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Quantitative Determination of Cerberin in Seed Extract of *Cerbera odollam* and Rat Serum by High Performance Thin Layer Chromatography

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ABSTRACT

Cerberin (2-o-Acetyl neriifolin) is the principal cardiac glycoside present in the seeds of *Cerbera odollam* (Fig 1) belonging to Apocynacea family. The seeds of *Cerbera odollam* are used as a poison for suicidal as well as homicidal purpose by people around the world. Its detection in the body fluids is somewhat difficult. The present study aims to develop a HPTLC method to identify and quantify Cerberin present in the seeds of *Cerbera odollam* and Cerberin present in Rat Serum. Chloroform and methanol in the ratio of 9.6:0.4 v/v was employed as mobile phase for Cerberin. Linear ascending development was carried out in a 10 x 10 cm twin trough glass chamber (CAMAG) saturated with the mobile phase. Detection and quantitation was performed by densitometric scanning at 254 nm, using deuterium lamp. The proposed HPTLC method provided a good resolution of Cerberin in ethylacetate/ethanol extract of seeds of *Cerbera odollam* and Cerberin present in rat serum, confirmed by overlaying UV absorption spectra with standard cerberin marker. The method found to be rapid, simple and precise and may be employed for the detection and quantification of cerberin in human serum, aspirates and other body fluids.

INTRODUCTION

Cerbera odollam is a tree belonging to the poisonous Apocynaceae family (Anantaswamy, 1940; Leeuwenberg *et al.*, 1998) where the seeds of the species are found to contain toxic principle Cerberin (Fig 2) as the main active cardenolide. *Cerbera venenifera*, a related species found in Madagascar, has a long history as an ordeal poison, and was responsible for the death of 3000 people per year in previous centuries (Ahamed *et al.*, 2008; Yamuchi *et al.*, 1987). The death is likely to occur within 6 h if more than one kernel of the seed is ingested due to cardiac failure (Gaillard *et al.*, 2004). The *Cerbera odollam* tree is responsible for about 50% of the plant poisoning cases and 10 % of the total poisoning cases in Kerala, India (Hien *et al.*, 1991).

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It is used both for suicide and homicide. It is a powerful toxic plant that is currently completely ignored by western physicians, chemists, analysts and even coroners and forensic toxicologists (Laphookhieo *et al.*, 2004). Cytotoxic activity of cardenolide principle from the seeds of *Cerbera odollam* has been studied (Chopra *et al.*, 1942). The Burmese use it for lighting, as a cosmetic, or mixed with other oils as an insecticide or insect repellent (Chen *et al*, 1942).

Cardiac properties in Cat show a rise in blood pressure and decrease in heart rate (Hasan *et al*, 2011). Triticustero l, 2, 6-Dihydroxy-4-methoxy benzoic acid, 2-Hydroxy-4-methoxy-6-methyl benzoic acid has been isolated from the stem bark of *Cerbera odollam*. Literature review shows that a TLC method is reported to detect the poisonous components from viscera after autopsy of people who consumed the *Cerbera odollam* kernels (Krishnamurthy *et al.*, 1978). Only one method has been reported so far for the determination of Cerberin that is by UPLC-MS (Carlier *et al.*, 2014).

Fig. 1: *Cerbera odollam* fruit.

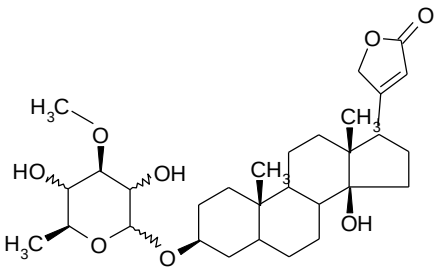

Fig. 2: Structure of Cerberin.

MATERIALS AND METHODS

For the present study CAMAG HPTLC system equipped with Linomat V applicator, TLC scanner 3 controlled by WinCATS software were used. All the solvents used for HPTLC analysis was obtained from MERCK. Reference Standard Cerberin (2-o-Acetyl neriifolin) was obtained from Sigma Aldrich, USA.

Preparation of Standard Solution

The 2 mg of marker Standard Cerberin (2-o-Acetyl neriifolin) was dissolved in 2 mL of methanol to get 1 µg/µl. Cerberin and this solution was used for HPTLC analysis as Standard solution.

Preparation of Plant seed Extract

Ripe seeds of *Cerbera odollam* were collected and about 14.2 g was extracted with ethyl acetate-ethanol mixture (1:1v/v). The extract was dried in a water bath maintained below 70 ^{o}C and the extract was evaporated to dryness. The residue was again added with ethyl acetate (Krishnamurthy *et al* 1978). The extract was again dried and to this 0.5 mL of ethanol was added and used as Plant seed extract for HPTLC.

Rat Serum Extract

Dried ripe seeds were macerated in distilled water and this solution was given orally to Albino Rats. After 30 min blood samples were collected from Tail Vein (LASA good practice guidelines 1998:1, 1) and serum was separated by Centrifuge. This serum is subjected to the same extraction procedure as mentioned in Plant seed extract. One more purification process with ethyl acetate was carried out. These two extracts were made available for HPTLC as Rat serum Extract.

Chromatographic Conditions for TLC

Samples of plant seed extracts and rat serum and standard Cerberin were spotted on a Pre-coated TLC aluminium sheets silica gel 60 F_{254} (10 x 10 cm, 0.2 mm thickness) as 8 mm wideband width by using automatic TLC applicator Linomat V,10 mm from the bottom. Several such Plates were prepared and kept in different Solvent Systems (Table 1) for development. The Mobile phase consisting of Chloroform: Methanol (9.6:0.4v/v) showed good movement of spots without tailing. Increasing the amount of Methanol led to poor discrimination. Hence Chloroform: Methanol (9.6:0.4 v/v) was selected as the mobile phase. The plates were kept for saturation in twin trough chamber for 10 min. After development the plates were dried in air and scanned at 254 nm by using CAMAG Scanner III. The plates were photographed at 254 nm and 366 nm by using CAMAG Reprostar instrument and shown in Fig 3, 4, 5, 6 and 7.

Table 1: Optimization of Solvent systems for estimation cerberin by HPTLC.

Solvent System	Ratio	Result
1-Dichloromethane: Methanol	5:5	Spot moved with tailing
2-Ethanol: Ethylacetate	7:3	No movement of Spots
3-Di-ethyl ether:Methanol	8:2	Spots moved along Solvent
4-n-hexane: Methanol	8:2	No movement of Spots
5-Chloroform:Methanol	9.6:0.4	Spots moved without tailing

Chromatographic Conditions for HPTLC

The standard and samples were spotted in the form of bands (6 mm width) with a Camag microlitre syringe on pre-coated silica gel HPTLC plate 60F_{254} (10× 10 cm with 250 µm thickness E. Merck, Darmstadt, Germany) using a Camag Linomat V (Switzerland). The plates were pre-washed by methanol and activated at 60 °C for 5 min prior to chromatography. The sample loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase and the plate was developed in the respective mobile phase up to 70 mm. The Chloroform-methanol (9.6:0.4 v/v) was employed as mobile phase (Krishnamurthy *et al* 1978). Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber (Camag, Mutenz, Switzerland) saturated with the mobile phase and the chromatoplate development for two times with the same mobile phase to get good resolution of photochemical contents.

The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ± 2 °C). The developed plate was dried by hot air to evaporate solvents from the plate. The plates were photographed at 254 nm and 366 nm by using CAMAG Reprostar instrument and shown in (Fig. 8, 9).

Calibration Curve for Standard Cerberin

The standard solutions (1, 3, 6, 9, 12 µg/spot) were applied on TLC plate and further it was developed and scanned as per the chromatographic conditions mentioned above and the peak areas were recorded (Fig 10, 11, 12, 13, 14). Calibration curve of Cerberin was prepared by plotting peak area against concentration of Cerberin (Fig 15).

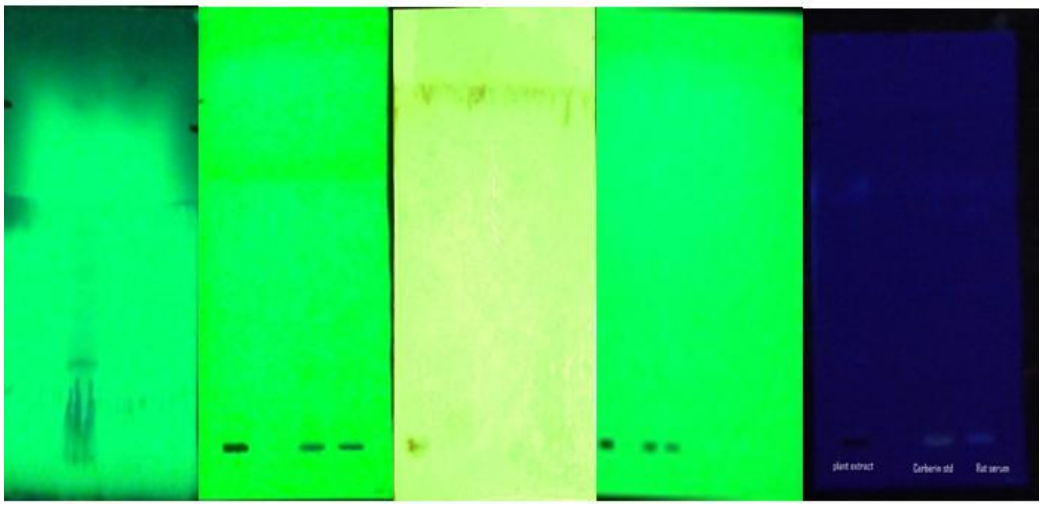

Fig. 3, 4, 5, 6 and 7 : Chromatograpm obtained with different solvent Systems 1, 2, 3,4 and 5

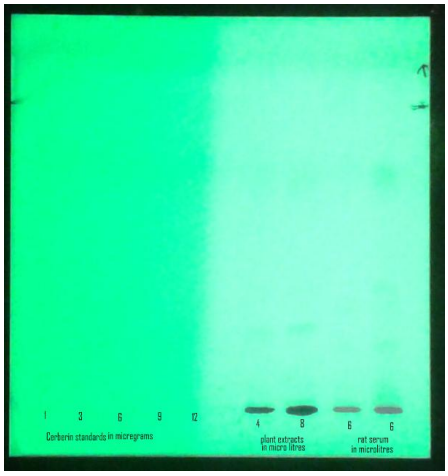

Fig. 8: HPTLC chromatogram at 254 nm.

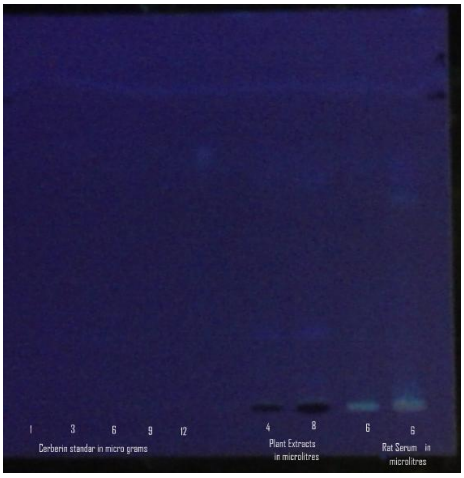

Fig. 9: HPTLC chromatogram at 366 nm.

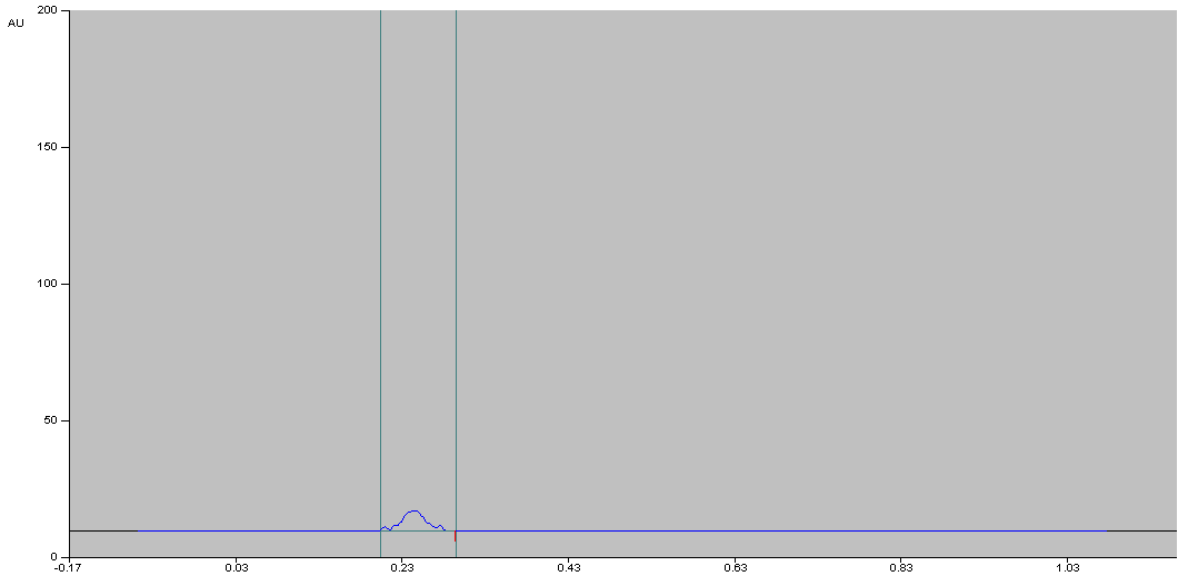

Fig. 10: Chromatogram of Standard Cerberin 1 µg/µl

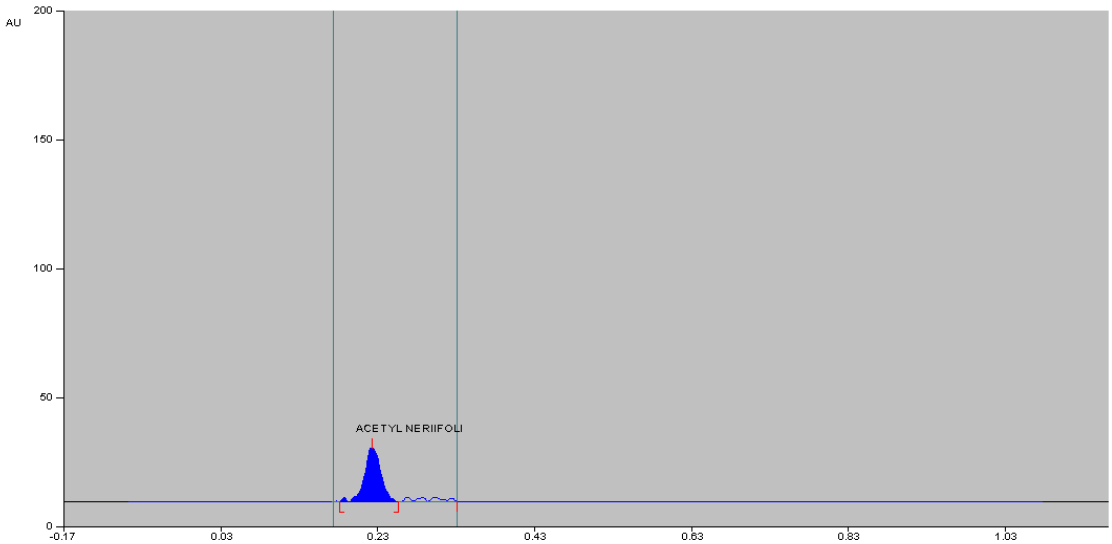

Fig. 11: Chromatogram of Standard Cerberin 3 µg/µl

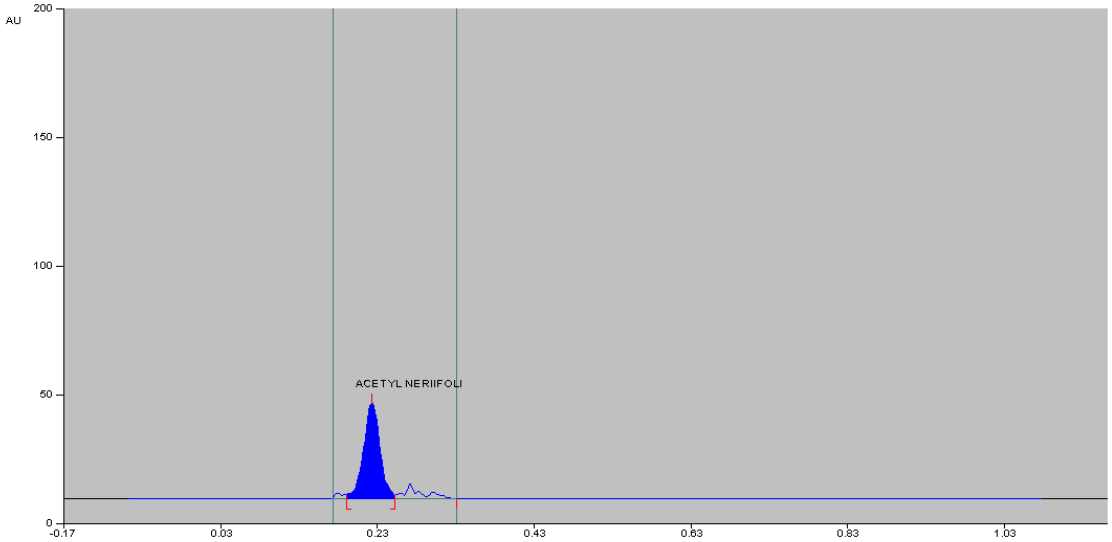

Fig. 12: Chromatogram of Standard Cerberin 6 µg/µl

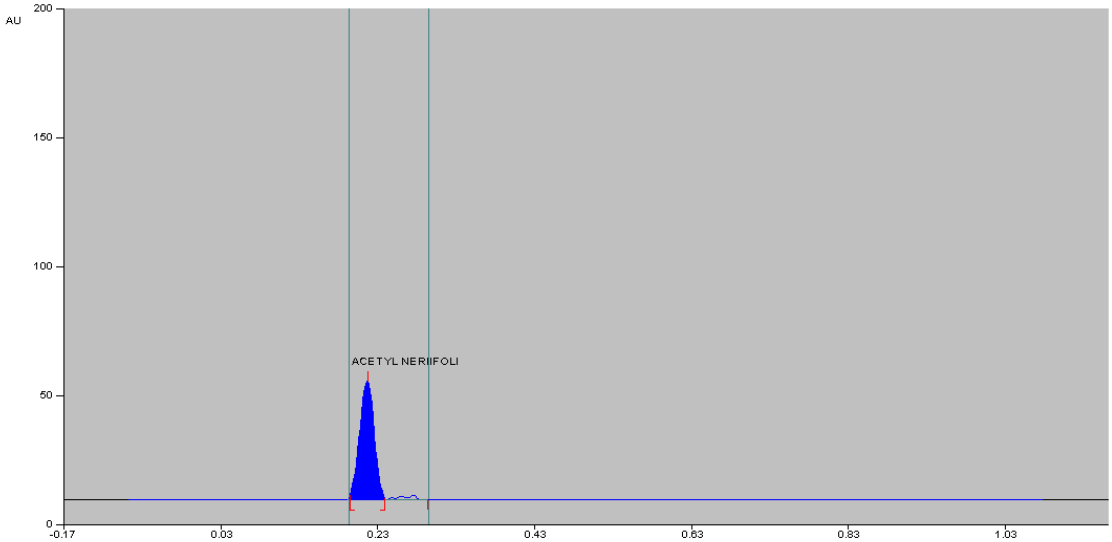

Fig. 13: Chromatogram of Standard Cerberin 9 µg/µl.

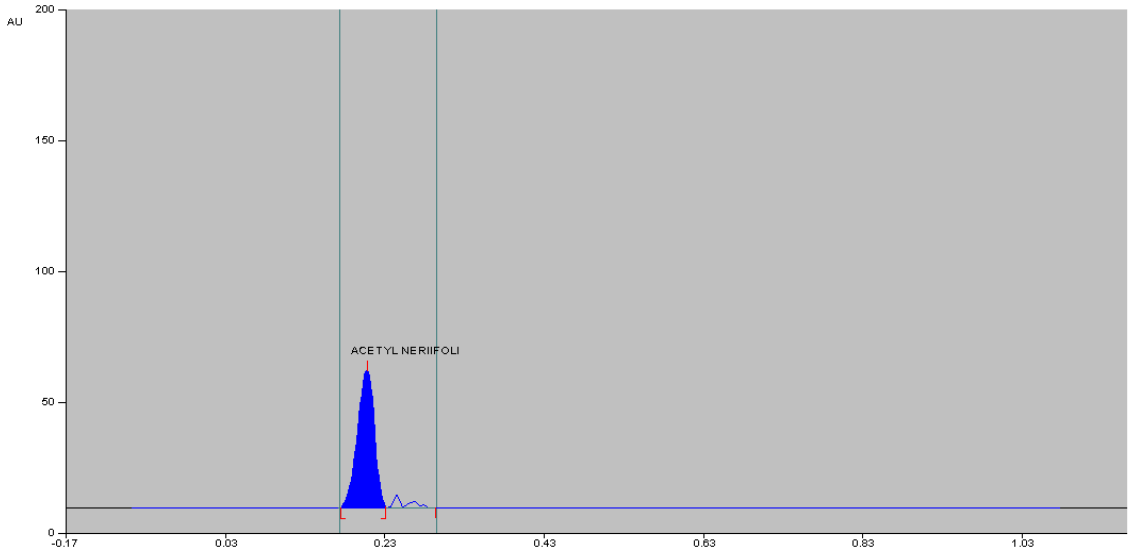

Fig. 14: Standard Cerberin 12 µg/µl.

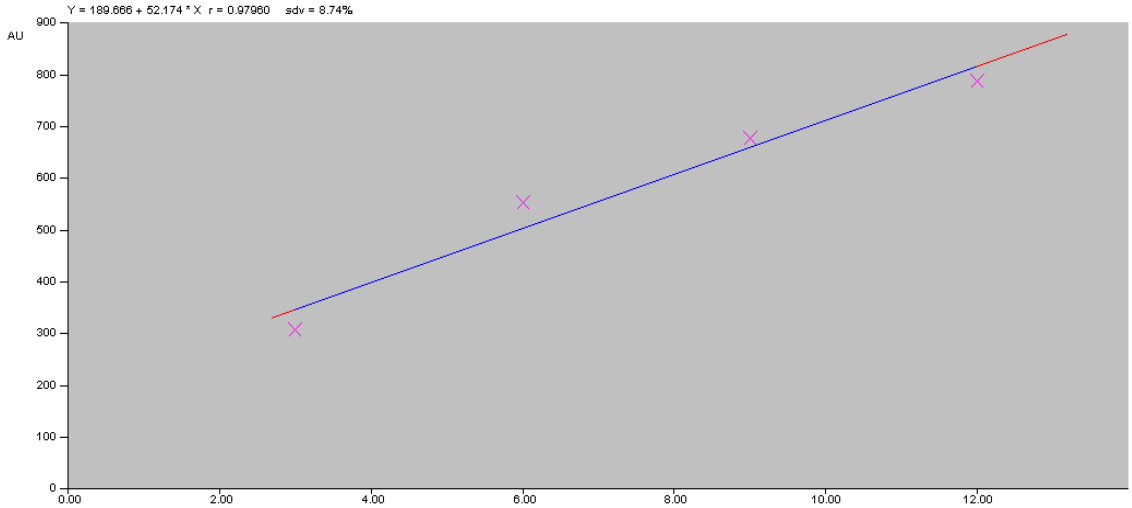

Fig. 15: Calibration Curve of Cerberin.

Quantification of Cerberin in Samples

Sample solutions of *Cerbera odollam* seed extract (4 µl, 8 µl) (Fig 16, 17) and rat serum crude extract (6 µl) and purified (6 µl) (Fig 18, 19) and standard solution (3 µl, 6 µl, 9 µl, 12 µl) were spotted on HPTLC plate (E. Merck). The percentage of Cerberin present in samples were calculated by comparison of the areas measured for standard solution (Table 2)

RESULTS AND DISCUSSION

Optimization of HPTLC Chromatographic Conditions

Standard Cerberin showed single peak in HPTLC chromatogram. The amount of Cerberin present in Seed extract and Rat serum were computed from the above calibration curve. The linearity of Cerberin was found to be 3-12 µg/spot. The correlation coefficient of Cerberin was found to be 0.9796. The LOD and LOQ of Cerberin were found to be 0.552 µg /spot and 1.675 µg/spot respectively. The method was found to be very specific. Since the overlay spectrums of standard Cerberin with sample were found to be same (Fig. 20).

Validation of the Proposed Method

ICH guidelines were followed for the validation of the analytical methods developed (precision, repeatability, accuracy, Ruggedness, Robustness, LOD and LOQ) (ICH, 1994; ICH, 1996). Summary of validation parameters was listed out in (Table 3).

Linearity

A representative calibration curve of Cerberin was obtained by plotting the peak area of spot against the concentration of Cerberin standard (3-12µg/µl). The correlation coefficient of Cerberin was found to be 0.9760 and thus exhibits the good linearity between concentration and peak area (Fig 15).

Table 2: uantification of Cerberin in Samples.

Sl No	Sample ID	Applied volume µl	Area	Standard Amount µg	Sample Amount µg
1	Cerberin standard	3	306.99	3	-
2	Cerberin standard	6	552.58	6	-
3	Cerberin standard	9	677.09	9	-
4	Cerberin standard	12	787.23	12	-
5	Plant seed extract	4	231.27	-	2.51
6	Plant seed extract	8	296.41	-	2.90
7	Rat serum extract	6	1278.76	-	16.99
8	Rat serum purified	6	2106.59	-	32.11

Table 3: Validation Parameters.

Linearity Range	3-12µg
Correlation coefficient	0.9767
Rf value	0.22
Interday Precision	0.692
Intraday Precision	0.952
Repeatability	0.890
LOD	0.552µg/spot
LOQ	1.675µg/spot
Robustness	Robust
Ruggedness	0.981

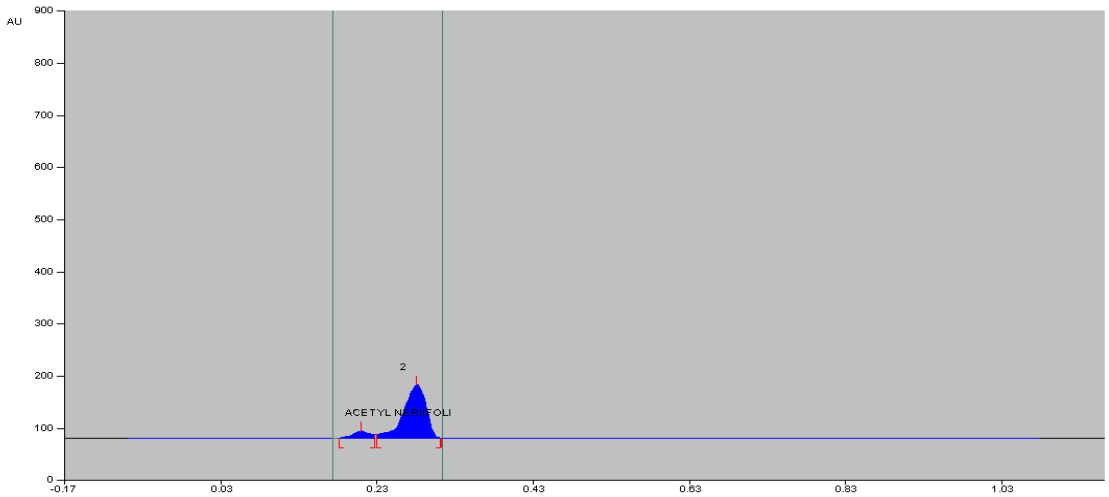

Fig. 16: Chromatogram of seed extract of *Cerbera odollam* 4 µl.

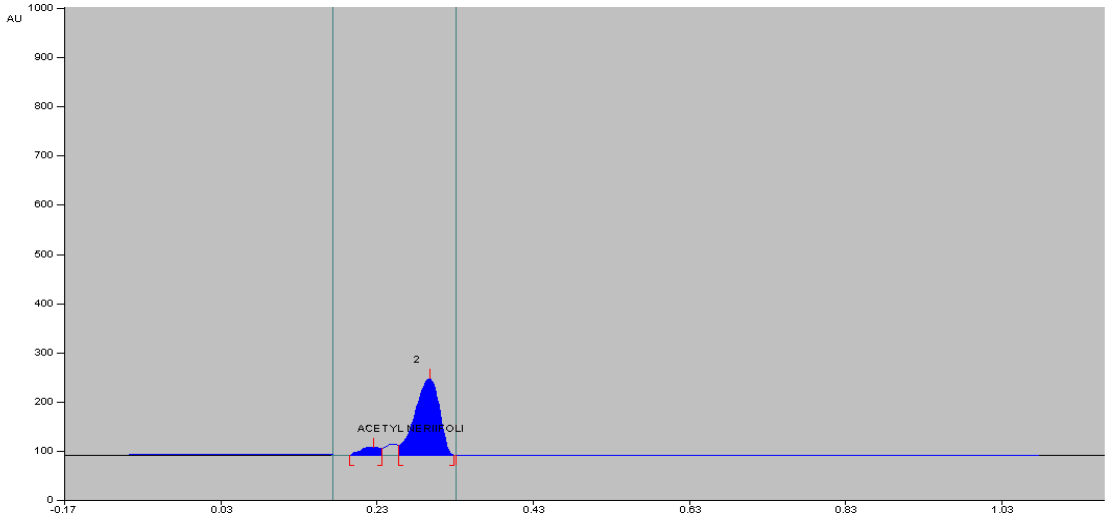

Fig. 17: Chromatogram of seed extract of *Cerbera odollam* 8 µl

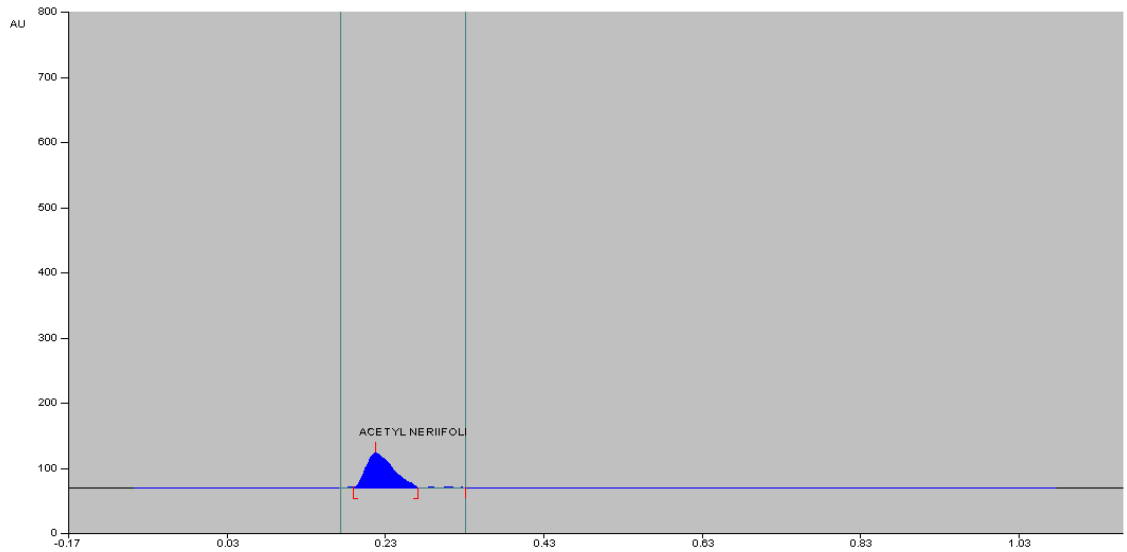

Fig. 18: Chromatogram of seed extract of *Cerbera odollam* in Rat Serum 6 µl.

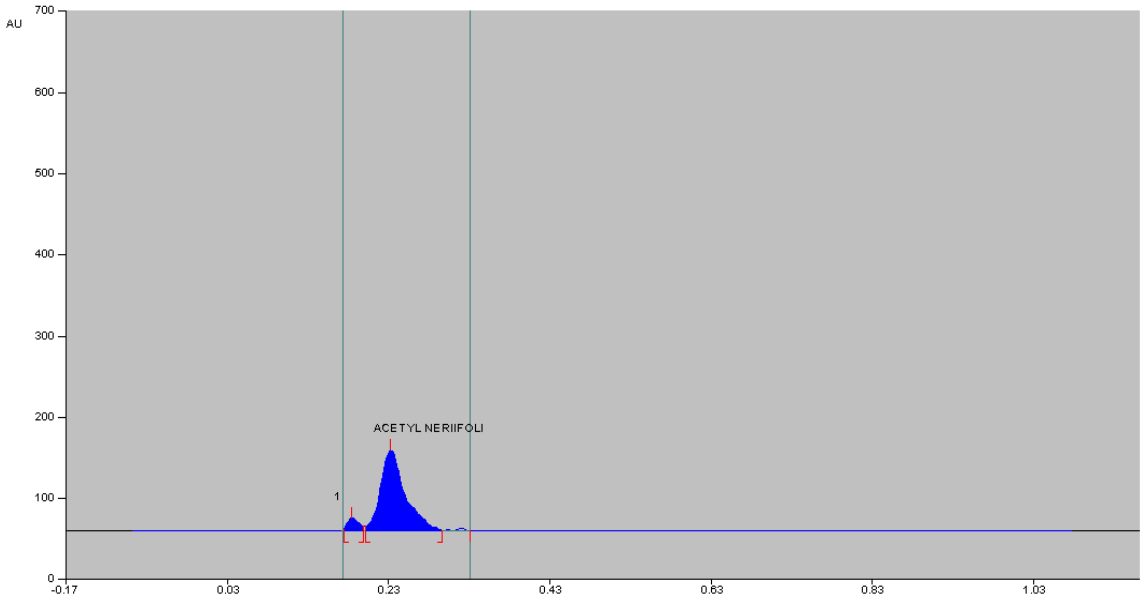

Fig. 19: Chromatogram of seed extract of *Cerbera odollam* in purified Rat Serum 6 µl.

Inter-day and intra-day precision

The inter-day precision (RSD) was determined by analyzing standard solution of Cerberin over the entire calibration range for three different days. The intra-day precision (RSD) was determined by analyzing standard solution of Cerberin over the entire calibration range for three times on the same day.

Repeatability

Repeatability of measurement of peak area and peak height: Standard Cerberin solution was spotted on a TLC plate, developed and dried. The separated spot was scanned for six times without changing plate position and RSD for measurement of peak area was computed.

Limits of detection and Quantification

According to ICH, limit of detection (LOD) is the lowest concentration of the analyte that can be detected and limit of quantification (LOQ) is the lowest concentration of analyte that can be detected with acceptable accuracy and precision. LOD and LOQ are calculated from the formulae 3.3σ/s and 10σ/s respectively. Where σ is the standard deviation of y-intercepts of the regression line and s is the slope of the calibration curve. The minimum detectable limit was found to be 0.552 µg/spot for Cerberin

Limit of Quantification

The minimum quantified limit was found to be1.675 µg/spot for Cerberin

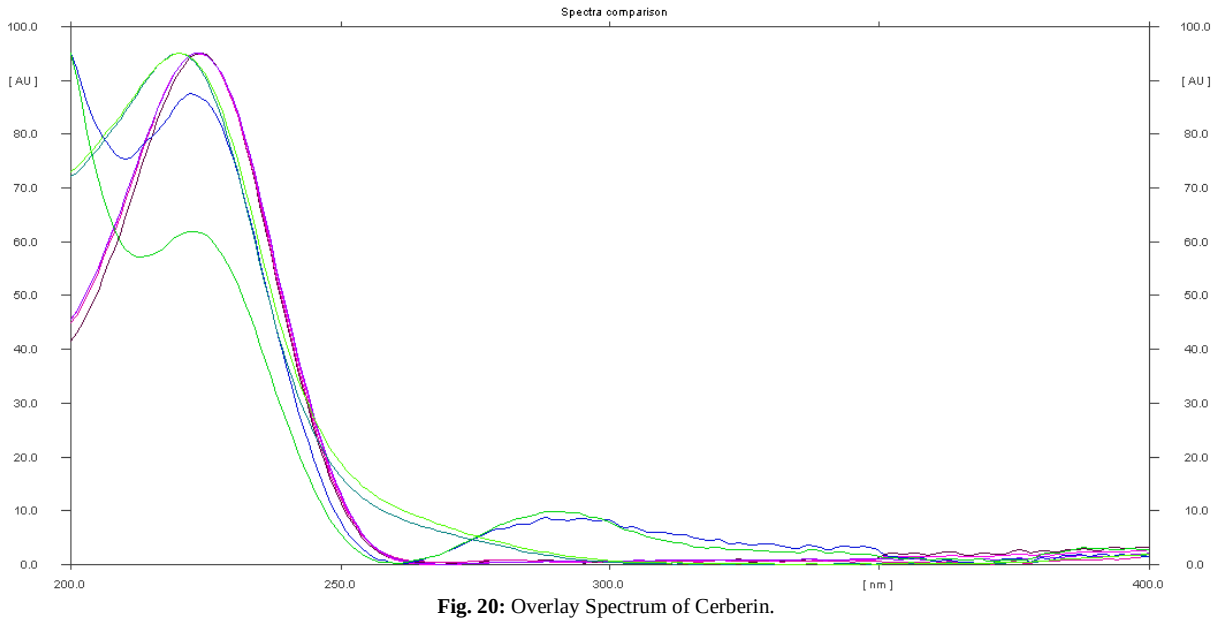

Fig. 20: Overlay Spectrum of Cerberin.

Specificity

It was observed that other constituent's presents in the Rat Serum did not interfere with the peak Cerberin. Therefore the method was specific. The overlay spectrum of standard Cerberin present in the samples was found to be similar on overlap. The peak purity of the Cerberin was assessed by comparing the spectra at three different levels, viz. peak start, and peak apex and peak end positions of the spot. The overlay spectrum was shown in (Fig 20).

Robustness

The mobile Phase composition, mobile phase volume, chamber saturation time was changed and effects in result were studied.(Table :4)

Table 4: Robustness Table.

Mobile phase	Ratio	Effect on bands
Diethyl ether : Methanol	7:3	Tailing effects
Chloroform : Methanol	9:1	Spreading of bands
Chloroform : Methanol	8:2	Spreading of bands

Ruggedness

It expresses the precision within laboratories variations like different days, different analyst. Ruggedness of the method was assessed by spiking the standard 6 times in two different days with different analyst. RSD was found..

CONCLUSION

The developed HPTLC technique is precise, specific, and accurate and stability indicating for the determination of Cerberin in Plant seed extracts and rat serum. Statistical analysis proves that the method is reproducible and selective for the analysis of Cerberin. The method can be used to identify and quantify Cerberin in plasma and other biological fluids of humans in case of accidental or intentional poisoning. This method can be used for detection *Cerbera odollam* poisoning by Clinical toxicologist. The quantification of Cerberin helps physician to judge the severity of poisoning and can select a suitable Antidote.

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