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The anti-feeding effect of different extracts derived from three moss species (*Atrichum undulatum*, *Kindbergia praelonga* and *Hypnum cupressiforme*) against Burgundy snail (*Helix pomatia*)

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ABSTRACT:
The anti-feeding effects of extracts from three bryophyte species, namely *Atrichum undulatum* (Polytrichaceae), *Kindbergia praelonga* (Brachytheciaceae), and *Hypnum cupressiforme* (Hypnaceae) were evaluated against Burgundy snails (*Helix pomatia*) under laboratory conditions. Ethanol, methanol, and dimethyl sulfoxide (DMSO) extracts and water decoction were used in four concentrations (1%, 3%, 5%, and 10% aqueous solutions). All the tested extracts demonstrated a certain level of anti-feeding effect. A positive relationship between extract concentration and anti-feeding effect was observed particularly for ethanol extracts of *A. undulatum*. Methanol extracts generally exhibited the weakest anti-feeding effects, except for *H. cupressiforme*, where the ethanol extracts showed the weakest performance. The potential of different bryophyte extracts as biocontrol agents is discussed.

Keywords: bryophytes, repellent, pest control, green solution, biocontrol

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INTRODUCTION

Bryophytes, a group comprised of hornworts, liverworts, and mosses, thrive across a diverse range of ecosystems, habitats, and specific microhabitats, including substrates where vascular plants are unable to grow. Many species are capable of surviving in nutrient-poor conditions and are adapted to respond rapidly to brief periods favourable for photosynthesis (Tuba *et al.* 2011). Like other plants, bryophytes have to protect themselves from microbial infections and herbivory. Due to their simple and delicate structure, lacking cuticles or bark which could provide protection, bryophytes employ "chemical weapons" as a means of defence. These "chemical weapons" consist of various secondary metabolites which often form part of their alternative poikilohydric life strategy (Frahm 2004; Xie & Lou 2009). It is widely recognised that one of the main factors contributing to the low levels of herbivory in bryophytes is the abundance of secondary compounds, some of which are unique

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or insufficiently known chemical constituents of mosses (e.g. Saxena & Harinder 2004; Sabovljević *et al.* 2016). Indeed, bryophytes are rarely consumed as a food source, and only a small number of animals and other organisms are capable of digesting them, with bryophytes rarely being their first dietary choice (Haines & Renwick 2009).

The apparent avoidance of bryophytes by insects (Markham *et al.* 2006) as a food source can be explained by three hypotheses: chemical defences, low digestibility, and low nutrient content. Most studies provide the strongest support and evidence for the first hypothesis, namely that bryophytes use chemical defences against herbivores (Davidson & Longton 1987; Davidson *et al.* 1990; Parker *et al.* 2007; Haines & Renwick 2009). Green bryophyte gametophytes are generally avoided by gastropods, whereas sporophyte capsules are considered suitable food sources (Glime 2007; Boch *et al.* 2013, 2015). Evidence suggests that endozoochory by slugs has a positive impact on bryophyte diversity (Speiser 2001; Boch *et al.* 2015) and that gastropod grazing can contribute to the stability and diversity of bryophyte communities (Türke *et al.* 2012; Boch *et al.* 2016).

In comparison to mosses, vascular plants, particularly crops, are often an excellent food source for various animal species. Invertebrates, such as molluscs, are recognised as pests (Kumar 2020) which damage field crops, vegetables, and fruit trees, thus reducing the productivity and yield of cultivated plants (Godan 1983). Therefore, snails, as significant pests, can be controlled in fields using various methods, including physical or mechanical, biological, and chemical approaches (Kumar 2020). In recent years, alternative control approaches using repellents or antifeedants have provided an efficient new strategy in green production and food safety (El-Zemity *et al.* 2001).

Helix pomatia Linnaeus, 1758 is one of the largest terrestrial molluscs in Europe, with a native range spanning across Central and Western Europe (Egorov 2015 and references therein). The species has also been widely introduced by humans to various regions worldwide, including northern Africa and America (Egorov 2015). This herbivorous snail is listed as endangered on several Red Lists, however, it is also recognised as a pest species (Tluste & Birkhofer 2023).

To test the hypothesis that bryophyte secondary metabolites exhibit an anti-feeding effect on Burgundy (Roman, escargot) snails (*Helix pomatia*), we evaluated various types of extract and their concentrations from three randomly selected moss species, representing three different families: *Atrichum undulatum* (Hedw.) P. Beauv. (Polytrichaceae), *Kindbergia praelonga* (Hedw.) Ochyra (Brachytheciaceae), and *Hypnum cupressiforme* Hedw. (Hypnaceae).

The primary goal of this pilot study was to investigate whether the bryophyte species described above exhibit any mollusc-repellent properties i.e. anti-feeding effects. Additionally, the results obtained will indicate the potential for using bryophyte extracts as natural repellents in the production of green vegetables, as well as their viability in the future development of commercial bio-repellent or even green pesticide products.

MATERIALS AND METHODS

Plant material. Three moss species were randomly selected to test their anti-feeding potential against snails. Samples of three common bryophyte species, namely *A. undulatum*, *K. praelonga,* and *H. cupressiforme*, were collected in July 2021 on the slopes of Pavlovac Hill (N44°14’ (44.23°), E20°48’ (20.80°) in the central part of the Republic of Serbia. The species were found in deciduous forests, as part of the *Quercetum frainetto-cerris* prov. association. The snails (*Helix pomatia*) used in the experiment were collected at the same location and released back into the wild after testing.

Atrichum undulatum is a robust, widespread acrocarpous moss species which can form extensive patches. Due to these characteristics, *A. undulatum* has been studied in various types of bryological research (e.g. Beckett *et al.* 2000; Bijelović *et al.* 2004; Hu *et al.* 2016). Moreover, numerous polyphenolic compounds have been documented from *A. undulatum*, including flavonoids and glycosides of three- and tetraoxygenated coumarins (Jung *et al.* 1994; Basile *et al.* 1999).

Kindbergia praelonga is a moss species widely distributed in lowland areas, usually growing on banks, on turf, on the ground in woodland, and tree trunks. Research on this species remains limited, and the chemical constituents of this species have only been partially investigated (Jocković *et al.* 2008; Pejin *et al.* 2010).

Hypnum cupressiforme is a very common, widespread pleurocarpous moss species which prefers acidic to slightly alkaline microhabitats such as tree trunks, logs, walls, and rocks. Due to its size, abundance, and frequency of occurrence, *H. cuppresiforme* is frequently used in air pollution monitoring studies (e.g. Adamo *et al.* 2011; Berisha *et al.* 2016; Capozzi *et al.* 2017). Its large biomass and abundance have also made it the subject of chemical studies and it is known to produce several compounds of practical interest (e.g. Lunić *et al.* 2022; Petkova *et al.* 2023).

Extract preparation. The moss extracts were prepared following a standardised procedure for all three species. The green moss material was cleared of mechanical impurities, dried to a constant weight at room temperature, and then finely ground using a hammer mill. A 0.1 g sample of moss material from each species was immersed in 10 ml of one of the following solvents: 80% methanol, 99% ethanol, DMSO (dimethyl sulfoxyde), or distilled water. The selection of solvents aimed to extract different classes of compounds, distinguishing between polar and nonpolar substances. DMSO was specifically chosen due to its inert properties, ensuring it did not exhibit biological activity in contrast to solvents such as methanol or ethanol, as discussed in Sabovljević *et al.* (2009, 2011). The water extracts were prepared by boiling water at 100°C, producing a decoction. The resulting extracts were then filtered through a cellulose-acetate membrane (0.45 μm), and subsequent water dilutions (1%, 3%, 5%, and 10%) were made for further experimentation.

Experimental design. In each of the identical-sized boxes, two lettuce leaves (commercially bought bio-farmed *Lactuca sativa* L.) were placed alongside a single starved Burgundy snail (Fig. 1A). The snails were deprived of food for 48 hours prior to the experiment but were maintained active with high humidity (above 75%) at room temperature (22°C). The leaves were labelled to distinguish them at the beginning of the experiment. One of the lettuce leaves was coated with a thin layer of moss extract (treatment), while the other was coated with distilled water (control). In total, there were 16 boxes for each moss species, with four boxes designated for each type of solvent (methanol, ethanol, DMSO, and water) and four for each of the different water dilutions of the extracts (1%, 3%, 5%, and 10%). The surface areas of the lettuce leaves were measured before placement in the boxes, with the shape of each leaf outlined on paper (Fig.1C left). These papers were then scanned, and the leaf areas were quantified using image analysis software (Digimizer version 3.7, MedCalc Software, Belgium). The boxes containing the test animals (snails) and lettuce leaves were maintained at a constant room temperature of 22°C and high humidity (above 75%) throughout the 24-hour testing period. After 24 hours of feeding, the remaining lettuce leaf material (Fig. 1B) was subjected to the same procedure for surface area measurement (Fig. 1C right). The amount of leaf consumed was calculated by subtracting the area of the

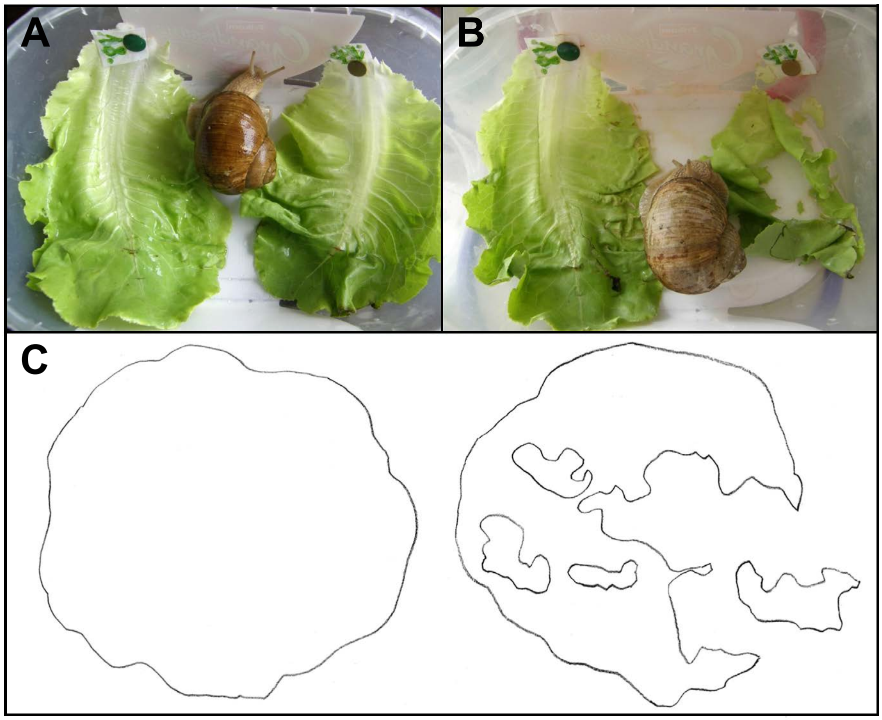

Fig. 1. The presentation of the experimental setup. A starved Burgundy snail and two lettuce leaves were placed in the box (A) and after 24 hours of feeding (B) the leaf areas were measured. The surface areas of the lettuce leaves were measured before placement in the boxes (C left), and after the experiment (C right) with the shape of each leaf outlined on paper.

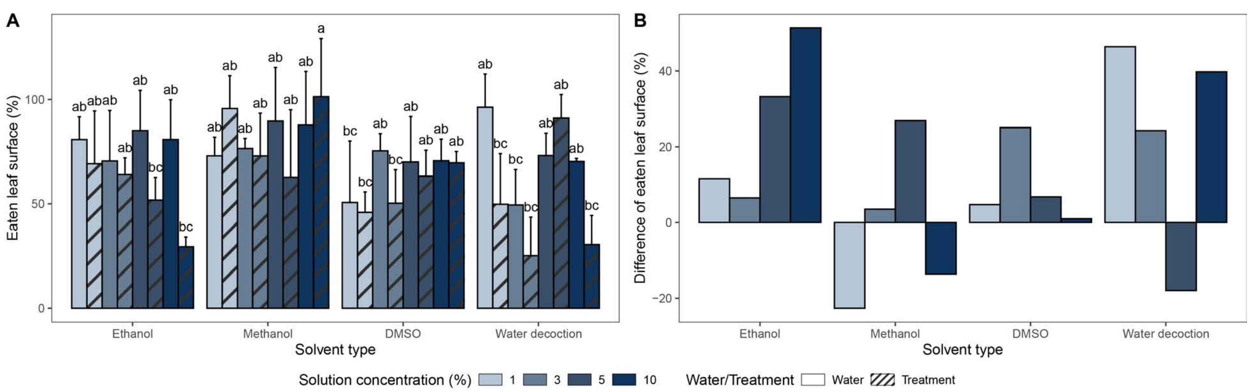

Fig. 2. Anti-feeding experiment results of four different extract types (ethanol, methanol, DMSO, and water decoction) of moss *A. undulatum*. A – The effects of different concentrations of various extract types (striped bars) and water-treated controls (non-striped bars) on the percentage of eaten leaf surface. B – Difference in eaten leaf surface between the water controls and treatments of different concentrations of various extract types. The results are presented as means with the error bars representing standard errors. The letters above the bars indicate the statistically significant differences ($p < 0.05$) between the experimental groups.

leaf after 24 hours (eaten leaf surface) from the area at the beginning of the experiment (initial leaf surface) and expressed as a percentage of eaten leaf area. The same procedure was applied to the extracts of all three species, and the entire experiment was repeated three times.

Statistical analysis. The obtained data were statistically analysed using analysis of variance (ANOVA), followed by the least significant difference (LSD) *post hoc* test with the Bonferroni *p*-value adjustment method in Statistica for Windows 5.1 (McCallum 1999). Data visualisation was done using the R programming language (v. 4.3.1) (R Core Team 2024).

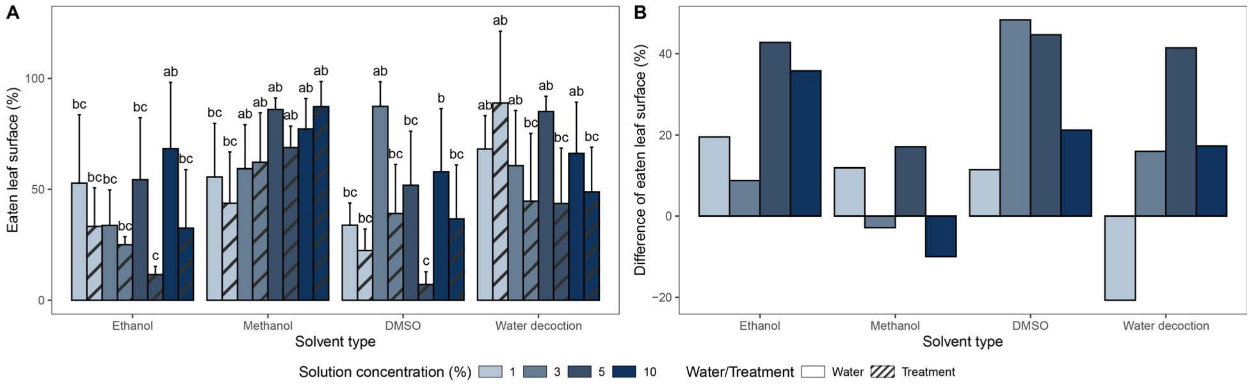

Fig. 3. Anti-feeding experiment results of four different extract types (ethanol, methanol, DMSO, and water decoction) of moss *K. praelonga*. A – The effects of different concentrations of various extract types (striped bars) and water-treated controls (non-striped bars) on the percentage of eaten leaf surface. B – Difference in eaten leaf surface between the water controls and treatments of different concentrations of various extract types. The results are presented as means with the error bars representing standard errors. The letters above the bars indicate the statistically significant differences ($p < 0.05$) between the experimental groups.

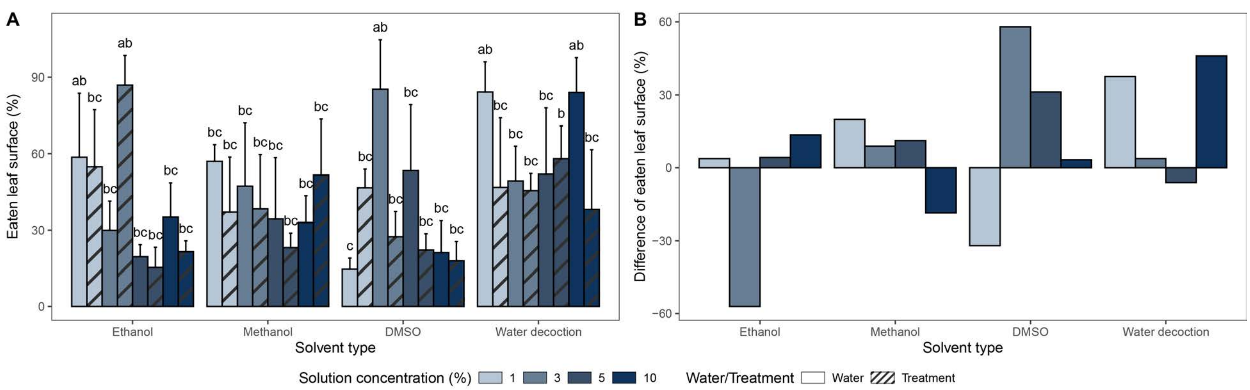

Fig. 4. Anti-feeding experiment results of four different extracts types (ethanol, methanol, DMSO, and water decoction) of moss *H. cupressiforme*. A – The effects of different concentrations of various extract types (striped bars) and water-treated controls (non-striped bars) on the percentage of eaten leaf surface. B – Difference in eaten leaf surface between the water controls and treatments of different concentrations of various extract types. The results are presented as means with the error bars representing standard errors. The letters above the bars indicate the statistically significant differences ($p < 0.05$) between the experimental groups.

RESULTS

The strongest anti-feeding effect of the *A. undulatum* extracts, characterised by the largest difference in the eaten surface area between the control and treated lettuce leaves, was observed in the case of the 10% ethanol extract (Fig. 2A, B). Furthermore, a strong anti-feeding effect was documented when 1% and 10% water decoction, 3% DMSO, and 5% methanol extracts were applied. A certain level of anti-feeding effect of *A. undulatum* extracts was documented across all the applied treatments, with the exception of the 1% and 10% methanol and 5% water decoction extracts (Fig. 2A, B).

The results for the *K. praelonga* extracts indicate that the 3% and 5% DMSO extracts exhibit the strongest anti-feeding effect (Fig. 3A, B). A strong anti-feeding effect against the Burgundy snails was also observed with the two highest concentrations of ethanol extracts (5% and 10%) and the 5% water decoction extract (Fig. 3A, B). Anti-feeding activity against the snails was noted in all treatments with the *K. praelonga* extracts, except for the 1% water decoction, and the 3% and 10% methanol extracts (Fig. 3A, B).

The most significant difference in eaten lettuce leaf surface (58%) between the control and treatment leaves among all the tested extract types and species was observed when a 3% DMSO extract of *H. cupressiforme* was applied, (Fig. 4B). A notable anti-feeding effect of *H. cupressiforme* was also evident with the application of 1% and 10% water decoction extracts and the 5% DMSO extract (Fig. 4A, B). In contrast, no anti-feeding effect was observed with 3% ethanol, 10% methanol, 1% DMSO, or 5% water decoction extracts (Fig. 4A, B).

DISCUSSION

A clear positive relationship between extract concentration and anti-feeding effect was observed for the ethanol extracts, particularly for *A. undulatum*. In contrast, the methanol extracts generally showed a lack of anti-feeding effects, with the weakest overall performance, except for *H. cupressiforme*, where the ethanol extracts were the least effective.

The variations in the anti-feeding performance of the tested extracts are likely attributable to differences in the extraction efficiency and yield of the solvents, as previously demonstrated by Klavina & Springe (2015) for polyphenolic compounds. For instance, DMSO was found to dissolve more polyphenolics than ethanol or methanol in *Rhytidiadelphus triquetrus*, while ethanol was the most efficient solvent for *Sphagnum rubellum*. Furthermore, the chemical structures of the compounds are closely related to their solubility in water or organic solvents. As a result, the extracts from the tested mosses contain a variety of chemical compounds with different properties, which may affect the snails in diverse ways.

All the snails used in our study survived, indicating that no molluscicidal effects were observed. Based on the results obtained, extracts from *A. undulatum*, *K. parelonga*, and *H. cupresssiforme* may be used as natural repellents for Burgundy snails. A similar anti-feeding effect, without biocidal activity, was observed for extracts from two moss species *Neckera crispa* and *Porella obtusata* against the slug *Arion lusitanicus* Mabille, 1868 (Frahm & Kirchhoff 2002). However, to further investigate the bio-repellent potential of the tested species, an analysis of the chemical content and biological activity of the extracts is needed.

Previous studies have detected phenolic compounds, such as phenolic acids and flavonoids, in extracts of *K. praelonga* (Jocković *et al.* 2008). Similarly, phenolic acids and flavonoids were identified in *H. cupressiforme* (Smolińska-Kondla *et al.* 2022), and phenolics were a major chemical component of *A. undulatum* extracts (Chobot *et al.* 2008). Given that phenolic compounds are well known for their potent antioxidant properties and diverse biological activities, it can be proposed that, at least to some extent, they contribute to the high anti-feeding effects observed in the species investigated. Phenolic components are shown to have insecticidal effects (e.g. Rodríguez *et al.* 2022). Bryophytes generally synthesise a wide array of secondary metabolites, including volatile compounds, many of which exhibit antioxidant activity (Asakawa & Ludwiczuk 2018). In addition to phenolics, volatile compounds have been identified in extracts of *H. cupressiforme* (Karataşa & Yayintasb 2024), which should be considered as contributing to the repellent effects this species exerts on snails. Investigating the chemical profiles of species showing anti-feeding effects is essential, as such plants can be subjected to simple and accessible tests which may lead to the development of environmentally friendly formulations for vegetable treatments. These formulations would deter but not harm snails, while simultaneously promoting human health, food production, and safety (Kumar 2020).

In order to produce commercial products suitable as natural repellents or even pesticides in agricultural fields from bryophytes, a large biomass is required (Dziwak *et al.* 2022). Collecting bryophytes from natural habitats is not feasible, both from a conservation perspective (due to potential population damage) and also because of the impossibility of obtaining the large quantities of pure axenic material necessary. Consequently, *in vitro*, and axenic propagation methods, including the use of bryo-reactors offer significant potential (Decker & Reski 2004, 2008). Experiments have demonstrated the feasibility of using *in vitro*-grown bryophytes for these purposes (Dziwak *et al.* 2022). The bioactivity of extracts from axenically grown bryophytes has been compared with those from wild-collected specimens, with the former generally showing superior results (Sabovljević *et al.* 2011; Mukhia *et al.* 2019). Furthermore, laboratory-grown material can be enhanced through the optimisation of growth conditions, which in turn can improve the speed of development and induce slight modifications in the synthesis of the specific target molecules naturally produced by the selected species (e.g. Sabovljević *et al.* 2017; Dziwak *et al.* 2022).

Bryophytes, often overlooked, offer significant potential for applied research (Sabovljević & Sabovljević 2010; Latinović *et al.* 2019) Considering the fact that only 5% of bryophyte species have been chemically studied to date (Asakawa 2007), this group of plants deserves greater attention. Further research is needed to increase our understanding of the biotic interactions between bryophytes and invertebrates. Moreover, it has been demonstrated that such research contributes novel insights and advances green technologies (e.g. Matić *et al.* 2024). However, knowledge regarding the chemical constituents of bryophytes remains insufficient, with many new and unique chemical structures being described every year (e.g. Asakawa *et al.* 2013; Asakawa & Ludwiczuk 2018).

CONCLUSION

The results obtained clearly show that all the extracts of the tested mosses exhibit repellent i.e. anti-feeding effects on burgundy snails, thus highlighting the huge potential of bryophytes as green factories of environmentally friendly products. Additionally, these findings underscore the relevance of bryophyte applications in biotechnology, biotechnical processes and pest control. Furthermore, this research represents a crucial first step towards the application of bryophytes in the development of environmentally friendly products and provides further elucidation of their applied potential.

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REZIME

Efekat ekstrakata tri vrste mahovina (*Atrichum undulatum*, *Kindbergia praelonga* i *Hypnum cupressiforme*) na ishranu vinogradskog puža (*Helix pomatia*)

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Efekat ekstrakata tri vrste mahovina – *Atrichum undulatum* (Polytrichaceae), *Kindbergia praelonga* (Brachytheciaceae), i *Hypnum cupressiforme* (Hypnaceae) na ishranu vinogradskog puža (*Helix pomatia*) je ispitivan u laboratorijskim uslovima. Ekstrakti etanola, metanola i dimetil sulfoksida (DMSO) i vodena dekokcija korišćeni su u četiri koncentracije (1%, 3%, 5% i 10% - vodeni rastvori). Svi testirani ekstrakti su pokazali određeni nivo efekta protiv hranjenja. Pozitivna veza između koncentracije ekstrakta i efekta protiv hranjenja primećena je posebno za etanolne ekstrakte *A. undulatum*. Metanolni ekstrakti su generalno pokazali najslabije dejstvo u supresiji hranjenja, osim kod *H. cupressiforme*, gde su etanolni ekstrakti pokazali najslabiji učinak. Potencijal različitih briofita kao sredstava za biokontrolu je diskutovan.

Ključne reči: briofite, repelent, kontrola štetočina, zelena rešenja, biokontrola