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ORIGINAL ARTICLE

Effect of 1,8-cineol on the biology and physiology of elm leaf beetle, *Xanthogaleruca luteola* (Col.: Chrysomelidae)

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Abstract

The effect of monoterpenoid 1,8-cineol on the toxicity and physiology of elm leaf beetle, *Xanthogaleruca luteola* Müller under laboratory conditions (26 ± 1°C, 65 ± 10% RH and 16L : 8D h) was investigated. Initially, LC_{30} and LC_{50} values of the constituent were estimated to be 23.5 ppm and 31.9 ppm for the last instar larvae after 48 h, respectively. Significant changes were observed in the values of relative growth rate (RGR), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), approximate digestibility (AD) and consumption index (CI) between control and treated larvae with 1,8-cineol. The amounts of protein, glucose and urea decreased in the treated larvae in comparison with control. Similar findings were observed in the activities of alkaline phosphatase and lactate dehydrogenase while the activities of glutathione S-transferase and esterase significantly increased in the treated larvae using CDNB and α-naphtyl acetates as the substrates. Morphological and histological changes brought about by 1,8-cineol in the present study are indicative of growth inhibition targeting specific organs such as those of reproduction. We believe that 1,8-cineol can be considered as a safe and environmentally friendly compound.

Keywords: biochemical parameters, 1,8-cineol, *Xanthogaleruca luteola*

Introduction

The elm leaf beetle *Xanthogaleruca luteola* Müller (Coleoptera: Chrysomelidae) is a serious pest of elm trees, causing considerable damage to *Elmus* spp. (Huerta *et al.* 2010, 2011; Chiffelle *et al.* 2013). The insect is cosmopolitan and is found in central and southern Europe, North Africa, west and central Asia, southern Australia, and temperate areas in North and South America (Romanyk and Cadahía 2002; Borowiec and Sekerka 2010). Both larvae and adults feed on elm leaves, and heavy infestation makes the tree susceptible to Dutch Elm disease (*Ceratocystis ulmi*) (Huerta *et al.* 2010). Intensive use of insecticides to control *X. luteola* has led to adverse effects on the environment and development of insecticide resistance in target pests. Hence, new approaches based on environmental safety are being encouraged as important components of pest management (Kaushik *et al.* 2009; Madhua *et al.* 2010). Plant extracts as well as essential oils and their components could be considered as alternatives to synthetic pesticides because of their low environmental pollution, side effects and persistence (Cohen *et al.* 2006; Isman 2008). Essential oils or their components affect insects through toxicity, repellency, growth and reproductive inhibitory properties (Arabi *et al.* 2008; Benzi *et al.* 2009).

The compound 1,8-cineol also called eucalyptol is a component of several plants including *Rosmarinus officinalis* L. (Kovar *et al.* 1987), *Psidium pohlianum*

(Andrade-Neto *et al.* 1994) and *Eucalyptus* spp. (Cloez 1870). This monoterpenoid has had promising results as a contact insecticide to control coleopteran pests (Liška *et al.* 2015). Similarly, Guo *et al.* (2015) reported strong toxicity by 1,8-cineol against *Tribolium castaneum* (Herbst) adults. Good acetylcholinesterase inhibition has also been reported by this compound (López and Pascual-Villalobos 2010).

Essential oils and their major constituents are being considered for integrated pest management (Isman 2008). However, if any compound is to be introduced for Integrated Pest Management (IPM), it is necessary to understand its various effects accompanying toxicity (Liu *et al.* 1990). Thus, in the present study the toxic effects of 1,8-cineol were taken into consideration as well as some physiological processes showing irreversible changes. These parameters included nutritional indices, energy reserves, detoxification enzymes and finally, its effect on the morphology and histology of the female reproductive system in *X. luteola*.

Materials and Methods

Tested compound

1,8-cineol (eucalyptol) 98% purity was purchased from the producers "Sigma-Aldrich" (Germany).

Insect rearing

Elm leaves containing *X. luteola* eggs and larvae were collected from the City Park of Rasht (37°1651N, 49°3459E), northern Iran. They were reared in plastic boxes (10–20 cm) in a rearing chamber set at 25 ± 2°C; 14L : 10D h; 65% relative humidity (RH). Fresh leaves of *Zelkova carpinifolia* (Pall.) (Ulmaceae) were provided daily for feeding until adulthood. The deposited eggs were similarly reared until final larval instar (<24 h) for bioassay and physiological experiments.

Bioassays

Oral toxicity was performed on newly ecdysed last instar of *X. luteola* with different concentrations of 1,8-cineol which were based on previous studies (Tripathi *et al.* 2001). In each experiment, five replications were considered in each concentration (15, 22, 33, 44 and 50 ppm) and a control (n = 50 for each concentration and in all 300 newly ecdysed last instar larvae were used). The leaf discs (10 × 20 cm) were dipped in the different concentrations for 30 s and then dried for 30 min. The controls received distilled water only and were treated as above. LC_{30} and LC_{50} values were estimated by POLO-PC software (LeOra 1987).

Biochemical analysis

Estimation of glucose

One hundred microliter of whole larval body homogenate was mixed with 0.3 N perchloric acid (500 µl) and then centrifuged for 10 min at 12,000 *g*. The supernatants were used for the estimation of glucose concentrations (Siegert 1987).

Estimation of total protein

The amount of proteins was determined according to Bradford (1976). First, the whole bodies of larvae were homogenized in 350 µl of distilled water and samples were centrifuged at 10,000 *g* at 4°C for 5 min. Then 10 µl of supernatant was mixed with 90 µl of distilled water and 250 µl of dye [10 mg of Coomassie Brilliant Blue (Bio-Red, Munich, Germany)] in 5 ml ethanol (96%) and 10 ml of phosphoric acid 85% (w/w). The final volume was then brought to 100 ml with distilled water and the absorbance was read at 630 nm.

Estimation of urea

Urease-GDH kit (Biochem. Co., Iran) was used for estimation of urea at 340 nm. The protocol of the manufacturer was followed.

Activity of lactate dehydrogenase (EC 1.1.1.27)

Briefly, 20 µl of NAD^+ solution and 20 µl of water were added to test and control tubes, separately. Then, 100 µl of the buffered substrate and 10 µl of the sample was added to the test tubes and incubated for 15 min at 37°C. Next, 100 µl of the reagent (2,4-dinitro phenyl hydrazine) was also added and again incubated for 15 min. Tubes were cooled to room temperature prior to adding 50 µl of NaOH (0.4 M). Finally, the absorbance was read at 340 nm after 60 s (King 1965).

Activity of alkaline phosphatase (EC 3.1.3.1)

Based on the method of Bessey *et al.* (1946), 10 µl of enzyme solution was added to the buffered substrate [Tris-HCl, 20 mM, pH 8 for alkaline phosphatase (ALP)] and incubated for 5 min. Next, 100 µl of NaOH (1 M) was added and the absorbance was read at 405 nm.

Determination of general esterase activity

Assay of the enzyme was carried out using α- and β-naphtyl acetate as substrates based on Han *et al.* (1998). Briefly, 20 µl of each substrate (5 mM) was added separately to 50 µl of fast blue RR salt (1 mM) prior to adding 10 µl of enzyme solution. The incubation was prolonged for 5 min and the absorbance was then read at 450 nm.

Determination of glutathione *S*-transferase (GST) activity (2.5.1.18)

Briefly, 20 µl of CDNB (1-chloro-2,4-dinitrobenzene, 20 mM) and DCNB (1,2-dichloro-4-nitro-benzene, 20 mM) were added separately to 50 µl of reduced glutathione solution (20 mM), then 10 µl of enzyme solution was added and the absorbance was read at 340 nm after 5 min of incubation (Habing *et al.* 1974).

Evaluation of feeding indices

Third instar larvae (<24 h) were starved for 4 h before the experiments began. Elm leaf discs were impregnated with 31.88 ppm concentration of 1,8-cineol (30 s) and dried at room temperature for 30 min. The leaf discs in the controls were impregnated with distilled water in a similar way. The experiments were replicated ten times with one larva of third instar in each replication. The initial weight of larvae was recorded and then they were allowed to feed on either treated or untreated leaves in the case of treatments and controls, respectively. Fresh treated leaves were provided every 24 h and the previous leaves were removed, weighed and oven dried at 45°C for 48 h and reweighed in order to calculate the dry weight of consumed food. The dry weight of consumed food is estimated on the basis of the dried weight of the total food provided to the insect. The feces of each dish were also collected each day and then oven dried and weighed to estimate the dry weight of excreta. Similarly, larval weights were recorded at the end of the day. A few similar fresh larvae were weighed initially and dried in an oven (at 45°C) for 48 h, reweighed for determining the dry weight of each larva. The duration of the experiment was set at 4 days and observations were recorded each day.

The following formulae of Waldbauer (1968) were used:

Approximate digestibility (AD) =
= [(E – F)/F] × 100 [%],

Efficiency of conversion of ingested food (ECI) =
= [P/E] × 100 [%],

Efficiency of digested food (ECD) =
= [P/(E–F)] × 100 [%],

Consumption index (CI) = E/TA,

Relative growth rate (RGR) = P/TA,

where: A = average dry weight of larvae during the experiment, E = dry weight of consumed food, F = dry weight of produced feces, P = dry weight of the biomass of larvae, T = duration of the experiment (4 days).

Gross morphology and histology of ovaries

Treated and control ovaries of adults were dissected out in insect saline under a stereomicroscope (Olympus, Japan) 24 h of post-emergence and their ovaries were removed and photographed. For histological studies, the ovaries were fixed in Buin's fluid and later dehydrated in an alcohol series. They were cleared in xylene, embedded in paraffin and serial sections were cut at 5 µm with a rotary microtome [CUT 4050 (4050F,R)]. Haematoxylin and eosin staining were done and photographed (Gurr 1958).

Statistical analysis

Polo-PC software (1987) was used to estimate LC values of 1,8-cineol. Biochemical data were compared by one-way analysis of variance (ANOVA). Differences in treatments were calculated at 5% by Tukey's multiple range tests using SAS software (SAS Institute, 1997).

Results

Susceptibility of the third instars larvae of *X. luteola* to 1,8-cineol was analyzed by leaf dip bioassay and physiological studies. The corresponding lethal concentrations (LC_{50} and LC_{30}), with 95% confidence limits and regression slope were determined after 48 h of exposure (Table 1). The LC_{50} and LC_{30} values were estimated as 31.9 ppm and 23.5 ppm, respectively. Amounts of total protein ($F = 9.56$; $df = 2, 6$; $p = 0.013$) (Fig. 1), glucose ($F = 54.18$; $df = 2$; $p = 0.0001$) (Fig. 2) and urea (Fig. 3) ($F = 36.20$; $df = 2$; $p = 0.0004$) declined in the treated larvae of *X. luteola* treated by both LC_{30} and LC_{50} concentrations of 1,8-cineol.

The activity of lactate dehydrogenase showed no statistical changes in LC_{50} and LC_{30} after 48 h ($F = 0.30$; $df = 2$; $p = 0.075$) (Fig. 4). Alkaline phosphatase was statistically affected by different concentrations of 1,8-cineol; it decreased at LC_{50} treated larvae compared with controls after 48 h, but no significant

Table1. LC_{50} and LC_{30} of 1,8-cineol on *Xanthogaleruca luteola* 3rd larvae (48 h after treatment)

Toxic material	LC_{50} [ppm] (95% CL)	LC_{30} [ppm] (95% CL)	Slope ± SE	χ^2 (df)
1,8-cineol	31.88 (27.84–36.62)	23.45 (19.09–26.94)	3.93 ± 0.64	2.76 (3)

CL = confidence limit which was calculated with 95% confidence

difference was observed in the LC_{30} treatment (F = 1.22; df = 2; p = 0.35) (Fig. 5). By measuring the activity of two detoxifying enzymes, general esterases and glutathione S-transferase, it was found that general esterases were the main detoxifying enzymes against 1,8-cineol in *X. luteola*. The LC_{30} and LC_{50} concentrations of 1,8-cineol significantly increased the activity of glutathione S-transferase estimated by CDNB (F = 1.34; df = 2; p = 0.035) (Fig. 6) while the activity of glutathione S-transferase using DCNB was decreased compared to the control (F = 5.35; df = 2; p = 0.0463) (Fig. 7). General esterase activity was increased especially when α-naphtyl was used as the substrate, and there was a significant difference in two concentrations (F = 1.76; df = 2; p = 0.025) (Fig. 8).

The feeding efficiency of treated insects was significantly affected by 1,8-cineol treated leaves. In Table 2, it can be observed that compared to the controls most parameters (RGR, ECI, ECD and AD) decreased when the larvae fed on the leaves treated with 1,8-cineol. Moreover, gross morphology of ovaries showed a great reduction in size compared to the controls (Figs 9 A and B). In comparison to the controls the ovarioles were undifferentiated and developing oocytes were not detected. Also, a significant reduction was observed in developing germinal cells which were loosely rranged compared to compact cells in the controls (Figs C and D).

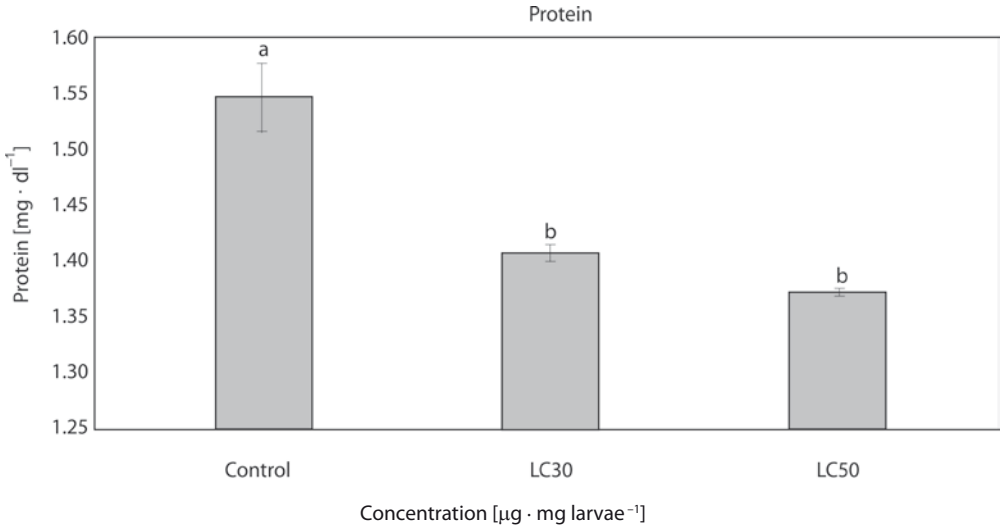

Fig. 1. Changes in the amount of protein caused by two concentrations of 1,8-cineol after 48 h of *Xanthogaleruca luteola* larval treatment. Statistical differences are shown by different letters in each interval (Tukey's test; p ≤ 0.05)

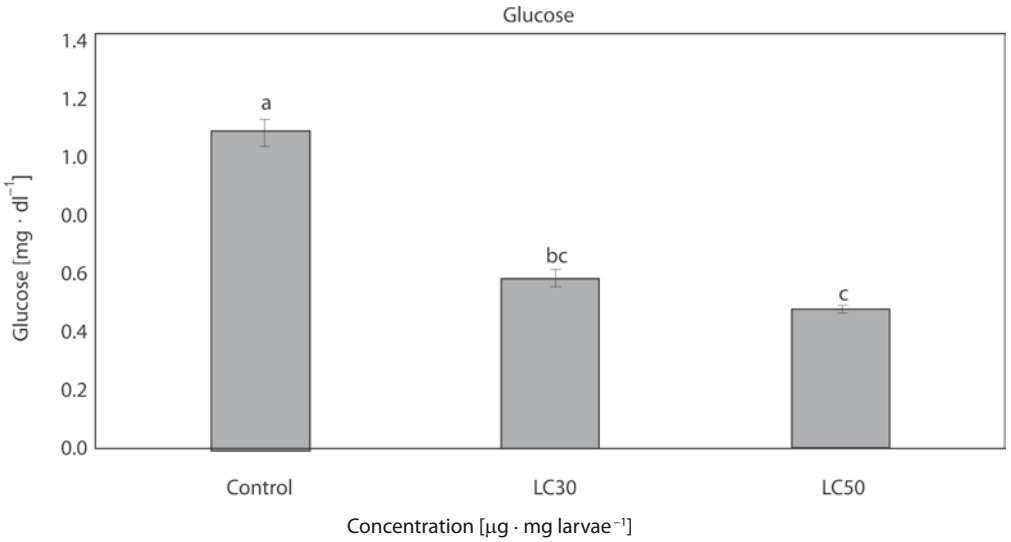

Fig. 2. Changes in the amount of glucose caused by two concentrations of 1,8-cineol after 48 h of *Xanthogaleruca luteola* larval treatment. Statistical differences are shown by different letters in each interval (Tukey's test; p ≤ 0.05)

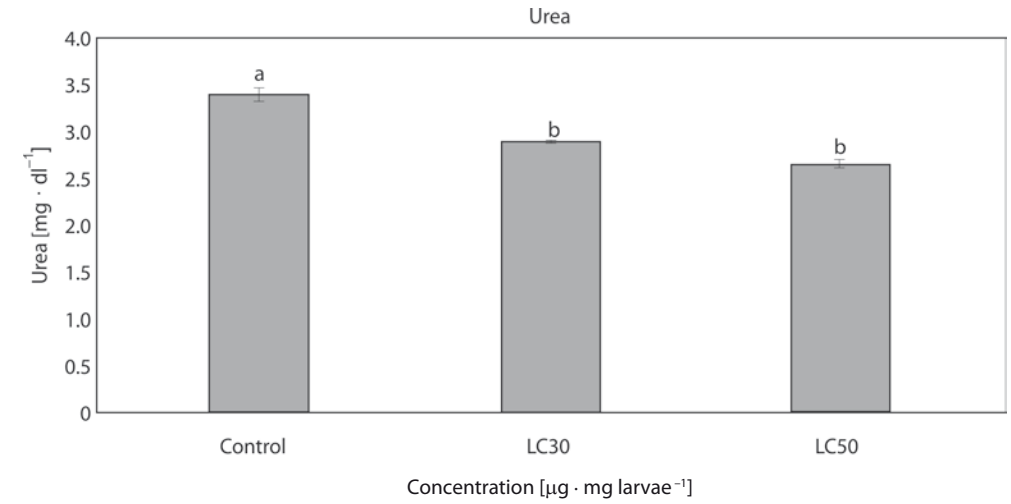

Fig. 3. Changes in the amount of urea caused by two concentrations of 1,8-cineol after 48 h of *Xanthogaleruca luteola* larval treatment. Statistical differences are shown by different letters in each interval (Tukey's test; $p \leq 0.05$)

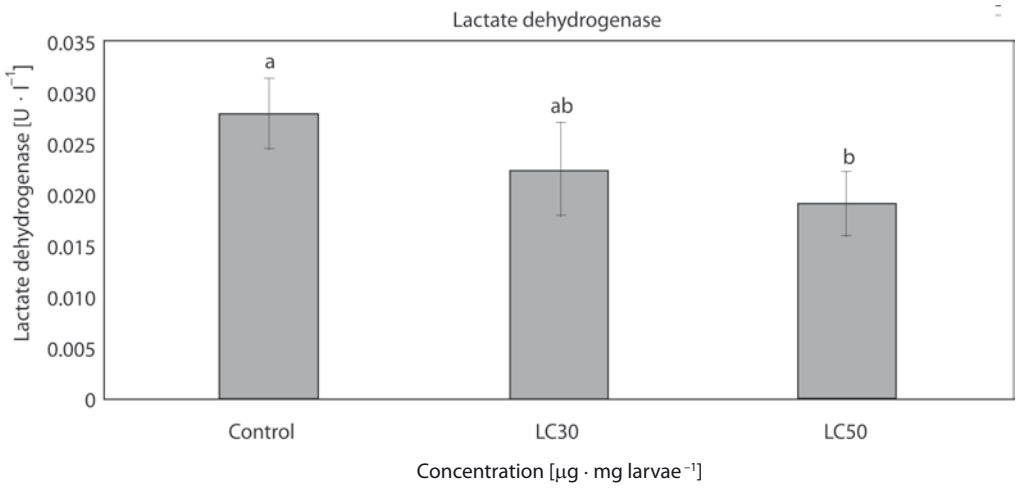

Fig. 4. Effect of 1,8-cineol on lactate dehydrogenase specific activities in *Xanthogaleruca luteola* larvae (3rd instar). Statistical differences are shown by different letters in each interval (Tukey's test; $p \leq 0.05$)

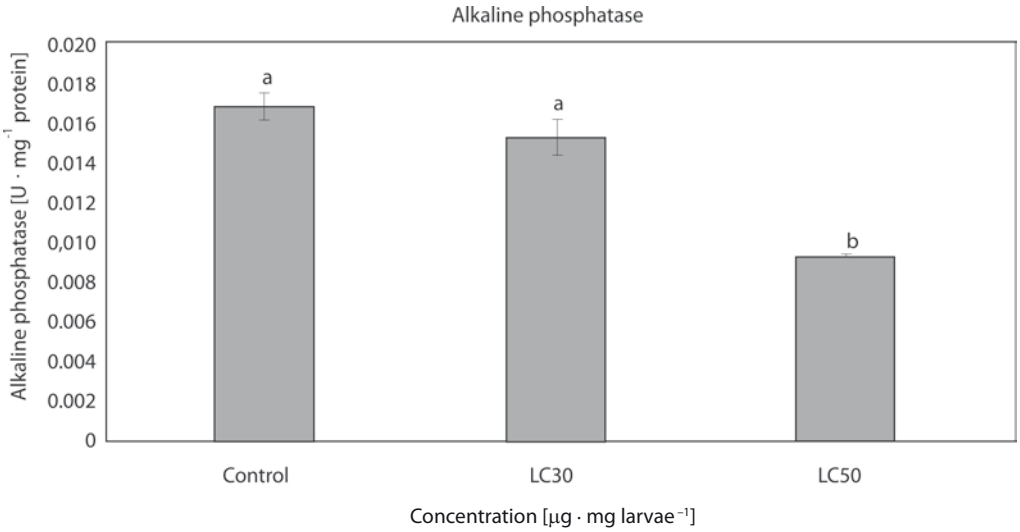

Fig 5. Effect of 1,8-cineol on alkaline phosphatase specific activities in *Xanthogaleruca luteola* larvae (3rd instar). Statistical differences are shown by different letters in each interval (Tukey's test; $p \leq 0.05$)

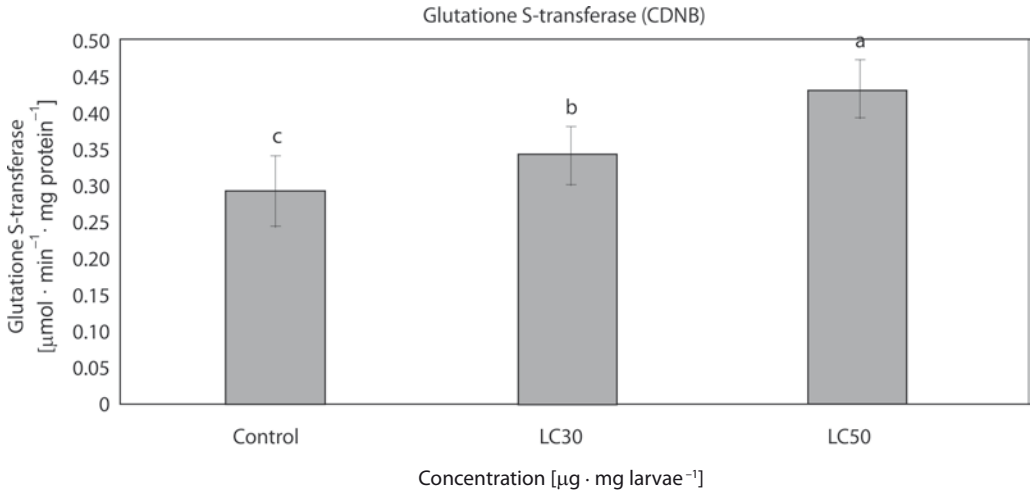

Fig. 6. The specific activities of glutathione S-transferase in the 3rd instar larvae of *Xanthogaleruca luteola* after treatment with 1,8-cineol. Statistical differences are shown by different letters in each interval (Tukey's test; $p \leq 0.05$)

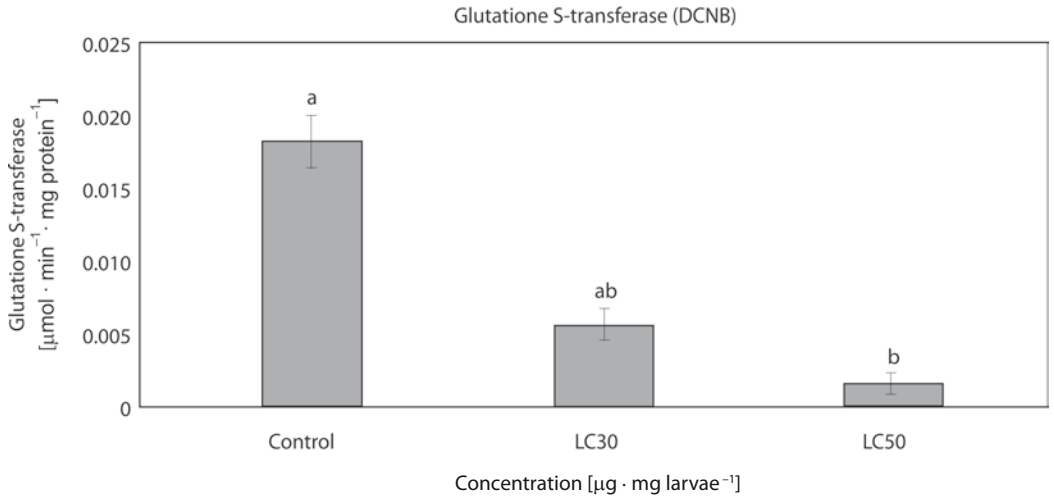

Fig 7. The specific activities of glutathione S-transferase in the 3rd instar larvae of *Xanthogaleruca luteola* after treatment with 1,8-cineol. Statistical differences are shown by different letters in each interval (Tukey's test; $p \leq 0.05$)

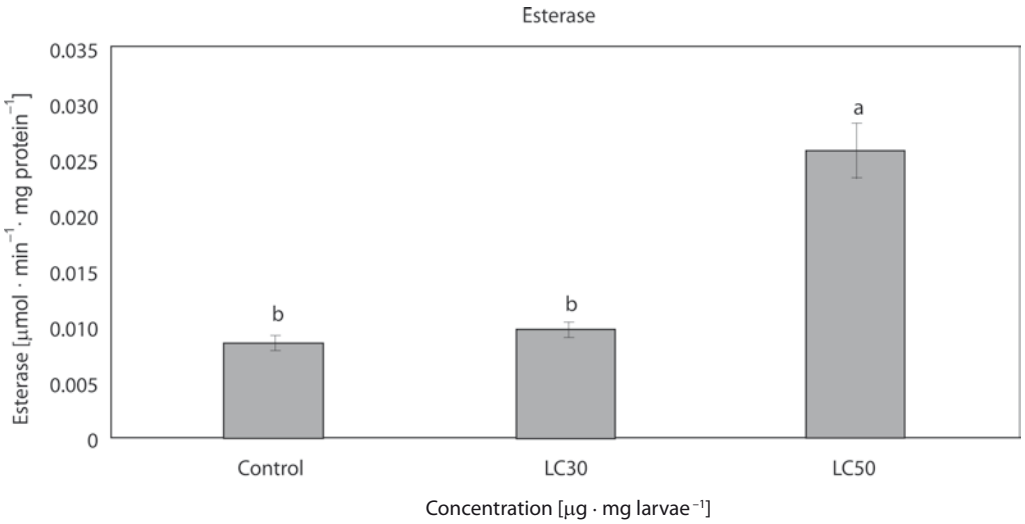

Fig 8. The specific activities of esterase in the 3rd instar larvae of *Xanthogaleruca luteola* after treatment with 1,8-cineol. Statistical differences are shown by different letters in each interval (Tukey's test; $p \leq 0.05$)

Table 2. Effect of 1,8-cineol on nutritional indices of *Xanthogaleruca luteola* 3rd instar larvae

Treatment	CI	AD [%]	ECI [%]	ECD [%]	RGR
Control	12.64 a	94.82 ab	2.69 a	2.83 a	0.34 a
1,8-cineol (31.88 ppm)	3.26 b	91.50 b	1.88 b	1.88 b	0.06 b

CI = consumption index; AD = approximate digestibility; ECI = efficiency of conversion of ingested food; ECD = efficiency of conversion of digested food; RGR = relative growth rate
Statistical differences are shown by different letters in each column (Tukey's test; $p \leq 0.05$)

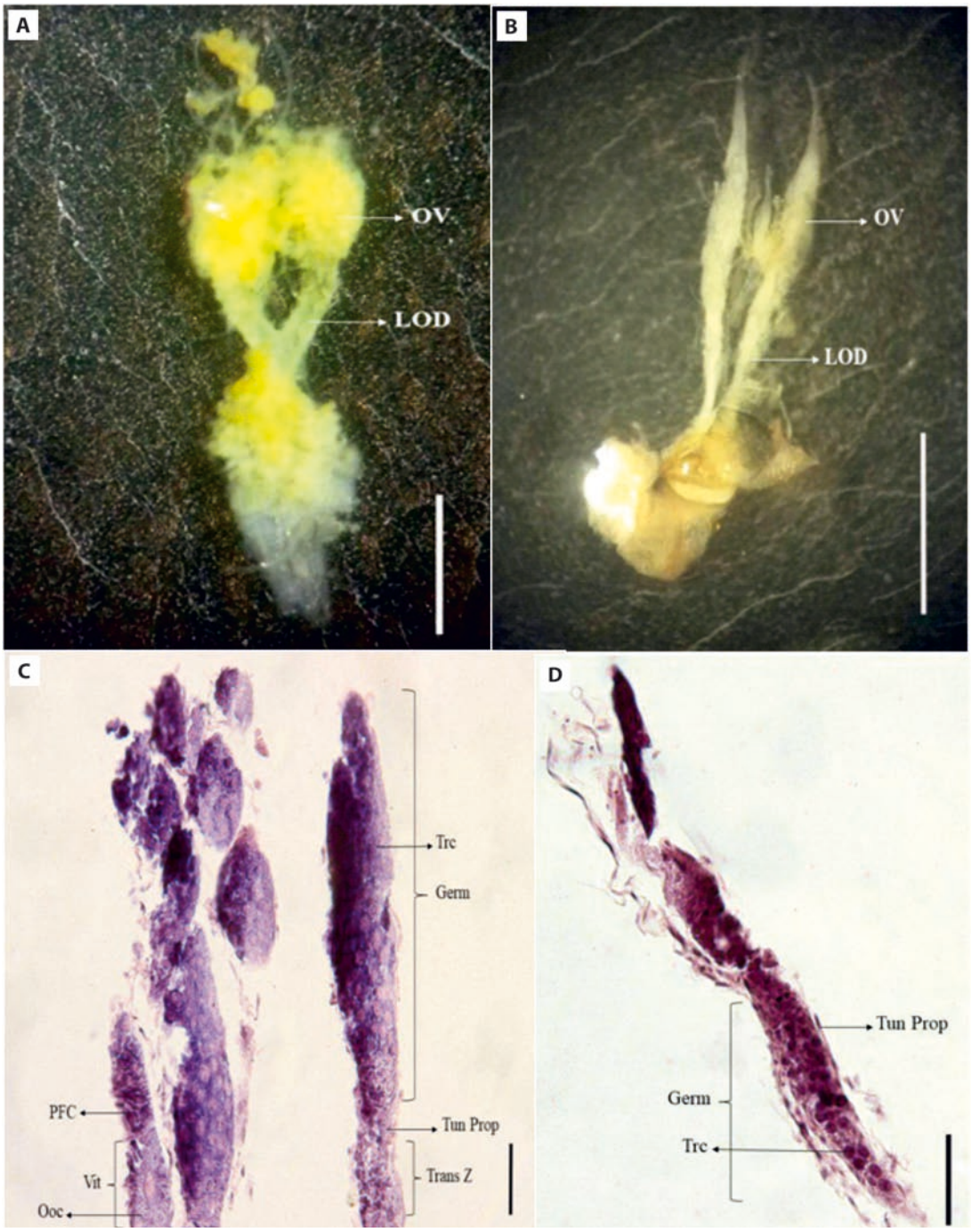

Fig. 9. (A) Gross morphology of a control female 24 h old, (B) gross morphology of a treated insect in larval stage with LC_{30} dose of 1,8-cineol, (C) histology of control insect ovary 24 h old showing compactly arranged germarium and (D) that of a LC_{30} of 1,8-ineol treated ovary with loosely arranged germarium (Hematoxylene-esosine). Scale = 1.15 mm for A and B, scale = 30 µm for C and D. Germ = Germarium, Ooc = Oocytes, PFC = prefolicular cells, Trc = Trophocytes, Trans Z = Transitional zone, Tun Prop = Tunica propria, Vit = Vitellarium

Discussion

Secondary products of plants are among important sources of insect development inhibitors which not only kill insects but also may affect insect fecundity, fertility, emergence, as well as development (Shaalan *et al.* 2005). Our results indicated insecticidal activity of 1,8-cineol on the last instar larvae of *X. luteola* in a concentration dependent rhythm. Similar reports are available on the toxicity of 1,8-cineol in dose- and time-dependent effect against other insects (Lee *et al.* 2003; Liška *et al.* 2015). Methanol extract of *Artemisia annua* containing 1.5–31.5% 1,8-cineol has been found to be responsible for larvicidal activity against cotton boll worm *Helicoverpa armigera* and elm leaf beetles (Shekari *et al.* 2008; Mojarab-Mahboubkar *et al.* 2015). Also, 1,8-cineol produced 100% contact toxicity in *C. maculatus, R. dominica* and *S. oryzae* adults at the highest dose of 0.1 µl · insect^{-1}. The LD_{50} values in the topical application assay were 0.03, 0.04 and 0.04 µl · insect^{-1} for *C. maculatus*, *R. dominica* and *S. oryzae,* respectively (Aggarwal *et al.* 2001).

Many botanical compounds have been reported to have antifeedant effects and decline insect feeding efficiency (Akhtar and Isman 2004). According to our results, 1,8-cineol significantly impaired relative consumption and growth of the treated larvae. These results are similar to previous research on plant essential oils or extracts rich in this compound (Senthil-Nathan 2006; Shekari *et al.* 2008; Khosravi *et al.* 2010; Hasheminia *et al.* 2011; Yazdani *et al.* 2013; Mojarab-Mahboubkar *et al.* 2015). Decreases in CI, ECI, ECD and RGR, without obvious reduction in AD, were indicative of changes occurring during digestion. These reductions might have been caused by damage to the peritrophic membrane, and/or damage to cellular surfaces of the midgut thus, hindering growth due to digestive damage by preventing the secretions of proteinases from gut epithelial cells (Timmins and Reynolds 1992; Senthil-Nathan *et al.* 2007; Mishra *et al.* 2015).

1,8-cineol affected the physiology of elm leaf larvae by altering the amount of protein and glucose. According to Chapman (2013) proteins apart from structural function have several other key roles in enzyme formation and transportation. They also function as storage molecules. The reason for the decline could be due to protein breakdown into amino acids (Etebari *et al.* 2007; Shekari *et al.* 2008; Yazdani *et al.* 2013). Carbohydrates, including simple sugars, are important components of the diet of most insects. They are the usual respiratory fuel, can be converted into lipid and provide the carbon skeleton for the synthesis of various amino acids (Chapman 2013). Glucose concentration is highly dependent on an insect's environmental conditions and its physiological and nutritional state. Hence decreased amounts of glucose following treatment with 1,8-cineol may have resulted from stress caused by the treatment (Nath 2003; Etebari *et al.* 2007; Da Silva *et al.* 2008; Valizadeh *et al.* 2013).

Lactate dehydrogenase (LDH) activates interconversion of pyruvate to lactate and vice versa (Pinto *et al.* 1969; Ford and Candy 1972; Kaplan and Pesce 1996). Moreover, if elevated it indicates damage or cytotoxic effects on tissues and organs (Senthil-Nathan 2006). There were no significant changes in LDH activity in our study which corresponds with lowered glucose and its rapid metabolism in tissues.

Alkaline phosphatases (ALPs; E.C.3.1.3.1) are enzymes involved mainly in cutting off phosphates from molecules. The considerable abundance of ALPs suggests their basic functions in animal cells. Alkaline phosphatases have several biological reactions and may affect stress, pathogenesis or infection (Sujak *et al.* 1978; Chang *et al.* 1993; Eguchi 1995; Sukhanova *et al.* 1996). Alkaline phosphatase is also an essential enzyme in the synthesis of tyrosine, the predecessor of dopamine and octopamine, which are known to participate in the control of insect growth and developmental hormones including juvenile hormone (JH), and 20-hydroxyecdysone (20E) (Wright 1987; Rauschenbach *et al.* 2007a, b). Our study showed decreased activity of ALP in the 1,8-cineol treated larvae which agrees with previous observations (Senthil-Nathan *et al.* 2004, 2006; Valizadeh *et al.* 2013). In fact, such a reduction suggests that this monoterpene affects the usual physiology of insect guts (e.g. ion delivery) which is controlled by the enzyme (Senthil-Nathan 2004).

Glutathione S-transferases are a major group of detoxification enzymes found in most organisms. They assist different insect cells in oxidative pressures caused by toxicants by helping in the removal of electrophilic and lipophilic compounds from the cell (Hayes and Pulford 1995). A sharp decrease was observed in DCNB indicating an overall decreasing trend. These results are consistent with incorporation of *Piper sarmentosum* essential oil and its primary compound, myristicin, which inhibited larval growth and development and were inhibitory to esterases and GSTs of *Brontispa longissima* (Qin *et al.* 2010). In contrast, the general esterases were activated after treatment thus showing a more pronounced role for these enzymes (Yazdani *et al.* 2014).

There are some reports on the reproductive inhibition of male or female insects under the influence of plant essential oils or their major constituents. Koul *et al.* (1987) reported dysfunction in ovaries of *Dysdercus kuengii* by treatment with *Origanum* oil and its benzene fraction (Rohdendorf and Sehnal 1972). Rao *et al.* (1999) reported underdeveloped ovaries in emerged adults from 5th instar nymphs treated with *Artemisia annua* oil. The ovarian follicles were still

intact in a bundle either without oocyte development or with poor oocyte development. Shekari *et al.* (2008) also reported juvenoid-like action of *A. annua* methanol extract in elm leaf beetles after larval treatment. The changes reported here were more drastic in that no vitellarium zone could be distinguished and the germarium contained few scattered developing germ cells. These adults never oviposited which was a clear indication that the ovaries were underdeveloped. Williams (1993) reported a 50% inhibition of egg laying and likewise a 65% and 80% hatching failure with 0.54 and 0.46 µg of *Artocarpus altilis* Park. and *Azadirachta indica* (A. Juss) on adult female *Boophilus microplus* (Canest.) ticks. Since in adults the growth of reproductive organs is complete, hence the extracts affected sequestration of proteins and fatty acids and the activity of Gené's organ is hindered which produces an extracellular lipid-protein complex important in the water retention capacity of eggs (Arthur 1962). Inhibition in fecundity but not fertility was reported in *Nezara viridula* L. females when nymphs were treated with azadirachtin (Riba *et al.* 2003). Both reduction and inhibition of fecundity and fertility were reported by Pulegone a monoterpene from mint on *Spodoptera eridania* (Gunderson *et al.* 1985). 1,8-cineol purified from *A. annua* affected egg hatchability in a dose dependent manner in *Tribolium* (Tripathi *et. al.* 2001).

Conclusions

In summary, 1,8-cineol, a plant-based monoterpene for *X. luteola* larval treatment had: (a) a high toxicity on *X. luteola*, (b) in sublethal doses, growth retardation of *X. luteola* occurred by interfering with digestion and assimilation and (c) sublethal doses reduced ovary development. This bio-insecticide is a safe alternative method for the control of this urban insect where application of synthetic insecticides is prohibited.

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