

Effect of *Eucalyptus citriodora* extract on *hsp70* expression and tissue damage in the third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg⁹

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Abstract

The effect of leaf extract of *Eucalyptus citriodora* was studied on *hsp70* expression by using soluble O-nitrophenyl- β D-galactopyranoside (ONPG) assay. The tissue damage was evaluated by trypan blue exclusion assay. The extract was studied at 1, 2, 4, 6, 8, 10 and 12 μ l/ml of food concentrations and third instar larvae were exposed to these concentrations for 24 and 48 hrs. The results showed a significant dose dependent increase in expression of *hsp70*. The tissue damage was not observed for the 24 hrs of exposure at all concentrations studied, very little damage was observed after 48hrs of exposure in salivary glands and malpighian tubules at 12 μ l/ml of food concentration of extract. The dose of 1 μ l/ml of extract did not induced significantly, expression of *hsp70* for 24 hrs of exposure, but expression was significantly higher as compared to control for 48 hrs of exposure at the same dose i.e. 1 μ l/ml.

Keywords: *Eucalyptus citriodora*; *Drosophila melanogaster* (*hsp70-lacZ*)Bg⁹, *hsp70*

Introduction

Volatile oils present in plants are the complex mixtures of terpenes. They give them characteristic odor, flavor, and a number of other properties (Batish et al., 2006). The essential oils have been used since antiquity in flavor and fragrances, attractants, herbivore deterrents, stress tolerant, insect/pest repellent, pesticidal agent etc. (Barton, 2000; Langenheim, 1994; Holopainen, 2004; Peñuelas and Llusià, 2004; Dorman and Deans, 2000; Isman and Machial, 2006; Bakkali et al., 2008; Batish et al., 2006). *Eucalyptus citriodora* or lemon scented *eucalyptus* is a good source of *eucalyptus* oil, which is not only use in pharmaceutical but also in perfumery and other industries (Brooker and Kleinig, 2006). *Eucalyptus* oil is generally regarded as non toxic and has been placed under GRAS (Generally Regarded as

Safe) category by Food and Drug Authority of USA (USEPA, 1993). Although various commercialized form of the oils are available in market as insecticides, repellants, insecticide, etc. (Kegley et al., 2007). At present there are less numbers of commercialized products based on eucalyptus oil due to non toxicological evaluation against non target organisms (Isman, 2006). The reports on the toxic effects of its leaf extract are warranted. All organisms under various physical or chemical stressors respond by the expression of heat shock proteins (Lindquist and Craig, 1988). In the recent years, *hsp70* has been considered to be one of the candidate genes for predicting cytotoxicity against environmental chemicals (Bierkens, 2000; Lis et al., 1983; Mukhopadhyay et al., 2003; Siddique et al., 2012; 2011a,b).

In the present study, the toxic effects of the leaf extract of *Eucalyptus citriodora* was studied on third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg⁹ by quantifying *hsp70* expression by O-nitrophenyl- β -D-galactopyranoside (ONPG) assay. The tissue damage was estimated by trypan blue exclusion assay.

Materials and methods

Fly strain

A transgenic *Drosophila melanogaster* line that expresses bacterial β -galactosidase as a response to stress was used in the present study (Lis et al., 1983). In the said strain of flies, the transformation vector is inserted with a β -element, the line contains wild type *hsp70* sequence upto the *lacZ* fusion point. The flies and larvae were cultured on standard *Drosophila* food containing agar, maize powder, sugar and yeast at 24°C \pm 1 (Siddique, 2012).

Preparation of leaf extract

The leaves of *Eucalyptus citriodora* were collected from the Aligarh Muslim University campus and were identified by Dr. Badaruzzaman Siddiqui, Associate Professor, Department of Botany, AMU, Aligarh (Specimen Voucher No: 47771). The leaves were air dried and grounded to fine powder. Extraction was performed by soaking samples (2g of dry weight) in 300 ml of acetone for 8 hrs at 60°C in soxhlet's apparatus. After filtration, the extract concentrations of 1, 2, 4, 6, 8, 10 and 12 μ l/ml of food were established (Siddique et al., 2008).

Experimental Design

The extract concentrations of 1, 2, 4, 6, 8, 10 and 12 μ l/ml of food were established and third instar larvae were allowed to feed on them for 24 and 48 hrs. The expression of *hsp70* was estimated by soluble-O-nitrophenyl- β -D galactopyranoside (ONPG) assay and tissue damage was evaluated by trypan blue exclusion assay.

Soluble O-nitrophenyl- β -D-galactopyranoside (ONPG) assay

The expression of *hsp70* gives a measure of cytotoxicity (Chowdhuri et al., 1996). We followed a method described by Nazir et al. (2003). Briefly, after washing the larvae in phosphate buffer, they were put in a microcentrifuge tube (20 larvae/tube; 5 replicates/grou-

p), permeabilized for 10 min by acetone, and incubated overnight at 37°C in 600 µl of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 µl of Na₂CO₃. The extent of the reaction was quantified by measuring the absorbance at 420 nm.

Trypan blue exclusion test

The extent of tissue damage in larvae caused by *E. citriodora* leaf extract was evaluated by a dye exclusion test (Krebs and Feder, 1997; Nazir et al., 2003). Briefly, the internal tissues of larvae were explanted in a drop of phosphate buffer (PB), rotated in trypan blue stain for 30 min, washed thoroughly in PB and scored immediately for dark blue staining. A total of 50 larvae per treatment (10 larvae/dose; 5 replicates per group) were scored for the trypan blue staining on an average composite index per larva: no color, 0; any blue, 1; darkly stained nuclei, 2; large patches of darkly stained cells, 3; or complete staining of most cells in the tissue, 4 (Krebs and Feder, 1997).

Statistical analysis

Statistical analysis was carried out by student's 't' test using commercial software statistical soft Inc. The regression analysis was also performed to see the dose effect on *hsp70* expression.

Results

β-galactosidase activity measured after the exposure of third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg⁹ to different doses of *E. citriodora* extract is shown in Table 1 and Fig. 1, for 24 and 48 hrs of exposure. For 24 hrs of exposure to 1 µl/ml of *E. citriodora* extract there was no significant increase in β-galactosidase activity as compared to control. The exposure of 2, 4, 6, and 8 µl/ml of *E. citriodora* extract was associated with 0.106±0.0030, 0.134±0.0025, 0.143±0.0023 and 0.173±0.0018, respectively and showed a dose dependent significant increase in the β-galactosidase activity as compared to control (Table 1; Fig. 1). The exposure to 10 and 12 µl/ml of *E. citriodora* extract showed a reduction (though significant) in β-galactosidase activity i.e. 0.116±0.0018 and 0.116±0.0015, respectively (Table 1; Fig. 1). For 48 hrs of exposure to third instar larvae at 1, 2, 4, and 6 µl/ml showed a dose dependent significant increase in activity of β-galactosidase i.e.

Table-1 β-galactosidase activity measured in the transgenic *Drosophila melanogaster* (*hsp70-lacZ*)Bg⁹ third larvae exposed to different concentrations of *Eucalyptus citriodora* leaf extract.

Treatments EC E	After 24 hrs O.D (Mean ± SE)	After 48 hrs O.D (Mean ± SE)
1µl/ml	0.083 ± 0.0025	0.123 ± 0.0014*
2 µl/ml	0.106 ± 0.0030*	0.174 ± 0.0025*
4 µl/ml	0.134 ± 0.0025*	0.195 ± 0.0023*
6 µl/ml	0.143 ± 0.0023*	0.196 ± 0.0015*
8 µl/ml	0.173 ± 0.0018*	0.135 ± 0.0027*
10 µl/ml	0.116 ± 0.0018*	0.114 ± 0.0023*
12 µl/ml	0.116 ± 0.0015*	0.112 ± 0.0015*
Control	0.080 ± 0.0029	0.075 ± 0.0025

*Significant at P<0.05 compared to control; O.D: optical density; S.E: standard error, EC E: *Eucalyptus citriodora* extract

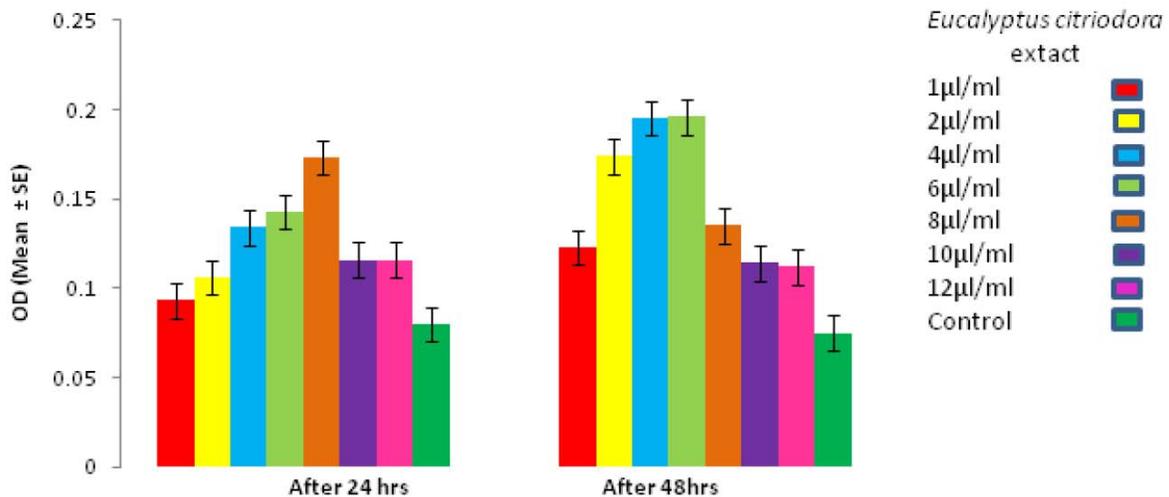


Figure 1. β -galactosidase activity observed after the exposure of *Eucalyptus citriodora* extract to the third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*^o.

Table-2 Regression analysis for the β -galactosidase activity in the third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*^o to study the dose effect of *Eucalyptus citriodora* extract for 24 and 48 hrs of exposure.

S. No.	Duration (hrs)	Regression Equation	β - coefficient	S.E	p-value	F-value
1.	24	$Y = 0.11227 + 0.00221X$	0.341	0.019	<0.002	0.657
2.	48	$Y = 0.17744 - 0.0045X$	-0.49	0.025	<0.001	1.59

0.123 \pm 0.0014, 0.174 \pm 0.0025, 0.195 \pm 0.0023 and 0.196 \pm 0.0015, respectively, as compared to control (0.075 \pm 0.0025) (Table 1; Fig. 1). The exposure of larvae to 8, 10 and 12 μ l/ml of *E. citriodora* extract showed a reduction (though significant) as compared to control for the activity of β -galactosidase i.e. 0.135 \pm 0.0079, 0.114 \pm 0.0023 and 0.112 \pm 0.0015, respectively

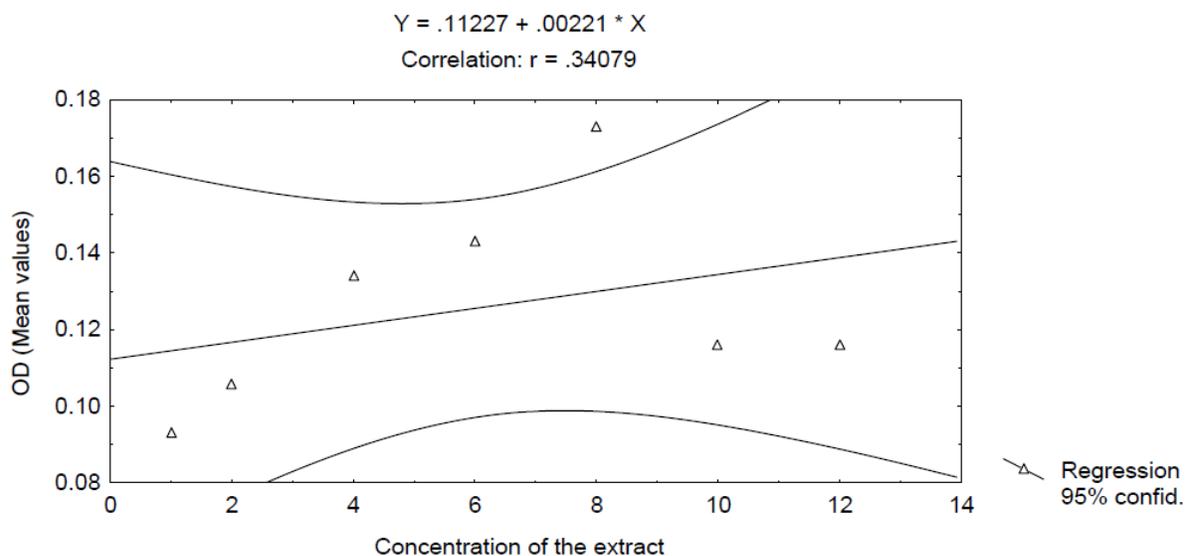


Figure 2. Regression analysis for the exposure of third instar larvae for 24 hrs to different doses of *Eucalyptus citriodora* (X μ l/ml)

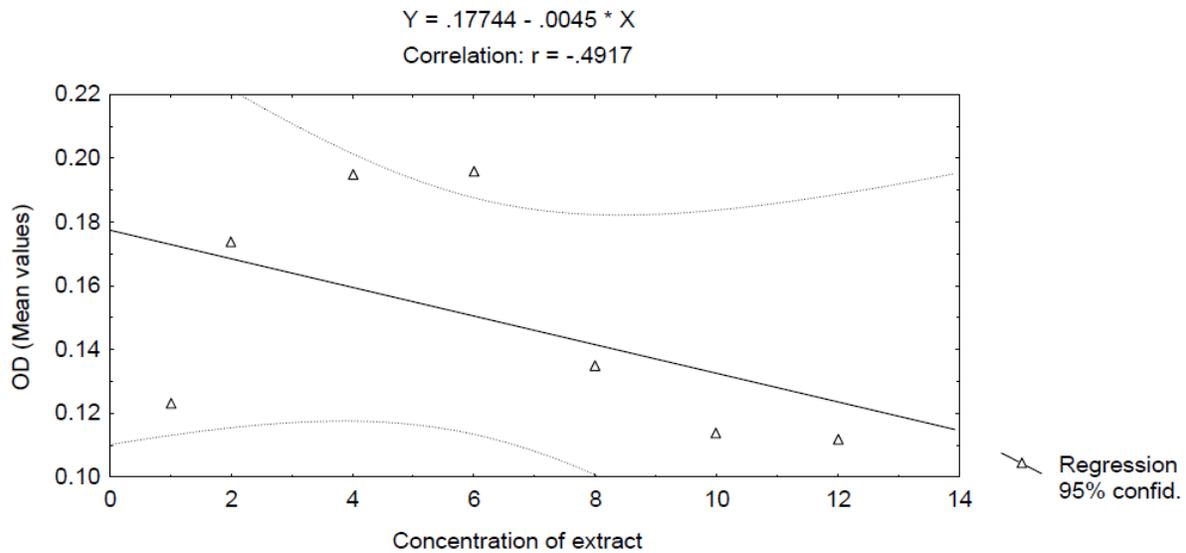


Figure 3. Regression analysis for the exposure of third instar larvae for 48 hrs to different doses of *Eucalyptus citriodora* (X μ l/ml)

(Table 1, Figure 1). Regression analysis was also performed to study the dose effect of *E. citriodora* extract on third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*)*Bg*⁹. For 24 and 48 hrs of exposure (Table 2; Fig. 2 and 3). For 24 hrs of exposure the β -coefficient was 0.341 (F=0.657) and for 48 hrs of exposure it was -0.49 (F=1.59) (Table 2; Fig 2). The reduction in the value of β -coefficient demonstrates a reduction in the β -galactosidase activity for the 48 hrs of exposure. Trypan blue staining was

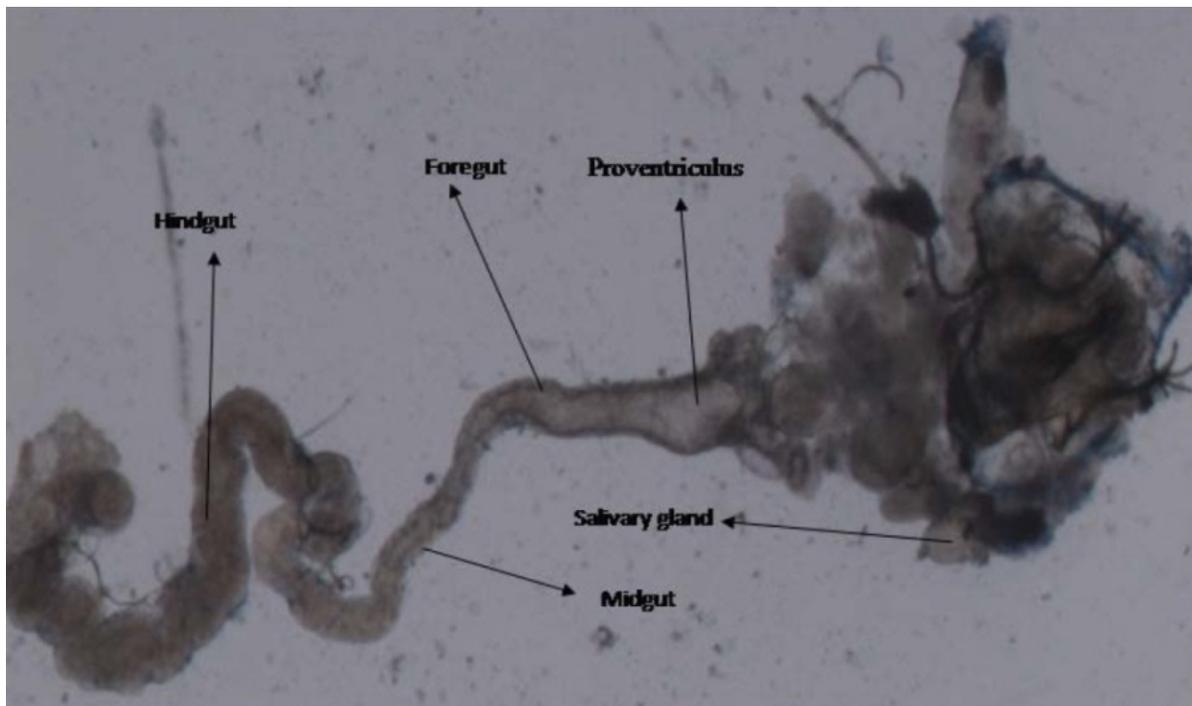


Figure 4. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ in untreated after 48 hours.

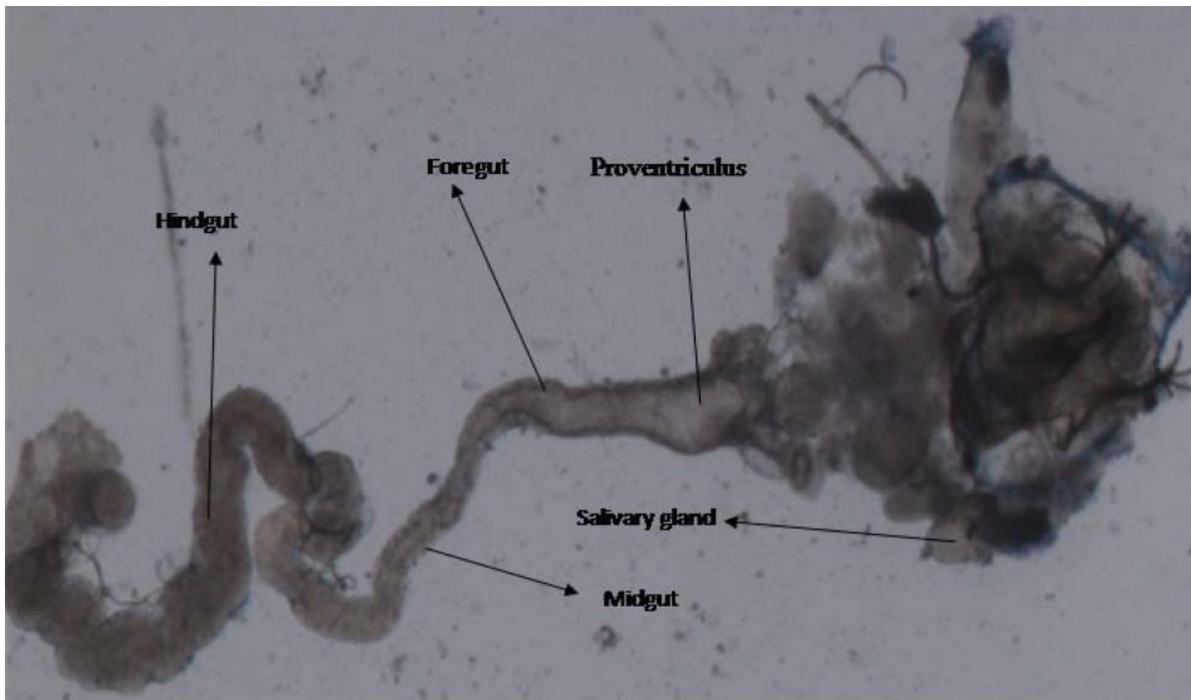


Figure 5. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ exposed to 2 µl/ml of *E. citriodora* extract (after 48 hours).

performed to study the tissue damage induced by *E. citriodora* extract. About 95% of untreated larvae were negative to trypan blue staining even. The larvae exposed to various doses of the extract did not show any tissue damage. However, only a slight tissue damage



Figure 6. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ exposed to 4 µl/ml of *E. citriodora* extract (after 48 hours).

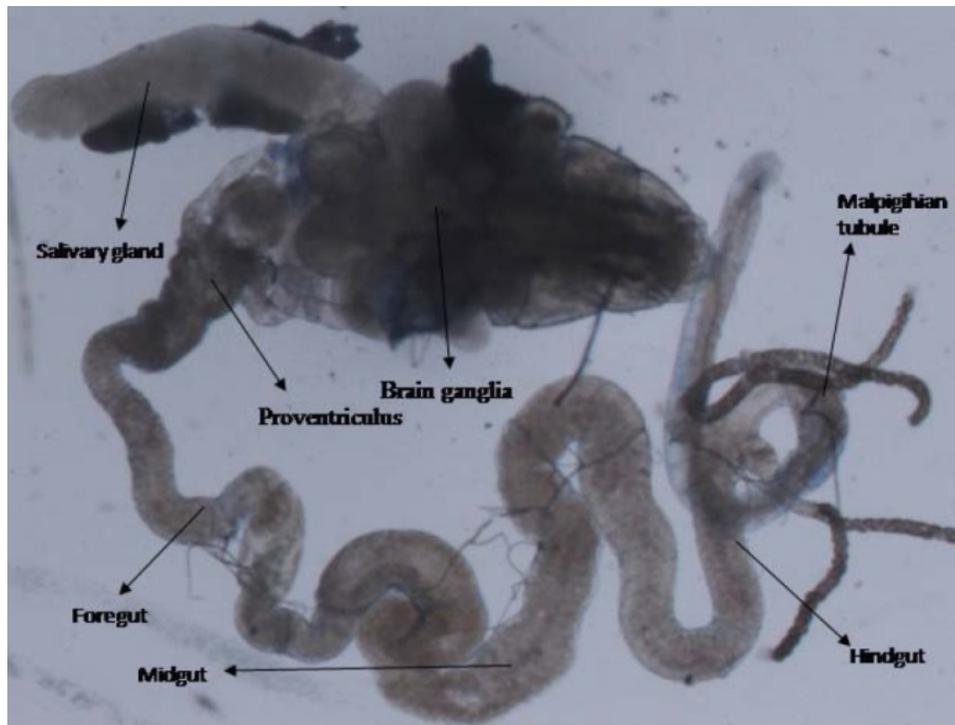


Figure 7. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ exposed to 6 μ l/ml of *E. citriodora* extract (after 48 hours).

was observed at the highest studied dose (12 μ l/ml) in salivary glands and malpighian tubules (Fig 4 -10).

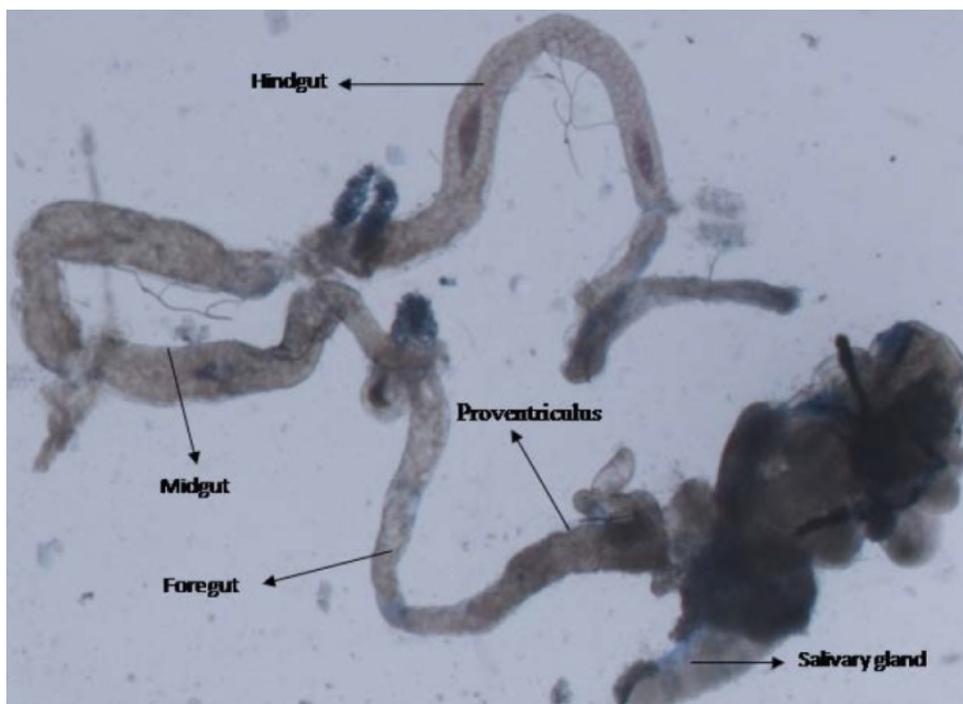


Figure 8. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ exposed to 8 μ l/ml of *E. citriodora* extract (after 48 hours).



Figure 9. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ exposed to 10 μ l/ml of *E. citriodora* extract (after 48 hours).

Discussion

The results obtained in the present study showed that 1 μ l/ml of leaf extract is not cytotoxic for 24 hrs of exposure but at 2, 4, 6 and 8 μ l/ml a significant dose dependent increase in the expression of *hsp70* was observed. Similarly for 48 hrs of exposure, a dose dependent increase in the *hsp70* expression was observed but a decrease was observed at 8, 10 and 12 μ l/ml of doses. A reduction in the activity of *hsp70* at the above concentrations may be due to reduction in the number of viable cells or due to auto repression of *hsp70* once its upper limit has been achieved. The higher doses or longer durations of exposure may also lead to the instability of the reporter gene, resulting in a decrease in *hsp70* expression. The eucalyptus oil/extract consist of oxides, aromatic phenols, ethers, monoterpenes and sesquiterpenes, esters, aldehydes, ketones and other minor components, but the exact activity composition and proportion, varies species to species (Brooker and Kleinig, 2006). For the activity of any plant extract, it has been suggested that the property of any plant extract should be studied as a whole because since antiquity various plants extract were/are being used to cure various ailments as a whole in Unani and Ayurveda (Chitravadivu et al., 2009). Hence, in the present study the effect of extract as a whole was studied to show its effect on *hsp70* expression. However, the main component of *E. citriodora* extract is citronellal (Ramezani et al., 2002; Batish et al., 2006; Su et al., 2006), but it also consist of cineole, citronellol, limonene, linalool, and α -terpinene (Brooker and Kleinig, 2004; Batish et al., 2006). It has been reported that various components of eucalyptus oil act synergistically to show the overall activity (Cimanga et al., 2002). The oral and acute LD₅₀ of eucalyptus oil in rat is 4440 mg/kg body weight (Regnault Roger, 1997). It is evident from the trypan blue assay that there is less tissue damage on the exposure to various doses of *E. citriodora* extract. The compound present in the extract may inhibit *hsp70* expression without causing the tissue

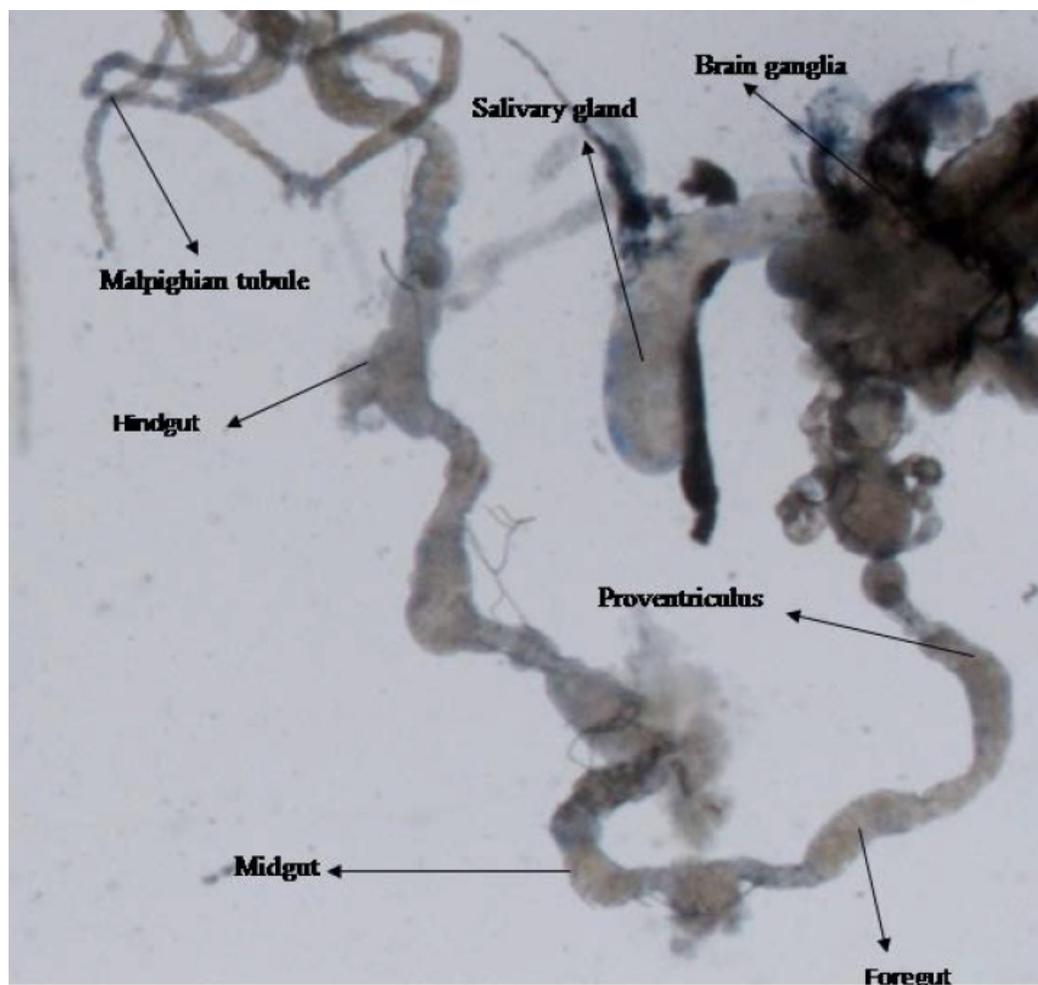


Figure 10. Trypan blue staining for third instar larvae of transgenic *D. malongaster* (*hsp70-lacZ*) *Bg*⁹ exposed to 12 µl/ml of *E. citriodora* extract (after 48 hours).

damage. It is evident from the ONPG assay that the reduction in the activity of β -galactosidase begins from 8µl/ml for 48 hrs of exposure. However, the exact mechanism at this stage is not clear but it will be the part of our future study. According to the National Toxicological Programme guidelines development and validation of alternative models is necessary to obtain reliable and sensitive results. For traditional toxicological studies a shift has taken place from the use of mammalian models to alternative models (Avanesian et al., 2009). Genetically modified models provide exact information with regard to the toxicity of the compounds or extracts (Avanesian et al., 2009). *Drosophila* as a model in toxicological evaluations is time and cost effective in comparison to rodents. The European Centre for the Validation of Alternative Methods (EVCAM) has recommended the use of *Drosophila* as an alternative model for scientific studies (Festing et al., 1998; Benford et al., 2000). In the present study the transgenic *Drosophila melanogaster* line that expresses bacterial- β -galactosidase as a response to stress was used. Increase in the levels of *hsp70* expression have been established as a measure of cellular assault (Siddique et al., 2011; Siddique et al., 2010; Siddique et al., 2012; Siddique, 2012). Hence, it is concluded that the *hsp70* expression in response to the environmental chemicals is a potential indicator of non-target toxicity. The present results are the suggestive of the cytotoxic potential of *E. citriodora* to non-targeted

organisms like *Drosophila* and also support the use of *hsp70* expression as a bioindicator of exposure to various environmental agents.

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