Original Paper

Forensic Implications of Viscera Analysis in Death due to *Cerbera odollam* Poisoning

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ABSTRACT

The main aim of this study was to perform thin layer chromatography (TLC) on plant extracts of *Cerbera odollam* and *Manihot esculenta*, and acertain as to whether similar patterns are obtained in the results. Subsequently, it was proposed to use high pressure liquid chromatography (HPLC) to find out whether it can differentiate between the two.

Most forensic science laboratories in India perform TLC assays for detecting active principles of *Cerbera odollam*, which is a common suicidal agent in some parts of the country, especially Kerala. There have been instances where a positive report for *Cerbera odollam* has been furnished, when no such history of ingestion was available. In some of these cases there was history of ingestion of cassava (*Manihot esculenta),* which is a staple food in some of the communities in Kerala. An earlier study had shown that the active principles of the two plants (one edible and other poisonous) could exhibit similar TLC profiles leading to confusion.

In this study, TLC patterns of the glycosides present in *Cerbera odollam* and *Manihot esculenta* were studied, and it was found that the results were similar to those of the other study. In order to develop an alternative method, which could be applied in suspected cases of *Cerbera odollam* poisoning, to avoid miscarriage of justice in medicolegal cases, HPLC was performed on the samples, which indicated that it could differentiate between the two.

Key Words: Thin layer chromatography; TLC; High pressure liquid chromatography; HPLC; Cassava; *Cerbera odollam*; *Manihot esculenta*

INTRODUCTION

Thin layer chromatography (TLC) is a common preliminary test performed on body fluids in medicolegal cases when poisoning is suspected in a living or dead victim, to detect evidence of toxins or chemicals. In the case of homicide, it is done on viscera samples also, e.g., stomach or intestinal contents, liver, kidney, etc.[1] It is a simple and economical method that can be done with relative ease in most government forensic science laboratories, which are eternally short of funds and expert manpower.

Thin layer chromatography or TLC is a multi-stage distribution process, and involves the use of solvents and solvent mixtures, sample molecule and a suitable adsorbent. It comprises a *stationary phase* consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide or cellulose immobilised onto a flat, inert carrier sheet. A *liquid phase* consisting of the solution to be separated, is dissolved in an appropriate solvent, and drawn through the plate via capillary action, separating the experimental solution. The method can be used to determine the active principles a plant contains, to detect pesticides or contaminants in food, and in forensics to analyze the body fluids or viscera for evidence of poisons.[2]

High pressure liquid chromatography (high performance liquid chromatography or HPLC) is an instrumentation technique of analytical chemistry that relies on the pressure of mechanical pumps on a liquid solvent to load a sample mixture on to a separation column, in which the separation occurs. An HPLC separation column is filled

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with solid particles (e.g., silica, polymers, or sorbents), and the sample mixture is separated into compounds as it interacts with the column particles. HPLC separation is influenced by the liquid solvent's condition (e.g., pressure, temperature), chemical interactions between the sample mixture and the liquid solvent (e.g., hydrophobicity, protonation, etc), as well as chemical interactions between the sample mixture and the solid particles packed inside of the separation column (e.g., ligand affinity, ion exchange, etc). HPLC is much superior to TLC and achieves finer separations of a sample mixture than ordinary liquid chromatography can.[3] This gives HPLC excellent resolving power when separating mixtures, which is why it is a popular chromatographic technique today.

Cerbera odollam (suicide tree) is a tree belonging to the Apocynaceae family, which also includes the yellow oleander and common oleander. The seed is highly toxic, containing cerberin as the main active principle. This tree is responsible for a significant number of poisoning cases in Kerala.[4] It is used for both suicide and homicide. The aim of this study was to bring to notice this powerful toxic plant that is currently completely ignored by Western physicians, chemists, analysts and even coroners and forensic toxicologists. Further, the commonest test employed by forensic science laboratories in India, i.e., TLC gives confusing results when the last meal of the deceased in unnatural deaths consisted of cassava. This has been observed in several medicolegal cases in Kerala state.[5]

Cassava or tapioca (*Manihot esculenta*), a perennial shrub of the world, currently is the sixth world food crop for more than 500 million people in tropical and subtropical Africa, Asia (including India, especially Kerala state) and Latin America.[6] It is cultivated mainly by resource-limited small farmers for its starchy roots, which are used as human food either fresh, when low in cyanogens, or in many processed forms and products, mostly starch, flour and for animal feed. Because of its inherent tolerance to stressful environments, where other food crops would fail, it is often considered a food-security source against famine, requiring minimal care. Under optimal environmental conditions, it compares favorably in production of energy with most other major staple food crops due to its high yield potential.[7]

In some cases of unnatural death subjected to autopsy it has been noted that when viscera have been submitted (as a routine procedure) for chemical analysis, the results have come positive for odollam glycosides, while there was nothing by the way of history or autopsy findings to suggest this. It was also observed that in many of these cases the last meal of the deceased consisted of cassava, which was present in the stomach/intestinal contents subjected to chemical analysis. When subjected to chemical analysis, the samples gave a false positive result suggesting the presence of odollam in those samples.[8] Hence in this study, it was decided to perform HPLC on these samples with the intent of differentiating between the two by this sophisticated technique, so that no miscarriage in justice takes place in medicolegal cases.

MATERIALS AND METHODS

Samples

Manihot esculenta and *Cerbera odollam*

Roots of the former were collected from Ernakulam and Idukki districts of Kerala state. Professional help was sought from a qualified botanist to identify the samples positively as *Manihot esculenta*. The samples were then shade-dried, powdered and preserved for further study.

Fruits of this *Cerbera odollam* were collected from Ernakulam district of Kerala state. Professional help was sought from a qualified botanist to identify the samples positively as *Cerbera odollam*. The samples were then shade-dried, powdered and preserved for further study.

TLC and high pressure liquid chromatography were used, both of which are ideal chromatographic methods for separating organic compounds. Silica gel slurry was prepared for TLC by dissolving 7 g of silica powder in 14 mL of water (1:2). HPLC system (Shimadzu) with LC1 AT VP pump, SPD-M10A, detector and C10-10 ASVP column were used.

Solvent Systems

TLC

1. Mobile phase 1 [Butanol: Acetic acid: Water (5:1:4)] Butanol, acetic acid and millipore water are mixed in the ratio 5:4:1, and kept in air tight container.
2. Mobile phase 2 [Butanol: Acetic acid: Water (3:2:5)] Butanol, acetic acid and millipore water are mixed in the ratio 3:2:5, and kept in air tight container.
3. Mobile phase 3 [Butanol: Acetic acid: Water (3:1:1)] Butanol, acetic acid and millipore water are mixed in the ratio 3:1:1, and kept in air tight container.
4. Mobile phase 4 [Chloroform: Water (19:1)] Chloroform and water are mixed in the ratio 19:1, and kept in air tight container.

HPLC

Solvent mixture for cyanogenic glycoside detection. Water: Acetonitrile (80:20).

Preservation of Samples

The collected samples were washed with tap water thoroughly and were dried in shade. They were then stored in dark place with very little moisture.

Extraction of Samples by First Method

1 g of cassava/odollam was taken in a beaker containing 20 mL Dioxan: Methanol: Dichloromethane mixture (8:1:1). This mixture was refluxed for 15 min at 40–45°C in a water bath. Subsequently, this mixture was centrifuged at 2000 rpm for 5 min. The supernatant was taken into a china dish. After drying, the sample was dissolved in a few drops of HPLC-grade methanol and preserved in small vials.

Exraction of Samples by Second Method

100 g of the product was taken in a beaker containing 100 mL methanol. This solution was filtered/centrifuged at 5000 rpm for 5 min. The filtrate was collected and the supernatant methanol was placed in a water bath at 40°C till the methanol was evaporated. 100 mL 5% aqueous acetic acid was added into the crude extract. This mixture was again filtered or centrifuged at 5000 rpm for 5 min. 10 mL dichloromethane (CH_2Cl_2) was added into the acetic acid-containing mixture. The aqueous phase was collected after clear separation. The aqueous phase was basified with 10% aqueous sodium carbonate (Na_2Co_3) to pH 10. This was again extracted with 100 mL CH_2Cl_2. The organic phase was collected and evaporated to dryness at 40°C in the water bath. After drying, the sample was dissolved in a few drops of methanol and preserved in small vials.

Extraction of Samples by Third Method

One litre of boiling water was added to 50 g of the dried products and allowed to settle for 1 hr at room temperature. The aqueous extract was filtered. In a separating funnel, the extract was mixed with isobutyl alcohol, maintaining a ratio of 40:60. The system was allowed to rest until complete phase separation was achieved. The butanolic extract was centrifuged at 2000 rpm for 15 min. The extract was dried and dissolved in a few drops of methanol and preserved in small vials.

Extraction of Samples by Fourth Method

50 g of product was taken and mixed with 10 mL methanol in a beaker. The mixture was vortex mixed for 10 min and left overnight. The supernatant solution was taken and centrifuged. The extract was evaporated and the crystals washed with a few drops of methanol, and preserved in small vials.

Extraction of Samples by Fifth Method

50 g of product was taken and mixed with 10 mL ethyl acetate in a beaker. The mixture was vortex mixed for 10 min and left overnight. The supernatant solution was taken and centrifuged. The extract was evaporated and the crystals washed with a few drops of methanol, and preserved in small vials.

Isolation

TLC

TLC silica gel 60 F254, 20 x 20 cm (Merck).

TLC plates - readymade: pore size 1.0554.0007 were taken and cleaned thoroughly. It is important to ensure that plates are clean and free from grease. 10 mg of each sample was applied to the TLC plate along the base line, and the plate was air dried for 2 min to dry the sample spot. The TLC plate was placed in the tank, which was saturated to the TLC plate along the baseline. The plate was run till the solvent attained $3/4^{th}$ of the plate, and then taken out and allowed to dry under gentle breeze. Viewing the plate was done by placing it in the iodine chamber. Spots of both cassava and odollam were compared by calculating Rf value. Five runs for each extract was done in each of four mobile phases to statistically validate them.

HPLC

HPLC system (Shimadzu) with LC1 AT VP pump, SPD – M10A detector and C10 – 10 AS VP column was used. The HPLC column was washed with millipore water. The temperature was adjusted to 40°C, pressure to 108 Kgf and flow rate to 0.8 mL/min. The detector was set at 267 nm. Twenty microlitres of extract was taken in a glass injector and injected into the column. The sample was run for 30 min. Graphical representations of these components were obtained in the form of peaks in a computer monitor.

RESULTS

TLC

Spots having similar Rf value were obtained in all the five mobile phases. Ethyl acetate extract did not give spots for odollam in the first mobile phase, both odollam

and cassava in the second and third and fourth mobile phase.

HPLC

All extracts except dioxan was subjected to HPLC. Dioxan being a thick extract was not considered to be suitable for HPLC. The following were the results:

1. **Extraction using dichloromethane -** A total of 13 peaks were obtained for cassava, and 19 peaks for odollam in the chromatogram. Out of these peaks, 1, 4, 6, 7 and 8th peaks of cassava were similar to the peaks 1, 6, 9, 10 and 16 of odollam with respect to retention time, i.e., similar peaks were obtained at 4.5 min, 7.4 min, 10 min and 13 min of the run.
2. **Extraction using isobutanol -** A total of four peaks were obtained for cassava and three for odollam. Peak1 was obtained for both at similar retention time (RT), i.e., at 4.7 min. All the other peaks were dissimilar.
3. **Extraction using methanol -** Six peaks were obtained for cassava and one for odollam. Only the first peak at RT at 4.8 min was similar with respect to the retention time.
4. **Extraction using ethyl acetate -** A total of 13 peaks were obtained for cassava and 17 for odollam. Peaks 1, 5, 7, 9, 10, 11 and 13 of cassava were similar to peaks 1, 7, 8, 11, 12, 15 and 17 of odollam with respect to the retention time. All the other peaks were dissimilar.

Table 1

Sample	Extraction 1 - Dioxan			
	Mobile Phase 1 (B:Ac:W-5:1:4) Mean value, Std deviation	**Mobile Phase 2** (B:Ac:W-3:1:1) Mean value, Std deviation	**Mobile Phase 3** (C:W-19:1) Mean value, Std deviation	**Mobile Phase 4** (B:Ac:W-3:2:5) Mean value, Std deviation
Cassava	0.703, 0.011	0.735, 0.526	0.465, 0.113	0.791, 0.341
Odollam	0.704, 0.0080	0.760, 0.514	0.395, 0.062	0.797, 0.052

Table 2

Sample	Extraction 2 - Ch_2Cl_2			
	Mobile Phase 1 (B:Ac:W-5:1:4) Mean value, Std deviation	**Mobile Phase 2** (B:Ac:W-3:1:1) Mean value, Std deviation	**Mobile Phase 3** (C:W-19:1) Mean value, Std deviation	**Mobile Phase 4** (B:Ac:W-3:2:5) Mean value, Std deviation
Cassava	0.512, 0.036	0.643, 0.046	0.206, 0.035	0.521, 0.028
Odollam	0.510, 0.044	0.606, 0.065	0.205, 0.059	0.520, 0.027

Table 3

Sample	Extraction 3 – Isobutanol			
	Mobile Phase 1 (B:Ac:W-5:1:4) Mean value, Std deviation	**Mobile Phase 2** (B:Ac:W-3:1:1) Mean value, Std deviation	**Mobile Phase 3** (C:W-19:1) Mean value, Std deviation	**Mobile Phase 4** (B:Ac:W-3:2:5) Mean value, Std deviation
Cassava	0.419, 0.008	0.624, 0.022	0.305, 0.015	0.558, 0.039
Odollam	0.499, 0.144	0.615, 0.022	0.322, 0.037	0.580, 0.012

CONCLUSIONS

1. It is well known that thin layer chromatography (TLC), which is popular as a method of analysing biological samples for the presence of toxins/drugs/chemicals can give confusing or misleading results in some cases. Much of this stems from its inability to discriminate clearly between closely related compounds. While such confusing or unreliable results can pose problems in clinical toxicology, it is in forensic cases that the maximum impact is felt.
2. One of the forensic situations where TLC has been known to give unreliable results is when the viscera/ body fluids of a dead body alleged to have died from odollam (*Cerbera odollam)* poisoning (common in Kerala) are subjected to chemical analysis, and the victim had partaken a meal containing cassava or tapioca (*Manihot esculenta*) just before his death. The glycosides of odollam and cassava have similarities that can be very difficult to discriminate by TLC.[8]
3. The rf value for both odollam and cassava are similar even when different extraction methods are applied. The mobile phase 19:1 is especially not a suitable mobile phase for interpretation of glycosides by TLC with odollam and cassava.
4. In order to find out whether a more advanced technique (high performance/pressure liquid chromatography or HPLC) can resolve the issue, this study was undertaken. While not as commonly employed for chemical analysis as TLC, HPLC is increasingly being used by Forensic Science Labs/ Chemical Examiner's Labs across India, and therefore could be easily used instead of TLC whenever there is likely to be confusion between cassava and odollam.[9]
5. This study has shown encouraging results for differentiating between odollam and cassava in both raw samples as well as biological fluids, especially gastric aspirate, when HPLC is employed.
6. However, further studies need to be undertaken to develop a refined method for HPLC for both cassava and odollam with high quality standards, so that the various glycosides, which are eluted can be identified with absolute certainty.
7. In the light of the foregoing, it is clear that in all cases of suspected odollam poisoning, a detailed history of the last meal consumed by the deceased should be taken before arriving at conclusions on the basis of TLC, in order to avoid false positive results, which can result from a cassava meal prior to death.
8. In all cases where cassava was part of the last meal of the deceased, it is better to undertake HPLC testing when there is a suspicion of odollam poisoning.

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