Original Paper

Identification of Active Principles of *Manihot esculenta* and *Cerbera odallum* by Thin layer Chromatography: The Potential for Misinterpretation In Forensic Cases

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ABSTRACT

The main aim of this study was to perform thin layer chromatography on plant extracts of *Cerbera odallum* and *Manihot esculenta*, and ascertain as to whether similar patterns are obtained in the results. Most forensic science laboratories in India perform TLC assays for detecting active principles of *Cerbera odallum*, which is a common suicidal agent in some parts of the country. There have been instances where a positive report for *Cerbera odallum* was furnished, where 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 communities. The suspicion naturally arose as to whether the active principles of these two plants (one edible, the other poisonous) exhibit similar TLC profiles leading to the confusion. In this study the TLC patterns of the glycosides present in *Cerbera odallum* and *Manihot esculenta* were studied, which revealed that this was indeed the case. This makes it imperative that alternative methods must be used in suspected poisoning with *Cerbera odallum*, in order to avoid confusion in medicolegal cases. It is clear that the glycosides present in both odallum and cassava cross-react with each other giving similar TLC results.

Key Words: Thin layer chromatography, TLC, Cerbera odallum, Manihot esculenta, Cassava, Suicide tree

Introduction

Thin layer chromatography (TLC) is the usual preliminary test done on body fluids in medicolegal cases where 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 or 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 insecticides in food, in forensics to analyze the body fluids or viscera for evidence of poisons, analysis of dye composition of fibers, or to identify compounds present in a given substance, among other uses.

Toxicological analysis of biological tissues involves firstly the separation of the drug from the biological tissue. For this, the contents of stomach/intestine are diluted in water, while the solid viscera are cut into small pieces and macerated in water. Then a solvent is used to extract

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the poison, purification procedures are undertaken using alkaline and acid solutions, and finally followed by analytical detection and quantitation**.** This can be done by thin layer chromatography (TLC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), or UV spectrophotometry.

It is the practice of most forensic science laboratories in India especially at regional and state levels to depend mainly on TLC because of the low cost involved, besides not requiring special training or expertise. Unfortunately, TLC results may mislead police and doctors in some cases, since it is not a highly specific or sensitive method. There have also been problems with regard to similar results obtained with different toxins, for instance the glycosides of *Cerbera odallum* (Suicide tree) often give similar results to those seen with *Manihot esculenta* (Cassava). The former is a common plant in many parts of Kerala bearing fruits that have a striking resemblance to unripe mango, while the latter is a staple food for many Keralite communities.

Cerbera odallum (Suicide tree) is a tree belonging to the Apocynaceae family, which also includes the yellow and common oleanders. The seed is highly toxic, containing cerberin as the main active principle. This tree is responsible for a significant number of the total poisoning cases in Kerala. It is used both for suicide and homicide. The aim of this retrospective study was to call attention to 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 (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.[2]

Many bioactive constituents of odallum have been identified. Biologically active components within the odallum exhibit a wide range of activities ranging from insecticidal, antimitotic, to cardiotonic effects, causing increased force and speed of contraction of cardiac muscle. The constituents of odallum responsible for exerting cardiotonic effects are the cardiac glycosides, which are of particular importance to the odallum's toxicity.

Cytotoxic cardenolide glycoside from the seed of *Cerbera odallum*

A cardenolide glycoside, 3 beta-O-(2-O-acetyl-1-thevetosyl)-15(14 8)-abeo-5 beta-(8R)-14-oxo-cardd-20(22)-enolide (2-O-acetyl cerleaside A), has been isolated from a methylene chloride extract of the seeds of *Cerbera odallum*, together with four known compounds: cerleaside A, 17 alpha-neriifolin, 17 beta-neriifolin, and cerberin. Their structures were elucidated by spectroscopic methods. All compounds except cerleaside exhibited cytotoxic activities against oral human epidermoid carcinoma (KB), human breast cancer cell (BC), and human small cell lung cancer.[3]

Cassava or tapioca (*Manihot esculenta*), a perennial shrub of the New 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. 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.[4]

The root of cassava is long and tapered, with a firm homogeneous flesh encased in a detachable rind, about 1 mm thick, rough and brown on the outside. Commercial varieties can be 5 to 10 cm in diameter at the top, and 50 to 80 cm long. A woody cordon runs along the root's axis. The flesh can be chalk-white or yellowish; it breaks like a carrot, and darkens quickly upon exposure to air. For this reason, the skinned root must be kept under water until it is ready to be cooked. The root's flavour spoils in a day or so, even if kept unskinned and under refrigeration, which is a problem for supermarkets.

Cassava is famous for the presence of free and bound cyanogenic glycosides, linamarin and lotaustralin. They are converted to HCN in the presence of linamarase, a naturally occurring enzyme in cassava.[5] Linamarase acts on the glucosides when the cells are ruptured. All plant parts contain cyanogenic glycosides with the leaves having the highest concentrations. In the roots, the peel has

a higher concentration than the interior. In the past, cassava was categorized as either sweet or bitter, signifying the absence or presence of toxic levels of cyanogenic glycosides. In cases of human malnutrition, where the diet lacks protein and iodine, underprocessed roots of high HCN cultivars may result in serious health problems.

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 as positive for odallum glycosides, while there was nothing by 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 submitted for chemical analysis. The suspicion naturally arose as to whether the glycosides present in both odallum and cassava were cross-reacting with each other giving similar TLC results. Since this was posing serious problems in the investigation of criminal cases, it was decided to undertake a study to ascertain whether this was true, and to suggest confirmatory methods of analysis be performed whenever samples tested positive for odallum glycosides before giving the final report. The main aim of the study is the TLC patterns of the glycosides present in *Cerbera odallum* and *Manihot esculenta* and ascertains as to whether similarities exist that can cause confusion in interpretation of results and also to suggest alternative methods if results suggest similarities that can cause confusion in medicolegal cases.

Materials

Samples

Manihot esculenta and *Cerbera odallum*

Samples of both these plants were collected from Alappuzha, Ernakulam and Idukki districts of Kerala State. The samples were identified as *Manihot esculenta* and *Cerbera odallum* by a botanist. It was then shade dried, powdered, and preserved for further study. Extraction was done with 1, 4-dioxin, Dichloromethane, Methanol, Acetic acid, and Sodium carbonate. Thin-layer chromatography (TLC) was used which is a chromatographic technique that is ideal for separating organic compounds. Silica gel slurry was prepared by dissolving 7 gm of silica powder in 14ml water (1:2).

Solvent Systems

1. **Butanol: acetic acid: water [5:1:4]**
   Mix 5ml of butanol in 1ml of acetic acid, and make up the final volume to 10ml distilled water.
2. **Butanol: acetic acid: water [3:1:1]**
   Mix 3ml of butanol in 1ml of acetic acid, and make up the final volume to 5ml distilled water.
3. Butanol: acetic acid: water [3:2:5]
   Mix 3ml of butanol with 2ml of acetic acid, and make up the final volume to 10ml with distilled water.
4. **Chloroform: methanol [19:1]**
   Mix 19ml of chloroform with 1ml methanol.

Characterisation

Two-dimensional Thin Layer Chromatography -

Two-dimensional TLC uses the TLC method twice to separate spots that are unresolved by only one solvent. After running a sample in one solvent, the TLC plate is removed, dried, rotated 90, and run in another solvent. Any of the spots from the first run that contain mixtures can now be separated. The finished chromatogram is a two-dimensional array of spots.

1. **Chloroform: pyridine [6:1]**
   Mix 85.71 ml chloroform with 14.29m pyridine.
2. **Butanol: acetic acid: water [5:1:4]**
   Mix 5ml of butanol with 1ml of acetic acid, and make up to 10ml with distilled water.

Methods

Preservation of Samples

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

Extraction of Samples by First Method

The sample was powdered and 1gm was weighed out, and mixed with 20 ml of dioxan, methanol, dichloromethane (8:1:1). The mixture was refluxed for 15 minutes on water bath at 40-45^{0}C using Soxhlet extraction apparatus. Later the sample was centrifuged at 2000rpm for 5 minutes.The supernatant was poured into a china dish for evaporation. Sediment particles were then re-extracted with 20 ml of dioxan: methanol: dichloromethane for 40-45^{0}C for the separation of cardenolide. It was again centrifuged, and the supernatant was poured into the china dish and evaporated to 10ml. The extracts were stored in glass bottles and placed in refrigerator to prevent evaporation.

Extraction of Samples by Second Method

Parts of the plants were prepared by air-drying at room temperature. 150g of dried plant material was extracted in 100 ml of CH_3OH. It was then filtered and centrifuged at 5000 rpm for 5 min and collected into a flask. The CH_3OH filtrate was evaporated at 40^0C by the help of Soxhlet apparatus for 2hr**.** After that, 100 ml 5% aq.CH3COOH was added into the crude extract. This was again filtered and centrifuged at 5000 rpm for 5 min. 100 ml CH_2Cl_2 was added, and extracted into a separating funnel, and the aqueous phase was collected after clear separation. The aqueous phase was basified with 10 % aq.Na_2 CO_3, to pH 10, and again extracted with 100 ml CH_2Cl_2. The organic phase was evaporated at 40^0C by the help of Soxhlet apparatus for 2hr. The crude extract was collected into glass bottles and placed in refrigeration to prevent evaporation.

Isolation

TLC plates were taken and cleaned thoroughly. It is important to ensure that the plates are clean and free from grease. The silica gel slurry was then quickly applied to the glass plate using a commercial spreader to form a film 0.25mm in thickness.The plates were dried in air, and activated by heating at 100°C for 30 minutes (this is helpful in maintaining performance). 10mg of each sample was applied to the TLC plate along the base line, and the plate was air dried for 2min to dry the sample spot. The TLC plate was placed in the tank, which was saturated to the TLC plate along the base line. The plate was run till the solvent attained $3/4^{th}$ of the plate, and then taken out and allowed to dry. Viewing of the plate was done under Iodine vapour chamber or UV lamp. The procedure was repeated with different solvent systems, and the most efficient solvent system was determined by comparing the Rf value of the sample.

Results

One-dimensional Thin Layer Chromatography

One-dimensional TLC of extracted samples under iodine vapour gave a characteristic red spot. Extraction of plants by two different extraction methods produced spots at the same level in the TLC. The Rf value of samples reflected the effectiveness of the used solvent system for the separation of the extract. Both the extracts gave similar spots on using different solvent systems. Clear similar spots were seen with the solvent system Butanol: Acetic acid: Water (3:1:1). The Rf values for the different types of solvents were tabulated. A graph was drawn for the two methods of extraction by taking solvent system on X-axis and Rf value on the Y-axis.

Two-dimensional Thin Layer Chromatography

For characterization, similar methods were repeated for the Two-dimensional Thin Layer Chromatography. The solvent system Butanol: Acetic acid; Water (5:1:4) and Chloroform: Pyridine (6:1) gave the best separation for the Two-dimensional TLC. All the samples showed similar spots regardless of the region from where they were procured (Alappuzha, Ernakulam or Idukki districts).

Table 1 Average Rf Value of Similar Spots by First Method of Extraction

Sl. No.	Type of Solvent	Rf Values (Alappuzha, Ernakulam and Idukki Districts of Kerala)		
1	Butanol: acetic acid: water (5:1:4)	0.282	0.284	0.280
2	Butanol: acetic acid: water (3:1:1)	0.326	0.322	0.323
3	Butanol: acetic acid: water (3:2:5)	0.281	0.279	0.283
4	Chloroform: methanol (19:1)	0.341	0.343	0.340

Table 2 Average Rf Value of Similar Spots by Second Method of Extraction

Sl. No.	Type of Solvent	Rf Values (Alappuzha, Ernakulam and Idukki Districts of Kerala)		
1	Butanol: acetic acid: water (5:1:4)	0.381	0.380	0.379
2	Butanol: acetic acid: water (3:1:1)	0.247	0.253	0.250
3	Butanol: acetic acid: water (3:2:5)	0.311	0.319	0.317
4	Chloroform: methanol (19:1)	0.391	0.387	0.384

Conclusion

1. TLC method gives similar spots for *Cerbera odallum* and *Manihot esculenta.* This can cause confusion in the interpretation of results, which could be of grave significance in forensic cases.
2. Because of such similarities in TLC results, it must not be used for deriving final conclusions in forensic cases.
3. Final report must always be based on more specific methods such as HPLC, GLC, Immunoassays, etc.

Acknowledgement

The authors wish to express their thanks to Dr. B. Umadethan, Professor and Head, Dept. of Forensic Medicine, Amrita Institute of Medical Sciences & Research, Cochin, Kerala for providing the impetus for the study, and to Ms. V. Sivasankari and Mr. N. Rathanakumar of the Dept of Biotechnology, KSR College of Arts & Science, Tiruchengode, Tamil Nadu for their guidance.

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