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Indian medicinal plant, *Coscinium fenestratum*- A new bio source for the multifunctional bio active molecule– ecdysterone

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Abstract
Phytochemical investigation on *Coscinium fenestratum* (Gaertn.) Collebr, an important Ayurvedic plant, revealed the presence of significant amounts of ecdysterone in the stem (0.22%) and leaves (0.12%), in addition to berberine. Ecdysterone was characterized using High Performance Liquid Chromatography (HPLC), Infrared Spectroscopy (FT-IR) and Liquid Chromatography-Mass Spectroscopy (LC-MS). Isolation of this multi- functional bioactive compound will throw light on the chemical basis for the various pharmacological effects of *Coscinium* plant extract.

Keywords: Coscinium fenestratum - ecdysterone -first report-ayurvedic-daru haridra

1. Introduction
Coscinium is a dioecious woody climber from the family Menispermaceae and in India it is endemic to the Western Ghats, especially in high rainfall wet evergreen and semi evergreen forests [1]. It is a highly traded medicinal plant and its stem is used in more than 60 ayurvedic preparations [2]. Ethno botanically the species has been used to treat a variety of ailments such as ulcers, skin diseases, eye disorders, inflammation, hypertension, jaundice, diabetes and snake bites [3-7]. Hypoglycemic activities of alcoholic stem extracts of *Coscinium* have been confirmed in rat models [8-12]. Yibchok- anun [11]. through *in-situ* pancreatic perfusion has proved that *C. fenestratum* stem extract had profound effect on stimulating insulin secretion and, the study established that Berberine, the major component of the extract, was not responsible for this activity. A phytochemical investigation was carried out on the stem and leaves of *C. fenestratum* for identifying bioactive molecules other than berberine. Though its stem is used extensively in Ayurveda, its leaves are usually discarded as waste. Ecdysterone (20E) is an analogue of the insect moulting hormone, expressed in some plant species with a wide range of beneficial pharmacological activities (adaptogenic, anabolic, antidiabetic [13-14]. hepatoprotective, immunoprotective, anti tumor *etc*.) and is extremely non toxic to mammals [15].

2. Materials and methods
2.1 Plant material
Plant material - Enough amounts of leaves and stem of *C. fenestratum* was collected from Boys town, Mananthavady, Wayanad District, Kerala in the month of July 2012 (11º84'18" N, 75º92'09" E) and was air dried at 50 ºC. Botanical voucher specimen has been deposited with the herbarium of M.S. Swaminathan Herbarium, Wayanad, Kerala (MSSH-0104).

2.2. Soxhlet extraction
The air dried leaves (100 g) and stem (50 g) were boorishly ground and was sequentially extracted in a Soxhlet extractor starting with Petroleum Ether (PE). Extract was filtered out, solvent removed under reduced pressure using a rotary evaporator (Buchi) at 35 ^{0}C and accurately weighed. The marc was put to further extraction sequentially with Chloroform ($CHCl_3$), Ethyl Methyl Ketone (MEK) and Methanol (MeOH). The extracts were collected, solvent removed and weighed accurately. The experiment was repeated three times and average yield in % w/w for each extract was taken (Table 1).

2.3. Column Chromatography

2.3.1. Leaves

MEK extract (3 g) was accurately weighed and washed with Diethyl ether for removing undesirable fractions which were 20E negative. The remaining extract was dissolved in 6 ml MeOH and pre adsorbed to 6 g acidic alumina (Merck) used for column chromatography. The pre adsorbed extract was loaded to a 60 g acidic alumina column. The elution was started with 100 % $CHCl_3$ and later on moved to MeOH/ $CHCl_3$ mixtures. A total of 25 fractions were collected. Fractions 13-18 (8% MeOH) were Berberine positive and fractions 19-25 were 20E positive. Solvent from 20E positive fraction were removed under reduced pressure and 215 mg of a yellow sticky mass was obtained. This was re suspended in MeOH /Di ethyl ether mixture and left overnight (10^0C). The resulting mixture on scratching yielded 120 mg of 20E crystals (Table 2).

2.3.2. Stem

MEK extract (650 mg) was accurately weighed out and dissolved in 3 ml MeOH and pre adsorbed to 3g acidic alumina as above. The same was loaded to a 35 g acidic alumina column and elution was started with 100% $CHCl_3$ and later on moved to MeOH/ $CHCl_3$ mixtures. A total of 16 fractions were collected. Fractions 3-5 (8% MeOH) were Berberine positive and fractions 7-16 (10% MeOH) were 20E positive. Solvent from 20E positive fractions were removed under reduced pressure and 200 mg of a yellow sticky mass was obtained. This was re suspended in MeOH /Di ethyl ether mixture and left overnight (10 ^{0}C). The resulting mixture on scratching yielded 110 mg of 20E crystals (Table 2).

2.4. HPLC conditions

The HPLC analyses were performed on a Shimadzu -SPD-M-204 instrument equipped with DAD (Diode Array Detector) with Phenomenex luna 5µ C_{18} (2) 100A, size 250×4.6 nm column. 1 mg of standard 20E (Sigma) and sample isolated from *Coscinium* were both dissolved in 1 ml MeOH (HPLC Grade) each. 10 µl of the both were injected separately into the system. All the compounds were detected at 254 nm at room temperature with an effluent rate of 1.2 ml/min. The mobile phase consisted of MeOH (A) and water (B) in 1:1 ratio.

2.5. IR spectrum conditions

The IR spectra of both standard 20E (Sigma) and sample isolated from *Coscinium* were recorded on a Shimadzu IR Affinity -1 machine in KBr.

2.6. LC-MS

Electro spray ionization mass spectra were collected on an Agilent 6340 series ion trap mass spectrometer coupled to an Agilent 1200 series HPLC system. The samples were infused to the mass spectrometer through a reversed phase column (Zorbax SB- C18, 2.1 × 35 cm) with solvent A (0.1% Formic acid in water) and solvent B (0.1% Formic acid in Acetonitrile). The flow rate was maintained at 0.2 ml/min and the UV absorbance was monitored at 254 nm. MS data were acquired over a range m/z, 100- 1500. MS/MS data were collected using collision induced dissociation (CID) with the mass spectrometer operated in a data-dependent mode. All data were acquired in positive ionization mode and were processed with Bruker Data Analysis software.

3. Results & Discussion

The leaves (100 g) and stem (50 g) of *Coscinium* were put to sequential hot extraction starting with Petroleum ether (PE) followed by Chloroform ($CHCl_3$), Ethyl Methyl Ketone (MEK) and finally Methanol (MeOH). The extracts were filtered, solvents removed below 40^0; and the yield obtained is presented in Table 1. Thin layer chromatography (TLC) of the MEK extracts of both leaf and stem showed distinct spots for 20E on comparison with an authentic sample of 20E, but PE, $CHCl_3$ and MeOH extracts were negative. The presence of berberine in MEK and MeOH extracts of both stem and leaves were confirmed by direct comparison with an authentic sample (Sigma) by colour reaction, TLC and HPLC. MEK extracts of leaves and stem were put to column chromatography on acidic alumina. 20E positive fractions were collected, solvent removed to yield crystals of 20E (Table 2). A 0.22% and 0.12% yield for 20E were obtained from the stem and leaves of *C. fenestratum* respectively. These levels of expression itself qualify *C. fenestratum* as a potential source of 20E, since 20E is usually expressed in very low concentration in plants.

The HPLC profiles of both standard 20E (Sigma) and 20E isolated from MEK extract of *Coscinium* leaves had a major peak at Retention time (Rt) 6.3 min with an UV absorbance around 245 nm which indicated the presence of α, β unsaturated carbonyl group, characteristic of 20E (Figure 1).

The IR spectra of both standard 20E (Sigma) and 20E isolated from the MEK extract of *Coscinium* leaves recorded in Potassium Bromide (KBr), revealed a broad hydroxyl absorption (3383 cm^{-1}) and strong conjugated carbonyl absorptions (1653 cm^{-1}) characteristic of an α, β unsaturated keto group and also corresponding to the *7-en-6-one* of ecdysteroids (Figure 2).

The Mass spectrum of 20E isolated from *Coscinium* leaves gave a peak at *m/z* 481 for $[M+H^+]$, which was consistent with the molecular formula $C_{27}H_{44}O_7$ (Figure 3). (Insert figure 3).The other prominent peaks observed were *m/z* 463 $[M+H-H_20]^+$, 445 $[M+H-2H_20]^+$ and 427 $[M+H-3H_20)^+]$ (Figure 4).

3.1 Tables and Figures

Table 1: Yield summary of the Soxhlet extraction for the leaves and stem of Coscinium

Part	Extract	% Yield	Presence of 20E +=Present -= Absent	Presence of Berberine += present -=Absent
Leaves	PE extract	1.3	-	-
	CHCl3 extract	0.9	-	-
	MEK extract	3.1	+	+
	MeOH extract	1.5	-	+
Stem	PE extract	0.8	-	-
	CHCl3 extract	1.5	-	-
	MEK extract	1.4	+	+
	MeOH extract	3.0	-	+

Table 2: The results of column chromatography of the 20E positive MEK extracts of Coscinium with percentage yield.

Sl No	Part used (weight)	Extract (weight)	Weight of 20E obtained	% yield
1	Leaf (100 g)	MEK (3 g)	120 mg	0.12 %
2	Stem (50 g)	MEK (650 mg)	110 mg	0.22%

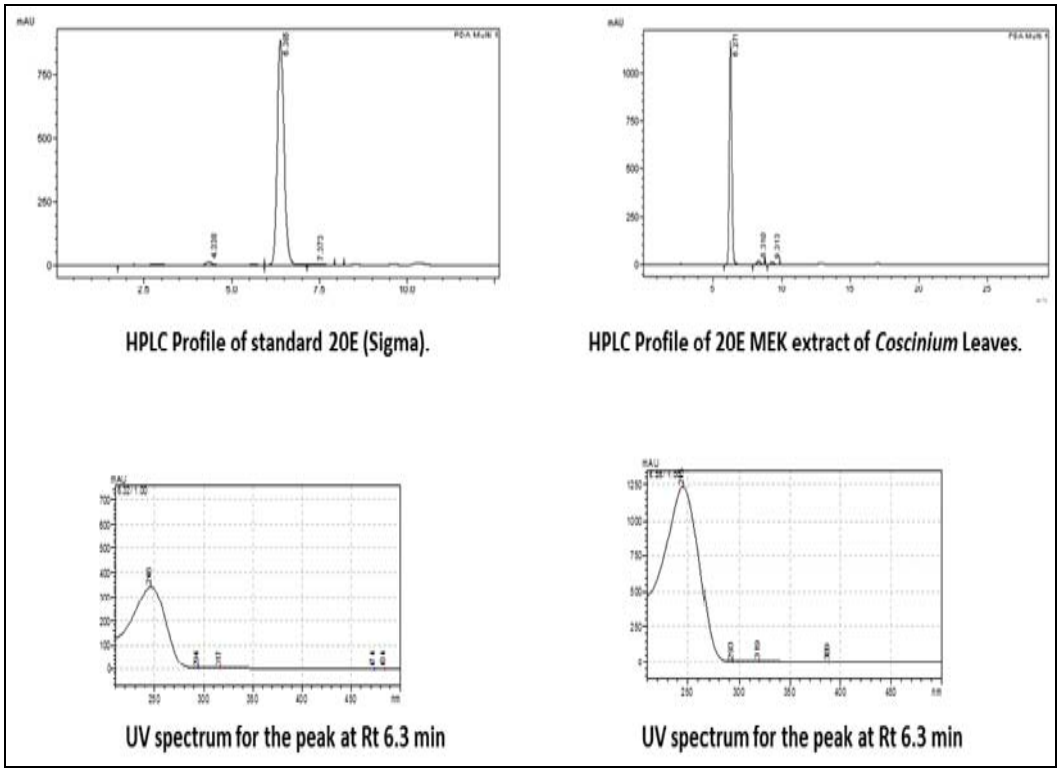

Fig 1: Comparison of HPLC Profiles of Std. 20E (Sigma) and 20E isolated from *Coscinium* leaves.

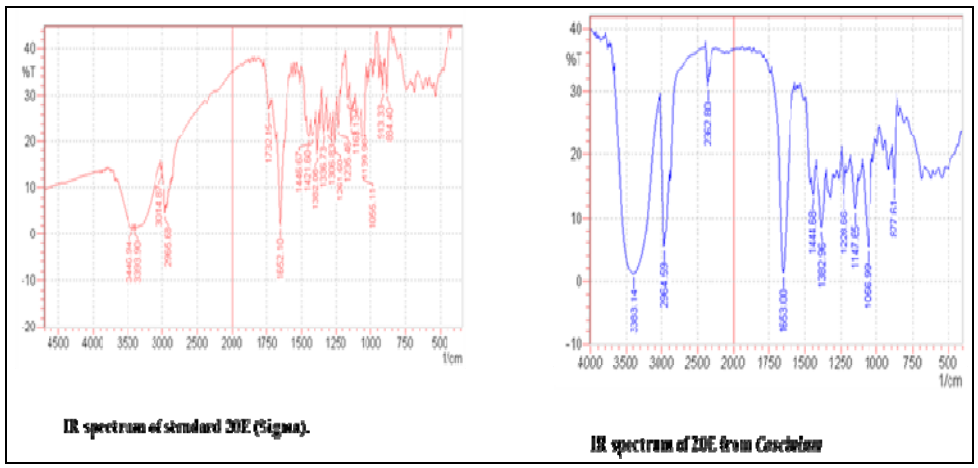

Fig 2: Comparison of FT-IR spectra of Std. 20E (Sigma) and 20E isolated from *Coscinium* leaves.

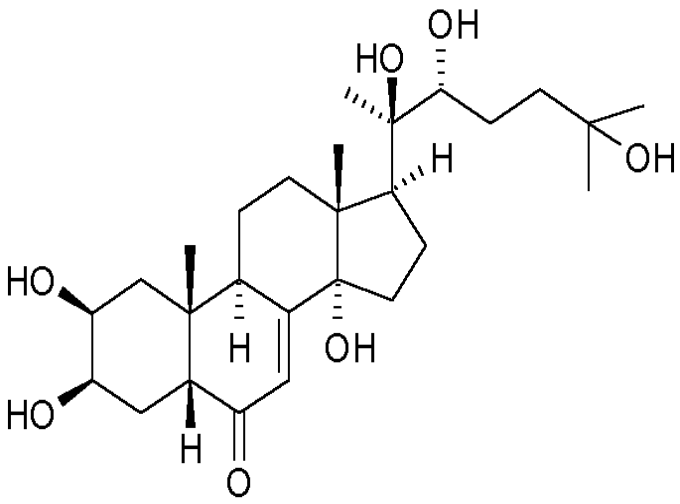

Fig 3: Structure of ecdysterone (20- hydoxy ecdysone)

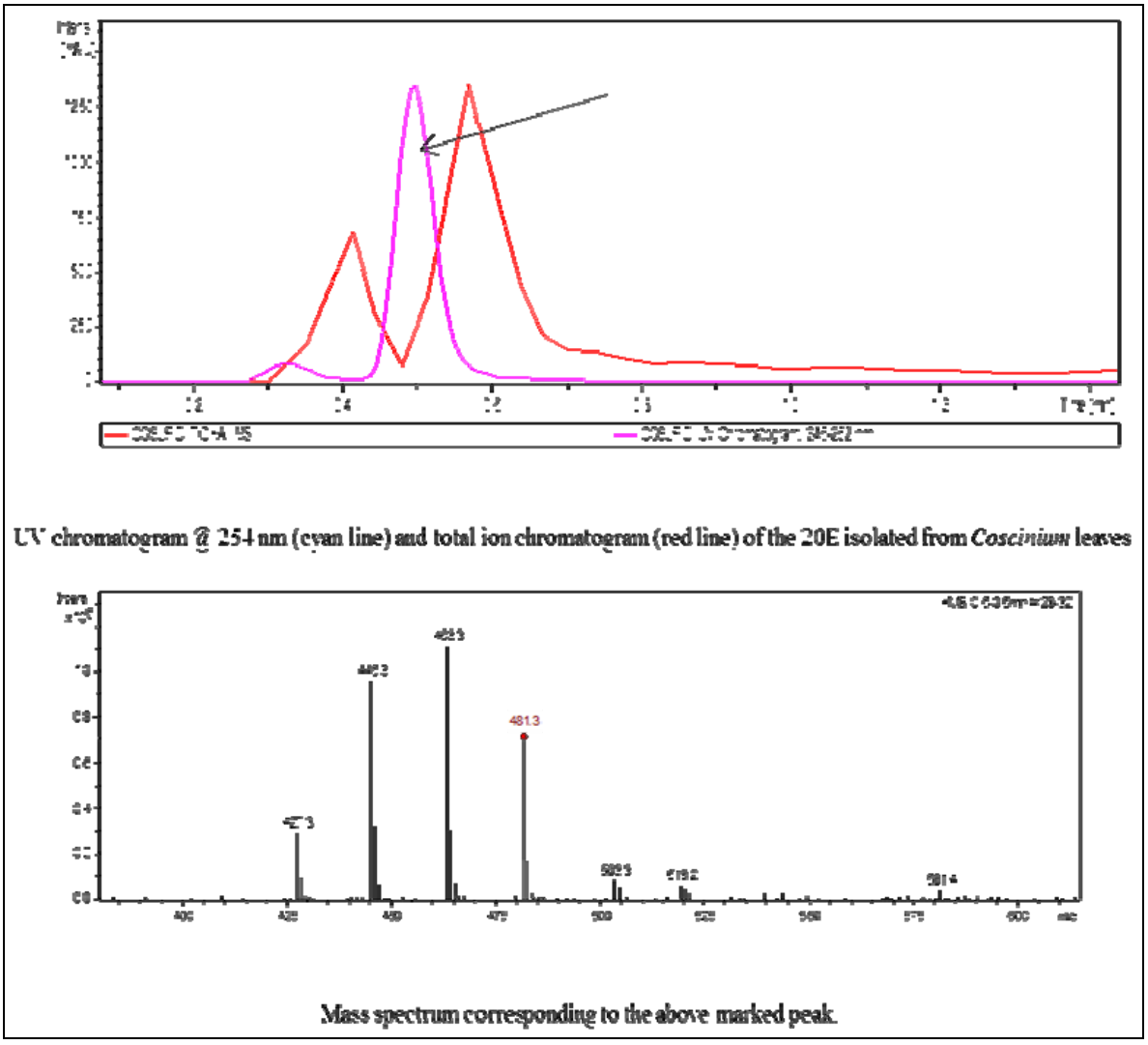

Fig 4: Mass spectrum of 20E isolated from *Coscinium* leaves.

4. Conclusions

Ecdysterone has been isolated, quantified and physically characterised from *C. fenestratum* for the first time. Ecdysterone being a multifaceted active bio molecule [15] may be the potential hypoglycemic active principle of *Coscinium* alcoholic extract reported in rat model [11]. *Coscinium* leaves, usually discarded as a waste from ayurvedic industry can be value added as a potential source for 20E.

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