



Original article

An insight into the skin glands, dermal scales and secretions of the caecilian amphibian *Ichthyophis beddomei*Damodaran Arun^a, S. Sandhya^b, Mohammad Abdulkader Akbarsha^c, Oommen V. Oommen^d, Lekha Divya^{a,*}^a Department of Zoology, Central University of Kerala, Kasaragod, Kerala, India^b Indian Institute of Science, Bangalore^c National College (Autonomous), Tiruchirappalli, Tamilnadu, India^d Department of Computational Biology and Bioinformatics, University of Kerala, Thiruvananthapuram, Kerala, India

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ABSTRACT

The caecilian amphibians are richly endowed with cutaneous glands, which produce secretory materials that facilitate survival in the hostile subterranean environment. Although India has a fairly abundant distribution of caecilians, there are only very few studies on their skin and secretion. In this background, the skin of *Ichthyophis beddomei* from the Western Ghats of Kerala, India, was subjected to light and electron microscopic analyses. There are two types of dermal glands, mucous and granular. The mucous gland has a lumen, which is packed with a mucous. The mucous-producing cells are located around the lumen. In the granular gland, a lumen is absent; the bloated secretory cells, filling the gland, are densely packed with granules of different sizes which are elegantly revealed in TEM. There is a lining of myo-epithelial cells in the peripheral regions of the glands. Small flat disk-like dermal scales, dense with squamulae, are embedded in pockets in the dermis, distributed among the cutaneous glands. 1–4 scales of various sizes are present in each scale pocket. Scanning electron microscopic observation of the skin surface revealed numerous glandular openings. The skin gland secretions, exuded through the pores, contain fatty acids, alcohols, steroid, hydrocarbons, terpene, aldehyde and a few unknown compounds.

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1. Introduction

In view of the limited distribution confining to the rain-fed tropical regions, the concealed fossorial life, and mistaken identity with snakes, caecilians are the least studied vertebrates (Gomes et al., 2012). Only 212 caecilian species are known, and are assigned to 10 families (www.amphibiaweb.org, 8th March 2019). Being a tropical country, India is home to diverse forms of caecilians (Taylor, 1968). Out of the 39 species of caecilians reported from India, 26 are from Western Ghats, a biodiversity hot-spot (Daniels, 1992; Kotharambath et al., 2013, 2017).

Ichthyophis beddomei is a comparatively small-bodied oviparous caecilian belonging to the family Ichthyophiidae. It is commonly called yellow-striped caecilian. The brownish body has two prominent lateral yellow stripes which become broad towards the head. Caecilians are a much-neglected group of animals in terms of research, little has been done with regard to this species. Its structural variation of oviduct in relation to the annual cycle (Masood-Parveez and Nadkarni, 1991), inter-renal gland structure and function (Masood-Parveez et al., 1992), ovarian cycle (Masood-Parveez and Nadkarni, 1993a), ovary (Masood-Parveez and Nadkarni, 1993b) and pituitary gland (Masood-Parveez et al., 1994) have been studied. Thus, *I. beddomei* became the animal of choice in this study.

Amphibian skin is an exocrine organ that produces skin secretion, which contributes to several aspects of biology, especially homeostasis, cutaneous respiration, conspecific communication, offence and defence (Fox, 1986; Duellman and Trueb, 1986). These roles are generally assigned to the wide variety of bio-active molecules that are present in the skin gland secretions (Zasloff, 1987, 2002; Erspamer, 1994; Fredericks and Dankert, 2000; Gomes et al., 2007; Pereira et al., 2018; Siano et al., 2018). Several

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bioactive peptides, amines (Erspamer, 1994) and over 800 alkaloids have been reported from anurans and salamanders (Daly et al., 2005). Since the caecilian amphibians are fossorial, the skin secretions from the cutaneous glands viz., mucous glands and poison/granular glands, play all these critical roles very efficiently (Jared et al., 2018). The cutaneous glands of *Ichthyophis orthoplicatus* and *Ichthyophis kohtaoensis* have been elaborately studied (Fox, 1983). Arun et al. (2018) reported the ultrastructural organization of the cutaneous glands of *Ichthyophis tricolor* and *Uraeotyphlus cf. oxyurus*. Unlike the skin of urodeles and anurans, the caecilian skin does not show any glandular accumulations (macro-glands) such as parotoids typically found in salamanders and toads (Brodie and Gibson, 1969; Jared et al., 1999, 2018; Regis-Alves et al., 2017). The secretions of the granular glands in *Ichthyophis glutinosus* were found to be non-venomous (Gabe, 1971), whereas a report that followed suggested that the granular gland secretion has a defensive role, as it contains repellent or toxic compounds that would distract the predators (Breckenridge and Murugapillai, 1974). There is no literature on the metabolomic study of the skin secretion of caecilians. In view of the several potential roles of the bio-active molecules, and the scope for research applications, it was conceived that it would be pertinent to investigate *I. beddomei* from this perspective.

Unlike the anurans and urodeles, most of the caecilian amphibians possess dermal scales which have been investigated in fair detail in the genera *Dermophis*, *Ichthyophis*, *Hypogeophis*, *Uraeotyphlus*, *Geotrypetes*, *Herpele*, etc. (Wake, 1975; Casey and Lawson, 1979; Zylberberg et al., 1980; Perret, 1982; Wake and Nygren, 1987; Zylberberg and Wake, 1990; Arun et al., 2018, 2019). The caecilian dermal scales are composed of a basal plate with layers of unmineralized collagen fibres. They are topped with mineralized squamulae (Zylberberg and Wake, 1990; Arun et al., 2019). The dermal scales of *I. beddomei* have not yet been looked into.

Therefore, the present study investigates the dermal glands, scales and secretome of *I. beddomei* using microscopic and metabolomics approaches.

2. Materials and methods

2.1. Sample collection

Specimens of *I. beddomei* (Fig. 1) were collected from Wayanad district, Kerala (1.7923° N, 76.1663° E) under permission from the Kerala State Forest Department (Order No-WL10-23554/2015). During transport to the lab, animals were kept in plastic bottles, with air holes, containing the humid soil collected from the area of collection, with earthworms as feed. Three animals (415 ± 17 mm long, inde-



Fig. 1. *I. beddomei*.

pendent of sex) were maintained in a terrarium in the lab. The mid-dorsal, ventral and lateral skins were dissected under 25% MS-222 (Tricaine methane sulfonate) anaesthesia. The procedures and practices of animal care and handling were in strict adherence to the policies of the Institutional Animal Ethical Committee.

2.2. Light and transmission electron microscopic (LM and TEM) observations of skin

The animal handling techniques were adopted from Beyo et al. (2008) and Arun et al. (2018). The dissected skin samples (n = 3) were washed in amphibian Ringer solution [6.6 g NaCl, 0.15 g KCl, 0.15 g CaCl₂, and 0.2 g NaHCO₃ added to 1L distilled water], followed by fixation in 2.5% glutaraldehyde solution, postfixation in 1% osmium tetroxide, and embedding in methacrylate (Sigma Chemical Co., St. Louis, MO). One-micron thick sections (semithin) were stained with toluidine blue O (TBO) and observed in an Axio-3 research microscope (Carl Zeiss AG, Oberkochen, Germany). Leica ultra-microtome (Jena, Germany) was used to cut ultrathin sections, which were stained with uranyl acetate and lead citrate and subjected to observation in a Tecnai 12G2 Biotwin transmission electron microscope.

2.3. Scanning electron microscopic (SEM) analysis of skin surface morphology

The skin samples, as mentioned *vide supra*, and dermal scales on dorsal side from three specimens were fixed in 10% neutral

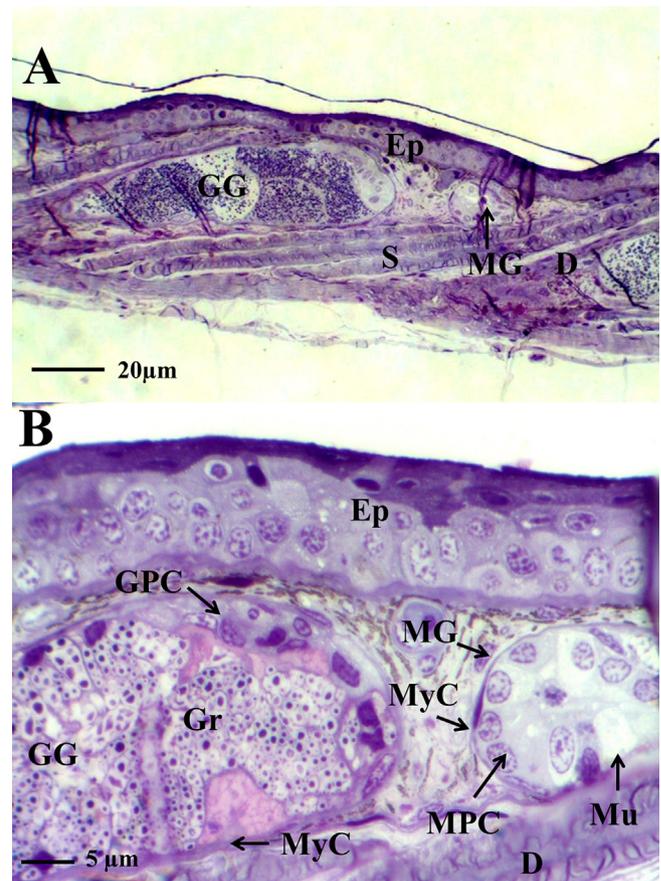


Fig. 2. Light micrographs of cutaneous glands and dermal scales in the skin of *I. beddomei*. A, B: Sections of mucous, granular glands and dermal scales of *I. beddomei*. Ep, Epidermis; D, Dermis; Gr, Granules; MyC, Myoepithelial cells; GPC, Granule producing cells; MPC, Mucous producing cells; Mu, Mucous vesicle; S, Scale.

buffered formalin and dehydrated for SEM (Robinson and Gray, 1990). The samples were dried adopting critical-point method, and mounted to visualize the skin surface morphology. After coating with gold palladium, the samples were observed in a Field Emission scanning electron microscope (SEM; Carl Zeiss, Jena, Germany).

2.4. GC MS analysis

Dichloromethane was used as solvent to extract out the skin gland secretion (Nowack et al., 2017). The dissected skin samples ($n = 3$) were separately kept in dichloromethane for 48 h. Thereafter, the skin was trashed and the dichloromethane was filtered to remove the debris (Poth et al., 2012, 2013) and injected to GC MS. Gas chromatography/mass spectrometry analysis was done on Agilent Technologies 7890A GC (Germany) with Column db5 MS (30 m 0.25 mm I.D., 0.25 μm), flow rate 1.5 mL/min and helium as carrier gas. Splitless injection was done at 250 $^{\circ}\text{C}$. The MS transfer line was maintained at 300 $^{\circ}\text{C}$, the ion source at 250 $^{\circ}\text{C}$ and was operated with 70 eV ionization energy. The mass spectrum was recorded in full scan mode. Identification of the compounds was achieved by comparison with MS-spectra provided by the software AMDIS/NIST/ NIH Mass Spectral Database.

3. Results

3.1. Light microscopic observations of cutaneous glands and dermal scales

There are two types of skin glands, mucous and granular, present in the dermis of *I. beddomei* (Fig. 2A, B). The mucous gland

appears oval in shape and the prominent mucous-producing cells are arranged around a distinct lumen, which is filled with mucous. The granular glands, which lack a lumen, are considerably larger than the mucous glands and are round or elliptical in shape (Fig. 2B). The secretory epithelial cells are compactly arranged, much in the form of cords, around the periphery, and the cytoplasm of cell body extends into the core of the gland. Granules of different sizes and densities are densely present in the granular glands (Fig. 2B). The dermal scales are arranged in the scale pockets along with the cutaneous glands (Fig. 2A).

3.2. TEM observations of cutaneous glands

There are myo-epithelial cells stretching along the lining of both granular and mucous glands (Fig. 3A, C). The granule-producing cells are located along the periphery of the gland (Fig. 3A). Granules of various sizes are present in the cytoplasm towards the core of the gland (Fig. 3A, B). The mucous gland is densely packed with mucous vesicles (Fig. 3C). The mucous-producing cells are darkly stained and located in the periphery of the gland (Fig. 3C). A few mucous vesicles are collapsed and the mucoid material is dispersed in the lumen, probably in preparation for discharge outside (Fig. 3C).

3.3. SEM observations of skin surface

The epithelial cells of the skin surface have micro-ridges with round, oval and polymorphic glandular openings (Fig. 4A, B). The latter are funnel type, where the epithelial cells lining the rim descend into the funnel (Fig. 4A, B, 5C, D). The glandular contents are

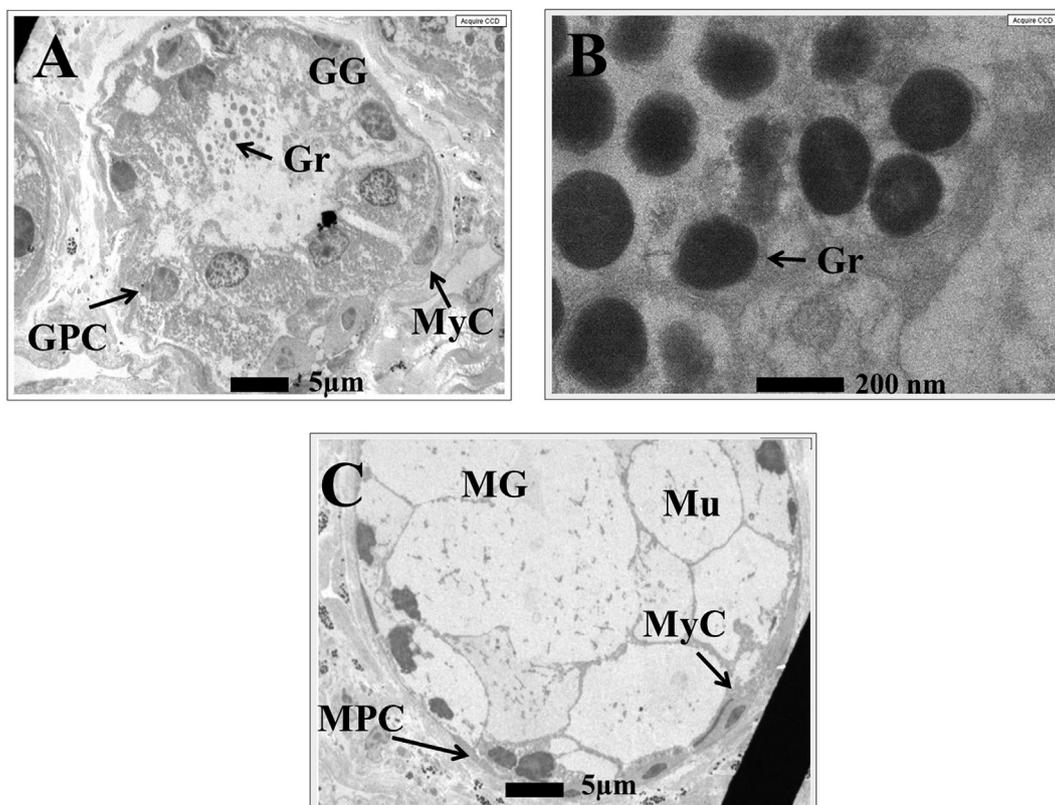


Fig. 3. TEM images of cutaneous glands in the skin of *I. beddomei*. A: Granular gland. B: Enlarged view of granules in the granular gland. C: Discharge of mucous droplet into the lumen of the mucous gland. Gr, Granules; MyC, Myoepithelial cells; GPC, Granule Producing Cells; MPC, Mucous producing cells; Mu, Mucous vesicle.

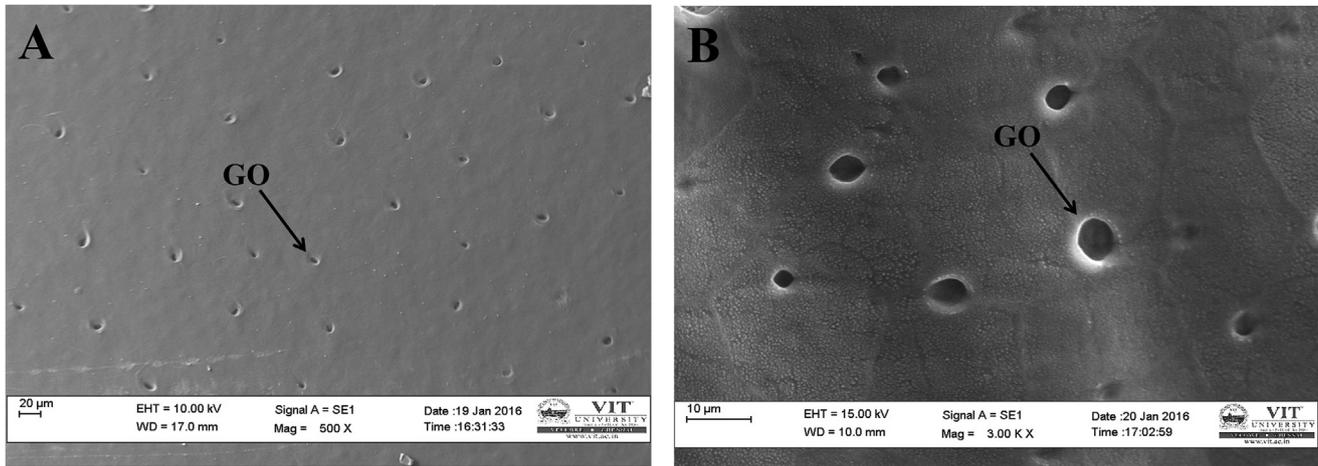


Fig. 4. SEM images of the skin surface of *I. beddomei*. A, B: Dorsal and ventral skin, respectively, showing glandular openings (GO).

released through these openings to be spread along the body surface (Fig. 5A, B).

3.4. SEM observations of dermal scales

The scales are embedded in scale pockets amidst the cutaneous glands in the dermis. The scales are oval in shape and measure a diameter of 700 – 1000 μm . Squamulae are present along the dorsal surface of the scale and are organized in a ring-like arrangement (Fig. 6A). The ventral surface of the scale lacks squamulae

(Fig. 6B). The squamulae are irregular in shape at the centre, and rectangular along the periphery (Fig. 6C). There are spherules located on the squamulae (Fig. 6D).

3.5. GC–MS analysis

The GC–MS analysis of skin extracts of *I. beddomei* yielded 27 compounds (Fig. 7, Table 1), which included three fatty acids, six alcohols, one terpene, five hydrocarbons, one steroid, one aldehyde and ten unidentified compounds (Table 1).

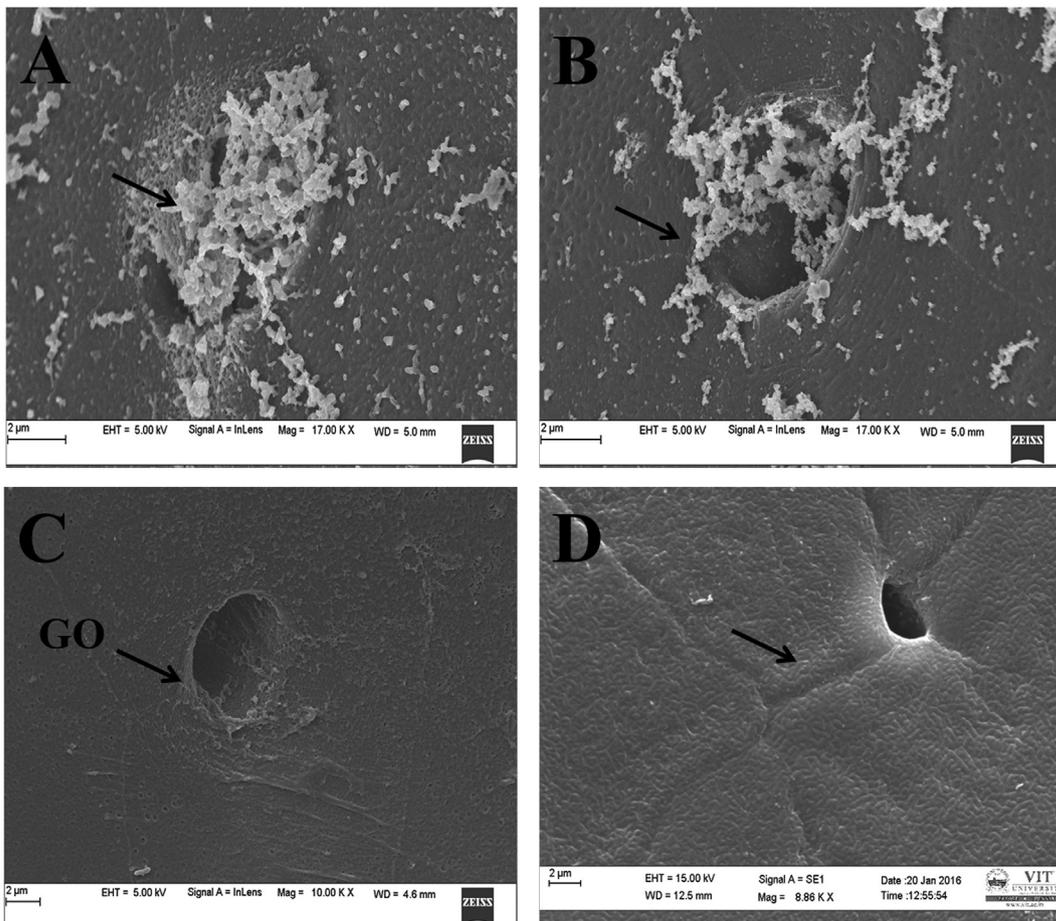


Fig. 5. SEM images of the glandular openings of *I. beddomei*. Dorsal (A, B) and ventral (C, D) skin showing glandular openings (GO).

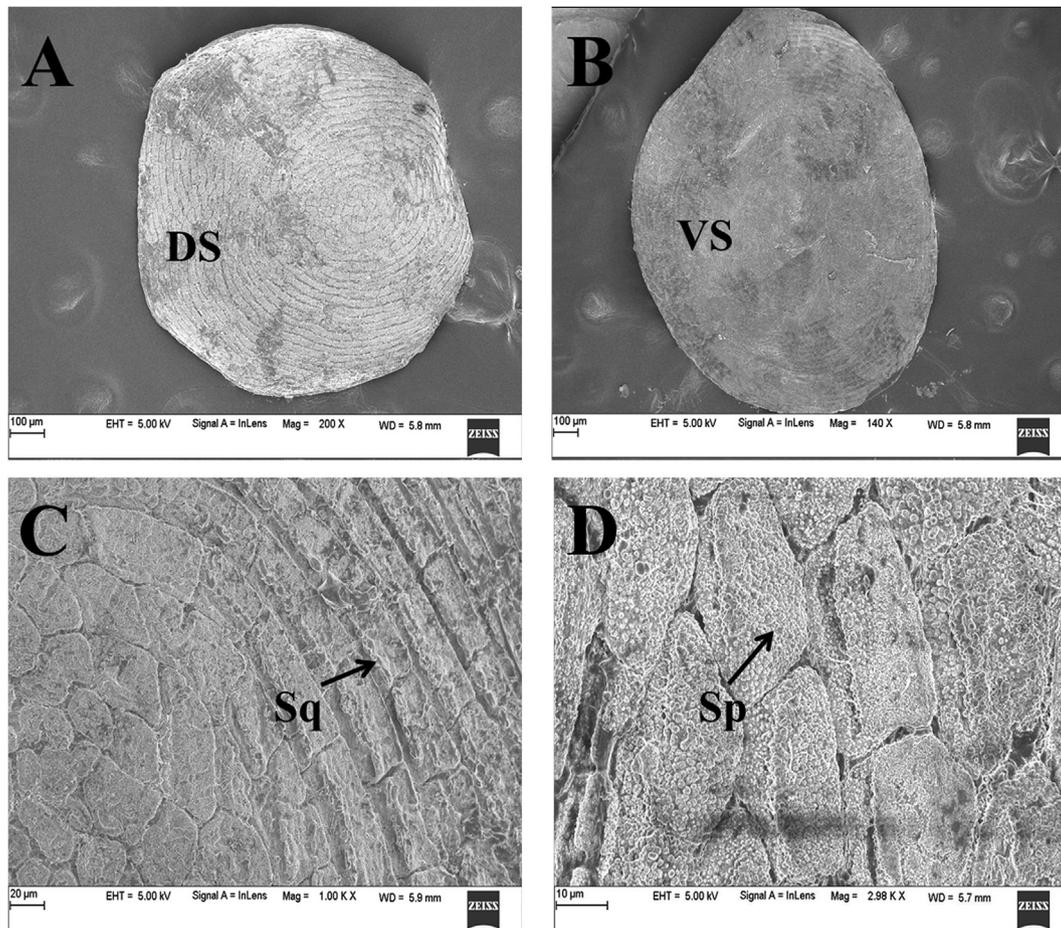


Fig. 6. SEM images of dermal scales of *I. beddomei*. A: Dorsal surface of the dermal scale. B: Ventral surface of the dermal scale. C: Enlarged view of a dermal scale showing squamulae. D: Enlarged view of a dermal scale showing spicules. DS, Dorsal surface; VS, Ventral surface; Sq, Squamulae; Sp, Spicules.

4. Discussion

The amphibian skin in general, and the topic of research in this article in particular, contains mainly two types of cutaneous glands. The mucous glands, possessing a central lumen, secrete a mucoid substance which until discharge accumulates in the cytoplasm in mucous vesicles and then released into the lumen. The vesicles at some stage mucify so as to be discharged outside as the mucous (Jared et al., 2018; Arun et al., 2018, 2019). Jared et al. (2018) reported two types of mucous glands in *Siphonops annulatus* each possessing two types of cells taking up stains differently. The mucous glands produce mucoproteoglycans and mucopolysaccharides (Heiss et al., 2009). The granular glands, sometimes known as poison glands (Jared et al., 2018), secrete a granular material (Duellman and Trueb, 1994; Toledo and Jared, 1995). The granular gland secretory material is rich in bioactive substances such as peptides, amines and alkaloids (Erspamer, 1994).

From the functional point of view, the mucous secreted by the mucous gland helps to keep the skin moist and controls the pH of the body surface (Fox, 1994; Toledo and Jared, 1995). Since mucous is a viscous material, it makes the animal slimy and slippery so as to render it difficult for predators to seize (Toledo and Jared, 1995; Stebbins and Cohen, 1997). The mucous in the skin of frog *Rana catesbeiana* is known to help in keeping the body cool via evaporation of moisture during high temperatures (Lillywhite, 1971). According to Gabe (1971) the mucus of *Ichthyophis glutinosus* contains a hydrophilic mucin, which helps in maintaining the skin moist and, thereby, facilitate cutaneous respiration.

Breckenridge and Murugapillai (1974) found the caecilian mucous gland secretion to be acidic, with sulphate and carboxyl groups, and contain vicinal glycols. According to Jared et al. (2018), as the granular gland matures, the number of vesicles in the granular glands increases and the cytoplasm becomes poorer in organelles, and it is ready to discharge the content. The cell membrane disappears from the core of the gland during the final stage of maturation. It results in a syncytial gland (Navas et al., 1982; Reyer et al., 1992). Williams and Larsen (1986) reported that the granular glands of *Ambystoma macrodactylum* may play role in poison production as well as nutrient storage. The secretory material of the granular glands is rich in biologically active molecules which would play eco-physiological roles such as antimicrobial and antipredatory (Duellman and Trueb, 1986). There are only a few reports on bioactive properties of caecilian skin secretion till now. Bioactive properties of the skin secretion of a caecilian amphibian *Siphonops annulatus* have been reported (Pinto et al., 2014). Schwartz et al. (1997) studied the haemolytic and cardiotoxic properties of the skin secretions of another caecilian – *Siphonops paulensis*. We also found the antimicrobial properties of skin secretions of *I. tricolor* and *U. cf. oxyurus* (unpublished data). The numerous funnel-shaped glandular openings with polymorphic profile on the surface have been reported in the tree frog *Hyla arborea* (Witalmska and Kubiczek, 1998), and caecilians *I. tricolor*, *U.cf. oxyurus* and *G. ramaswamii* (Arun et al., 2018, 2019).

The dermis of caecilian amphibians is unique in possessing scales in dermal pockets (Fox, 1983). The morphology of caecilian dermal scales has been described in *Dermophis mexicanus* (Ochoterena, 1932), *Ichthyophis orthoplicatus* and *Ichthyophis*

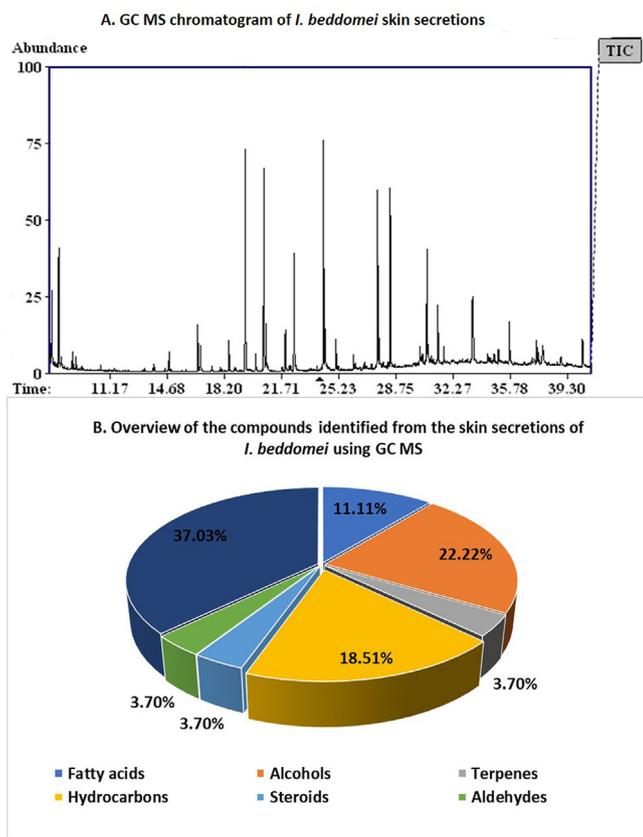


Fig. 7. 7A. GC MS chromatogram of *I. beddomei* skin secretion. 7B. Overview of the compounds identified from the skin secretions of *I. beddomei* using GC MS.

kohtaoensis (Fox, 1983), *I. tricolor* and *U. cf. oxyurus* (Arun et al., 2018), *Dermophis* species (Cockerell, 1912), *Ichthyophis glutinosus* (Gabe, 1971) and *Dermophis mexicanus* and *Microcaecilia unicolor*

(Zylberberg and Wake, 1990). Species belonging to Scoleomorphae and Typhlonectidae do not possess dermal scales, though *Typhlonectes compressicauda* apparently possesses minute scales in the hinder region of the body (Wake, 1975). A common feature of squamulae in all species examined so far is the mineralized material deposited as globules in the dermal scales. We also found mineralization in *I. beddomei*. Mineralization does not occur in the basal plate (Casey and Lawson, 1979). The shapes and distribution of squamulae in the dorsal surface of the scales in *I. beddomei* are similar to the other *Ichthyophis* sp. reported. The peripheral squamulae in *Ichthyophis* are rectangular (Zylberberg et al., 1980; Arun et al., 2018) while in *Geotrypetes* (Perret, 1982) and *Dermophis* (Wake and Nygren, 1987) they are circular. The squamulae are almond shaped in *G. Ramaswamii* (Arun et al., 2019). The squamulae present in the dermal scales of *I. tricolor* and *U. cf. oxyurus* are rectangular in the peripheral region and irregular centrally and scales are present from the anterior to posterior region of the body (Arun et al., 2018), the same as in *I. beddomei* found in this study.

In the present study we adopted metabolomics profiling of *I. beddomei* skin secretion using GC MS. Communication within and between species is fundamental to life at all levels (Wyatt, 2003). How caecilians communicate with each other is a mystery. The caecilians are fossorial in nature. They may communicate through sensory tentacles and/or volatiles. We found five hydrocarbons in the skin secretion of *I. beddomei*. Hydrocarbons like decane, 2,3,5,8-tetramethyl, 1-nonene, 4,6,8-trimethyl and tetradecane, 2,6,10-trimethyl have been reported in the skin secretions of the Brazilian frog *Bokermannohyla alvarengai* (Centeno et al., 2015). So far, role of hydrocarbons in chemical communication is not yet reported in any amphibian species. However, in insects air-borne communication occurs mainly through cuticular hydrocarbons (Wyatt, 2003; Leonhardt et al., 2016).

Squalene is an intermediate metabolite in the synthesis of cholesterol (Gregory and Kelly, 1999). It is an effective oxygen scavenging agent (Bargossi et al., 1994). Therefore, it may be suggested that squalene present in the skin secretion of *I. beddomei* may act as an antioxidant and protect the skin from oxidative damages.

Table 1
Compounds identified from the skin secretions of *I. beddomei* using GC MS.

No	Name	Class	RT*	Formula	MW (Da)**
1	Unknown 1	–	7.60	–	–
2	Unknown 2	–	8.09	–	–
3	Unknown 3	–	8.18	–	–
4	Unknown 4	–	8.82	–	–
5	Unknown 5	–	8.90	–	–
6	1-Tridecene	Hydrocarbon	16.58	C ₁₃ H ₂₆	182.35
7	Pentanedioic acid	Fatty acid	17.45	C ₅ H ₈ O ₄	132.15
8	8-Heptadecene- 9-octyl	Hydrocarbon	18.49	C ₂₅ H ₅₀	350.69
9	Unknown 6	–	19.50	–	–
10	Unknown 7	–	20.65	–	–
11	Hexadecane	Hydrocarbon	20.78	C ₁₆ H ₃₄	226.26
12	1-Bromo tridecane	Hydrocarbon	21.97	C ₁₃ H ₂₇ Br	263. 29
13	Unknown 8	–	22.50	–	–
14	2-Isopropyl-5-methyl-1-heptanol	Alcohol	22.61	C ₁₁ H ₂₄ O	172.18
15	E-15-Heptadecenal	Aldehyde	24.31	C ₁₇ H ₃₂ O	252. 45
16	Octadecane	Hydrocarbon	24.42	C ₁₈ H ₃₈	254.49
17	Unknown 9	–	26.16	–	–
18	1-Eicosanol	Alcohol	27.98	C ₂₀ H ₄₂ O	298.52
19	Hexadecanoic acid	Fatty acid	28.41	C ₁₆ H ₃₂ O ₂	256.47
20	1-Dodecanol, 2-octyl	Alcohol	30.25	C ₂₀ H ₄₂ O	298.52
21	Behenic alcohol	Alcohol	30.68	C ₂₂ H ₄₆ O	326.35
22	Octadecanoic acid	Fatty acid	31.34	C ₁₈ H ₃₆ O ₂	284.31
23	n-Tetracosanol	Alcohol	33.48	C ₂₄ H ₅₀ O	354.68
24	Unknown 10	–	34.81	–	–
25	Octacosanol	Alcohol	37.42	C ₂₈ H ₅₈ O	410.44
26	Squalene	Terpene	37.54	C ₃₀ H ₅₀	410.71
27	Cholesterol	Steroid	38.68	C ₂₇ H ₄₆ O	386.65

* Retention Time.

** Molecular Weight.

Achiraman et al. (2011) reported that squalene produced from the clitoral gland of the rat acts as a semi-volatile and may serve as a chemo-signal. According to Centeno et al. (2015) the major lipid components present in the skin secretion of *Bokermannohyla alvarengai* are hexadecanoic acid methyl ester and octadecanoic acid methyl ester. Hexadecanoic acid, pentanedioic acid, octadecanoic acid and cholesterol were also identified in the skin secretions of *I. beddomei*. Kupfer et al. (2006) also found skin secretions of the caecilian amphibian *Boulengerula taitanus* as rich in lipids. The lipids present in *Rana tigerina* skin play an important role during the proliferative phase of wound healing (Sai and Babu, 1998).

Alkanols and lactones as pheromonal compounds were reported in the femoral gland secretion of mantellid frogs by Poth et al. (2012, 2013). Alcohols, sesquiterpenes and macrolides may have an important role in sexual communication of hyperoliids (Starnberger et al., 2013). We report six alcohols from the skin secretion of *I. beddomei*. Aromatic alcohol, methyl branched alcohols and straight-chain primary alcohols were reported from the skin secretion of two species of hypsiboas tree frogs (Brunetti et al., 2015).

There are two mechanisms which can explain the presence of secretory compounds in the skin of an amphibian, (i) dermal uptake, and (ii) dietary uptake (Smith et al., 2004). Some caecilians (*Afrocaecilia taitana*) were found to have vegetable matter in their diet (Hebrard et al., 1992). Similar observations were made in *Chthonerpeton indistinctum* by Gudynas and Williams (1986) and *Typhlonectes compressicaudus* by Exbrayat et al. (1985). We also found the presence of plant materials along with ants, mites, soil and remnants of earthworms in the gut. Thus, we suggest that the dermal uptake, dietary uptake or both mechanisms may exist in *I. beddomei*.

Our study revealed the presence of alcohols, hydrocarbons, cholesterol, aldehyde, fatty acids and terpene in the skin secretion of *I. beddomei*. Further, there were few compounds that were not identified. The fact is that, the unidentified portion may contain potential volatile molecules. We conclude that caecilian skin glands in general, and those of *I. beddomei* in particular, secrete substances which would play multiple roles including cutaneous respiration, offence and defence, free radical scavenging and conspecific communication. Since very little is known about the volatile compounds present in the skin secretions of caecilian amphibians, investigation of skin secretion of these amphibians adopting secretomic approach offers enormous scope.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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