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Research article

Developed and validated method for the simultaneous estimation of ferulic acid and chlorogenic acid in four Indian *ananas comosus* merrill cultivars through HPTLC

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ABSTRACT

Ananas comosus (L) Merrill fruit is used for the treatment of urinary tract infection, and jaundice. The fruit has anti-obesity activity and used as antihelmithic agent for the treatment of intestinal worms. The *Ananas comosus* fruit contains many phytoconstituents; among them are Ferulic acid and Chlorogenic acid exhibiting various pharmacological activities. To ensure the content of uniformity of biomarkers, the aim of the present study was to develop a HPTLC method for simultaneous estimation of Ferulic acid and Chlorogenic acid in *Ananas comosus* fruit. The standard marker compounds and four different fruit extract solutions were applied on the HPTLC plate (20 mm × 10 mm dimension) with the help of Linomat 5 applicator by using WinCATS software. The solvent system toluene, ethyl acetate, methanol, formic acid (4:4:1.2:0.8, v/v) was used as mobile phase. The λ_{max} was set for Ferulic acid and Chlorogenic acid at 280 nm. The calibration range of Ferulic acid was found to be 300-900 ng/ml, with the linear equation Y= 338.485+6.238x with coefficient of determinants (r^2) of 0.992 and the calibration range of Chlorogenic acid was found to be 200-800 ng/spot with the linear equation Y= 282.512+ 4.932x with coefficient of determinants (r^2) of 0.996. The limit of detection and limit of quantification were found to be for Ferulic acid 14.35, 40.12 ng/spot, and 10.26, 29.51 ng/spot, for Chlorogenic acid respectively. The relative standard deviation of precision and recovery of Ferulic acid and Chlorogenic acid was < 2.0%. The developed method was accurate, specific, precise and reproducible.

Keywords: Ananas comosus cultivars, Ferulic acid, Chlorogenic acid, Simultaneous estimation, Method Validation.

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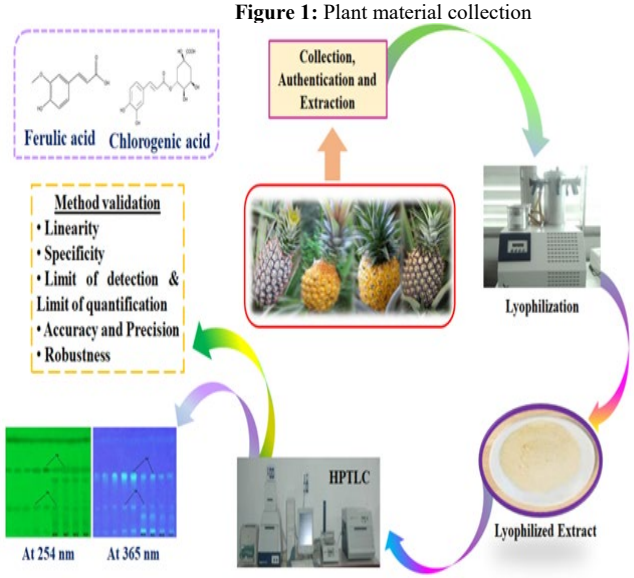
INTRODUCTION

Ananas comosus (L.) Merrill, belongs to family: Bromeliaceae (common name- pineapple) are mainly found in Central and South America, India, Brazil, Hawaii, Philippines, Malaysia, Thailand and China[1]. There are different cultivars found in *Ananas comosus* species. Giant Kew is the most common commercial *Ananas comosus* variety in India. Charlotte Rothchild Kew, Mauritius, Desi, Lakhat, Queen Jaldhup and others are important verities [2]. The size, shape, colour, and flavour of the fruits define the cultivars [3]. Their morphological and geographic heterogeneity has the potential to influence secondary metabolite composition as well as therapeutic advantages. Because of its appealing sweet flavour, *Ananas comosus* is consumed as a juice. It is also used as an ingredient in several exotic foods [1]. The *A. comosus* fruit used in the treatment of urinary tract infection [4]. *A. comosus* is used as folk medicine to treat the dysuria. In China, the *A. comosus* cortexes were used as antidiarrheal agent [5]. The leaf extract is used to treat hiccups and as a vermifuge. Fruits that are ripe are used to treat jaundice [6]. The *A. comosus* fruit contains some phytoconstituents reported to have anti-obesity activity [7]. In folk medicine, *A. comosus* leaf was used as an antimicrobial agent [8]. The *A. comosus* is also used as anthelminthic agent for the treatment of intestinal worms [9]. The *Ananas comosus* fruit contains various phytochemicals including flavonoid, alkaloid, phenol, tannins and carotenoid [6]. Ferulic acid and chlorogenic acid are found in the fruit juice and have a variety of pharmacological properties, including antioxidant and anti-cancer properties [10]. It also protects against coronary artery disease, lowers cholesterol, and improves sperm viability [11]. N-L-glutamyl-S-sinapyl-L-cysteine, S-sinapyl-L-

cysteine, and S-sinapyl glutathione are all sinapyl derivatives found in the fruit juice [12]. The proteolytic enzyme, bromelain is present in the *A. comosus* fruit in a significant amount. The enzyme has several clinical applications, which includes anti-obesity, antitumor, immune response modulator, anti-inflammatory, blood coagulation, mucolytic and gastrointestinal action and also used in the cardiovascular and circulatory diseases. The enzyme also employed in surgical procedures and wound care [7,13] .

For determining the concentration of bioactive chemicals contained in herbal plants, advancement in marker profiling and standardization of plants is critical [14]. Therefore, appropriate quantitative estimation of ferulic acid and chlorogenic acid (Figure 1) from different pineapple is essential to find out the content uniformity. The chromatographic separation of bioactive compounds presents in the different plants mainly based on their structural resemblance [15]. The current study aims to create a validated HPTLC method for simultaneous measurement of ferulic acid and chlorogenic acid from four distinct pineapple cultivars in this context.

Figure 1: Plant material collection



MATERIALS AND METHODS

Plant material collection and extract preparation

The several pineapple varieties were collected in Assam and other sections of West Bengal, India. For future reference, they were authenticated at the Bengal College of Pharmaceutical Sciences and Research, Durgapur, under the voucher specimen number BCPSR-1874. The flesh of the fruit section was cut into small pieces and pulverized using a motorized grinder after the stalk (central core) of the ripe pineapple was detached from the fleshy fruits. To eliminate the fibrous debris, the juice was filtered through a white cloth. To eliminate insoluble elements, the filtrate was centrifuged for 10 minutes. Whatman filter paper was used to filter the clear supernatant once more.

HPTLC standardization of *Ananas comosus* plant extracts

HPTLC Instrumentation

Automatic sample injector (Linomat 5, 100 µl Hamilton syringe, Camag, Switzerland), scanner III, win CATS planar chromatography manager software version 1.4.2.8.21 (All form CAMAG, Muttenz, Switzerland, UV chamber (Camag, Switzerland), Stationary phase: Aluminum backed precoated silica gel 60 F_{254} (20 × 10 cm, thickness, Merck, Mumbai, India) were used in the study.

Chemicals and reagents

Merck Ltd provided the toluene, ethyl acetate, methanol, and formic acid (analytical grade) (Mumbai, India). Sigma Aldrich provided standard ferulic acid (FA) and chlorogenic acid (CA) (St. Louis, MO, USA). Merck Ltd provided additional analytical grade regents and solvents (Mumbai, India).

Sample and standard preparation

In methanol, a standard stock solution of FA and CA was produced at a concentration of 1 mg/ml. Standard solutions with varying concentrations (200, 400, 600, and 1000 ng/ml) were made by diluting the stock solution. The sample solution was made by dissolving 10 mg of extract in 10 ml of methanol, resulting in a concentration of 1 mg/ml. Whatman NYL 0.45 m syringe filter was used to filter the standard and sample solutions. The reactions were plotted versus concentration and measured as peak areas.

Application of standard and sample solutions

Using the Linomat 5 applicator and Win CATS software, the standards (FA and CA) and extract solutions (10 L) were applied to the HPTLC plate (20 mm x 10 mm dimension). The band length and distance between tracks of applied each standard and extract solutions are 8 mm and 12 mm, respectively.

Development of HPTLC plate

The HPTLC plate was dried in the air for a while after the standard and extract solutions were applied. For FA and CA, the solvent system toluene, ethyl acetate, methanol, and formic acid (4:4:1.2:0.8, v/v) was utilized as the mobile phase. The plate was developed in a twin trough chamber after 30 minutes of soaking with mobile phase. To allow the solvent to run, the plate was housed in a twin trough chamber. The plate was removed from the twin trough chamber and dried for 10 minutes in the air. The plate was then ready to be scanned densitometrically.

Densitometric scanning

Using a densitometric HPTLC scanner III from Camag, Switzerland, the produced plate was scanned at 280 nm wavelength for both FA and CA. The Win CATS software was used to combine the data, and the area under the curve, as well as the amount of FA and CA, were correlated and determined using this information.

Method validation

On the basis of the International Conference on Harmonization (ICH) Q2 (R1) recommendations, linearity, specificity, accuracy and precision, limit of quantification, and limit

of detection were used to validate the method (ICH, 2005).

Specificity

The method's specificity was determined by comparing the extract samples to the standard marker chemicals (FA and CA). This primarily verifies the analyte's identity and purity, as well as minimizing errors caused by sample contamination.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Sensitivity was determined by establishing the Limit of Detection (LOD) and Limit of Quantification (LOQ), as well as the SD of the response and the slope of the linear equation, as per the ICH guidelines. The LOD and LOQ were determined using the following equation: LOD = 3.3/S and LOQ = 10/S, where S is the slope of the calibration curve and 3.3 is the standard deviation [12].

Precision

Intra-day and inter-day precision were achieved by applying 400 and 800 ng of standard marker compounds (FA and CA) to each location, developing the plate in the same manner as the previously proposed procedure. Both precision parameters were evaluated by comparing data from within a single run (n = 6) and were expressed as a percent RSD value [12].

Accuracy

The method's accuracy was measured using the standard addition procedure and represented as a percent RSD for mean recovery of the theoretical concentration. In this experiment the known equivalent number of standard compounds (FA and CA) was added in the different varieties of pineapple extracts. The 5 µl of the different pineapple extracts were applied on HPTLC plate. The first spot was left unspiked, while the second, third, and fourth spots were spiked with 200, 400, and 600 ng equivalent mixtures of standard chemicals, respectively, and examined using the previously established ideal conditions [12].

Robustness

The robustness of the system was tested by varying the mobile phase composition, spotting duration, development distance (5.0 mm), and scanning approach [12].

Statistical analysis

Graph Pad Prism Version 5.0 was used to conduct the statistical analysis. The average percent RSD has been used to depict the result.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The marker compounds spot separation was achieved at ambient (25ºC) conditions. The mobile phase consisted for FA and CA is toluene, ethyl acetate, methanol, formic acid (4:4:1.2:0.8, v/v). The λ_{max} was set for FA and CA at 280 nm.

Linearity

FA and CA were found to have calibration ranges of 300-900 ng/spot and 200-800 ng/spot, respectively. The linear equation for FA was found to be Y= 338.485+ 6.238X with coefficient of determinants (r^2) of 0.992 (Figure 4a). The linear equation for CA was found to be Y= 282.512+4.932X with coefficient of determinants (r^2) of 0.996 (Figure 4b).

Specificity

The specificity test was evaluated by analyzing standard marker compounds and extracts. The specificity test, R_f value of standard FA and CA (Figure 5) comparing with the R_f value of the FA and CA present in the extracts (Figure 6 - 9). The peak purity of FA and CA was investigated by comparing spectra at three different levels, namely the peak start, peak middle, and peak end positions of the spot/bands. The method's specificity was indicated by the well-shaped peak. By comparing the UV absorption spectra of samples to the standards at 280 nm, the purity of the standards (FA and CA) peak in four varieties of pineapple extract samples was established. (Figure 11).

Limit of detection (LOD) and Limit of quantification (LOQ)

FA has a LOD and a LOQ of 14.35 and 40.12 ng/spot, respectively. CA's LOD and LOQ were discovered to be 10.26 and 29.51 ng/spot, respectively.

Accuracy

A recovery study was used to assess the method's accuracy. The method's accuracy was demonstrated by the high recovery values for FA (96.26-99.96%) and CA (96.07-99.95%). (Table 1).

Precision

The percent RSD of intra-day and inter-day precision was found to be less than 2%, indicating that the procedure is highly repeatable. (Table 2).

Robustness

Within the limits (5%), the robustness was assessed by adjusting selected parameters such as mobile phase composition, volume, and length time of mobile phase saturation. There were no significant modifications in technique development, and the RSD of the data was less than 2% for any variable condition.

Determination of FA and CA in four different Indian *A. comosus* cultivars

The developed HPTLC plate (observed at 254 nm and 366 nm) of FA with CA and different varieties of *A. comosus* extracts were showed in Figure 2 - 3. The mean retardation factor (R_f) was observed at 0.68 (for FA) and 0.32 (for CA) by comparing between standard and extracts chromatograms (Figure 5 - 9). The amount of FA and CA found in different Indian *A. comosus* cultivars are showed in Table 3.

Table 1: Recovery studies of FA and CA in of A. comosus fruit extract (n=6)

Excess FA and CA added to extract (ng)	Expected content of FA and CA in mixture (ng)		Average amount of FA and CA found (ng)		Average recovery (%)	
	FA	CA	FA	CA	FA	CA
0	444.38	444.22	444.23	444. 02	99.96	99.95
200	644.38	644.22	643.30	642.05	99.83	99.66
400	844.38	844.22	818.34	811.10	96.92	96.07
600	1044.38	1044.22	1005.35	1012.13	96.26	96.93

Table 2: Intra- and inter-day precision of HPTLC method (n=6)

Amount (ng/spot)	FA				CA			
	Intra-day precision		Inter-day precision		Intra-day precision		Inter-day precision	
	Mean area	%RSD	Mean area	%RSD	Mean area	%RSD	Mean area	%RSD
400	1985.83	0.92	1998.25	0.94	1768.56	0.88	1780.08	0.99
800	3542.74	0.94	3588.93	0.96	3298.52	0.92	3302.55	0.98

Table 3: FA and CA Content in different variety of Indian A. comosus cultivars

Variety of A. comosus Cultivars	FA Content in Extract (% w/w)	CA Content in Extract (% w/w)
Ananas comosus var Giant Kew (ACG)	1.02	0.99
Ananas comosus var Queen (ACQ)	0.98	0.84
Ananas comosus var Charlotte Rothschild (ACC)	1.06	0.88
Ananas comosus var Desi (ACD)	0.92	0.76

Figure 2: Structure of FA and CA

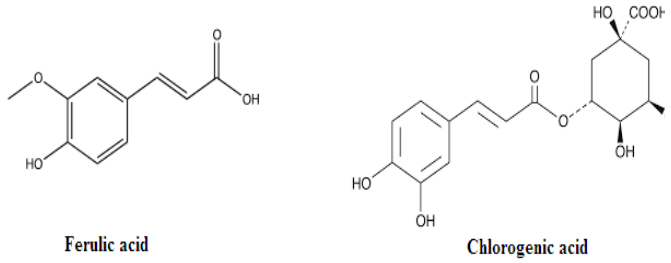


Figure 3: HPTLC plate of FA and CA at 254 nm

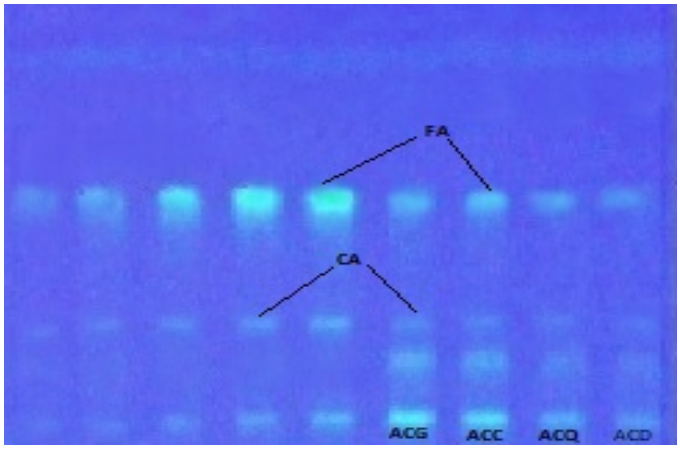


Figure 4: HPTLC plate of FA and CA at 366 nm

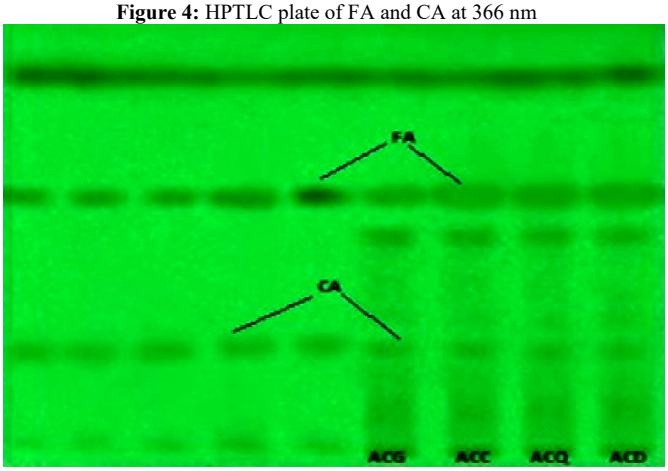


Figure 5a: Linear range of calibration curve for FA

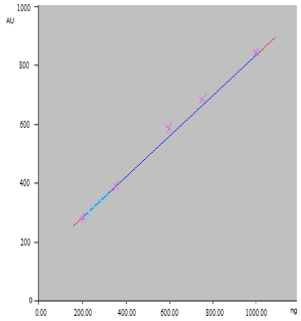


Figure 5b: Linear range of calibration curve for CA

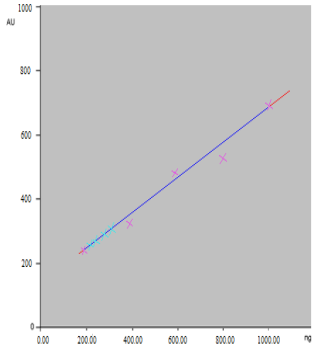


Figure 6: HPTLC chromatogram of FA and CA standard

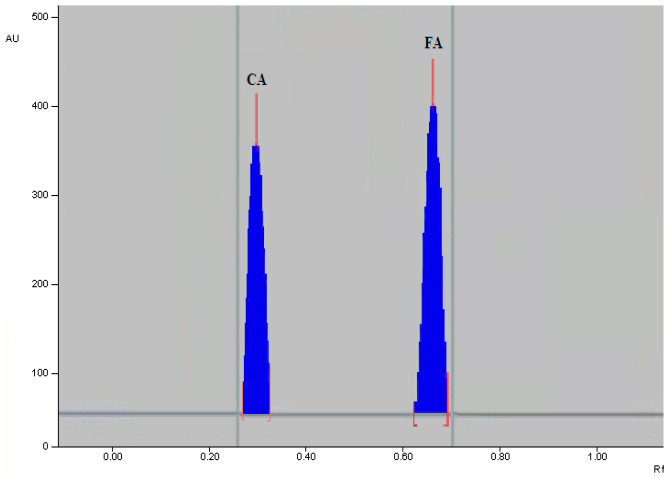


Figure 7: HPTLC chromatogram of Ananas comosus var Giant Kew extract

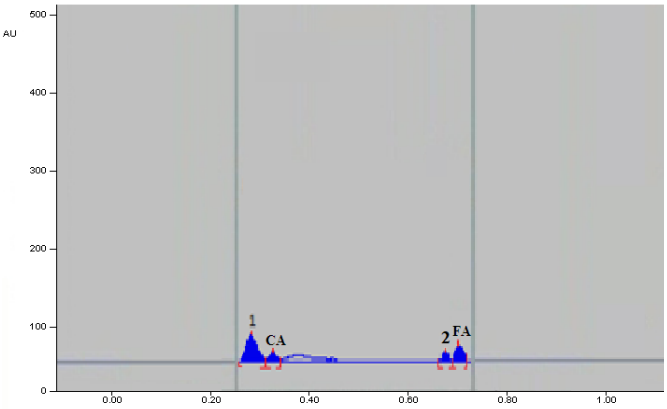


Figure 8: HPTLC chromatogram of *Ananas comosus* var Charlotte Roths child extract

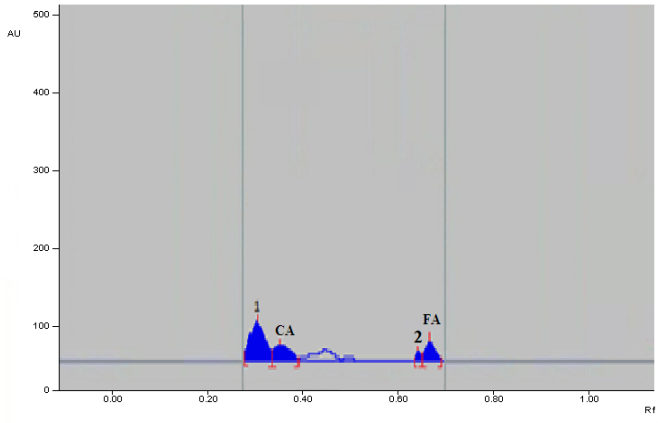


Figure 9: HPTLC chromatogram of Ananas comosus var Queen extract

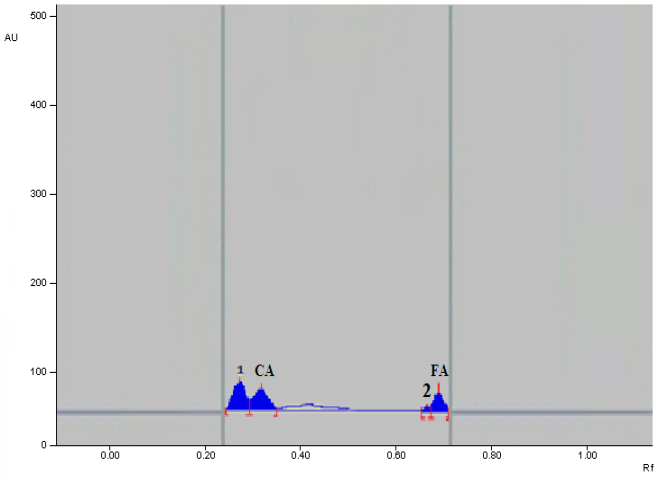


Figure 10. HPTLC chromatogram of Ananas comosus var Desi extract

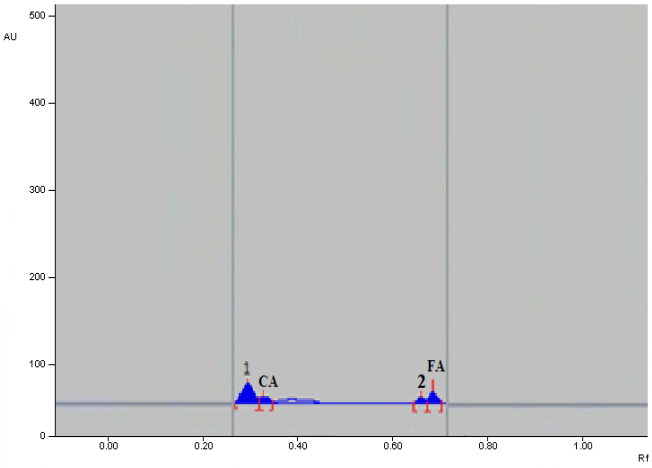
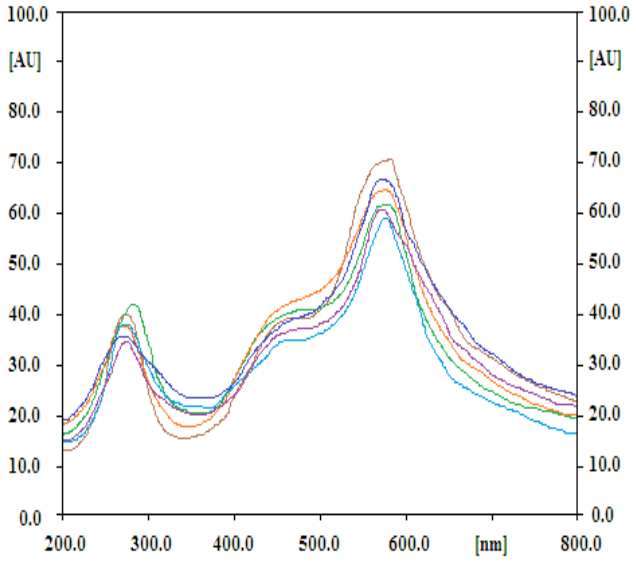


Figure 11: Overlaid spectra of standard FA and CA with four varieties of pineapple cultivar



FA and CA content were found to be highest in *Ananas comosus* var Charlotte Rothschild fruit extracts and lowest in *Ananas comosus* var Desi fruit extracts, which could be attributable to morphological and soil differences. With a small linear range, the HPTLC established method for simultaneous assessment of FA and CA in distinct Indian kinds of *Ananas comosus* cultivars was accurate, precise, and reproducible. As a result, this technology may be industrialized at the industrial level to ensure the highest quality pineapple as a raw material for culinary and pharmaceutical preparations.

CONCLUSIONS

This research focused on estimating FA and CA in distinct *Ananas comosus* varieties based on their geographical distribution. This research will aid in identifying the content of FA and CA in medicinal plants, which could be valuable in the future in pharmaceutical and food preparation. This proven method can also be used to assess quality, ensuring that the content of FA and CA in various pineapples is consistent.

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CONFLICT OF INTEREST

The authors declare that they don't have any conflict of interest.

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