medical sciences forum

MDPI

Proceeding Paper

Antimicrobial Activity of Defensive Secretion of Terrestrial Invertebrates (Diplopoda, Spirobolida, *Rhinocricus*) from the Insular Neotropics †

Juan Enrique Tacoronte Morales [1,*], Joseph Cruel Sigüenza [1], María Elizabeth Canchingre Bone [1], Carla Bernal Villavicencio [1] and María Teresa Cabrera Pedroso [2,*]

[1] Chemical Engineering Coordination and Multidisciplinary Research Group-GIM-FACI, Faculty of Science & Technology, Campus New Horizons, Technical University of Esmeraldas, Esmeraldas PC 08102, Ecuador; joseph.cruel@utelvt.edu.ec (J.C.S.); elizabeth.canchingre@utelvt.edu.ec (M.E.C.B.); elizabeth.canchingre@utelvt.edu.ec (C.B.V.)

[2] Faculty of Health Sciences, UDLA, University of Americas, Udla-Park, Quito CP 170124, Ecuador

* Correspondence: juan.tacaronte.morales@utelvt.edu.ece or juan.tacaronte.morales@utelvt.edu.ec (J.E.T.M.); maria.cabrera@udla.edu.ec (M.T.C.P.); Tel.: +593-9-9170-2915 (J.E.T.M.); +593-9-8434-3740 (M.T.C.P.)

† Presented at the 2nd International Electronic Conference on Antibiotics—Drugs for Superbugs: Antibiotic Discovery, Modes of Action And Mechanisms of Resistance, 15–30 June 2022; Available online: https://eca2022.sciforum.net/.

Abstract: In the last 30 years, a significant increase in resistance of pathogenic microorganisms to conventional therapeutic strategies has been observed and it constitutes a drive force for the search of, in plants and microorganisms, more effective bioactive molecular systems. In this context, terrestrial invertebrates associated with mega edapho-fauna has not been considered as a source of poly-component systems or molecular entities with potential antimicrobial action. The defensive secretions of Diplopods, given the presence of benzenoids and monoterpenes, possess recognized antimicrobial activity. In the present communication we report the isolation of repugnatorial secretions from millipedes gen. *Rhinocricus* inhabiting in Cuban neotropical island conditions and the analysis, by GC/Ms, of their composition pattern (hydroxylated quinonoids) that show, under in vitro conditions, a significative antimicrobial activity against pathogenic microorganisms (*Candida albicans*, *E. coli* and *S. aureus*). These natural derivatives can be used, as bioactive components, in formulations for topical treatment of epidermal infections of microbial origin.

Keywords: Diplopoda; *Rhinocricus*; defensive secretions; antimicrobial activity

Citation: Tacoronte Morales, J.E.; Cruel Sigüenza, J.; Canchingre Bone M.E.; Bernal Villavicencio, C.; Cabrera Pedroso, M.T. Antimicrobial Activity of Defensive Secretion of Terrestrial Invertebrates (Diplopoda, Spirobolida, *Rhinocricus*) from the Insular Neotropics. *Med. Sci. Forum* **2022**, *2*, x. https://doi.org/10.3390/xxxxx

Academic Editor:

Published: date

1. Introduction

The extraordinary advances in metabolomics [1–4] and chemogenomics [5,6] of natural products and secondary metabolites, their structural characterization, modelling and derivatization, during the last decade, including novel structural elucidation techniques, have led to the development of a new approach to the study of natural products molecular biodiversity, their ecological significance, and potential applications in the field of applied microbiology for controlling human and animal pathogens.

The molecular potentiality of biodiversity of the (edaphic) fauna of terrestrial invertebrates of the Cuban neotropical archipelago has not been properly chemo-prospected in searching for new potential pharmacological entities (NPE) and antimicrobial agents as well. There are just few reports detailing biological (antimicrobial) activity and structural-compositional patterns of defensive secretions of Millipedes (Diplopoda) inhabiting in different eco-geographical regions of Cuban tropical archipelago [7–11]. The reported results detail the microbiocidal action vs. microbial pathogens of defensive secretions

(repugnatorial ejections) studied and isolated from some populations of endemic millipede gen. *Rhinocricus* sp., inhabiting in Cuban neotropical archipelago.

2. Materials and Methods

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

2.1. FTIR 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.

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 CDCl3. The chemical shifts (δ) for 13C refer to the central peak of the CDCl3 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, USA), PMW_TOX2 (Wiley Library and Pfleger Maurer Weber (PMW), National Metrology Institute of Japan (NMIJ); and National Institute of Advanced Industrial Science and Technology (AIST).

2.4. Biological Material

Adult individuals (males) of the millipede species gen. *Rhinocricus* sp. (Diplopoda, order Spirobolida, fam. Rhinocricidae) were collected in eco-geographical region of Banao Forest (geographical coordinates: −79.5686 W; 21.8286 N, Sancti Spíritus), during the months of May–July 2019. The specimens were kept in the GIM Laboratory of the Faculty of Science & Technology at Technical University of Esmeraldas, Ecuador (voucher 001–003). In the laboratory, millipedes were kept in plastic boxes (24 cm × 18 cm) filled with soil and litter from the collecting site or prepared one. The boxes were regularly sprayed with water to maintain high humidity.

2.5. Collection of Repugnatorial Secretion

The defense secretions were collected by gently pressing the producing glands located in the middle of dorso-lateral region of the animals. The ejected liquid was collected with the aid of a micropipette (0.5–10 μL) and, conditioned. After determining the collected volume, the fluid was transferred to Eppendorf tubes containing 50 μL of methanol, concentrated with N_2 flow (g) to dryness, and stored at −10 °C. To each Eppendorf vial, with the dry extract, dichloromethane (1 mL) is added, filtered through 0.45 μm frit, and injected into the GC-MS (2–20 μL).

2.6. Determination of Percentage of Growth Inhibition of Microorganisms

The percentage of growth inhibition of microorganisms was determined by reading the absorbance in a spectrophotometer for microplates SLT Spectra (Tecan SLT Spectra Shell B 039.053 Microplate Reader/Spectrophotometer, Madison, WI, USA) at 630 nm

2.7. Microbiocidal Activity

For each assay, the microorganisms were previously incubated in inclined tubes with Tryptose Soya Agar (TSA, Difco) for bacteria at 37 °C and Sabouraud Dextrose Agar (SDA, Difco) for fungi at 25 °C for 24 h. The cultures obtained from the tubes were re-suspended in 0.85% saline solution. The microbial suspensions obtained were serially diluted with saline solution and inoculated in TSA and SDA medium to determine the concentration of microorganisms. The microbial suspension of the appropriate concentration was then diluted in TSB (Tryptose Soya Broth) and SDB (Sabouraud Dextrose Broth) liquid culture medium so that the final concentration of microorganisms in each well of the microplate had a number of 200 to 400 CFU/200 µL. The antimicrobial activity was determined through serial dilution of the repellent liquids in DMSO/MeOH (1:1 *v*/*v*), being inoculated 10 µL of these dilutions in 190 µL of the respective microbial suspensions, the positive controls used were chloramphenicol and amikacin for bacteria and nystatin for the fungus, all at a concentration of 1 mg/mL and as negative control were used 10 µL of the dilution vehicle of the defensive secretion. After distribution of the respective samples and microorganisms, the microplates were incubated in an incubator for 48 h at 25 °C for C. albicans and 24 h at 35 °C for the other microorganisms. After the incubation period, the percentage of growth inhibition of microorganisms was determined by reading the absorbance in a spectrophotometer for microplates (SLT Spectra) at 630 nm. The lowest concentration of the tested sample capable of inhibiting microbial growth was considered the Minimum Inhibitory Concentration (MIC) [12,13]. After determining the MIC, the content of the wells, where no growth was observed, was plated in TSA culture medium for bacteria and SDA for fungus and subsequently incubated at the appropriate temperature and time for the respective microorganisms to obtain the Minimum Bactericidal Concentration (MBC) [14,15] and the Minimum Fungicidal Concentration (MFC) [16] of the tested samples. The MBC and MFC were considered as the lowest concentration of the sample capable of reducing the number of viable bacteria or fungi by 99.9% [17]. The overview of the process is represented, schematically, in Figure 1.

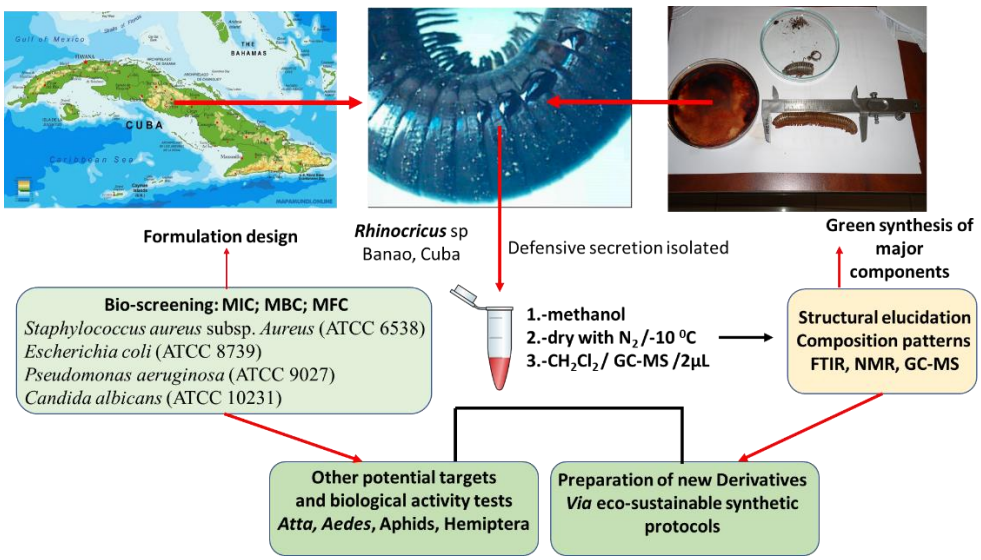

Figure 1. General process of molecular characterization and determination of biological activity of the defensive secretion of *Rhinocricus sp.* from Banao Forest, Cuba.

3. Results

The analysis of the chemical composition of defensive secretions of *Rhinocricus* sp., collected in situ in the Banao Forest, Cuba, by thin layer chromatography (chromogenic reactions on SiO_2 plates doped with oxalic acid and silver nitrate [18]), revealed that this repugnatorial fluid is a polycomponent mixtures. The uses of specific reagents suggest the presence of quinonoid metabolites (phenols and benzoquinones) with a certain degree of substitution [19].

The compositional-structural analysis, by GC-MS and FTIR, of the millipede defensive secretions of the gen. *Rhinocricus* sp. that inhabits the Banao Forest in the central ecogeographical zone of the Cuban archipelago, revealed a great heterogeneity both in composition and in structural variations and molecular patterns. The results of the TLC-GC/MS analysis (Retention Time (Rt, min.) and %) in the defensive secretion are detailed in Figure 2.

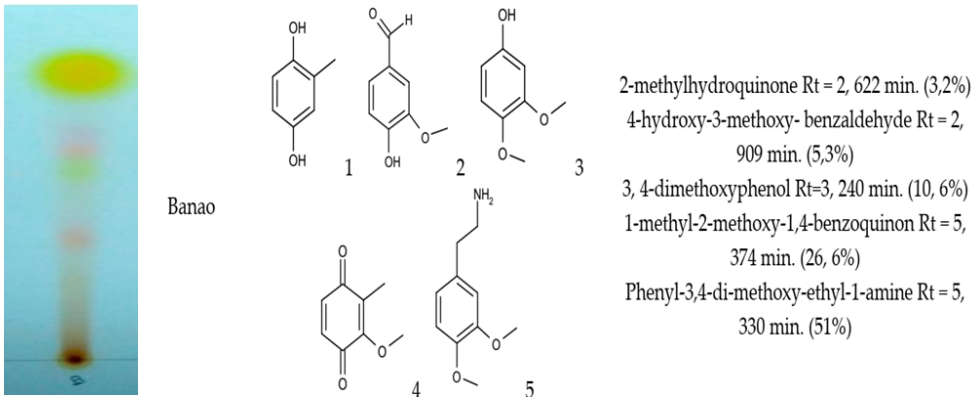

Figure 2. TLC & composition of the defensive secretion of *Rhinocricus sp.* from Banao Forest, Cuba.

Determination of the Antimicrobial Activity of defensive secretion of *Rhinocricus* sp. from Banao Forest.

All the test microorganisms showed susceptibility to the repugnatorial fluid of *Rhinocricus* sp. millipede. The bacterium *P. aeruginosa* showed the highest resistance to the toxic effects of these secretions. The results are illustrated in Tables 1 and 2.

Table 1. Antibacterial activity of defensive secretion of *Rhinocricus* sp. from Banao Forest, Cuba.

taxa	*S. aureus* (nL/mL)		*E. coli* (nL/mL)		*P. aeruginosa* (nL/mL)	
Rhinocricus sp.	**MIC***	**MBC***	**MIC**	**MBC**	**MIC**	**MBC**
(Diplopoda, Spirobolida) Banao Forest (-79.5686 W; 21.8286 N) Cuban archipelago	**< 135,5**	**>300**	**< 40.5**	**>70,0**	**<45,0**	**n/a**

* MIC: Minimum Inhibitory Concentration; * MBC: Minimum Bactericidal Concentration.

Table 2. Antifungal activity of defensive secretion of *Rhinocricus* sp. from Banao Forest, Cuba.

Taxa	***C. albicans* (nL/mL)**	
Rhinocricus sp. (Diplopoda, Spirobolida) Banao Forest (−79.5686 W; 21.8286 N) Cuban archipelago	MIC *	MFC *
	>4000.5	>4000.5

* MIC: Minimum Inhibitory Concentration; * MFC: Minimum Fungicidal Concentration.

4. Discussion

In arthropods, a significative amount of antibacterial and antifungal substances and compositions [20] have been described and characterized. They differ in regard to their mode of action, activity, tissue of formation and chemical composition. The search for antimicrobials from natural sources as important medical need especially for Gram-negative infections, has received much attention and efforts have been made to identify compounds that can act as suitable antimicrobial agents. The defensive secretion of *Rhinocricus* sp. individuals from Banao Forest in the Cuban neotropical archipelago [21] has a multicomponent molecular system of great heterogeneity (Figure 1, *vide supra*) where the molecular complexity ranges from derivatives of 1,4-benzoquinones and their respective hydroquinones [22] to aldehydes and an unexpected primary alky-aromatic amine. The presence of these structures was expected in species belonging to the families Spirobolidae and Julida, according to the phylogenetic pattern previously found for the chemical composition of defense secretions within Diplopoda [23,24].

The caustic and irritant properties of these odorous, and extremely volatile, compounds and their physical mixture, can cause to invertebrate predators to desist from attack and perform vigorous cleaning activities in the areas of contacting with this noxious poly-component mixture [25], as well as other physiological effects [26]. It should be noted that these described compounds, individually, have been detected and quantified in other species of millipedes, as well as in other families of arthropods, which supports, from the point of view of chemical evolution and functionality, their significance as volatile defensive compounds of rapid effect by contacting [27,28] in repelling spiders and species of ants.

Taking into consideration the ecology of millipedes [29,30] and their significance as detritivores for ecosystems [30], inhabiting in a hot and humid environment also exposes *Rhinocricus* sp. to attack by pathogenic microorganisms such as entomopathogenic bacteria and fungi. Previously, was described the growth inhibition capacity of *E. coli* for 1,4-benzoquinone and 2-methyl-1,4-benzoquinone (toluylquinone) [31], isolated from the repugnatorial secretions of several Arthropoda species.

The results presented in this study demonstrate that the complex poly-component mixture of benzoquinonoids (p-benzoquinones substituted and hydroquinones + and primary alkylaromatic amine) presents in the defensive secretion of *Rhinocricus* sp. possess a potent antimicrobial activity for both Gram positive and negative bacteria and fungi. In general, the secreted repugnatorial fluid from ozopores of *Rhinocricus* sp. showed a more pronounced toxicity against the Gram-positive bacterium *S. aureus*, which was completely eliminated with a concentration of the defensive secretion in the order of parts per million (ppm). In [32] is considered that most species with a quinonic-hydroquinone chemoprofile live in the soil and litter, where they are in direct contact with many pathogenic microorganisms and MIC values for benzoquinones are low and very effective at deterring microorganisms

On the other hand, fungus was less sensitive to the secretions. For these microorganisms, it was possible to observe growth inhibition in concentration ranges similar to those found for *S. aureus*, but the toxic effects were only observed at much higher concentrations. However, in order to confirm the possible role of defense against microorganisms it is necessary to test the activity of these substances against pathogenic bacteria and fungi of these species.

5. Conclusions

This study represents the first report devoted the antimicrobial activity of defensive secretions isolated from *Rhinocricus* sp., inhabiting in Banao Forest located in the central eco-region of the Cuban neotropical archipelago. In general, the tested extract showed antimicrobial activity against both type of bacteria. The defensive secretion also exhibited

an antifungal potential. The given extract contains antimicrobial components potentially useful as therapeutic agents in the pharmaceutical and agricultural industries.

Author Contributions: Conceptualization, J.E.T.M. and C.B.V.; methodology, M.T.C.P., J.E.T.M.; formal analysis, M.E.C.B.; investigation, J.E.T.M., M.E.C.B., C.B.V., M.T.C.P.; resources, J.C.S.; data curation, J.C.S., J.E.T.M.; writing—original draft preparation, M.T.C.P.; writing—review and editing, J.E.T.M., M.E.C.B.; supervision, C.B.V.; project administration, J.C.S.; funding acquisition, J.C.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable. The studies were developed without participation of humans or animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments:

Conflicts of Interest: The authors declare no conflict of interest. The Technical University of Esmeraldas, Ecuador had no role in the design, development and implementation, at laboratory scale of the study; nor in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Lindsay, K.; Montaser, R.; Kellerb, N.; Kelleher, N. Metabolomics and genomics in natural products research: Complementary tools for targeting new chemical entities. *Nat. Prod. Rep.* **2021**, *38*, 2041–2065. https://doi.org/10.1039/D1NP00036E.
2. Zhao, Q.; Zhang, J.L.; Li, F. Application of Metabolomics in the Study of Natural Products. *Nat. Prod. Bioprospect*. **2018**, *8*, 321–334. https://doi.org/10.1007/s13659-018-0175-9.
3. Floros, D.J.; Jensen, P.R.; Dorrestein, P.C.; Koyama, N. A metabolomics guided exploration of marine natural product chemical space. *Metabolomics* **2016**, *12*, 145. https://doi.org/10.1007/s11306-016-1087-5.
4. Doroghazi, J.R.; Albright, J.C.; Goering, A.W.; Ju, K.S.; Haines, R.R.; Tchalukov, K.A.; Labeda, D.P.; Kelleher, N.L.; Metcalf, W.W. A roadmap for natural product discovery based on large-scale genomics and metabolomics. *Nat. Chem. Biol.* **2014**, *10*, 963–968. https://doi.org/10.1038/nchembio.1659.
5. Brown, J.B. Practical Chemogenomic Modeling and Molecule Discovery Strategies Unveiled by Active Learning. *Syst. Med. Integr. Qual. Comput. Approaches* **2021**, *2*, 154–162. https://doi.org/10.1016/B978-0-12-801238-3.11533-8.
6. James, T.; Sardar, A.; Anighoro, A. Enhancing chemogenomics with predictive pharmacology. *J. Med. Chem.* **2020**, *63*, 12243–12255. https://doi.org/10.1021/acs.jmedchem.0c00445.
7. Tacoronte, J.; Tchervas, T.; Prieto, D.; Rodríguez, C.; Aspiazu, M.; Gonzalez, V. A natural benzoquinone isolated from defensive secretions of Cuban endemic millipede. *Rev. CNIC. Cienc. Quím. (Chem. Sci.)* **2005**, *36*, 115–116.
8. Tacoronte, J.; Cabrera Pedroso, M.T. Defensive secretions of terrestrial invertebrates. Applied Chemical Ecology. A molecular and Eco sustainable perspective of the Ecuadorian biodiversity. In *Un Espacio para la Ciencia*; Manglar: Quito, Ecuador, 2018; Volume 1, pp. 51–100.
9. Mesa, J.A.; Tacoronte, J.; Montes de Oca, R.; Tobellas, J.; Garrido, R. 3,4-dimethoxyphenol, in defensive secretions of Cuban endemic millipedes (Spirobolida, Rhinocricidae, *Rhinocricus*). A study case *Rhinocricus duvernoyi* Karch 1881, geographical population of La Palma. *Rev. CENIC Cienc. Quím. (Chem. Sci.)* **2009**, *40*, 32–35.
10. Morales, J.E.T.; Cabrera, M.T. Diplodomica II. Repugnatorial Secretion of Ecuadorian Endemic Millipede *Rhinocricus* sp. (Diplopoda, Spirobolida, Rhinocricidae) from Orellana Province, Amazonia, Ecuador. *Chem. Proc.* **2021**, *3*, 90–97. https://doi.org/10.3390/ecsoc-24-08435.
11. Morales, J.E.T.; Pedroso, M.T.C.; Diplodomica, I. Chemical Composition of Repugnatorial Secretions of Cuban Endemic Millipede gen. Rhinocricus sp. *Proceedings* **2019**, *41*, 73–79. https://doi.org/10.3390/ecsoc-23-06522.
12. Kowalska-Krochmal, B.; Dudek-Wicher, R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. *Pathogens* **2021**, *10*, 165. https://doi.org/10.3390/ pathogens10020165.
13. Binish, M.B.; Binu, P.; Gopikrishna, V.; Mohan, M. Potential of anaerobic bacteria in bioremediation of metal-contaminated marine and estuarine environment. In *Microbial Biodegradation and Bioremediation Techniques and Case Studies for Environmental Pollution*, 2nd ed.; Das, S.; Dash, H.R., Eds.; Elsevier Inc.: Amsterdam, The Netherlands, 2022; Volume 15, pp. 305–326, doi.org/10.1016/C2020-0-01871-2.
14. Santos, N.C.S.; Scodro, R.B.L.; Sampiron, E.G.; Ieque, A.L.; Carvalho, H.C.; Santos, T.D.S.; Ghiraldi Lopes, L.D.; Campanerut-Sá, P.; Siqueira, V.L.D.; Caleffi-Ferracioli, K.R.; Teixeira, J.J.V.; Cardoso, R.F. Minimum Bactericidal Concentration Techniques

in *Mycobacterium tuberculosis*: A Systematic Review. *Microb. Drug Resist*. **2020**, *26*, 752–765. https://doi.org/10.1089/mdr.2019.0191.

15. Rodríguez-Melcón, C.; Alonso-Calleja, C.; García-Fernández, C.; Carballo, J.; Capita, R. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for Twelve Antimicrobials (Biocides and Antibiotics) in Eight Strains of Listeria monocytogenes. *Biology* **2022**, *11*, 46. https://doi.org/10.3390/biology11010046.
16. Borman, A.; Fraser, M.; Palmer, M.; Szekely, A.; Houldsworth, M.; Patterson, Z.; Johnson, E. MIC Distributions and Evaluation of Fungicidal Activity for Amphotericin B, Itraconazole, Voriconazole, Posaconazole and Caspofungin and 20 Species of Pathogenic Filamentous Fungi Determined Using the CLSI Broth Microdilution Method. *J. Fungi* **2017**, *3*, 27. https://doi.org/10.3390/jof3020027.
17. Lenczewski, M.E.; McGavin, S.T.; VanDyke, K. Comparison of automated and traditional minimum inhibitory concentration procedures for microbiological cosmetic preservatives. *J. AOAC Int*. **1996,** 79, 1294–1299.
18. Yan, Y.; Wang, X.; Liu, Y.; Xiang, J.; Wang, X.; Zhang, H.; Yao, Y.; Liu, R.; Zou, X.; Huang, J.; Jin, Q. Combined urea-thin layer chromatography and silver nitrate-thin layer chromatography for micro separation and determination of hard-to-detect branched chain fatty acids in natural lipids. *J. Chromatogr. A* **2015**, 1425, 293–301. https://doi.org/10.1016/j.chroma.2015.11.011.
19. Harrison, V.J.; Weatherston, J. Thin-layer chromatography of simple naturally occurring benzoquinones. *J. Chromatogr. A* **1967**, *31*, 258–259. https://doi.org/10.1016/s0021-9673(01)86062-3.
20. Mudalungu, C.M.; Tanga, C.M.; Kelemu, S.; Torto, B. An Overview of Antimicrobial Compounds from African Edible Insects and Their Associated Microbiota. *Antibiotics* **2021**, *10*, 621–644. https://doi.org/10.3390/antibiotics10060621.
21. Gibb, T. Insect Identification Techniques. *Contemp. Insect Diagn.* **2015**, *4*, 67–151. https://doi.org/10.1016/b978-0-12-404623-8.00004-1.
22. Bodner, M.; Vagalinski, B.; Makarov, S.E.; Antić, D.Ž.; Vujisić, L.V.; Leis, H.J.; Raspotnig, G. "Quinone Millipedes" Reconsidered: Evidence for a Mosaic-Like Taxonomic Distribution of Phenol-Based Secretions across the Julidae. *J. Chem. Ecol.* **2016**, *42*, 249–258. https://doi.org/10.1007/s10886-016-0680-4.
23. Dettner, K. Chemical Defense and Toxins of Lower Terrestrial and Freshwater Animals. *Compr. Nat. Prod. II* **2010**, 387–410. https://doi.org/10.1016/b978-008045382-8.00100-3.
24. Bojan, I.; Unković, N.; Ćirić, A.; Glamočlija, J.; Ljaljević, M.; Raspotnig, G.; Bodner, M.; Vukojević, J.; Makarov, S. Phenol-based millipede defence: Antimicrobial activity of secretions from the Balkan endemic millipede *Apfelbeckia insculpta* (L. Koch, 1867) (Diplopoda: Callipodida). *Sci. Nat.* **2019**, *106*, 37. https://doi.org/10.1007/s00114-019-1631-z.
25. Shear, W.A. The chemical defenses of millipedes (diplopoda): Biochemistry, physiology and ecology. *Biochem. Syst. Ecol.* **2015**, *61*, 78–117. https://doi.org/10.1016/j.bse.2015.04.033.
26. Taira, J.; Tamashiro, M.; Naka, K.; Gakiya, S.; Taira, K. Initial defensive secretory compounds emitted from the live millipede and the induction of apoptotic cell death. *Sci. Rep.* **2021**, *11*, 8222. https://doi.org/10.1038/s41598-021-87390-w.
27. Stanković, S.; Dimkić, I.; Vujisić, L.; Pavković-Lučić, S.; Jovanović, Z.; Stević, T.; Sofrenić, I.; Mitić, B.; Tomić, V. 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* **2016**, *11*, e0167249. https://doi.org/10.1371/journal.pone.0167249.
28. Eisner, T.; Rossini, C.; Gonzalez, A.; Eisner, M. Chemical defense of an opilionid (*Acanthopachylus aculeatus*). *J. Exp. Biol*. **2004**, *207*, 1313–1321. https://doi.org/10.1242/jeb.00849.
29. David, J.-F. Ecology of millipedes (Diplopoda) in the context of global change. *Soil Org.* **2009**, *81*, 719–733.
30. Alagesan, P. Millipedes: Diversity, Distribution and Ecology. In *Arthropod Diversity and Conservation in the Tropics and Sub-Tropics*; Chakravarthy, A., Sridhara, S., Eds.; Springer: Singapore, 2016; pp. 119–137. https://doi.org/10.1007/978-981-10-1518-2_7.
31. Shrestha, J.P. Synthesis, Structure-Activity Relationship Study, and Mode of Action Study of 1,4-Naphthoquinone Based Anticancer and Antimicrobial Agents. Ph.D. Thesis, Utah State University, Logan, Utah, May 2016; p. 4925. Available online: https://digitalcommons.usu.edu/etd/4925 (accessed on).
32. Rocha, D.F.; Wouters, F.C.; Zampieri, D.S.; Brocksom, T.J.; Machado, G., Marsaioli, A.J. Harvestman phenols and benzoquinones: Characterization and biosynthetic pathway. *Molecules* **2013**, *18*, 11429–11451. https://doi.org/10.3390/molecules180911429.