DOI: 10.14260/jemds/2014/3700

ORIGINAL ARTICLE

CEREBRA ODOLLAM POISONING: FORENSIC IMPLICATIONS OF VISCERA ANALYSIS

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HOW TO CITE THIS ARTICLE:
Mohandas S. "Cerebra Odollam Poisoning: Forensic Implications of Viscera Analysis". Journal of Evolution of Medical and Dental Sciences 2014; Vol. 3, Issue 56, October 27; Page: 12813-12820,
DOI: 10.14260/jemds/2014/3700

ABSTRACT: Cerbera odollam is a common plant suicidal agent in Kerala, next to the insecticidal group of poisons. It is a cardiac poison and death is due to 'Heart attack' caused by the toxic principles, especially Cerberin. Of the available methods for the identification of the toxic principles of Cerbera odollam, colour reactions are non-specific. Chromatography can be accepted as a reliable, speedy and cost effective procedure if TLC plates are large and the solvent is allowed to run sufficiently long. Even TLC can be misleading if the stomach contains tapioca unless extreme care is taken.

KEYWORDS: Cerbera odollam poisoning, Thin Layer Chromatography, Toxicological Analysis.

INTRODUCTION: Cerbera odollam/Suicide tree/Budha tree/Sea mango/Jungle mango is a poisonous shrub seen all over India. Its Botanical name is Cerbera odollam Gaertn. It is known as 'othalanga' or 'chatanga' in Malayalam. The fruit resembles mango- the kernel contains various toxic principles, the important one is Cerberin, a cardiac glycoside almost similar in toxicity to digitalis found in Foxglove. It is the most common plant suicidal agent in the coastal areas of Southern Kerala in India.[1] The kernel is taken as such or after grinding it with jaggery or after preparing a 'curry' with it.

It is occasionally used as a perfect homicidal agent- the powdered kernel may be added to toddy or liquor or food. The incidence is next to insecticidal poisoning.[2,3,4,5,6,7,8] Chemical colour reactions using concentrated H2SO4 which produces crimson colour with the precipitated glycosides obtained in the Stas-Otto procedure, which are conventionally followed for its detection in Kerala, are not specific for Cerbera odollam.[9,10] Such colour reactions are given also by tapioca which is a common food in Kerala.

While the author was working on the histo-pathological changes in the conducting tissue of heart in Cerbera odollam poisoning, in one unrelated case of homicide, where stomach contained tapioca, there was a positive report for Cerbera odollam, based on chemical reactions, where there was no history of consumption of the same.

Object of this study was to assess (1) Whether the conventional chemical reactions used for the identification of toxic principles in biological materials in Cerbera odollam can be misleading; (2) Can Thin layer chromatography (TLC) be used as a reliable, affordable and convenient identification procedure; (3) Limitations of TLC if any.

PHARMACOLOGICAL ASPECTS: Cerbera odollam is a cardiac poison. Its effects resemble those produced by digitalis found in Foxglove. Cerberin has some advantage without the disadvantages of digitalis. Cerberin is more active than Cerberoside. Both produce negative chronotropic, negative bathmotropic and negative dromotropic effect on the heart. Ionotropic action varies with the dose, so is blood pressure. Both Odollin and Odollotoxin stimulate the intestinal musculature and act as

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cathartic. Kernel of one fruit is lethal for man. Page (1964) stated that cardiac glycosides specifically inhibit the active transport of Na^+ out of the heart muscle cells resulting in a net cellular accumulation of Na and net cellular loss of K.

Narendranathan et al (1975) gave a postulate that the toxins lead to inhibition of membrane ATPase resulting in loss of intra cellular K and increase in extra cellular K. An increase in extra cellular K concentration causes a decrease in the ratio of diastolic depolarization so that the distance between two action potential is increased leading to brady-arrhythmias and slowing of conduction. This action is secondary to its depression on the Na pump as well as its parasympathetic action.[1,11,12,13,14]

Cerbera Odollam Fruit

The author has studied the conducting tissue of heart in 20 cases of Cerbera odollam poisoning: haemorrhage was noted in 80% of cases in the conducting tissue of the heart- SA node(14/20), AV node(15/20), AV bundle(2/20) or bundle branches(6/20). Any structural change in the SA node or AV node or the peripheral parts of conducting tissue is likely to cause alteration in the impulse production and conduction. Haemorrhage was detected in those areas of heart having abundant vascular supply.

GASTRO INTESTINAL:

1. Direct irritation of stomach mucosa.
2. Parasympathetic.

CENTRAL NERVOUS: Depression of central synaptic transmission.

CARDIO VASCULAR:

1. Through Vagus nerve.
2. Direct depressant action on the myocardium.
3. Decreasing the slope of phase 4 of action potential by inducing hyperkalemia.
4. Changes in the conducting tissue.[1,14]

CYTOTOXIC: Neriifolin and Deacetyl tanghinin have anti-proliferative / cytotoxic effect- tried in Oral epidermoid Ca, Small cell lung Ca, Breast Ca (anti-estrogenic). Anti-oxidant.

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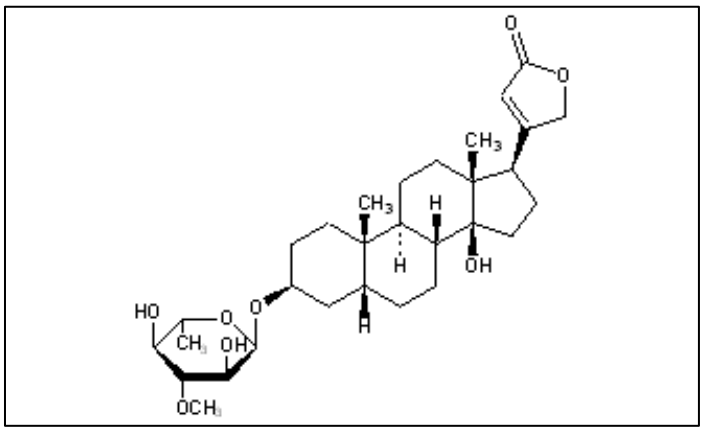
TOXINS: IMPORTANT ONE: Cerberin (Monoacetyl neriifolin). Isolated by D'Vry in 1864. On hydrolysis, yields a sugar called cerberose- a methyl pentose, C6H12O5. Crystallises in different colourless forms-short needles, long needles, disc-shaped, thromboid or coarse prisms. Soluble in ethanol, methanol, ethyl acetate, chloroform, phenol, amyl alcohol, glacial acetic acid and acetone.



TLC Spots: Glycosides of Cerbera odollam

Molecular formula of Cerberin: Others: Cerberoside, Cerleaside A, Odollin, Odollotoxin, Thevetin, Cerapain, 17-alpha neriifolin, 17-beta neriifolin, Cardenolide glycoside, Tanghinin, Deacetyl tanghinin.[15,16,17,18,19,20]

Thin Layer Chromatography (TLC): Chromatography was discovered in 1906 by Michail Tswett, a Russian Botanist. TLC is a simple, quick and in-expensive chromatographic technique used to separate non-volatile mixture of substances, especially when the components are in small amounts and have more or less the same physical and chemical properties. TLC is performed on a sheet of glass, metal or plastic which is coated with a thin layer (0.1 to 0.25 mm) of solid absorbent material, either silica or aluminia.

This layer of absorbent is known as stationary phase. A small amount of the mixture (usually 1%) to be analyzed is spotted 0.5 to 1 cm above the bottom of the plate. This plate is placed in a shallow pool of solvent (0.5 cm) in a developing chamber (jar with a lid or beaker with a watch glass). This liquid is the mobile phase and it slowly rises up the plate by capillary action. As the solvent begins to soak up the plate, it first dissolves the compound in the spot on the base line; continues to move past the spot carrying up the compound; an equilibrium is established for each component of the mixture between the mobiles of that component which are absorbed on the solid and the molecules which are in the solution.

The components will differ in solubility and in the strength of their absorption to the absorbent and some components will be carried farther up than the others. When the solvent has reached 0.5 cm below the top of the plate, the plate is removed from the chamber, dried and the separated components are visualized, if colourless by UV lights or showing the spots up chemically.[10,20,21]

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Retention factor or Rf values are calculated and compared:

$$Rf = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent}}$$

Rf value for a compound is constant only if the chromatographic conditions - solvent system, absorbent, thickness of the absorbent, amount of material spotted and temperature- are constant. Hence relative Rf values are generally considered. The compound with large Rf value is less polar and it will travel larger distance. Rf can give corroborative evidence as to the identity of a compound.

If the identity is suspected, but not proven, an authentic sample (standard) is spotted side by side with the compound in question in the TLC plate. If the two substances have the same Rf values, they are likely, but not necessarily, the same compound. If they have different Rf values, they are definitely different compounds.

Silica gel is silicon dioxide: Silicon atoms are joined via oxygen atoms in a giant covalent structure. At the surface of the silica gel, silicon atoms are attached to –OH groups. So at the surface of the silica gel, there will be Si-O-H instead of Si-O-Si bonds; this surface is very polar and because of the –OH groups, can form hydrogen bonds with suitable compounds around it as well as under vander Waals disperson forces and diapole-diapole attractions. Aluminia is aluminium oxide; its surface has –OH groups attached. High Performance TLC (HPTLC) is one where enhancements are made to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative analysis.

ADVANTAGES OF TLC: Simple, speedy, inexpensive, >1 compound can be used, reliable. When the solid stationary phase is taken as a column, it is known as column chromatography. The column is a glass tube with a diameter 5 to 50 mm and a height of 5 cm to 1 meter with a tap and some kind of a filter at the bottom. Dry method and wet method are used to prepare a column.

For the dry method, the column is first filled with dry stationary phase powder, followed by addition of mobile phase, which is flushed through the column until it is completely wet. For the wet method, slurry is prepared of the eluent with the stationary phase powder and then carefully poured in to the column. A solution of the compound is pipetted on top of the stationary phase. Eluent is slowly passed through the column.

The components are absorbed at different regions depending on their ability for separation. High Performance Liquid Chromatography was developed to solve some of the short comings of liquid chromatography. The use of high pressure here in a narrow column allows for more effective separation in much lesser time.

I R SPECTROSCOPY: IR spectroscopy deals with IR region of the electromagnetic spectrum of light. It can be used to identify and study chemicals. An IR spectrometer(spectrophotometer) providing an IR spectrum is required. IR spectrum is a graph of IR light absorbance or transmittance on the vertical axis vs frequency or wave length on the horizontal axis. IR spectroscopy is based on the principle that molecules absorb specific frequencies that are characteristic of their structure.

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X-RAY DIFFRACTION STUDIES: X-ray powder diffraction is used for the study of (partially) crystalline materials. Each crystalline material shows a distinct X-ray diffraction pattern due to the differences in the crystalline lattice parameters, atom types or packing of molecules.

MATERIALS & METHODS: 20 cases of Cerbera odollam poisoning having reliable history, autopsied were included in this study. This group comprised of 10 males and 10 females; aged between 16 & 38 years. Cerbera odollam kernels, both fresh and darkened, were also used. The study was undertaken in Medical College, Trivandrum.

METHOD OF EXTRACTION:

1) **Solid viscera (Stomach + upper intestine with contents; Liver + kidney):** Samples, 50 gm of each, were taken in a china dish; acidified with acetic acid and extracted with petroleum ether 2 or 3 times. Petroleum ether extracts containing fat were rejected. After removing fat by the above process, ethyl acetate was added, gently shaken and allowed to separate the layers. It was evaporated to dryness and the residue was dissolved in ethanol; filtered again and evaporated. The residue was tested for glycosides of Cerbera odollam ie, colour tests and thin layer chromatography.

2) **Body fluids (Blood, Urine, Brain, CSF, Vitreous):** From the samples, proteins were first precipitated with tungstic acid. To the serum, ethyl acetate was added and proceeded as above.

3) **TLC:** Ethanol was the solvent used. A beaker with a watch glass was the container. Commercially available silica gel plates were used as TLC plates. 1 mg of extract residue dissolved in 1 ml of ethanol was used for spotting.

   A. **Colour reactions:** Chemical colour reactions employed for the identification of the toxic glycosides of Cerbera odollam were non-specific as they gave positive results with other plant materials like tapioca.[10,14]

   B. **Micro-chemical studies:** Micro-chemical studies for the crystals of Cerberin were undertaken from an ethanol extract. The alcoholic extract was evaporated to dryness. The residue was again treated with petroleum ether repeatedly to remove any fat left with it. The residue again dissolved in absolute alcohol and evaporated in room temperature to dryness. It was examined under a microscope for the crystals of Cerberin. Re-crystallization from alcohol enabled to obtain different shades of crystals-rhomboid & needle shaped.

   C. **Thin layer chromatography:** Well macerated viscera was extracted with a 1:1 mixture of ethanol and ethyl acetate. After separation of the phases by centrifugation, the upper layer was removed and evaporated to dryness on a water bath, re-extracted with ethyl acetate and again evaporated to dryness. The residue was washed well with light petroleum to remove any fat extracted along with it, dried and re-dissolved in 1 ml of

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ethanol. Ethanol was poured in a beaker for 0.5 cm; a portion of the inner side was lined with filter paper for saturation of TLC chamber with ethanol vapour.

In a commercially available silica gel TLC plate, using a pencil, a horizontal line was marked 1 cm from the bottom.1 mg of residue (material) was dissolved in 1 ml of ethanol; a micro capillary was dipped in to this solution and gently touched on the spot chosen on the marked line in the TLC plate. The solvent was allowed to evaporate off fully. The prepared TLC plate was placed in the developing beaker and covered with watch glass and left undisturbed until the solvent was about 0.5 cm below the top. The TLC plate was taken out and the upper level of solvent marked with a pencil. Dried in room temperature; sprayed with conc H2SO4 and heated at 100°C for 3 minutes to get purple to brown spots.

Kernels of fresh and darkened Cerbera odollam were similarly treated and finally dissolved in ethanol and used as the standard sample. Distance of the spots from the initial spotting site and the distance travelled by the solvent ethanol from the same base were measured. Rf values were calculated.

RESULTS: In the ascending technique, an alcohol extract of Cerbera odollam gave 4 spots having Rf values 0.46, 0.57, 0.80, 0.97. These 4 spots were obtained in all the viscera including brain, CSF and Vitreous in similar conditions. Similar 4 spots were obtained with ethanol extracts of the kernel-both fresh & darkened in similar conditions.

The Rf values were found to vary with the condition prevailing at the test- temperature, humidity, saturation of the jar, inclination of the plate, thickness and homogeneity of the gel. The 2nd & 4th spots from below were more prominent.

TLC spots – Glycosides of Cerbera odollam

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DISCUSSION: Cerbera odollam is a poisonous plant present in the southern coastal parts of Kerala. The kernel of the fruit contains cardiac toxins like Cerberin, Cerberoside; intestinal irritants like Odollin, Odollotoxin and cytotoxic molecules. It is a common suicidal agent in Kerala with 25% mortality rate, its incidence is next to the insecticidal poisoning. It comprises 50% of the plant poisoning cases.(1) Death is due to 'heart attack' produced by the poison. It can be a perfect murder weapon especially outside Kerala where it is least suspected and the identification is difficult unless sophisticated tests are routinely employed. Recently the kernel is exported from Kerala.

Toxicological analysis is the only confirmatory evidence of poisoning. Using, TLC, toxic principles could be isolated from all the internal viscera. An alcoholic extract of defatted tapioca (Manihot esculenta) gave positive colour reactions with conc H2SO4. Even with TLC, Rf values of tapioca extracts were misleading if the length of TLC plate is less or the time allowed is less. The conventional chemical analytical methods cannot be accepted as standard procedures. If the stomach contains tapioca, even TLC can be misleading unless extreme care is taken. This is of vital significance in Kerala, where tapioca is a common food.

Of the available methods for the identification of the toxic principles of Cerbera odollam, chromatography can be accepted as a reliable procedure. The method employed in the present study was sensitive, rapid and gave positive results with all the tissues and body fluids. 4 principles with different Rf values could be isolated by this method. These 4 spots were identical with those obtained with alcoholic extract of fresh kernel and darkened kernels. It has also been found that these principles are stable in nature and could be isolated up to 9 months both from the viscera and the seed extract. Chromatography can be employed for routine analysis. The method is simple and inexpensive.

CONCLUSION: Cerbera odollam is the most common plant suicidal agent in the coastal areas of Southern Kerala in India, more so among the females. It can be a perfect murder weapon especially in places out side Kerala; death is due to 'heart attack' caused/precipitated by the poison, where poisoning is least suspected and even in suspected cases, unlikely to have subjected to rare sophisticated techniques of detection. Of the available methods for the identification of the toxic principles of Cerbera odollam, colour reactions are non-specific; chromatography can be accepted as a reliable, speedy and cost effective procedure if the TLC plates are large and the solvent is allowed to run sufficiently long. TLC is more reliable than colour reactions. Even TLC can be misleading if the stomach contains tapioca unless extreme care is taken.

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Date of Submission: 13/10/2014.
Date of Peer Review: 14/10/2014.
Date of Acceptance: 22/10/2014.
Date of Publishing: 27/10/2014.