Antimicrobial activity of skin secretions isolated from Indian toad, *Bufo melanostictus* Schneider 1799

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Vertebrates of nearly all kinds secrete broad-spectrum antimicrobial substances, which play an important task in operations of immunity inside them. Amphibians like toads secrete such antimicrobial secretions both inside their body (e.g. in intestinal tracts) like other organisms as well as outside their body into the environment, through skin pores and parotid glands [1]. Toxins or compounds secreted from their skin pores are of special interest for experimental evaluation, since they help toads to endure in habitats full of pathogenic microbes. Toad skin-secretions contain four types of compounds namely, biogenic amines, bufadienolides, alkaloids & steroids and peptides & proteins [2].

Bulk of research relating to amphibian antimicrobial secretions has been carried out on frogs. In toads, such research is in short availability and has been carried out only on *Bufo rubescens* [3], *Bombina orientalis* [4] and *Bufo arenarum* [5]. Skin-secretions of toad especially of those belonging to genus *Bufo* contain as many as 86 different types of active compounds [6], each of which has potentials of becoming a potent drug. Research on this theme has exposed enormous promise for future recently. These secretions have been shown to exert bactericidal effects possibly by disrupting and permeabilizing the target cell membrane [7]. Such investigations haven't been

carried out on skin secretions of Indian toad, *Bufo melanostictus* Schneider 1799, which is a part of a unique Indian sub-continent biodiversity. Composition and constitution of compounds found in toad skin-secretions varies significantly from specie-to-specie [8, 9]. This implies that skin-secretions of *B. melanostictus* may have many novel and unique compounds with previously unexplained mechanisms. This makes it imperative to carry out preliminary examination of effectiveness of *B. melanostictus* skin-secretions to recognize whether it exhibits antimicrobial action or not, before extensive research in this regard can be initiated.

In this groundwork investigation, we tried to examine the potential of *B. melanostictus* skin-secretions as source of future antimicrobial agents. *Bufo melanostictus* (Figure 1) required for this experimentation were collected during breeding season (spring-summer) from Nagpur region in Maharashtra situated at 20.30^0 N - 21.45^0 N & 78.15^0 E – 79.45^0 E. Fresh skin secretions were secured from the toad by squeezing the "parotid glands" of the toad according to procedure given by Abel & Macht [10]. The semi fluid and viscous secretions thus obtained were not allowed to dry-up and were immediately scrapped and mixed in sterile distilled water. Since these secretions are highly viscous, they were mixed in distilled water by mechanical shearing, which was carried out with the help of sterile syringe. Mechanical shearing was repeated several times till the solution mixture was obtained. A 3% solution of crude toad skin-secretion was initially created by the above methodology. This concentration was suppose to be later made up to 0.18% for this preliminary examination since best results were expected to be obtained around this concentration based upon the earlier research [3, 4, 5].

The antimicrobial activity of *B. melanostictus* skin-secretions was tested against the gram-negative bacilli, *Escherichia coli* (Strain tested for Lac^+), which is a major enteric pathogen, particularly in developing countries [11]. Lac^+ *E. coli* cells were maintained on nutrient media. The antimicrobial activity was elucidated using two differential media-based antimicrobial screening assays namely MacConkey agar-based and Eosin-methylene blue (EMB) agar-based. MacConkey agar and EMB agar were prepared and steam sterilized at 120^0C for 20 min. Respective agar were

then mixed with 1 ml of 3% toad skin-secretion solution such that, each Petri dish has 15 ml media and 1 ml toad skin-secretion solution; this would bring down the final concentration of toad skin secretions in the medium to 0.18%, as proposed previously. After this setup had settled, *E. coli* were sector-streaked onto the surface of MacConkey and EMB agar. Thus, two types of experimental systems were obtained i.e. (1) MacConkey agar + toad skin-secretions + streaked *E. coli* culture and (2) EMB agar + toad skin-secretions + streaked *E. coli* culture. MacConkey agar and EMB agar with streaked *E. coli* culture only, served as controls for the above systems respectively. Three replicates for each system were also assayed simultaneously. The plates were examined after incubation at 37^0 C for 24h. Bacterial growth inhibition was defined based upon the differential nature of Lac^+ *E. coli* growth on respective media. Existence of *E. coli* is usually indicated by presence of pink colored colonies (on MacConkey agar) and green metallic sheen growth (on EMB agar). Absence of these two characters implies Lac^+ *E. coli* growth inhibition [12].

Results obtained after above assays showed that *E. coli* growth was inhibited on both the systems. In MacConkey agar-based system, while *E. coli* growth was observed by the agency of pink colored growth in control Petri dishes (Figure 2 A. right), the same wasn't seen on the experimental media containing the toad skin-secretions, which was yellow in appearance (Figure 2 B. right). However, presence of some non-lactose fermenting organism (source suspected being toad skin), evident because of yellow color raised a question regarding the antagonism of this Lac^- organism towards Lac^+ *E. coli*. To rule out this possibility, results on specific medium (i.e. EMB agar) proved useful. Since EMB agar is selective for Lac^+ bacteria, there will be no growth of Lac^- bacteria, thereby eliminating the question of antagonism. In the EMB agar-based system, *E. coli* growth was observed in the form of green metallic sheen in the control media (Figure 2 A. left); however no such sheen was recordable on the experimental media containing toad skin-secretions (Figure 2 B. left). Identical results were obtained in all the replicates (Table 1). These results signify substantial inhibition of *E. coli* growth by *B. melanostictus* skin-secretions only. In

order to confirm whether the antibacterial action seen here was bacteriostatic or bactericidal, surface inoculants were obtained from the surface of EMB agar experimental system. These surface inoculants were streaked again on fresh EMB agar systems, not containing any toad skin secretions. This confirmatory system was examined after incubating at 37^0 C for 24h. Results obtained after this were same as obtained previously i.e. there wasn't any growth of *E. coli* on the fresh EMB agar system. Thus, the retesting of *E. coli* growth suggested that the action of *B. melanostictus* skin-secretions on *E. coli* was bactericidal.

Results elucidated here clearly confirm that *B. melanostictus* skin secretions do have some potent anti-microbial as well as bactericidal activity, which needs further comprehensive investigation. Similarly, examination of skin-secretions of *Bufo himalayanus* and *Bufo stomaticus*, other widely found *Bufo* sp. of Indian sub-continent must also be carried out. Amphibians evolved about 363 million years ago [13, 14] while, toads evolved about 200 million years ago. In terms of genetic-immunological evolution, this means that the toad immune system, of which these skin-secretions are a part, has been exposed to a wide range of microbial diversity for 200 million years. This makes constituents of toad skin-secretions one of the most promising sources of "medicine-by-evolution", a therapeutic product especially required to tackle the threat of super bugs or the rapidly evolving multi-drug resistant microbes. Since potency of these toxins differs from specie-to-specie among Anurans, we may have about 1'00'000 such compounds still waiting to be discovered. But, we may loose more than 50% of them in next 20 years itself, if we don't seize the human inflicted destruction of Anuran habitats. Saving fledging populations of these Anurans can help us maintain ecological integrity and also lend us a chance to discover and examine these amphibians for more pharmacologically and therapeutically important compounds.

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Table 1. Comparison of experimental and control results across all replicates of differential media-based antimicrobial screening assay for evaluation of toad skin-secretion's antimicrobial activity.

Assay type	**System Type**	**Lac$^+$ *E. coli* growth in replicates**		
		1	**2**	**3**
MacConkey agar	Control	Pink growth *	Pink growth *	Pink growth *
	Experimental	No growth **	No growth **	No growth **
EMB agar	Control	Green metallic sheen growth *	Green metallic sheen growth *	Green metallic sheen growth *
	Experimental	No sheen **	No sheen **	No sheen **

* Presence of pink growth on MacConkey agar and green metallic sheen growth on EMB agar signifies presence of Lac$^+$ *E. coli*.

** Absence of Lac$^+$ *E. coli* growth is signified by yellowish color (i.e. absence of pink color) on MacConkey agar and lack of metallic sheen growth on EMB agar.

Figure 1. A photographic image of *Bufo melanostictus*, model organism used during this investigation.

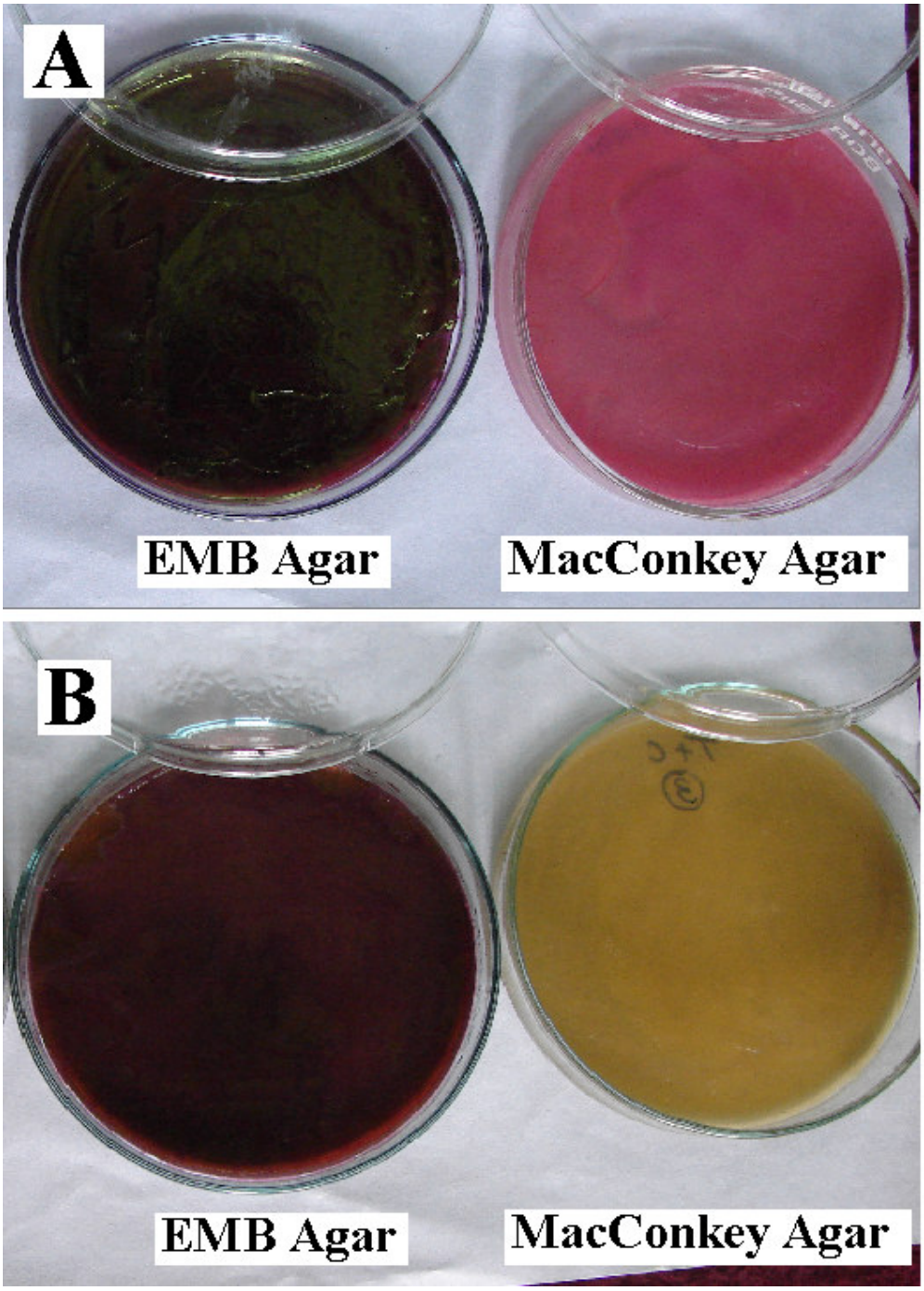

Figure 2. Results of differential-media based antimicrobial screening assays.

(A) This is the image of assays, which served as controls. Growth of *E. coli* can be seen in the form of green metallic sheen on EMB agar (left) and pink colored colonies on MacConkey agar (right). **(B)** This is the image of assays, which served as experimental systems. Absence of green metallic sheen on EMB agar (left) as well as absence of pink colored colonies on MacConkey agar (right) implies that growth of *E. coli* was inhibited by *B. melanostictus* skin secretions.