

COMPARATIVE TOXICOLOGICAL EVALUATION OF VERONICA AND SALIX EXTRACTS USING *DAPHNIA PULEX* AND *HETEROCYPRIS INCONGRUENS*

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Manuscript received: August 2026

Abstract

Botanical extracts are increasingly investigated as sources of bioactive compounds for pharmaceutical, nutraceutical, and cosmetic applications; however, their biological activity should be accompanied by an adequate safety profile. This study compared the acute toxicity of aqueous, hydroethanolic, and ethanolic extracts from three *Veronica* species (*V. prostrata*, *V. officinalis*, and *V. hederifolia*) and *Salix* × *fragilis* using two complementary freshwater invertebrate models, *Daphnia pulex* and *Heterocypris incongruens*. Acute toxicity was assessed after 24 and 48 h of exposure by determining median lethal concentration (LC₅₀) values. Aqueous extracts exhibited negligible toxicity in both models, whereas hydroethanolic and ethanolic extracts induced higher mortality. In *D. pulex*, the 96% ethanolic extract of *V. officinalis* showed the highest toxicity, whereas the hydroethanolic extract of *S. × fragilis* produced the most pronounced response in *H. incongruens*. *D. pulex* generally appeared to be more sensitive than *H. incongruens*, allowing clearer discrimination between extracts with different toxicological profiles. These findings show that extraction solvent influences the acute toxicity of botanical extracts and support using freshwater invertebrate models for preliminary toxicological evaluation of plant-derived preparations.

Rezumat

Extracțele vegetale sunt investigate din ce în ce mai frecvent ca surse de compuși bioactivi cu aplicații farmaceutice, nutraceu-tice și cosmetice, însă activitatea lor biologică trebuie însoțită de un profil de siguranță adecvat. Acest studiu a comparat toxicitatea acută a extractelor apoase, hidroetanolicе și etanolice obținute din trei specii de *Veronica* (*V. prostrata*, *V. officinalis* și *V. hederifolia*) și din *Salix* × *fragilis*, utilizând două modele complementare de nevertebrate acvatice, *Daphnia pulex* și *Heterocypris incongruens*. Toxicitatea acută a fost evaluată după 24 și 48 de ore de expunere prin determinarea concentrației letale medii (LC₅₀). Extracțele apoase au prezentat o toxicitate neglijabilă în ambele modele biologice, în timp ce extracțele hidroetanolicе și cele etanolice au indus, în general, o mortalitate mai mare. La *D. pulex*, extractul etanolic 96% de *V. officinalis* a prezentat cea mai mare toxicitate, în timp ce extractul hidroetanolic de *S. × fragilis* a produs cel mai pronunțat răspuns la *H. incongruens*. *D. pulex* a prezentat, în general, o sensibilitate mai mare decât *H. incongruens*, permițând o diferențiere mai clară între extracțele cu profile toxicologice diferite. Rezultatele demonstrează că solven-tul de extracție influențează toxicitatea acută a extracțelor vegetale și susțin utilizarea combinată a celor două modele de nevertebrate pentru evaluarea toxicologică preliminară a preparatelor vegetale.

Keywords: *Veronica* spp., *Salix* × *fragilis*, *Daphnia pulex*, *Heterocypris incongruens*, acute toxicity bioassay

Introduction

Natural products are increasingly recognised as valuable sources of biologically active compounds for pharmaceutical, nutraceutical and cosmetic applications (1–3). Plant-derived extracts contain a wide variety of secondary metabolites, including phenolic acids, flavonoids, tannins and terpenoids, which have been associated with antioxidant, anti-inflammatory, antimicrobial and photoprotective activities (4–6). Owing to these properties, botanical extracts are investigated as multifunctional ingredients for the development of

products intended to promote human health and protect against oxidative stress-related disorders (7–9). Although plant extracts' biological activities support their potential applications, efficacy alone is insufficient to justify practical use; an appropriate safety profile must also be established (10–13). Plant extracts are complex mixtures whose qualitative and quantitative phytochemical composition depends on several factors, including botanical species, geographical origin, harvesting conditions, and extraction solvent (4, 14–22). These variables can markedly influence not only biological efficacy but also toxicological properties,

making safety assessment an essential early step in natural product development (8, 15, 18, 19, 22).

The increasing demand for rapid and ethically acceptable safety assessment has encouraged the use of alternative biological models capable of providing useful preliminary toxicological data while reducing the use of vertebrate animals (23-25). Freshwater microcrustaceans are among the most widely employed alternative organisms for this purpose because they are inexpensive to maintain, highly sensitive to biologically active compounds, and suitable for standardised laboratory assays (23, 24). *Daphnia pulex* L., a planktonic cladoceran, is extensively used for acute toxicity testing and has become a well-established model for evaluating the biological effects of pharmaceuticals, environmental contaminants and natural products (26-29). *Heterocypris incongruens* R., a benthic freshwater ostracod, represents a complementary ecotoxicological model with distinct physiological and ecological characteristics that may provide additional information regarding the biological effects of complex plant extracts (30-33). The combined use of these two invertebrate species may improve the preliminary toxicological evaluation of plant-derived extracts by identifying differences in species-specific sensitivity (21, 34, 35).

Among medicinal plants investigated for their pharmacological potential, species belonging to the genera *Veronica* L. (*Plantaginaceae*) and *Salix* L. (*Salicaceae*) have attracted considerable scientific interest in recent years owing to their rich phytochemical composition (4, 36, 37). *Veronica* species contain iridoid glycosides, phenolic acids and flavonoids that have been associated with antioxidant, anti-inflammatory and antimicrobial activities (4, 38). Likewise, *Salix* species are recognised as important sources of phenolic compounds, flavonoids and salicylate derivatives responsible for their well-documented antioxidant and anti-inflammatory effects (39, 40).

Despite extensive research on the phytochemical composition and biological activities of *Veronica* and *Salix* extracts, data regarding their acute toxicity remain limited, particularly for extracts obtained using solvents of different polarity (4, 38, 39). Although the acute toxicity of a dry extract of *V. officinalis* has previously been investigated, few studies have comparatively evaluated different plant species and extraction solvents using complementary freshwater invertebrate models (41). Because solvent polarity substantially influences extract composition, such comparisons are necessary to identify potential differences in toxicological profiles.

This study compared the acute toxicity of aqueous, hydroethanolic, and ethanolic extracts from three *Veronica* species (*V. prostrata*, *V. officinalis*, and *V. hederifolia*) and *S. × fragilis* using two complementary freshwater invertebrate models, *D. pulex* and *H. incongruens*. Toxicity profiles were descriptively

compared across plant species, extraction solvents, and exposure durations (24 and 48 h) to identify differences in mortality responses and provide preliminary toxicological data on the investigated botanical extracts. Additionally, binomial generalised linear models were used to investigate the associations of extract concentration, extraction solvent, and botanical species with 48-h mortality in both test organisms. This statistical approach allowed a more comprehensive comparison of the toxicity profiles of the investigated extracts.

Materials and Methods

Plant extracts

This study investigated twelve lyophilised extracts from the aerial parts (herba) of *V. prostrata*, *V. officinalis*, and *V. hederifolia*, and from the female inflorescences (catkins) of *S. × fragilis*, collected during the flowering stage. For each species, three extracts prepared using different extraction solvents, namely water, 50% (v/v) ethanol, and 96% (v/v) ethanol, were evaluated, resulting in a total of twelve samples. Throughout the manuscript, the investigated extracts are referred to using the following abbreviations: VPw, VPhe and VPe (*V. prostrata* aqueous, hydroethanolic and ethanolic extracts, respectively); VOw, VOhe and VOe (*V. officinalis* extracts); VHw, VHhe and VHe (*V. hederifolia* extracts); and SFw, SFhe and SFe (*S. × fragilis* extracts).

Although willow bark is traditionally used for medicinal purposes, this study focused on the female inflorescences of *S. × fragilis* to provide additional insight into the toxicological profile of this less-investigated plant material.

The aerial parts (herba) of *V. prostrata*, *V. officinalis*, and *V. hederifolia* were collected during the flowering period, between April and May 2023, from spontaneous populations in Buzău County, Romania, at the following GPS coordinates: 45.458051° N, 27.004770° E (*V. prostrata*), 45.492050° N, 26.972924° E (*V. officinalis*), and 45.444874° N, 27.013277° E (*V. hederifolia*). The female inflorescences of *S. × fragilis* were also collected during the flowering period, between April and May 2023, from a spontaneous population in Buzău County, Romania (45.444929° N, 27.013372° E). Botanical identification and authentication of all four species were performed by Octavian Tudorel Olaru. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, under accession numbers 23-503 (*V. prostrata*), 23-504 (*V. officinalis*), 23-502 (*V. hederifolia*), and 23-501 (*S. × fragilis*). The plant materials were extracted by reflux, then filtered and concentrated under reduced pressure. The resulting extracts were subsequently lyophilised to obtain dry

preparations for toxicological evaluation. The extraction and lyophilisation procedures were performed as previously described (42). The present study focuses exclusively on the comparative evaluation of the acute toxicity of the obtained extracts using two alternative freshwater invertebrate models, rather than on the assessment of extraction efficiency.

Acute toxicity assay using Daphnia pulex

The acute toxicity of the investigated extracts was evaluated using *D. pulex*, a freshwater cladoceran commonly employed as an alternative model for preliminary toxicological screening. The assay was adapted from a previously described protocol (43).

D. pulex neonates (< 24 h old) were obtained from a parthenogenetic laboratory-renewed culture maintained from 2024 at the Department of Pharmaceutical Botany and Cell Biology, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania. The cultures were maintained in artificial freshwater (Elendt M7-modified medium) under controlled laboratory conditions at $25 \pm 1^\circ\text{C}$, with a photoperiod of 16 h light and 8 h darkness. Prior to testing, the organisms were acclimatised for 24 h in the test medium. Ten *D. pulex* specimens were transferred into each well of sterile 12-well culture plates, with the test solution adjusted to a final volume of 4 mL *per* well. A similar experimental setup has been previously reported in *Daphnia* toxicity studies (44).

Lyophilised plant extracts were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted with the culture medium to obtain six final concentrations ranging from 30 to 960 $\mu\text{g/mL}$ (30, 60, 120, 240, 480 and 960 $\mu\text{g/mL}$). A 1% DMSO solution served as the negative (solvent) control (45) with mortality remaining below 10%, in accordance with the validity criterion specified in OECD Test Guideline 202 (46). Each concentration was tested in duplicate. No visible precipitation was observed at the highest tested concentration (960 $\mu\text{g/mL}$).

Mortality was assessed after 24 h and 48 h of exposure. Organisms showing no movement for 30 seconds following gentle mechanical stimulation were considered non-viable. Mortality percentages recorded at each concentration were used to construct concentration-response curves and determine the LC_{50} .

Acute toxicity assay using Heterocypris incongruens

The acute toxicity of the investigated extracts was further assessed using *H. incongruens*, a freshwater ostracod increasingly used as an alternative ecotoxicological model because it is sensitive to a wide range of environmental contaminants. *H. incongruens* specimens were obtained from a continuously maintained laboratory culture established from Ostracodtookit F at the Department of Pharmaceutical Botany and Cell Biology, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania. Prior to testing, the organisms

were acclimatised to the experimental medium and subsequently used for acute toxicity assessment. To enable direct comparison of species-specific sensitivity, *H. incongruens* was tested under the same concentration range, exposure periods, and solvent conditions as *D. pulex*.

Ten *H. incongruens* specimens were introduced into each well of sterile 24-well culture plates, with the test solution adjusted to a final volume of 2 mL *per* well. The experiments were conducted under controlled laboratory conditions at $25 \pm 1^\circ\text{C}$, with a photoperiod of 16 h light and 8 h darkness. Lyophilised extracts were dissolved in DMSO and diluted in the exposure medium to obtain final concentrations between 30 and 960 $\mu\text{g/mL}$ (30, 60, 120, 240, 480 and 960 $\mu\text{g/mL}$). A 1% DMSO solution served as the negative (solvent) control, with mortality remaining below 10% (45). Each experimental condition was performed in duplicate. No visible precipitation was observed at the highest tested concentration (960 $\mu\text{g/mL}$). Mortality was recorded after 24 h and 48 h of exposure, and individuals showing no detectable movement for 30 seconds after gentle stimulation were considered non-viable. The obtained mortality data were subsequently used for LC_{50} calculation.

Statistical analysis

The results are expressed as the mean percentage mortality of duplicate determinations. Median lethal concentration (LC_{50}) values together with their 95% confidence intervals (95% CI) were estimated by nonlinear regression using GraphPad Prism version 5.1 (GraphPad Software Inc., San Diego, CA, USA). LC_{50} values were calculated separately after 24 and 48 h of exposure. For samples that did not reach the 50% mortality threshold at the maximum tested concentration, empirical determination of the LC_{50} was not possible. Consequently, LC_{50} values for these samples were theoretically estimated by extrapolation beyond the tested concentration range.

Hierarchical agglomerative cluster analysis was performed using IBM SPSS Statistics 26.0 software to identify groups of extracts with similar toxicity profiles. Each extract was characterised by 12 variables corresponding to the mean mortality values recorded at six concentrations after 24 and 48 h of exposure. The variables were not standardised prior to clustering, as all measurements were expressed on the same percentage scale (0 - 100%). Euclidean distance was used as the dissimilarity measure, and clusters were generated using the between-groups linkage method. The clustering results were visualised as dendrograms.

Additionally, binomial generalised linear models (GLMs) with a logistic link were fitted separately for *D. pulex* and *H. incongruens* using 48-h mortality data. Log_2 -transformed concentration, extraction solvent, and botanical species were included as predictors. Adjusted odds ratios (ORs) with 95% confidence intervals were calculated, and statistical

significance was assessed using HC3 robust Wald tests to account for overdispersion. Probit regression was performed as a sensitivity analysis. Exploratory comparisons between extracts were conducted using concentration-stratified permutation tests with Holm correction, while differences between organisms were assessed using the Wilcoxon signed-rank test. Statistical analyses were performed using Python (version 3.10.12) with the statsmodels package (version 0.14.0), with $p < 0.05$ considered statistically significant.

Results and Discussion

Acute toxicity in *Daphnia pulex*

Mortality responses in *D. pulex* varied by extract type, concentration, and exposure time. Table I presents the LC_{50} values determined after 24 and 48 h, while Figure 1 shows representative concentration–response curves.

None of the aqueous extracts reached the mortality threshold required to determine LC_{50} within the tested concentration range. The aqueous extracts of *V. officinalis*, *V. hederifolia*, and *S. × fragilis* did not induce sufficient mortality to determine LC_{50} after 24 or 48 h of exposure, indicating low acute toxic potential under the investigated conditions. Similarly, the aqueous extract of *V. prostrata* produced only limited mortality, reaching a maximum of 20% after 48 h at 480 $\mu\text{g/mL}$, which remained well below the threshold required for LC_{50} estimation.

A marked influence of the extraction solvent on acute toxicity was observed. Compared with the aqueous extracts, both hydroethanolic and ethanolic extracts generally induced higher mortality, particularly after 48 h of exposure. This trend was most evident for *V. officinalis*, whose 96% ethanolic extract showed the highest toxicity among all samples investigated, with LC_{50} values of 378.5 $\mu\text{g/mL}$ and 158.7 $\mu\text{g/mL}$ after 24 and 48 h, respectively. The

corresponding 50% hydroethanolic extract also showed considerable toxicity, although to a lesser extent, with LC_{50} values of 513.8 and 405.1 $\mu\text{g/mL}$ after 24 and 48 h, respectively.

Table I

Median lethal concentration (LC_{50}) values of the investigated *Veronica* spp. and *S. × fragilis* extracts against *D. pulex* after 24 h and 48 h of exposure

Extract	24 h		48 h	
	LC_{50}	95% CI	LC_{50}	95% CI
VPw	NC	NC	NC	NC
VPhe	NC	NC	NC	NC
VPe	NC	NC	381.9	340.5 - 428.4
VOw	NC	NC	NC	NC
VOhe	513.8	321.0 - 822.5	405.1	228.1 - 719.5
VOe	378.5	244.0 - 587.1	158.7	96.68 - 260.6
VHw	NC	NC	NC	NC
VHhe	NC	NC	1035*	NC
VHe	NC	NC	NC	NC
SFw	NC	NC	NC	NC
SFhe	NC	NC	478.6	282.0 - 812.3
SFe	NC	NC	NC	NC

NC = not calculated ($LC_{50} > 960 \mu\text{g/mL}$, the highest tested concentration; could not be reliably estimated from the observed concentration–response data).

* LC_{50} extrapolated above the highest tested concentration

The ethanolic extract of *V. prostrata* also showed a concentration- and time-dependent toxic response, reaching an LC_{50} of 381.9 $\mu\text{g/mL}$ after 48 h. In contrast, the corresponding hydroethanolic extract did not induce sufficient mortality to determine a reliable LC_{50} . In contrast, *V. hederifolia* exhibited a substantially lower acute toxic potential. Only the hydroethanolic extract produced mortality approaching the LC_{50} threshold after 48 h, yielding an extrapolated LC_{50} of 1035 $\mu\text{g/mL}$, which exceeded the highest tested concentration and suggests relatively low acute toxicity under the experimental conditions investigated.

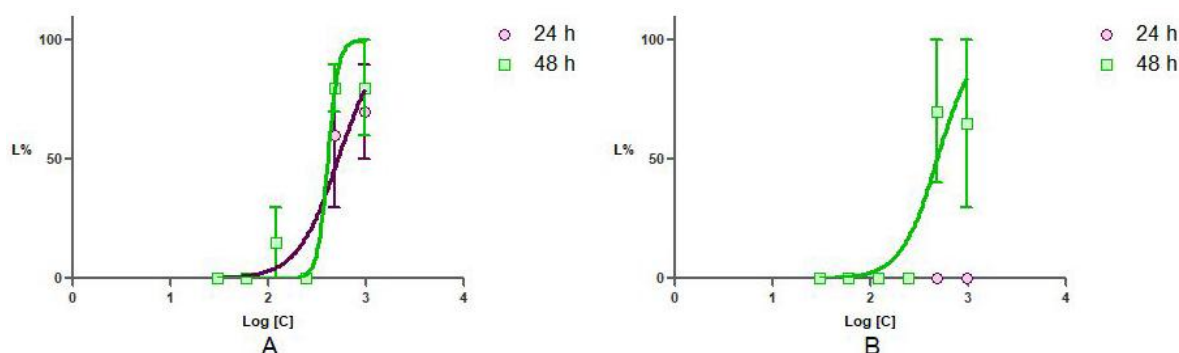


Figure 1.

Representative concentration–response curves of the hydroethanolic extract of *V. officinalis* (VOhe, A) and the hydroethanolic extract of *S. × fragilis* (SFhe, B) in *D. pulex* after 24 h and 48 h of exposure. Curves were generated by nonlinear regression using GraphPad Prism 5.1 for LC_{50} estimation. Error bars represent the standard error of the mean (SEM)

Among the *S. × fragilis* extracts, only the 50% hydroethanolic extract exhibited measurable acute toxicity, with an LC₅₀ value of 478.6 µg/mL after 48 h. Both the aqueous and ethanolic extracts produced negligible mortality throughout the experiment, preventing reliable LC₅₀ determination.

For nearly all active extracts, mortality increased between 24 and 48 h of exposure, indicating a clear time-dependent toxic response. The corresponding reduction in LC₅₀ values after prolonged exposure suggests an exposure-time-dependent biological response. Similar time-dependent responses have previously been reported for plant-derived extracts rich in phenolic compounds and other bioactive secondary metabolites, whose biological activity often becomes more pronounced following prolonged exposure (12, 15). Differences in acute toxicity among the investigated extracts may reflect variations in phytochemical composition due to the use of extraction solvents with different polarities. Such variations could contribute to the distinct toxicological profiles observed among the investigated preparations. Although phenolic compounds, flavonoids, and iridoid glycosides are commonly reported in *Veronica* species, while phenolic compounds and salicylate derivatives are characteristic of *Salix* species, the contribution of individual phytochemical constituents to the observed toxicological responses remains to be established (15).

The results demonstrate that *D. pulex* is a sensitive and suitable model for the preliminary assessment of the acute toxicity of plant extracts (26, 34, 47). According to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS), all estimated LC₅₀ values exceeded 100 mg/L, indicating low acute aquatic toxicity and falling outside the GHS acute aquatic hazard categories.

Acute toxicity in *H. incongruens*

The biological response of *H. incongruens* to the investigated extracts was subsequently analysed under the same exposure conditions. Except for VPe and SFhe, all extracts exhibited lethalities below 40%. The LC₅₀ values determined after 24 and 48 h are presented in Table II.

Compared with *D. pulex*, *H. incongruens* generally exhibited lower sensitivity to several of the investigated extracts. For VPe, an LC₅₀ value of 1075 µg/mL was estimated after 48 h, whereas no LC₅₀ value could be determined after 24 h. SFhe showed the most pronounced toxic response in *H. incongruens*, with LC₅₀ values of 1026 µg/mL after 24 h and 885.3 µg/mL after 48 h.

The sensitivity of *H. incongruens* varied depending on the investigated extract and exposure duration. Most extracts induced only limited mortality, and LC₅₀ values could not be determined because mortality remained below 50% even at the highest tested concentration (960 µg/mL). Similar to *D. pulex*,

mortality generally increased after 48 h of exposure, indicating a time-dependent toxic response.

Table II

Median lethal concentration (LC₅₀) values of the investigated *Veronica* spp. and *S. × fragilis* extracts against *H. incongruens* after 24 h and 48 h of exposure

Extract	24 h		48 h	
	LC ₅₀	95% CI	LC ₅₀	95% CI
VPw	NC	NC	NC	NC
VPhe	NC	NC	NC	NC
VPe	NC	NC	1075*	323.4 - 3570
VOw	NC	NC	NC	NC
VOhe	NC	NC	NC	NC
VOe	NC	NC	NC	NC
VHw	NC	NC	NC	NC
VHhe	NC	NC	NC	NC
VHe	NC	NC	NC	NC
SFw	NC	NC	NC	NC
SFhe	1026*	970.3 - 1086	885.3	589.7 - 1329
SFe	NC	NC	NC	NC

NC = not calculated (LC₅₀ > 960 µg/mL, the highest tested concentration).

*LC₅₀ extrapolated above the highest tested concentration.

The differences between the two invertebrate models likely reflect species-specific variations in physiology, toxicant uptake, and metabolic processing. *D. pulex* is generally considered a more sensitive organism for detecting the toxicity of waterborne compounds, whereas *H. incongruens*, owing to its benthic lifestyle and distinct biological characteristics, appears to be less susceptible to certain classes of plant-derived bioactive compounds (27, 34).

These findings indicate that *H. incongruens* provides complementary toxicological information to that obtained with *D. pulex*. Despite its lower sensitivity to several extracts, *H. incongruens* provided complementary toxicological information by revealing a distinct response pattern, with the hydroethanolic extract of *S. × fragilis* exhibiting the most pronounced toxicity. However, this model did not reproduce the higher toxicity observed for the *V. officinalis* extracts in *D. pulex*, highlighting differences in sensitivity between the two invertebrate species. The combined use of both invertebrate models provides a more comprehensive assessment of the acute toxicity profile of botanical extracts by accounting for species-specific differences in sensitivity and biological response (23, 34).

Comparative toxicity profile

The comparative evaluation of the two invertebrate models revealed differences in acute toxicity among extracts obtained using solvents of different polarities. Regardless of the biological model, aqueous extracts generally showed negligible toxicity, whereas hydroethanolic and, in particular, ethanolic extracts induced higher mortality and lower LC₅₀ values (15,16,22). The 96% ethanolic extract of *V. officinalis* showed the highest toxicity in *D. pulex*,

whereas SFhe produced the most pronounced response in *H. incongruens*.

To further evaluate similarities among the investigated extracts, hierarchical agglomerative cluster analysis was performed using the mortality

responses obtained at all tested concentrations and exposure times (Figure 2). The dendrograms revealed differences in acute toxicity profiles among the investigated extracts.

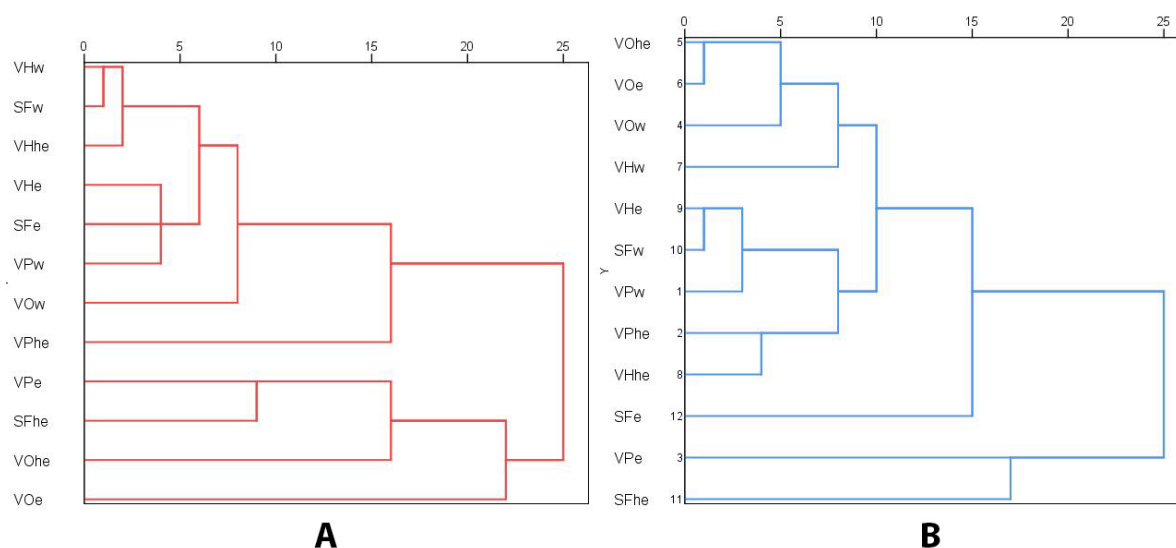


Figure 2.

Hierarchical clustering of the investigated extracts based on the mortality responses of *D. pulex* (A) and *H. incongruens* (B)

In *D. pulex* (Figure 2A), two major clusters were identified. One cluster comprised predominantly aqueous extracts (VPw, VOw, VHw, and SFw), together with SFe and the *V. hederifolia* extracts (VHhe and VHe), reflecting their generally low mortality. VPhe also belongs in this cluster, but it is less similar to the other extracts. The second cluster grouped the more toxic extracts, including VOhe, VOe, VPe, and SFhe. In Figure 2A, VPe and SFhe formed the closest pair within the higher-toxicity cluster, followed by VOhe. In contrast, VOe joined the cluster at a greater rescaled distance, indicating a more distinct toxicity profile.

The clustering pattern obtained for *H. incongruens* (Figure 2B) was less pronounced, consistent with the overall lower sensitivity of this model. VOhe and VOe extracts formed a closely associated pair, while the corresponding aqueous extract (VOw) joined this group at a relatively low rescaled distance. This grouping suggests that botanical origin may contribute to similarities in toxicity profiles, even among extracts prepared using different solvents. In contrast, VPe and SFhe formed a separate pair that merged with the remaining extracts only at the highest clustering distance. The other extracts showed mixed clustering patterns, with no consistent separation by extraction solvent.

Hierarchical clustering analysis provided additional insight into similarities and differences among the investigated extracts, supporting the trends observed

in the acute toxicity assays. Although aqueous extracts generally showed lower mortality, the clustering patterns did not consistently separate based solely on extraction solvent. Instead, the results suggest that both botanical origin and extraction conditions may contribute to the observed toxicological profiles, while also highlighting the greater discriminatory capacity of *D. pulex* compared with *H. incongruens*.

Among the extraction solvents investigated, aqueous extracts consistently showed the lowest acute toxicity regardless of plant species. None of the aqueous extracts reached the mortality threshold required for LC₅₀ determination in either biological model, indicating a favourable preliminary safety profile under the investigated experimental conditions. In contrast, ethanol-based extraction markedly increased the toxicological response, particularly for the *Veronica* species. The 96% ethanolic extract of *V. officinalis* showed the highest toxicity, yielding LC₅₀ values of 378.5 µg/mL after 24 h and 158.7 µg/mL after 48 h in *D. pulex*. Similarly, the 96% ethanolic extract of *V. prostrata* showed pronounced toxicity after prolonged exposure, with an LC₅₀ of 381.9 µg/mL after 48 h. These findings are consistent with previous reports indicating that extraction solvent strongly influences the phytochemical profile of plant extracts and, consequently, their biological activity and toxicological behaviour (15).

Hydroethanolic extracts generally exhibited an intermediate toxicological profile (21, 22). Although they

were less toxic than the corresponding ethanolic extracts for most *Veronica* species, the hydroethanolic extract of *S. × fragilis* represented a notable exception, exhibiting the highest toxicity among the investigated *Salix* preparations in both experimental models (49). This extract yielded LC₅₀ values of 478.6 µg/mL in *D. pulex* and 885.3 µg/mL in *H. incongruens* after 48 h of exposure, suggesting that the hydroethanolic solvent system extracted a phytochemical profile differing from that obtained with 96% ethanol, resulting in greater biological activity under the investigated experimental conditions (15, 39).

Marked interspecific differences were also observed among the investigated plant species. *V. officinalis* demonstrated the highest acute toxicity, particularly for the ethanolic extracts, followed by *V. prostrata*, whereas *V. hederifolia* exhibited minimal toxicity irrespective of the extraction solvent. These findings suggest considerable interspecific differences in phytochemical composition and indicate that closely related species may display substantially different toxicological profiles despite belonging to the same genus (49, 50). The low mortality observed for *V. hederifolia* is particularly noteworthy, suggesting relatively low acute toxicity under the experimental conditions used in this study.

Exposure duration also influenced the biological response. For most active extracts, mortality increased between 24 and 48 h, resulting in lower LC₅₀ values after prolonged exposure. This time-dependent effect suggests that the biological activity of the investigated extracts becomes more pronounced following continuous exposure rather than inducing immediate lethality. Similar time-dependent responses have been reported for plant extracts rich in phenolic compounds and other bioactive secondary metabolites, whose toxicological effects may become more pronounced following prolonged exposure owing to the cumulative action of multiple phytochemicals (15, 34, 51).

Comparison of the two freshwater invertebrate models revealed clear differences in sensitivity (28, 29, 31, 52, 53). *D. pulex* responded to lower concentrations for several extracts, although this pattern was not consistent across all experimental conditions. SFhe produced no mortality in *D. pulex* after 24 h, whereas an extrapolated LC₅₀ value of 1026 µg/mL was estimated in *H. incongruens*. However, direct comparisons between the two models should be interpreted cautiously, given the limited number of extracts with estimable LC₅₀ values in both species. This difference was particularly evident for the hydroethanolic and ethanolic extracts of *V. officinalis*. Such differences most likely reflect species-specific physiological characteristics, including differences in toxicant uptake, detoxification capacity and metabolic processing, which contribute to variable

sensitivity toward plant-derived bioactive compounds among freshwater invertebrates (24, 27, 34, 54).

These findings show that both freshwater invertebrate models provide valuable information for preliminary safety evaluation of botanical extracts while highlighting important differences in sensitivity. *D. pulex* appeared particularly suitable for distinguishing differences in toxicity among the investigated extracts, whereas *H. incongruens* represented a complementary model, highlighting variations in species-specific toxicological responses. The combined use of these alternative bioassays therefore provides a robust strategy for early ecotoxicological characterisation of plant-derived preparations intended for pharmaceutical, nutraceutical, and cosmetic applications. Similar applications of *Daphnia* bioassays for the preliminary toxicological screening of medicinal plant extracts have also been reported for *Euphorbia* species and pyrrolizidine alkaloid-containing botanical extracts, further supporting the suitability of this model for the safety evaluation of botanically derived preparations (55, 56).

This study assessed acute toxicity in two aquatic invertebrate models after 24 and 48 h of exposure. While these models provide useful information for preliminary toxicological screening, the findings cannot be directly extrapolated to human safety. Moreover, the study did not address the potential effects of prolonged exposure or the underlying mechanisms of toxicity. Further investigations using complementary experimental models, including mammalian systems, are therefore needed to provide a more comprehensive toxicological characterisation of the investigated extracts.

Effects of Experimental Factors on 48-h Mortality: Binomial Logistic Regression Analysis

Binomial logistic regression revealed significant associations of concentration ($p < 0.0001$), extraction solvent ($p = 0.0003$), and botanical species ($p < 0.0001$) with 48-h mortality in *D. pulex*. Compared with aqueous extracts, 50% and 96% ethanolic extracts had higher adjusted mortality odds (OR = 9.09, 95% CI: 2.60 - 31.81; OR = 12.06, 95% CI: 3.62 - 40.16). In *H. incongruens*, botanical species was significantly associated with mortality ($p < 0.0001$), whereas concentration ($p = 0.3758$) and extraction solvent ($p = 0.1862$) were not. Probit regression confirmed these overall patterns. The results of the regression analyses are summarised in Table III.

Additional exploratory comparisons in *D. pulex* showed higher mortality for VOe than VOhe (Holm-adjusted $p = 0.0278$), VPe ($p = 0.0093$), and SFhe ($p = 0.0278$). However, no statistically significant difference in sensitivity was observed between the two test organisms (Wilcoxon test, $p = 0.312$). These findings suggest organism-dependent toxicity responses, although the small number of experimental replicates limits interpretation.

Table III

Associations of experimental factors with 48-h mortality in *D. pulex* and *H. incongruens* assessed using binomial logistic regression

Experimental factor	<i>D. pulex</i> p-value	<i>H. incongruens</i> p-value
Extract concentration	< 0.0001	0.3758
Extraction solvent	0.0003	0.1862
Botanical species	< 0.0001	< 0.0001

Note: P-values were obtained from HC3 robust Wald chi-square tests in separate additive binomial logistic regression models. Each model included log₂-transformed concentration, extraction solvent, and botanical species; p < 0.05 was considered statistically significant.

Conclusions

This study comparatively evaluated the acute toxicity of aqueous, hydroethanolic, and ethanolic extracts from *V. prostrata*, *V. officinalis*, *V. hederifolia*, and *S. × fragilis* using two freshwater invertebrate models, *D. pulex* and *H. incongruens*. The results revealed differences in acute toxicity among extracts prepared using different solvents and between the two aquatic invertebrate models. Aqueous extracts generally exhibited low acute toxicity, whereas hydroethanolic and ethanolic extracts induced higher mortality, although the association with extraction solvent differed between the two test organisms.

In *D. pulex*, the 96% ethanolic extract of *V. officinalis* exhibited the highest acute toxicity, whereas the hydroethanolic extract of *S. × fragilis* produced the most pronounced response in *H. incongruens*. The comparative evaluation also highlighted species-specific differences, supporting the complementary use of both invertebrate models for preliminary toxicological screening. Binomial logistic regression revealed significant associations between concentration, extraction solvent, and botanical species and 48-h mortality in *D. pulex*, whereas only botanical species showed a significant association in *H. incongruens*.

The present findings provide preliminary information on the acute toxicity of the investigated botanical extracts in aquatic invertebrate models. Further studies integrating detailed phytochemical characterisation and additional toxicological assessments are needed to better understand the observed biological effects and to provide a more comprehensive evaluation of their toxicological profiles.

Acknowledgement

This study was supported by Carol Davila University of Medicine and Pharmacy, Bucharest, Romania.

Funding

Not applicable.

Availability of data and materials

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the authors used ChatGPT (GPT-6, OpenAI) to improve the manuscript's clarity, grammar, and linguistic quality and to assist with language editing. All scientific content, interpretation of results and final decisions remained entirely the responsibility of the authors.

Conflict of interest

The authors declare no conflict of interest.

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