



Identification and Characterization of Lipids by TLC in Parotoid Gland Secretion and Its Extract of *Bufo melanostictus* (Schneider)

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ABSTRACT

The present study was undertaken to analyze the lipids qualitatively in the secretions of parotoid gland and its extracts of *Bufo melanostictus* (Common Indian toad) for the presence of various types of lipids by thin layer chromatography using chloroform, methanol and water (65:25:4) as solvent system. The chromatograms revealed many lipid spots with different R_f values indicating the presence of different types of lipids such as Glycolipids, Phospholipids, and sterols in parotoid gland secretion and its extract.

Keywords: *Bufo melanostictus*, parotoid gland, TLC, Lipids, Chromatogram, R_f value.

INTRODUCTION

The presence of cutaneous granular glands is a characteristic feature of amphibians and are considered to be the source of the bioactive compounds in its skin.¹ The integument of amphibian glandular cells of the skin secretes slimy mucus which is constantly replenished. These cells are organized into two different glandular structures viz., i) mucus secreting glands that help to keep the skin moist and slippery and protect the skin from the mechanical damages and prevent microbial settlement on the skin. This mucus contains a variety of glycoproteins such as mucin, musinogen, and carbohydrate residues such as galactose, fucose, and sialic acid². These glands secrete glycoprotein rich material which plays a part in cutaneous respiration and defense and ii) granular glands generally associated with chemical defense and against predators and microbial infection³. The secretions of these glands contain rich components like biogenic amines, bufo toxins, oligopeptides, proteins, guanidine derivatives, steroids and alkaloids in terms of pharmacological effects.³⁻⁷ In toads, they form the parotoid glands located between eyes and tympanum.⁸ The venomous secretions of the parotoid glands of the *Bufo* species are known to contain several bioactive compounds, which are species specific.⁹⁻¹¹

Lipids are essential to living systems which are a diverse group of compounds with a variety of functions like structural components in cellular membranes; they are most potent energy storage source or cell signaling molecules. Lipids are hydrophobic or amphiphilic small molecules whose biosynthesis originates from two building blocks, a keto acyl group or isoprene group.¹² Lipid classes are divided into natural lipids such as triglycerides, polar lipids such as phospholipids and cholesterol. TLC can be used as a separation technique for analysis of lipids. Bagrov *et al.*, (1995) reported that the steroid bufadienolides are synthesized by many species of toads of the genus *Bufo*. A mixture of steroidal

compounds was separated by TLC in the venom of *Bufo marinus*.¹³ The bufadienolides are biosynthesized from cholesterol in toads and are structurally similar to the toxic luci bufagisin of fire flies and the monarch butterfly cardenolides which are sequestered from dietary milkweed sources.¹⁴ *In vitro* and animal models have suggested bufadienolides could be used clinically in place of orin combination with cardenolides (such as Digoxin) to improve the treatment of congestive heart failure and atrial fibrillation.¹⁵ In the present study, chloroform, methanol and water (65:25:4) has been used as solvent system to extract the various lipid components of the parotoid gland secretion and its extract of *B. melanostictus* by using silica gel thin layer chromatography (TLC) plates.

MATERIALS AND METHODS

Animal materials chosen for study

The toads (7cm to 10 cm in length, weighing about 50 to 75 grams) were collected from vicinity of Kakatiya University hostel buildings, Warangal.

Extraction and Collection of Samples

The parotoid gland secretion and its extracts were collected by the method of Folch *et al.*, (1957).¹⁶ The parotoid gland secretion of *B. melanostictus* was collected in a petri dish with the help of sterile forceps, by gently pressing the parotoid gland to release the secretion.

Experimental procedure for preparation of TLC

These secretions and gland extracts were weighed to the nearest milligram and homogenized in chloroform, methanol mixture 2:1 ratio. The mixture was centrifuged at 2000 rpm for 5 minutes and filtered. The supernatant containing organic layer was collected and evaporated. The particulate matter (dry matter) of the secretion and gland extracts were determined by heating at 110°C for 48 to 72 hours until a constant weight of the dry material was obtained.



The residue was dissolved in 0.5 ml of chloroform, methanol (2:1) solvent. The solutions were spotted at a distance of 1 cm on to readymade TLC silica gel plates (E-merc, Germany) and run in a solvent system of chloroform, methanol and water (65:25:4). The plates were dried at room temperature.

Staining for identification and characterization of lipid spots

For the identification of separated lipid classes. The TLC plates were treated with specific reagents for identification and characterization specific lipid types. Viz., 1). iodine vapors, 2). sulphuric acid (H₂SO₄), 3).

ninhydrine, 4). dittmer-leister reagent and 5) dragendroff's reagent according to the procedure of Kates.¹⁷ R_f values of all the spots were determined immediately.

RESULTS

The results obtained on the characterization and identification of lipids by the TLC in parotoid gland secretion and its extract of *B. melanostictus* are presented in Figs.1 and 2 respectively. The Retardation Factor (R_f) values of lipid spots were determined according to the procedure¹⁶ and presented in Tables.1 and 2.

Table 1: Parotoid gland secretion Staining of lipid spots with different reagents

S.No	Reagent	Rf X100											
		96.6	88.3	83.3	80	70	60	53.3	41.7	30	26.7	16.7	10
L1	Dittmer-leister reagent	-	-	-	-	++	++	-	-	++	++	±	-
L2	Dragendroff's reagent	-	+	-	-	-	-	+	++	-	++	-	-
L3	Sulphuric acid reagent	++	++	++	++	-	++	+++	++	++	++	++	++
L4	Iodine reagent	++	++	+++	++	-	++	+++	++	++	++	++	++
L5	Ninhydrine reagent,	++	++	+++	++	+	++	++	+	-	++	+	+

+++ = high intensity; ++ = moderate intensity; + = low intensity ± = not clearly visible

Table 2: Parotoid gland extraction Staining of lipid spots with different reagents

S.No	Reagent	Rf X100											
		96.6	88.3	83.3	80	70	60	53.3	41.7	30	26.7	16.7	10
L1	Dittmer-leister reagent	-	-	-	-	-	++	-	-	+	-	±	-
L2	Dragendroff's reagent	-	++	++	++	-	-	++	++	+	+	-	+
L3	Sulphuric acid reagent	-	++	+	-	++	+	+	±	-	++	-	-
L4	Iodine reagent	-	++	++	+	-	++	+	±	-	++	+	-
L5	Ninhydrine reagent,	-	-	++	-	-	-	-	-	++	++	+	+

+++ = high intensity; ++ = moderate intensity; + = low intensity ± = not clearly visible

The results obtained indicate that the parotoid gland secretion and its extract stained with molybdenum blue staining reagent¹⁸ showed five blue coloured spots (Lane 1) of phospholipids at R_f values 70, 60, 30, 26.7 and 16.7. The spots of R_f 70, 60, 30 and 26.7 were stained with more intensity. The spots at R_f at 60, 30 and 16.7 were also present in the parotoid gland extract (Fig.2&Table.2), whereas staining was more intense for spot at R_f 60 in parotoid gland extract. The spot at R_f 16.7 was not clearly visible in the parotoid gland secretion and its extract. The spots at R_f value 70 and 26.7 were completely absent in the parotoid gland extract.

The parotoid gland secretion stained with Dragendroff's staining reagent showed four orange spots of choline lipids (Lane 2) at R_f 88.3, 53.3, 41.7 and 26.7 (Fig.1&Table.1). The spots at R_f 41.7 and 26.7 were stained with more intensity. In the parotoid gland extract showed the spots at R_f 88.3, 83.3, 80, 53.3, 41.7, 30, 26.7

and 10 (Fig.2 & Table.2). The staining was more intense for the spots at R_f 88.3, 83.3, 80, 53.3 and 41.7 in parotoid gland extract. Parotoid gland extracts showed more number of lipid spots as compared to the parotoid gland secretion.

The sulphuric acid staining reagent for lipids yielded charred eleven black glycolipid spots in parotoid gland secretion and its extract (Lane 3) spots at R_f 96.6,88.3, 83.3, 80, 60, 53.3, 41.7, 30, 26.7,16.7 and 10 present in parotoid gland secretion (Fig.1&Table.1) and the R_f 53.3 was with high intensity (+++) and the remaining ten spots were stained with moderate intensity (++) . The spots at R_f 88.3, 83.3, 70, 60, 53.3, 41.7, and 26.7 were present in the parotoid gland extract (Fig.2 & Table.2), whereas staining was more intense at R_f 88.3, 70, and 26.7 in parotoid gland extract, the spot at R_f 41.7 was not clearly visible. The spots at R_f 96.6, 80, 30, 16.7 and 10 were

completely absent in the parotoid gland extract. The R_f 70 was absent in parotoid gland secretion.

The Iodine is a general stain for lipids. Staining of TLC plates under iodine vapours resulted in the appearance of eleven yellow spots in parotoid gland secretion (Lane 4) (Fig.1&Table.1) spots at R_f 96.6, 88.3, 83.3, 80, 60, 53.3, 41.7, 30, 26.7, 16.7 and 10 present in parotoid gland secretion and the spots at R_f 83.3 and 53.3 were with high intensity and the remaining eight spots were stained with more intensity. The spots at R_f 88.3, 83.3, 80, 60, 53.3, 41.7, 26.7 and 16.7 were present in the parotoid gland extract (Fig.2&Table.2), whereas staining was moderate intense spots at R_f 88.3, 83.3, 60, and 26.7 in parotoid gland extract, the spot at R_f 41.7 was not clearly visible. The spots at R_f 96.6, 30, and 10 was completely absent in the parotoid gland extract. The spots at R_f 80, 53.3 and 16.7 have shown low intensity in the parotoid gland extract compared to gland secretion.

On staining for lipids with Ninhydrine, lipids containing amino group appeared as eleven purple spots in parotoid gland secretion (Lane 5) (Fig.1&Table.1) Spots at R_f 96.6, 88.3, 83.3, 80, 70, 60, 53.3, 41.7, 26.7, 16.7 and 10 present in parotoid gland secretion and the R_f 83.3 showed high intensity and the remaining six spots were stained with more intensity. The spots at R_f 70, 41.7, 16.7 and 10 were with low intensity. The spots at R_f 83.3, 30, 26.7, 16.7 and 10 present in the parotoid gland extract (Fig.2&Table.2), whereas staining was more intense spots at R_f 83.3, 30, and 26.7 in parotoid gland extract, The spots at R_f 16.7 and 10 were showed low intensity in the parotoid gland extract. The spots at R_f 96.6, 88.3, 80, 70, 60, 53.3, 41.7 were completely absent in the parotoid gland extract. The spot at R_f 30 absent in parotoid gland secretion.



Figure 1: Parotoid gland secretion of *Bufo melanostictus*

Fig. 1 TLC patterns of lipid Lanes:

L1 Parotoid gland secretion in the presence Dittmer-leister reagent; L2 Parotoid gland secretion in the presence Dragendroff's reagent; L3 Parotoid gland secretion in the presence of Sulphuric acid reagent; L4

Parotoid gland secretion in the presence of Iodine reagent; L5 Parotoid gland secretion in the presence of Ninhydrin reagent.



Figure 2: Parotoid gland extraction of *Bufo melanostictus*

Fig. 2 TLC patterns of lipid Lanes:

L1 Parotoid gland extract in the presence Dittmer-leister reagent; L2 Parotoid gland extract in the presence Dragendroff's reagent; L3 Parotoid gland extract in the presence of Sulphuric acid reagent; L4 Parotoid gland extract in the presence of Iodine reagent; L5 Parotoid gland extract in the presence of Ninhydrin reagent.

DISCUSSION

Qualitative studies on characterization and identification of lipids by thin layer chromatography in parotoid gland secretion and its extract of *Bufo melanostictus*, showed a considerable variation between the parotoid gland secretion and its extract. The number of spots was highest in the parotoid gland secretion compared to its extract. The R_f values of the spots indicated the regions of similarity between the parotoid gland secretion compared to gland extract. The pattern observed with Iodine/Sulphuric acid stains indicated that the parotoid gland secretion and its extract was same (Lane 3 and 4).

The changes in the TLC separations of various neutral lipids and phospholipids components are illustrated in figures 1 and 2 respectively. The number of spots stained for phosphate group was same for parotoid gland secretion and its extract but with Ninhydrine, the numbers of spots were more (Fig.1 and Fig.2). Four additional spots between R_f 53.3 to 83.3 were stained with Ninhydrine in the secretion. These spots were also detected with iodine and sulphuric acid but were not stained with phosphate stain. The staining pattern indicates that phosphotides are predominant lipids. Spots with R_f 53.3, 26.7 and 16.7 corresponded more or less with Phosphotidylethanolamine, Phosphatidylcholine and phosphotidyleserine respectively. The fast moving spots are probably neutral lipids or cholesterol esters. The spots lying between R_f 53.3 and 88.3, which were stained with Ninhydrine in the parotoid gland secretion of the toad,

could be biogenic amines. The gland extract did not exhibit these spots. Several of the biogenic amines like bufotenines, indolealkylamines, which were derivatives of aromatic amino acids like phenylalanine and tryptophan are secreted into the venom by gland.¹⁹ It is possible that some of these compounds extracted into the solvent (chloroform: methanol) lipid containing amino groups are stained positively with Ninhydrine, so it is particularly useful for verification of Phosphotidyl ethanolamine and Phosphotidyleserine.²⁰

The thin layer chromatographic separation of parotoid gland secretion indicated that the lipids contained phosphorous moiety in Phosphatidyl choline with Dittmer-leister reagent; Spingomyelin, Phosphoric acid with Dragendroff's reagent; monoacylglycerides, diacylglycerides, triacylglycerides, which were stained with H₂SO₄ reagent; cholesterol, cholesteryl esters and free fatty acids. Unsaturated lipid compound spots were identified through Iodine reagent. At a comparative level, quantitatively triacylglycerides occurred in maximum concentration in parotoid gland secretion followed by diacylglycerides, monoacylglycerides and cholesterol. The alterations occurring in various neutral lipid components run parallel to those described above for the total neutral lipids, with some minor differences.

Gao *et al.*,²¹ reported that the chemical profiles of lipids, number and types of biochemical constituents of toad venoms are different in *Bufo* species and these are differed significantly and forms different geographic locations. The biochemical components of lipids relative and absolute concentrations were varied based on the different solvent extraction methods.²²

CONCLUSION

The TLC study of lipids in *B. melanostictus* indicated that the number of lipid spots found in parotoid gland secretion is more compared to gland extract. Comparison of R_f values with those of standards indicated that the presence of Phosphatidylethanolamine and phosphotidyleserine among the phosphatides present in gland extract.

The present study showed that the parotoid gland secretion and its extract consists of different lipid components with different staining reagents. The selection of solvent system is also very important for the extraction of targeted lipid components and several bioactive compounds, which are species specific. This needs further investigations in various solvent and extraction methods in *Bufo melanostictus*.

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REFERENCES

1. Clarke BT., (The Natural History of Amphibian Skin secretions, Their Normal Functioning And Potential Medical Applications, Biol. Rev., 72, 1997, 365-379.
2. Abhishek DG, Hippargi RV, Gandhare AN, Toad skin-secretions: potent source of pharmacologically and therapeutically significant compounds, The Internet Journal of Pharmacology. 5(2), 2008.
3. Toledo RC and Jared C, Cutaneous granular glands and amphibian venoms, *Comparative Biochemistry and Physiology A*, 111(1), 1995, 1–29.
4. Eraspmer V, Half a century of comparative research on biogenic amines and active peptides in amphibian skin and molluscan tissues. *Comp. Biochem. Physiol.*, 79(C), 1994, 1-7.
5. Lyttle T, Goldstein D, and Gartz J, *Bufo* toads and bufotenine: Fact and Fiction surrounding an alleged psychedelic. *J. Psychoactive Drugs*, 28(3), 1996, 267-290.
6. Maciel NM, Schwartz CA, Rodrigues Pires O, *et al.*, Composition of indolealkylamines of *Bufo rubescens* cutaneous secretions compared to six other Brazilian bufonids with phylogenetic implications. *Comparative Biochemistry and Physiology B*, 134(4), 2003, 641–649.
7. Daly JW, Wilham JM, Spande TF, Alkaloids in bufonid toads (*Melanophryniscus*): temporal and geographic determinants for two Argentinian species," *Journal of Chemical Ecology*, 33(4), 2007, 871–887.
8. Jared C., Antoniazzi M. M., Jordao A. E. C., Silva J. R. M. C., Greven H., and Rodrigues M. T, Parotoid macroglands in toad (*Rhinella jimii*): their structure and functioning in passive defence. *Toxicon*, 54(3), 2009, 197–207.
9. Habermehl GG, Antimicrobial activity of amphibian venoms. *Studies in Natural Products Chemistry, Part C*, 1995, 327-339.
10. Matsukawa M, Akizawa T, Marinoic acid, A novel bufadienolides-related substance in the skin of the giant toad, *Bufo marinus*; *Chem. Pharm. Bull. Tokyo*, 45(2), 1996, 249- 254.
11. Weil AT, and Davis W, *Bufo alvaris* a potent hallucinogen of animal origin, *J. of Ethano pharmacol.* 41(1-2), 1995, 1-8.
12. Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH Jr, Murphy RC, Raetz CR, Russell DW, Seyama Y, Shaw W *et al* A comprehensive classification system for lipids. *Journal of Lipid Research* 46, 2005, 839–862.
13. Bagrov AY, Roukoyatkine NI, Pinaev AG, Dmitrieva RI and Fedorova OV, Effects of two endogenous Na⁺, K⁺-ATPase inhibitors, marinobufagenin and ouabain, on isolated rat aorta, *Eur J Pharmacol*, 151, 1995, 274.
14. Barrueto, Fermin, Jr., Cardioactive Steroid Poisoning: A Comparison of Plant- and Animal Derived Compounds. *Journal of Medical Toxicology*, 2(4), 2006.
15. Daly JW, Myers CW, and Whittaker N, Further classification of skin alkaloids from neotropical poison frogs (*Dendrobatidae*), with a general survey of toxic/noxious substances in the Amphibia. *Toxicon*, 25, 1987, 1023–1095.



16. Folch J, Lees M, and Sloane-Stanely GH A simple method for the isolation and Purification of total lipids from animal tissues. *J.Biol.Chem*, 226, 1957, 497-509.
17. Kates M, *Techniques of Lipidology, Isolation, Analysis, and Identification of Lipids*, 2nd ed. Elsevier, Amsterdam, 1986.
18. Dittmer JC and Lester RL, A simple, specific spray for the detection of phospholipids on thin-layer chromatography. *J. Lipid Res*, 5, 1964, 126–127.
19. Habermehl, GG, *venomous animals and their toxins-amphibians*. New York: Springer-Verlag 1974 (quoted by Lyttle *et al* 1986).
20. Christie WW and Han X, *lipid analysis-isolation, separation, identification and lipidomic Analysis* (4th edition), 2010, 446 (Oily Press, Bridgewater, U.K).
21. Gao H, Zehl M, Leitner A, Wu X, Wang Z, Kopp B, Comparison of toad venoms from different *Bufo* species by HPLC and LC-DAD-MS/MS. *J Ethnopharmacol*, 131(2), 2010, 368-376.
22. HyoJL, Fan-PK, Ki-RK, Dae-In K, Lorenzo C, Pei-Y Y, Hwa-SY, Comparative Analysis of the Bufois Venenum by Using TLC, HPLC, and LC-MS for Different Extraction Methods. *J of Pharmacopuncture*, 15(4), 2012, 52-65.

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