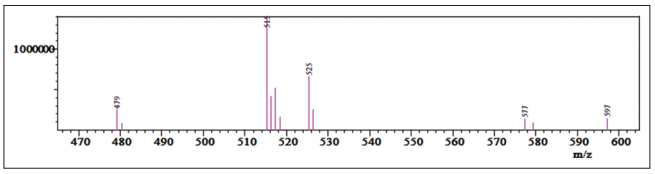
Line #: 1
Retention time (RT): 12.621 (Scan#: 11833) scan
positive
Mass value: 481
Mass peaks: 18
Spectrum mode: Single 12.621 (11,833)
Base peak: 463 (5,121,396)
BG mode: Averaged 13.414-21.905 (12,577-20,537)
Segment 1-Event 1



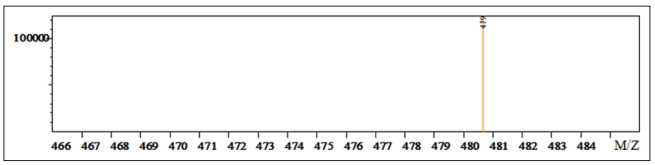
Line #: 2
RT: 12.622 (Scan#: 11834) positive selective ion monitoring
Mass value: 481
Mass peaks: 1
Spectrum mode: Single 12.622 (11,834)
Base peak: 482 (527,477)
BG mode: Averaged 13.416-21.907 (12,578-20,538)
Segment 1-Event 2



Line #: 3
RT: 12.623 (scan#: 11835) scan negative
Mass value: 479
Mass peaks: 11
Spectrum mode: Single 12.623 (11,835)
Base peak: 515 (1,407,349)
BG mode: Averaged 13.417-21.907 (12,579-20,539)
Segment 1-Event 3

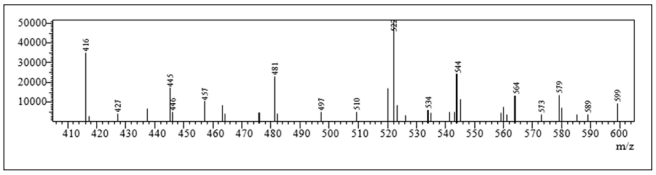


Line #: 4
RT: 12.625 (Scan#: 11,836) negative selective ion monitoring
Mass value: 479
Mass peaks: 1
Spectrum mode: Single 12.625 (11,836)
Base peak: 480 (123,906)
BG mode: Averaged 13.418-21.909 (12,580-20,540)
Segment 1-Event 4

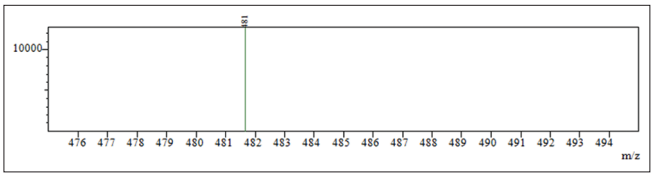


MS spectrum

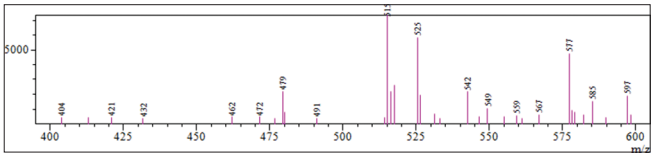
Line#: 1
RT: 0.000 (Scan#:1) scan positive
Mass value: 481
Mass peaks: 34
Spectrum mode: Averaged 12.378-12.843 (11,605-12,041)
Base peak: 522 (51,776)
BG mode: Averaged 12.800-21.926 (12,001-20,557)
Segment 1-Event 1



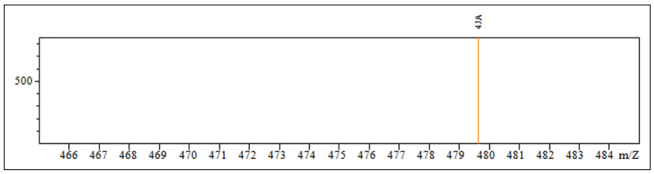
Line #: 2
RT: 0.002 (Scan#: 2) positive selective ion monitoring
Mass value: 481
Mass peaks: 1
Spectrum mode: Averaged 12.379-12.844 (11,606-12,042)
Base peak: 482 (12,711)
BG mode: Averaged 12.802-21.928 (12,002-20,558)
Segment 1-Event 2



Line #: 3
RT: 0.002 (Scan#: 3) scan negative
Mass value: 479
Mass peaks: 33
Spectrum mode: Averaged 12.380-12.845 (11,607-12,043)
Base peak: 515 (7332)
BG mode: Averaged 12.802-21.929 (12,003-20,559)
Segment 1-Event 3



Line #: 4
RT: 0.004 (Scan#: 4) negative selective ion monitoring
Mass value: 479
Mass peaks: 1
Spectrum mode: Averaged 12.381-12.846 (11,608-12,044)
Base peak: 480 (849)
BG mode: Averaged 12.804-21.930 (12,004-20,560)
Segment 1-Event 4



Results and Discussion

Beta ecdysone was analyzed in *A. aspera* extract by HPLC method. Principal peak due to beta ecdysone in reference solution at RT 12.5 min was also present in the chromatogram of *A. aspera* extract sample solution. Content of beta ecdysone in *A. aspera* extract was 0.05% w/w.

Mass spectrometric analysis (ESI) was carried out on a Shimadzu LC-MS/MS-8030 in positive mode and negative mode. Samples analyzed were beta ecdysone and *A. aspera* extract. Spectra generated for beta ecdysone reference standard in positive selective ion monitoring gave protonated molecule $[M+H]^+$ 481 and in negative selective ion monitoring gave deprotonated molecule $[M-H]^-$ 479. Mass value (m/z) of beta ecdysone is 480.65. Same mass of $[M+H]^+$ 481 and $[M-H]^-$ 479 in spectra generated for *Achyranthes aspera* extract eluted at RT 12.5 min, confirms the presence of beta ecdysone.

Conclusion

In this study, a sensitive, rapid, and selective HPLC and LC-MS/MS method was developed and validated for the simultaneous determination of beta ecdysone in *A. aspera* leaf extract. In addition, application of several chemical and physical methods to evaluate the quality of this drug should be established for its antimalarial efficacy. The methods in this study can provide a standard procedure for quantity control of herbal pharmaceutical products.

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