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Original paper

Efficacy of *Echium* spp. water extracts as post-harvest grain protectants against *Plodia interpunctella*

FILIP N. VUKAJLOVIĆ[1,*], SNEŽANA B. PEŠIĆ[1], SNEŽANA T. TANASKOVIĆ[2], DRAGANA Z. PREDOJEVIĆ[1], SONJA M. GVOZDENAC[3], DEJAN M. PRVULOVIĆ[4], VOJISLAVA P. BURSIĆ[4]

[1]University of Kragujevac, Faculty of Science, Radoja Domanovića 12, 34000 Kragujevac, Serbia
[2]University of Kragujevac, Faculty of Agronomy, Cara Dušana 34, 32000 Čačak, Serbia
[3]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia
[4]University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, 21101 Novi Sad, Serbia

Abstract Water extracts of three plant species of the genus Echium (*E. italicum* L., *E. vulgare* L. and *E. rubrum Jacq.*) were tested under laboratory conditions for their activity as potential post-harvest grain protectants against Plodia interpunctella (Hübner, 1813) (*Lepidoptera: Pyralidae*) larvae. Antioxidative activity and total phenolic, flavonoid and tannin contents in plant extracts were determined. The larvicidal assays were conducted with the local population of *P. interpunctella* from Central Serbia on kernels of Takovčanka winter wheat cultivar. The experiment was set as factorial 3x3x3x3 block design type. Three groups of larvae of different maturity were used. The larvae were exposed to three different concentrations of water extracts (1%, 2% and 5%) of three listed plant species. Mortality was registered daily during 96 hours and the efficacy was calculated using Schneider-Orelli's formula. The largest number of dead larvae was recorded in the treatment of 1% extract of *E. italicum* on youngest group, after 72 and 96 h. Extracts of *E. vulgare* and *E. rubrum* did not show high larvicidal effect.

Keywords Indian meal moth, wheat, plant extract, efficacy, *Echium.*

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*Corresponding author: FILIP N. VUKAJLOVIĆ, University of Kragujevac, Faculty of Science, Radoja Domanovića 12, 34000 Kragujevac, Serbia
E-mail: fvukajlovic@kg.ac.rs

Introduction

Wheat and its products are among the most important components of human and animal diet. It is strategically very important cereal in Republic of Serbia, where it is the 2nd most produced and exported cereal (Anonymous 1, [1]). In Republic of Serbia, wheat production is increasing in rural, mountainous areas, mainly economically undeveloped areas of the country, where its low input requirements and cold resistance make the crop economically viable (Anonymous 2, [2]). Stored wheat can be damaged or destroyed by a number of different pests, mostly insects, in facilities/warehouses, where storage conditions suit their development.

One of the major insect pests of stored, raw or processed food, including wheat, is Indian meal moth (IMM), *Plodia interpunctella* (Hübner, 1813) (Lepidoptera: Pyralidae) (reviewed in S. Mohandass & al [3]). IMM larvae cause the most of the damage to the stored food. They reduce the quality of stored food by leaving silk cover, feces and exuviae in infested products. In the same time, they reduce the quantity of stored food by feeding themselves (S. Mohandass & al [3]). Small farms in Serbia have a number of problems when storing products in mini-warehouses. Family agricultural holdings make 99.6% of total number of holdings in Serbia (D. Marković, [4]) and they usually have inadequate storage places (loft, woody places, cellars), which favors the development of IMM. In such conditions, synthetic insecticides are usually applied to mitigate damages caused by storage pests (J.J. Menn, [5], L.M. Redlinger & al [6]). However, the use of conventional insecticides can cause numerous side effects, such as toxicity to consumers of stored wheat, development of pest resistance, increased cost of insecticide applications and therefore the increase in wheat production costs. Also, due to lack of funds, conditions in mini storage systems are inadequate for effective conventional chemical control. All this implicates that safe, biodegradable, nontoxic compounds should be used as a control measure. Due to increased environmental and health demands, the promotion of pest control agents of botanical origin (plant-based insecticides) is gaining in importance (S. Gvozdenac et al [7]).

Plants from family Boraginaceae, including *Echium* species, are rich in pyrrolizidine alkaloids – PA (Stegelemeier, [8]). Those compounds are secondary metabolites which plant produces as chemical defense against herbivores, especially insects (T. Hartmann, [9]). PAs are commonly called "natural pesticides", because they are often toxic to insects (T. Hartmann, [9]) and therefore they represent a competitive advantage for plants that produce them. The only three species of the genus *Echium* present in the flora of Serbia are *E. vulgare* L., *E. italicum* L. and *E. rubrum* Jacq. (M. Josifović, [10]). Extracts of these species have good antimicrobial and antioxidative activity (N. Nićiforović & al [11], [12]).

The aim of this work was to determine the efficacy of water extracts from three *Echium* species on stored wheat against IMM larvae and to estimate their potential as post-harvest grain protectants.

Materials and Methods

Insect culture

Indian meal moth larvae used in this study, originate from mass rearing laboratory population, bred for several generations in Laboratory for General and Applied Entomology, at the Faculty of Science, University of Kragujevac, Republic of Serbia. Larvae were reared in a controlled laboratory conditions – in transparent, plastic containers (1.5 L in volume), on standard laboratory diet for *P. interpunctella* (D.L. Silhacek & G.L. Miller, [13]), in thermostat chamber, at 28 ±1°C, r.h. 60 ±10% and 14:10 (L:D) photoperiod.

Wheat

Takovčanka winter wheat cultivar kernels were used as nutritive substrate for assays, because it is one of the most grown cultivar in Serbia (S. Denčić & al [14]). This cultivar belongs to the group of very good bread cultivars, according to technological quality of grain, milling, flour, baking and bread (M. Madić & al [15]). In our assays, we used 10 g of Takovčanka cultivar kernels per replication as nutritive substrate. Kernels (10 g) for assays were sodden in 1 mL of adequate extract solution, while for untreated control 1 mL of distillated water was used. After that kernels were air dried.

Collection and preparation of plant material

Plant species were identified and the voucher specimens were deposited at the Department of Botanics, Faculty of Biology, University of Belgrade (BEOU). Plant specimens of *E. vulgare* were sampled in Gamzigrad, East Serbia (Voucher No 16283, BEOU), *E. rubrum* on Goč mountain, Central Serbia (Voucher No 16284, BEOU) and *E. italicum* on Vidlič Mountain, East Serbia (Voucher No 16283, BEOU). Collected material was dried in the windy, dark place, at 20°C and chopped afterwards. Extraction was carried out using a Soxhlet device. After 24 hours, filtration was performed with Whatman filter paper No. 1 and the supernatant was matched on a rotary vacuum evaporator. Preparation of plant extracts was performed according the method of Nićiforović et al (2009). Extracts were applied at rates 1, 2 and 5%.

Analytical methods

For biochemical analysis, 10 mg of dry matter was diluted with 50 mL of 70% methanol.

Tests of antioxidative activity

The antioxidative activity of plant methanol extracts was assessed using five different tests.

The total antioxidant capacity (TAC) of plant extracts was evaluated by phosphomolybdenum method (P. Prieto & al [16]). An aliquot of 100 µL of plant extract solutions were combined with 3 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). All tubes were capped and incubated in a

boiling water bath at 95°C for 90 min. Tubes were allowed to cool at room temperature. Absorbance of the test and standard solutions was measured at 695 nm against blank consisting 0.1 mL of 70% methanol and 3 mL of reagent. The standard curve for TAC was plotted using butylated hydroxytoluene (BHT) solution. Antioxidant capacities of plant extracts were expressed as mg BHT equivalents (BHTE) per gram of dry weight of extract (DWE).

The DPPH (1,1-Dyphenyl-2-picrylhydrazyl) radical scavenging assay is employed as described in N. Abe & al [17]. The degree of decoloration of solution indicates the scavenging efficiency of the substance added. DPPH is a stable free radical and accepts an electron or hydrogen to become a stable diamagnetic molecule. Plant extracts (200 μL) were added to 2.0 mL of 50 μM methanol DPPH solution. The mixture was left in the dark for 30 min before reading the absorbance at 517 nm with 70% methanol as blank. Radical scavenging activity was expressed as mg Trolox equivalents (TE) per gram of DWE.

The FRAP (ferric reducing antioxidant power) assay was performed according to the method of P. Valentão & al [18]. FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} into Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming a blue Fe^{2+}-TPTZ complex. This reaction is pH-dependent, with an optimum at pH = 3.6. The absorbance increase is proportional to the antioxidant content in samples. The working FRAP reagent was prepared daily by mixing 10 volumes of 300 mM acetate buffer, pH = 3.6, with 1 volume of 10 mM TPTZ in 40 mM hydrochloric acid and with 1 volume of 20 mM ferric chloride. Results were expressed as mg TE per gram of DWE.

The superoxide free radical scavenging activity was carried out by NBT (nitroblue tetrazolium) test (K.G. Mohan & S.J. Sanjay [19]). The 200 μL of EDTA, 100 μL of NBT, 50 μL of riboflavin, 2.5 mL of phosphate buffer pH = 8.0 and 200 μL of plant extract solutions were mixed in the glass tubes. Reaction commenced by illuminating the reaction mixture for 20 min using a fluorescent lamp. After illumination, the absorbance was measured at 590 nm. The same procedure followed for control by replacing methanol in place of samples. The percent inhibition of superoxide anion generated was calculated using the following formula:

Scavenging activity (%) = (1 – absorbance of sample/ absorbance of control) x 100

A reducing power assay (RP) was performed according to the method of M. Oyaizu [20]. Plant extract solutions (200 μL) were mixed with 3 mL of potassium ferricyanide (1% w/v) in phosphate buffer (pH = 6.6) and incubated at 50°C for 20 min. 1.5 mL of trichloroacetic acid (10% w/v) was added and centrifuged for 10 min. 3 mL of supernatant was mixed with 500 μL of ferric chloride (0.1% w/v) and absorbance was measured at 700 nm. Trolox was used as a standard. Results were expressed as mg TE per gram of DWE.

Determination of total phenolics, tannins and flavonoids content in plant extracts

The total phenolic content was determined according to Folin-Ciocalteu's method (G.T. Kroyer [21]). Extract (0.02 mL) was mixed with 3.36 mL of deionized water and 0.2 mL of 33% Folin-Ciocalteau reagent. Ten minutes later, 0.4 mL of 20% sodium carbonate was added. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 720 nm on a spectrophotometer. Quercetin was used as a standard and results were expressed as milligrams of quercetin equivalents (QE) per gram of DWE.

Total tannins content was determined by the Folin-Ciocalteu procedure, after removal of tannins by their adsorption on insoluble matrix (polyvinylpolypyrrolidone) (H.P.S. Makkar & al [22]). Calculated values were subtracted from total phenolic contents, and total tannin contents were expressed as milligrams of quercetin equivalents (QE) per gram DWE.

Total flavonoid content was estimated according to the method described by K.P. Markham [23]. Extract (0.4 mL) was mixed with 1 mL of deionized water and 2.5 mL of aluminum chloride hexahydrate solution. After incubation at room temperature for 15 min, the reaction mixture absorbance was measured at 430 nm. The total flavonoid content was determined using a standard curve with quercetin as a standard. The mean of three readings was used and expressed as mg of quercetin equivalents (QE) per gram of DWE.

Experimental procedure

The experiment was set up as factorial 3x3x3x3 block design type. We used water extracts of three mentioned *Echium* species, in three different concentrations (1, 2 and 5%). Three groups of larvae of different maturity were used (S_1< 7, S_2 7-14 and S_3> 14 days old). Each treatment was repeated three times. One treatment, placed in Petri dish (ø 8 cm), contained 10 larvae from the same maturity group and 10 g of dry wheat kernels treated with extracts. For each maturity larval group three replications of untreated control were used. One untreated control, placed in Petri dish, contained 10 larvae and 10 g of dry wheat kernels treated with distilled water. Mortality was recorded after 24, 48, 72 and 96 h.

Statistical analysis

The efficacy was calculated using O. Schneider-Orelli [24] formula. The differences between the efficacy of extracts regardless on concentration and larval stage were processed using Duncan's multiple range test for confidence interval of 95%, or non-parametric Kruskal-Wallis test in cases when variances were not homogenous and distribution diverged from normal, Gausian distribution. Two-way ANOVA was used to analyze the main effects of single factors and interactions as follows: concentration, larval stage, concentration x larval stage; concentration, exposure, concentration x exposure. Statistical software IBM SPSS 21 was used.

Results

Antioxidative activity and total phenolic, flavonoid and tannin content in plant extracts

The antioxidative activity of plant extracts was assessed using five tests (TAC, DPPH, FRAP, NBT and RP). The highest total antioxidative capacity(TAC) was registered for *E. vulgare* and was on significantly higher level compared to *E. italicum* and *E. rubrum* (F= 25.30**, P<0.01). However, in DPPH, FRAP and RK test, *E. rubrum* expressed the highest antioxidative activity. The differences between test values were highly significant (F= 44.14**; 59.90**, 21.11**, P<0.01, respectively) (Tab. 1).

Table 1. Antioxidative activity of *Echium* sp. water extracts using five different tests

Extract	TAC[1]	DPPH[2]	FRAP[2]	NBT[3]	RP[1]
E. vulgare	477.58 ±36.70[a]	47.98 ±6.34[b]	71.89 ±14.90[b]	81.52 ±19.40[a]	464.32 ±81.20[b]
E. italicum	334.73 ±12.90[b]	47.28 ±8.59[b]	49.82 ±16.30[c]	92.72 ±8.62[a]	439.61 ±64.70[b]
E. rubrum	328.24 ±78.00[b]	78.18 ±8.73[a]	122.07 ±11.30[a]	81.52 ±19.40[a]	638.74 ±65.40[a]
F value	**25.30****	**44.14****	**59.90****	**1.36**	**21.11****

Mean values ± *SD*. [a-c] values without the same superscripts are on the same level of significance for confidence interval 95%, **P < 0.01; * P< 0.05; [1]mg BHTE/g DW; [2]mg TE/g DW; [3] %.

Phenolic content was detected in highest amount in *E. italicum* (178.25 mg/g) and *E. rubrum* extract (168.78 mg/g), flavonoid content was the highest in *E. vulgare* extract (22.06 mg/g), while the content of tannins was the highest in *E. italicum* (155.06 mg/g) and *E. rubrum* (132.04 mg/g). The differences between phenol, flavonoid and tannin contents in tested extracts were highly significant (F = 13.93**; 14.77**; 136.66**, P < 0.01, respectively) (Tab. 2).

Table 2. Content of total phenols, flavonoids and tannins (mg/g) in *Echium* spp. water extracts

Extract	Phenols	Flavonoids	Tannins
E. vulgare	116.41 ± 28.32[b]	22.06 ± 3.46[a]	78.77 ± 5.89[c]
E. italicum	178.25 ± 30.86[a]	12.74 ± 1.99[c]	155.06 ± 6.96[a]
E. rubrum	168.78 ± 19.90[a]	18.40 ± 4.93[b]	132.04 ± 14.8[b]
F value	13.93**	14.77**	136.66**

Mean values ±SD.[a-c] values without the same superscripts are on the same level ofsignificance for confidence interval 95%, **P<0.01; *P <0.05.

The results of Correlation and Regression analysis (Tab. 3) indicate at strong dependence of TAC, DPPH, FRAP and RP test results on phenol content which is also expressed in high values of determination coefficient. The dependence of tests on flavonoid and tannin content was not strong, which is indicated in low values of determination coefficient (< 0.700). RP test was not flavonoid dependent at all (Tab. 3).

Table 3. Correlation between phenol, flavonoid and tannin content and test and Regression models

Active substance	Test	Pearson Correlation	*P*	Determination Coefficient - R	R Square	Adjusted R Square	SE of the Estimate
Phenols	TAC	0.553**	0.000	0.553	0.306	0.286	141.692
	DPPH	0.765**	0.000	0.765	0.585	0.573	25.239
	FRAP	0.773**	0.000	0.773	0.598	0.586	80.830
	RP	0.652**	0.000	0.652	0.426	0.409	113.427
Flavonoids	TAC	- 0.356*	0.033	0.356	0.127	0.101	158.950
	DPPH	- 0.476**	0.003	0.476	0.227	0.204	34.464
	FRAP	- 0.515**	0.001	0.515	0.265	0.244	109.277
	RP	- 0.291	0.085	0.291	0.085	0.058	143.206
Tannins	TAC	- 0.660**	0.000	0.660	0.435	0.419	127.840
	DPPH	- 0.400*	0.016	0.400	0.160	0.135	35.925
	FRAP	- 0.486**	0.003	0.486	0.236	0.213	111.449
	RP	- 0.332*	0.048	0.332	0.110	0.084	141.192

Mean values ±SD. **P<0.01; *P<0.05.

Tests of insecticidal activity

Results of the efficacy of applied extracts with significant differences, regarding larval stage and concentrations are presented in Tab. 4-6.

Echium italicum

Results of the efficacy of *E. italicum* water extract is presented in Tab. 4. The highest efficacy of *E. italicum* on S_1 larvae was achieved when applied at 1% concentration after 72 and 96 h (59.26%, 58.33%, respectively) and was statistically higher compared to the efficacy after 24 and 48 h (35.00; 32.78%, respectively). Differences between efficacy of 1% extract achieved after different exposure periods are statistically highly significant (F = 504.08**, P < 0.01), and increased in time. When applied at 2% concentration, there were no significant differences in efficacy among exposure periods (F = 1.11, P > 0.05). At 5% the statistically highest efficacy was registered after 96 h. The efficacy of 2 and 5% extract were not satisfactory (22.41-37.43%), regardless exposure. The increase of concentration resulted in significant decrease of the efficacy on S_1 larvae, after 24, 72 and 96 h (F / H = 44.45**; 332.28**; 6.40*, P > 0.01 respectively), while after 48 h, the differences were not significant (F = 1.58, P > 0.05).

In assays with S_2 larvae, the only significant efficacy (41.44-66.67%) was achieved at 1% concentration after 48, 72 and 96 h and at 2% after 72 and 96 h. In other treatments and exposure periods efficacy ranged from 21.11 to 36.79% and was statistically at lower level compared to the above mentioned. However, the efficacy significantly decreased with the increase of concentrations after all exposure periods (F / H = 54.49*; 63.26**; 4.62*; 5.76*, P > 0.01, respectively) which was due to increasing mortality in the control treatment. When observed from the aspect of exposure, at all applied concentrations, the efficacy increased in time and differences were significant (H = 10.26*; 10.53*; 9.68*, P < 0.05, respectively).

The efficacy on S_3 larvae was in general very low (17.78-43.33%), but the highest (42.06, 43.33%, respectively) was registered in treatments with 2 and 5% extracts after 96 h and was at statistically higher level compared to 1% (21.48%) after the same exposure period (F = 4.12*, P < 0.01). With the increase of exposure, the efficacy increased at all applied concentrations, and it was the highest after 72 and 96 h with statistically significant differences (F = 75.91*, 5.25*, 195.77**, P < 0.05, respectively).

Table 4. Efficacy (%) of *E. italicum* water extract applied at different concentrations (1, 2, 5%) on mortality of three larval stages of IMM

Larval stage	Exposure (h)	Concentrations (%)			F / Hvalue
		1	2	5	
S_1	24	35.00 ± 8.66 aB	22.41 ± 2.50 cA	28.51 ± 5.70 bB	44.45**
	48	32.78 ± 7.51 aB	30.55 ± 4.81 aA	30.55 ± 4.81 aAB	1.58
	72	59.26 ± 6.1 aA	27.78 ± 4.81 bA	27.78 ± 4.81 bB	332.28**
	96	58.33 ± 9.4 aA	24.21 ± 9.54 cA	37.43 ± 7.2 bA	6.40*
	Fvalue	504.08**	1.11	9.02*	
S_2	24	32.50 ± 4.33 aB	35.18 ± 3.90 aB	21.11 ± 9.49 bC	54.49*
	48	41.11 ± 8.39 aAB	38.88 ± 9.62 abAB	21.11 ± 9.49 bC	63.26**
	72	44.81 ± 5.01 aAB	44.44 ± 9.62 aA	25.28 ± 9.30 bB	4.62*
	96	66.67 ± 11.5 aA	47.78 ± 2.55 bA	36.79 ± 6.46 cA	5.76*
	F value	10.26*	10.53*	9.68*	
S_3	24	17.78 ± 5.87 aB	17.78 ± 5.87 aB	23.33 ± 9.50 aB	0.49
	48	17.78 ± 5.87 cB	35.03 ± 7.20 aA	26.67 ± 5.77 bB	32.58**
	72	21.48 ± 1.20 bA	35.33 ± 4.04 aA	33.33 ± 5.30 aAB	61.39*
	96	21.48 ± 5.18 bA	42.06 ± 8.36 aA	43.33 ± 6.74 aA	4.12*
	F value	75.91*	5.25*	195.77**	

The results represent mean ± SD; Mean values with the same lowercase letters indicate the same level of significance in rows-between concentrations (α = 0.05); Mean values with the same uppercase letters indicate the same level of significance in columns-between different exposure period (α = 0.05); ** P < 0.01; * P < 0.05.

Echium vulgare

Results of the efficacy of *E. vulgare* water extract is presented in Tab. 5. Water extract of *E. vulgare* expressed very low efficacy on S_1 larvae, regardless on the exposure and concentration (0-14.80%) (Tab. 5). The highest efficacy was registered when applied at 2% concentration after 48 h, although it significantly decreased in time (F = 8.67*, P < 0.05). Higher efficacy was achieved on S_2 larvae (13.70-26.43%) when applied at 5% concentration after 96 h (26.43%). The efficacy on S_2 larvae increased with the increase of concentration, with statistically significant differences after 48, 72 and 96h of exposure (F = 177.12**, 135.87**, 60.02**, P < 0.01, respectively), but with no practical importance because the final mortality was very low. S_3 larvae were resilient to this extract, because the only and very low efficacy (6.67-13.33%) was registered in 5% treatment after 48, 72 and 96 h.

Table 5. Efficacy (%) of *E. vulgare* water extract applied at different concentrations (1, 2, 5%) on mortality of three larval stages of IMM

Larval stage	Exposure (h)	Concentrations (%)			F / H value
		1	2	5	
S_1	24	0	0	0	0
	48	3.33 ±0.33 cC	14.80 ±1.33 aA	11.11 ±1.33 bA	69.67**
	72	6.67 ±0.43 bB	11.11 ±1.60 aB	11.11 ±1.33 aA	8.55*
	96	7.41 ±0.37 bA	11.11 ±1.60 aB	8.47 ±0.94 bA	10.27*
	F value	99.81**	8.67*	2.68	
S_2	24	0	0	0	0
	48	13.70 ±0.91 bB	13.33 ±0.40 bB	21.18 ±0.53 aB	177.12**
	72	17.40 ±0.54 bA	13.33 ±0.40 bB	21.80 ±0.41 aB	135.87**
	96	17.40 ±0.54 cA	20.37 ±0.36 bA	26.43 ±0.89 aA	60.02**
	F value	38.18*	329.17**	153.46*	
S_3	24	0	0	0	0
	48	0	0	6.67 ±0.31 B	0
	72	0	0	10.00 ±0.30 A	0
	96	0	0	13.33±0.41 A	0
	F value	0	0	253.92**	

Mean ± SD; Mean values with the same lowercase letters indicate the same level of significance in rows -between concentrations (α = 0.05); Mean values with the same uppercase letters indicate the same level of significance in columns - between different exposure period (α = 0.05); ** P < 0.01; * P < 0.05.

Echium rubrum

Results of the efficacy of *E. rubrum* water extract is presented in Tab. 6. Water extract of *E. rubrum* in general achieved very low efficacy on S_1 larvae, which ranged from 3.70 to 21.48% (Tab. 6). There is slight inconsistence when observed the efficacy in different exposure periods, as well as concentrations; in 5% treatment, the efficacy is decreasing with in time, which can be attributed to increased mortality in the control. S_2 larvae were more resilient to extract and the efficacy was insignificant (3.33-10.74%). S_3 larvae were totally unaffected by this extract, but only in 5% concentration after 48, 72 and 96 h a slight efficacy (3.33-6.67%) was registered, but with no practical importance.

Factor analysis

Due to very low efficacy of *E. vulgare* and *E. rubrum* extracts on IMM larvae, the Two-way ANOVA was performed only for *E. italicum* (Tab. 7).

Table 6. Efficacy (%) of *E. rubrum* water extract applied at different concentrations (1, 2, 5%) on mortality of three larval stages of IMM

Larval stage	Exposure (h)	Concentrations (%)			F / H value
		1	2	5	
S1	24	10.00 ± 0.85 aB	6.67 ± 0.42 bA	10.37 ± 1.05 aA	17.43**
	48	10.37 ± 0.85 aB	7.41 ± 0.38 bA	7.41 ± 0.38 bB	19.28**
	72	21.48 ± 2.56 aA	3.70 ± 0.44 bB	3.71 ± 0.39 bC	5.61*
	96	12.50 ± 0.81 aB	7.41 ± 0.28 bA	3.71 ± 0.39 cC	197.98**
	F value	237.69**	78.02**	103.51**	
S2	24	0	0	0	0
	48	0	0	10.74 ± 0.69 A	0
	72	3.33 ± 0.11 bA	3.33 ± 0.11 bB	7.41 ± 0.76 aB	187.23**
	96	3.70 ± 0.21 bA	7.04 ± 0.37 aA	7.04 ± 0.37 aB	68.14**
	F value			51.21**	
S3	24	0	0	0	0
	48	0	0	3.33 ± 0.31 B	0
	72	0	0	3.33 ± 0.34 B	0
	96	0	0	6.67 ± 0.37 A	0
	F value	0	0	82.76**	

Mean ± SD; Mean values with the same lowercase letters indicate the same level of significance in rows-between concentrations (α = 0.05); Mean values with the same uppercase letters indicate the same level of significance in columns-between different exposure period (α = 0.05); ** P < 0.01; * P < 0.05.

Table 7. Effect of concentrations and exposure periods on efficacy of *E. italicum* calculated with a Two-Way ANOVA

Larval stage	Source of varition	Sum of Squares (SS)	df	Mean Square	F	*P*
S_1	concentration	2643.586	2	1321.793	16.946	0.000
	exposure	800.694	3	266.898	3.422	0.033
	concentration x exposure	1363.043	6	227.174	2.912	0.028
S_2	concentration	2556.102	2	1278.051	9.570	0.001
	exposition	1705.931	3	568.644	4.258	0.015
	concentration x exposure	825.573	6	137.595	1.030	0.430
S_3	concentration	1350.236	2	675.118	7.324	0.003
	exposure	1263.690	3	421.230	4.570	0.011
	concentration x exposure	528.044	6	88.007	0.955	0.476

A two-way ANOVA examined the effect of concentrations and exposure periods on efficacy of tested extracts, for each larval stage separately. The efficacy of extracts on S_1 larvae was affected significantly by both concentration and exposure, as well as their interaction ($F = 16.94^{**}$; 3.42^{*}; 2.91^{*}, $P < 0.01$; $P < 0.05$; $P < 0.05$, respectively). Simple main effects analysis showed that concentration had significantly stronger effect on the efficacy of all extracts on S_1 larvae than the exposure and their interaction, according to SS and P value.

However, according to the Two-factor analysis of variance in the case of S_2 and S_3 larvae, only concentrations and exposure were factors of influence, while their interaction was not significant ($F = 1.03$; 7.32, $P > 0.05$, respectively). Dominant factor in both cases was the concentration ($SS = 2556.102^{**}$; 1350.236^{**}, $P < 0.01$, respectively).

Discussion

Aromatic and medical plants are very important source of potential natural pesticides, especially insecticides (M.R. Fakoorziba & al [25]). Plant extracts are attracting the attention of researchers as an environmentally friendly alternative for pest control. Natural products are biodegradable, significantly less toxic than synthetic insecticides and they are target-specific (D.M.C. Nguyen & al [26]).

Efficacy of tested extracts varied among both larval groups and concentrations of extracts of the analyzed species. The highest efficacy was recorded among the larvae of the first, S_1 maturity group (the youngest, up to seven days old), followed by the S_2 and S_3 groups.

Differences in efficacy on larval maturity groups can be attributed to morphological and behavioral differences among age groups. We hypothesized that the sensitivity of the larval stage influenced efficiency of applied plant extracts, regardless of the treatment. Inspections define S_1 larvae as active, especially during feeding, but also very vulnerable, which explains very high mortality of S_1 larvae recorded in untreated controls. Larvae belonging to S_2 maturity group are the most active and they made the greatest damage to the wheat kernels. Larvae of the S_3 group were poorly active, as they prepare to become pupae. During the experiment, we also registered a significant occurrence of cannibalism, especially among larvae of S_2 group. Cannibalism is often increased in stressful situations, when population density is increased or food availability or nutritional value is reduced (G.A. Polis [27], S. Via [28]). Since wheat is not the most suitable food for this moth due to very hard pericarp, low content of polyunsaturated fatty acids, vitamins and steroids in the kernels (D.P. Locatelli & L. Limonta [29]), increased cannibalism is not surprising in our results.

Differences in insecticidal activity of water extracts could be explained by presence of different biologically active substances in extracts.

Phenols are one of the most active groups of allelochemicals which effects growth, development and behavior of whiteflies (A. Wójcicka [30]). Stressful conditions in plants, such as UV radiation, injuries and infections, induce the biosynthesis and accumulation of phenolic components (Y. Sakihama & al [31], K.D. Asami & al [32]). Thus, external factors can have a significant impact on the content of phenolic acids and flavonoids in plants (S. Häkkinen [33], A. Michalak [34]).

Flavonoids, which are located on the surface of leafs, have a physiological role in the protection of the plant from fungus infections and UV irradiation (H.K. Sandhar & al [35]). The most important role of phenolic compounds is plant protection against attacks of pathogens and predators, such as phytophagous insects.

Tannins are known as phagorepellents for insects and the extracts of tested *Echium* species could be used for the protection of stored grains from IMM. Based on our results *E. italicum* extract contained the highest concentration of tannins and expressed the highest insecticidal efficacy against larvae.

According to the literature data, three tested *Echium* species are exceptionally rich in secondary metabolites pyrrolizidine alkaloids (PA) (D.J. Robins [36]), which play important role in plants chemical defense, especially against herbivorous insects (M. Boppre [37], T. Hartmann & D. Ober [38]). It can be especially the case with the *E. italicum*, where we recorded the most important insecticidal effects. According to the data given by P.R. Cheeke [39] and M. Maham & al [40], the effects of PA of this species have led to significant liver toxicity and mortality in calves.

PA are liver-toxic for vertebrates (P.R. Cheeke [39], M. Maham & al [40]) and mutagenic for insects (H. Frei & al [41]). Microsomal liver cytochrome P-450 enzymes of vertebrates bioactivate this compounds into unstable pyloric intermediates which are highly reactive (C.K. Winter & H.J. Segall [42]). Insects should also be affected by potentially toxic PAs, because they also have microsomal cytochrome P-450 enzymes (L.B. Brattsten [43]), as do vertebrates. Therefore, we assume that the concentration of these particular alkaloids, together with tannins that this plant contains and different complexes which it creates, led to a significant increase in mortality of larvae in our experiment.

Conclusion

Based on the results of this study we can conclude that the tested plant extracts had different efficacy on *Plodia interpunctella* larvae. The highest efficacy showed *Echium italicum* extracts, among the larvae of the youngest group (up to seven days old). Further research in this field is required, especially in terms of implementation of tannins, and other bioactive substances, such as pyrrolizidine alkaloids as bioinsecticides. If these attempts confirm the insecticidal efficacy, mentioned compounds of *E. italicum* could be applied as an effective post-harvest grain protectant against *P. interpunctella*.

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