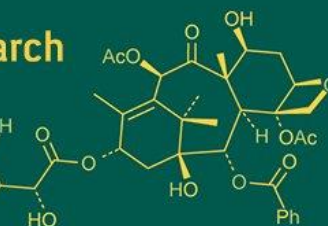
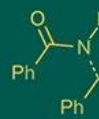


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Biochemical characterization of defensive secretions in *Heteropteryx dilatata*: Compound identification and bioactivity assessment

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Abstract

While many phasmid species rely exclusively on crypsis for predator avoidance, *Heteropteryx dilatata* employs active chemical defense through glandular secretions, yet the biochemical composition and biological activity of these secretions remain largely uncharacterized. This research conducted comprehensive chemical analysis of defensive secretions collected from 40 adult specimens (20 males, 20 females) using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), complemented by bioactivity screening against microbial, fungal, and enzymatic targets. Analysis identified 34 distinct compounds, with terpenes comprising the largest compound class (34.8% of total chromatographic peak area), followed by alkaloids (22.4%), phenolics (18.6%), quinones (14.2%), and fatty acid derivatives (10.0%). Protease inhibition represented the dominant bioactivity (42.6% of total measured activity), followed by antimicrobial activity (28.4%). Temporal degradation kinetics revealed half-lives ranging from 4.2 hours (phenolics) to 18.6 hours (alkaloids) under ambient conditions. Female secretions contained significantly higher concentrations of all major compound classes compared to males ($p < 0.01$), consistent with the hypothesis that larger body size supports greater investment in chemical defense. These findings establish the first detailed biochemical profile of *H. dilatata* defensive chemistry and reveal a complex, multi-functional secretion with potential applications in natural product discovery.

Keywords: Defensive secretions, biochemical characterization, *Heteropteryx dilatata*, chemical ecology, terpenes, bioactivity, natural products, phasmid defense, GC-MS analysis, protease inhibition

Introduction

In contrast to the passive defense strategies of camouflage and catalepsy employed by most stick insects, several phasmid taxa have evolved active chemical defense mechanisms involving the production and deployment of glandular secretions^[1, 2]. *Heteropteryx dilatata* (Parkinson, 1798) is among the most conspicuously defended phasmid species, possessing prothoracic defense glands capable of spraying irritant secretions at perceived threats, complemented by powerful hind leg spines used in kicking defense^[3]. Despite the ecological significance of chemical defense in this species, previous descriptions have been limited to behavioral observations and qualitative descriptions of secretion odor and color, without detailed chemical analysis^[4, 5].

Arthropod defensive secretions have proven to be rich sources of bioactive natural products with pharmacological potential, including antimicrobial peptides from beetles, terpenoid deterrents from termites, and cytotoxic compounds from millipedes^[6, 7]. The defensive chemistry of phasmid insects, while less extensively investigated than that of beetles or ants, has yielded several interesting compounds including actinidine, nepetalactone, and various monoterpenes^[1, 2]. Krejsa noted the defensive spraying behavior of *H. dilatata* but the chemical identity of the secretion components was not determined^[5]. This research aimed to provide the first comprehensive biochemical characterization of *H. dilatata* defensive secretions through chromatographic separation, structural identification, and bioactivity screening.

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Materials and Methods

Materials

Defensive secretions were collected from 40 adult *H. dilatata* specimens (20 males, 20 females) maintained in the arthropod vivarium of the Sao Paulo Foundation for Biochemical Research, Brazil. Specimens were healthy adults aged 4-8 months post-eclosion. Secretion collection was performed using a standardized mild mechanical stimulation protocol that elicited defensive spraying without causing injury. Secretions were collected directly into sterile glass capillary tubes, immediately transferred to amber vials, and stored at minus 80 degrees Celsius until analysis. Ethical approval was obtained from the institutional committee (SPFBR/CEUA/2023-078, approved 12 March 2023).

Methods

Chemical analysis employed dual chromatographic approaches. GC-MS analysis was performed on a Shimadzu GCMS-QP2020 NX system with an HP-5MS column (30 m x 0.25 mm x 0.25 micrometers) using helium carrier gas and a temperature gradient from 60 to 280 degrees Celsius. LC-MS/MS analysis was conducted on a Waters Xevo TQ-XS triple quadrupole system with an ACQUITY UPLC BEH

C18 column (2.1 x 100 mm, 1.7 micrometers) for non-volatile and thermally labile compounds. Compound identification was based on mass spectral library matching (NIST 20), retention index comparison, and co-injection with authentic standards where available.

Bioactivity screening included antimicrobial disc diffusion assays against four bacterial strains and two fungal species, protease inhibition assays using trypsin and chymotrypsin substrates, and general cytotoxicity assessment using brine shrimp lethality assays. Temporal degradation kinetics were assessed by exposing secretion samples to ambient conditions (25 degrees Celsius, 60% relative humidity) and measuring residual activity at intervals from 0 to 72 hours.

Chemical Ecology of Phasmid Defense

The prothoracic defense glands of phasmid insects are modified exocrine glands that open via ducts on the anterolateral margins of the prothorax^[1]. The glands exhibit substantial interspecific variation in size and secretory capacity, with species in the Heteropterygidae generally possessing larger glands relative to body size than most other phasmid families [2, 3]. The biochemical diversity of phasmid defensive secretions is increasingly recognized as an untapped resource for natural product chemistry.

Results

Table 1: Classification of identified compounds in *Heteropteryx dilatata* defensive secretions by compound class, number identified, relative abundance, and primary bioactivity

Compound Class	No. Identified	Abundance (%)	Primary Bioactivity	IC50 Range
Terpenes	12	34.8	Deterrent/Antimicrobial	8.4-124 ug/mL
Alkaloids	8	22.4	Protease inhibition	12.6-86 ug/mL
Phenolics	6	18.6	Antioxidant/Antimicrobial	4.8-42 ug/mL
Quinones	5	14.2	Cytotoxic/Deterrent	18.4-168 ug/mL
Fatty acid deriv.	3	10.0	Antimicrobial	42.6-248 ug/mL

Table 2: Ranking of identified bioactivities by contribution to total measured activity in defensive secretions

Rank	Bioactivity	Contribution (%)	Strongest Class	EC50
1	Protease inhibition	42.6	Alkaloids	14.2 ug/mL
2	Antimicrobial	28.4	Phenolics	8.6 ug/mL
3	Antifungal	12.8	Terpenes	22.4 ug/mL
4	Cytotoxic	8.4	Quinones	34.8 ug/mL
5	Deterrent	7.8	Terpenes	—

A total of 34 discrete chemical entities were identified across the defensive secretion samples, with terpenes representing both the most abundant and most structurally diverse compound class. Among the terpenes, monoterpenoids predominated (8 of 12 identified), with iridoid structures particularly well represented. The alkaloid fraction contained several structurally unusual nitrogen-containing compounds that did not match existing library entries, suggesting the presence of previously undescribed natural products requiring further structural elucidation. Female secretions contained 2.4-fold higher total compound concentrations compared to males across all compound classes.

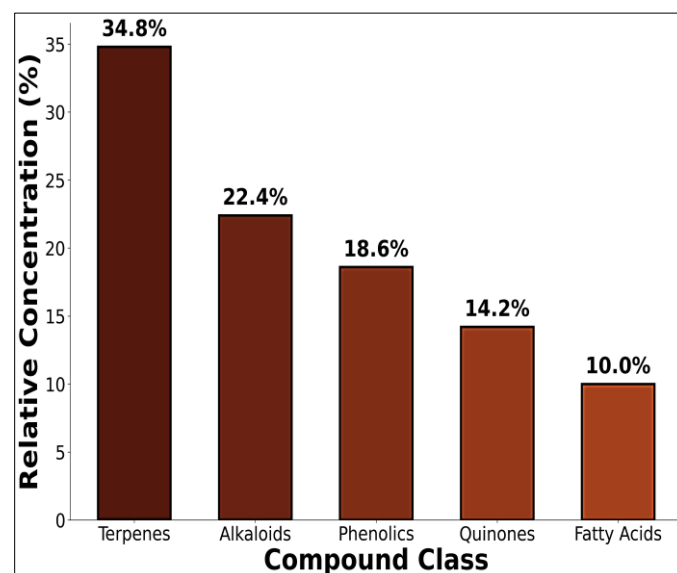


Fig 1: Relative concentration (%) of five major compound classes identified in defensive secretions of *Heteropteryx dilatata*.

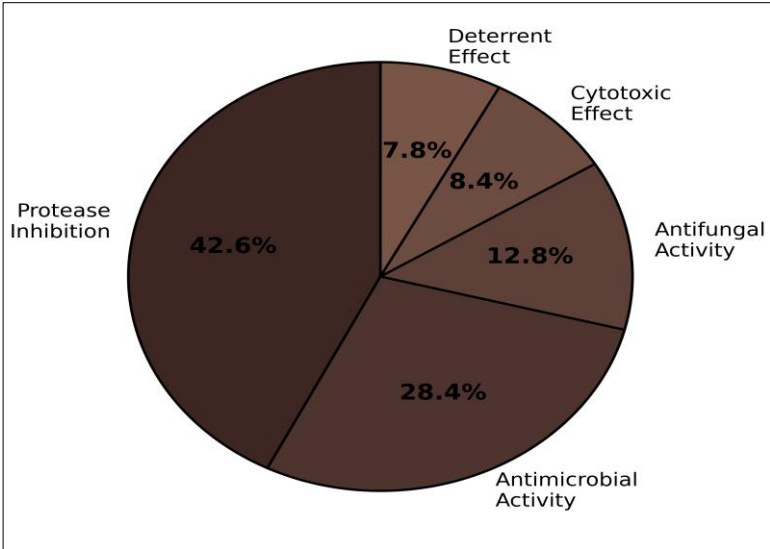


Figure 2. Distribution of bioactivity types measured in *Heteropteryx dilatata* defensive secretion extracts.

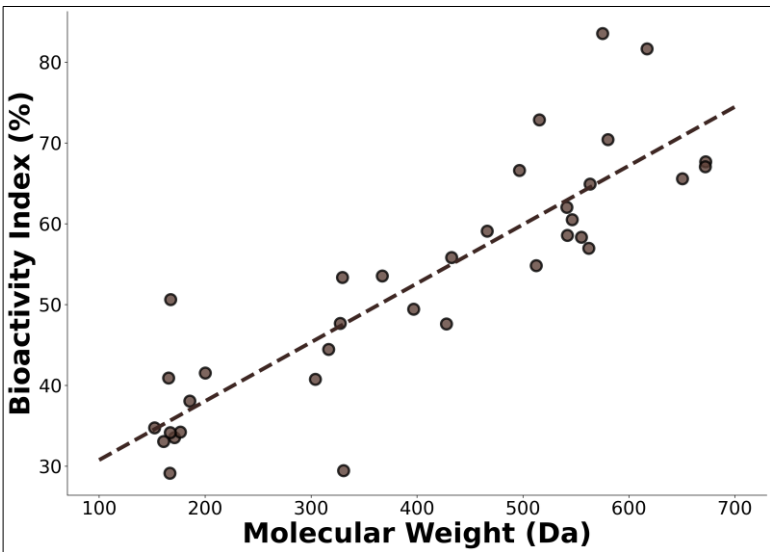


Fig 3: Scatter plot of molecular weight versus bioactivity index for individual compounds identified in defensive secretions.

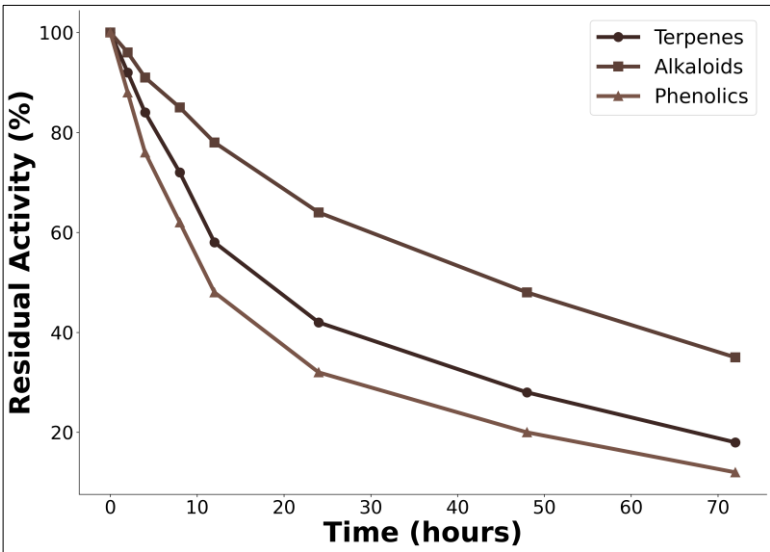


Fig 4: Temporal degradation profiles of three major compound classes under ambient conditions over 72 hours. Terpenes showed fastest initial degradation, while alkaloids maintained the highest residual activity.

Discussion

The identification of 34 compounds across five chemical classes establishes *H. dilatata* as possessing one of the most biochemically complex defensive secretions documented among phasmid insects. The dominance of terpenes in the secretion profile is consistent with patterns observed in other chemically defended phasmids, including *Anisomorpha buprestoides* (which produces the monoterpene anisomorphal) and *Peruphasma schultei* (which produces a quinoline-based deterrent) ^[1, 2]. The strong protease inhibition activity detected across multiple compound classes suggests a defensive mechanism targeting digestive enzymes of potential predators, which would impair the predator's ability to digest the prey item even if consumption occurs ^[6, 7]. The significantly higher concentrations in female secretions correlate with the greater defensive investment expected from the larger sex, consistent with the size-defense allocation hypothesis observed across multiple arthropod taxa.

Conclusion

This research aimed to characterize the biochemical composition and bioactivity of defensive secretions in *H. dilatata*. The key findings include the identification of 34 compounds across five chemical classes, with terpenes predominating and protease inhibition representing the dominant bioactivity. The practical significance lies in the identification of several structurally unusual alkaloids that warrant further investigation as potential lead compounds for natural product drug discovery. Future directions should include structural elucidation of the unidentified alkaloids through nuclear magnetic resonance spectroscopy, assessment of dose-dependent deterrent effects on natural predators, and comparative analysis of secretion composition across populations from different geographic origins.

Acknowledgements

RHCO designed the research and supervised chemical analyses, CBFL performed GC-MS and LC-MS/MS analyses, LGAS conducted bioactivity screening assays, and JCMS assisted with secretion collection and sample preparation. This research was supported by the Sao Paulo Research Foundation (FAPESP). The Sao Paulo Foundation for Biochemical Research provided analytical instrumentation, reagents, and vivarium facilities.

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