المميزات التصنيفية والتصنيفية الكيميائية لضفادع جنس البوفو في منطقة شندي .

حسام الدين الحاج أبوجبر[1]، نصر عثمان حاج رحمة[*]، عبدالله إسحاق عبدالله[*]، محمد يعقوب محمد[*]، ٢٠٠٨م .

ملخص :

أثبتت الدراسات التصنيفية لجنس البوفو (برمائيات) في منطقة شندي وجود عدة أنواع وهي *بوفو زيروس وبوفو ماكيولاتاس وبوفو ريغيولاريس*. النوعان *ب. زيروس* و *ب.ماكيولاتاس* أظهرا وجوداً دائما بالقرب من البيئات المائية بينما أظهر ب. ريغيولاريس وجوداً في مناطق أبعد نسبيا من البيئات المائية في المناطق الأكثر جفافا .

أوضحت الدراسة أنَّ المظاهر المورفولوجية للنوعين *ب. ماكيولاتاس* و*ب. ريغيولاريس* متشابهة تقريبا ، ومن الناحية الأخرى يمتلك النوع *ب. زيروس* بقع حمراء كبيرة في الجزء الأعلى من الفخذ.

وفي دراسة للمركبات الكيميائية في الافرازات الغدية النكفية للأنواع الثلاثة من الضفادع، تم استخدام تقنية الفصل الكروماتوغرافي باستخدام الشرائح الدقيقة للحصول على طرز الببتيدات والبروتينات من جهة، وطرز الستيرويدات والالكلويدات من جهة أخرى ، ووجد أنَّ طرز الببتيدات والبروتينات والستيرويدات والالكلويدات قريبة الشبه بين النوعين *ب. ماكيولاتاس* و*ب. ريغيولاريس* بينما خالفهما *ب. زيروس*.

النتائج تدل على تقارب تطوري بين *ب. ريغيولاريس* و*ب. ماكيولاتاس* أكبر من بين أي واحد منهما مع *ب. زيروس* ودعمت نتائج الدراسات التصنيفية في بداية البحث باستخدام القياسات الكلاسيكية.

تم تحليل نتائج التصنيف الكلاسيكي بواسطة SPSS باستخدام اختبار ANOVA واختبار t .

ABSTRACT

Taxonomical investigation of *Bufo* species in Shendi area illustrated the existence of various *Bufo spp.*, these species showed similar morphological features of *B. xeros, B. regularis* and *B. maculatus*.

[1] محاضر – كلية العلوم والتقانه – جامعة شندي
[*] كلية العلوم والتقانه – جامعة شندي

This study recorded for the first time the existence of Bufo maculatus in Northern Sudan, and stated that the tympanum eye ratio is a significant morpho-functional adaptation in *B. xeros* and *B. maculatus*, because both species live in sympatry.

Chemotaxonomical investigation of the parotoid gland secretions of *Bufo spp.*, using Thin Layer Chromatography (TLC), revealed various spots of amino acid/peptide/ protein components and alkaloid/steroid components corresponding to the morphological features to each species, these patterns provided evidence that *B. regularis* and *B. maculatus* are relativity closer than *B. xeros*. The findings of this study strengthened the fact that parotoid gland secretions of amphibians, especially *Bufo spp*., have a significant role in taxonomical and evolutionary studies.

OBJECTIVE

This study investigated the diversity of the indigenous *Bufo spp.* in Shendi area, by studying several morphological parameters and by applying TLC to investigate the amino acid/peptide/protein and alkaloid/steroid components in the parotoid glands secretions.

INTRODUCTION AND LITERATURE REVIEW

Members of the order Anura (Amphibia) are characterized by the absence of the tail, protruding eyes, and other distinct morphological features. Over 350 extant species in approximately 26 genera are recognized and divided into toads and frogs. Toads are distinguished from frogs by their appearance prompted by the convergent adaptation, especially their thick and warty skins; they are cosmopolitans, distributed throughout both temperate and tropical regions, and they are also terrestrial with partial life in streams (Pough *et al*, 1998; Rödel, 2000; Cogger *et al*, 2004).

African *Bufo* species are widespread in savanna regions south of the Sahara from Senegal through West Africa to Central Africa and through North Africa (Rödel, 2000).

Toads of the genus *Bufo* are typically large and compact with a warty skin and basically a dark olive brown color at the dorsal, often turning lighter towards the Venter. They have compact parotoid glands situated on the neck, on sides of the head or shoulder regions, which accumulate a milky secretion. These parotoid glands are considered histologically parotoid macroglands (Toledo and Jared, 1995) and have large prominent kidney or parallel rod shape, with a relatively smooth appearance because the warts are quite flat in this region (Perry, 2000; Rödel 2000).

These parotoid glands are granular glands that produce toxic and repellent secretions, which are addressed as venoms, affect various vertebrate species. These secretions possess pharmacological and antimicrobial effects and are released in response to stress, injury and predator attack onto the skin. The secretions consist of a plethora of biologically active components such as guanidine derivatives, biogenic amines, steroids, alkaloids and distinct sets of peptides and proteins (Lazarus and Attila, 1993; Toledo and Jared, 1995; Amiche *et al*, 1999; Perry, 2000; Rollins-Smith *et al*, 2002).

The *Bufo spp*. venom contains bufotoxins, bufotenine, bufothionine, epinephrine, nor-epinephrine, serotonin (Siperstein *et al*, 1957); cardiotonic steroids such as bufotalin, marinobufagen, marinoic acid and resibufogenin (Hirai and Morishita, 1992), and flavianates (Jensen & Chen, 1936). Cholesterol was also identified and evaluated in the skin secretions of *Xenopus laevis, Bombina variegata, Bufo viridis, Bufo marinus, Triturus cristatus carnifex* and its concentration varied from 1% in Xenopus laevis to 0.27% in *Triturus cristatus carnifex* (Croce and Bolognani, 1975)

Bufo spp. Individuals are particularly convenient and useful sources of granular gland secretions for biochemical investigation compared with sampling from other types of amphibians (Clarke, 1997), one of such studies was the application of two-dimensional paper chromatography with multiple staining techniques to separate a variety of compounds in secretions from different *Bufo spp*., the patterns obtained was used in evolutionary studies (Low, 1972).

Individuals of *Bufo spp.* in Khartoum state exhibited different morphological features, and the TLC patterns of their parotoid gland secretion depicted richness in the amino acid and peptide components, further taxonomical investigations and biochemical studies on the secretion components were suggested (Abugabr, 2006).

MATERIALS AND METHODS

Classical taxonomy of *Bufo* species

Ninety individuals of the African toad (*Bufo spp.*) were collected from stagnant pools in Shendi area, 150 North Khartoum, ($16^{o}41'N$, $33^{o}26'E$) during August - September 2006 and kept in wet glass and plastic aquaria for this study.

Morphological features such as the colour of the skin; the shapes of the parotoid glands; arrangement of warts; colour and shape of dorsal patches and colour of the outer parts of the thighs were examined. Depending on the results obtained, the toads were distributed to categories. The toads were divided into males and females and each individual was weighed using a sensitive balance.

The members of each category were examining by using classical parameters, these parameters included the measurement of the Snout-Ventral Length (SVL) of each

individual using a ruler, and the diameters of the tympanum and eye by using a slide gauge, the ratio between the tympanum and the eye was calculated (Rödel 2000).

Chemotaxonomy of *Bufo* species

From each category, Parotoid glands' secretions were obtained by applying manual compression on the glands and immediately dissolved in 10 ml de-ionized water.

Thin layer chromatography (TLC) was applied to study the amino acid, peptide and protein components and the alkaloid and steroid components of the crude secretion, pre-coated silica gel plates were used; these plates were 0.2 cm thick coated on aluminum sheets (Merick, Kebo-Lab) (Stahl, 1969).

BAW solution [n-Butanol: Glacial Acetic Acid: Distilled water (100ml: 10 ml: 30 ml)] was used as a solvent system to investigate the amino acid, peptide and proteins components in the crude secretions. The solvent system for studying the steroids and alkaloids in the crude secretions was made by the addition of 10 ml of acetone to the BAW solution.

In both investigations, chromatographic development was regarded complete, when the mobile phase ascended approximately 10 cm; the residual was removed from the plates at room temperature until the plates were completely dried.

The plates were sprayed with Ninhydrin 0.5% to detect the amino acid/peptide/protein composition, and left for about 20 minutes until the chromatograms became visible. To detect the alkaloid/steroid components on other plates, the plates were exposed to Iodine vapour for 10 minutes until the chromatograms became visible. Retardation factor percentages (*Rf* %) of the chromatograms were illustrated.

Statistical analysis

Results were analyzed using Statistical Package for Social Sciences (SPSS) by applying One-Way ANOVA test and t-Test (one population and two populations).

RESULTS AND DISCUSSION

Classical parameters

Ninety Individuals of the *Bufo spp.* collected from Shendi area exhibited various distinct morphological features and parameters, the varieties of these features directed the taxonomical investigation to sort the individuals into three categories, each one of the three categories expressed morphological features similar to *Bufo xeros*, *B. maculatus* and *B. regularis*.

The collected samples showed that the *B. regularis* is the abundant species in Shendi area, followed by B. *maculatus* then *B. xeros* (table 1).

species	All samples		*Bufo xeros*		*Bufo maculatus*		*Bufo regularis*	
Total number	90		11		21		58	
Sex	M=18	F=72	M=8	F=3	M=5	F=16	M=5	F=53

Table (1): Size of collected samples, classification and sex determination of individuals

The species also exhibited different behaviours in choosing the habitat; *B. xeros* and *B. maculatus* were found mainly in the stagnant ponds, small canals… etc, while *B. regularis* was mostly found in dryer areas nearby stagnant ponds.

Even though the morphological features of *B. maculatus* and *B. regularis* were quite similar, the former displayed distinct black dots on its warts, while the later didn't. *Bufo xeros* obtained a distinguishable red colour patches on both thighs, but was not

found neither on *B. maculatus* nor *B. regularis* thighs; this was in agreement with Rödel, (2000).

Taxonomical features of *B. xeros*

Bufo xeros specimens showed the red colour on the outer parts of the thighs, the colour of skin varied between olive green, yellow and brown, the warts were mostly well defined and with black dots. The patches were dark and symmetric. The parotoid glands were kidney shaped (figure 1). Individuals weighed 11 to 40 gm (22.54 ± 9.99), the males ranged between 11 and 40 grams (22.62 ± 10.67 gm), while the females ranged between 11 grams and 30 (22.33 ± 10.01 gm). The SVL of the males ranged between 45 mm and 70 (57.62 ± 9.60 mm), while the females ranged between 49 mm to 96 mm (70.66 ± 0.06 mm). The tympanum eye ratio for all the specimens ranged between 0.58 and 0.81 (0.63 ± 0.06), the ratio for the males was 0.58 to 0.64 (0.61 ± 0.01), and for the female 0.63 to 0.81 (0.69 ± 0.10).

Taxonomical features of *B. maculatus*

Bufo maculatus skin varied between yellow and brown; the warts were well defined with dark spots on the top. The patches were less symmetric. The parotoid glands were flat, elongated, paralleled and warty (figure 2). Individuals weighed between 15 and 51 gm (32.19 ± 10.44 gm), the males ranged between 16 and 35 gm (29.4 ± 7.73 gm), and the females ranged between 15 and 51 gm.(33.06 ± 11.23 gm) The SVL of the males ranged between 50 and 62 mm (58.4 ± 4.82 mm), while the SVL of the females ranged between 54 and 80 mm (67.18 ± 7.7 mm). The tympanum eye ratio for all specimens ranged between 0.6 and 0.9 (0.77 ± 0.08), the ratio for the males was 0.6 to 0.82 (0.74 ± 0.01), and for the females 0.61 to 0.9 (0.78 ± 0.08).

Taxonomical features of *B. regularis*

Bufo regularis possessed warts without black dots and no red colour on the outer thighs, the colour of the skin varied between dark olive green and brown, the warts were well defined and more flat. The patches were dark and symmetric. The parotoid glands in majority of the specimens were kidney shaped (figure 3). The individuals weighed between 15 and 58 gm (29.01 ± 9.99 gm), the males ranged between 17 and 43 gm (23.80 ± 10.82 gm), and the females ranged between 15 and 58 gm (29.50 ± 9.87 gm). The SVL of the males ranged between 55 and 63 mm (59.20 ± 4.02), while the SVL of the females ranged between 44 and 85 mm (63.26 ± 7.06). The tympanum eye ratio for all specimens was 0.66 to 0.85 (0.77 ± 0.06), the ratio of males was 0.66 to 0.83 (0.78 ± 0.07), and for the female 0.64 to 0.82 (0.78 ± 07).

Figure (1): Individual classified as *Bufo xeros* (note the red patches on the outer sides of the thighs)

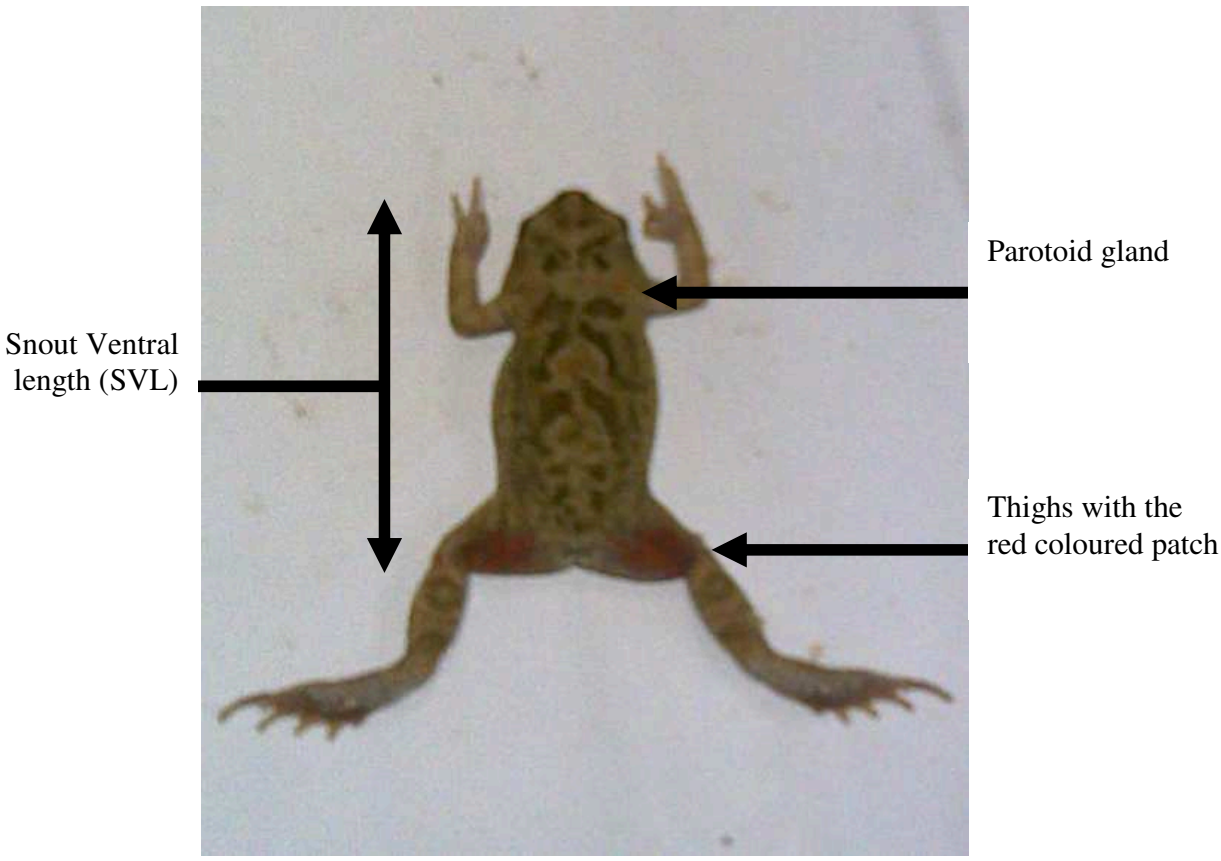


Figure (2): Individual classified as *Bufo maculatus*

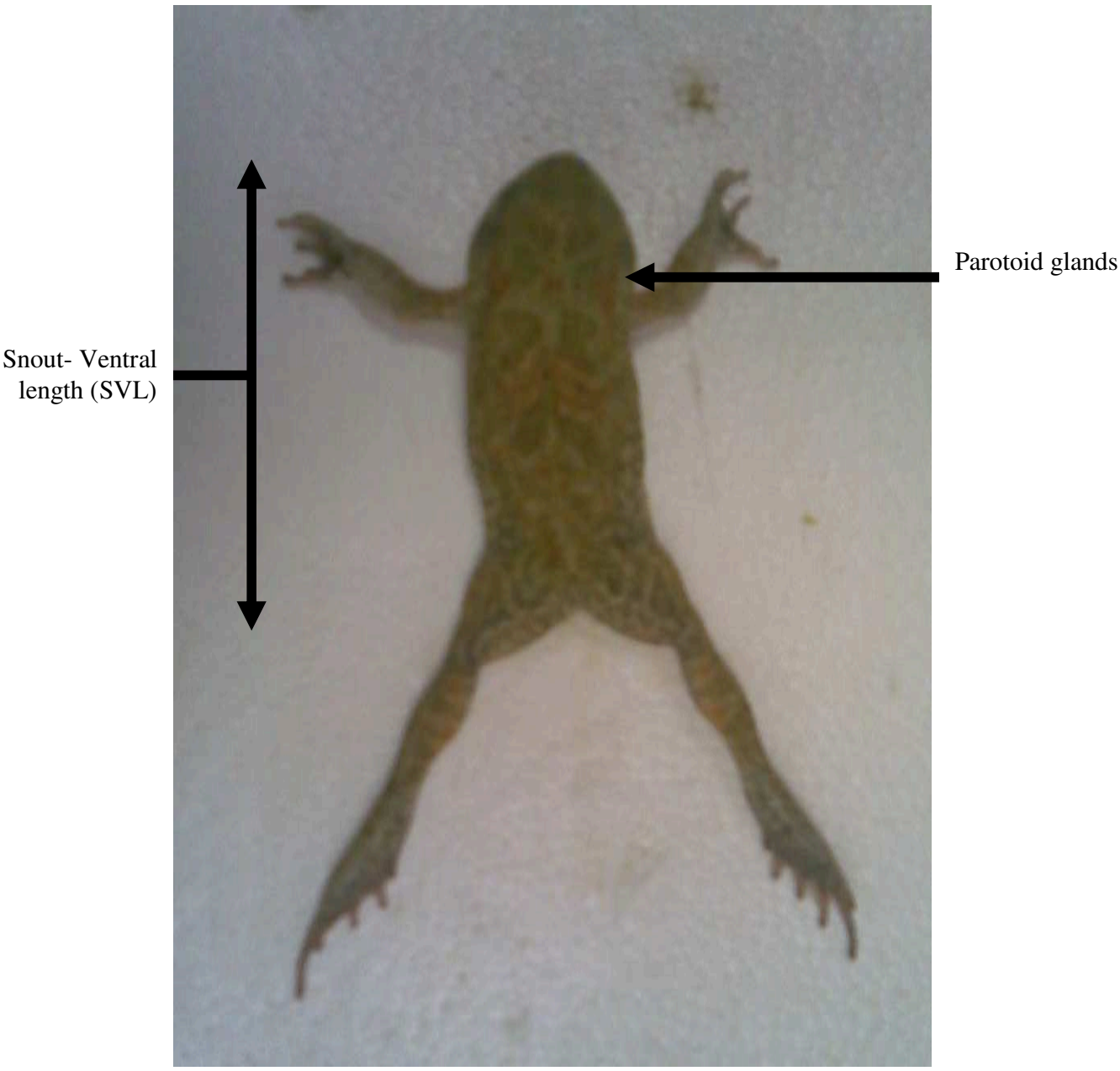
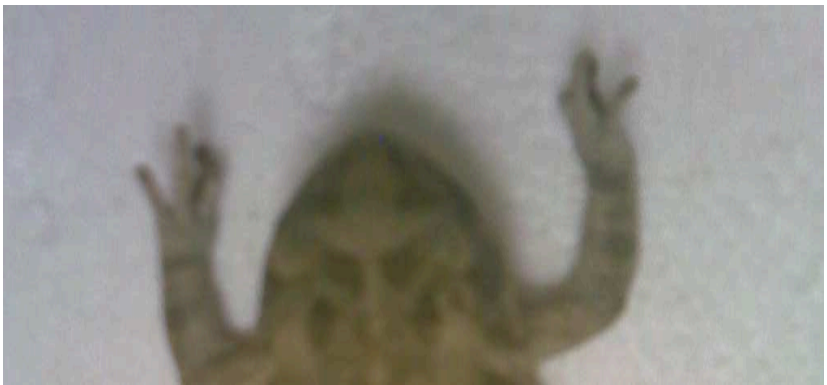
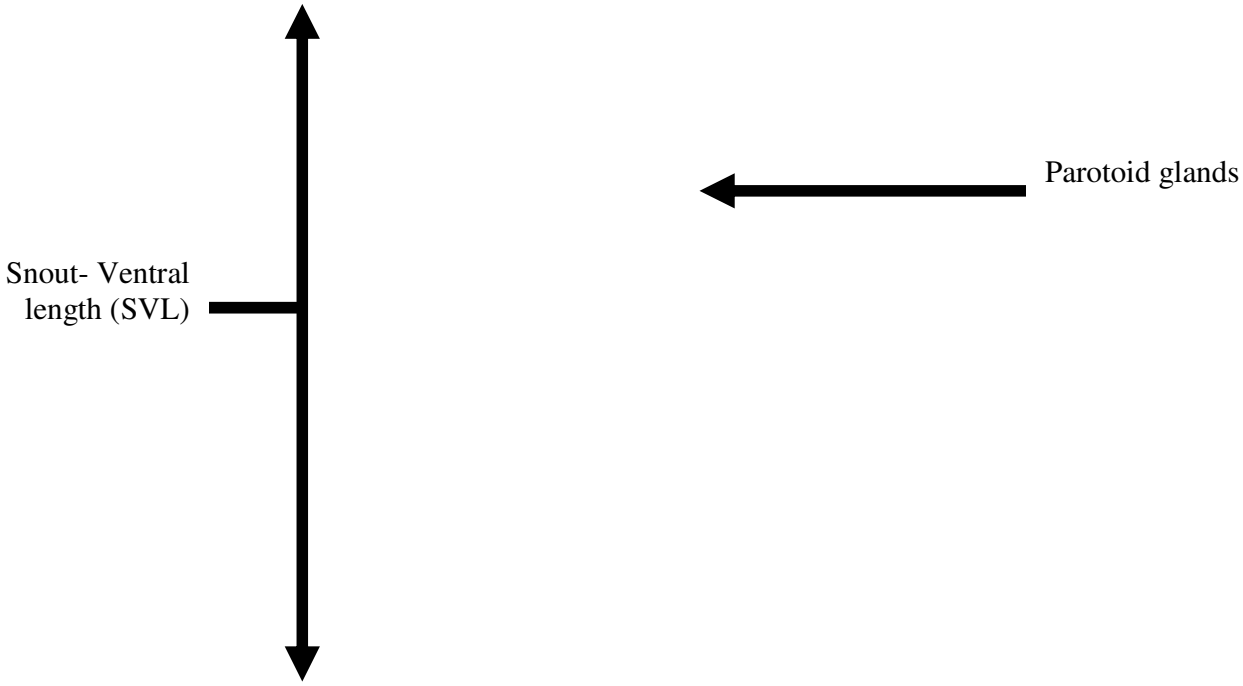


Figure (3): individual classified as *Bufo regularis*





From the records in this study, it was found that the tympanums of *B. xeros* and *B. maculatus* female are larger than those of the males of same species. This fact suggests that the tympanum eye ratio is a significant factor between the males and females of *B. xeros* and *B. maculatus* but not for the *B. regularis*.

This could be explained by that both species are found in sympatry; it was observed that they share the wet habitats; the females have to distinguish the vocal calls of the correspondent male of the same species from other males of other species, which

leads to successful mating. This study records for the first time that the tympanum eye ratio of *Bufo spp*. in Shendi area is significant in taxonomical investigations and suggests that that this characteristic should be considered as an morpho-physiological adaptation.

The tympanum and eye ratio showed no significancy between males and females of the *B. regularis*, which implies less need for strengthening the listening sense. This species is found mainly in groups, in wet habitats, in dryer areas or near light sources for feeding.

This present study provides the first record of *B. maculatus* existence in a Sub-Saharan area in Northern Sudan; *B. maculatus* is recorded as the African forest toad that exists in Southern Sudan. It is probably that individuals of this species were drifted by the northerly flow of the great Nile over the years; the high vegetation of the Nile banks in Shendi area seems to be a suitable for the species survival.

Chemotaxonomy

In this study, TLC separation of parotoid gland secretion of *Bufo spp.* showed varieties in the Ninhydrin-positive spots and Iodine-positive spots detected in the crude secretion, it revealed that the patterns of amino acid/peptide/protein and alkaloid/steroid components, obtained from the parotoid gland secretions of *B. xeros* differs from the patterns, of *B. maculatus* and *B. regularis* which both showed a kind of similarity, even with the absence of one chromatogram in crude secretion of the *B. maculatus*.

Abugabr *et al*, (2008) stated the existence of eight Ninhydrin-positive spots in the secretions of the whole population of *Bufo* species in Khartoum area by using TLC.

TLC patterns for amino acids, peptides and proteins in the crude parotoid gland secretions from *Bufo maculatus* and *B. regularis* revealed respectively 7 and 8 Ninhydrin

chromatograms (including the origin), while the pattern from *B. xeros* revealed 5 Ninhydrin chromatograms (figure 4); while the TLC patterns of alkaloid and steroid components revealed 4, 5 and 3 Iodine - positive chromatograms (including the origin) in *B. maculatus*, *B. regularis* and *B. xeros* respectively (figure 5).

The TLC patterns strengthened the suggestion that both *B. maculatus* and *B. regularis* are related closer in the evolutionary scale than *B. xeros.* The chemotaxonomical findings reinforced the results of the classical taxonomical findings; the *B. maculatus*, and *B. regularis* are quite similar in there morphological features, despite few parameters mentioned.

The results agree with in the general view with the accumulative literature reports on the nature of toad secretion (Low, 1972; Lazarus & Attila, 1993; Toledo & Jared, 1995; Perry, 2000). The arrays of proteinacious components in *Bufo* parotoid gland secretions suggest a more complex role for these secretions than simply anti-predator defense. Such arrays of amino acid/peptide/ protein components and alkaloid/steroid components in the parotoid gland secretion could be useful in taxonomical or evolutionary studies for the local *Bufo spp*. in Sudan, which agrees with (Low, 1972).

Figure 4: TLC of amino acid/ peptide/ protein system of parotoid gland secretion of *Bufo* species. Silica gel, solvent: BAW (100:10:30). M: *Bufo maculatus*, X: *Bufo xeros*, R: *Bufo regularis*

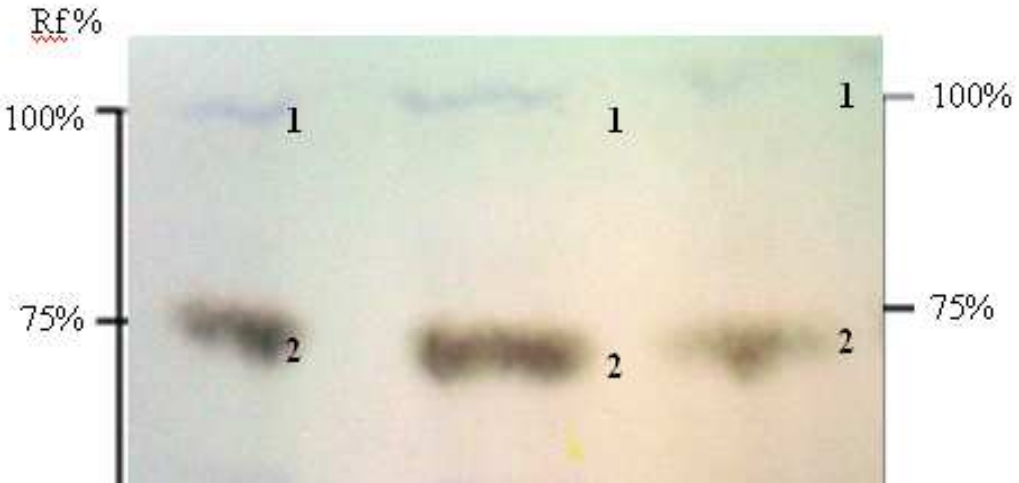
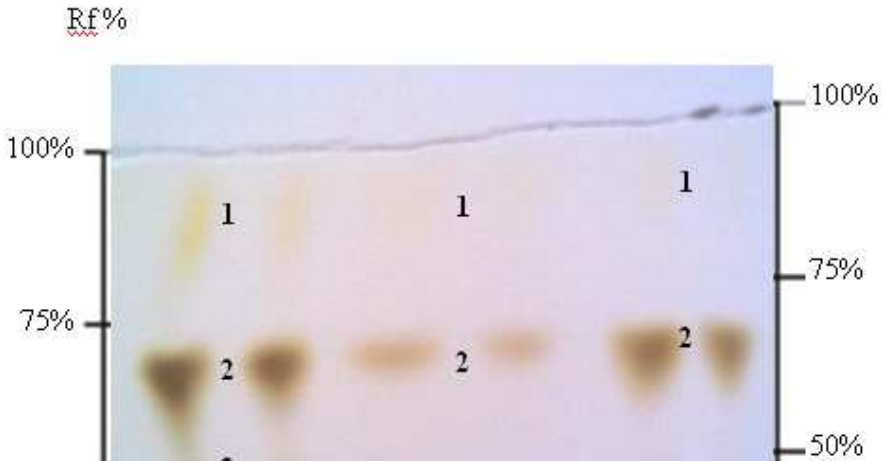


Figure 5: TLC of parotoid gland secretion of *Bufo* species. Silica gel, solvent: BAW. Acetone (100:10:30:10). M: *Bufo maculatus*, X: *Bufo xeros*, R: *Bufo regularis*



CONCLUSION

- The *Bufo* species population in Shendi area showed the existence of three species, *B. xeros*, *B. maculatus* and *B. regularis*; this study records for the first time the existence of *B. maculatus* in Shendi area, the study also depicts that the tympanum eye ratio shows differences between males and females in the species *B. maculatus* and *B. xeros*, while it didn't show any differences in the *B. regularis*.
- Other classical parameters stated a base of taxonomical keys for classification of *Bufo spp.*, and suggest that the *B. maculatus* and *B.regularis* closely related on the evolutionary scale than *B. xeros*.
- Chemotaxonomy study, using Thin Layer Chromatography (TLC), investigated the

amino acid/peptide/protein components and the alkaloid/steroid components of the parotoid glands secretions of each species, illustrated that each species obtains a different pattern of chromatograms in these secretions. These results strengthened the findings of the classical taxonomy method.

- The patterns showed similarity between *B. maculatus* and *B. regularis* secretions, while the patterns obtained from the secretions of *B. xeros* was quite different; this reinforced the suggestion that the *B. maculatus* and *B. regularis* are closely related on the evolutionary scale than *B. xeros*. The chemotaxonomy study succeeded with its simplicity and proved that these secretions could be used as a taxonomical parameter and for further evolutionary studies, which agreed with the findings of Low (1972).

RECOMMENDATIONS

- Taxonomical documentation of genus *Bufo* in Shendi area and other parts of Sudan**.**
- Further biochemical investigation of the parotoid glands secretions from *Bufo* species using more sophisticated technique.
- Deeper investigation of the evolutionary role of the parotoid glands secretions of *Bufo* species

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