HPTLC method for estimation of isolated derivative in fractions of seeds of *Ensete superbum*

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Abstract

Isolated chroman derivative from the seeds of *Ensete superbum* Cheesm have been reported to possess anti-implantation activity. The literature survey reveals that no HPTLC methods are reported for the present drug. Hence, an attempt was made that the active derivative can be selected as a standard for the presence of same in different fractions respectively. The isolated chroman derivative along with the fractions was subjected to multi-wavelength scanning and spectrum analysis. The detection and quantification were observed at wavelengths of 256 and 550 nm. HPTLC analysis indicated that the maximum amount of isolated derivative was present in ethanol fraction (1.83%) followed by ethyl acetate (1.74%) and methanol fraction (0.70%).The petroleum ether fraction of the extract was found to contain no traces of the isolated compound. Linearity studies indicated that isolated derivative was present in the linear range of 100 -500 ng. Since this method resolves and quantifies the isolated chroman derivative effectively, therefore it can be used as a marker compound to quantify the concentration of this active principle in its different extracts.

Key words: *Ensete superbum* Cheesm, HPTLC, chromane derivative.

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Introduction

The rapid growth in the global population with a persistently increasing birth rate has intensified research in the direction of regulation of fertility. Due to certain common side effects which

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include, nausea, vomiting, weight gain, cardiovascular problems; the use of steroidal contraceptives is less acceptable. This led to search for safe non -steroidal post-coital contraception [1]. One approach being pursued to identify new antifertility agents is the search for their presence in natural sources. Many plant preparations are reported to have fertility regulating properties in ancient Indian literature [2-4].

So far no single plant is available which can be used on humans as a potent anti-fertility agent. Hence, the search needs to be continued, *Ensete superbum* stands in the order of priority for the activity [5]. *Ensete superbum* Cheesm reported as occurring naturally only in India was suggested by Fransworth for reinvestigation for anti fertility activity[6].

The fraction (VIDR-2GD) isolated from its seeds has been reported to possess anti-implantation activity [7].

In another study the isolated compound a chroman derivative 4-(4-hydroxy-3-methyl-hex- 5-enyl)-chroman-2, 7-diol, possess significant anti-implantation activity and anti-estrogenic activity [8].

Since the seeds of *Ensete superbum* Cheesm are not easily available and the yield of the isolated compound by chemical method [7] was very less (0.1%) for elaborate anti –fertility studies. Keeping this in view this work was carried using column chromatography method, taking solvents of different polarity and passing it through the silica column.HPTLC analysis of the extract and its fractions was standardized [9]. The extract and its fractions were subjected to multi-wavelength scanning, and spectrum analysis using isolated chroman derivative (ICD) as the marker compound [10]. Hence the proposed method has been validated as per ICH guidelines [11, 12 &13].

Materials and Methods

Equipment
A CAMAG TLC system comprising of a Linomat-5 applicator and CAMAG TLC scanner III , Reprostar and Wincat 4.02, integrated software(3.3.1,Anchrome Switzerland)

Chemicals
Analytical grade petroleum ether, ethyl acetate, ethanol, methanol, toluene, formic acid, acetic acid were obtained from Merck, India. Silica gels G 60-120 mesh (for column chromatography (CDH, New-Delhi). Pre-coated silica gel $G60F_{254}$, 10 X 10 cm aluminium plates obtained from (Merck India)

Drugs
Seeds of *Ensete superbum* Cheesm were collected from the forest area of Waynard district in Kerala during the month of May-June. Seeds were dried in the shade at the temperature of $28\pm2^{o}C$ and were authenticated at NISCAIR (National Institute of Science Communication and Information Resources) CSIR, New Delhi, (Ref No. NISCAIR/RHM/F-3/2005 Conslt. No. 544/19).

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Isolation of standard chroman derivative
The kernels of the dried seeds of *Ensete superbum* (2.5 kg) was extracted with 95% (v/v) ethyl alcohol in Soxhlet apparatus, a red colored substance was obtained (crude extract, 1.92%)which was further dissolved water and treated basic lead, sodium sulphate and calcium carbonate to obtain a yellowish white solid (0.1%)[7]. The isolated compound was further characterized using FTIR,IH NMR,13C NMR and GC-MS and presumed to be a chroman derivative, 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol of molecular formula $C_{16}O_4H_{22}$[8].

Isolation of different fractions of ethanolic extract
The dried alcoholic extract of *Ensete superbum* Cheesm (10 gm) was column chromatographed over silica-gel (60-120 mesh).Elution of the column was carried out with petroleum ether, (1%); ethyl acetate(5%), methanol (5%)and ethanol(25%)respectively.
The extract and its fraction were then subjected to TLC using different solvent systems and the presence of ICD (Isolated chroman derivative) in each was detected in Iodine vapors**.**

Preparation of standard chroman derivative
A stock solution of ICD was prepared by dissolving 1mg of isolated compound in 10ml of methanol to give a concentration of 100mcg/ml.The stock solution was further diluted with methanol and 0.5-4μl of the solution was spotted in duplicates and subjected to HPTLC. The chromatogram were developed and scanned at 254nm to detect its limit of detection.
The stock solution was further diluted with methanol and 2.5-7 μl were spotted in duplicate. The chromatogram was developed and scanned at 254nm to detect its limit of quantification.

Preparation of calibration curve of standard chroman derivative
Linearity of an analytical method is its ability to elicit test result that are directly or by a well defined mathematical transformation proportional to the concentration of analyte in the sample within a given range. Linearity is determined by a series of three to six application of standard**.**
A stock solution of ICD was prepared by dissolving 1mg of isolated compound in 10ml of methanol to give a solution of 100μg/ml concentration**.** Calibration curve from 300-900 ng /spot was prepared by plotting peak area vs. concentrations and checked for reproducibility, linearity and validating the proposed method. The correlation coefficient, coefficient of variance and the linearity of results were calculated.

Chromatographic conditions
Stationary Phase - Precoated silica gel G_{254} plates (10 x 10cm,0.2 mm thickness)
Mobile Phase – Toluene: Ethyl acetate: formic acid (5.0: 4.0: 1 v/v)
Development Chamber –Twin Trough Glass Chamber
Scanning range –190-800 nm (with 20 nm interval)
               200-320 nm (with 10 nm interval)
Software – Wincat software

Preparation of fractions from ethanolic extracts of *Ensete superbum* Cheesm
All the extract/fractions of *Ensete superbum* Cheesm were subjected to HPTLC analysis to quantify the amount of ICD in the extract and its fractions. Different concentration of the ICD 1mg/ml (4ul, 8ul, 10ul, 10ul) was spotted along with the extract and its fractions ethyl acetate, methanol and ethanol (5ul).along different tracks respectively. The chromatogram were

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developed using mobile phase (toluene: ethyl acetate: formic acid in the ratio of 5.0:4.5:0.5), and scanned. The tracks were scanned at 256nm and the R_f region 0.70 to 0.73 of isolated compound in each track was selected and plotted. The amount of ICD in the extract/fractions was determined from the linear graph.

Analytical procedure

Silica gel 60 F254 precoated plates (10 x 10 cm) were used with toluene: ethyl acetate: formic acid in the ratio of 5.0:4.5:0.5v/v as solvent system. Sample was spotted on precoated TLC plates by using Linomat V spotter. Ascending mode was used for development of thin layer chromatography. The chromatogram depicting the TLC pattern and presence of the isolated derivative at 254nm and 366nm for crude ethanolic extract and its fractions in petroleum ether, ethyl acetate, methanol and ethanol. The plate was detected for the no. of bands in Iodine vapors. In our study multiwavelength scanning was done for the ICD considered as a standard by scanning the chromatogram in the range of 190-800 nm wavelength using D2 and W lamps. The wavelength showing maximum number of peaks and maximum area and height of peaks was selected. 256 nm was selected and its suitability was further confirmed by scanning the chromatogram between 190-320 nm at 10 nm intervals.

The plate was sprayed with Lieberman Burchard reagent and heated at 150^0C for the presence of phytosterols, multiwavelength scanning was done for the ICD. The wavelength showing maximum number of peaks and maximum area and height of peaks was selected.550 nm was selected and its suitability was further confirmed by scanning the chromatogram between 500-700 nm at 10 nm intervals.

Results and Discussion

Isolated compound showed a significant band in TLC at R_f 0.63 ethyl acetate, methanol and ethanol fraction showed a significant band at R_f 0.62 ,0.64 and 0.63 respectively(Table:1).

Table: 1 Rf values of various bands of extract and fractions in Iodine vapors

S. No	Extract/fraction	No. of spots	Rf values
1	Crude ethanolic ext	7	0.9850, 0.826, 0.73, 0.65, 0.52, 0.30, 0.12
2	Pet. ether fraction	2	0.965, 0.822
3	Ethyl acetate fraction	3	0.814, 0.743, 0.62
4	Methanol fraction	5	0.720, 0.638, 0.520, 0.365 0.248
5	Ethanol fraction	5	0.63, 0.55, 0.35, 0.22, 0.17
6	Isolated compound	1	0.63

The presence of ICD was seen in all the fractions except petroleum ether fraction.at 254nm and 550nm (after spraying Libermann Burchard reagent) respectively (Fig.1 and 2).
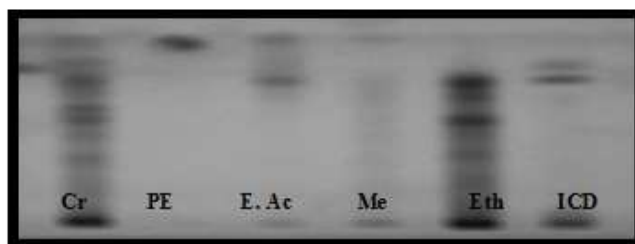
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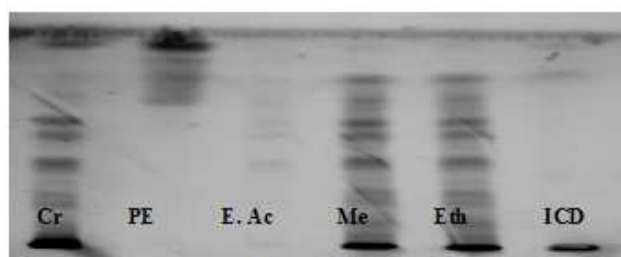Fig: 1 TLC pattern for different fractions and the ICD at 254nm

Fig: 2 TLC pattern for different fractions and the ICD at 550nm

Cr-crude ethanolic extract PE-Petroleum ether fraction of ethanolic extract E Ac-Ethyl acetate fraction of ethanolic extract Me- Methanolic fraction of ethanolic extract Eth-Ethanolic fraction of ethanolic extract ICD-Isolated chroman derivative.

The HPTLC profile with mobile phase (Toluene: ethyl acetate: formic acid 5.0: 4.5: 0.5), showed a single spot with max R_f of 0.69(Fig.3). The spectrum of all the tracks in R_f region 0.70 to 0.73 was found to correspond to each other and exhibited maxima at 256nm and 550nm respectively, with ICD peaks overlapping each other(Fig.4 and 5). Even though ethanol fraction of the extract was found to contain maximum amount of ICD as compared to the ethyl acetate fraction of the extract, ethyl acetate fraction depicted lesser number of other compounds eluted with it as compared to more bands observed in the ethanol fraction. Quantification of ICD in the extracts was done from the AUC from the graph (Fig.6)

HPTLC analysis indicated that the maximum amount of ICD was present in ethanol fraction of the ethanolic extract (1.83%) of *Ensete superbum* Cheesm followed by ethyl acetate (1.74%) and methanol fraction (0.70%) (Table 3). The petroleum ether fraction of the extract was found to contain no traces of the isolated compound.

Table 2: Linear range, co-relation coefficient and standard deviation for ICD

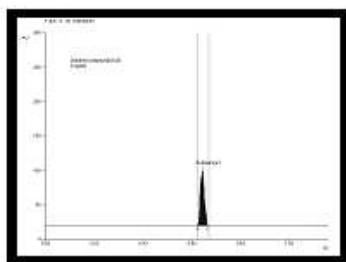| Parameters	Values
Linear range	300-900 ng
Standard Deviation (According to area)	3.76
Correlation coefficient (According to area)	0.99338

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Fig.: 3 HPTLC chromatogram of the isolated chroman derivative
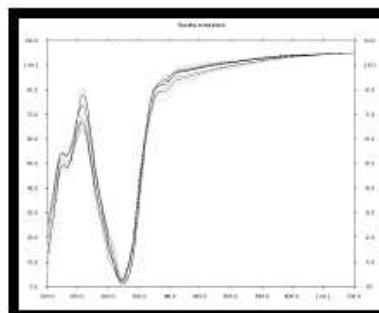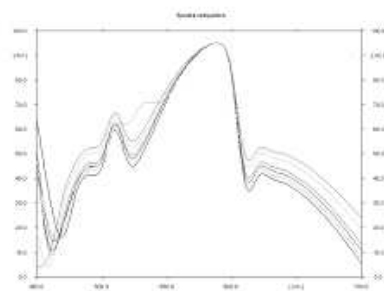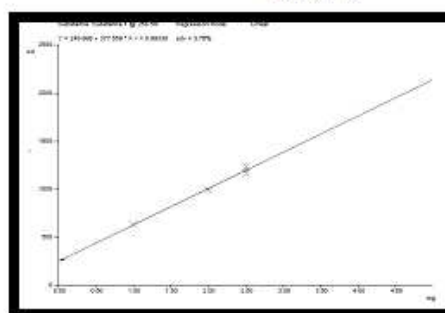

Fig.: 4 Chromatogram depicting λ max of the ICD in all fractions at 256nm

Fig.: 5 Chromatogram depicting λ max of the ICD in all fractions at 550nm

Fig.:6 Linear graph for ICD in all tracks (conc.vs area)

Table: 3 Percentage yield of ICD in different fractions of ethanolic extract of Ensete *superbum*, Cheesm by HPTLC

S.No	Fractions	Percentage isolated compound by HPTLC
1	Petroleum Ether	0
2	Ethyl acetate	1.74
3	Methanol	0.70
4	Ethanol	1.83

Validation of HPTLC method

1. **Limit of Detection:** The Limit of detection was found to be 150ng
2. Limit of Quantification: The Limit of quantification was found to be 450 ng
3. Linearity: A representative calibration curve of the ICD as standard was obtained by plotting peak area against its concentration over the range of 300- 900 ng/spot. The correlation coefficient was found to be 0.99338(Table 2).
4. Specificity: It was observed that the other constituents present in the extract and its fractions as an excipients did not interfere with the peak of ICD.
Therefore the method was specific. The spectrum of standard ICD spot and it's presence in other sample was found to be similar or overlapping (Fig.4 and 5).

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Conclusion

The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of the isolated chroman derivative i.e ICD in the extract and it's fractions from column chromatography. The percentage yield of the various fractions obtained by column chromatography were 1% (petroleum ether), 5.0% (ethyl acetate), 5.0% (methanol) and 25% (ethanol) and the percentage of the isolated compound calculated by HPTLC in each fraction was found to be 0, 1.74, 0.70, 1.83 % respectively. The limit of detection and limit of quantification was found to be 150ng and 450 ng respectively. The petroleum ether fraction of the extract was found to contain no traces of the isolated derivative. The method was found to be useful in deciding the chroman derivative as the marker compound.

Acknowledgement

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