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Composition and Antimicrobial Activity of Defensive Secretions of the Giant Millipede *Anurostreptus sculptus* (Diplopoda, Spirostreptida, Harpagophoridae)

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ABSTRACT. – This paper reports on the chemical composition and antimicrobial activity of the defensive secretions of the giant millipede, *Anurostreptus sculptus* (Spirostreptida: Harpagophoridae). The chemical composition of the defensive secretions was analyzed by gas chromatography-mass spectrometry, demonstrating the presence of at least 13 identifiable compounds, including two major compounds, viz. (1) 2,3-dimethoxy-1,4-benzoquinone, and (2) 2-methyl-1,4-benzoquinone, as well as (3) 8 other quinone derivatives, and (4) 3 fatty acid esters, which may enhance the biological effects of the quinone compounds. Antimicrobial activity was tested with three gram-positive bacteria (*Bacillus cereus, Staphylococcus aureus*, and *Staphylococcus aureus* DMST20654), four gram-negative bacteria (*Escherichia coli, Escherichia coli* ATCC25922, *Pseudomonas aeruginosa*, and *Salmonella* ser. Typhi ATCC16122), and two yeasts (*Candida albicans* and *Candida albicans* ATCC10231), with streptomycin and fluconazole as positive controls. Disc diffusion assays showed that fresh secretions inhibited the growth of all these microorganisms. In line with this, a broth microdilution analysis indicated that fresh secretions yielded Minimum Inhibitory Concentrations (MIC) of 0.0019 to 0.2500% (v/v) and Minimum Bactericidal/Fungicidal Concentrations (MBC/MFC) of 0.0039 to 0.5000% (v/v), i.e.fresh secretions were more effective in inhibiting yeast than the antifungal medicine, fluconazole. The gram-positive bacteria and *C. albicans* were also inhibited by three types of extracted secretions with MIC values in the range of 0.25 to 2.00 mg/ml and MBC values of 2.00 mg/ml for gram positive-bacteria.

KEYWORDS: antimicrobial activity, chemical defense, 2,3-dimethoxy-1,4-benzoquinone, disc diffusion test, 2-methyl-1,4-benzoquinone, Minimum Inhibitory Concentration

INTRODUCTION

Millipedes (Diplopoda) constitute a megadiverse group of terrestrial arthropods comprising more than 11,000 species (Enghoff et al., 2015). This evolutionary success looks somehow at odds with the apparently limited physical protection of millipedes against predators, viz. their more or less calcified exoskeleton and/or their protective spiral coiling or rolling-up behaviour. However, numerous millipede taxa have repugnatorial glands (ozadenes) that produce chemical defensive secretions, which are released via ozopores when the animals are attacked or disturbed (Vujisić et al., 2014; Shear, 2015). The composition of these chemical defense secretions is highly variable and differs among taxonomic groups: for example, the order Polydesmida produces cyanogenic compounds, the superorder Juliformia (orders: Julida, Spirobolida and Spirostreptida) produces benzoquinones and hydroquinones (therefore the Juliformia are also referred to as the "quinone millipedes"), the order Callipodida produces phenols, the orders Glomerida and Polyzoniida produce alkaloids, etc. (e.g. Vujisić et al., 2011, 2014; Shear, 2015). In addition to these main compounds, the defensive secretions of millipedes often contain various other substances, thus forming "cocktails" of sometimes up to ca. 60 different components (Ilić et al., 2019b), some of which may assume other than strictly defensive functions. Such other functions may include antioxidant effects, chemical communication, anti-inflammatory effects, antimicrobial activity, etc. (e.g. Ômura et al., 2002; Dandawate et al., 2010; Ilić et al., 2019b). Hence, the study of the defensive secretions of millipedes is challenging as it must consider (1) their overall biological function(s) as "cocktails", (2) the biological function(s) of their individual compounds, and (3) the taxonomic and phylogenetic framework that underpins their chemical composition and/or patterning (e.g. Rodriguez et al., 2018). Moreover, current knowledge on millipede defensive secretions is based on less than 3% of the known species (Ilić et al., 2018). So, a huge amount of research remains to be done before a more or less comprehensive picture of the chemistry and biological functions of the defensive secretions of millipedes will become available for potential medicinal, pharmaceutical and other applications.

Against this background, the present paper reports on the chemical composition and antimicrobial activity of the defensive secretions of the giant millipede *Anurostreptus sculptus* Demange, 1961. This species

belongs to the Harpagophoridae (Juliformia: order Spirostreptida), a highly speciose family (Pimvichai et al., 2010, 2014, 2016) with > 44 genera and > 220 species (Enghoff et al., 2015), whose defensive secretions have hitherto only been studied superficially in one single, unidentified species (Deml and Huth, 2000). Since *A. sculptus* belongs to the Juliformia ("quinone millipedes") it is predicted that its defensive secretions should contain a substantial amount of benzoquinones and hydroquinones. Hence, this prediction will be tested here. In addition, this paper aims at (1) elucidating the other chemical compounds in the defensive secretions of *A. sculptus*, and (2) exploring the antimicrobial activity of these defensive secretions.

MATERIALS AND METHODS

Collection and handling of millipedes

Samples of *A. sculptus* were collected during the rainy season (August and September 2021) in Thep Sathit District (15° 38' 28"N, 101° 23' 20"E) and Nong Bua Daeng District (16° 00' 01"N, 101° 52' 36"E), Chaiyaphum Province, Thailand. The millipedes were kept alive in plastic boxes with soil and leaf litter for 2 days, after which they were stored in a freezer at -20 °C for 7 days before analysis of the defensive secretions. This research was conducted under the approval of the Animal Care and Use regulations (numbers U1-09054-2563 and IACUC-MSU-25/2021) of the Thai government.

Extraction of defensive secretions

The repugnatorial glands (Fig. 1) of 13 individuals were excised from the body rings, starting from body ring 6 to the last body ring (55−60 rings). The glands of 10 individuals were pooled and air-dried in a Petri dish for 72 h at room temperature. In order to extract the defensive secretions from the repugnatorial glands, the dried glands were sequentially soaked in 80 ml of (by increasing polarity) hexane for three days, ethyl acetate for the next three days, and methanol for the last three days. This soaking sequence was repeated three times, so that the dried glands were soaked 3 x 3 days in each of the three solvents. After each soaking step of three days the extracts were filtered on Whatman® qualitative filter paper. Finally, the filtered extracts from the three solvents were completely dried with a rotary evaporator at the Central Lab, Mahasarakham University. The fresh secretions were obtained directly from the repugnatorial glands, by pooling the fresh, thick secretion liquids of three individuals in a vial, without further air-drying.

FIGURE 1. The giant millipede *Anurostreptus sculptus* from Chaiyaphum Province, Thailand. A: Living female. B: Location of the repugnatorial glands (arrows) inside a body ring.

Preparation of defensive secretions for analysis

For the chemical analysis by gas chromatography-mass spectrometry (GC-MS), the three dried filtered extracts were dissolved again in 2 ml of their original solvents, i.e. hexane, ethyl acetate, or methanol. The fresh secretions (fractions of 10 µl) were also dissolved in 2 ml of hexane, ethyl acetate, and methanol.

For the antimicrobial activity assays, the three dried filtered extracts were dissolved in 3% dimethyl sulfoxide (DMSO) at a final concentration of 2 mg/ml. The fresh secretions were tested directly without dissolving in solvents.

Chemical analyses

GC-MS was done at the Central Lab, Mahasarakham University and analyses were performed on a QP 2010 Shimadzu with an Rtx®-5MS column (Restek, 30 m × 0.25 mm ID, 0.25 µm). The injection volume was 1 µl and the injector temperature was 250 °C. The temperature of the GC program followed the method of Arab et al. (2003) with a slight modification, initially 70 ºC, hold for 2 min, ramped to 250 ºC at a rate of 5 ºC/min then to 280 ºC at a rate of 5 ºC, and finally hold for 16 min. The MS condition was operated in the full-scan range: m/z 40-550 amu, and the electron ionization (EI) mass spectra were produced at 70 eV. The *n*-alkanes standard solution (C7-C30) was analyzed with the same GC conditions to obtain the retention index (RI). The mass spectra of the compounds were identified by comparing them with the mass spectra in the NIST standard reference database NIST14 libraries and with the data published in the literature of the compounds previously identified (Bodner et al., 2016; Stanković et al., 2016; Vujisić et al., 2011).

Antimicrobial activity assays

Tested microbial organisms and inoculum preparation

The microbial organisms used in this study include: three gram-positive bacteria viz., *Bacillus cereus, Staphylococcus aureus* and *Staphylococcus aureus* DMST20654; four gram-negative bacteria viz., *Escherichia coli, Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* and *Salmonella* ser. Typhi ATCC16122; and two yeasts viz., *Candida albicans* and *Candida albicans* ATCC10231. The tested bacterial and *Candida* strains were obtained from the collection of the Microbiology Laboratory, Department of Biology, Faculty of Science, Mahasarakham University. Bacteria were grown on Mueller Hinton Agar (MHA) (Himedia, India) and yeasts were grown on Sabouraud Dextrose Agar (SDA) (Himedia, India) at 37 °C for 24 h. The cell suspensions were then prepared with a density of 1.5×10^8 CFU/ml (0.5 McFarland standard).

Disc diffusion method

The antimicrobial activity of the three extracts and the fresh secretion against the selected microbial organisms were first examined by the disc diffusion method based on the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2012a). The suspensions of bacteria or yeast (1.5×10^8 CFU/ml) were spread over the surface of the MHA medium plates for bacteria and SDA medium plates for yeasts. Sterile filter paper discs with a diameter of 6 mm were impregnated with 15 µl of fresh secretion or 2 mg/ml of either hexane extract, ethyl acetate extract, or methanol extract. Then the impregnated discs were placed on the plates. The positive controls were 250 µg/ml streptomycin for bacteria and 200 µg/ml fluconazole for yeast. The negative controls were 3% DMSO and sterile distilled water. The plates were incubated at 37 °C for 24 h. The inhibition zone was measured in mm.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined in 96 well microtiter plates using the microdilution method based on the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2012b). The concentrations of the three extracts ranged from 2.000 to 0.001 mg/ml, that of the fresh secretion from 2.000 to 0.001% (v/v), according to a two-fold serial dilution series, with Mueller Hinton Broth (MHB) for bacteria, and Sabouraud Dextrose Broth (SDB) for yeast. Then, each well was filled with 100 µl of 1.5×10^8 CFU/ml tested microorganisms. The negative controls were 100 µl of MHB or SDB. The positive controls were 100 µl of 250 to 0.122 µg/ml streptomycin for bacteria and 200 to 0.097 µg/ml fluconazole for yeast. All the microbial cultures were incubated at 37 °C for 24 h. Optical densities (OD) were measured with a multimode plate reader at a wavelength of 600 nm. The lowest concentration of fresh secretions or extracts that inhibit the growth of microorganisms was recorded as the MIC value.

Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by serial sub-cultures of the wells from the 96 wells plate showing no bacterial growth in the MIC test. Each well was sub-cultured on MHA medium for bacteria and SDA medium for yeast using loopfuls. These sub-cultures were incubated at 37 °C for 24 h. Streptomycin served as the positive control for bacteria, and fluconazole served as the positive control for yeast. The lowest concentration with no colony growth was defined as the MBC or MFC.

TABLE 1. Chemical composition of the defensive secretions of the giant millipede *Anurostreptus sculptus*. The % refer to the peak areas characterizing the compounds in the gas chromatography-mass spectrometry spectra.

No.	Compound	RI measured*/ authentic reference**	Fresh secretions (%)	Hexane extract (%)	Ethyl acetate extract (%)	Methanol extract (%)
1	Not identified	794	3.14	-	-	40.33
2	Not identified	875	-	2.37	-	-
3	1,4-Benzoquinone	913/917	8.22	-	-	-
4	Not identified	943	-	6.05	-	-
5	Not identified	1009	-	2.69	-	-
6	2-Methyl-1,4-benzoquinone	1116	15.17	-	-	-
7	Not identified	1154	-	-	-	0.35
8	2-Methoxy-3-methyl-1,4-benzoquinone	1189/1191	6.07	-	-	-
9	2-Methoxy-1,4-benzoquinone	1235/1240	-	-	40.23	-
10	3,4-Dimethoxyphenol	1279	3.08	-	-	-
11	2,3-Dimethoxy-1,4-benzoquinone	1324/1322	58.58	-	-	-
12	2-Methylhydroquinone	1334/1341	1.84	-	-	-
13	2-Methoxy-5-methylhydroquinone	1350/1350	0.36	-	-	-
14	2-Methyl-3,4-methylenedioxyphenol	1392/1390	0.24	-	-	-
15	2-Methoxy-3-methylhydroquinone	1428/1425	0.48	-	-	-
16	Not identified	1822	-	-	-	1.00
17	Hexyl pentadecanoate[a]	2228/2223	2.08	80.02	54.87	58.33
18	Hexyl pentadecanoate[a]	2264/2266	-	-	4.90	-
19	Hexyl hexadecanoate	2390/2386	-	1.83	-	-
20	Hexyl octadecanoate	2596/2582	-	7.04	-	-

* Retention index relative to *n*-alkanes on elite-5MS capillary column
** Bodner et al. 2016, Stanković et al. 2016 and Vujisić et al. 2011
[a] Isomer compound of Hexyl pentadecanoate
- = Compound not detected

RESULTS

Composition of defensive secretions

GC-MS analyses of the fresh secretions and three extracts demonstrated the presence of 20 different compounds, including 10 quinone compounds, four hexyl esters of fatty acids (C_{15}, C_{16} and C_{18}) and six unidentified compounds (Table 1). The fresh secretions extracts gave the same results and are therefore further treated jointly. They yielded 11 compounds, among which two major quinones, viz. 2,3-dimethoxy-1,4-benzoquinone (Fig. 2A) and 2-methyl-1,4-benzoquinone (Fig. 2B), together with all other listed quinone components except for 2-methoxy-1,4-benzoquinone (Table 1). In addition, they contain hexyl pentadecanoate and one unidentified compound (Table 1).

The three extracts from the dried glands confirmed the major prevalence of hexyl pentadecanoate (Fig. 2C), and disclosed the presence of eight compounds that were not detected in the fresh secretions, including 2-methoxy-1,4-benzoquinone (Fig. 2D) as a major one, hexyl hexadecanoate, hexyl octadecanoate and five unidentified compounds (Table 1).

Antimicrobial activity assays by the disc diffusion method

The disc diffusion assays (Table 2) showed that the fresh secretions were more effective against the tested bacteria and yeasts than the positive controls (streptomycin and fluconazole). As such, fresh secretions completely inhibited the growth of *S. aureus*, *S. aureus* DMST20654, *C. albicans*, and *C. albicans* ATCC10231 (Fig. 3). In addition, they strongly inhibited the growth of *B. cereus* (Fig. 4A; Table 2 inhibition zone diameter of 76.60 ± 0.05 mm), and that of the four gram-negative bacteria, *E. coli*, *E. coli* ATCC25922, *P. aeruginosa*, *Salmonella* ser. Typhi ATCC16122 (Fig. 4B-E; Table 2 inhibition zone diameters of 71.80 ± 0.10 mm, 69.30 ± 0.07 mm, 63.70 ± 0.20 mm, and 69.30 ± 0.09 mm, respectively).

The three extracts inhibited the growth of the gram-positive bacteria but to a lesser extent than streptomycin, while they inhibited the growth of *C. albicans* more effectively than fluconazole. They showed no inhibitory effects on the gram-negative bacteria and *C. albicans* ATCC10231.

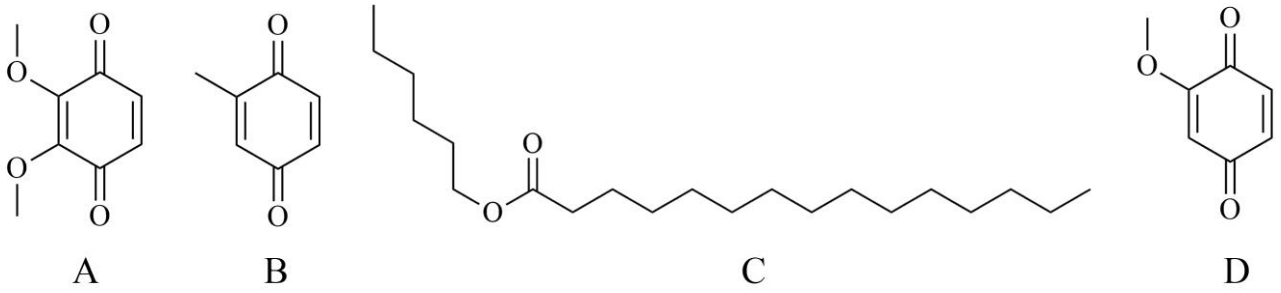

FIGURE 2. Chemical structure of the main compounds of the defensive secretions of the giant millipede *Anurostreptus sculptus*. A: 2,3-dimethoxy-1,4-benzoquinone. B: 2-methyl-1,4-benzoquinone. C: hexyl pentadecanoate. D: 2-methoxy-1,4-benzoquinone.

TABLE 2. Mean diameters of the microbial inhibition zones provoked by the defensive secretions of the giant millipede *Anurostreptus sculptus* as determined by the disc diffusion method.

Microbial strains		Mean diameter of inhibition zone in mm ± SD					
		Hexane extract (2 mg/ml)	**Ethyl acetate extract (2 mg/ml)**	**Methanol extract (2 mg/ml)**	**Fresh secretions**	**Streptomycin (250 µg/ml)**	**Fluconazole (200 µg/ml)**
Gram-positive bacteria	*B. cereus*	9.30 ± 0.05	9.70 ± 0.05	6.70 ± 0.05	76.60 ± 0.05	13.20 ± 0.04	N/A
	S. aureus	12.30 ± 0.05	14.00 ± 0.05	7.00 ± 0.08	85.00 ± 0.00*	19.60 ± 0.38	N/A
	S. aureus DMST20654	11.60 ± 0.05	13.00 ± 0.09	6.30 ± 0.05	85.00 ± 0.00*	16.20 ± 0.06	N/A
Gram-negative bacteria	*E. coli*	0	0	0	71.80 ± 0.10	14.80 ± 0.34	N/A
	E. coli ATCC25922	0	0	0	69.30 ± 0.07	12.10 ± 0.03	N/A
	P. aeruginosa	0	0	0	63.70 ± 0.20	10.10 ± 0.03	N/A
	Salmonella ser. Typhi ATCC16122	0	0	0	69.30 ± 0.09	6.20 ± 0.04	N/A
Yeast	*C. albicans*	10.80 ± 0.13	9.10 ± 0.09	7.00 ± 0.05	85.00 ± 0.00*	N/A	6.80 ± 0.04
	C. albicans ATCC10231	0	0	0	85.00 ± 0.00*	N/A	0

* Fresh secretions completely inhibit microbial growth on the plates (85.00 mm).
0 = No inhibition zone
N/A = Not Applicable

MIC, MBC and MFC

The MIC values of the fresh secretions ranged from 0.0019 to 0.2500% (v/v) (Table 3). The lowest MIC value, viz. of 0.0019% (v/v), inhibited the growth of *C. albicans*, followed by 0.0039% (v/v) inhibiting the growth of *C. albicans* ATCC10231. Hence, the fresh secretions are more effective than fluconazole. The highest MIC value was 0.2500% (v/v) which inhibited the growth of *P. aeruginosa*.

The MIC values of the three extracts ranged from 0.25 to 2.00 mg/ml (Table 3). The lowest MIC value (0.25 mg/ml in ethyl acetate) inhibited the growth of *S. aureus*. The highest MIC value was 2.00 mg/ml (for each solvent), which inhibited the growth of *C. albicans*.

Since the disc diffusion assay showed that neither of the three extracts revealed any inhibitory effect against gram-negative bacteria and *C. albicans* ATCC10231, these microorganisms were not tested for MIC.

The MBC and MFC values (Table 4) were in line with the MIC results. For fresh secretions they ranged from 0.0039 to 0.5000% (v/v). The lowest concentration, viz. MFC = 0.0039% (v/v), inhibited the growth of *C. albicans*, followed by MFC = 0.0078% (v/v) inhibiting the growth of *C. albicans* ATCC10231. The highest MBC value was 0.25% (v/v), which inhibited the growth of *P. aeruginosa*.

The MBC and MFC values of the three extracts ranged from 2.00 to > 2.00 mg/ml (Table 4). The

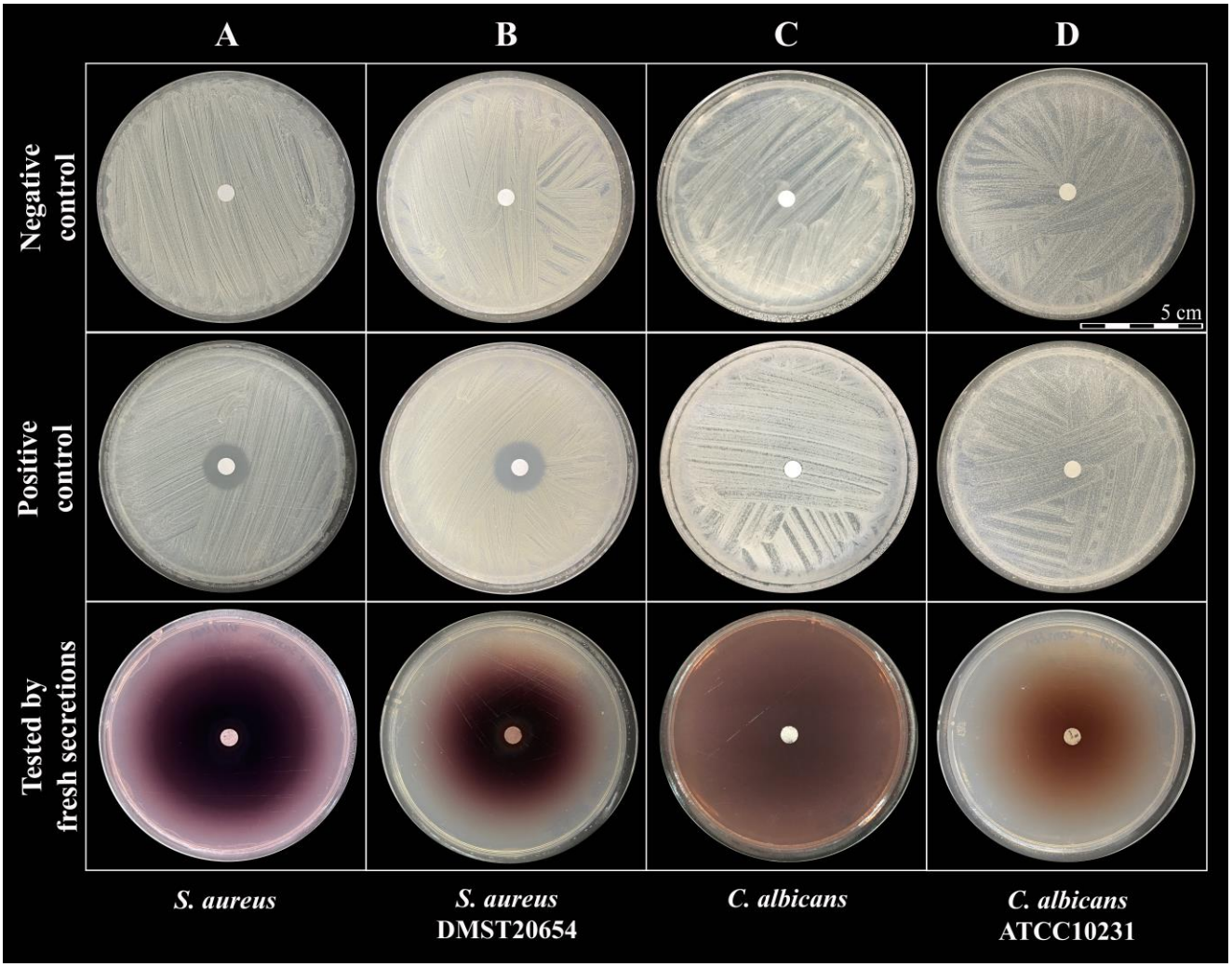

FIGURE 3. Antimicrobial activity against: A: *Staphylococcus aureus*. B: *S. aureus* DMST20654. C: *Candida albicans*. D: *C. albicans* ATCC10231. Negative control, top row; Positive control, middle row; Fresh defensive secretions of the giant millipede *Anurostreptus sculptus*, bottom row, none of the four tests showed any colony growth on the plates.

lowest values, viz. MBC = 2.00 mg/ml in hexane and ethyl acetate, inhibited the growth of gram-positive bacteria. The highest values, viz. MBC > 2.00 mg/ml in methanol, also inhibited the growth of gram-positive bacteria, while MFC > 2.00 mg/ml (for each solvent) inhibited the growth of *C. albicans*.

DISCUSSION

Chemical composition of the defensive secretions of *Anurostreptus sculptus*

The defensive secretions of *A. sculptus* contain at least 20 chemical compounds, several of which are in line with those of other Juliformia species. Yet, the most prevalent compound in the fresh secretions of *A. sculptus* is 2,3-dimethoxy-1,4-benzoquinone (but not detected in the three extracts), which shows a much lower prevalence in other Juliformia species (e.g. Bodner et al., 2016; Kania et al., 2016; Ilić et al., 2018). Conversely, in other Juliformia species, the main compound is 2-methoxy-3-methyl-1,4-benzoquinone (e.g. Deml and Huth, 2000; Valderrama et al., 2000; Vujisić et al., 2011, 2014; Bodner et al., 2016; Stanković et al., 2016), which in *A. sculptus* accounted for only 6.07% of the peak areas. In contrast, the second main compound in the fresh secretions of *A. sculptus*, viz. 2-methyl-1,4-benzoquinone, is also a major compound in other Juliformia species. Still other quinone compounds produced by *A. sculptus*, such as 1,4-benzoquinone, 2-methoxy-1,4-benzoquinone, 2-methylhydroquinone and 2-methoxy-3-methylhydroquinone are consistent with many previous reports dealing with other Juliformia species (e.g. Eisner et al., 1978; Deml and Huth, 2000; Vujisić et al., 2011, 2014; Shear, 2015; Bodner et al., 2016; Stanković et al., 2016; Morales and Pedroso, 2019).

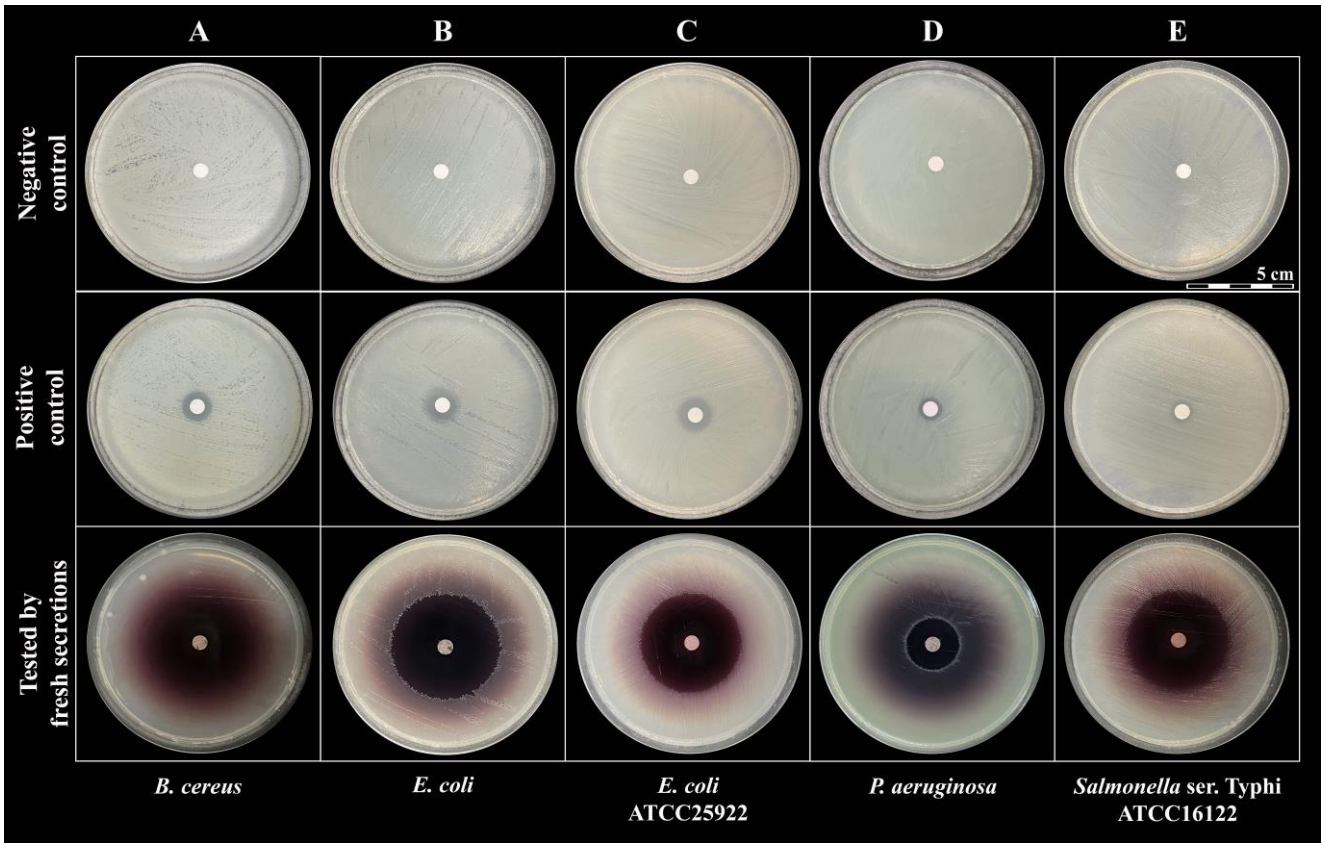

FIGURE 4. Antimicrobial activity against: A: *Bacillus cereus*. B: *Escherichia coli*. C: *E. coli* ATCC25922. D: *Pseudomonas aeruginosa*. E: *Salmonella* ser. Typhi ATCC16122. Negative control, top row; Positive control, middle row; Fresh defensive secretions of the giant millipede *Anurostreptus sculptus* bottom row.

The most striking compound secreted by *A. sculptus* is 3,4-dimethoxyphenol, which, like other phenols, is an antimicrobial defensive compound (Davidson and Branden, 1981; Furtado et al., 2002; Shahidi and Yeo, 2018). It is the first report of this compound in Spirostreptida and the second report in the Juliformia. This compound was earlier found in *Rhinocricus* (Spirobolida) (Morales and Pedroso, 2019; Morales et al., 2022).

Quinones are an important group of natural products that assume many biological functions, such as repelling other organisms (e.g. Peschke and Eisner, 1987; Valderrama et al., 2000; Weldon et al., 2003) and inhibiting fungal or bacterial growth (e.g. Alibi et al., 2021). In this respect, they are even more bioactive than phenol (Beheshti et al., 2012; Campos-Xolalpa et al., 2021; Ilić et al., 2019a).

Another group of compounds in the defensive secretion of *A. sculptus* are fatty acid esters (hexyl pentadecanoate, hexyl hexadecanoate and hexyl octadecanoate), of which hexyl pentadecanoate (numbers 17 and 18 in Table 1) was prevalent in the three extracts. The presence of fatty acid esters in defensive secretions has been reported in several Juliformia millipedes (e.g., Shimizu et al., 2012; Stanković et al., 2016; Ilić et al., 2018). It is assumed that the antimicrobial effects of quinones may be enhanced by other, non-quinone, compounds such as fatty acids esters (Vujisić et al., 2014). Ilić et al. (2018) noted that arthropods that use benzoquinones as defensive secretions often also contain non-quinones that may act as solvent or that facilitate the diffusion of defensive secretions. Some of these non-quinone compounds, like fatty acids esters, may also play a role in chemical communication (Stanković et al., 2016). This perhaps explains the presence of non-quinone compounds in the defensive secretions of *A. sculptus* (Table 1).

Several compounds, including the two most prevalent ones observed in the fresh secretions, were not detected in the three extracts from the dried glands. Conversely, some compounds were only detected in the extracts, but not in the fresh secretions. This observation illustrates the need of using both approaches in a complementary way and at the same time points to two methodological issues: (1) the degree by which the compounds in the defensive secretion will be detected in the extracts, will depend

TABLE 3. Minimum inhibitory concentrations (MIC) of the defensive secretions of the giant millipede *Anurostreptus sculptus* against the selected microorganisms.

Microbial strains		MIC					
		Hexane extract (mg/ml)	Ethyl acetate extract (mg/ml)	Methanol extract (mg/ml)	Fresh secretions (% v/v)	Streptomycin (µg/ml)	Fluconazole (µg/ml)
Gram-positive bacteria	*B. cereus*	0.50	0.50	1.00	0.0156	62.50	N/A
	S. aureus	0.50	0.25	1.00	0.0078	31.25	N/A
	S. aureus DMST20654	0.50	0.50	1.00	0.0156	62.50	N/A
Gram-negative bacteria	*E. coli*	-	-	-	0.0625	62.50	N/A
	E. coli ATCC25922	-	-	-	0.0625	62.50	N/A
	P. aeruginosa	-	-	-	0.2500	125.00	N/A
	Salmonella ser. Typhi ATCC16122	-	-	-	0.0625	125.00	N/A
Yeast	*C. albicans*	2.00	2.00	2.00	0.0019	N/A	200.00
	C. albicans ATCC10231	-	-	-	0.0039	N/A	200.00

- = Not tested
N/A = Not Applicable

TABLE 4. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the defensive secretions of the giant millipede *Anurostreptus sculptus* against the selected microorganisms.

Microbial strains		MBC/MFC					
		Hexane extract (mg/ml)	Ethyl acetate extract (mg/ml)	Methanol extract (mg/ml)	Fresh secretions (% v/v)	Streptomycin (µg/ml)	Fluconazole (µg/ml)
Gram-positive bacteria	*B. cereus*	2.00	2.00	>2.00	0.1250	250.00	N/A
	S. aureus	2.00	2.00	>2.00	0.0620	125.00	N/A
	S. aureus DMST20654	2.00	2.00	>2.00	0.1250	250.00	N/A
Gram-negative bacteria	*E. coli*	-	-	-	0.2500	125.00	N/A
	E. coli ATCC25922	-	-	-	0.2500	250.00	N/A
	P. aeruginosa	-	-	-	0.5000	250.00	N/A
	Salmonella ser. Typhi ATCC16122	-	-	-	0.2500	250.00	N/A
Yeast	*C. albicans*	>2.00	>2.00	>2.00	0.0039	N/A	>200.00
	C. albicans ATCC10231	-	-	-	0.0078	N/A	>200.00

- = Not tested
N/A = Not Applicable

on the solubilities and eventual alterations of the compounds after the air-drying procedure in relation to the different polarities of the extraction solvents (Tomsone et al., 2012); (2) the high temperatures to

which the repugnatorial glands are exposed during the sample preparation procedures (air-drying, rotary evaporator) can cause oxidation reactions that may alter the characteristics of the compounds (Stanković et al., 2016) and thus could affect the results.

Antimicrobial potential of the defensive secretions

The fresh secretions showed the highest inhibitory potential against *S. aureus*, *S. aureus* DMST20654, *C. albicans* and *C. albicans* ATCC10231 (no colony growth at all). They also inhibited, to a lesser extent, *B. cereus* and the tested gram-negative bacteria. Hence, the defensive secretions of *A. sculptus* appear to show a high antimicrobial activity. In this respect they have a significantly higher potential to inhibit *S. aureus* and *E. coli* than the African millipedes *Ophistreptus guineensis* (Silvestri, 1897) (Spirostreptidae) and *Pachybolus ligulatus* (Voges, 1878) (Pachybolidae) (Billah et al., 2015). Moreover, the three extracts of *A. sculptus* also showed a much lower MIC value than these two African millipedes. Maybe even more impressive is the observation that the defensive secretions of *A. sculptus* are more effective against *C. albicans* and *C. albicans* ATCC10231 than the commercial antifungal medicine, fluconazole, a result that was also reported for the millipede *Pachyiulus hungaricus* (Karsch, 1881) (Julidae) (Stanković et al., 2016).

In conclusion, the strong antimicrobial activity of the defensive secretions of *A. sculptus* appears promising for potential medicinal applications, the more so as *A. sculptus* is a very large (up to 20 cm long), common and widespread species in NE Thailand. The ease by which these animals can be maintained and handled makes them potentially attractive for culturing and mass production. In this way they are also excellent model organisms for further research on the chemistry and biological functions of their defensive secretions.

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LITERATURE CITED

Alibi, S., Crespo, D. and Navas, J. 2021. Plant-derivatives small molecules with antibacterial activity. Antibiotics, 10: 231.

Arab, A., Zacarin, G.G., Fontanetti, C.S., Camargo-Mathias, M.I., Dos Santos, M.G. and Cabrera, A.C. 2003. Composition of the defensive secretion of the Neotropical millipede *Rhinocricus padbergi* Verhoeff 1938 (Diplopoda: Spirobolida: Rhinocricidae). Entomotropica, 18: 79–82.

Beheshti, A., Norouzi, P. and Ganjali, M.R. 2012. A simple and robust model for predicting the reduction potential of quinones family; electrophilicity index effect. International Journal of Electrochemical Science, 7: 4811–4821.

Billah, M.K., Kwang, D., Adofo, C., OlutaİwoM.A. and Pesewu, G. A. 2015. Antibacterial activities of Millipede extracts against selected bacterial pathogens. Journal of Microbiology and Antimicrobial Agents, 1: 30–35.

Bodner, M., Vagalinski, B., Makarov, S.E., Antić, D.Z., Vujisić, L.V., Leis, H.J. and Raspotnig, G. 2016. "Quinone millipedes" reconsidered: evidence for a mosaic-like taxonomic distribution of phenol-based secretions across the Julidae. Journal of Chemical Ecology, 42: 249–258.

Campos-Xolalpa, N., Pérez-Ramos, J., Esquivel-Campos, A., Pérez-González, C., Sánchez-Pérez, L. and Pérez-Gutiérrez, S. 2021. Cytotoxic and Antimicrobial Activities of Quinones Isolated from Different Organism. In Soloneski, S. and Larramendy, M.L. (Eds) Cytotoxicity - New Insights into Toxic Assessment, IntechOpen, 37 pp.

CLSI. 2012a. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Eleventh Edition. CLSI document M02-A11. Wayne, PA. Clinical Laboratory Standard Institute.

CLSI. 2012b. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard-Ninth Edition. CLSI document M07-A9. Wayne, PA. Clinical Laboratory Standard Institute.

Dandawate, P.R., Vyas, A.C., Padhye, S.B., Singh, M.W. and Baruah, J.B. 2010. Perspectives on Medicinal Properties of Benzoquinone Compounds. Mini-Reviews in Medicinal Chemistry, 10: 436–454.

Davidson, P.M. and Branden, A.L. 1981. Antimicrobial activity of non-halogenated phenolic compounds. Journal of Food Protection, 44: 623–632.

Deml, R. and Huth, A. 2000. Benzoquinones and hydroquinones in defensive secretions of tropical millipedes. Naturwissenschaften, 87: 80–82.

Eisner, T., Alsop, D., Hicks, K. and Meinwald, J. 1978. Defensive secretions of millipedes. In Bettini, S. (Ed.), Arthropod Venoms, Handbook of Experimental Pharmacology, 48: 41–72.

Enghoff, H., Golovatch, S., Short, M., Stoev, P. and Wesener, T. 2015. Diplopoda – Taxonomic overview. In Minelli, A. (Ed.), The Myriapoda 2. Treatise on Zoology – Anatomy, Taxonomy, Biology. pp. 363–453.

Furtado, N.A.J.C., Said, S., Ito, I.Y. and Bastos, J.K. 2002. The antimicrobial activity of *Aspergillus fumigatus* is enhanced by a pool of bacteria. Microbiological Research, 157: 207–211.

Ilić, B., Dimkić, I., Unković, N., Ljaljević Grbić, M., Vukojević, J., Vujisić, L., Tešević, V., Stanković, S., Makarov, S. and Lučić, L. 2018. Millipedes vs. pathogens: Defensive secretions of some julids (Diplopoda: Julida) as potential antimicrobial agents. Journal of Applied Entomology, 142: 775–791.

Ilić, B., Unković, N., Ćirić, A., Glamočlija, J., Ljaljević Grbić, M., Raspotnig, G., Bodner, M., Vukojević, J. and Makarov, S. 2019a. Phenol-based millipede defence: antimicrobial activity of secretions from the Balkan endemic millipede *Apfelbeckia*

insculpta (L. Koch, 1867) (Diplopoda: Callipodida). The Science of Nature, 106: 1–9.

Ilić, B., Unković, N., Knežević, A., Savković, Ž., Ljaljević Grbić, M., Vukojević, J., Jovanović, Z., Makarov, S. and Lučić, L. 2019b. Multifaceted activity of millipede secretions: Antioxidant, antineurodegenerative, and anti-*Fusarium* effects of the defensive secretions of *Pachyiulus hungaricus* (Karsch, 1881) and *Megaphyllum unilineatum* (C. L. Koch, 1838) (Diplopoda: Julida). PLoS ONE, 14(1): e0209999.

Kania, G., Kowalski, R. and Pietraś, R. 2016. Defensive secretions in millipede species of the order Julida (Diplopoda). Acta Societatis Zoologicae Bohemicae, 80: 17–20.

Morales, J.E.T. and Pedroso, M.T.C. 2019. Diplodomica I. Chemical Composition of Repugnatorial Secretions of Cuban Endemic Millipede gen. *Rhinocricus* sp. Multidisciplinary Digital Publishing Institute Proceedings, 41(1).

Morales, J.E.T., Siguenza, J.C., Villavicencio, C.B. and Canchingre, M. 2022. Diplodomica III. Chemical Ecology of defensive secretions from neotropical archipelago. Isolation of 3,4-dimethoxyphenol from ejected secretion of endemic Cuban millipedes (Spirobolida, Rhinocricidae, *Rhinocricus*). Study case *Rhinocricus duvernoyi* Karch 1881, La Palma population. In Proceedings of the 2nd International Electronic Conference on Diversity (IECD 2022)—New Insights into the Biodiversity of Plants, Animals and Microbes, 15–31 March 2022, MDPI: Basel, Switzerland.

Ômura, H., Kuwahara, Y. and Tanabe, T. 2002. 1-Octen-3-ol together with geosmin: new secretion compounds from a polydesmid millipede, *Niponia nodulosa*. Journal of Chemical Ecology, 28: 2601–2612.

Peschke, K. and Eisner, T. 1987. Defensive secretion of the tenebrionid beetle, *Blaps mucronata*: Physical and chemical determinants of effectiveness. Journal of Comparative Physiology, 161: 377–388.

Pimvichai, P., Enghoff, H. and Panha, S. 2010. The Rhynchoproctinae, a south-east Asiatic subfamily of giant millipedes: cladistic analysis, classification, four new genera and a deviating new species from north-west Thailand (Diplopoda: Spirostreptida: Harpagophoridae). Invertebrate Systematics, 24: 51–80.

Pimvichai, P., Enghoff, H. and Panha, S. 2014. Molecular phylogeny of the *Thyropygus allevatus* group of giant millipedes and some closely related groups. Molecular Phylogenetics and Evolution, 71: 170–183.

Pimvichai, P., Enghoff, H. and Panha, S. 2016. A revision of the *Thyropygus allevatus* group. Part 5: the *T. allevatus* subgroup (Diplopoda: Spirostreptida: Harpagophoridae). European Journal of Taxonomy, 199: 1–37.

Rodriguez, J., Jones, T.H., Sierwald, P., Marek, P.E., Shear, W.A., Brewer, M.S., Kocot, K.M. and Bond, J.E. 2018. Step-wise evolution of complex chemical defenses in millipedes: a phylogenomic approach. Scientific Reports, 8: 3209.

Shahidi, F. and Yeo, J. 2018. Bioactivities of phenolics by focusing on suppression of chronic diseases: A review. International Journal of Molecular Sciences, 19: 1573.

Shear, W.A. 2015. The chemical defenses of millipedes (Diplopoda): biochemistry, physiology and ecology. Biochemical Systematics and Ecology, 61: 78–117.

Shimizu, N., Kuwahara, Y., Yakumaru, R. and Tanabe, T. 2012. n-Hexyl laurate and fourteen related fatty acid esters: new secretory compounds from the julid millipede, *Anaulaciulus* sp. Journal of Chemical Ecology, 38: 23–28.

Stanković, S., Dimkić, I., Vujisić, L., Pavković-Lučić, S., Jovanović, Z., Stević, T., T, Sofrenić, I., Mitić, B. and Tomić, V. 2016. Chemical defence in a millipede: Evaluation and characterization of antimicrobial activity of the defensive secretion from *Pachyiulus hungaricus* (Karsch, 1881) (Diplopoda, Julida, Julidae). PLoS One, 11(12).

Tomsone, L., Kruma, Z. and Galoburda, R. 2012. Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (*Armoracia rusticana*). International Scholarly and Scientific Research & Innovation, 6: 236–241.

Valderrama, X., Robinson, J.G., Attygalle, A.B. and Eisner, T. 2000. Seasonal anointment with millipedes in a wild primate: a chemical defense against insects? Journal of Chemical Ecology, 26: 2781–2790.

Vujisić, L.V., Makarov, S.E., Ćurčić, B.P.M., Ilić, B.S., Tešević, V.V., Godevac, D.M., Vučković, I.M., Ćurčić, S.B. and Mitić, B.M. 2011. Composition of the defensive secretion in three species of European millipedes. Journal of Chemical Ecology, 37: 1358–1364.

Vujisić, L.V., Antić, D.Ž., Vučković, I.M., Sekulić, T.L., Tomić, V.T., Mandić, B.M., Tešević, V.V., Ćurčić, B.P.M., Vajs, V.E. and Makarov, S.E. 2014. Chemical defense in millipedes (Myriapoda, Diplopoda): do representatives of the family Blaniulidae belong to the 'quinone' clade? Chemistry and Biodiversity, 11: 483–490.

Weldon, P.J., Aldrich, J.R., Klun, J.A., Oliver, J.E. and Debboun, M. 2003. Benzoquinones from millipedes deter mosquitoes and elicit self-anointing in capuchin monkeys (*Cebus* spp.). Naturwissenschaften, 90: 301–304.