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STUDIES ON THE SUDANESE INDIGENOUS AFRICAN TOAD *BUFO SPP.* (Amphibia): PARTIAL CHARATERIZATION OF ANTIBACTERIAL PEPTIDES AND PROTEINS OF THE PARATOID GLAND

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ABSTRACT

The parotoid gland secretion of *Bufo spp.*, obtained by manual compression, was found to contain 23.1-41.2% (w/w) of total protein. Gel filtration chromatography showed the existence of four fractions of peptides and proteins responsible for antibacterial activity. Thin layer chromatography showed seven Ninhydrin-positive spots in addition to the origin, in the parotoid gland secretion of *Bufo spp.* Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis revealed the existence, in the crude secretion, of 2 bands of proteins (P_1 and P_2) and 2 bands of peptides (P_3 and P_4) with various molecular weights.

OBJECTIVES

We have previously shown that the parotoid glands of the indigenous toad *Bufo spp.* contain antibacterial components likely to be proteins. The main objectives of this study was to confirm the initial finding and to further characterize the active compound(s).

INTODUCTION

Since the discovery of Bombinin in the skin of the frog *Bombina variegata*, amphibian skin has become an important source of new antimicrobial agents and several novel peptide molecules were reported, often with unprecedented structural features (Csordás and Michl, 1969; Hancock and Lehrer, 1998). These biologically active compounds have diverse physiological and defensive functions (Clarke, 1997; Kreil, 1996; Perry, 2000) and are characterized by selectivity, broad spectrum and low toxicities to normal eukaryotic cells (Lazarus and Attila, 1993; Barra and Simmaco, 1995; Nicolas and Mor, 1995: Hancock and Chapple, 1999; Epand and Vogel, 1999; Rinaldi, 2002; Rollins Smith *et al*., 2002), research on this subject is upsurging.

Bufo spp. members have compact parotoid glands situated on the neck, on sides of the head or shoulder regions. These glands accumulate a milky venom (Rödel, 2000 and Perry, 2000) that contains antibiotic substances (Hancock and Lehrer, 1998; Lazarus and Attila, 1993).

In a previous study (to be published) we have demonstrated considerable morphological variability among indigenous Sudanese *Bufo spp.* and we also showed the antibacterial activity of parotoid glands and that the active compounds were proteinaceous in nature.

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In this study chromatographic and SDS electrophoresis were applied to investigate the nature of the antibacterial peptides in the parotoid gland secretions of the Sudanese toad, hitherto not investigated.

MATERIALS AND METHODS

Analytical grade reagents were used, together with standard laboratory equipments for weighing, centrifugation, … etc.

Individuals of the toad (*Bufo spp).* were collected from stagnant pools in Khartoum state, the parotoid gland secretion was obtained by manual compression, and the secretion was dissolved in deionized water (Perry, 2000; Batista *et al*, 2001). The compression method constitutes a positive development over the method we initially used which relied on removal of the animal skin followed by solvent extraction.

Protein was determined using the Biuret method, ovalbumin solution was the standard (Rybicki and Purves, 1996).Thin Layer Chromatography: Pre-coated silica gel sheets (0.2 mm thick) were used to separate amino acids and peptides. The solvent system was butanol/ acetic acid/ water (100:10:30) as was used by Stahl, 1969. After chromatographic development, plates were sprayed with Ninhydrin 5% to reveal peptide spots.

Gel filtration chromatography was used to separate parotoid gland peptides (Johnson and Stevenson, 1978; Bauer *et al*., 1978). Sephadex G25 was swelled in distilled water overnight then packed onto columns (500mm length X 10 mm in diameter) to a height of 200 mm, with distilled water used as eluant. Fractions (2 ml) were manually collected at a constant flow rate of 0.5 ml/min. Antibacterial activity of each fraction was tested against *S. aureus* (ATCC 29213) using filter paper technique (Hunter, 1977; Hugo and Russell, 1998).

SDS PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis was used to separate and characterize parotoid gland proteins (Laemmli, 1970; Rybicki and Purves, 1996).

Stock solution containing 29% (w/v) acrylamide and 1% (w/v) N'N'-methylen bisacrylamide was prepared, the pH was adjusted to 7.0. A solution of SDS (10%) was also prepared. Resolving gel buffer was prepared by dissolving 18.20 gm Tris OH (Tris base) in 80 ml distilled water, the volume was completed to 100 ml by adding 20 ml of distilled water and the pH was adjusted to 8.8. Stacking gel buffer was prepared by dissolving 3.00 gm Tris HCl (Tris acid) in 40 ml distilled water, the volume completed to 50 ml and the pH was adjusted to 6.8. Resolving gel (10%) was prepared by mixing 3.1 ml acrylamide, 4.2 ml distilled water, 2.5 ml resolving buffer, 100 µl of 10% SDS, 100 ml ammonium persulphate (APS) solution and 14 ml of TEMED. Stacking gel (4%) was prepared by mixing 1.3 ml acrylamide, 6.0 ml distilled water, 2.5 ml stacking buffer, 100 ml of 10% SDS, 100 ml of ammonium persulphate solution and 10 ml of TEMED. Running buffer was prepared by dissolving 3.00 gm Tris OH, 14.40 gm glycine and 1 gm SDS in 500 ml of distilled water, the volume completed to 800 ml and the pH adjusted to 8.3. Loading buffer solution was prepared by mixing 4.00 ml distilled water, 1 ml of 0.50 µl stacking buffer, 1.6 µl of 10% SDS, 0.80 ml glycerol and 0.20 ml of 0.05% bromophenol blue.

Glass plates of a mini-vertical electrophoresis apparatus were washed and assembled together with a spacer inserted between each two glasses at each side, clips held the two plates together. The lower part of the assembled plates was dipped in a heated 2% agarose solution for sealing, then the lower end of the gel was left to dry. Using an electric micropipette, the resolving gel was poured into the gap between the glass plates. Methanol was added to level the gel. Sufficient space was left to hold the stacking gel. The resolving gel was left for about 45 minutes to polymerize. The stacking gel was added on the top of the

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polymerized resolving gel after methanol removal. A Teflon comb was inserted carefully into the stacking gel solution, the gel was placed in a vertical position to polymerize.

After the removal of the Teflon comb, the gel was mounted in the electrophoresis apparatus. The running buffer was added to the top and bottom reservoirs. Five samples of parotoid gland secretion obtained from five toads, separately, were loaded in predetermined order into the bottom of the gel wells by using a micropipette (20 µl). Protein markers, of known molecular weights were loaded as standards (ovalbumin: 4.5 kDa and a 2.1 kDa peptide). A voltage of 100 v/cm was applied to the gel. When the Bromophenol blue reached the bottom of the resolving gel (in about 2 hours), the run was considered as complete.

Plates were pried apart carefully; the gel was immersed in the Coomassie brilliant blue solution and left to stain (over-night), and then immersed in the destaining solution, which was changed 4 to 5 times until clear protein bands were seen in the gel. The protein bands were photographed and the molecular weights of the bands were compared to the protein markers. The molecular weights of the peptides and proteins were determined using following formula:

$Log_{10}MW_{peptide\ or\ protein} = mD + b$

Where m: slope, D: distance moved by the peptide/ protein, b: constant

RESULTS AND DISCUSSION

The milky secretions of the parotoid glands varied between thin to thick liquid (Fig.1). The amount of secretion obtained from each toad was nearly equal (0.4 gm). The protein concentration (%w/w) varied between 23.08 and 41.18% of the fresh weight of the secretion of the parotoid gland (Table 1). This may be due to the nature of secreted components and/or inter and intra individual differences. The protein concentration is consistent with that reported by Perry (2000) for other *Bufo spp.* namely *B. mauritanicus* and *B. calamita* for which the protein concentration found varied from 25% to 35%. Taken with the apparent variability in morphological features (Figs. 2 & 3), the protein range we obtained points to chemical variability as well.

Table 1: Peptides and Proteins concentration (%, W/W) in the secretion of the parotoid glands of African *Bufo* species (n= 8).

Samples	Estimated weight of proteins (gm)	Weight of the secretion (gm)	Percentage of peptides and proteins in secretion (%)
1	0.09	0.39	23.08
2	0.11	0.40	27.50
3	0.22	0.54	40.74
4	0.15	0.39	38.46
5	0.13	0.45	28.89
6	0.14	0.34	41.18
7	0.13	0.48	27.08

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8	0.14	0.41	34.15
M±SD	0.14 ± 0.04	0.42 ± 0.06	32.63 ± 6.49

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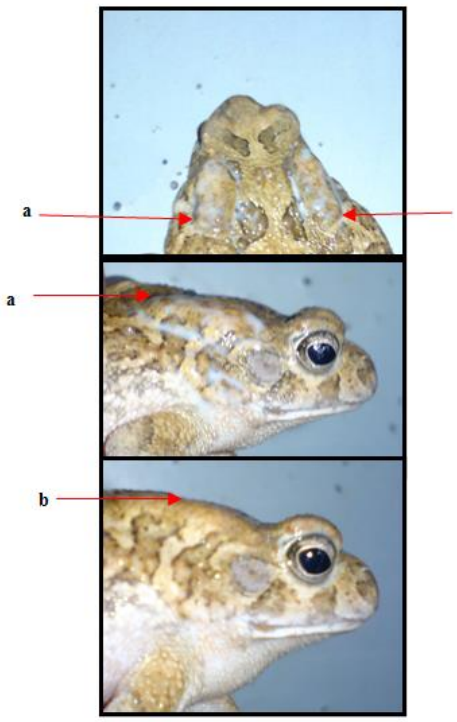

Fig.1. Photographs showing the secretion from parotoid glands (arrowed) in African toad *Bufo spp.* a. Parotoid gland secreting the venom (note the milky appearance)

b. Parotoid gland before secreting

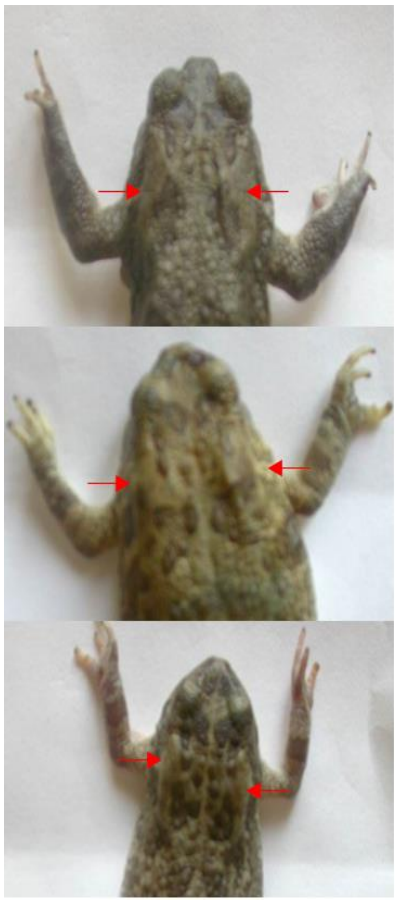

Fig. 2. Position of the paratoid gland (arrowed) in th indigenous African toad (*Bufo spp.*).

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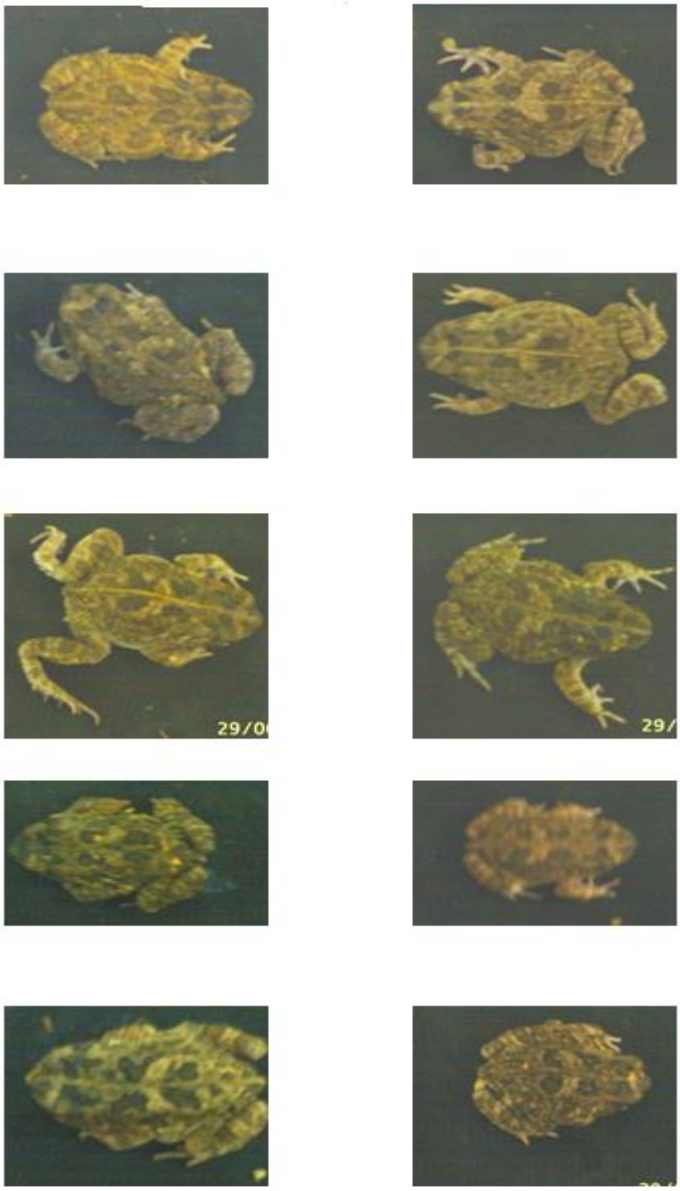

Fig.3. Photographs showing morphological features of African toad *Bufo spp.* Toads were collected from Khartoum State.

We have used gel filtration chromatography (Sephadex G25) to separate the peptide components of the parotoid gland secretions of *Bufo spp.* The fractions (2ml in volume) were obtained as described in Materials and Methods, totaling 80 fractions. Each fraction was assayed for protein content (Biuret method) and for antibacterial activity against *Staphylococcus aureus*. The results are shown in Fig. 4. Several protein peaks were detected in the different sephadex fractions. Four peaks of antibacterial

activity were detected associated with certain protein fractions. The peaks were each comprised of more than one gel fraction collected.

Thin layer chromatography of crude parotoid gland secretions using a solvent system that separates peptides and proteins and detecting with ninhydrin reagent, revealed 7 spots (Fig.5). When comparing the secretion with standard peptides and proteins (Fig.6), the components of parotoid secretion showed both types of peptides and proteins.

The above mentioned TLC system was also used to separate the components of some of the sephadex gel fractions that had antibacterial activity. These sephadex fractions contained two to four peptides with *Rf* values of 0.5 or more. Thus it seems that high molecular weight polypeptides of the crude parotoid gland secretion (spots 0, 1 and 2 in Fig. 7) have no antibacterial activity. This is in agreement with Perry (2000) who stated that high molecular weight polypeptides of the parotoid gland secretions have no involvement in the antimicrobial activity.

SDS PAGE demonstrated the presence of 4 peptide/protein compounds (Fig. 8) in the crude parotoid gland secretions of 5 randomly selected toads. We referred to these as P_1, P_2, P_3, P_4 and for which we calculated the molecular weight using the standards shown in Fig. 8. Our calculations (Table 2) indicate that P_1 and P_2 are proteins, while P_3, P_4 were smaller peptides. P_1 had a high MW (3700 kDa). The compounds P_3 and P_4 (MW<9 PDA) are likely two of the antibacterial peptides of the Sudanese toad.

Literature on the reports on the molecular weights of toad peptides and proteins, not necessarily evaluated for antibacterial activity, are variable for *Bufo spp.* in general. For example Perry (2000) reported a range of approximately 12-200 kDa, and also found that the parotoid secretions from *B. mauritanicus* and *B. calamita* were approximately 58 kDa and 30.5 kDa respectively. Barberio *et al.* (1987) found a peptide of 6.7 kDa in *Bombina variegata pachypus*.

Further work is underway to further characterize morphological and biochemical aspects of the Sudanese toad.

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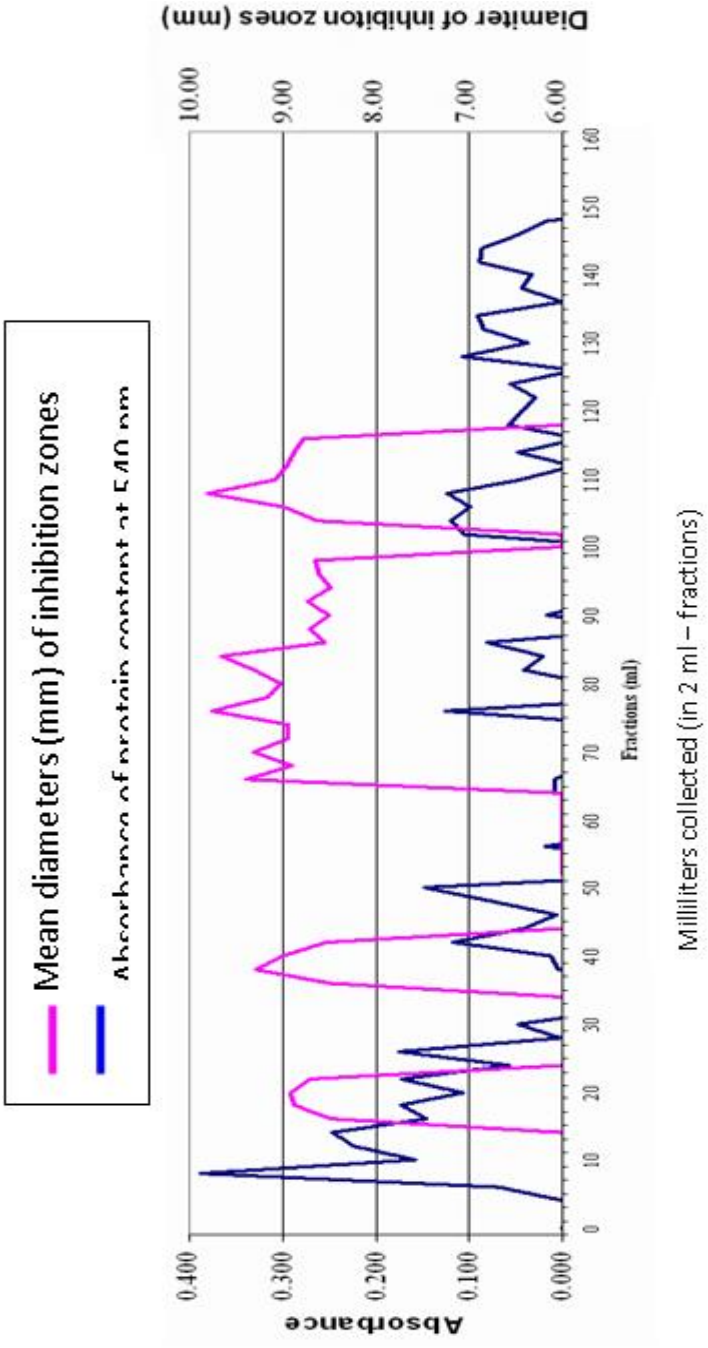

Fig. 4. Antibacterial activity (diameter of inhibition zone, mm) and relative protein content (Absorbance at 540 nm) of sephadex fractions collected.

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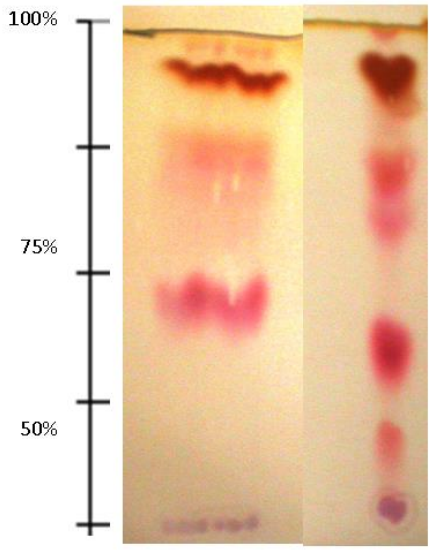

Fig. 5. TLC of *Bufo spp.* parotoid gland secretion. Adsorbent :
Silica gel, solvent system: BAW (100:10:30).

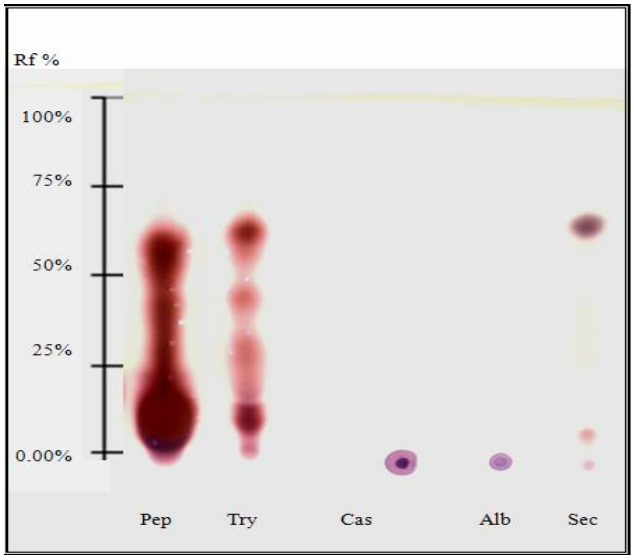

Fig. 6. TLC of standard peptides and proteins in comparison with the parotoid gland secretion. Silica gel, solvent system: BAW (100:10:30).

Pep: Peptones Try: Tryptones Cas: Casein

Alb: Ovalbumin Sec: Secretion

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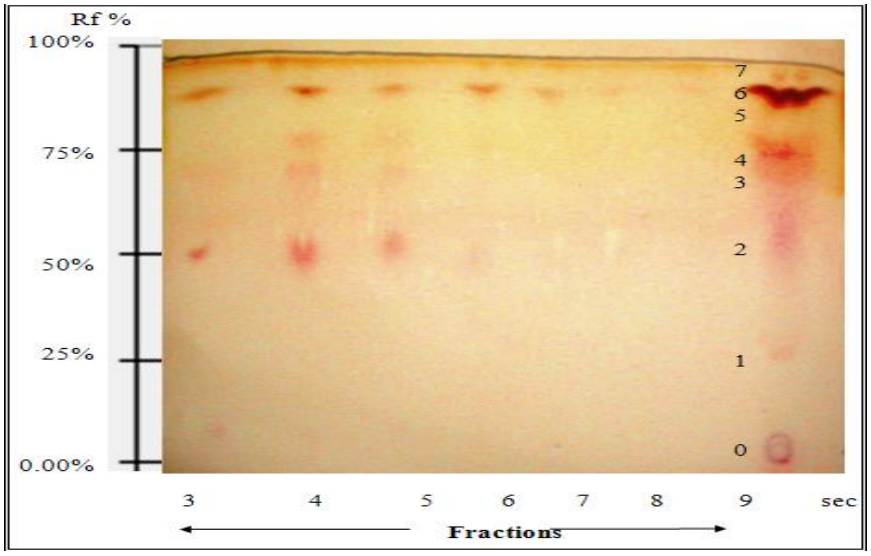

Fig. 7. TLC of sephadex gel fractions (of the paratoid gland secretion) that had antibacterial activity (fractions 3-9) and of the crude secretion. TLC conditions were as indicated in Figs. 5 and 6. The spray reagent was nin-hydrin (to reveal peptides and proteins). In this experiement the fraction collected from the sephadex column was 5 ml in volume (C.F. 2 ml in Fig. 4).

Table 2: Molecular weights of the proteins and peptides in parotoid gland secretion of *Bufo spp.* (P_1 to P_4) and standard MW markers (protein (M_1) and a peptide (M_2).

Bands	Molecular weight (Da and kDa)
P_1	≥3700 kDa
P_2	≈ 20 kDa – 40 kDa
P_3	≈ 4.4 kDa – 8.7 kDa
P_4	≈700 – 1100 Da
Ovalbumin egg (M_1)	45 kDa
Peptide (M_2)	2.1 kDa

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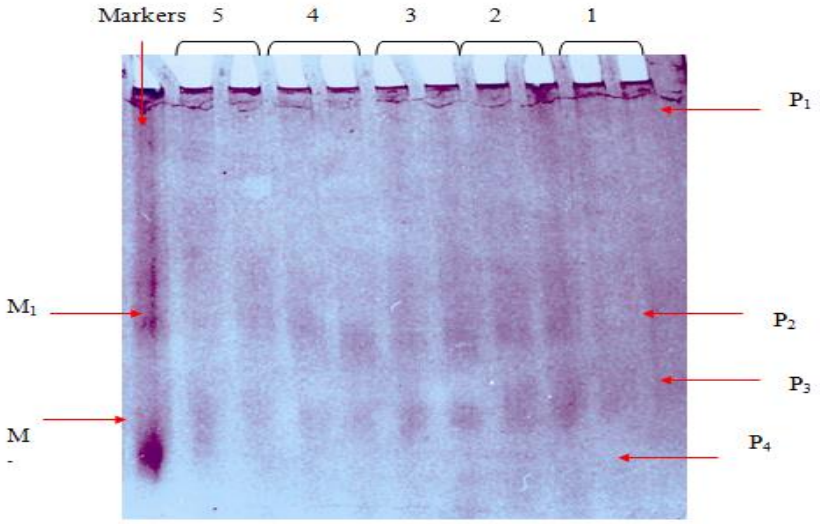

Fig. 8. SDS PAGE of peptides and proteins in parotoid glands secretions of *Bufo spp*. (1 to 5 represent secretions of individual toads).

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