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Research Article

Eastern Fire Salamander (*Salamandra infraimmaculata*) Skin Mucus Metabolites – LC-MS/MS Analysis

Gad Degani[1], Gad Ish Am[2], Neria Yatom[2], Soliman Khatib[1]* and Sanaa Musa[1]*

[1]MIGAL-Galilee Research Institute, Kiryat Shmona, Israel; Faculty of Science and Technology, Tel-Hai Academic College, Kiryat Shmona, Israel

[2]Kibbutz Sasa, Upper Galilee, Israel

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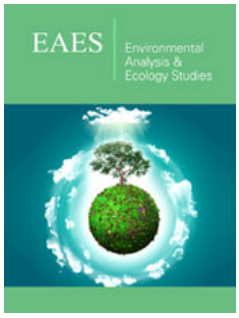

***Corresponding author:** Soliman Khatib, MIGAL-Galilee Research Institute, Kiryat Shmona, Israel; Faculty of Science and Technology, Tel-Hai Academic College, Kiryat Shmona, Israel

Sanaa Musa, MIGAL-Galilee Research Institute, Kiryat Shmona, Israel; Faculty of Science and Technology, Tel-Hai Academic College, Kiryat Shmona, Israel; Email: sanaa@migal.org.il

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Abstract

Metabolites were determined in skin mucus of the Near Eastern fire salamander (*Salamandra infraimmaculata*) by LC-MS/MS analysis. The salamanders were sampled from three different sites at the southern border of their distribution in Israel: Kibbutz (Sasa) in the Upper Galilee and (Manof) in the Lower Galilee-both semi-arid mountain areas, and Dan River (Dan), where water is available year-round at a constant temperature. In total, 1112 metabolites were detected in the secreted mucus on the salamanders' backs. Many of them were known protective substances (SSP) found in *Salamandra salamandra*. Of the metabolites, 891 were found in all of the *S. infraimmaculata* populations, 119 were found only in the salamanders from Manof, 84 only in the Sasa population and 7 only in the Dan salamanders. The percentage of metabolites that were common to salamanders from Dan and Manof, and to those from Dan and Sasa, was significantly lower than that between the two mountain populations (Manof and Sasa). The chemical names and formulas of 14 metabolites found in *S. infraimmaculata* are described. Comparison of these 14 metabolites among populations from the different habitats showed no significant difference in metabolite level, except for 2 metabolites between Sasa and Dan-cholic acid and dihydronandrolone, and one between Manof and Dan-samanine.

Keywords: *Salamandra infraimmaculata*; Skin mucus; Protective Substances (SSP); Metabolites

Graphical Abstract

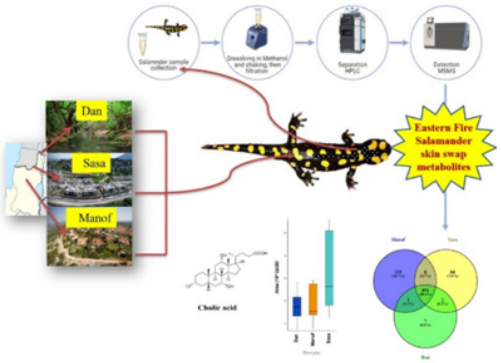


Figure 1:

Introduction

Salamander species of the genus Salamandra are common in Europe, and the southern limit of their distribution is in North Africa, where the species Salamandra *algira* is found, and in the Near East, where Salamandra *infraimmaculata* is found [1]. The yellow-spotted black species of *Salamandra* in Europe have been described in detail in several articles [1],

as has the species *S. infraimmaculata* at the southern limit of its distribution [1,2]. All species of the genus Salamandra secrete poisons, termed Salamandra Skin Poison (SSP), which serves to protect them in conjunction with the salamander's warning colors. SSP has been characterized as steroidal alkaloids of the samandarine type [3]. Most of the studies on the mucus secreted from the skin of salamanders have been conducted on *S. salamandra* from Europe. Gas Chromatography-Mass Spectrometry (GC-MS) and Electron Spray Ionization Mass Spectrometry (ESI-MS) analyses have identified a group of secreted substances that are steroidal alkaloids: samandarine, samadarone, O-acetylsamandarine, samanone and O-3-hydroxybutanoyl samandarine [3]. Plácido et al. [4] identified peptides, of which salamandrin-I, [M+H]+ = 1406.6 Da, was selected for solid-phase synthesis; it showed free radical-scavenging activity against DPPH and ABTS radicals, but no antimicrobial activity against Gram-positive or Gram-negative bacteria [4]. The biological activities of these steroidal alkaloids from *S. salamandra* and other organisms, including blockbuster drugs such as abiraterone acetate, have been described [5].

The genetic structure of the salamander population at the southern limit of its distribution in Israel, belonging to the species *S. infraimmaculata*, differs from that of the salamanders in Europe (*S. salamandra*) [6-10]. In Israel, the salamanders are found in isolated populations, and various differences have been found among those populations, including ecological [11-14], morphological [15,16], physiological [17,18], behavioral [12] and genetic [19-21] differences. The purpose of this study was to examine the metabolites in the mucus secreted by *S. infraimmaculata* from different habitats at the southern limit of its distribution.

Material and Methods

Sample collection

Salamander skin-excreted mucus samples were collected from salamander populations in three sites in Israel: Dan, Sasa, and Manof (Figure 2). The distance between Sasa and Dan is 50km, between Sasa and Manof 36km and between Dan and Manof 86km. Each animal was picked up in one hand, and the secretion was collected by applying filter paper with gentle thumb pressure.

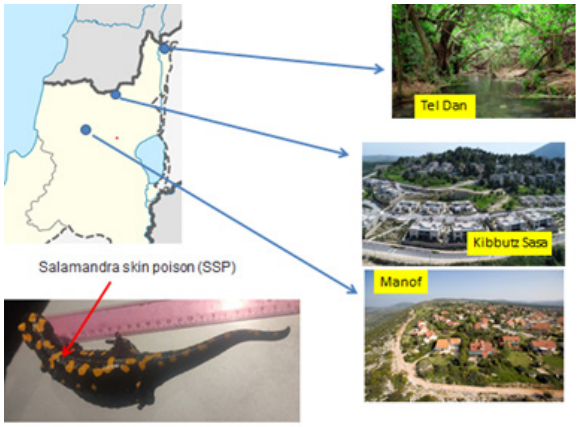


Figure 2: The three sites from which the salamander mucus samples were collected. The illustration shows a salamander secreting the poisonous white mucus. The distance between Sasa and Dan is 50km, between Sasa and Manof 36km and between Dan and Manof 86km. The breeding places of the salamanders in Sasa are in man made pits and caves, in Manof in man made pool, and in Dan in a river flowing year round [19-21].

This secretion was extracted in 3mL of methanol for 10min with gentle vortexing. The samples were filtered through a 0.22-µm membrane, and the solvent was evaporated to dryness under nitrogen flow and kept at -80 °C, until analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Figure 3). The samples were freshly prepared by dissolving them to a concentration of 2mg/mL in methanol. For untargeted metabolomics analysis, Quality Control (QC) procedures were used to ensure data quality. A pooled matrix was prepared by mixing a small volume (20µL) of each experimental sample and this served as the QC sample with four replicates. The blank contained methanol.

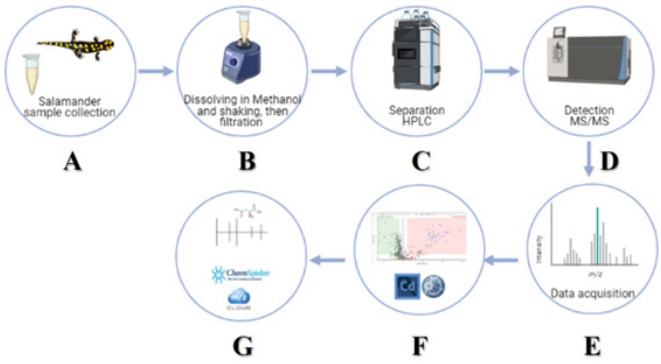


Figure 3: Scheme of the study protocol. (A) Mucus is sampled from the salamander's back and head; (B) the sample is extracted in methanol, and (C) separated by HPLC; (D) detection of substances by MS/MS; (E) data acquisition; (F) data analysis by Compound Discoverer 3.3; (G) identification of the metabolites using MzCloud and ChemSpider databases.

Metabolomics instrumentation

The QC samples, blanks, and *Salamandra* samples were analyzed by injecting 5µL of the extracted solutions into an Ultra-High-Performance LC (UHPLC) apparatus connected to a photodiode array detector (Dionex Ultimate 3000), with a reverse-phase column (ZORBAX Eclipse Plus C18, 3.0x100mm, 1.8µm) (Figure 3). The QC and blank samples were injected first in the sequence, and then after every 10 samples and at the end of the sequence to ensure data quality. The mobile phase was comprised of (A) DDW with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The gradient began with 2% B which was kept for 1.5min, then increased to 30% B over 2.5min, then to 40% B over 1min and kept isocratic at 40% B for 3min. It was then increased to 50% B over 6min and kept isocratic at 50% B for 4min. Solution B was then increased to 95% over 5min and kept for 9min more at 95%, then returned to 2% over 2min, and the column was allowed to equilibrate at 2% B for 4min before the next injection.

The flow rate was 0.4mL/min. The LC-MS/MS analysis was performed with a Heated Electrospray Ionization (HESI-II) source connected to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific). The ESI capillary voltage was set to 3900V, capillary temperature to 350 °C and gas temperature to 350 °C. Nitrogen gas (N_2) was used. The MS conditions were set as follows: the flow rate of sheath gas, aux gas, and sweep gas were kept at 35L/min, 10L/min and 1L/min, respectively. For MS2 (tandem mass spectrometry) analysis, the collision energy was set to 15, 50 and 100EV. The mass spectra (m/z 67-1000) were obtained in negative-and positive-ion mode with high resolution (FWHM=70,000at m/z=200) and mass tolerance=5ppm.

Metabolomics data processing

Peak determination and peak area integration were performed with Compound Discoverer 3.3 (version 3.1.0.305; Thermo Xcalibur). Auto-integration of peak areas was manually inspected and corrected where necessary. Peak areas from each sample were normalized to the QC as follows: peak areas that exhibited a Relative Standard Deviation (RSD) above 50% in the pooled QC samples were removed from the analysis. RSD<50% was corrected using QC samples as reported previously (Dunn et al., 2011). For some compounds, identification was based on the MzCloud database using MS2 data (to obtain accurate qualitative and relative quantitative results) and the Chem Spider database using High-Resolution Mass Spectrometry (HRMS).

Data processing calculations and statistics

For the LC-MS/MS results, peak area medians, SD and RSD were calculated using the Compound Discoverer 3.3 program. Significant differences between groups ($p<0.05$) were determined by one-way analysis of variance (ANOVA).

Results

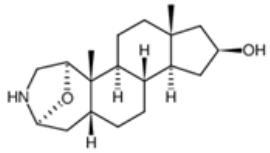
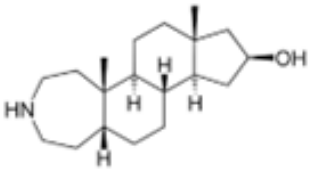
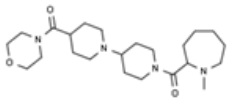
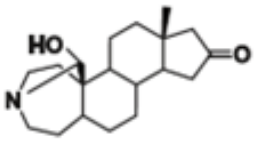
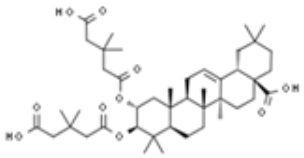
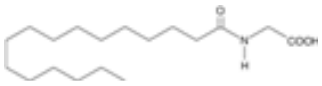
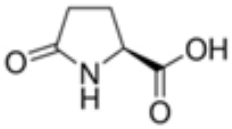
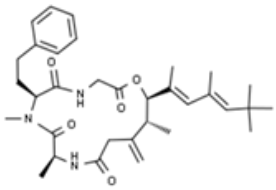
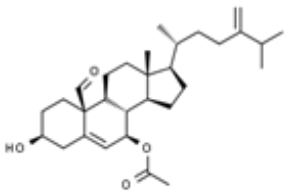
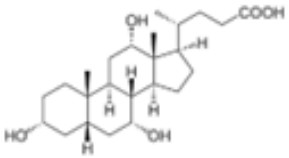
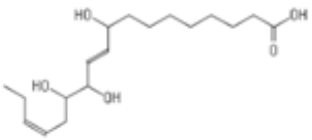
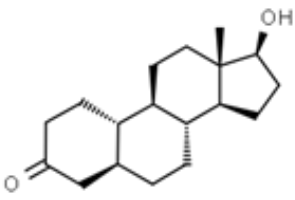
In total, 1112 metabolites were detected. Volcano plots comparing the untargeted metabolites in the mucus of the three populations are shown in Figure 4, and a Venn diagram is shown in Figure 5. Some of these metabolites are further described in Tables 1 & 2. Mucus composition was significantly different for the three salamander populations (Figure 4). A list of 14 metabolites that were found in the three populations-Manof, Sasa and Dan-is given in Table 1. In total, about 1112 different metabolites were found in the mucus secreted from the salamanders' body (Figure 5). Many of the metabolites found in the mucus were known SSPs. 891 metabolites were common to the three *S. infraimmaculata* populations, 119 were unique to the Manof population, 84 to Sasa and 7 to Dan (Figure 5). The percentage of metabolites shared between the salamanders from Dan and Manof (0.1%), and between those from Dan and Sasa (0.2%), was significantly lower than that between the two mountain populations (Manof and Sasa, 0.7%) (Figure 5).

Table 1: Fourteen metabolites in the mucus of *S. infraimmaculata*. D, Dan; M, Manof, S: Sasa.

Compound Name	Chemical Formula	Calculated MW	ΔMass [ppm]	P-value			Log^2 FC		
				M / D	S / D	S / M	M / D	S / D	S / M
Samandarone	$C_{19}H_{29}NO_2$	303.2198	-0.88	0.261	0.236	0.96	2.15	0.43	-1.73
Samanine	$C_{19}H_{33}NO$	291.2559	-1.06	0.04	0.39	0.317	2.61	0.84	-1.77
(1-Methyl-2-azepanyl) [4-(4-morpholinylcarbonyl)-1,4'-bipiperidin-1'-yl] methanone	$C_{23}H_{40}N_4O_3$	420.3094	-1.64	0.083	0.08	0.905	1.11	0.64	-0.47
Samandridine	$C_{21}H_{31}NO_3$	345.2395	-1.26	0.002	0.05	0.042	7.12	3.65	-3.47
(2α,3β)-2,3-Bis[(4-carboxy-3,3-dimethylbutanoyl) oxy] olean-12-en-28-oic acid	$C_{44}H_{68}O_{10}$	756.4813	1.01	0.842	0.401	0.256	0.22	-4.19	-4.41
Palmitoyl glycine	$C_{18}H_{35}NO_3$	313.2616	0.23	0.77	0.578	0.992	-0.52	0.09	0.61
L-pyroglutamic acid	$C_5H_7NO_3$	129.0427	0.86	0.444	0.267	0.999	1.91	0.75	-1.16
Antillatoxin B	$C_{33}H_{47}N_3O_5$	565.3521	0.87	0.999	0.038	0.121	0.52	2.1	1.59
Nebrosteroid L	$C_{40}H_{46}O_4$	470.3395	-0.07	0.592	0.979	0.688	0.33	-0.07	-0.4

Cholic acid	$C_{24}H_{40}O_5$	408.2878	0.69	0.864	0.028	0.245	-0.18	0.6	0.78
(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	$C_{18}H_{34}O_5$	330.2405	-0.16	0.802	0.451	0.938	-0.81	-1.68	-0.88
Dihydronandrolone	$C_{18}H_{28}O_2$	276.2089	-1.28	0.3197	0.044	0.858	-1.35	-2.19	-0.84
Anatoxin-a	$C_{10}H_{15}NO$	165.1153	-0.11	0.246	0.295	0.908	1.81	1.36	-0.45

Table 2: Chemical structures and compound names of metabolites identified in the mucus of *Salamandra infraimmaculata* (Table 1).

Compound Name	Chemical Structure	Compound Name	Chemical Structure
Samandarone		Samanine	
(1-Methyl-2-azepanyl) [4-(4-morpholinylcarbonyl)-1,4'-bipiperidin-1'-yl]methanone		Samandridine	
(2α,3β)-2,3-Bis[(4-carboxy-3,3-dimethylbutanoyl) oxy]olean-12-en-28-oic acid		Palmitoyl glycine	
L-pyroglutamic acid		Antillatoxin B	
Nebrosteroid L		Cholic acid	
(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid		Dihydronandrolone	

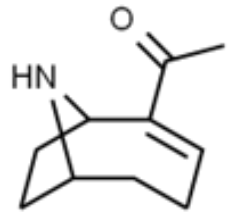
Anatoxin-a			

Figure 4: Volcano plots of the untargeted metabolites for (A) Manof vs. Dan, (B) Sasa vs. Dan, (C) Sasa vs. Manof. P = 0.01, Log2 FC = 2; n = 15 (Dan), 7 (Manof), 16 (Sasa). Red zone: significant and higher than the upper FC threshold (upregulated). Green zone: significant and lower than the FC threshold (downregulated).

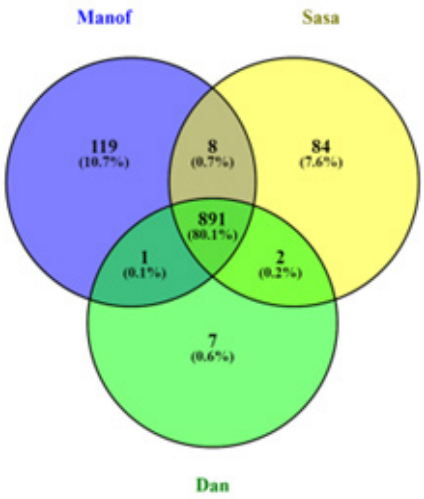


Figure 5: Venn diagram of the untargeted metabolites in the mucus of *S. infraimmaculata* from the three sites (Manof, Sasa and Dan).

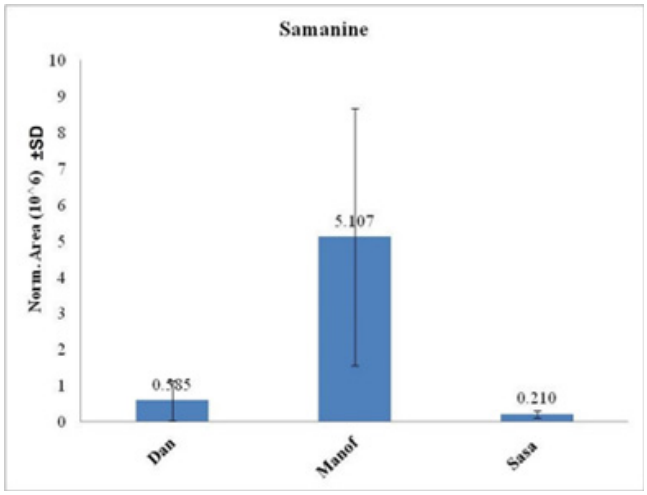


Figure 6: Comparison between the normalized areas of samanine in the mucus of the three populations of salamanders, measured by auto-integration using Compound Discoverer 3.3. Results are presented as mean±SD.

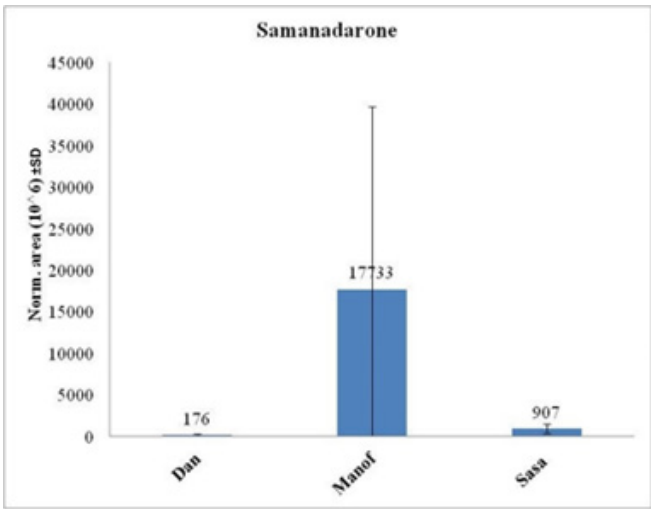


Figure 7: Comparison between the normalized areas of samandarone in the mucus of the three populations of salamanders, measured by auto-integration using Compound Discoverer 3.3. Results are presented as mean ±SD.

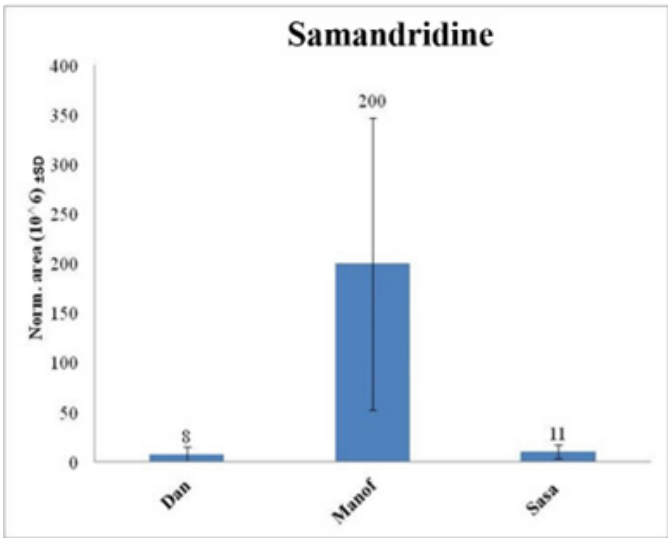


Figure 8: Comparison between the normalized areas of samandridine found in the mucus of the three populations of salamanders, measured by auto-integration using Compound Discoverer 3.3. Results are presented as mean ± SD.

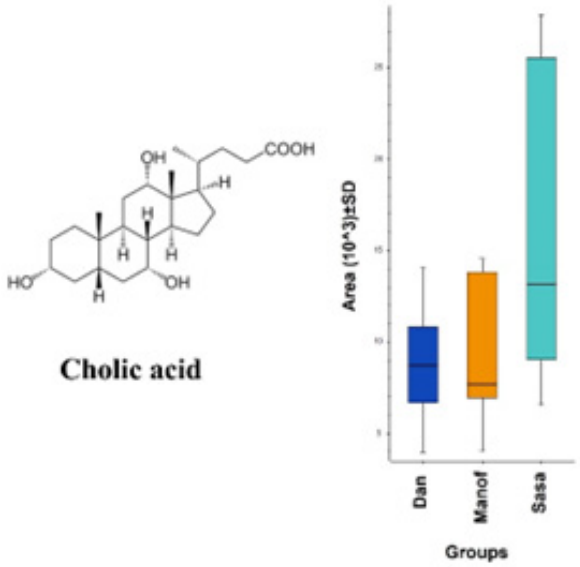


Figure 9: Comparison between the normalized areas of cholic acid found in the mucus (mean ± SD) of the three populations of salamanders.

Names and chemical formulas of 14 of the metabolites found in S. infraimmaculata in Israel (Manof, Sasa and Dan) are presented in Table 1. Some of these metabolites have also been described in S. salamandra in Europe [1,5]. The chemical structures and compound names of the metabolites identified in the mucus of S. infraimmaculata are presented in Table 2. No significant differences in normalized area were found for most of these metabolites among the different populations of salamanders, except for cholic acid and dihydronandrolone between Sasa and Dan, and samanine between Manof and Dan. Samanine and samandarone were present at different levels in the mucus of the three populations of salamanders from different habitats (Figure 6 & 7). Both were higher in the Manof population than in the Dan and Sasa populations, but the difference was only significant ($p<0.05$) between Manof and Dan. No significant differences were found in the comparison of the 14 different metabolites' level from the different salamander populations (Table 1), except for 1 metabolite between Manof and Dan and between Sasa and Dan-samandridine; (Figure 8), and 2 metabolites between Sasa and Dan_cholic acid (Figure 9) and dihydronandrolone (Figure 10).

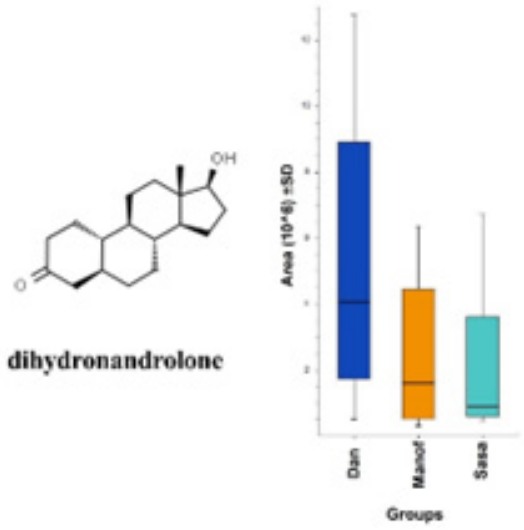


Figure 10: Comparison between the normalized areas of dihydronandrolone in the mucus (mean ± SD) of the three populations of salamanders.

Discussion

Many studies have been published on SSP-the toxic mucus secreted from Salamandra skin, most characterized steroidal alkaloids of the sandarin type [3-5] belonging to the species S. *salamandra*. The data published here on the species S. *infraimmaculata* are preliminary in this field, as not much work has been done on this species [1]. This species is found in isolated populations at the southern limit of its distribution, where the ecological conditions are different from the habitats of S. *salalmandra* in Europe [1,21]. The habitats of the S. *infraimmaculata* studied in this work included a high mountain (Sasa population), a low mountain (Manof population) and Dan River, a stable habitat where there is water year-round [21]. We present here a first study to examines substances secreted from the skin of this species of salamander, and also to compares them between geographically isolated populations. The differences found in the mucus secretions of *S. infraimmaculata* from the various habitats paralleled differences in many biological parameters, such as: morphological [14-16], physiological [14,15], genetic [6,17,15] and behavioral [11,12]. Most of the metabolites found here in the *S. infraimmaculata* mucus have also been found in *S. salamandra* [3-5]. However, the comparison between the populations from different habitats in Israel [21] indicated metabolite differences. Although in most cases no significant difference was found between the three populations regarding the 14 metabolites tested in the mucus composition, nevertheless, a difference was found for 2 metabolites between Sasa and Dan-cholic acid and dihydronandrolone, and 1 between Manof and Dan-samanine. The number of metabolites discovered in this work in the population was much larger. Therefore, much more work is needed to define the differences between these populations from different habitats. The black-yellow coloration of *S. infraimmaculata* probably serves to deter predators [1], as described in other species of Salamandra, but solid evidence for aposematism in *Salamandra* is still lacking. Different color-pattern phenotypes for the yellow spots on the black back of the S. *infraimmaculata* populations studied here have been found [16]. The differences between the color patterns for the salamander populations in Dan and Sasa, and the concomitant variation found here in SSP composition may indicate a connection between these two variables.

Acknowledgement

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