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Diplodomica II. Repugnatorial Secretion of Ecuadorian Endemic Millipede *Rhinocricus* sp. (Diplopoda, Spirobolida, Rhinocricidae) from Orellana Province, Amazonia, Ecuador †

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Abstract: Millipedes (Myriapoda: Diplopoda) represents one of the oldest and most evolutionary successful classes of invertebrates. The order Spirobolida is dominantly tropical, and the family Rhinocricidae is widely distributed in the Ecuadorian edaphic fauna inhabiting the Amazonia region. The millipedes collected were stimulated mechanically, and an ejected repugnatorial fluid was subjected to structural analysis. The analysis of the defensive–repugnatorial secretion of the endemic millipede *Rhinocricus* sp., inhabiting in the Amazonian region of Orellana, Republic of Ecuador, and chemical evidence (chromogenic reactions: KI starch paper and rodanine test in aqueous ammonia) as well, revealed the quinonoide composition of the secretion. We identified 2-methyl-3-methoxy-1,4-benzoquinone as an active major component.

Keywords: millipede; *Rhinocricus*; Ecuador; defensive secretion; quinonoids; benzoquinones

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1. Introduction

Comprising probably about 80,000 species, of which only 10% have been described, millipedes (Myriapoda: Diplopoda) represent one of the oldest, largest, and evolutivelly successful classes of the Animal Kingdom and invertebrates [1,2]. The group contains forest floor-dwellers, stratobions, few cavernicoles, edaphobionts, and epiphytobionts [3]. Within their biotopes, millipedes function ecologically as detritivores and play major roles in the reduction of plant litter and formation of organic soil. The order Spirobolida is dominantly tropical in its distribution and diversification and the family Rhinocricidae is widely distributed in the Ecuadorian edaphic fauna inhabiting in the Amazonia region [4]. The defensive allomones substances and volatile mixtures of about 80 species of millipedes have been analyzed, and about 40 substances of different chemical nature as, e.g., hydrocyanic acid, esters, acids, aromatic and aliphatic aldehydes, substituted phenols and their derivatives, polysubstituted 1,4-benzoquinones, and benzoquinoids in general, quinazolines, spiro-pyrrolizidines and their oximes, aromatic nitriles, and even an alicyclic amino-nitro compound and monoterpenes have been isolated and identified [5–10]. The most frequently detected are 1,4-benzoquinones, substituted, generally, by one or two methyl, ethyl or methoxyl groups, and/or even their combination, and they have been found in several millipede orders, more often in those belonging to Juliformia. [5,11–13].

The repugnatorial glands, from which defensive noxious policomponent organic mixtures are ejected, are located, dorso-laterally, approximately at the dorsum on most

diplosegments (except for the first five anterior and the last three posterior ones). Anatomically, *Rhinocricus* sp., has glands similar to those found in many species of the orders Spirobolida, Spirostreptida, Julida, and Callipodia.

There are no reports detailing chemical ecology, biological activity and structural–compositional aspects of defensive secretions of Millipedes inhabiting in the Amazonian region of Orellana, Republic of Ecuador. The reported results constitute the first chemical analysis of repugnatorial ejections isolated and collected from individuals of Orellana endemic millipede gen. *Rhinocricus* sp. (Figure 1).

Figure 1. *Rhinocricus* sp. (Spirobolida, Rhinocricidae) from Palmar del Rio, Orellana, Ecuador.

2. Materials and Methods

All reagents used were supplied by MERCK, Darmstadt, Germany, and were used without prior purification.

2.1. IR Spectroscopy

The infrared spectra were recorded on a PHILIPS ANALYTICAL FTIR PU-9600 spectrophotometer, Germany; the samples were prepared in potassium bromide (KBr) tablets at 25 °C. Alternatively, the spectra were recorded in a JASCO-Canvas 4600, Japan system in CsBr tablets at 25 °C.

2.2. NMR Spectroscopy

NMR spectra were recorded on a BRUKER AC-250 instrument, Germany, at 25 °C. The protonic chemical (δ) shifts are given in ppm, using tetramethylsilane as internal reference (TMS, δ = 0.0) and as a solvent $CDCl_3$. The chemical shifts (δ) for 13C refer to the central peak of the $CDCl_3$ solvent at 77.03 ppm.

2.3. Gas Chromatography Coupled to Mass Spectrometry (GC-MS)

A Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) with 5973 quadrupole detection system (GC-MS) was used. The separations were carried out through a capillary column of Ultra 2 type (J & W Scientific, Folsom, CA, USA), 12 m long and 0.22 mm of internal diameter. As carrier gas, He was used at a flow of 1 mL/min. Temperature ramp: 60 °C with increments of 10 °C/min up to 300 °C (isothermal 5 min.). Run time 30 min. Injection volume 2 μL at a temperature of 280 °C, in split mode (1:10 ratio). The ionization source was IE at 70 eV operating at 230 °C. Acquisition mode: Full Scan. Range of m/e 40–700.

The following databases were used for structural characterization: Nist98 (National Institute of Standards and Technology, Gaithersburg, MD, USA), PMW_TOX2 (Wiley Library and Pfleger Maurer Weber (PMW), National Metrology Institute of Japan (NMIJ,

Tokyo, Japan), and National Institute of Advanced Industrial Science and Technology (AIST, Tokyo, Japan). In addition, the databases that report the chemical composition of the invertebrate defensive secretions (https://www.pherobase.com, accessed 16 October 2020) and their GC-MS (libraries for the rapid identification of metabolites in complex biological samples, Max-Planck Institute of Plant Molecular Physiology, Postdam, Germany) were considered. Using the reported sources and m/e data from the GC-MS, the most likely structures and their fragmentation mechanisms are postulated. Identification of the components was based on comparison of GC retention data and mass spectra. Each component was quantified using n-hexadecane as internal standard.

2.4. Biological Material

The millipede studied, *Rhinocricus* sp., (Rhinocricidae, Spirobolida), is one of the largest endemic species of this genus inhabiting in the Ecuadorian edaphic fauna of Amazonian macroinvertebrates and representing a largest group in biomass. Adult specimens reach over 150–170 mm with a diameter of about 10–16 mm and weight of 25–28 g. Adult individuals (males) of the millipede species gen. *Rhinocricus* sp. were collected, gently, by hand picking, in Palmar del Rio, Francisco de Orellana Province (0°19′ S latitude and 77°04′ W longitude, at altitude of 280 m) during the months of May–July 2019, from raked forest soil at the edge of the pluvial tropical evergreen forest, consisting mostly of *Heliconia* sp., *Cecropia* sp., *Clusia* sp., and *Ficus* sp., 175 Km East from Quito City, and maintained in laboratory conditions for 20 days by feeding on litter at 25 °C under 16L:8D photoperiod and reared in plastic boxes (*terrariums* 30 × 15 × 15 cm), which were kept humid by spraying with water every other day. Voucher specimen RA-002 was conserved in the Laboratory of Ecology at Centre of Biology, Central University of Ecuador (Prof. Franklin Gavilánez), and several individuals (5) were kept in the Organic Chemistry Laboratory of the Faculty of Science & Technology at Technical University of Esmeraldas, Ecuador (voucher 004-007).

Millipedes gen. *Rhinocricus* respond to disturbance by coiling up, and they produce, after mechanical irritation or predator disturbance, a considerable quantity of defensive noxious secretion that could be ejected to a distance of 20–25 cm.

After mechanical stimulation with a pincette, 850 μg of a deep brown-red secretion with intense *phenolic* odor was released from lateral ozopores and obtained by means of glass capillary from the individual studied. The secretion (650–800 μg/individual) is absorbed on Whatman 40 filter paper and cooled to −10 °C. The filter paper is extracted with diethyl ether (5 × 2 mL), and the ether extracts are concentrated with N_2 flow (g) to dryness, stored at −10 °C in 1 mL Eppendorf vials, and sealed with parafilm for further analysis. To each vial, with the dry extract, dichloromethane (1 mL) is added, the mixture is stirred, filtered through 0.45 μm frit, and injected into the GC-MS (2–20 μL). The ability of the secretion to react strongly with KI starch paper suggested the presence of quinones. This was corroborated by spot analysis using a positive test for a p-quinone with rhodamine and aqueous ammonia (Figure 2).

Figure 2. Droplets of defensive repugnatorial secretion ejected from ozopores after mechanical stimulation with a pincette and prior its collecting by means of glass capillary.

3. Results and Discussion

The molecular and basic peaks of the mass spectrum is formed by the ions of a substance m/z **M⁺ 152** (corresponding to **$C_8H_8O_3^+$**) from which the fragmentation pattern shows two paths: the first one is an elimination of the neutral molecule **CH_2O** (from methoxyl), generating the most intensive peak of the spectrum of m/z 122 (**$C_7H_6O_2^+$** Rel.int.73%) and then successively two CO molecules peaks m/z 94, (**$C_6H_6O^+$**) and m/z 66 (**$C_5H_6^+$**). In the second path, first, **C_2H_3O** will be eliminated whereby ions m/z 109 (**$C_5H_3O_2^+$**) arise, and then, again, we observe the elimination of two neutral molecules CO i.e., m/z 81 (**$C_5H_3O^+$**) and m/z 51. In addition to the described fragmentations, the elimination of methyl and water from molecular ion occurs in parallel, the last ion **$(M\text{-}H_2O)^+$** forming the peak of a relative intensity of only 2%. It can be judged from the described fragmentation patterns (and from the composition of the individual ions) that the analyzed compound is a typical substituted benzoquinone by one CH3- group and one OCH3- group.

The NMR spectra ($CDCl_3$ ^{1}H-250 MHz and ^{13}C-100 MHz) shown: for ^{1}H NMR, δ, ppm: 6.72–6.67 [d, 1H], 6.62–6.57 [d,1H], 4.03 [s, 3H], 1.95 [s, 3H]; for ^{13}C NMR, δ,ppm: 188, 34 (C-1); 129, 40 (C-2); 156, 15 (C-3); 175,52 (C-4); 136,45 (C-5); 137.85 (C-6); 11.22 (C-7); 59.48 (C-8).

The FTIR (Fourier Transform Infrared Spectroscopy) spectrum shows the valence vibrations of the C=O bond at 1645 and 1662 cm^{-1}; C=C at 1590 cm^{-1}; C-H (-OCH_3) at 2840 cm^{-1}; (-CH3) at 2940 cm^{-1}. When comparing the spectrum of three isomers of methyl-methoxy-1,4-benzoquinones from the collection of spectra, it was explicitly proved that the substance, isolated as a major component, is 2-methyl-3-methoxy-1,4- benzoquinone (Figure 3).

The biological effects of this substance were evaluated experimentally, together with several further substances isolated from the defensive secretions of the millipedes, on forest ants as chief enemies of the millipedes in natural conditions [5,14] and some pathogenic fungus and bacteria [15]. The results of the preliminary experiments showed a quick repellent effect vs. ants gen. *Paraponera* and *Atta* and a microbicide action vs. *Pseudomonas* and *Staphylococcus* [16,17]. The secretions also irritate the more sensitive parts of epidermis, vertebrate skin and human mucoses, and especially the cornea of the eyes.

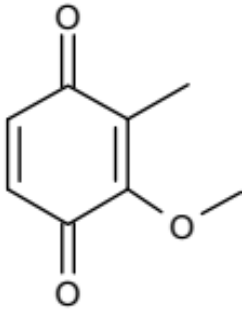

Figure 3. Major component, isolated from defensive repugnatrial secretion of *Rhinocricus* sp. inhabiting in Orellana, an Amazonian region from Ecuador: 2-methyl-3-methoxy-1,4- benzoquinone.

2-methyl-3-methoxy-1,4-benzoquinone is the most prevalent active substance in the defensive secretions of millipedes, and it has been found in about 30 species of the subclass Helminthomrpha. It is not present in only the orders Callipodia, Polyzoniida, and Polydesmida, as they have a different type of gland. Its occurrence in the orders Spirobolida, Spirostreptida, and Julida appears very often.

4. Conclusions

The analysis (FTIR-NMR-GC/MS) of the defensive–repugnatorial secretion of the endemic millipede *Rhinocricus* sp., inhabiting in the Amazonian region of Orellana, Republic of Ecuador, and chemical evidence (chromogenic reactions: KI starch paper and rodanine test in aqueous ammonia) as well, revealed the quinonoide composition of the secretion. We identified 2-methyl-3-methoxy-1,4-benzoquinone as an active major component. This is the first report related to analysis of chemical composition of repugnatorial secretion of Ecuadorian Rhinocricidae from the Amazonian region. The results concerning biological action (repellent and microbiocidal effects) will be reported soon.

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Conflicts of Interest: The authors declare no conflict of interest.

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