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# Activities of two Major Lichen Compounds, Diffractaic Acid and Usnic Acid against *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae)

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## ABSTRACT

Two major lichen compounds (diffractaic and usnic acids), isolated from *Usnea longissima* Ach. were tested against 4th instar larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* Say for 24, 48, 72 and 96 h under laboratory conditions. Durations and mortalities were recorded at various concentrations (1.25, 2.5, 5, and 10 mg. $ml^{-1}$). Results showed that secondary metabolites of *U. longissima* had a significant insecticidal potential against larvae and adults of *L. decemlineata*. Mortality rates after 96 h of treatment, with the highest concentration (10 mg. $ml^{-1}$) of diffractaic and usnic acids, were 100 and 70% for adults and 100 and 80% for larvae, respectively. No mortality was observed in the control treatment. Bioassay tests with diffractaic and usnic acids revealed that the 96 h median lethal concentration ($LC_{50}$) values were 1.783 and 4.048 mg. $ml^{-1}$ for adults and 1.509 and 2.759 mg. $ml^{-1,}$ for larvae of *L. decemlineata*, respectively. The present results suggest that the lichen secondary metabolites may have a potential action for control of *L. decemlineata* 4th instar larvae and adults.

**Key words:** Diffractaic acid, Usnic acid, Lichen, *Leptinotarsa decemlineata*.

## INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae) is a serious pest of potatoes. It may cause significant damage to tomatoes and eggplants as well. Both adults and larvae feed on foliage and may completely destroy the crop. Insecticides are currently the worldwide main control method, but unfortunately many chemicals are often unsuccessful when used against this pest because of the beetle's ability to develop, rapidly, insecticide resistance (Gillott, 2005). *L. decemlineata* has developed resistance to all major insecticide groups, although not every population is resistant to every chemical (Alyokhin *et al*., 2008). Therefore, in recent years, researchers are looking for new biological insecticides.

Lichens, organisms formed through symbiosis between fungi and algae and/ or cyanobacteria, a very significant insecticidal source within biological insecticides (Emmerich *et al*., 1993). Antibiotic substances are known in more than 60 species of lichens. Many researches indicated that some lichen acids, such as; usnic and vulpinic have powerful antibiotic effect against some bacteria (Galun, 1988). Furthermore, it was reported that the lichens *Letharia vulpina* (L.) Hue and *Vulpicida pinastri* (Scop.) J.-E. Mattsson had been used to poison to death wolves and foxes that harmed herds during the winter in some countries of Europe and Scandinavia (Aslan *et al*., 1998).

Lichens usually contain only one or two major substances, often found in high concentrations. Concentrations of lecanoric acid (2.6 – 4.8% of *Parmelia* spp. dry weight) were found (Culberson *et al*., 1977). In *Cetraria islandica*, contents of fumarprotocetraric acid could reach 11% (Gudjonsdottir and Ingolfsdottir, 1977), while *Pertusaria alaianta* contains up to 20% of a mixture of chloroxanthones (Huneck and Hoefle, 1978). Many experiments proved that lichen metabolites have insecticidal effects; some have antifeedant and/or lethal characteristic on the insects (Emmerich *et al*., 1993; Bombuwala, 2001; Nimis and Skert, 2006; Cetin *et al*., 2008 and Silva *et al*., 2009).

Because of the economic importance of *L. decemlineata* in Turkey and many other countries, the present study was carried out to evaluate the insecticidal action of two secondary metabolites of *Usnea longissima* (diffractaic and usnic acids) against the 4th instar larvae and adults of the pest *in-vivo* conditions.

## MATERIALS AND METHODS

### Insects and rearing conditions

Adults of *L. decemlineata* were collected from potato plantations at Erzurum in Turkey and were reared in the Laboratory of the Department of Plant Protection, Atatürk University, Turkey at 25±1 °C, 64±5 % R.H. and 12:12h L/D. Fourth instar larvae (determined according to their morphological characteristics) and 3-5 day-old adults were used as

test insects. In order to define the age of adults, newly emerged adults were collected soon after their emergence from the pupae and placed in separate insect cages.

**Plant material and isolation of lichen secondary metabolites**

*U. longissima* (Fungi, Ascomycetes, Parmeliaceae) was collected in July 2009 from Trabzon in Turkey, then kept to dry under indoor conditions. All samples were identified and stored at the herbarium of Kazim Karabekir, Education Faculty, Atatürk University-Erzurum, Turkey. An air-dried sample of 250g *U. longissima* was extracted by 500 ml diethyl ether using a Soxhlet apparatus at 40°C. The crude extract of lichen sample was filtered and stored at 4°C, for 24 h to precipitate usnic acid (UA). The UA precipitates were collected and subjected to silica gel (70-230 mesh) column chromatography (CC) by eluting it with a $CHCl_3$: *n*-hexane (8:2) solvent system. At the end of this process, 2.10 g of usnic acid was obtained with a yield 0.84% (w/w). After the usnic acid precipitates were removed, the solution was concentrated using an evaporator under reduced pressure. The extract (18.75 g) was subjected to CC using silica gel (70-230 mesh), eluted with $CHCl_3$: *n*-hexane (7:3, 7.5:2.5, 9:1 and 10:0) and $CHCl_3$:$CH_3OH$ (9:1) solvent systems. Thus, 5.75 g of diffractaic acid were purified. The spectral data have been previously reported by Bayir *et al*. (2006) and Odabasoglu *et al*. (2006).

**Preparation of the lichen compound solutions**

100 mg of each of the diffractaic and usnic acids were dissolved separately in 10 ml of 80% acetone solvent to obtain stock solutions with a concentration of 10 mg. $ml^{-1}$ of each. Solutions with the concentrations of 1.25, 2.5, and 5 mg. $ml^{-1}$ were prepared by dilution with 80% acetone.

**Bioassay**

For bioassay, 4$^{th}$ instar larvae and adults of *L. decemlineata* were placed in Petri dishes (9 cm) with a potato leaf. Each replicate consisted of 10 individuals. A dose of 0.8 ml of solution was used for each Petri dish. Initial tests were done to estimate the appropriate dose and exposure time ranges. The concentrations of 1.25, 2.5, 5, and 10 mg. $ml^{-1}$ were applied in the Petri dishes. After exposure, mortality of adults and 4$^{th}$ instar larvae was determined at 24, 48, 72, and 96 h. Petri dishes applied with only 80 % acetone solution were used as control. Three replicates were used for each combination of dose and exposure time. Insecticidal activity of the solutions was expressed as percent mean mortality of the 4$^{th}$ instar larvae and adults.

**Statistical analysis**

The differences among insecticidal activities of the two tested lichen metabolites were determined according to analysis of variance (ANOVA) test by using the SPSS 15.0 software package. Duncan's test was used for comparison between means. Significance of differences between means were determined at $p<0.01$. The median lethal concentration ($LC_{50}$) values were calculated according to the method of Finney (1971). Probit analysis of concentration-mortality data was conducted to estimate the $LC_{50}$ values and associated 95 % confidence limits for each treatment (EPA Probit Analysis).

## RESULTS AND DISCUSSION

Toxicity effects of the two secondary metabolites (diffractaic and usnic acids) obtained from *U. longissima* on 4$^{th}$ instar larvae and adults of *L. decemlineata* are summarized in tables (1 and 2). Presented results showed that secondary metabolites of *U. longissima* had an insecticidal effect on both the larvae and adults of *L. decemlineata* compared with the control (Figs. 1-4). Higher concentration and longer exposure time resulted to highest toxicity on both larvae and adults. Mortality rates, after 24, 48, 72, and 96 h post treatment with different concentrations of lichen secondary metabolites, are given in figs. 1 and 2.

Analysis of variance demonstrated that the effects of these two acids on the mortality rates among *L. decemlineata* adults and 4$^{th}$ instar larvae were highly significant on the basis of concentration and exposure time tested (Tables 1 and 2). Higher concentrations and longer exposure times resulted in high toxicity of *L. decemlineata.* Diffractaic acid was more potent and possessed higher mortality rates than that obtained with usnic acid on both of the larvae and adults (Tables 1 and 2).

Mortality rates after 96 h of treatment, with the highest concentration (10 mg. $ml^{-1}$) of diffractaic and usnic acids, were estimated at 100 and 70 % of adults and 100 and 80 % of larvae, respectively. There was no mortality in the control of each metabolite (Figs. 1 and 2).

Total mortality rate increased as the concentration increased. Diffractaic solution with 10 mg. $ml^{-1}$ concentration showed highest insecticidal effect for both larvae and adults (Fig. 3). Meanwhile, diffractaic solution proved to be more toxic to larvae rather than to adults. The highest total mortality was obtained after 96 h exposure period and again the highest larvicidal and adulticidal activities resulted from diffractaic treatment (Fig. 4).

Table (1): Effects of two lichen secondary metabolites on adults of the Colorado potato beetle, *Leptinotarsa decemlineata* under laboratory conditions

| Treatments | Concentration ($mg.ml^{-1}$) | Mean mortality (%)[a] | | | |
|---|---|---|---|---|---|
| | | 24[b] | 48[b] | 72[b] | 96[b] |
| Control | - | 0.00±0.00 a $a_1$ | 0.00±0.00 a $a_1$ | 0.00±0.00 a $a_1$ | 0.00±0.00 a $a_1$ |
| Usnic acid | 1.25 | 0.00±0.00 a $a_1$ | 6.67±0.67 ab $a_1$ | 16.67±0.33 b $a_1b_1$ | 30.00±0.00 b $b_1$ |
| | 2.5 | 0.00±0.00 a $a_1$ | 20.00±0.58 bc $b_1$ | 23.33±0.33 bc $b_1$ | 40.00±0.00 bc $c_1$ |
| | 5 | 0.00±0.00 a $a_1$ | 23.33±0.33 bc $b_1$ | 26.67±0.33 bc $b_1$ | 50.00±0.58 c $c_1$ |
| | 10 | 0.00±0.00 a $a_1$ | 30.00±0.00 c $b_1$ | 53.33±0.66 e $c_1$ | 70.00±0.58 d $c_1$ |
| Diffractaic acid | 1.25 | 0.00±0.00 a $a_1$ | 20.00±0.00 bc $b_1$ | 23.33±0.33 bc $b_1$ | 40.00±0.00 bc $c_1$ |
| | 2.5 | 0.00±0.00 a $a_1$ | 26.67±0.33 c $b_1$ | 33.33±0.33 cd $b_1c_1$ | 50.00±0.58 c $c_1$ |
| | 5 | 0.00±0.00 a $a_1$ | 36.67±0.33 c $b_1$ | 46.67±0.33 de $b_1$ | 100.00±0.00 e $c_1$ |
| | 10 | 3.33±0.33 a $a_1$ | 80.00±0.58 d $b_1$ | 100.00±0.00 f $c_1$ | 100.00±0.00 e $c_1$ |

[a] Mean ±SE of three replicates, each set-up with 10 adults [b] Exposure time (h)
a, b, c, d, e, f: Values followed by different letters in the same column differ significantly at $p<0.01$
$a_1$, $b_1$, $c_1$: Values followed by different letters in the same row differ significantly at $p<0.01$

Table (2): Effects of two lichen secondary metabolites on 4th instar larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* under laboratory conditions

| Treatments | Concentration ($mg.ml^{-1}$) | Mean mortality (%)[a] | | | |
|---|---|---|---|---|---|
| | | 24[b] | 48[b] | 72[b] | 96[b] |
| Control | - | 0.00±0.00 a $a_1$ | 0.00±0.00 a $a_1$ | 0.00±0.00 a $a_1$ | 0.00±0.00 a $a_1$ |
| Usnic acid | 1.25 | 0.00±0.00 a $a_1$ | 10.00±0.58 ab $a_1b_1$ | 20.00±0.00 ab $b_1$ | 36.67±0.33 b $c_1$ |
| | 2.5 | 0.00±0.00 a $a_1$ | 23.33±0.33 bc $b_1$ | 26.67±0.33 b $b_1$ | 43.33±0.33 bc $c_1$ |
| | 5 | 0.00±0.00 a $a_1$ | 30.00±0.00 bcd $b_1$ | 33.33±0.33 b $b_1$ | 60.00±1.00 cd $c_1$ |
| | 10 | 6.67±0.33 a $a_1$ | 33.33±0.88 cd $a_1b_1$ | 56.67±0.88 cd $b_1c_1$ | 80.00±0.00 e $c_1$ |
| Diffractaic acid | 1.25 | 0.00±0.00 a $a_1$ | 23.33±0.33 bc $b_1$ | 30.00±0.58 b $b_1c_1$ | 46.67±0.33 bcd $c_1$ |
| | 2.5 | 0.00±0.00 a $a_1$ | 30.00±0.00 bcd $b_1$ | 40.00±0.58 bc $b_1$ | 63.33±0.33 de $c_1$ |
| | 5 | 0.00±0.00 a $a_1$ | 46.67±0.33 d $b_1$ | 66.67±0.33 d $c_1$ | 100.00±0.00 f $d_1$ |
| | 10 | 10.00±0.58 a $a_1$ | 86.67±0.33 e $b_1$ | 100.00±0.00 e $b_1$ | 100.00±0.00 f $b_1$ |

[a] Mean ±SE of three replicates, each set-up with 10 4th instar larvae [b] Exposure time (h)
a, b, c, d, e, f: Values followed by different letters in the same column differ significantly at $p<0.01$
$a_1$, $b_1$, $c_1$, $d_1$: Values followed by different letters in the same row differ significantly at $p<0.01$

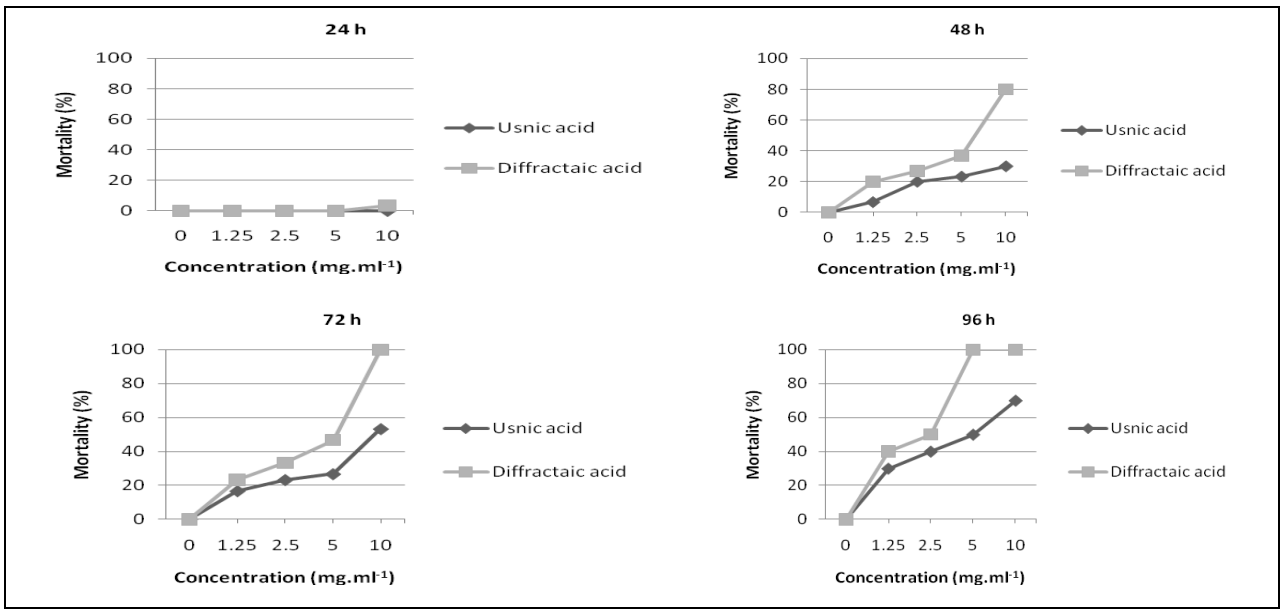


Fig. (1): Mortality rates among adults of *Leptinotarsa decemlineata* in relation to exposure time and concentration of two lichen secondary metabolites under laboratory conditions.

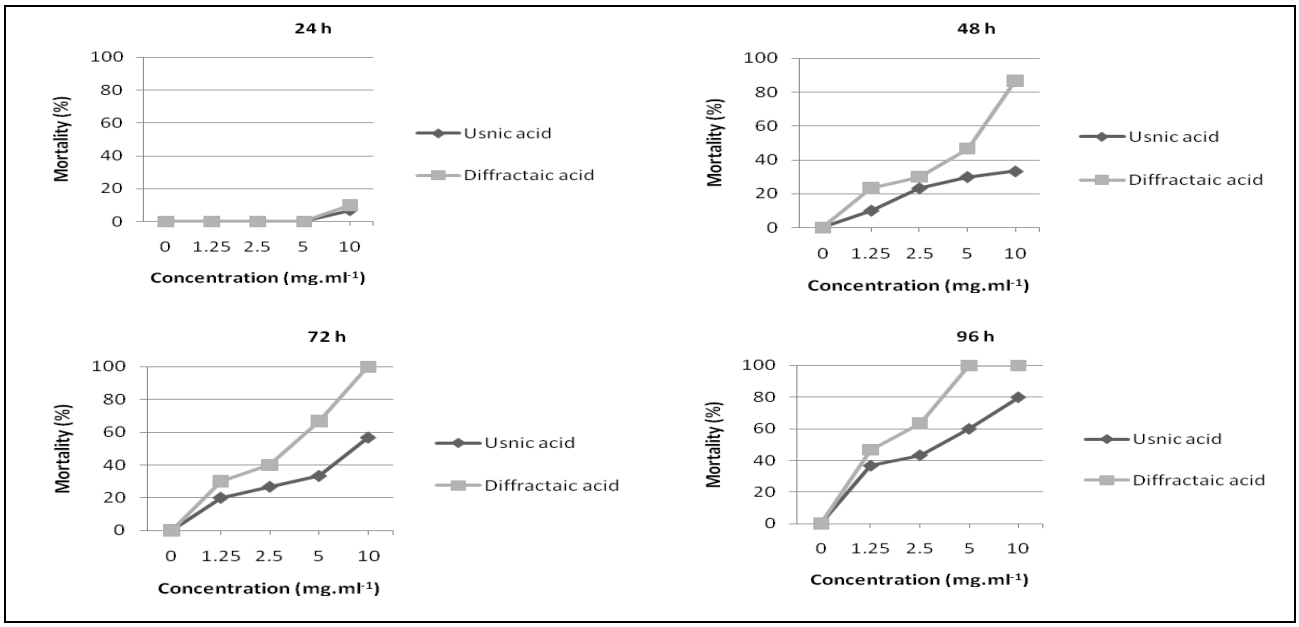


Fig. (2): Mortality rates among fourth instar larvae of *Leptinotarsa decemlineata* in relation to exposure time and concentration of two lichen secondary metabolites under laboratory conditions.

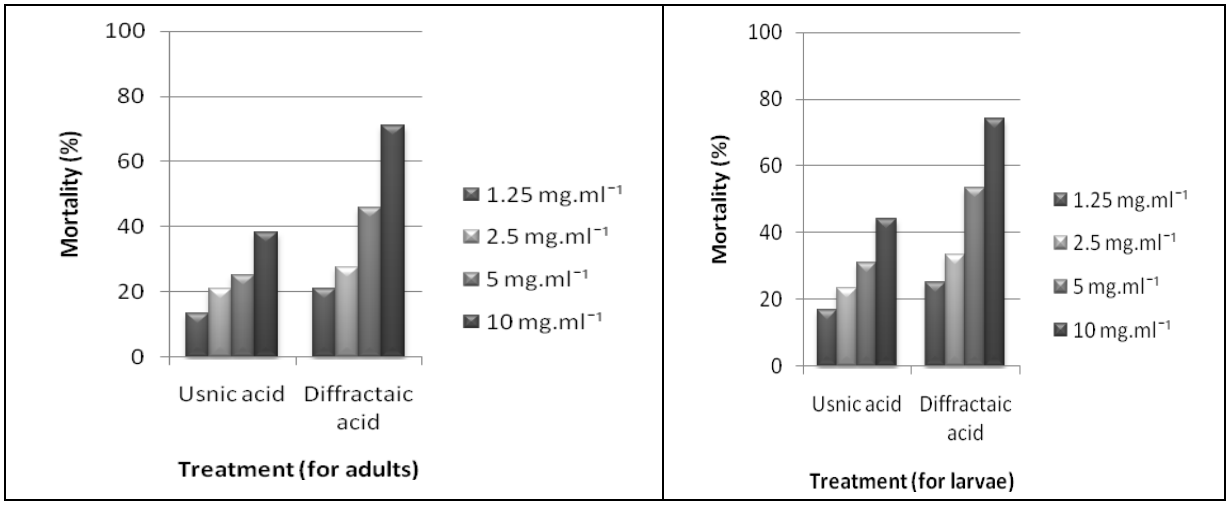


Fig. (3): Total mortality rates among adults and fourth instar larvae of *Leptinotarsa decemlineata* exposed to different concentrations of two lichen secondary metabolites under laboratory conditions.

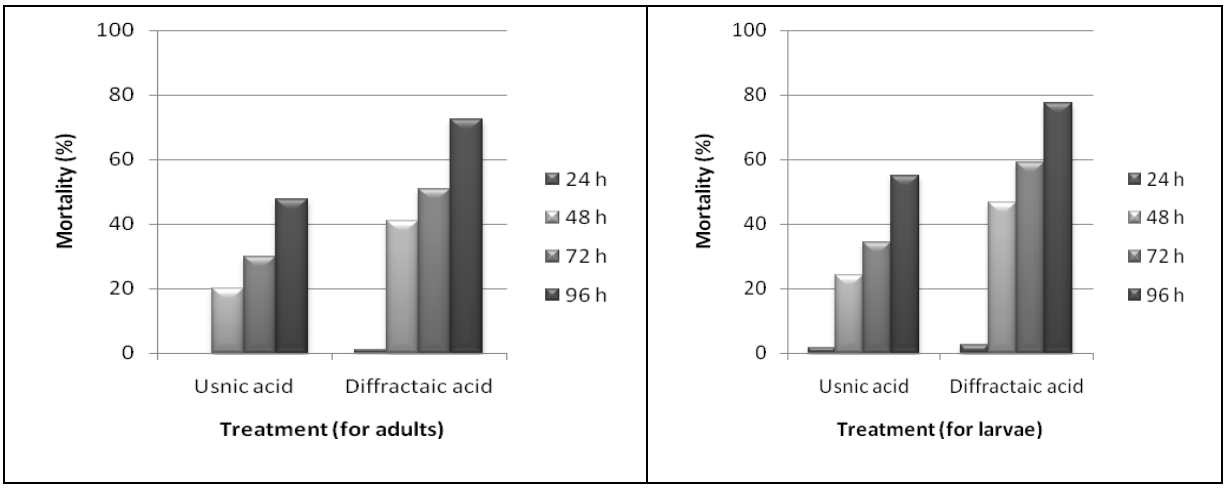


Fig. (4): Total mortality rates among adults and fourth instar larvae of *Leptinotarsa decemlineata* according to exposure period of two lichen secondary metabolites under laboratory conditions.

Table (3): $LC_{50}$ values ($mg.ml^{-1}$) of two lichen secondary metabolites on adults of *Leptinotarsa decemlineata* under laboratory conditions

| Treatments | Exposure Time (h) | $LC_{50}$ (Limits) | Slope (±SE) (Limits) |
|---|---|---|---|
| Usnic acid | 72 | 11.278 (6.407–82.161) | 1.170 (0.381) (0.422–1.917) |
| | 96 | 4.048 (2.370–8.032) | 1.127 (0.355) (0.431–1.823) |
| Diffractaic acid | 72 | 3.362 (2.612–4.342) | 2.421 (0.422) (1.595–3.248) |
| | 96 | 1.783 (1.375–2.176) | 3.398 (0.631) (2.160–4.636) |

Table (4): $LC_{50}$ values ($mg.ml^{-1}$) of two lichen secondary metabolites on 4th instar larvae of *Leptinotarsa decemlineata* under laboratory conditions

| Treatments | Exposure Time (h) | $LC_{50}$ (Limits) | Slope (±SE) (Limits) |
|---|---|---|---|
| Usnic acid | 72 | 8.838 (5.172–53.859) | 1.091 (0.367) (0.372–1.811) |
| | 96 | 2.759 (1.500–4.236) | 1.299 (0.361) (0.591–2.008) |
| Diffractaic acid | 72 | 2.590 (1.948–3.291) | 2.469 (0.428) (1.629–3.309) |
| | 96 | 1.509 (1.080–1.876) | 3.289 (0.654) (2.007–4.571) |

The $LC_{50}$ values, at 72 and 96 h, were calculated for *L. decemlineata* adults (Table 3) and 4th instar larvae (Table 4). The 96 h $LC_{50}$ values were the lowest. After 96 h exposure period, the $LC_{50}$ values calculated for usnic acid and diffractaic acid were 4.048 and 1.783 mg. $ml^{-1}$ for adults and 2.759 and 1.509 mg. $ml^{-1}$ for 4th instar larvae, respectively.

Natural products are now being considered as alternatives to the currently available arsenal synthetic compounds (Dayan *et al*., 1999). The present results confirmed that diffractaic and usnic acids from lichen secondary metabolites had varying degrees of larvicidal and adulticidal activities against *L. decemlineata*. In agreement with the present results, some previous studies demonstrated that, in general, the toxicity of extracts isolated from lichen samples against pests is related to their secondary components (Emmerich *et al*., 1993; Bombuwala, 2001; Nimis and Skert, 2006; Cetin *et al*., 2008 and Silva *et al*., 2009). These results suggested that extracts isolated from different lichen species might have different toxicity levels that might be attributed to their different components and different chemical composition (Sahip *et al.*, 2008).

In this study, diffractaic solutions, with 5 and 10 mg. $ml^{-1}$ concentrations, showed 100 % mortality of larvae and adults. These results differed, significantly at $p<0.01$, than other concentrations (1.25 and 2.5 mg. $ml^{-1}$) at 96 h (Tables 1 and 2). These disparities (at $p<0.01$) attract the attention about mortalities occurred after some exposure periods for the same concentration (Tables 1 and 2). Low values of $LC_{50}$ at 96 h (1.783 mg. $ml^{-1}$ for adults (Table 3) and 1.509 mg. $ml^{-1}$ for 4th instar larvae (Table 4)) indicated that diffractaic acid was highly toxic to the tested stages of *L. decemlineata*.

Lichens are known as biological indicator organisms. They survive better in regions having unpolluted air and produce secondary metabolites which are likely to be adaptive and not harmful to the environment but they have only an effect on the phytophagous insects. For this reason, lichen acids isolated from lichen extracts are functional substances that are suitable to have effect only on the target organisms.

It can be concluded that diffractaic and usnic acids obtained from *Usnea longissima* had an insecticidal activity, therefore they may have potential insecticidal actions against adults and 4th instar larvae of *L. decemlineata*.

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