Agriculture and Food

AIMS Agriculture and Food, 8(1): 105–118.
DOI: 10.3934/agrfood.2023005
Received: 16 September 2022
Revised: 21 November 2022
Accepted: 10 December 2022
Published: 23 December 2022

http://www.aimspress.com/journal/agriculture

Research article

Essential oils rich in pulegone for insecticide purpose against legume bruchus species: Case of *Ziziphora hispanica* L. and *Mentha pulegium* L.

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Abstract: *Ziziphora hispanica* L. and *Mentha pulegium* L. are aromatic shrubs known to produce essential oils demanded by food industries. This study reports on the insecticidal activity of these two oils harvested in Khenifra (Morocco) and of its major compound (Pulegone) against the main legume bruchids. Chromatographic analysis revealed the presence of 33 compounds in the essential oil of *Ziziphora hispanica* (EOZH) and 24 compounds in the essential oil of *Mentha pulegium* (EOMP), with pulegone being the most dominant in both with percentages respectively of 79.1% and 63.8%. According to the insecticidal activities exerted, the two essential oils, as well as the pulegone, showed significant results against all the bruchids tested of legumes. Pulegone has shown good insecticidal activity, thus the lethal doses of 50% are between 61.6mg/g and 74.4mg/g, which explains the remarkable activity of the two essential oils which are rich in this molecule. moreover, the addition of essential oils in the storage of legumes does not have a significant effect on the legumes and does not diminish their nutritional qualities. This study indicates that essential oils and their main constituent have the potential to be developed into botanical insecticides.

Keywords: essential oils; pulegone; insecticidal activities; bruchids; legumes

1. Introduction

Grain legumes are abundant in proteins, oligosaccharides, slow-digesting starches, dietary fiber and phenolic content, with just a minor quantity of lipids [1,2]. These elements could contribute to the nutrition advantages, such as their effects on chronic diseases due in particular resulting from deficiencies in biochemical elements [3].

Legumes storage loss assessment and mitigation strategies have occupied a prominent position on the scientific research agenda during the past few decades, but farmers in underdeveloped countries continue to suffer enormous losses despite the attention the framed governments have given to these issues [4–6]. Adults of certain insect species such as Bruchidae beetles (Coleoptera: Bruchidae) attack legume grains and cause serious damage, reducing the quality and quantity of stored biomass [7].

Bruchus lentis Frölich, *Bruchus pisorum* L., *Bruchus rufimanus* Boheman are an important pest on lentils, peas and faba beans respectively, and is now distributed in all countries of the world, particularly North Africa [8–10].

Several options have proved effective in controlling storage this insect pests, including environmental manipulations to discourage their growth, development and reproduction [11,12]. Such experimental options can be intervened by employing a number of control measures such as the use of chemical insecticides, biological and physical control methods or a combination of these operations [13–15]. Chemical pesticides work well against pests of storage insects, but they are also always accompanied by a number of disadvantages, including high prices of this products and worries about environmental contamination and food safety [16].

Previous studies pointed out the possibility of an integrated pest insects management strategy by using other constituents such as essential oils to control bruchids development in food legume stocks [17–19]. In these regards, many studies reported the efficacy of various plant species essential oils for the control of various beetles. These natural products are extracted from plants using traditional and innovative techniques [20]. Their excellent efficiency against a variety of pests, numerous modes of action, low toxicity of residues after application, and relatively inexpensive production procedure make them a potential and suitable option [21–23].

In this study, we investigated the insecticidal effects of the two essential oils of *Ziziphora hispanica* (EOZH) and *Mentha pulegium* (EOMP) against the main legume bruchids such as: *Bruchus lentis*, *Bruchus pisorum*, *Bruchus rufimanus*. The two essential oils and its main constituent, the pulegone, were evaluated by several tests, in order to determine certain parameters to know the effects of the compounds tested on the nutritional values.

2. Materials and methods

2.1. Essential oils: Extraction and analysis

The essential oils used in this study were extracted from aerial parts of both aromatic plants: *Ziziphora hispanica* L. and *Mentha pulegium* L. Plant materials collected from Morocco (Midle

Atlas), were air-dried at room temperature (20–25 ℃) and under the dark for one month and then stored in glass boxes. The species was identified by ESTK-USMS team and it is classified in the herbarium with the accession numbers: ZH4-2021 and MP7-2021. Essential oils were extracted by hydro-distillation of the dried plant leaves (250 g of each sample in 1L of distilled water) using a Clevenger-type apparatus for 3 h.

Essential oils were characterised by GC-MS, whose apparatus operating conditions were as follows: A Shimadzu 2010 with injector temperature 200 ℃; detector interface at 250 ℃; ion source 200; carrier gas helium; OV-1 column (non-polar, WCOT); Column flow: 1 ml/min; column ramp: 60 °C (no hold), 5 ℃ per min then held at 280 ℃ for 5 min. The all compounds were identified by comparing they mass spectra and retention indices (derived relative to n-alkanes) with the National Institute of Standards and Technology (NIST) collection [24].

2.2. Insecticidal assay

Bruchus adults were obtained from the breeding stock maintained at the laboratory of EST-Khenifra (University Sultan Moulay Slimane) at 28 ± 2 ℃ in glass containers containing leguminous and closed with non-woven fabric (*Bruchus lentis* in lentils, *Bruchus pisorum* in peas and *Bruchus rufimanus* in faba bean). The insecticidal assay of essential oils and the pure pulegone molecule consisted of an adaptation of the method proposed by several previous works [25–27]. For each bioassay, a weight in mg of each sample was added to 2.0 g of the leguminous biomass of each species, and the mixture was grinded for 5 min in order to obtain a final powder.

Next, five aliquots of 200 mg of the powder were placed in a Petri plate (90 × 100 mm), which was then incubated at 25 ℃ for 2 h. After this period, 10 insects were transferred to the plate, and the bioassay was maintained in the dark at 28 ± 2 ℃. Each assay was performed in triplicate, and the weight of leguminous and insects was determined at time zero and after 7 days. Mortality rates (%) were recorded after 7 days of experiment. The final concentrations of all samples in the disks were 20–400 mg/g (mg per g of leguminous). The determination of the lethal doses of 50% (LD50) are determined by linear interpolation in curves giving the percentage of mortality as a function of the logarithm of the concentrations tested.

2.3. Nutritional Indices

Four nutritional indices, including Feeding-Deterrence Index (FDI), Relative Consumption Rate (RCR), Relative Biomass Gain Rate (RBGR), and Efficiency of Conversion of Ingested food (ECIF) were calculated respectively on the basis of formulas [28,29]:

$$FDI(\%) = \frac{A-B}{A} \times 100 \tag{1}$$

$$RCR(mg.mg^{-1}.day^{-1}) = \frac{C}{(D \times days)} \tag{2}$$

$$RBGR(mg.mg^{-1}.day^{-1}) = \frac{E}{(D \times days)} \tag{3}$$

$$ECIF(\%) = \frac{E}{C} \times 100 \tag{4}$$

where:
A is the mass of food ingested by insects in the control;
B is the mass of food ingested by insects in the test;
C is the mass (mg) of ingested food;
D corresponds to the initial insect biomass (mg);
E corresponds to the biomass gained (mg).

According to the FDI, the samples were classified as: no-deterrent (FDI < 20%), weakly deterrent (50% > FDI ≥ 20%), moderately deterrent (70% > FDI ≥ 50%), or strongly deterrent (FDI ≥ 70%).

2.4. Evaluation of major biochemical constituents

After storing of 50 g of leguminous grains for a period of three months with a concentration of 100mg/g of each essential oil and pulegone, the different major biochemical components of leguminous, such as proteins, carbohydrates, and total fat, were measured using standards: ISO 26642:2010, ISO 16634-2:2016 and 11085:2015, respectively [30–32].

2.5. Statistical analysis

LD50s, Nutritional parameters and nutritional values of leguminous are expressed as mean of three tests with uncertainty at significance level (alpha) = 5 %. Analysis of variance (ANOVA) followed by Tukey's test was employed to compare the means of LD_{50} of each product (Essential oils and Pulegone) and nutritional values of leguminous (lipids proteins and sugars). ANOVA was performed to check the significant relationship between treatment sets in various bioassays [33].

3. Results

After obtaining the two essential oils EOZH and EOMP by hydrodistillation, quantitative analyses were made by the GC-MS technique. The qualitative chromatograms for GC-MS analyses of the two essential oils are reported in the Figures 1 and 2. The analysis data are mentioned in Table 1.

The chemical compositions of the two essential oils reveal the presence of pulegone as a major molecule with a percentage of 79.1% for EOZH and 63.8% for EOMP.

In addition, these results accompanied by the presence of other compounds with moderate percentages, we note the presence of limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) (3.5%) and 4-Menthen-8-ol (2-methyl-2-(4-methylcyclohexen-1-yl)propan-1-ol) (2.8%) in EOZH, and of the presence of menthone ((2*S*,5*R*)-5-methyl-2-propan-2-ylcyclohexan-1-one) 9.1%), limonene (1-methyl-4-(1-methylethenyl)-cyclohexene) (5.4%), germacrene-D ((1*E*,6*E*,8*S*)-1-methyl-5-methylidene-8-propan-2-ylcyclodeca-1,6-diene) (4.1%), p-menthane-3-ol (2-methyl-2-(4-methylcyclohexen-1-yl)propan-1-ol) (2.1%) and (E)-β-caryophyllene ((1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene) (2.1%) in EOMP.

Other compounds were detected with different percentages and in a quantity lower than 2%, as well as some in common between the two essential oils studied.

Four components accounting about 1–1.5% were found only in EOZH: carvacrol (2-methyl-5-propan-2-ylphenol) was 1.6%; thymol (5-methyl-2-propan-2-ylphenol) was 0.9%; menthofuran (3,6-dimethyl-4,5,6,7-tetrahydro-1-benzofuran) was 0.9%; trans-isopulegone

(5-methyl-2-prop-1-en-2-ylcyclohexan-1-one) was 0.8%.

Several previous works have been done on the essential oils of these two species and which confirm the present identification, among these studies, we note the research of Bekhechi et al. [34] and research by Stoyanova et al. [35] who show the presence of pulegone as the main molecule respectively in the essential oils of *Ziziphora hispanica* and *Mentha pulegium*.

The results of the insecticidal activity of the two essential oils and of pulegone against the three species of bruchids: *Bruchus lentis*, *Bruchus pisorum* and *Bruchus rufimanus* are given in Table 2.

These results are expressed by the lethal dose of 50% (LD_{50}) in mg/g. the reading of the data shows the interesting activity of the products tested, particularly of the molecule of pulegone, moreover all the results are significantly different after the statistical test of ANOVA, from where, the activities noticed in the two essential oils returned to the presence of pulegone with high concentrations. EOZH always was more effective with respect to EOMP.

In fact, the LD_{50} against *Bruchus lentis* was 121.4 mg/g for EOZH and 148.1 mg/g for EOMP. Similarly, the LD_{50} against *Bruchus pisorum* was 135.5 mg/g for EOZH and 163.4 mg/g for EOMP. The same prevalent effect was found against *Bruchus rufimanus* for which the LD_{50} was 133.6 mg/g by EOZH and 137.9 mg/g by EOMP (Table 2).

Other parameters were studied to complete the insecticide study, these are the determination of Feeding-Deterrence Index (FDI), Relative Consumption Rate (RCR), Relative Biomass Gain Rate (RBGR), and Efficiency of Conversion of Ingested food (ECIF). The values obtained are displayed in Figures 1, 2, 3 and 4.

The results obtained confirm the previous results, so the two essential oils EOZH and EOMP, and the pulegone have positive values compared to the controls. Moreover, all the FDI values are between 50% and 70%, which proves that the two essential oils and the pulegone are moderately deterrents [36].

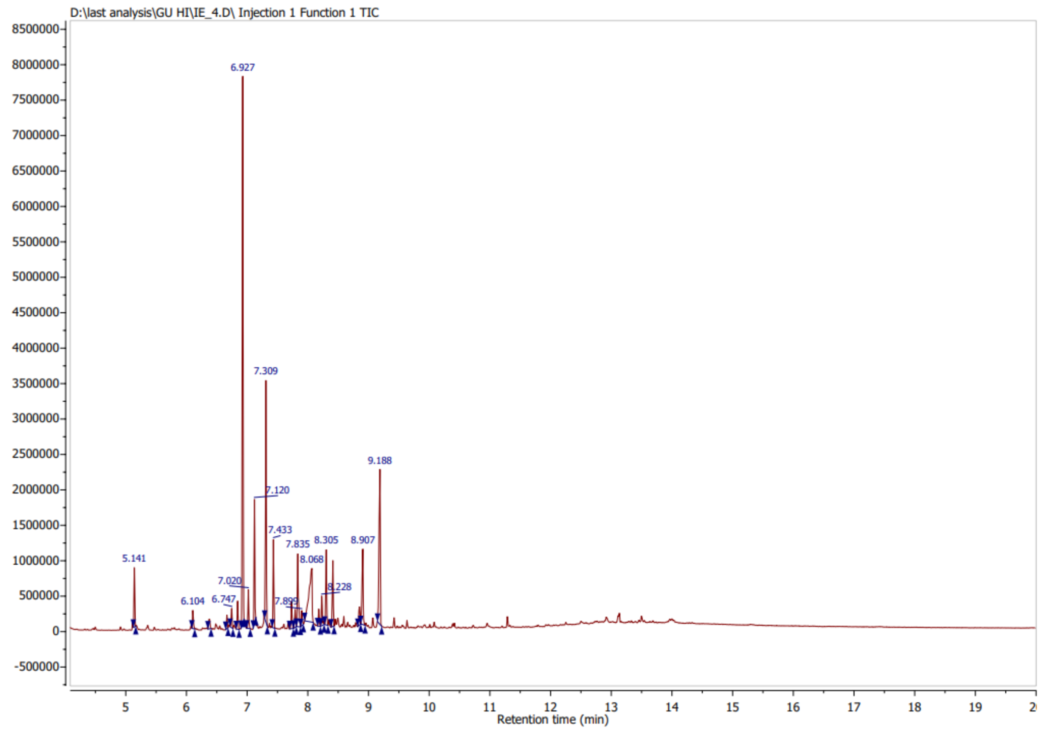

Figure 1. Chromatogram of the *Ziziphora hispanica* essential oil.

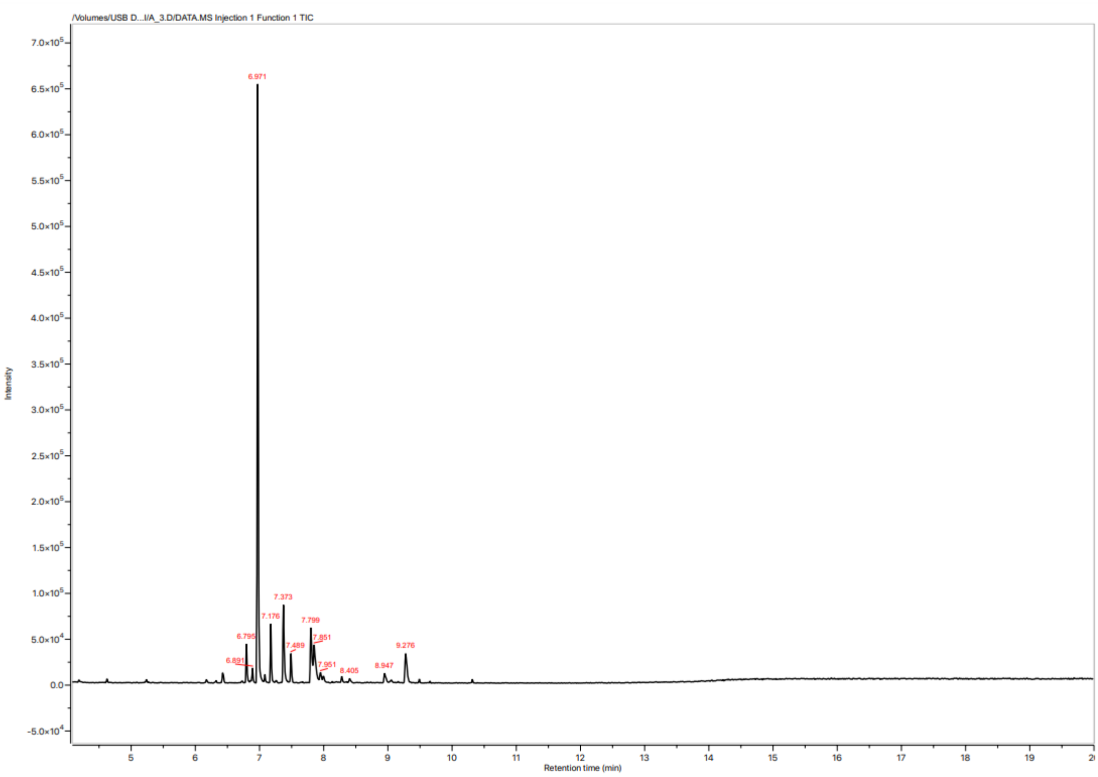

Figure 2. Chromatogram of the *Mentha pulegium* essential oil.

Table 1. Chemical composition of EOZH and EOMP.

Compounds	RI_{exp}	RI_{th}	EOZH$^{(*)}$	EOMP(**)
α-Pinene	930	942	0.2	1.1
Oct-1-en-3-ol	958	970	0.1	-
β-Pinene	970	974	0.4	-
Sabinene	977	977	0.1	-
Myrcene	979	987	0.1	1.2
α-Phellandrene	1008	1000	-	1.4
p-Cymene	1010	1007	0.2	-
1,8-Cineole	1019	1016	0.2	-
Limonene	1010	1021	3.5	5.4
Eucalyptol	1021	1016	0.3	0.3
γ-Terpinene	1047	1252	0.2	0.1
p-Mentha-3,8-diene	1058	1060	0.2	-
Isomenthone	1171	1170	-	0.5
Terpinolene	1077	1077	0.1	0.8
Linalool	1081	1085	0.1	0.4
p-Mentha-2,8-diene-1-ol	1101	1124	0.2	0.5
Menthone	1129	1127	-	9.1
Verbenol	1137	1138	0.1	0.2
4-Menthen-8-ol	1140	1140	2.8	-

Continued on the next page

Compounds	RI$_{exp}$	RI$_{th}$	EOZH(*)	EOMP(**)
Pinocarveol	1142	1141	0.6	0.2
Borneol	1145	1143	-	0.7
Menthofurane	1146	1147	0.9	0.1
trans-Isopulegone	1148	1152	0.8	-
p-Menthane-3-ol	1155	1158	0.2	2.1
cis-Isopulegone	1159	1159	1.2	-
Terpineol-4	1160	1162	0.1	0.1
α-Terpineol	1172	1172	0.2	-
Myrtenol	1175	1173	-	0.2
trans-Carveol	1196	1192	0.2	-
Pulegone	1208	1208	79.1	63.8
Piperitone	1223	1221	0.2	0.7
cis-Carveol	1226	1225	0.1	0.3
cis-Piperitone oxide	1255	1257	0.2	-
Carvacrol	1272	1279	1.6	-
Thymol	1282	1288	0.9	-
Mintlactone	1314	1314	0.3	-
Piperitenone	1318	1317	0.6	-
(E)-β-Caryophyllene	1417	1416	-	2.1
Germacrene-D	1471	1473	-	4.1
Caryophyllene oxide	1569	1561	0.2	0.4
		Total	96.2	95.8

(*) 33 compounds in the essential oil of *Ziziphora hispanica* (EOZH); (**) 24 compounds in the essential oil of *Mentha pulegium* (EOMP).

Table 2. Insecticidal activity of the two essential oils and the pulegone.

Sample concentration (mg/g of biomass of legume)	*Bruchus lentis*	*Bruchus pisorum*	*Bruchus rufimanus*	F-ratio	p-value
LD$_{50}$ (mg/g)					
EOZH	121.4 ± 4.2	135.5 ± 5.1	133.6 ± 1.6	18.89	< 0.0001*
ZOMP	148.1 ± 5.4	163.4 ± 4.5	137.9 ± 8.5	20.33	< 0.0001*
Pulegone	61.6 ± 5.4	74.4 ± 4.0	63.2 ± 4.9	9.73	< 0.0001*

Different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$). * Values are significant at $p < 0.05$.

More in detail, the FDI of EOZH was highest against *Bruchus pisorum* (61) and lowest against *Bruchus rufimanus* (43). The FDI of EOMP was 63, 62 and 53, respectively against *Bruchus pisorum*, *Bruchus lentis* and *Bruchus rufinanus*. The highest values against all insects were found for pulegone: 67 against *Bruchus pisorum*, 65 against *Bruchus lentis* and 63 against *Bruchus rufimanus* (Figure 3).

The RCR of EOZH was highest in *Bruchus pisorum* and *Bruchus rufimanus* whereas was lowest in *Bruchus lentis*. Also for the EOMP the highest RCR values were found for *Bruchus pisorum* and *Bruchus rufimanus* whereas for *Bruchus lentis* was found a half value compared to the one of *Bruchus pisorum.* The RCR of pulegone for all insects was lower than EOZH and EOMP (Figure 4).

The control showed the significantly highest values for all the studied species. Data of RGBR are reported in the Figure 5.

All treatments evidenced an effect in reduction of the biomass gain rate of insects. As expected, for all species, the control showed the highest RGBR values. Pulegone was the most effective. EOZH and EOMP showed, by and large, the same effect against *Bruchus lentis* and *Bruchus pisorum* whereas EOMP prevailed against *Bruchus rufimanus*. The ECIF is described in the Figure 6. EOMP was more effective against *Bruchus pisorum* and *Bruchus rufimanus* whereas EOZH showed the highest effect against *Bruchus lentis*. As for all the nutritional indexes, pulegone was the most effective against the three studied species.

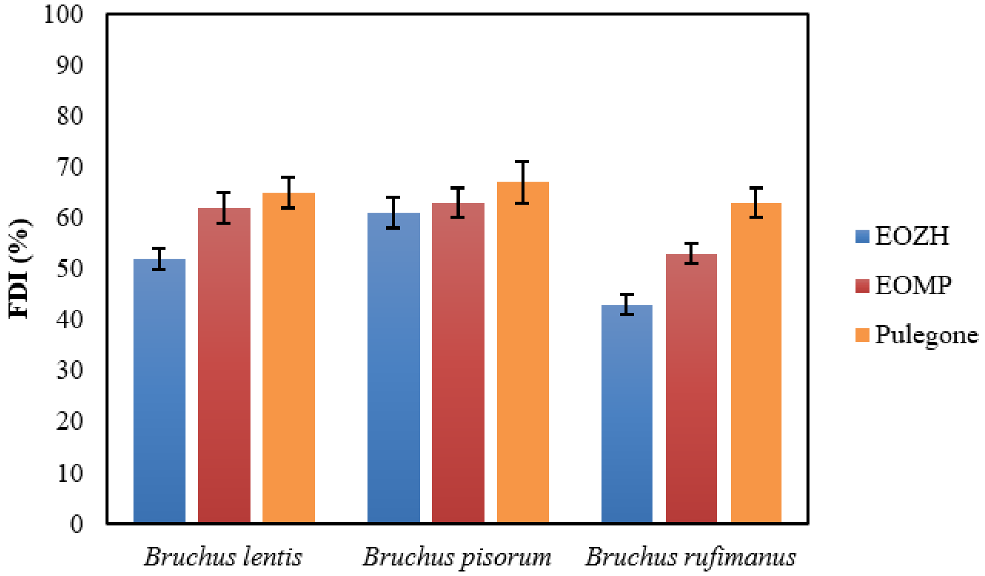

Figure 3. Determination of Feeding-Deterrence Index (FDI).

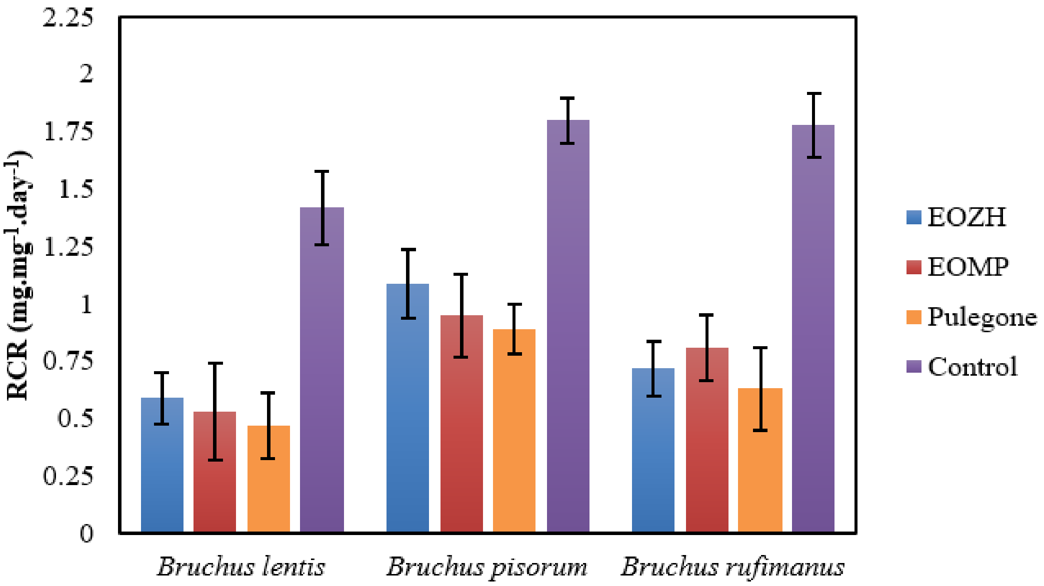

Figure 4. Determination of Relative Consumption Rate (RCR).

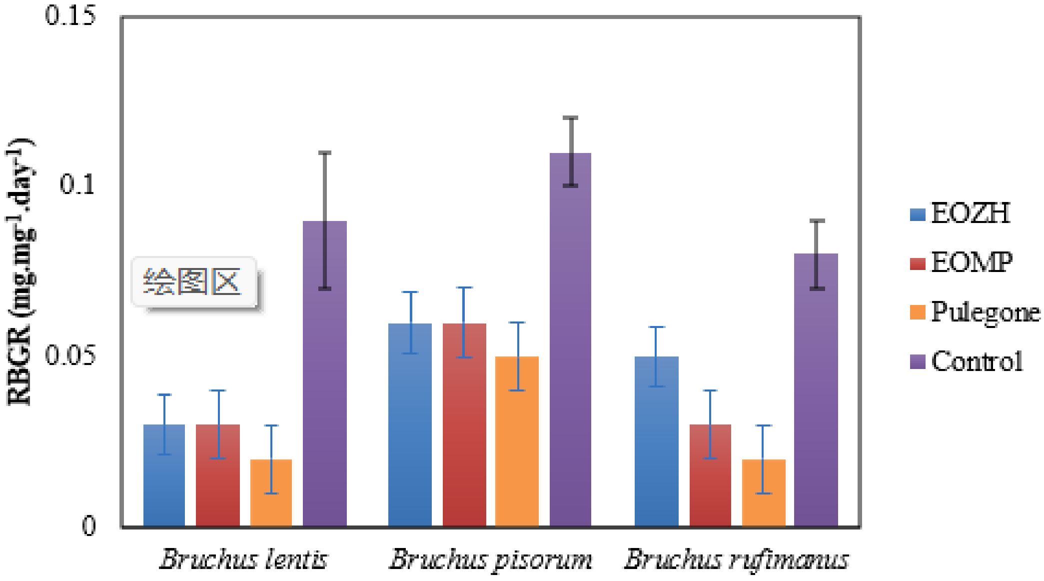

Figure 5. Determination of Relative Biomass Gain Rate (RBGR).

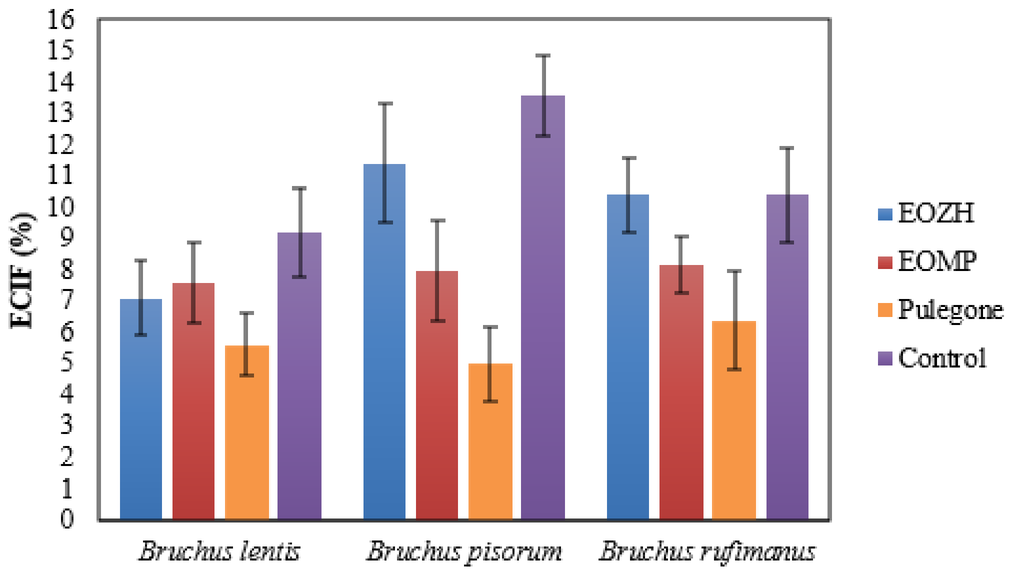

Figure 6. Determination of Efficiency of Conversion of Ingested food (ECIF).

To deepen the study, an additional test was carried out which concerns the biochemical analysis of the essential nutritional values of legumes after a storage period of three months with the essential oils EOZH and EOMP. These analyzes aim to determine the content of proteins, carbohydrates, and total fat, and they were measured according to two modes: without treatment and with treatment with essential oil. All the results are displayed in Table 3.

The statistical analysis by ANOVA of this biochemical contents shows that all the values do not have any significant difference, which proves that the essential oils added do not have a difference on the nutritional quality of stored foods.

Table 3. Biochemical analysis of the essential nutritional values of stored legumes.

Leguminous vs insect	Treatment/statistical analysis	Proteins content	Carbohydrates content	total fat content
Lentil (*Lens culinaris* L.) vs *Bruchus lentis*	Without treatment	9.1 ± 0.1	19.9 ± 0.2	0.41 ± 0.02
	Treatment with EOZH	9.0 ± 0.2	20.1 ± 0.4	0.40 ± 0.01
	After treatment with EOMP	9.0 ± 0.2	20.0 ± 0.4	0.41 ± 0.03
	F-ratio	0.83	0.02	0.04
	p-value	0.458	0. 974	0.959
Pea (*Pisum sativum* L.) vs *Bruchus pisorum*	Without treatment	4.9 ± 0.1	14.4 ± 0.1	0.41 ± 0.03
	Treatement with EOZH	4.9 ± 0.1	14.2 ± 0.2	0.41 ± 0.02
	After treatment with EOMP	4.9 ± 0.1	14.4 ± 0.4	0.39 ± 0.02
	F-ratio	0.28	0.95	0.85
	p-value	0.756	0.411	0.450
Faba bean (*Vicia faba* L.) vs *Bruchus rufimanus*	Without treatment	8.1 ± 0.1	18.3 ± 0.3	0.73 ± 0.03
	Treatment with EOZH	8.0 ± 0.2	18.2 ± 0.3	0.72 ± 0.03
	After treatement with EOMP	8.1 ± 0.1	18.2 ± 0.2	0.73 ± 0.03
	F-ratio	1.31	0.61	0.02
	p-value	0.303	0.612	0.983

Different letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

4. Discussion

Natural products remain an inexhaustible source of complex and diverse structures given the role that certain pure compounds can play in many applications, including the food industry. Plants synthesize several substances of secondary metabolism [37,38]. These molecules can have different effects in insects (repellent, attractive, disruptive of development, inhibitor of reproduction, etc.). Their toxicity can be direct or indirect on the target organs [39].

In recent years, and in the face of climate change and restrictive international legislation on the application of synthetic insecticides, the search for natural alternatives is part of a vision adapted to the requirements of the consumer and the environment [40]. In this strategy, our study directed towards the evaluation of the insecticidal activity of essential oils of *Ziziphora hispanica* L. and *Mentha pulegium* L. which are rich in pulegone. The insecticidal effectiveness of these two oils was confirmed by the presence of this molecule, whose activities are important appeared in all bruchids of storage legumes.

According to previous works this molecule is reported as a natural insecticide [41–43], so it has presented good effects according to the observed mortalities and high toxicity and is lethal listed in dozens of insect species. The pulegone has specific physico-chemical characters, it is well known that it is not volatile than other organic compounds, but it is highly lipophilic, tending to be more toxic, moreover this type of compounds is less selective in the binding to proteins, being in some cases chemically reactive and extensively metabolized [44,45].

This study concluded that the essential oils of *Ziziphora hispanica* L. and *Mentha pulegium* L. well controlled the development of bruchids of leguminous plants with greater attention can be paid to the existence of pulegone as the active ingredient.

The results obtained in this study showed that the use of essential oils as a biological insecticide could be incorporated into the management program for the control of weevil pests as a safe alternative form. Studies conducted in Algeria on the essential oil extracted by hydrodistillation of aerial parts of *Ziziphora hispanica* revealed pulegone as the major component (78.6%) and menthofurane and limonene accounting respectively for 1.26% and 1.06% of the total essential oil composition [46]. The authors found that essential of the *Ziziphora hispanica* showed an inhibition activity against Gram positive and Gram negative bacteria and also against fungus and that the inhibition effect was higher in Gram positive bacteria with respect the Gram negative ones [46]. Attia et al. showed an insecticidal effect of *M. pulegium* essential oil against three scale insects in Le Kef region citrus orchards of Tunisia. They found that hydrodistilled extracts had high toxicity toward *Planococcus citri*, *Aonidiella aurantii* and *Chrysomphalus aonidum* nymphs, whereas they generated low mortality rates of *Cryptolaemus montrouzieri* adults, a coccinellid predator [47].

5. Conclusions

In conclusion, our data in this study showed that pulegone could act as a potential insecticide, as it exhibited higher activity in insecticidal tests against legume bruchids as well as their specific effects during food storage through the preservation of nutritional biochemical values. Moreover, essential oils rich in this compound revealed a direct relationship with these insecticidal activities. All the products tested were potential tools to control bruchids during storage, but the presence of pulegone improved the bioactivity, which suggests using these natural substances for the control of legumes during the storage period.

Conflict of interest

The authors declare no conflict of interest.

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