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Quantification of *Allethrin* using HPTLC from *Annona Squamosa* (*Custard Apple*)

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Abstract

Annona squamosa (Custard Apple) is delicious fruit and it is traditionally used for curing a variety of ailments such as Ulser, dysentery, Antiovulatory, hair problems, burning sensation, hysteria (fearful state of mind) and fainting spells. There are no reports of quantification of Allthrine from this plant. Hence a HPTLC densitometric method has been developed and validated for quantification of this marker compound. Allethrine was quantified from methanolic extract using the solvent system of Petroleum ether: Diethyl ether (7: 3, v/v). The method was validated using ICH guidelines in terms of precision, repeatability and accuracy. Linearity range for Allethrine was 40–320 ng/spot and its content was 0.01046% w/w in peel and 0.01042% w/w in seed. This simple, precise and accurate method gave good resolution from other constituents of extract. It can be adopted for routine quality control of herbal material and formulations containing Annona squamosa (Custard Apple).

Keywords: Annona squamosa (Custard Apple); HPTLC; Alltherine; Densitometric method

Introduction

Annona squamosa (Custard Apple) is delicious fruit. The important features of Annona squamosa is their adaptability to soil and climatic conditions and freedom from pests and diseases. Due to their hardy nature and escape from animal damage, custard apples have become naturalized in many tropical and sub tropical parts of world. This plant is reputed to possess several medicinal properties [1,2]. Folkloric record reports its use as an insecticide and an anti tumor agent [3-13], anti-diabetic [14], anti-oxidant and anti-lipidimic activity [5], anti-inflammatory activities due to presence of cyclic peptides [14,15]. In addition, the crushed leaves are sniffed to overcome hysteria and fainting spells, they are also applied on ulcers and wounds, and a leaf decoction is taken in case of dysentery [15]. Custard apple is consumed as table fruits. They are also used in ice-creams and other milk products and preserved as jam, jelly or other products. The edible portion or pulp is creamy with good blend of sweetness and acidity which vary with the species. The pleasant flavor and mild aroma have a universal liking [7].

Material and Methods

Collection of the plant material

Variations in collection site, altitude, plant age, climate and soil can affect the concentration of secondary metabolites among different batches of the same plant, collected at different times. Hence it is necessary to carry out the collection of plant material with utmost care. The plant materials and their different parts are collected when they contain maximum amount of active constituents. Flowering tops of the plants are collected before they reach the flowering stage. The fruits are collected ripe.

Standard compound

Allethrin (purity 93%) was purchased from Sumitomo Chemical India Pvt. Ltd.

Experimental reagents

Analytical grade petroleum ether, 1-2 diethyl ether and methanol were obtained from E Merck. All other chemicals used were of analytical grade.

TLC conditions

TLC plates: 20×10 cm, precoated with silica gel 60 F254 TLC plate (E. Merck) (0.2 mm Thickness)

Spotting device: CAMAG Linomat V Automatic Sample Spotter; CAMAG (Muttenz, Switzerland)

Syringe: 100 µL (Hamilton)

Developing chamber: CAMAG glass twin trough chamber (20×10 cm)

Densitometer: CAMAG TLC Scanner 3 linked to winCATS software; CAMAG

Experimental condition

Temperature 25 ± 2°C, relative humidity 40%

TLC fingerprinting profile

Sample solution: Preparation of sample solution was optimized to achieve good fingerprinting and also to extract the marker compounds efficiently. Of these, the preparation of selected sample solution is given below.

Methanolic extract: Accurately weighed, exact 10 gms dried peel and seed of Annona squamosa was loaded for extraction by super critical fluid extraction method conditions are mentioned as follows.

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Temp:	800°C
Pressure:	170 Kg/cm^2
CO_2	2.0 cm^3/min
Co-solvent used	Ethanol
Co-solvent flow	0.2 cm^3/min
Collection time	45 min

Further 10 cm^3 solvent added in extract and collected in dry amber color bottle tightly closed and used as sample for carrying out the experiment.

Standard solution of Allethrin: 20 mg of accurately weighed stock of standard Allethrin was dissolved separately in methanol and the volume was made upto 10 ml with methanol in volumetric flask.

Preparation of working standard solution: Working Standard (B) solution prepared by using 0.5 cm^3 of standard (A) diluted to 10 cm^3 with methanol in 10 cm^3 standard volumetric flask.

Standard solution used on plate: Further 2 cm^3 aliquot from working standard is taken and diluted to 10 cm^3 in standard volumetric flask with methanol. This standard is used.

Solvent system: Petroleum ether: Diethyl ether solvents were mixed in 7:3 v/v and used as mobile phase.

Procedure: For co-chromatography with Allethrin, 10 µL of sample solution of methanolic extract along with the standard was applied on a TLC plate and the plate was developed in Petroleum ether: Diethyl ether (7:3, v/v) solvent system to a distance of 8 cm. The plates were dried at room temperature in air and observed in UV cabinet approximate R_f values is noted and colour of the resolved bands were noted., after the densitometric scanning R_f values resolved bands were noted.

Quantification of Allethrin using HPTLC

Preparation of Standard Solutions of Allethrin: A Stock Solution (A) of Allethrin (2 µg/µl) was prepared by dissolving 20 mg of accurately weighed Allethrin in 10 ml with methanol in volumetric flask. After that preparation of Working Standard (B) solution prepared by using 0.5 cm^3 of standard (A) diluted to 10 cm^3 with methanol in 10 cm^3 standard volumetric flask. Further dilution is required for that 2 cm^3 aliquot from working std (B) is taken and diluted to 10 cm^3 in std volumetric flask with methanol. This standard is used.

Preparation of calibration curve of Allethrin: 10 µl each of the Standard Solutions of Allethrin (40 to 320 ng/spot) were applied (band width: 6 mm, distance between the tracks: 14 mm) on a TLC plate using automatic sample spotter Linomat V. The plates were developed in a twin trough chamber (20×10 cm) up to a distance of 8 cm using Solvent System of Petroleum ether: Diethyl ether (7:3 v/v) at 25 ± 2°C and 40% relative humidity. After development, the plates were dried at room temperature in air and scanned densitometrically at 325 nm in reflectance–fluoresce mode using mercury lamp. The area of the resolved peaks was recorded. Calibration curve of Allethrin was obtained by plotting peak areas vs. concentrations of Allethrin applied.

Quantification of Allethrin in the sample: 3 µl of suitably diluted Sample Solution of methanolic extract was applied in triplicates on a TLC plate. The plate was developed and scanned as mentioned above. The peak areas were recorded and the amount of Allethrin was calculated using the calibration curve.

Validation of the method: ICH guidelines were followed for the validation of the analytical method developed (CPMP/ICH/281/95 and CPMP/ICH/381/95) for precision, repeatability and accuracy. Instrumental precision was checked by repeated scanning (n=7) of the same spot of Allethrin (160 ng/spot) and expressed as relative standard deviation (% R.S.D.). The repeatability of the method was affirmed by analyzing 160 ng/spot of Allethrin individually on TLC plate (n=5) and expressed as % R.S.D. Variability of the method was studied by analyzing aliquots of standard solution containing 80, 160, 240 ng/spot of Allethrin on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % R.S.D. For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of Allethrin were applied along with methanol as blank and determined on the basis of signal to noise ratio. The accuracy of the method was assessed by performing recovery study at three different levels (80%, 100% and 120% addition of Allethrin). The percent recoveries and the average percent recoveries were calculated.

Result and Discussion

There is no report of quantification of Allethrin in Annona squamosa (Custard Apple). Hence we developed a simple and precise method for quantification of this marker compound.

TLC fingerprint and co-chromatography

Quality control and quality assurance of herbal drugs remains a challenge as they contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy. Hence a systematic consideration of all its constituents is as important as the quantification of the active constituents present in it. TLC fingerprint profile of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality and ensuring the consistency and stability of herbal drugs and their products. In the present study, we developed TLC fingerprint profile for Annona squamosa (Custard Apple) and carried out co-chromatography with marker compound Allethrin. Allethrin was resolved at R_f 0.3 (Table 1, Figures 1-4) from Sample Solution of methanolic extract. When the plate was developed in Solvent System and observed in UV cabinet.

S. No.	R_f value	Colour of the band
1	0.12	black
2	0.3 (Allethrin)	Blue
3	0.52	black

Table 1: TLC fingerprinting profile of Extract from Peel and seed of Annona squamosa and Allethrin Standard Solution; under UV 254 nm.

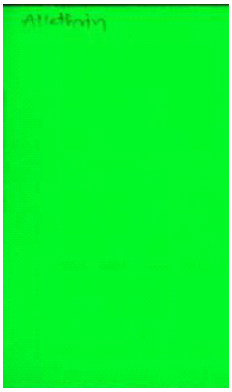

Figure 1: Allethrin content estimated in extract of peel and seed of Annona squamosa: TLC plate image at 254 nm.

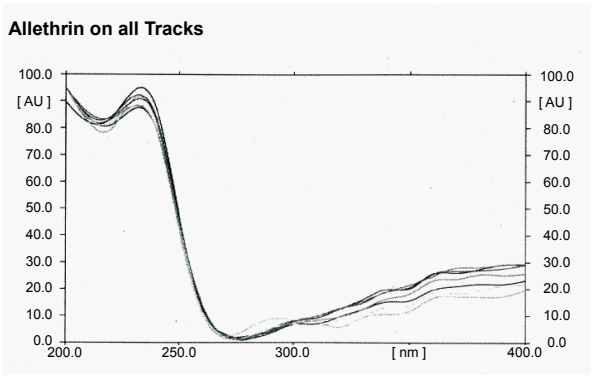

Figure 2: Specta of Allethrine.

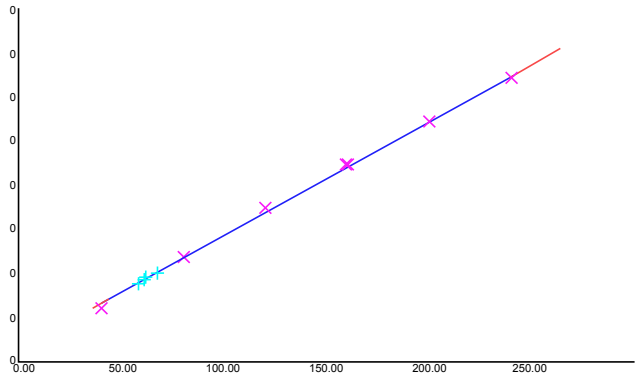

Figure 3: Linearity of Allethrine in peel of Annona Squamosa.

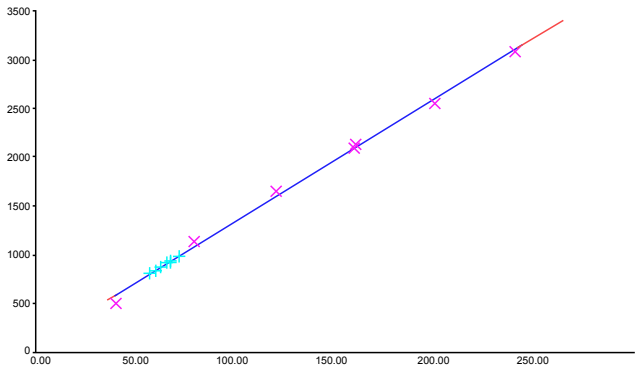

Figure 4: Linearity of Allethrine in Seed of Annona Squamosa.

TLC densitometric quantification of Allethrin using HPTLC

The simplicity of the sample preparation, and the possibility of analyzing several sample of herbal products simultaneously in a short time, make HPTLC the method of choice. In the present method Allethrin was quantified from Annona squamosa (Custard Apple) by TLC densitometric method using HPTLC.

The TLC densitometric method was validated in terms of precision, repeatability, and accuracy (Tables 2-4). The linearity range for Allethrin was 40–320 ng/spot with correlation coefficient (r values) of 0.998. The TLC densitometric method was found to be precise with R.S.D for intraday in the range of 0.15 to 0.46 and for interday in the range of 0.26 to 0.61 for different concentrations of Allethrin (Table 3). This indicates that the proposed method was precise and reproducible. The limit of detection (LOD) value for Allethrin was found to be 13 ng, and limit of quantification (LOQ) value was 40 ng (Table 2). The average percent recoveries at 3 different levels of Allethrin were found to be 99.88% (Table 4).

S. No.	Parameter	Allethrin
1	Instrumental precision (% CV, n=7)	0.85
2	Repeatability (% CV, n=5)	1.51
3	Accuracy (average % recovery)	99.88
4	Limit of detection (ng)	13
5	Limit of quantification (ng)	40
6	Specificity specific	Specific
7	Linearity (Correlation coefficient)	0.99831
8	Range (ng/spot)	40-320

Table 2: Method validation parameters for the quantification of Allethrin by the proposed TLC densitometric method.

Marker	Concentration (ng/spot)	Intra-day precision*	Inter-day precision*
Allethrin	40	0.15	0.26
	120	0.52	0.43
	200	0.46	0.61

* % R.S.D.; Mean (n=3)

Table 3: Intra-day and Inter-day precision of Allethrin.

Marker	Amount of extract present (µg)	Amount of marker added (µg)	Amount of marker found (µg)	Recovery* (%)	Average Recovery (%)
	0.062	0.16	0.22376	100.81	
	0.062	0.2	0.26212	99.72	99.92
	0.062	0.24	0.29964	99.22	

*Mean ± SD (n=3)

Table 4: Recovery studies of Allethrin at 80%, 100% and 120% addition by the proposed TLC densitometric method.

S. No.	Sample/Standard Solution	Allethrin (% w/w)*
1	1 (peel)	0.01046%
2	2 (seed)	0.01042%

Table 5: Allethrin content estimated in extract of Peel and seed of Annona squamosa by proposed TLC densitometric method.

The content of Allethrin quantified using TLC densitometric method was found to be 0.01046% w/w (Table 5, Figure 2) in the peel of Annona squamosa and 0.01042% w/w (Table 5, Figure 2) allethrin in the peel of Annona squamosa.

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