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HPTLC METHOD FOR QUANTITATION OF ALLETHRIN FROM DRIED PEEL AND SEED OF *Annona squamosa.*

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Abstract: A precise and straightforward high-performance thin-layer chromatography (HPTLC) technique has been created to quantitatively measure Allethrin in the dried seed and peel powder of Annona squamosa. The chromatographic assessment involved the use of a Hexane extract obtained from the dried seed and peel powder of Annona squamosa on silica gel 60 F254 TLC plates, utilizing a solvent mixture composed of petroleum ether and diethyl ether in a ratio of 7:3 (v/v). The detection and quantification of Allethrin were achieved by scanning densitometry at a wavelength of 237nm. The method's precision was validated, and its accuracy was confirmed through recovery experiments at three different Allethrin levels. This proposed HPTLC method effectively separated Allethrin from other components present in the Hexane extract of the dried seed and peel powder of Annona squamosa, making it suitable for Allethrin quantification. A comprehensive literature review revealed that no prior method existed for detecting or quantifying Allethrin in the dried peel and seed of Annona squamosa fruit. Consequently, in this research, we developed and validated an HPTLC method to quantify Allethrin in the dried peel and seed of Annona squamosa fruit.

Keywords: High-performance thin layer chromatography, quantification, herbal, Annona squamosa

Introduction*:* *Annona squamosa* (Custard Apple) is a delicious fruit. The important features of Annona squamosa, are 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 the world. 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 a good blend of sweetness and acidity which vary with the species. The pleasant flavor and mild aroma have a universal liking. The immature fruits, seeds, leave, and roots are of considerable medicinal value. seeds are abortifacient and roots are drastic purgative. The leaf poultice is used for suppuration. The seed contains about 30% oil which can be used in the soap and paint industry. At least thirty-three compounds are present in the essential oil extracted from the peel**.**

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There are medicinal applications of the custard apple tree. The bark and leaves contain annonaine, an alkaloid. A bark decoction is used to stop diarrhea, while the root is used in the treatment of dysentery. A decoction of the leaves is used as a cold remedy and to clarify urine. *Annona squamosa* Linn, family Annonaceae, is said to show varied medicinal effects, including insecticide, and antiovulatory. The fruits of Annona are Haematinic, cooling, sedative, stimulant, expectorant, maturant, and tonic. They are useful in anaemia, a burning sensation. The seeds are abortifacient and insecticidal and are useful in destroying lice in the hair. Leaves are used to overcome hysteria and fainting spells. Fruit is used in making ice creams & milk beverages.

The herbal extract is an extract of herbs made from alcohol. It is a strong medicinal dose of herbs. It consists of the active ingredient of a particular herb. The extraction process is used to obtain the extract from the authenticated herb.

High-Performance Thin Layer Chromatography (HPTLC) is the most commonly used method for obtaining chemical fingerprints and identification of crude plant extracts High-performance thin-layer chromatography is a useful chromatographic technique, used in quantitative analysis of herbals and herbal formulations. HPTLC is a planar chromatographic technique, where the separation of sample components is achieved on high-performance layers, which are precoated plates, coated with a sorbent of small particle size. The reduction in thickness of the layer and particle size results in increasing plate efficiency and the nature of separation.

The analysis is carried out using silica gel as a sorbent, with a small particle size, which increases resolution and sensitivity. The particle size of silica is 60 A and is applied on the aluminium plate, using a sample applicator. The plate is then placed vertically in a solvent system, in a suitable container. The plate after development is dried and then scanned, at a suitable wavelength.

Classification:

- Family Name: *Annonaceae*
- Genus: Annona
- Species: *Annoa squamosa*
- Common Name: Custard Apple, Sugar Apple, Sweetsop, Kaner'apra, Pomme Canelle, Tapotapo, Fun Li Chi, Anon, Anona Blanca, Ati, Ates Northen India Sharifa, southern part Sitaphal.
- Sanskrit-Synonyms-Seetaphalam, Sudha,Subha
- PLANT NAME IN DIFFERENT LANGUAGES English-Custardapple, Sugarapple
- Hindi - Seetaphal
- Malayalam: Athachakka, Atha, Seetapazham
- Part Used: Annona Seeds, Annona Fruit

Botanical description of *Annona squamosal:* *Annona squamosa* is a deciduous, tall, woody shrub or small tree of about 5 to 6 meters in height with irregularly spreading branches. The alternate double-ranked leaves are lanceolate and acuminate. The greenish-yellowish flowers arise at an extra auxiliary position, usually in clusters and rarely solitary. Fruit is a globose, flashy, composite berry. Consisting many flashy carpels all fused together in one mask. The black seeds are surrounded by white creamy pulp which is very sweet and pleasantly flavoured.

Medicinal-Properties: Plant pacifies vitiated pitta, constipation, burning sensation, anemia, vomiting, cough, malignancy, and general tonic. Root decoction is a powerful purgative. Powdered seed kills body and hair lice on external application. The ethanolic extract of the leaves and stem of *Annona squamosa* was found to possess anticancer activity. Alkaloids isolated from *Annona squamosa* were also found to possess larvicidal and insect growth regulating and chemo sterilant activities against the mosquito.

Materials and Methods: Experimental Reagent: Analytical grade petroleum ether, 1-2 diethyl ether methanol were obtained from E Merck

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

Preparation of dry powder from seed and peel of Annona squamosa: The peel and seed of *Annona squamosa* were collected, they were washed with water, shade dried finely powdered, and sieved through a BSS mesh No.85 sieve. The sieved powder was stored in commonly available airtight polyethylene containers, labelled with details such as the date of collection, the weight of the powder, the time of collection, and the region of collection.

Preparation of sample

Accurately weighed, about 10 gms dried peel and seed of *Annona squamosa* was loaded for extraction by supercritical fluid extraction method conditions are mentioned in table

Table 1. Supercritical fluid extraction conditions

Temp:	80^0 C
Pressure:	170 Kg/cm^2
CO_2	2.0 cm3/min
Co-solvent used	Ethanol
Co-solvent flow	0.2 cm3/min
Collection time	45 min

Further 10 cm3 solvent was added in the extract and collected in a dry amber color bottle tightly closed and used as the sample for carrying out the experiment.

Instrumentation: The analysis was performed on aluminium plates, precoated silica gel 60F254 with a thickness of 200 µm (Merck, Darmstadt, Germany, supplied by Anchrom Lab, Mumbai) The standard and sample solutions were applied on plates, using a CAMAG Linomat V automatic sample applicator (Switzerland), provided with a CAMAG 100 µl (0.1cm3). The plates were developed using a CAMAG twin trough chamber and Densitometric scanner Camag scanner III, win Cats software.

Preparation of solutions:

Preparation of mobile phase

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

Preparation of standard solution

Preparation of standard solution (A) of Allethrin was prepared in 10 cm3 standard volumetric flask by dissolving 20 mg of accurately weighed stock of standard Allethrin in 5cm3 methanol followed by shaking. The volume of the solution was made up to 10 cm3, with methanol

Preparation of working standard solution

Working Standard (B) solution prepared by using 0.5 cm3 of standard (A) diluted to 10 cm3 with methanol in a 10 cm3 standard volumetric flask.

Preparation of sample

Accurately weighed, exact 10 gms dried peel and seed of *Annona squamosa* were loaded for extraction by supercritical fluid extraction method conditions are given in Table 1. Further 5 cm3cm3 methanol solvent was added in the extract and collected in a dry amber color bottle tightly closed. And used as the sample for carrying out the experiment.

Optimized Chromatographic conditions maintained in the HPTLC method used for the quantitation of Allethrin from the dry powder of peel and seed of *Annona squamosa* are given in Table No. 2

Table 2. Optimized Chromatographic conditions maintained in the HPTLC method used for the quanitation of Allethrin from dry powder of peel and seed of Annona squamosa

PARAMETERS	CHROMATOGRAPHIC CONDITIONS
Instrument	Camag Linomat V Automatic sample applicator, Camag TLC Scanner III.
Stationary phase	Aluminium plates, pre-coated with Silica Gel 60 $F_{254,}$ with a thickness of 200 µm (E Merck, Mumbai)
Mobile phase	Petroleum ether and 1-2, Di-ethyl ether
Pre development conditions	Filter paper saturation for 15 minutes.
Spotting Volume	10µl
Detection wavelength	237 nm
Software	winCATS

Results: High-Performance Thin Layer Chromatographic Separation of Standard Allethrin and Extract from Peel and seed of Annona squamosa

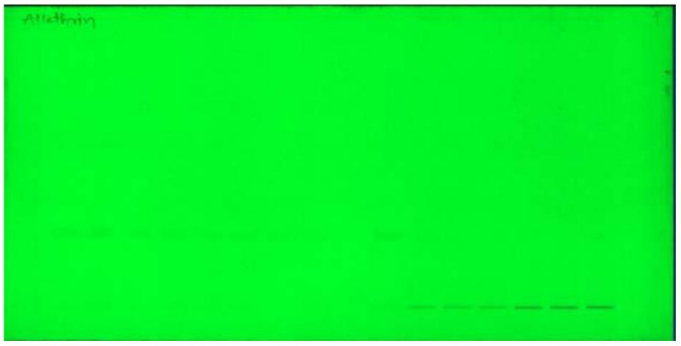

Figure 1. Separation of Standard Allethrin and Extract from Peel and seed of *Annona squamosa*

Method validation parameters were studied for the determination of Allethrin by the HPTLC method.

Determination of linear dynamic range for Allethrin

Allethrin Standard stock is prepared by weighing 20mgs standard Allethrin and diluting it to 10 cm3 with methanol in a standard volumetric flask.

Working std is prepared by taking aliquots 0.5 cm3 of the Allethrin std stock solution is transferred to 10 cm3 volumetric flasks and the volume of the flask was adjusted to 10.0 cm3 with methanol. Further 2cm3 aliquot from working std is taken and diluted to 10 cm3 in a std volumetric flask with methanol. this standard is used.

A series of the standard solution of Allethrin, in the concentration range of 0.04µg/cm3 to 0.32µg/cm3, were thus prepared and used for the determination of linear dynamic range .16,14,12,10,8,6,4,2 µl of the standard solution of Allethrin, in the concentration range of 0.32µl/cm3 to 0.04µl/cm3, and 10gms of dried powder of peel and seed of *Annona squamosa* extracted to5 cm3 Aliquot of 1cm3 diluted to 10cm3 with methanol 3 µl in three times were applied on same 20cm×10cm silica gel60 F254 pre coated aluminium TLC plate, as a band. The bandwidth for each application was 6mm and the bands were 14mm apart from each other and 10mm from the bottom edge of the plate. The mobile phase, comprising of Petroleum ether and Di ethyl ether 7:3 (v/v), was prepared and poured on the filter paper in the CAMAG glass twin trough chamber on one side of the twin trough and kept for 15 minutes saturation, The TLC plate was then placed in the other side of the chamber and the chamber was closed with a lid. the mobile phase was allowed to ascend to a distance of 80mm above the position of the sample application. After development, the plate was removed from the twin trough chamber and dried. Densitometric scanning of the plate was then performed on a CAMAG TLC scanner III in the reflectance–fluoresce mode, at 325 nm for all measurements and it was operated by Win CATS software the dendrograms were recorded and the Determination of linear dynamic range for Allethrin Standard stock is prepared by weighing 20mgs standard Allethrin and diluting to 10 cm3 with methanol in a standard volumetric flask. Working std is prepared by taking aliquots 0.5 cm3 of the Allethrin std stock solution is transferred to 10 cm3 volumetric flasks and the volume of the flask was adjusted to 10.0 cm3 with methanol.

Further 2cm3 aliquot from working std is taken and diluted to 10 cm3 in a std volumetric flask with methanol. this standard is used. A series of the standard solution of Allethrin, in the concentration range of 0.04µg/cm3 to 0.32µg/cm3, were thus prepared and used for the determination of linear dynamic range .16,14,12,10,8,6,4,2 µl of the standard solution of Allethrin, in the concentration range of 0.32µl/cm3 to 0.04µl/cm3, and 10gms of dried powder of peel and seed of *Annona squamosa* extracted to5 cm3 Aliquot of 1cm3 diluted to 10cm3 with methanol 3 µl in three times were applied on same 20cm×10cm silica gel60 F254 pre-coated aluminium TLC plate, as a band. The bandwidth for each application was 6mm and the bands were 14mm apart from each other and 10mm from the bottom edge of the plate. The mobile phase, comprising Petroleum ether and Di ethyl ether 7:3 (v/v), was prepared and poured on the filter paper in the CAMAG glass twin trough chamber on one side of the twin trough and kept for 15 minutes saturation, The TLC plate was then placed in the other side of the chamber and the chamber was closed with a lid. The mobile phase was allowed to ascend to a distance of 80mm above the position of the sample application. After development, the plate was removed from the twin trough chamber and dried. Densitometric scanning of the plate was then performed on a CAMAG TLC scanner III in the reflectance–fluoresce mode, at 325 nm for all measurements and it was operated By Win CATS software the dendrograms were recorded and the, and the peak area of Allethrin was determined for each applied concentration of Allethrin.

The response factor was calculated for each concentration of Allethrin, by dividing the peak area of Allethrin by the applied concentration of Allethrin. The results are tabulated below:

Table 3. Response factor was calculated for each concentration of Allethrin

Observation	Weight of sample taken	Amount of extracted sample taken	Peak area of Allethrin	Amount of Allethrin found in dried powder of peel of Annona squamosa ng

1	600 mg	3µl	914.75	65.95
2	600 mg	3µl	874.77	62.77
3	600 mg	3µl	835.70	59.67

Observation	Weight of sample taken	Amount of extracted sample taken	Peak area of Allethrin	Amount of Allethrin found in dried powder of peel of Annona squamosa ng
1	600 mg	3µl	931.51	67.28
2	600 mg	3µl	802.18	57.02
3	600 mg	3µl	986.36	71.63

Limit of Detection (LOD)and limit of Quantitation (LOQ)

The limit of Detection (LOD) is related to both the signal and the noise of the system and is defined as a peak, whose signal–to–noise (S/N) ratio is at least 3:1. the Limit of Quantitation (LOQ) is defined as a peak, whose signal–to–noise (S/N)ratio is at least 10:1.The limit of detection for Allethrin was found to be 0.50µg/cm3 and the limit of quantitation for Allethrin was found to be 5.00µg/cm3.

Precision: The precision of the method was studied by determining repeatability (instrumental precision and intra–assay precision) and intermediate precision. Instrumental precision was studied by analyzing the standard solution of Allethrin with a concentration of 7.00µg/cm3, ten times, using the optimized chromatographic conditions the values of the peak area of Allethrin were recorded, and the values of the mean peak area of Allethrin, standard deviation (S.D.) and percent relative standard deviation (%R.S.D) were calculated the results are listed in the table given Table No. 4.

Table 4. Values of mean peak area of Allethrin, standard deviation (S.D.), and percent relative standard deviation (%R.S. D) calculated.

Observation	Weight of sample	peak area of Allethrin	Mean Peak Area	S. D	% R. S. D
1	600 mg	914.75 874.77 895.70	875.07	8.24	0.94%
2	600 mg	931.51 802.18 986.36	906.68	10.51	1.16%

Calculations for assay of mean amount of allethrin found in dried powder of peel and seed of Annona squamosa

The mean weight of sample =600.00mg

The amount of allethrin in mg in 600.00g of peel and seed powder of *Annona squamosa* was determined as follows:

For the calibration curve,

Y= mx+c

Where,

Y= peak area of allethrin

M=Slope of the regression line

X=concentration of allethrin in µg/cm3

C=Intercept

For the sample solution, the mean peak area of allethrin (y)=875.07 for the peel sample

The mean weight of sample =600.00mg of peel

From the result of the regression analysis of calibration data,

Slope m =12.61

Intercept c = 83.49

The concentration of allethrin in 1 cm3 of sample solution = $\frac{c-y}{m}$

$$= \frac{875.07 - 83.49}{12.61}$$

= 62.77ng

The concentration of allethrin in the peel of *Annona squamosa* =

$$= \frac{0.06277X100}{600}$$

=0.01046 %

The mean weight of sample =600.00mg

The amount of allethrin in mg in 600.00g of peel and seed powder of *Annona squamosa* was determined as follows:

For the calibration curve,

Y=mx+c

Where,

Y= peak area of allethrin

M=Slope of the regression line

X=concentration of allethrin in µg/cm3

C=Intercept

For the sample solution, the mean peak area of allethrin (y)=906.68 for the seed sample

The mean weight of sample =600.00mg of seed

From the result of the regression analysis of calibration data,

Slope m =12.61

Intercept c = 83.49

Concentration of allethrin in 1 cm3 of sample solution

$$= \frac{y-c}{m}$$

$$= \frac{906.68 - 83.49}{12.61}$$

= 65.28 ng

. The concentration of allethrin in the peel of *Annona squamosa*

$$= \frac{0.06528X100}{600}$$

=0.01042 %

Conclusion:

The present research work was carried out to develop analytical instrumental methods which can be used as quality control methods for standardization of Allethrin HPTLC method was found sensible method for quantitation of allethrin in dried powder of peel and seed of Annona squamosa.

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