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# Exploring the Antioxidant and Bioinsecticidal Activity of Spontaneous Flora Vegetal Extracts for Plant Protection and Prevention of Soil Contamination

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**Abstract:** The purpose of this article was to evaluate the application of different plant extracts with bioinsecticidal action and antioxidant activity for plants and soil protection, by substitution of the application of synthetically formulated pesticides with eco-friendly compounds. In this framework, this research focused on the utilization of plant extracts from the spontaneous flora of Moldova (Romania) as bioinsecticides for the control of field pests of the species *Leptinotarsa decemlineata* and their antioxidant activity. Plant extracts of oregano (*Origanum vulgare*), yarrow (*Achillea millefolium*), wormwood (*Artemisia absinthium)*, and cowslip (*Primula veris*) were assessed for their antioxidant activity by the microplate spectrophotometric-based method (for polyphenols and flavonoids content and for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenger activity) and were characterized by FTIR spectroscopy spectra. To evaluate the bioinsecticidal properties of the plant extracts, the mortality (%) and neuroleptic manifestations appearing in the middle of the monitoring period for larvae and adults of the *Leptinotarsa decemlineata* species were identified. Mortality (%) was statistically analyzed using a one-way analysis of variance (ANOVA) and the resulting experimental results were compared with the LSD-Fisher's test ($p < 0.05$). The highest mortality (%) was observed after 24 h of treatment with extracts of *Origanum vulgare* at 100% concentration, while the maximum effect was recorded after 48 h for *Origanum vulgare* at 60% and 100% concentrations.

**Keywords:** antioxidant and bioinsecticidal activity; *Leptinotarsa decemlineata* species; mortality; plant extract; soil pollution prevention



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## 1. Introduction

An important aspect of the environmental, economic and social policies currently being developed is ensuring a high quality and security of life. The principles of sustainable development and ecological farming seek to secure the very best raw materials for the food industry [1]. The practice of intensive farming results in the necessity to employ large amounts of various chemical compounds, from pesticides, soil enhancers and fertilizers, which causes the long-term deterioration of the health of the soil, of agricultural products and thus foodstuffs. Considering the regulations of international organizations and agriculture norms regarding the limitation of the use of chemicals in agriculture, attempts are being made to introduce new types of substances, such as bioinsecticides, in order to remove a number of disadvantages caused by chemical insecticides [2–4].

Even though plant extracts are currently less widely used in micro-farms and by organic food producers, they are able to eliminate a number of disadvantages of synthetic

pesticides. Several advantages should be mentioned, such as: lack of toxicity, selectivity, avoidance of resistance, ease of preparation and safe handling [5–7].

Today, plant-based products have come to be used across a broad spectrum in a variety of sectors, with new applications continually emerging. Botanical products frequently work at new and multi-target sites and mitigate the growth resistance potential of insects. Botanical insecticides are encouraged for use in organic farming as alternatives to conventional insecticides for the management of a variety of pests. They are specific to a particular target, biodegradable and have low or no toxic effect on the natural habitat [8,9]. With many phytochemical pesticides, there is a wide range of possible effects on pests and other diseases. In addition, the manufacturing costs of biopesticides are considerably less than those of synthetically produced chemical pesticides. Biopesticides account for just 5% of the overall pesticide trade worldwide, in spite of their qualities, with 175 currently registered biopesticide active ingredients and 700 commercially available products [10–13]. It was identified that out of more than 6500 plant species studied, more than 2500 species belonging to 235 families showed biopesticide action [14]. Several bioassays have consistently found that botanical extracts have insecticidal, larvicidal and mosquitocidal properties. In addition, multiple studies recommend targeted interventions which could assist in maximizing the use of both essential oils and plant extracts within integrated pest management programmes [11,12,15–19]. Therefore, the use of plant extracts as bioinsecticides is a new topical field with encouraging results in scientific research and future perspective progress [20–25].

In this context, for the preparation plant extracts, solid-liquid extraction has been used in different variants which depend mainly on their efficiency and selectivity [26–28]. The quick qualitative and quantitative characterization was performed by using modern analysis methods, such as spectrophotometric-based methods, high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS) [29–31].

Therefore, in order to obtain a large amount of bioactive compounds from plants, efficient extraction procedures are required. Methods frequently used include maceration, percolation, Soxhlet extraction, distillation, ultrasonic-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, etc. [32–35]. To the best of our knowledge, the evaluation of the bioinsecticidal action against *Leptinotarsa decemlineata* species and antioxidant activity of plant extracts derived from oregano *(Origanum vulgare)*, yarrow *(Achillea millefolium)*, wormwood *(Artemisia absinthium)* and cowslip *(Primula veris)* belonging to Romanian spontaneous flora has not yet been studied.

Antioxidant activity is the result of a combination of compounds. In addition to the antioxidant effect they exert, some compounds also have a pesticidal, repellent, larvicidal, etc. effect through a multitude of mechanisms. Many studies show a number of compounds present in plants that have antioxidant activity, therefore at least we can speculate a directly proportional relationship between the high content of antioxidant substances and the biopesticidal effect. As suggested in the literature, many substances with an antioxidant character are present in the plants considered in this study, *Origanum vulgare, Achillea millefolium, Artemisia absinthium* and *Primula veris*, which are used to control pests of the *Leptinotarsa decemlineata* species, among others. Such compounds are represented by carvacrol, α- and β-pinene, α- and β-thujone, borneol, eugenol, carvone, among others etc. [36–40].

The goal of this study was to obtain an eco-friendly product with bioinsecticidal activity able to ensure the quality of raw materials in the food industry. To this purpose, extracts from the above-mentioned plants were evaluated for their bioinsecticidal activity against *Leptinotarsa decemlineata* species, linking with their antioxidant activity by identifying the polyphenols and flavonoids content and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenger activity.

## 2. Materials and Methods

### *2.1. Plant Materials*

The selected plants were *Origanum vulgare* (Order: Lamiales, Family Lamiaceae), *Achillea millefolium* (Order: Asterales, Family: Asteraceae), *Artemisia absinthium* (Order: Asterales, Family: Asteraceae) and *Primula veris* (Order: Ericales, Family: Primulaceae). Plants were collected from Strambu Bosanci hill, on the administrative territory of the Bosanci municipality (314 m.d.m. altitude), Suceava county located by GPS coordinates: 47°35′12″ N 26°19′0.2″ E/47.58667° N 26.31722° E. Harvesting was done during the period of maximum development and the identification was made according to Barnes et al. [41] and Bujor [42]. *Origanum vulgare* was harvested in early summer (July) in the early morning due to the high concentration of essential oils. *Achillea millefolium* was harvested at flowering, in summer (July), at noon, in full sun. *Artemisia absinthium* was harvested in summer (August), in full bloom. In the case of *Primula veris*, the plants were harvested in spring (May), at the full flowering stage. The whole plants (stems, flowers and leaves) were dried by placing them in a layer of about 2 cm on a sheet of paper in a shady, moisture-free and well-ventilated chamber for 2 months. After drying, they were ground in a food mill and subsequently deposited in paper or cloth bags prior to further use.

### *2.2. Reagents*

All necessary reagents used in the following described methods were of analytical quality (p.a.) and were purchased from Merck Co. (Darmstadt, Germany).

### *2.3. Extraction Procedure*

In the extraction process, 96% ethanol was used as solvent, since it fulfills the quality conditions required and is accepted by the food and agriculture industries. The established weighing of the powder from each plant was done using an analytical balance type RADWAG AC 230 V/400 W, 50 Hz.

The procedure of preparation the plant extracts consisted of plant harvesting, drying, grinding, packaging and solvent extraction.

After the preliminary studies on the extraction process, heat reflux extraction (using an Soxhlet extractor) was chosen as the extraction variant. The solid-liquid extraction process took place in a Soxhlet apparatus, with a series of physical operating parameters: the solid/liquid ratio phases: 1/10; 1/15 and 1/20, extraction time = 45 min, 1 h and 2 h [43].

For the identification of the extraction yield Equation (1), a 5 mL sample of every plant extract was allowed to evaporate to dryness (at a constant temperature of up to 60 °C) using a thermostatic oven Poleko SLW53 model (Pol-Eko-Aparatura sp.j., Wodzisław Śląski, Poland).

$$\eta\% = \frac{m_{residue} \cdot V_{extract}}{n_{extract} \cdot m_{solid\ sample}} \cdot 100 \quad (1)$$

where, $\eta$%—extraction yield, $m_{\text{residue}}$—the mass of the residue obtained after evaporation to dryness, (g); $V_{extract}$—the volume of the extract sample for evaporation to dryness, (mL); $n_{extract}$—the total volume of extract obtained after the liquid-solid extraction, (mL); $m_{solid\ sample}$—the mass of vegetal powder introduced in liquid-solid extraction process (g).

### *2.4. Evaluation of the Antioxidant Activity*

#### 2.4.1. Total Polyphenols Content

A microplate spectrophotometric method based on the Folin-Ciocalteu method was used for total polyphenol content determination [44].

Folin-Ciocalteu reagent (25 µL) was added to 10 µL of plant extracts of *Origanum vulgare, Achillea millefolium, Artemisia absinthium* and *Primula veris*. Samples were kept at room temperature, and afterwards 25 µL of a 20% aqueous sodium carbonate solution was added, followed by ultrapure water after 5 min, until the final volume reached 200 µL.

Blank samples consisting of bidistilled water were also used in the assays to ensure the correctiveness of the working procedure.

Gallic and tannic acid were used as standard references, and the results are given in equivalents of gallic and tannic acid per 1 g of sample. Standards and samples were recorded after 30 min at 760 nm using a multiwell plate reader (Tecan InfinitePro 200, Männedorf, Switzerland). Experimental values were calculated using the linear regression equation of the calibration curve.

Taking into account the fact that the phenolic acids present in plants can be free molecules (gallic acid), or linked in complex structures (tannic acid), two standards were chosen. The literature data concerning the polyphenols content frequently give the same standards [45–47].

#### 2.4.2. Total Flavonoids Content

The determination of total flavonoids content was achieved by using a microplate spectrophotometric method, based on the complexation of flavonoids with aluminum chloride [48].

A total of 2% aqueous aluminum chloride solution (100 µL) was mixed with the probe (100 µL). The absorbance was obtained after 15 min of incubation at room temperature, by reading it at 415 nm using the multiwell plate reader (Tecan InfinitePro 200). The reference standards used for flavonoids determination were quercetin and rutin, and the results are given in equivalents of quercetin or rutin per 1 g of sample, considering the fact that these compounds can be in a form of flavones (quercitin) or glycosides (rutin); literature reports gave the same standards [46,47].

#### 2.4.3. DPPH Free Radical Scavenging Activity

The stable chromogenic free radical, 1,1′-diphenyl-2-picrylhydrazyl (DPPH) has been reported for the antioxidant activity scanning [49]. The DPPH radical gives a purple color at 517 nm [50]. The oxidized form of DPPH shifts from a purple color to a reduced yellow form. The ethanol—soluble antioxidant compounds were analyzed in a 96-well plate. Briefly, 100 µL of a DPPH solution (100 µg/mL concentration) was added to 100 µL methanolic extract of each sample (the same concentration used for polyphenols and flavonoids determination). The resulting solutions were then homogenized by slightly shaking them, and were then left in the dark at room temperature for 20, 35, and 50 min. Data acquisition was recorded at 517 nm after each period. A 1:1 mixture of methanol and DPPH solution was used as blank sample.

The antioxidant activity of extracts which consists in the DPPH inhibition percentage was calculated using the Equation (2):

$$\%\ \text{inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \cdot 100\% \quad (2)$$

### *2.5. FTIR Analysis*

The ethanolic plant extracts were analyzed using the Thermo Scientific Nicolet 6700 FT-IR Spectrometer which is a fully upgradeable, routinely and scientifically advanced FT-IR spectrometer that focuses on strength and versatility. Samples were scanned in the absorption range of 4000 and 600 $cm^{-1}$. The analysis was repeated twice for the spectrum confirmation.

### *2.6. Evaluation of Bioinsecticidal Activity*

The prepared extracts were used to establish the bioinsecticidal action against the adults and larvae of *Leptinotarsa decemlineata* (Colorado potato beetle) species.

Our previously established working protocol [44,48,51] includes: (i) spraying the plant extracts in the premises where the pests are found together with the food source; (ii) pest monitoring carried out at well-established intervals (2, 8, 12, 24, 48, 72, 96, 120, 144, 168 h)

after the application of the treatments (spraying with plant extracts in raw form). The method used for monitoring was adapted after Asawalam et al. [52], the mortality (%) was calculated using Equation (3).

$$\% \text{ mortality } = \frac{N_d}{N_0} \cdot 100 \quad (3)$$

where $N_d$ is the number of dead individuals and $N_0$ is the number of initial test individuals.

Beetles were collected from spots of land where no chemical treatments were administered in the last 5 years, in order to exclude a possible resistance to treatments from previous generations. The same condition was maintained in the case of food. All individuals were placed in growth cages (10 L volume) provided with ventilation holes, and were daily supplied with food (potato leaves).

The experimental variants were accompanied by a control sample. In each growth cage, the number of individuals consisted of 10 adults and 10 larvae. The introduction of the individuals in the growth cages was done 24–48 h before treatment administration, so that they can adapt to the new conditions. The administration of the extract solution was done by controlled release, and the spraying was done on an adsorbent cellulosic material placed at the top of the cage. In all experiments, extract solutions of two concentrations were used: 60% and 100%.

All treatments with the plant extracts were performed in triplicate. The differences obtained between the experimental results were highlighted in the graphs by error bars, which represent the standard error of the mean ± SEM (<5%).

### *2.7. Statistical Analysis*

The obtained results consisting of flavonoids and polyphenols content (performed in triplicate) were subsequently subjected to statistical analysis in order to determine whether the content of flavonoids and polyphenols are significantly different in each plant species. A one-way analysis of variance (ANOVA) analysis was performed to detect the differences between the averages of the concentrations of the compounds from each of the four groups (*Origanum vulgare, Achillea millefolium, Artemisia absinthium, Primula veris*) and compared with the LSD-Fisher's test ($p < 0.05$), its results being highlighted by letters.

In the case of bioinsecticidal activity induced over time by plant extracts on the adults and larvae of *Leptinotarsa decemlineata* species, the results (each with three replicates) were statistically examined with a one-way analysis of variance (ANOVA) via Minitab 17 software. The obtained data were compared with the LSD-Fisher's test ($p < 0.05$) and significant differences across the treatments were indicated by distinct letters above the columns. The 3D clustered column graphical representation was chosen to have a clearer view of low mortality as a result of treatments applied at certain time intervals.

## 3. Results

### *3.1. Antioxidant Activity*

#### 3.1.1. Total Polyphenols and Flavonoids Assay

The plant extracts obtained by heat reflux extraction were analyzed in order to determine the total polyphenols content using gallic and tannic acid as calibration standards. The obtained results are presented in Table 1.

#### 3.1.2. DPPH Free Radical Scavenging Assay

The antioxidant activity has been measured for the ethanolic plant extracts obtained by heat reflux extraction. The concentration of compounds necessary to reduce the free radical DPPH with 50% represents the $IC_{50}$ value. It was determined graphically by plotting the inhibition's percentage against the inhibitor concentration (Figure 1).

**Table 1.** Total polyphenols and flavonoids content found in the plant extracts.

| Vegetal Extract | Polyphenols, mg g$^{-1}$ (Dilution 1:1) | | | Flavonoids, mg g$^{-1}$ | | |
|---|---|---|---|---|---|---|
| | C $_{gallic\ acid}$ | C $_{tanic\ acid}$ | Total | C $_{quercetin}$ | C $_{rutin}$ | Total |
| Oregano *(Origanum vulgare)* | 12.7624 ± 0.4477 $^{a}$ | 3.4511 $^{a}$ ± 0.1855 | 16.2134 ± 0.3974 $^{a}$ | 1.3858 ± 0.3434 $^{b}$ | 5.6211± 0.5608 $^{b}$ | 7.0068 ± 0.5531 $^{b}$ |
| Wormwood *(Artemisia absinthium)* | 3.2638± 0.2273 $^{c}$ | 0.8533 $^{b,c}$ ± 0.2919 | 4.1170 ± 0.2415 $^{c}$ | 1.4944 ± 0.3042 $^{a,b}$ | 6.0652± 0.5099 $^{b}$ | 7.5596 ± 0.4513 $^{b}$ |
| Yarrow *(Achillea millefolium)* | 4.1577± 0.3750 $^{b}$ | 1.0861 $^{b}$ ± 0.0954 | 5.2438± 0.4554 $^{b}$ | 2.0325 ± 0.2871 $^{a}$ | 8.2537 ± 0.5086 $^{a}$ | 10.2863 ± 0.7360 $^{a}$ |
| Cowslip *(Primula veris)* | 2.3807 ± 0.2077 $^{d}$ | 0.6267 $^{c}$ ± 0.0503 | 3.0073 ± 0.1704 $^{d}$ | 1.0544 ± 0.3074 $^{b}$ | 4.2852± 0.3999 $^{c}$ | 5.3396± 0.0943 $^{c}$ |

The values are mean ± SD. Different letters in each column indicate significant differences among the mean of flavonoids/polyphenols content according to LSD Fisher's test ($p < 0.05$).

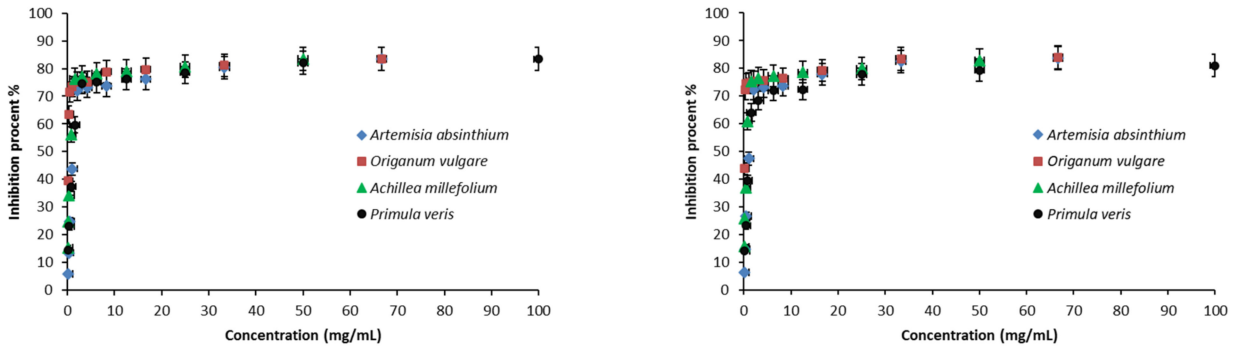



**Figure 1.** DPPH inhibition percentage of the ethanolic extracts after 20 min of incubation (**a**), and after 50 min of incubation (**b**). Values are means of three replicates ± standard deviation.

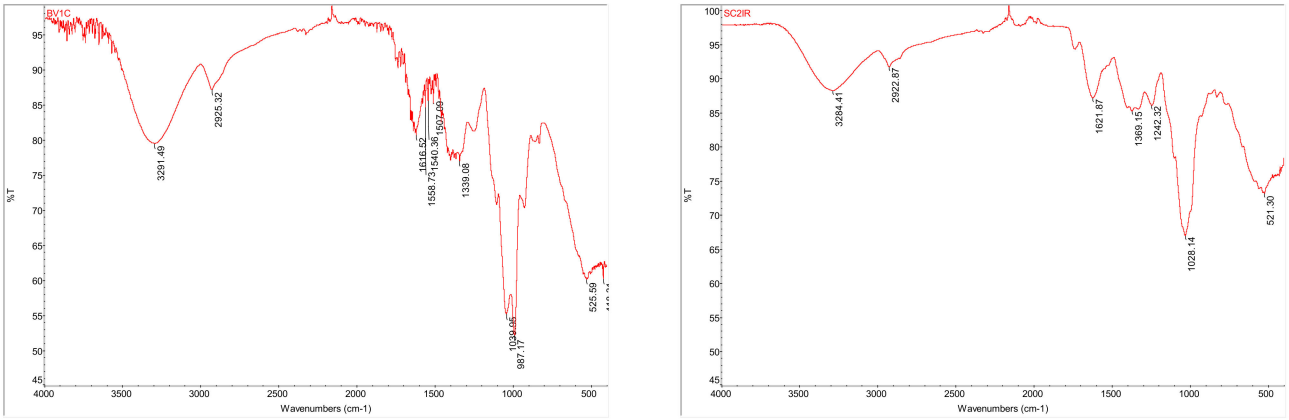



**Figure 2.** *Cont.*

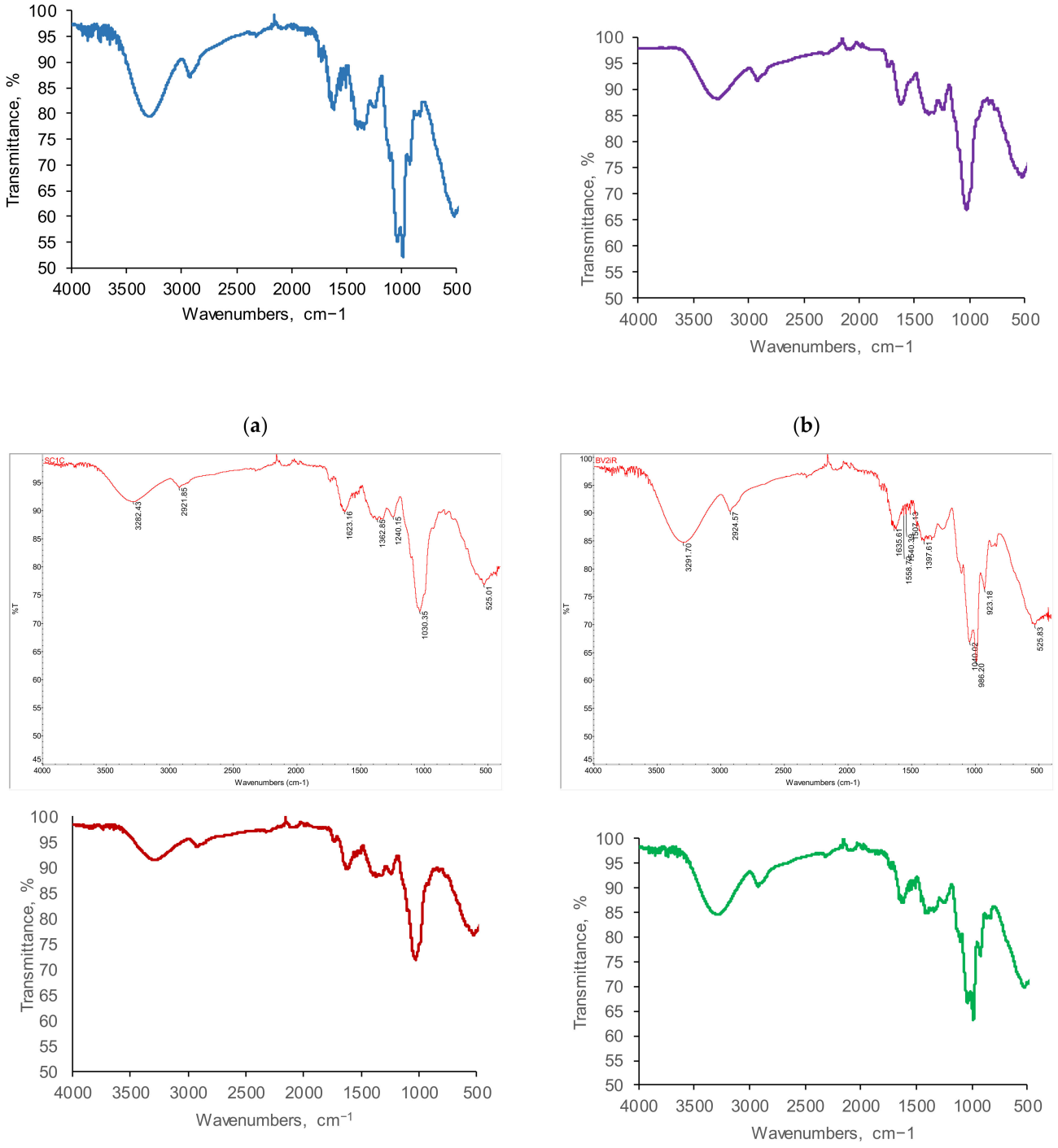
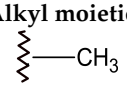
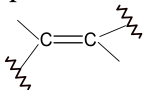
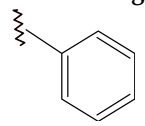
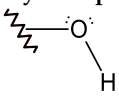
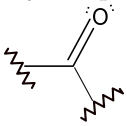
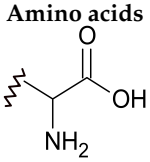
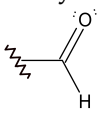
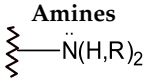
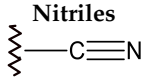
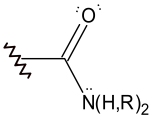
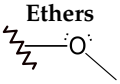



**Figure 2.** FT-IR spectra of analyzed plant extracts: (**a**) *Origanum vulgare*; (**b**) *Artemisia absinthium*; (**c**) *Achillea millefolium*; (**d**) *Primula veris*.

**Table 2.** The chemical functional groups found in the analyzed ethanolic plant extracts.

| Functional Group | Chemical Bond Vibration (Stretching/ Bending) | Range | Ethanolic Extracts | | | |
|---|---|---|---|---|---|---|
| | | | *O. vulgare* | *A. absinthium* | *A. millefolium* | *P. veris* |
| **Alkyl moieties**  | C–H stretch<br>C–H bend | 3000–2850<br>1500–1440 | + | + | + | + |
| **Olephinic moieties**  | C–H stretch<br>C=C stretch | 3200–3000<br>1680–1600 | + | + | + | + |
| **Aromatic rings**  | C–H stretch<br>C=C stretch | 3200–3000<br>1600–1400 | + | + | + | + |
| **Hydroxyl compounds**  | O–H stretch | 3600–3200 | + | + | + | + |
| **Carbonyl compounds**  | C=O stretch | 1610–1550 | + | + | + | + |
| **Amino acids**  | C=O stretch | 1600–1660 | + | + | + | + |
| **Aldehydes**  | C=O stretch<br>C–H stretch of C=O | 1750–1625<br>2700–2850 | + | + | + | + |
| **Amines**  | N–H stretch<br>N–H Bend | 3500–3100<br>1640–1550 | + | + | + | + |
| **Nitriles**  | CN stretch | 2500–2000 | + | + | + | + |
| **Amides**  | N–H stretch<br>C=O stretch<br>N–H bend | 3500–3100<br>1670–1600<br>1640–1550 | + | + | + | + |
| **Ethers**  | C–O stretch | 1040–1260 | + | + | + | + |

### *3.2. Assessing the Bioinsecticidal Activity of Plant Extracts*

The monitoring involved identifying both the number of adults and larvae in the storage cages during the setted time, and the behavior differences that may occur (the neuroleptic manifestations caused by pests such as hyperactivity, inconsistency of the hind limbs, unnatural behaviors, etc.) as a result of applying the treatment with plant extracts

(represented by dilute crude extract samples at the selected concentration: 60% and 100%) (Figures 3 and 4).

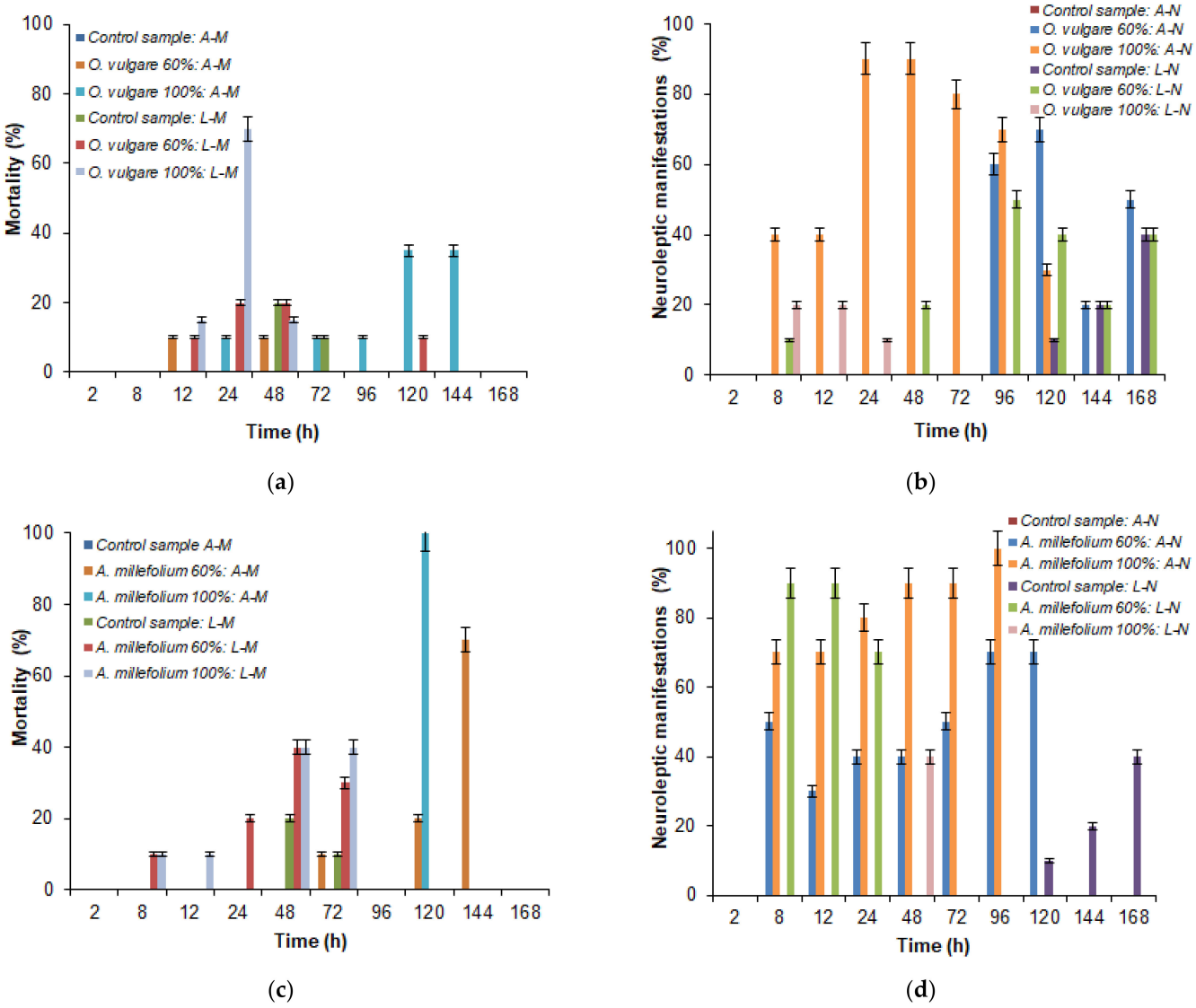



**Figure 3.** The results of the action of *Origanum vulgare* (**a**,**b**) and *Achillea millefolium* (**c**,**d**) plant extracts on a population of individuals belonging to *Leptinotarsa decemlineata* species (adults, larvae) (A-M: adults mortality; L-M: larvae mortality; A-N: adults neuroleptic manifestations; L-N: larvae neuroleptic manifestations). The columns error bars are mean ± SEM.

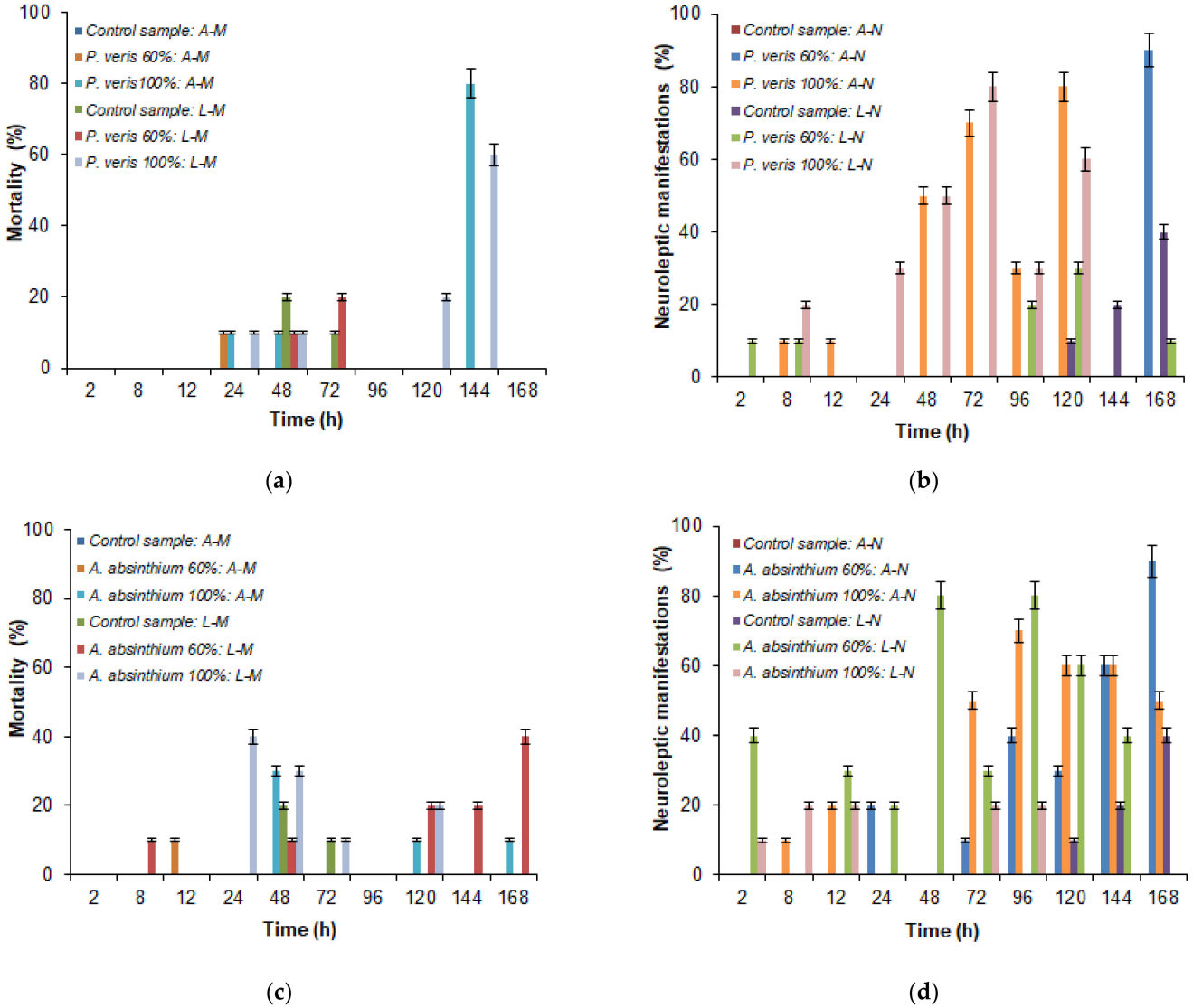



**Figure 4.** The results of the action of *Primula veris* (**a**,**b**) and *Artemisia absinthium* (**c**,**d**) plant extracts on a population of individuals belonging to *Leptinotarsa decemlineata* species (adults, larvae) (A–M: adults mortality; L–M: larvae mortality; A–N: adults neuroleptic manifestations; L–N: larvae neuroleptic manifestations). The columns error bars are mean ± SEM.

### *3.3. Statistical Analysis the Bioinsecticidal Activity of Plant Extracts*

The results of the statistical analysis concerning the registered mortality on Leptinotarsa decemlineata species (adults and larvae) in time, as a result of the Origanum vulgare 60%, Origanum vulgare 100%, Achillea millefolium 60%, Achillea millefolium 100%, Primula veris 60%, Primula veris 100%, Artemisia absinthium 60% and Artemisia absinthium 100% treatments application, are shown in Figure 5. The results of the LSD-Fisher's test are highlighted in the graphs by the letters above the columns that represent the total mortality (%) recorded as a function of time (h).

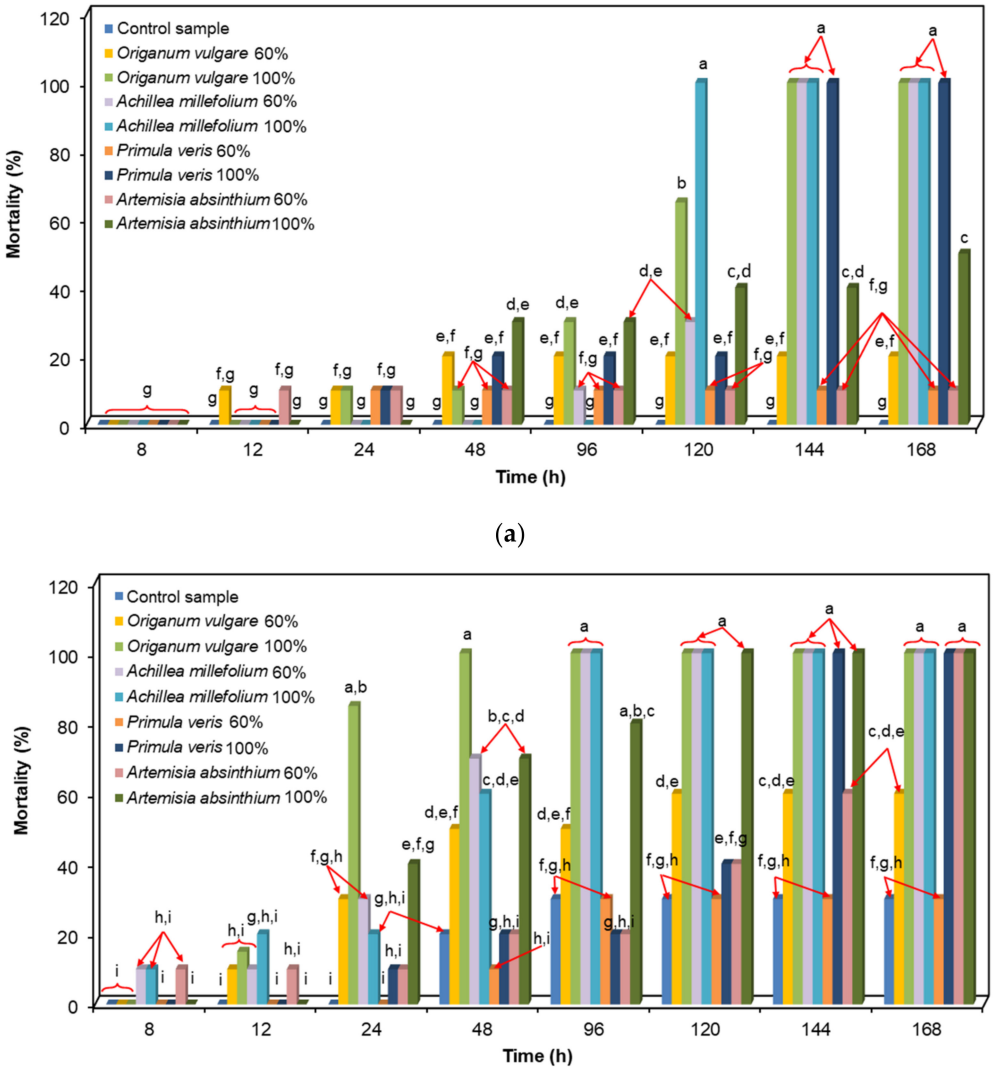



**Figure 5.** Results of statistical analysis on mortality (%) of Leptinotarsa decemlineata species: (**a**) adults and (**b**) larvae.

## 4. Discussions

The selection of the four plants used in the experiments was made following a preliminary study [43] to find those plants from the Romanian spontaneous flora that have a potential bioinsecticidal potential on crop pests (*Leptinotarsa decemlineata* species) and/or on seeds in storage (*Acanthoscelides obtectus* species). The study was initially based on extracts obtained by maceration, which allowed the selection of the most effective plants based on their extraction yields and bioinsecticidal effects [43,53].

These results were supported by early results from preliminary tests using solid-liquid extraction in the Soxhlet apparatus [54].

An analysis of the experimental data obtained emphasizes that the extraction process studied, for each plant considered, depends on the solid/liquid ratio and the extraction time. Taking into account this information (about their preparation conditions), the experimental studies were continued using those extracts characterized by the highest values of extraction yield, i.e., oregano (*Origanum vulgare*)—2 h and ratio S/L = 1/15; wormwood (*Artemisia absinthium*)—1 h and ratio S/L = 1/15; yarrow (*Achillea millefolium*)—2 h and ratio S/L = 1/20 and cowslip (*Primula veris*)—2 h and ratio S/L = 1/10.

### *4.1. Antioxidant Activity*

#### 4.1.1. Total Polyphenols and Flavonoids Assay

The experimental results for the total polyphenols content showed values ranging from 12.76 (*Origanum vulgare* extract) to 2.38 (*Primula veris* extract) mg EqGA/1 g of extract and, respectively, 3.45 to 0.62 mg EqTA/1 g of extract.

For the total flavonoids content, quantified with quercetin and rutin as calibration standards, the recorded values ranged from 8.25 (*Achillea millefolium* extract) to 4.28 (*Primula veris* extract) mg EqR/1 g of extract and 2.03 to 1.05 mg EqQ/1 g of extract. For oregano and wormwood extracts, the total flavonoids content does not significantly differ.

The yarrow extract presents the highest content in total flavonoids, respectively, 8.25 mg EqR/1 g of extract, and 2.03 mg EqQ/1 g of extract (Table 1).

Following statistical analysis, it was found that there were no statistically significant differences between the total flavonoid content of *Origanum vulgare* and *Artemisia absinthium*, but significant differences were obtained between the total polyphenol content of each plant. Statistical analysis also showed that the quercetin and rutin content of *Achillea millefolium* is significantly higher compared to the content extracted from *Origanum vulgare* and *Primula veris*. The polyphenol content (eq. GA/g extract) is significantly different in each plant, and no statistically significant differences were observed for the polyphenol content expressed using eq. tannic acid/g of extract between *Artemisia absinthium* and *Achillea millefolium* or between *Artemisia absinthium* and *Primula veris*. Fisher's LSD test recorded a $p < 0.05$ in all cases, meaning that the averages in the content of analyzed compounds (polyphenols, or flavonoids) are significantly different for at least two of the four groups analyzed.

These data demonstrated that the plant extracts have significant antioxidant activities, based on our results on polyphenols and flavonoids, compounds that are known to be important antioxidants.

#### 4.1.2. DPPH Free Radical Scavenging Assay

It has been reported that antioxidants inherently protect cells from oxidative stress, by eliminating reactive oxygen species (ROS) from biological systems [55].

Data recorded from the experiments showed that all samples had a very high antioxidant activity, with a radical scavenging capacity (RSC) of about 83%. The $IC_{50}$ values for the ethanolic extracts are as follows: oregano—8.33 mg $mL^{-1}$, wormwood—12.5 mg $mL^{-1}$, yarrow—6.25 mg $mL^{-1}$, and cowslip—12.49 mg $mL^{-1}$ (Figure 1a). After 50 min of incubation, for all extracts, the percentages of DPPH inhibition proved that the antioxidant activity of the active compounds is stable over time (Figure 1b).

The relationship between the antioxidant capacity of plant extracts and various phenolic compounds, including flavonoids, is well known. The measurement of antioxidant activity of our samples was significantly positively correlated with both free polyphenol content ($p < 0.05$; gallic acid $R^2$: 0.99827; tannic acid $R^2$: 0.99935) and total flavonoid content ($p < 0.05$; quercetin $R^2$: 0.99955; rutin $R^2$: 0.99623), suggesting that the antioxidant properties of plant extracts are due to their high polyphenol and flavonoid content.

### *4.2. FTIR Analysis*

Although the information provided by the FTIR spectrum study can be used in both qualitative assessments and quantitative determinations, in this study it was used for qualitative interpretations. Figure 2 and the data in Table 2 show that the same specific groups (carbonyl, hydroxyl) of bioactive compounds such as polyphenols, flavonoids exist in all samples used, and we used it to confirm their presence. Literature data show that FTIR analysis can be a method used to confirm the presence of bioactive compounds [56].

The FT-IR spectra (Figure 2) show the main peaks of the plant extracts analyzed in the range 4000 to 500 $cm^{-1}$. Similar trends were observed in all plant extracts. These suggest the presence of the following functional groups, which are also shown in Table 2: alkyl moieties, olefinic moieties, aromatic rings, hydroxyl and carbonyl groups, and also amino,

cyano, amide and ether groups. The characteristic bands identified are also an argument for the presence of polyphenolic and flavonoid compounds in the analyzed ethanolic extracts.

### *4.3. Assessing the Bioinsecticidal Activity of Plant Extracts*

The experimental results (Figures 3 and 4) show that in the control samples there were only a few manifestations that deviated from the normal constant and natural feeding of individuals, meaning that the deaths that occurred were considered natural.

For *Origanum vulgare* extract at 60% and 100% concentration (Figure 3a,b), the results of the applied treatments were mostly seen in the middle part of the monitoring observation, which at the end was evidenced by the high mortality (%) in both cases. In this case, the time taken for the death of individuals is directly proportional to the concentration of the treatment administered, highlighting the *Origanum vulgare* extract at 100% concentration with a mortality of 100%, for both adults (after 144 h) and larvae (after 48 h). Larvae showed the most susceptibility to treatment, while adults showed diverse neuroleptic manifestations, which did not cause immediate deaths. In Figure 3a, in the case of *Origanum vulgare*, in 100% of adults (*Origanum vulgare* 100% A-M), at 24 h, 10% had already died, and in the adjacent graph (Figure 3b) where neuroleptic manifestations are shown, it can be seen that the rest of the insect population (90%) in that interval exhibited neuroleptic manifestations. The situation remained the same at the 48 h interval, while at the 72 h interval 10% of the population had died, while neuroleptic manifestations persisted for the rest of the population (80%) remaining alive. Further, we can see that at the 96 h interval the trend continues, 10% of the remaining population had died, while for neuroleptic manifestations the value remained high (70%). While individuals did not die immediately after the administration of the treatments, and even though a mortality of more than 60% was not achieved in the case of *Origanum vulgare* extract at 60% concentration, the goal of plant protection was still reached as a lack of appetite was noticeable. This thus inhibited the individual's development, with death occurring through lack of nutrition during the monitoring period and shortly thereafter, judging by the condition of individuals 168 h after the first treatment.

In the case of *Achillea millefolium* extract at both 60% and 100% concentrations (Figure 3c,d), different manifestations occurred towards the middle part of the monitoring interval. The condition of the individuals is characterized by lethargy, showing lack of appetite. This first manifested in the initial part of the interval and more obviously towards the middle part of the 168 h of monitoring.

In the case of *Primula veris* extract samples at 60% and 100% concentration (Figure 4a,b), towards the end of the monitoring period, the individuals showed signs of weakness manifested by inactivity and subsequent death. At 100% concentration, total mortality for both adults and larvae was observed within 120–144 h. On the other side, various manifestations occurred from the second half of the monitoring interval towards the end of the period, described by sluggishness and later by lack of a feeding appetite.

When *Artemisia absinthium* extract is used in concentrations of 60% and 100% (Figure 4c,d), the impacts of the treatments administered on the *Leptinotarsa decemlineata* individuals become more apparent from the middle to the end of the monitoring period. This indicates high mortality in the case of larvae which reached 100% in 120 h, while adults reached 50% mortality after 168 h. In all cases, the mortality is directly proportional to the concentration of extract administered, meaning that plant extracts at 100% concentration have better results compared to plant extracts at 60% concentration.

In all scenarios considered, the administration of the plant extract treatment caused a range of symptoms that that emerged both at the beginning and in the middle of the monitoring interval, characterized by fatigue of individuals with lack of appetite in the second part of the monitoring period. For all plant extracts at 100% concentration, except for *Artemisia absinthium* extract, adults were found dead at the end of the monitoring period. However, in pest control the ultimate goal is to prevent pests from causing a decrease in productivity, not necessarily to kill them.

The presence of bioactive compounds in different amounts in the extracts analyzed can be correlated with the intensity of the bioinsecticidal action, which manifested differently on *Leptinotarsa decemlineata* species.

The results obtained confirm the properties of these alcoholic extracts of biopesticides. They are also in agreement with other information from the specialized literature regarding the alcoholic extracts and essential oils of the plant species studied in combating other types of pests. Thus, the study investigated the insecticidal effect of *Artemisia annua, A. absinthium, A. camphorata, A. dracunculus* and *A. vulgaris* extracts against the melonworm *Diaphania hyalinata* (Lepidoptera: Crambidae) larvae, a pest of Cucurbitaceae, and their selectivity for fire and *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) and jataí bee *Tetragonisca angustula* (Latreille) (Meliponinae). In these cases, a mortality of 42–96% was recorded, depending on the plant species and the type of pest control [57]. The team of researchers led by Dancewicz [58] used *Artemisia absinthium* L. extract and essential oils alone or mixed with other essential oils or soap solutions in pest control (peach potato aphid *Myzus persicae* (Sulz.)). The use of *A. absinthium* oil led to a mortality of 80–90% within 48 h of application. The essential oils of three species of Tunisian *Artemisia* genus were studied in order to reduce the contamination of stored cereals by *Tribolium castaneum* [59]. It was found that the essential oil of *Artemisia absinthium* had a very fast repellent action, and induced a mortality greater than 60% after 24 h of application, at an essential oil dose of 200 mL/L [59]. The extract and essential oils obtained from *Achillea millefolium* and *Origanum vulgare* were studied for their repellent action against *Acrobasis advenella* (Zinck.) (Lepidoptera, Pyralidae) which is the most dangerous pest of black chokeberry (*Aronia melanocarpa* [Michx.] Elliot) [60]. The effects of plant extracts and essential oils on the behavior of *Acrobasis advenella* (Zinck.) caterpillars and females was studied [60]. The essential oil of *Achillea millefolium* has been shown to exhibit a contact and fumigant toxicity against the adults of the species *Tetranychus urticae* Koch [61], while the essential oil of *Origanum vulgare* L. was studied for their effect on toxicity, physiology and biochemical characteristics against diamondback moth, *Plutella xylustella* L. (Lepidoptera: Pyralidae)—a major and cosmopolitan pest of crucifer crops [62]. All the essential oils of *Origanum vulgare* L. were investigated for their toxicity and physiological aspects on the lesser mulberry pyralid *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) in controlled conditions [63]. The essential oil of another species of Origanum—*Origanum glandulosum (Desf.)* was investigated for their insecticidal activities against *Rhizopertha dominica* [64].

### *4.4. Statistical Analysis of the Bioinsecticidal Activity of Plant Extracts*

The analysis of results with a one-way analysis of variance (ANOVA) and comparison of experimental results with the LSD-Fisher's test are some of the most widely used statistical methods by researchers in the field [49,65,66].

Since neuroleptic effects occurred and disappeared during monitoring intervals, these results could not be statistically analyzed. Therefore, the final effect of bioinsecticide treatment (mortality) was considered for statistical analysis.

According to the statistical analysis, the mortality recorded in adults compared to the control is significantly different after 48 h for the treatments with *Origanum vulgare* 60%, *Primula veris* 100% and *Artemisia absinthium* 100%, and after 96 h for the treatment with *Origanum vulgare* 100%. The application of *Primula veris* 60% and *Artemisia absinthium* 60% treatments did not cause significantly statistical differences in adults' mortality compared to the control, not even after 168 h. From the comparison of the mortality registered in time, it can be seen that for the treatments with *Origanum vulgare* 100%, *Achillea millefolium* 60% and *Primula veris* 100% after 144 h, no more differences in adult mortality of *Leptinotarsa decemlineata* have been registered. The same effect over time was observed for *Achillea millefolium* 100% treatment after 120 h. For *Leptinotarsa decemlineata* larvae, it was observed that the treatments have more effectiveness and comparatively significant differences to the control samples, starting with the 24th hour of treatment. The highest mortality was recorded, after 24 h, for the treatment with *Origanum vulgare* 100% followed by the treatment

with *Artemisia absinthium* 60%, *Origanum vulgare* 60% and *Achillea millefolium* 60%. The statistical analysis of the results also showed that the treatment with *Primula veris* 60% has no significant effect either on *Leptinotarsa decemlineata* larvae. The maximum effect that *Origanum vulgare* 60% and *Origanum vulgare* 100% registered starting with the 48th hour of treatment, and for *Achillea millefolium* 60% and *Achillea millefolium* 100% treatments with the 96th hour of treatment.

### *4.5. Insecticidal and Repellent Effect of Plant Compounds and Their Mechanism of Action*

The literature shows that the four plants considered in this study exhibit a number of compounds with insecticidal, repellent, vermicidal, and pesticidal effects, etc. In the following we outline the most important compounds found in significant amounts and their insecticidal effect for each plant species, as reported in the literature.

In the case of the species studied, *Origanum vulgare, Achillea millefolium, Arthemisia absinthium* and *Primula veris*, a series of chemical compounds were identified using mainly HPLC-MS and GC-MS analysis. The compounds with potential were α- and β-pinen, α-terpineol, α- and β-thujone, borneol, eugenol, carvone, and sabinene, etc., which in some cases exhibited a pesticidal effect in addition to their antioxidant action [36–40,67].

According to Alkan (2020), the essential oil extracted from *Achillea millefolium* showed promising results against *R. dominica* pests 24 h after treatment, with 99.2% mortality recorded at a dose of 0.15 (*v*/*v*), and 83.4% mortality for *S. granari* pests. For both pests, in addition to the high mortality recorded, the repellent effects of the essential oil extracted from *Achillea millefolium* were also recorded [40]. In another study, Zhou et al. (2019) demonstrated the insecticidal, germicidal and repellent effects of the sabinene compound against *Sitophilus granaries* pests. It showed a repellent effect at low doses, and the authors concluded that the main compound responsible for the effects of insecticidal activity was the sabinene compound [67]. Adrianjafinandrasana et al. (2013) also reported promising results of the sabinene compound on pests of the plant species *Lepidium sativum* and *Vigna radiate* [68]. The potential of the sabinene compound was evaluated in another study by Wang et al. (2011), the authors concluding that it exhibited a strong toxic effect on adults of *Sitophilus zeamais* species at a dose of 9.12 mg/L [69]. Tampe (2015) demonstrated the efficacy of α- and β-thujone compounds with a repellent effect against the pest *Aegorhinus nodipennis* [36]. Terpenes are also compounds considered to have insecticidal and repellent effects against several pests, such as *Blatta orientalis* and *Musca domestica*, and against specific grain storage pests. The manifestations of pests under neuroleptic action of terpene compounds include hyperactivity, convulsions and tremors, followed by paralysis [70]. Monoterpenoid compounds also target insect-specific octopamine receptors, with α-terpineol shown to have a high binding affinity to the octopamine receptor in the species *Periplaneta americana* [71].

It has also been reported that 1-2-cineole inhibits the enzyme acetylcholinesterase, blocking the octopamine receptor pathway. Another mechanism of action of compounds with desired effects in pest control is inhibition of the most important neurotransmitter, the gamma-aminobutyric acid (GABA)-gated neurotransmitter in the central nervous system of insects, which causes hyperexcitation, convulsions and insect death [72]. In addition, α-thujone is a potent neurotoxin that inhibits the GABA system and is considered a neurotoxic insecticide [36]. Nasr et al. (2017) concluded that *O. vulgare* essential oil possesses larvicidal effects on *P. xylutella*, and in sub-lethal concentrations acts as an antifeedant [62].

*Primula veris* is notable for its high content of saponins and flavonoids, with these compounds having a role in pest control. Saponins are known to have a high toxicity, which increases the results obtained by administering such treatments based on *P. veris*. The main substances identified in *Primula veris* with insecticidal effect were quercetin 3-O-rutinoside (rutin) and isorhamnetin 3-O-rutinoside [73].

According to Goławska et al. [74], the intake of flavonoids constituted by the compound quercetin in the diet of *Acyrthosiphon pisum* insects led to a significant rise in the reproductive period, a reduction in fecundity, a mean generation development time, de-

creased intrinsic natural growth rate and increased adult mortality. It has also been found that flavonoids bind the ecdysone receptor of insects.

## 5. Conclusions

To avoid soil contamination with emerging pollutants such as pesticides, this study focused on the use of plant extracts obtained by the process of thermal reflux extraction from *Origanum vulgare, Achillea millefolium*, and *Artemisia absinthium* and *Primula veris*, for the assessment of their bioinsecticidal action on pests and antioxidant activity.

The content in polyphenols and flavonoids in the analyzed extracts could be associated with the intensity of bioinsecticidal action manifested differently in adults and larvae of *Leptinotarsdecemlineata* species.

These results demonstrate that the studied plant extracts presented noteworthy potential as development disruptors, being considered inducers of the lethal effect against *Leptinotarsa decemlineata* species. Thus, the studied plant extracts could fulfill an important double role: the bioinsecticidal action makes them protectors of the soil from synthetic chemicals, also ensuring the protection of plants from specific pests.

Following the results, it was found that the extracts have good bioinsecticidal action and also the advantage that they can be used as raw extracts, without considering further steps such as separation and purification. Moreover, the plant extracts studied could be used successfully in the control of the adults and larvae *Leptinotarsa decemlineata* species in farming systems, being recommended as a source of bioinsecticides for organic agriculture.

These results could be a starting point for the expansion of studies on biopesticides obtained from spontaneous or cultivated flora and their use to control pests in deposits, considering the specific climate conditions of each geographical region.

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