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Bioactivity of the Tree of Heaven Leaf Extracts Incorporated into Biopolymer Matrix Against Spongy Moth Larvae

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Abstract: The bioactivity of the *Ailanthus altissima* crude leaf extract (CLE) and a leaf extract incorporated into a biopolymer matrix (BPM) was tested against *Lymantria dispar* larvae. The crude leaf extracts and those incorporated into a chitosan–gelatin polymer matrix were examined in choice and non-choice assays at 0.01, 0.05, 0.5, and 1% concentrations for feeding deterrent activity, contact, and digestive toxicity. The CLE exhibited moderate deterrent activity at all concentrations, whereas the BPM showed a very strong deterrent effect at 0.5% and 1% and a strong effect at 0.1% and 0.01%. No significant differences in digestive or contact toxicity were observed between the CLE and BPM groups and the control groups. The BPM also influenced larval behavior after digestion, decreasing consumption and growth and increasing development time. The higher bioactivity of the CLE compared to the control group is attributed to its high content of total phenols, flavonoids, and tannins, whereas the enhanced bioactivity of the BPM is due to its incorporation into the biopolymer matrix. Given its very strong deterrent activity, and absence of contact and digestive toxicity, the BPM can be recommended as a potential environmentally friendly bioproduct for forest pest control after field evaluation.

Keywords: *Lymantria dispar*; antifeedant activity; nutritional indices; *Ailanthus altissima*; bioproducts

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1. Introduction

The spongy moth, formerly known as the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae) is an extremely polyphagous caterpillar that feeds on over 500 trees [1,2]. This pest spread from Japan to the Far East through Asia, Europe, North Africa [3], and North America after its introduction in the 19th century [4,5]. During outbreaks, which have been very frequent in southern Europe [6,7], spongy moths completely defoliated thousands of hectares of broadleaved forest in Serbia [8,9]. All tree species can be ranked from the extremely suitable for spongy moth development, such as oaks (*Quercus* spp.) [10–13],

to the extremely unsuitable, such as different American and European ash species (*Fraxinus* spp.) [14–16].

Control of the spongy moth relies on biological or growth-regulating insecticides because chemical suppression is prohibited in the certified forests of many countries [14–16]. The efficacy of biological insecticides strongly depends on weather conditions during and after their application [17–19], whereas growth regulators have a serious negative impact on non-targeted organisms [20]. Therefore, novel environmentally friendly solutions for combating the spongy moth are urgently required. Over the last two decades, great efforts have been made to identify plant products with suitable antifeedant properties against the spongy moth. For this purpose, the antifeedant and toxic activities of essential oils (EOs), extracted from several plants, such as *Ocimmum basilicum* L. [21,22], *Athamanta haynaldii* Borb. et Uecht., *Myristica fragrans* Houtt. [23], *Pimpinella anisum* L., *Anethum graveolens* L., *Foeniculum vulgare* Mill. [24], *Tanacetum vulgare* L. [25], *Chamaecyparis lawsoniana* (A. Murray) Parl., *Thuja plicata* Donn ex Don [26], *Calocedrus decurrens* (Tor.) Florin, and *Cupressus arizonica* (Greene) [27] were tested against spongy moth larvae. In parallel, the bioactivity of some of the dominant components of previously evaluated EOs was tested [21,28]. Additionally, the effects of leaf extracts of green ash, *Fraxinus pennsylvanica* Marsh, on spongy moth feeding and development have been tested [29]. The antifeedant activity of the plant extracts derived from the leaves of the tree species *Morus alba* L. and *Aesculus hippocastanum* L. and the entire herbaceous plants *Ambrosia artemisiifolia* L., *Erigeron canadensis* L., and *Daucus carota* L. on spongy moth larvae was tested by Gvozdenac et al. [30]. A similar study was conducted to evaluate the antifeeding and insecticidal activities of the tree of heaven (*Ailanthus altissima* (Mill.) Swingle) bark and leaf extracts as well as white mulberry (*M. alba*) on spongy moth larvae [30,31].

Both essential oils and extracts rich in bioactive compounds are based on the secondary metabolites that plants have developed during coevolution as part of various defense mechanisms against pests and pathogens [32–34]. Given that both types of botanicals are rich in diverse chemical compounds, they are considered the most viable alternatives to chemical insecticides that rely on single chemicals, owing to their diverse modes of action that impede the evolution of pest resistance [35]. Previous studies on the spongy moth have demonstrated low to very high antifeedant activity and low contact and digestive toxicity in some cases, which nominate some of the tested oils and extracts as promising candidates for environmentally friendly control of this pest. Despite the promising efficiency of plant products, some physical characteristics, such as high volatility and low stability to heat, sun, moisture, and oxygen, may limit their large-scale use in forest protection [36–38]. One possible way to reduce volatility and enhance the stability of eco-friendly plant products is to encapsulate them in appropriate biopolymer matrices [39–42]. Choosing the suitable matrix material for the intended application is of great importance, since it affects the encapsulation efficiency and the stability of the formed nanostructures. Chitosan, a cationic polysaccharide characterized by its film-forming and gelling properties, has been extensively utilized as a matrix for the encapsulation of plant compounds due to its biocompatibility, biodegradability, and low toxicity—in conjunction with its amine and hydroxyl groups—rendering it suitable for encapsulating various active ingredients [43]. In the field of pesticides, chitosan nanoparticles have been used to encapsulate citronella oil to target *Spodoptera littoralis* (Boisd.) [44], carvacrol and linalool have been used to combat *Helicoverpa armigera* (Hüb.) and *Tetranychus urticae* Koch [45], and *Achillea millefolium* (L.) essential oil has been used to control adult *T. urticae* [46]. The encapsulation of these active substances in chitosan nanoparticles has demonstrated the effectiveness of chitosan nanoencapsulation in prolonging insecticidal and acaricidal effects through slow and sustained release. Jovanović et al. [47] demonstrated that combining chitosan and gelatin is

an effective polymer matrix for encapsulating essential oils and gradually releasing their main active components.

Ailanthus altissima (Mill.) Swingle, commonly known as the tree of heaven (Simaroubaceae), is an invasive woody plant species [48]. Originally from northern and central China [49], it has spread to Europe, America, Australia, and other areas, where it has become a noxious weed [50]. In Europe, *A. altissima* is one of the most invasive species that harm local biodiversity [51]. It competes with native plants, inhibits seed germination and seedling growth [52], and is less susceptible to herbivorous insects [53]. In previous studies, leaf extracts of *Ailanthus altissima* were found to exhibit antibacterial and antifungal properties [54], deter oviposition by *Spodoptera frugiperda* [55], and demonstrate insecticidal and antifeeding activities against larvae of *Spodoptera littoralis* and *Lymantria dispar* [31,56], emphasizing its potential as a natural pesticide and its significant impact on the life history traits of *Spodoptera frugiperda* [57].

To the best of our knowledge, this is the first study to investigate the bioactivity of leaf extracts of the tree of heaven (*Ailanthus altissima*), integrated into a biopolymer matrix, against spongy moth (*Lymantria dispar*) larvae. The primary objective of this research is to evaluate the feeding deterrent properties, digestive and contact toxicity, and the influence of the extract on larval behavior, growth, feeding indices, and molting. Furthermore, this study aims to assess the role of the biopolymer matrix in prolonging the bioactivity of the leaf extract, ensuring sustained efficacy and offering a more environmentally friendly alternative for pest management. By combining the natural bioactive potential of *Ailanthus altissima* with advanced biopolymer technology, this research will provide new insights into sustainable approaches for controlling forest pests.

2. Materials and Methods

2.1. Plant Material

Leaf samples of *Ailanthus altissima* (Mill.) Swingle trees of heaven were collected from five randomly selected mature trees in the forest of Košutnjak (N: 44°46′34″, E: 20°25′56″) in Belgrade at the beginning of June 2024. Per each tree, 500 g of fresh leaves were gathered. Plant material was identified at the Faculty of Forestry, University of Belgrade. After collection, leaves were air-dried and processed. For the preparation of the tested extracts, the conventional method was used: percolation, 50% EtOH, and a solvent: extract ratio of 1:2. After obtaining the corresponding percolate, EtOH was evaporated under vacuum. The extracts were characterized by total phenolic, total flavonoids, and tannin content, as well as the "fingerprint" using HPLC.

2.2. Determination of Total Phenolic (TP), Tannins (TT), and Flavonoids (TF) Content

TP content was determined using the Folin–Ciocalteu reagent by the method previously described, with slight modification [58]. The absorbance was measured at 725 nm, while gallic acid (GA) was used as a standard (0.01–0.1 mg/mL, equation of the calibration curve: $y = 1.281x + 0.02$). The calibration curve showed the linear regression at $r > 0.99$, and the results were expressed as milligrams of gallic acid equivalents per gram of plant extract dry weight (mg GAE/g DW). Triplicate measurements were taken, and data were presented as mean ± standard deviation (SD).

The percentage content of TT was calculated using the method described in the European Pharmacopoeia 11.0 [59]. The absorbance was measured by UV–VIS spectrophotometer HP 8453 (Agilent Technologies, Santa Clara, CA, USA) at a max of 760 nm. The percentage content of tannins expressed as pyrogallol (%, *w*/*w*) was calculated from the

difference in absorbance of total polyphenols (A_1) and polyphenols not adsorbed by PVPP (polyvinylpolypyrrolidone) reagent (A_2) using the following expression:

$$TT = (62.5\ (A_1 - A_2) \times m_2)/(A_3 \times m_1)\ [\%], \quad (1)$$

where m_1 and m_2 represent the mass of the sample to be examined and pyrogallol in grams, respectively, and A_3 represents the absorbance of standard solution (pyrogallol). The results represent the mean of three determinations.

The content of total flavonoid was calculated using the method described in the European Pharmacopoeia 11.0. [59]. The content of flavonoid (mean of three determinations) was spectrophotometrically determined, expressed as the hyperoside percentage, and calculated using the following expression:

$$TF = A \times 1.25/m\ [\%], \quad (2)$$

where A is absorbance at 425 nm, and m is the mass of the extracts to be examined in grams.

HPLC analysis "fingerprinting" of the investigated phenolic compounds was achieved by an Agilent Technologies 1200 HPLC machine (Agilent Technologies, Santa Clara, CA, USA), equipped with Lichrospher® 100 RP 18e column (Agilent Technologies, Santa Clara, CA, USA), applying gradient elution of two mobile phases, i.e., "A/B" ("A"—consisting of 1 M phosphoric acid—and "B"—being a pure acetonitrile) at flowrates of 1 mL/min, with photodiode array (PDA) detection (UV at 360 nm), always within 70 min. The suitable separation of the presented constituents was achieved by applying the following scheme of elution: 89–75% A (0–35 min); 75–60% A (35–55 min); 60–35% A (55–60 min); and 35–0% A (60–70 min). The amount of the tree of heaven sample was 3.51 mg/mL 50% EtOH. Prior to injection, samples were filtered through a 0.45 µm PTFE membrane filter. For standards used in the investigation, the concentrations were 0.54 mg/mL for gallic acid, 0.524 mg/mL for quercitrin, 0.21 mg/mL for neochlorogenic acid, 0.96 mg/mL for chlorogenic acid, 0.468 mg/mL for syringic acid, 0.504 mg/mL for ellagic acid, 0.34 mg/mL for isoquercitrin, and 0.28 mg/mL for kaempferol-3-*O*-glucoside. The volume of the standard solutions being injected, as well as that of the tested sample extracts, was 10 µL. Identification was based on the retention times and overlay curves. Once spectral matching was successful, the results were confirmed by spiking with the respective standards to achieve complete identification using the so-called peak purity test. Peaks that did not fulfill these requirements were not quantified. Quantification was performed by external calibration using standards.

2.3. Formulation Preparation

For the synthesis of the biopolymer solutions, the following biopolymers were used: chitosan (powder, low molecular weight, Sigma-Aldrich, Burlington, MA, USA) and bovine gelatine type B (powder, bio reagent, Sigma-Aldrich, Burlington, MA, USA). Acetic acid glacial (ACS, Reag. Ph. Eur.) was obtained by AnalaR Normapur®, VWR Chemicals, Darmstadt, Germany. Leaf extract of *Ailanthus altissima* (Mill.) Swingle tree of heaven (TH) was obtained according to the previously described procedure.

The biopolymer solution was obtained by combining gelatine (G) and chitosan (C) solutions in a weight ratio of C:G = 80:20. The chitosan solution was prepared by dissolving chitosan powder in 1% acetic acid aqueous solution by intensive stirring for 30 min using OV5 Homogenizer (VelpScientifica, Usmate, Italy). The gelatine solution was prepared by dissolving gelatine powder in distilled water at 30–35 °C and stirring for 30 min at a magnetic stirrer. The gelatine solution was added to the chitosan solution with intensive stirring for 10 min. Different formulations were obtained by adding leaf extracts of the tree of heaven at concentrations of 0.01, 0.1, 0.5, and 1% (*w*/*v*) in a previously prepared

biopolymer solution under intensive homogenization (10,000 rpm, OV5 homogenizer, Velp® Scientifica, Usmate, Italy) for 10 min.

2.4. Release Study of the Bioactive Component from the Formulation

The slow release of the main bioactive components of the tree of heaven extract from the chitosan–gelatin polymer matrix was determined using a UV–VIS spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). The intensity value at 270 nm (the absorption maximum for gallic acid) was used to determine the content of the extract [60]. The UV–VIS spectrum of the pure extract was recorded for different concentrations, and then a calibration curve was made based on the intensity of the peaks. The blind test was prepared the same way as the formulation but without the tree of heaven extract to eliminate responses of the other components (except those from the extract) in the formulation. A procedure similar to the one used in our previous research was applied [61]. The formulation was left in an open laboratory beaker at room temperature under ambient conditions (24 ± 1 °C, relative humidity of 40%). The samples for analysis were prepared by dissolving a certain amount of the formulation in distilled water immediately before measurement. The absorption spectrum was measured every 24 h for the next 6 days.

2.5. Insect Material

Spongy moth egg masses were collected during the Autumn of 2022 from the Turkey oak forest situated in Eastern Serbia (N: 44°20′36″, E: 22°22′22″). After collection, 30 egg masses were transported to the Entomological Laboratory (University of Belgrade Faculty of Forestry), mechanically cleaned from hairs, sterilized, and stored in a refrigerator at 4 °C to prevent hatching [62] until the experiments were performed in the following Spring. After hatching, 100 randomly selected larvae per egg mass were fed a spongy moth artificial diet until molting to the second or third larval instar, depending on the experiment. All experiments were conducted under controlled environmental conditions at a temperature of 23 °C, relative humidity of 65%, and a photoperiod of 16 h light and 8 h dark in an environmental cabinet (Sanyo MLR-350, Osaka, Japan).

2.5.1. Feeding Deterrent Activity

The bioactivity of the tree of heaven crude extract (CLE) and leaf extracts incorporated into the biopolymer matrix (BPM) was tested in the feeding choice and non-choice tests, whereas the growth and nutritional indices were tested only in non-choice tests. For the choice test, two leaf discs, 30 mm in diameter, were placed on opposite sides of a Petri dish (9 × 12 mm) over seed testing filter paper (Whatman, grade 181) to prevent desiccation of the leaf discs. The first disc was dipped in 0.01, 0.1, 0.5, or 1% solution of the tree of heaven CLE or BPM for three seconds and air-dried for 30 min before being introduced into the Petri dish, whereas the second disc was dipped in distilled water instead of CLE or BPM. The positions of both the treated and control discs were marked with different colors on the upper part of the Petri dish. For the non-choice experiment, only the treated or control discs were introduced into the Petri dishes. After the feeding arena was set up, a sample of second-instar spongy moth larva, starved for 24 h, was introduced to each Petri dish. For the tree of heaven CLE or BPM, 25 replicates per concentration were performed in both experiments, including the control group in the non-choice test. Both experiments ended after 48 h when the remains of the leaf discs were scanned at a resolution of 200 dpi in jpg format. The remaining area of the leaf disc was calculated using SigmaScan Pro software (version 5.0; SPSS Inc., Chicago, IL, USA). The area of treated or control discs consumed by the spongy moth larvae was calculated by subtracting the initial area from the remaining area after 48 h of feeding.

Based on the consumed area of the pairs of treated and controlled discs in the choice test, the relative deterrent index (RDI) was calculated using the following formula:

$$RDI = (C - T)/(C + T) \times 100\ [\%], \quad (3)$$

The absolute deterrence index (ADI) was calculated based on the area of the treated and control discs consumed by the spongy moth larvae from the non-choice test using the following formula:

$$ADI = (CC - TT)/(CC + TT) \times 100\ [\%], \quad (4)$$

The total deterrence index (TDI) was calculated using the following formula:

$$TDI = ADC + RDC\ [\%], \quad (5)$$

where C and T are the areas consumed by spongy moth larvae of the control and CLE- or BPM-treated leaf discs, respectively, in the choice test; the CC and TT are the average consumed area by spongy moth larvae from the control and CLE- or BPM-treated groups, respectively, in the non-choice test.

The evaluation of the selected CLE or BPM deterrent activity was conducted using the scale proposed by Szczepanik et al. [63,64], which categorizes the activity as follows: very strong (TDI value range: 150–200), strong (TDI value range: 101–150), moderate (TDI value range: 51–100), or weak (TDI value range: 0–50). Negative TDI values indicate that the tested CLE or BPM exhibit attractive properties.

2.5.2. Larval Growth and Consumption

Larval growth and consumption indices were tested in a non-choice experiment with 25 freshly molted third-instar spongy moth larvae per concentration of CLE, BPM, or control. Growth and consumption indices were calculated according to Waldbauer [65]:

$$RCR = C/(2 \times Win)\ [mm/mg/day], \quad (6)$$

$$RGR = (Wfin - Win)/(2 \times Win)\ [mg/mg/day], \quad (7)$$

where Wfin is the weight of a larva at the end of the experiment, Win is the weight of the larvae at the beginning of the experiment, 2 is the duration of the experiment in days, C is the area of the consumed food, RCR is the relative consumption rate, and RGR is the relative growth rate of spongy moth larvae.

2.5.3. Digestive and Contact Toxicity

Digestive toxicity was tested in the non-choice type of experiment described above on freshly molted third-instar larvae. For each concentration, including CLE, BPM, and the control group, the experimental design incorporated 25 replicates. After 48 h of feeding on the treated leaf discs, the larvae were transferred to an artificial spongy moth diet. Larval mortality or molting to the fourth instar was monitored and recorded daily until all larvae died or molted.

Contact toxicity was tested on freshly molted third-instar spongy moth larvae. On the dorsal thoracic segments, 200 µL of the selected BPMs was applied to 10 spongy moth larvae per concentration in five replicates. After the application of CLE or BPM, the larvae were kept in Petri dishes and fed an artificial diet. Larval mortality or molting to the fourth instar was monitored and recorded daily until all larvae died or molted.

2.6. Statistical Analysis

All tested data were checked for normality using the Shapiro–Wilk test and homogeneity using Levene's test but did not meet the ANOVA assumptions of normal data distribution and homogeneous variances. Therefore, all data related to deterrent activity (RDI, ADI) and larval performance (RCR, RGR, and DL3) were analyzed by two-way PERMANOVAs to reveal the main and interaction effects of CLE or BPM type and corresponding concentrations. One-way PERMANOVAs followed by pairwise comparisons were used to explore the significance of differences between specific experimental groups. Figures were created using the OriginPro 2024 software (OriginLab Corporation, Northampton, MA, USA).

3. Results

The total phenol content of the tree of heaven leaves was 721.1 mg GAE/g DW, while the percentages of flavonoids and tannins were 0.38% and 4.41%, respectively. HPLC analysis revealed that gallic acid was abundant in the investigated leaf extracts, whereas derivatives of *p*-hydroxycinnamic acid (chlorogenic and neochlorogenic acid) were present in significant quantities (Table 1, Figure S1). Additionally, 18.74 mg/dg DW *p*-hydroxybenzoic acid (syringic acid) was detected in the *A. altissima* extract. The predominant flavonoid in the extract was isoquercitrin (14.27 mg/g DW *of A. altissima* extract), followed by kaempferol-3-*O*-glucoside and quercitrin. A significant presence of ellagic acid (37.32 mg/g DW *A. altissima* extract) was confirmed, and this finding is interesting considering the biological activities that might be ascribed to this polyphenolic compound (Table 1, Figure S1).

Table 1. The content of the identified components in the tested *A. altissima* extract.

The Number Ascribed to the Corresponding Peak in Figure S1	The Identified Compound	Content in the Investigated *A. altissima* Extract (mg/g DW)
1	gallic acid	30.97 ± 0.22
2	neochlorogenic acid	19.84 ± 0.12
3	chlorogenic acid	3.36 ± 0.05
4	syringic acid	18.74 ± 0.09
5	ellagic acid	37.32 ± 0.19
6	isoquercetin	14.27 ± 0.08
7	kaempferol-3-*O*-glucoside	9.66 ± 0.11
8	quercitrin	4.39 ± 0.04
*	quassinoid 1	Tentative identification
*	quassinoid 2	Tentative identification

* [66].

The changes in the intensity of the absorption spectrum of the main bioactive component of the tree of heaven plant extracts incorporated into the chitosan–gelatin polymer matrix for six days are depicted in Figure 1. It was shown that the presence of a polymer matrix significantly prolonged the release of the active component, that is, the concentration of the main bioactive compound of the tested extracts incorporated into the biopolymer matrix changed very little over time. After six days of analysis, the percentage content of the main bioactive compounds in the BPM was 71.4% of the initial concentration, whereby the CLE was released completely after 48 h.

There were significant effects regarding the incorporation of the leaf extract into a biopolymer matrix ($F_{1, 199} = 26.30$; $p < 0.001$) and the concentrations ($F_{3, 199} = 4.03$; $p < 0.05$), whereas their interactions ($F_{3, 199} = 0.23$; $p = 0.965$) did not significantly influence the RDI values in the choice test based on the two-way PERMANOVA. Pairwise comparisons be-

tween the same CLE and BPM concentrations, following one-way PERMANOVA, revealed significant differences in the RDI across all tested concentrations. The highest RDI value was recorded at 0.5% BPM, which was significantly different from the other BPM concentrations, whereas no differences were observed among the tested CLE concentrations (Figure 2a).

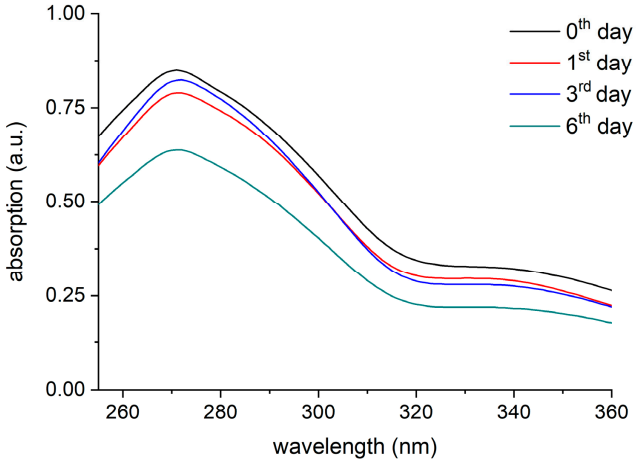

Figure 1. The absorption spectrum of the main bioactive component of the tree of heaven leaf extract incorporated into the chitosan–gelatin polymer matrix for 6 days.

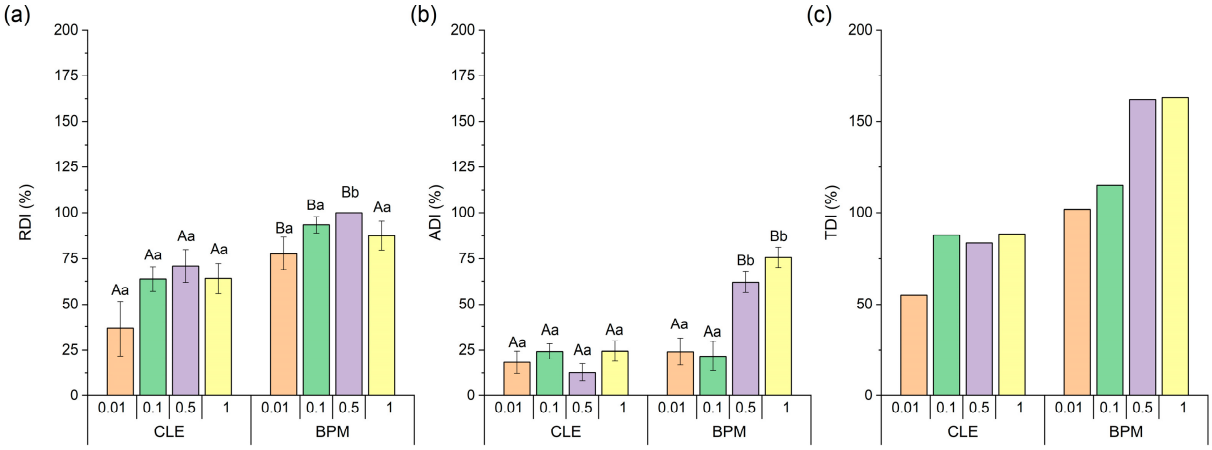

Figure 2. Feeding deterrent activity of the tree of heaven crude (CLE) and leaf extracts incorporated into a biopolymer matrix (BPM): (**a**) relative deterrence index (RDI) (choice assay); (**b**) absolute deterrence index (ADI) (non-choice assay); and (**c**) total deterrence index (TDI). Experimental groups that do not share the same capital letters above bars indicate significant differences between the same concentrations of CLE and BPM. Experimental groups that do not share the same small letters above bars indicate significant differences among the tested concentrations within CLE or BPM.

Significant effects of the incorporation of leaf extract into a biopolymer matrix ($F_{1, 199} = 37.59$; $p < 0.001$), concentration ($F_{3, 199} = 10.19$; $p < 0.001$), and their interactions ($F_{3, 199} = 11.17$; $p < 0.001$) on ADI values in the non-choice test were revealed by two-way PERMANOVA (Figure 2b). Pairwise comparisons between the same CLE and BPM concentrations, following one-way PERMANOVA, showed significant differences in the ADI values across all tested concentrations. The highest ADI values were recorded at concentrations of 0.5% and 1% BPM, which were significantly different from the other BPM concentrations, whereas no differences were observed among the tested CLE concentrations (Figure 2b).

Based on the total deterrence index (TDI), the tree of heaven BPM exhibited strong feeding deterrent properties at a concentration of 0.1% and very strong deterrent properties against spongy moth larvae at concentrations of 0.5% and 0.1%. In contrast, the CLE showed moderate deterrent properties at all tested concentrations (Figure 2c).

There were also significant effects regarding the incorporation of leaf extract into a biopolymer matrix ($F_{1, 199} = 21.74$; $p < 0.001$), concentrations ($F_{3, 199} = 12.05$; $p < 0.001$), and their interactions ($F_{3, 199} = 10.90$; $p < 0.001$) on the RCR based on the two-way PERMANOVA. The control group had significantly higher RCR values than all the other experimental groups, except for the BPM at a concentration of 0.01%. Pairwise comparisons between the same CLE and BPM concentrations, following one-way PERMANOVA, revealed significant differences in the RCR values at concentrations of 0.01% and 0.1%. Significantly higher RCR values were recorded at concentrations of 0.01% and 0.1% BPM, whereas no differences were observed among the tested CLE concentrations (Figure 3a).

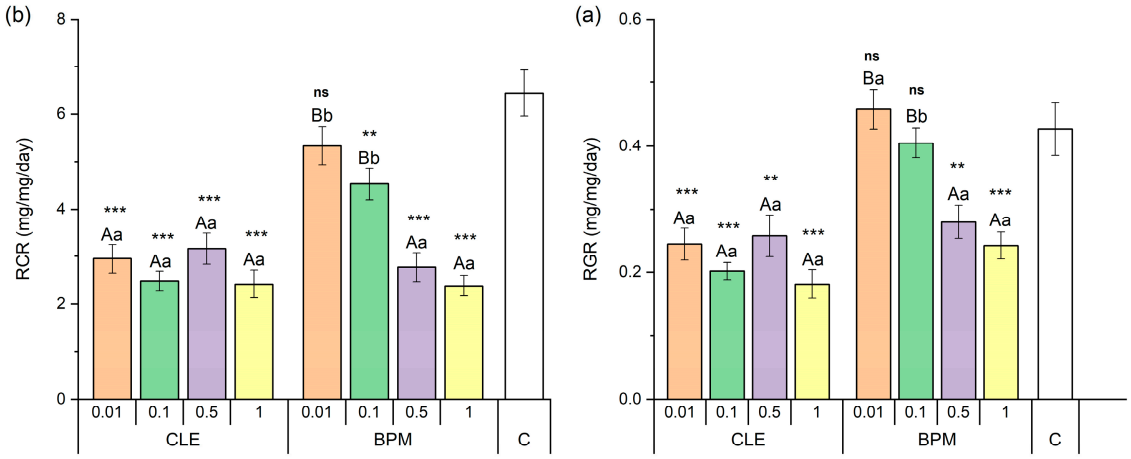

Figure 3. Effect of the tree of heaven crude (CLE) and leaf extracts incorporated into a biopolymer matrix (BPM) on spongy moth larval performance: (**a**) relative consumption rate (RCR); (**b**) relative growth rate (RGR). **, *** indicate significant differences in treatment groups from the control group ($p < 0.01$, $p < 0.001$, respectively), while ns indicates no significant differences in treatment groups from the control group. Experimental groups that do not share the same capital letters above bars indicate significant differences between the same concentrations of CLE and BPM. Experimental groups that do not share the same small letters above bars indicate significant differences among the tested concentrations within CLE or BPM.

The incorporation of leaf extract into a biopolymer matrix ($F_{1, 199} = 48.56$; $p < 0.001$), the concentrations ($F_{3, 199} = 10.70$; $p < 0.001$), and their interactions ($F_{3, 199} = 7.42$; $p < 0.01$) had significant effects on the RGR values, as indicated by the two-way PERMANOVA analysis. The control group had significantly higher RGR values than all the other experimental groups, except for the BPM, at concentrations of 0.01% and 0.1%.

A similar pattern was observed for the RGR values, where significant differences at concentrations of 0.01% and 0.1% were detected after pairwise comparisons, following a one-way PERMANOVA, between the same CLE and BPM concentrations. The highest RGR value for the BPM was recorded at 0.01%, followed by 0.1%, with both concentrations being statistically different from each other and the other tested BPM concentrations. In contrast, no significant differences were observed among the tested CLE concentrations (Figure 3b).

The two-way PERMANOVA revealed significant effects of the incorporation of leaf extract into a biopolymer matrix ($F_{1, 199} = 48.56$; $p < 0.001$), concentration ($F_{3, 199} = 10.70$; $p < 0.001$), and their interactions ($F_{3, 199} = 7.42$; $p < 0.01$) on the duration of the spongy moth third larval instar (DL3) in the digestive toxicity test. The control group had a significantly shorter DL3 than all experimental groups and was shorter than the CLE only at the 1%

concentration. Pairwise comparisons between the same CLE and BPM concentrations, following one-way PERMANOVA, revealed significant differences in the DL3s across all tested concentrations. A significantly longer DL3 was recorded at 1% concentrations for both the CLE and BPM compared to the 0.01% and 0.1% concentrations (Figure 4a).

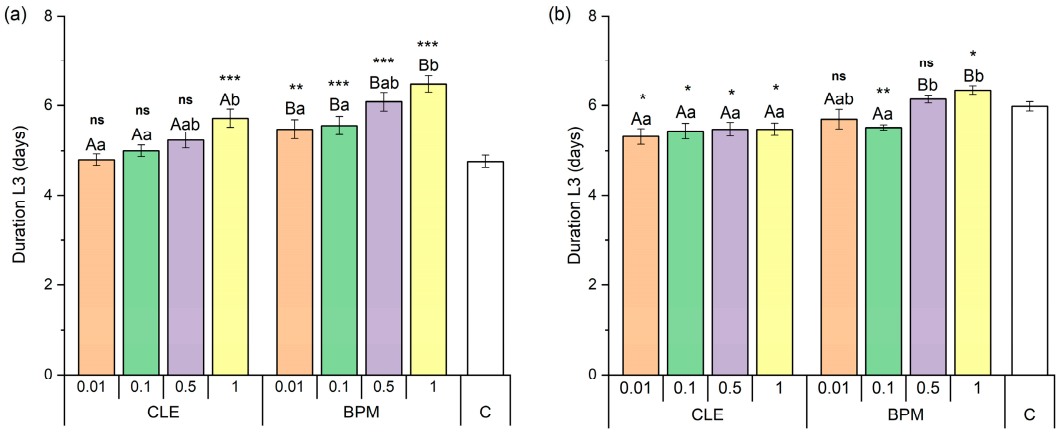

Figure 4. The effect of the tree of heaven crude (CLE) and leaf extracts incorporated into a biopolymer matrix (BPM) on the spongy moth larval performance: (**a**) Duration of third larval instar in the test for digestive toxicity (DL3D). (**b**) Duration of third larval instar in the test for contact toxicity (DL3C). *, **, *** indicate significant differences in treatment groups from the control group ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively), while ns indicates no significant differences in treatment groups from the control group. Experimental groups that do not share the same capital letters above bars indicate significant differences between the same concentrations of CLE and BPM. Experimental groups that do not share the same small letters above bars indicate significant differences among the tested concentrations within CLE or BPM.

According to the two-way PERMANOVA results, the duration of the spongy moth third larval instar (DL3) in the contact toxicity test was significantly influenced by the incorporation of leaf extract into a biopolymer matrix ($F_{1, 199} = 26.30$; $p < 0.001$) and the concentrations ($F_{3, 199} = 4.03$; $p < 0.05$), while their interactions ($F_{3, 199} = 0.23$; $p = 0.965$) had no significant effect. The control group had significantly shorter DL3 than all experimental groups of CLE and BPM at a concentration of 1%, while the BPM at concentration of 1% had a longer development than the control group of the digestive toxicity test. Pairwise comparisons between the same CLE and BPM concentrations, following one-way PERMANOVA, revealed significant differences in the DL3s at concentrations of 0.5% and 1%. A significantly shorter DL3 was recorded at 0.1% BPM. In contrast, there were no differences in the DL3s across the tested concentrations of CLE during the contact toxicity test (Figure 4b).

4. Discussion

Biopesticides should be naturally occurring, effective, affordable, readily degradable, and exhibit diverse modes of action to effectively manage pests [67]. Moreover, successful plant-based pest control requires a balance between high feeding deterrence, low contact, and digestive toxicity. Chemical analysis revealed that *A. altissima* leaf extract (CLE) exhibited high concentrations of tannins and polyphenols, correlating with significant antioxidant activity (Table 1, Figure S1, [68]). Polyphenols, particularly tannins, are recognized as crucial defense mechanisms against herbivores, functioning as growth regulators or feeding deterrents [69]. The effectiveness of foliar tannins in deterring herbivory varies depending on their chemical structures. For instance, hydrolyzable tannins (such as ellagitannins or gallic acid derivatives) exhibit stronger pro-oxidant activity in the guts of

spongy moth larvae than condensed tannins [70]. This pro-oxidant activity, particularly pronounced in the alkaline environment of insect midguts, leads to the generation of reactive oxygen species. These reactive species can degrade nutrients within the gut lumen and induce oxidative stress in the midgut tissues, resulting in slower growth and prolonged development [71]. Consistent with these findings, *A. altissima* demonstrated significant levels of tannins and phenolic compounds, which acted as feeding deterrents for the spongy moth larvae (Figure 1). Consequently, CLE, which is rich in polyphenols and tannins, significantly reduced larval growth and prolonged larval development (Figures 3b and 4a). Antioxidant analysis, presented by Žugić et al. [68], revealed moderate activity in the ABTS assay, with an IC50 value higher than that of the reference antioxidant, BHT, reported by Wangsawat et al. [72]. However, the CUPRAS assay demonstrated strong antioxidant activity comparable to that of BHT [73]. This discrepancy may be attributed to the presence of various phenolic compounds, including flavonoids, phenolic acids, and derivatives of cinnamic acid and *p*-hydroxybenzoic acid, as identified by HPLC analysis (Table 1, Figure S1).

The bioactivity of *A. altissima* leaf extracts is species-specific, exhibiting significant variability in efficacy depending on the pest species tested and the extraction method employed [74]. The leaf extract from *A. altissima* negatively affects *Spodoptera frugiperda* [57]. It prolongs pupation time, reduces larval and pupal biomass and growth rates, decreases food consumption, and lowers the survival rate. Adult moths from larvae fed the extract showed reduced biomass and smaller wings. Moreover, *A. altissima* extract deters egg deposition of *S. frugiperda* on *Zea mays* [55]. Tanasković et al. [31] found no antifeedant activity of the leaf extracts of *A. altissima* on spongy moth larvae at a concentration of 1% in the non-choice test, while the results from the same test in our study depicted a very strong feeding deterrent activity (Figure 2b). In contrast to our results, a study by Tanasković et al. [31] demonstrated 30% digestive mortality in spongy moth larvae after 48 h at the same concentration. The chemical composition and identified compounds of *A. altissima* leaves in our study differed from those reported in previous studies [31,54]. This discrepancy can be attributed to variations in harvesting season, sample preparation, and the type of solvents used for extraction [75] Namely, in plants, secondary metabolite production depends on genetic and environmental conditions (photoperiod, soil pH, moisture and nutrient availability, herbivory, and atmospheric CO_2 and O_3). Along with their total quantity, the biological reactivity of polyphenols is closely regulated by their chemistry (substitution pattern of the phenolic ring, degree of polymerization, etc.). In addition, the changes in the composition of polyphenols between green and senesced tissues are primarily influenced by the overall resorption metabolism during tissue aging [76]. In addition, many studies have reported the influence on the content of secondary metabolites and/or their biological activities of different solvents used in the extraction process as fundamental for bioactive compound separation and recovery for plants [77]. These factors likely explain the contrasting findings observed in our study compared to those of Tanasković et al. [31]. The essential oils were used more often than other secondary metabolites incorporated into polymers to mitigate volatility. A literature survey revealed that the effects of chitosan-based bioactive formulations of essential oils on several lepidopteran species varied from repellent activity and digestive enzyme interruption to enhanced toxicity [44,78–80]. Gelatin can additionally help to incorporate and retain EOs within the film matrix, and chitosan might function as a carrier and delivery system for EO, thereby improving their bioavailability and, at the same time, retaining their efficacy [81–83].

5. Conclusions

In conclusion, the bioactivity of *Ailanthus altissima* (tree of heaven) leaf extracts incorporated into a biopolymer matrix was evaluated for the first time against *Lymantria dispar* (spongy moth) larvae. At concentrations of 0.5% and 1%, the tree of heaven biopolymer matrix (BPM) demonstrated significant deterrent activity. The tested BPM exhibited no significant difference in digestive and contact toxicity compared to the control groups. Furthermore, the tested BPM affected spongy moth behavior only after ingestion of the applied formulations, resulting in decreased consumption and growth and increased larval development time. Given that the tested BPM demonstrated excellent deterrent activity and very low digestive toxicity with no contact toxicity, it can be recommended as a prospective environmentally friendly bioproduct for forest pest management. Further research is warranted to optimize these formulations for specific insect pests and to evaluate their long-term efficacy and environmental impact under field conditions. Additionally, the utilization of *Ailanthus altissima* could offer a dual benefit, providing a source for plant product extraction while simultaneously addressing a significant invasive species control issue.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f16020375/s1: Figure S1: Fingerprint of tested *A. alissima* extract. The numbers ascribed to peaks correspond to the identified constituents given in Table 1 (1: gallic acid; 2: neochlorogenic acid; 3: chlorogenic acid; 4: syringic acid; 5: ellagic acid; 6: isoquercitrin; 7: quercitrin; 8: kaempferol-7-O-glucoside; *: tentative identification as quassinoids [66].

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Data Availability Statement: The data recording the antioxidative activity of the tree of heaven leaves (*Ailanthus altissima* (Mill.) Swingle) can be found at https://data.mendeley.com/datasets/7bkycv63pc/1 (accessed on 7 January 2025). All other data generated within this study are available under reasonable request.

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

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