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Original article

Abiotic factors and anti-reproductive action of bait containing eugenol against *Lymnaea acuminata*

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ABSTRACT

The effect of abiotic factor (temperature, pH, dissolved oxygen, carbon dioxide) on the reproduction of snail L. acuminata fed to bait containing 40% and 80% of 24h LC50 eugenol have been reported in this paper. Feeding of baits containing sub-lethal concentration of eugenol caused a significant reduction in fecundity, hatchability and survival of young snails. Maximum (1644 egg/20 snails) and minimum (228 eggs/ 20 snails) fecundity was observed in month of May and December, respectively. There was a significant ($P<0.05$) change in the endogenous level of protein, amino acid, DNA and RNA in the ovotestis of L. acuminata. Treatment of 80 % of 24h LC50 with eugenol in bait caused maximum reduction in protein (52.12% of control), amino acid (11.89% of control), DNA (30.90% of control) and RNA (13.12% of control) level. Simultaneous, inhibition in acetylcholinesterase (AChE) activity in nervous tissue was also noted. Maximum inhibition in AChE activity (36.19% of control) was observed in snail exposed to 80 % of 24h LC50 of eugenol.

1. Introduction

Fasciolosis is an important helminthes disease caused by Fasciola hepatica and F. gigantica (Singh and Agarwal, 1981; Mas-Coma et al., 2009). Several species of fresh water snail belonging to the family Lymnaeidae are

intermediate host of genus Fasciola. This species occurs throughout the world and act as intermediate host of Fasciola (Agarwal and Singh, 1988; Sierra et al., 2011). Presently, it is now considered to be an emerging disease in human all over the world (Mas-Coma et al., 2005). Lymnaea acuminata breeds all the year round and lays eggs on the lower surface of aquatic vegetation. Earlier studies have shown that the reproductive capacity of snail significantly altered from one season to other (Maat et al, 1983; Wayne, 2001; Jigyasu and Singh, 2010). Ovipositions in snails are induced by neuroendocrine hormone of Caudo-Dorsal Cells (CDC) in the cerebral ganglion (Maat et al, 1982; Takeda, 1977; Singh et al, 2008). Several mechanisms are involved in the release of ovipository hormone (Brink et al, 1992). Environmental factor such as temperature, pH, dissolved oxygen, and carbon dioxides are major seasonal variant that affect the morphological characteristics of CDCs (Wayne, 2001). One of the possible approaches to control the incidence of fasciolosis is to interrupt the life cycle of the parasitic trematodes by eliminating intermediate host. Baits are the best approach for long term management of slugs and snails (Angelis, 2007). One of the main advantages of baits that there is no direct release of pesticides in to the environment. Use of a combination of snail attractant and molluscicides in bait formulation is an effective tool for the pest management (Kumar et al, 2011; Agrahari et al, 2012). The objective of the present study is to explore the possibility that whether abiotic factors (temp, pH, CO2, DO) and bait containing eugenol can affect the reproductive capacity of snail L. acuminata and simultaneously, biochemical parameters in each month of the year 2011-12 or there is no such effect.

2. Materials and methods

2.1. Test animals

Adult L. acuminata (2.25±0.20 cm in length) were locally collected in each month of the year Nov- 2011- Oct-2012. The snails were acclimatized for 72 hours in dechlorinated tap water .The pH of the water was 7.1-7.3 and dissolved oxygen, free carbon dioxide and bicarbonate alkalinity were 6.5-7.2, 5.2-6.3, and 102.0-105.0 mg/l, respectively.

2.2. Pure compound

Agar-agar, amino acids (starch and serine purchase from Qualigens Fine Chemicals and Sisco Research Laboratories Pvt. Ltd. Mumbai, India), Eugenol (2-Methoxy-4-(2 propenyl) phenol) was procured from Sigma Chemical Co. (USA)

2.3. Preparation of bait formulations with plant molluscicide

Bait formulations were prepared by the method of Madsen (1982) , as modified by Tiwari and Singh (2004). In brief, 0.02 g of amino acid (20 mM) added in to 2% agar solution. After boiling plant molluscicides eugenol (40% and 80% of 24h LC50) was added to the solution. The mixture was stirred constantly for 30 minute and spread a uniform thickness (5mm). After cooling, the bait pellets were cut out from the layer with a corer (5 mm diameter).

2.4. Assay apparatus and procedure

The bioassay was performed by the method of Tiwari and Singh (2004). Each regimen of 5 liter water was kept in six aquaria separately, containing 20 snails in each aquarium. Simultaneously, one of the prepared bait of active component eugenol (molluscicide) was added in each aquarium. After every 24 hours up to 96 hours, the total number of eggs oviposited by the snails were counted in each aquarium. Temperature, pH, dissolved oxygen and dissolved free carbon dioxide of different regimen of water were measured simultaneously. Temperature, pH were measured by thermometer and digital pH meter, respectively. Dissolved O2 and CO2 were estimated according to methods prescribed by APHA (2005). After 96 hours, the snails exposed to different regimen of water were removed from aquaria and washed with fresh water. The ovotestis /nervous tissue were dissected out and placed on filter paper for removal of adherent water and weighed. Protein, free amino acids, DNA, RNA and enzyme AChE activity were then measured.

2.5. Estimation of total protein and free amino acid

Total protein (μg/mg) was estimated according to Lowry et al. (1951) using bovine serum albumin as a standard. Homogenates of ovotestis were prepared in 10% (w/v) trichloro acetic acid (TCA). Total free amino acids (μg/mg) were determined according to the method of Spies (1957).

2.6. estimation of nucleic acids

DNA and RNA in ovotestis of L. acuminata were estimated according to Shneider (1957) using diphenylamine and orcinol reagents, respectively. Homogenates (1.0 mg/ml, w/v) were prepared in 10%TCA at 90ºC and centrifuged at 5000 g for 20 minutes. The supernatants were used for DNA and RNA estimations.

2.7. Estimation of acetylcholinesterase (AChE)

Acetylcholinesterase activity was measured according to the method of Ellman et al.,(1961) as modified by Singh et al (1982). Fifty milligram of nervous tissue of L. acuminata was taken around the buccal mass and homogenized in 1.0 ml of 0.1M phosphate buffer pH 8.0 for 5 minutes in an ice bath then centrifuged at 1000 g for 30 minutes at 40C. The supernatant was used as an enzyme source. Enzyme activity was measured in a 10 mm path length cuvette using an incubation mixture consisting of 0.1 ml of chromomeric agent DTNB (5,5-dithiobis–2-nitrobenzoic acid), and 0.02 ml of freshly prepared ATChI (acetylthiocholine iodide) solution in distilled water. The change in optical density at 412 nm was recorded for 3 minutes after every 30 second interval at 250C. Enzyme activity has been expressed as μ mole/ 'SH'/ hydrolyzed/ min/ mg protein.

2.8. Statistical analysis

Each experiment was replicated at least six times, Values of temperature pH, dissolved O2 and CO2 are expressed as mean of six replicates. Values of fecundity and biochemical parameter (protein, amino acids, DNA, RNA and AChE activity) were expressed as Mean ± SE. Product moment correlation coefficient was applied to determine significant ($P<0.05$) difference between environmental factors (temperature, pH, dissolved O2 and CO2) and fecundity of snail fed with bait containing eugenol. Student t-test was applied in between altered biochemical parameters and enzyme activity (Sokal and Rohlf, 1973) in control and treated snails.

3. Results

Lymnaea acuminata laid their egg in gelatinous string, each egg floating in albuminous fluids bounded externally by a membrane. The ovoid eggs were laid in 2 rows. There was significant ($P<0.05$) variation in the fecundity of L. acuminata kept in different concentration of bait formulations (40% and 80% of 24h LC50) with molluscicide eugenol. In the control group of snails, the maximum fecundity in 24h was observed in the month of May (2631 eggs/20 snails) and minimum (739 egg/ 20 snails) in March (Table 1). 24h treatment with Eu+Se+Ag (40% of 24h LC50) caused maximum (1593 eggs/ 20 snails) and minimum (228 eggs/20 snails) fecundity in month of June and December (Table 1). Whereas, snail treated with Eu+St+Ag (40% of 24h LC50) maximum (1644 eggs/20 snails) and minimum (241eggs/20 snails) fecundity in the month of May and December (Table 2). A significant ($P<0.05$) (+) positive / (*) negative correlation was noted between dissolved oxygen/CO2/pH of water and fecundity, hatchability and survival of young snail in each month. In contrast, a significant negative correlation was observed in between temperature and fecundity (Table 1, 2). The control group of snails hatched in to young snail within 7-9 days (Table 1, 2). Complete embryonic development was lacking in the egg of snails kept in bait formulation of 80% of 24h LC50. Percent hatchability in the treated group was between 87.6-65.4% and 90.3-62.1% in control group (Table 1, 2). The hatching period was prolonged 9 to 18 days than the control group (7- 9 days). Maximum prolongation (9-18 days of control) of hatchability was noticed in eggs laid in December month, whereas minimum (7-9 days of control) in the month of June (Table 1, 2).

There was a significant ($P<0.05$) change in the endogenous level of protein, amino acid, DNA and RNA in ovotestis of exposed snails. The acetylcholinesterase activity in nervous tissue of snail fed to 80% of 24h LC50 of bait containing eugenol was significantly inhibited in different month of the year 2011-2012. Maximum reduction in level of DNA (21.65 % of control) was noted in the month of July and RNA (21.95% of control) in the month of June. Reduction in ovotestis protein (49.97% of control) and amino acid level (11.89 % of control) was observed in the month of June to August (Table 3). Treatment with Eu+St+Ag in bait formulation caused maximum reduction in the level of amino acid (13.90 % of control) in the month of June and protein (52.12 % of control) was observed in the month of September. Reduction in the level of DNA (30.90 % of control) was observed in the month of July and

RNA (18.89 % of control) in the month of June (Table 4). With the treatment of Eu+Se+Ag there was a highest inhibition in AChE activity (36.14 % of control) within the nervous tissue of L. acuminata was noted when they fed to 80% of 24h LC50. in the month of December (Table 4). Whereas, treatment of Eu+Se+Ag in bait formulation cause inhibition of the AChE activity up to (38.03 % of control) in the month of December (Table 3). There is no significant difference between control and treatment of agar alone in bait formulation.

4. Discussion

The active component eugenol (Syzygium aromaticum) is very effective molluscicide against L. acuminata. Although its molluscicidal activity in different months has been reported earlier (Agrahari et al, 2012), yet in the present study its anti-reproductive property of eugenol is noticed. Seasonal fluctuations in the secretory neuroendocrine cells of Aplysia californica inhibited the protein Kinase A and C which play a significant role in regulation of egg laying hormone (Wayne et al, 1998). According to their cAMP and diacylglycerol second messenger pathways are regulated on a seasonal basis (Wayne et al, 1998)).

Dissolved oxygen is one of the major components required by snail's metabolic activity (Watten, 2004). In normal condition metabolic demand for oxygen increases substantially with temperature (Portner, 2002). At higher temperature the increasing rate of snails metabolism CO2 affects the pH of water (Berge et al, 2006). This was evident from the elevated concentration of CO2 which causes decrease in pH of water during the summer season. It seems that cumulative effect of these abiotic factors on the level of protein, amino acids and nucleic acids in ovotestis of L. acuminata directly or indirectly through caudo dorsal cells (CDCs), which release ovulation hormone and ultimately affect the reproduction of snails in different months of the year.

The reduction in protein levels may be due to indirect interference of the environmental abiotic factors with protein synthesis. pH is one of the crucial environmental factor that have significant effect on number of enzymes involved in protein synthesis (Madshus, 1988). Amino acids are the building blocks for structural protein and enzyme. Change in the level of DNA and RNA in ovotestis of L. acuminata were significantly influenced by the water temperature (Schlechtriem et al, 2008). Small change in DNA/RNA levels can have a tremendous impact on biological parameters (Danilowicz et al, 2007). and elevated temperature from 5-25ºC induces spermatogenic, DNA synthesis and formation of spermatid and spermatozoa in ovotestis of snails (Gomot and Gomot, 1991) . RNA translation efficiency is lower at higher temperature (Pannevis and Houlihan, 1992). Therefore animals need higher concentration of RNA to maintain their metabolic function at lower temperature (Schlechtriem et al, 2008). The synthesis of DNA and RNA also influenced by the intracellular pH physiological range. The activity increases with increasing pH from 7.0-8.0 the process of cellular growth and divisions requires the synthesis of nucleic acids and protein (Jigyasu et al, 2010). Dohi et al. (2009) reported that eugenol is a potent actylcholinesterase inhibitor. Kumar et al. (2009) noted that In vitro exposure of eugenol is competitive non-competitive inhibitor of acetylcholinesterase. Possibly, exposure of eugenol start the accumulation of acetylcholine in the synapse may affect the caudo dorsal cell. There is a significant positive correlation between the AChE activity and the fecundity of snail. It indicates that the reproductive capacity of snail up to some extent is mediated through cholinergic stimuli in the brain of snail. Earlier, it has been reported that prostaglandins have significant role in the egg laying of snail L. acuminata (Singh and Agarwal, 1981; Singh et al, 2010). There is no report that in invertebrate eugenol affect the prostaglandin synthesis metabolism. Raghvendra et al. (2006) reported that eugenol inhibit the prostaglandin synthesis by inhibiting 5-lipoxygenase activity and leukotrienes. It may be possible that eugenol inhibit the prostaglandins metabolism in exposed snail, so that fecundity is reduced in different month of the year 2011-12.

5. Conclusion

Conclusively, it can be states that eugenol significantly altered the reproductive capacity of snails. Their anti-reproductive action against L. acuminata significantly altered with respect to the change in the abiotic factors in different months of the year. In this way eugenol can be used precisely in different concentration of eugenol in different months to check the population of snail below a threshold level, so that incident of fasciolosis can be controlled. Certainly, this study will provide a data that can be utilized to reduce the number of snails to reduce the level of fasciolosis in Eastern Uttar Pradesh.

Table 1
Effect of sub-lethal concentration (40% and 80% of 24 h LC50) of eugenol + serine + agar bait formulation on the reproduction of snail L. acuminate.

Month	Treatment	24h LC_{50}	Sub-lethal dose of 24h LC_{50} mg/l	Abiotic Factor				Fecundity/ 20 snails/ Day				Hatchability (%)	Survival (%)		
				Temp	pH	DO ppm	CO_2 ppm	24h	48h	72h	96h		24h	48h	72h
November 2011	Control	-	-	24 ºC	9.34	0.7	18.0	1646±0.85	284±0.92	254±0.81	196±0.84	100(7-9)	100	100	100
	Eu+Se+Ag	3.03	40% (1.21)	24 ºC*	9.13+	0.7*	18.0*	942±0.65	252±0.45	184±0.63	147±0.85	85.6±0.85(8-9)	64.2±0.41	32.1±0.65	16.5±0.65
			80% (2.42)	24 ºC+	9.02+	0.5*	14.0+	644±0.58	242±0.52	119±0.85	78±0.56	67.3±0.78(10-12)	48.6±0.25	18.7±0.52	0
December 2011	Control	-	-	18 ºC	8.92	0.6	18.0	1234±0.85	164±0.96	168±0.74	93±0.85	100(7-10)	100	100	100
	Eu+Se+Ag	4.14	40% (1.65)	18 ºC*	8.91+	0.5*	16.0*	392±0.32	126±0.62	92±0.65	28±0.63	87.6±0.85(7-16)	64.2±0.85	35.2±0.56	22.5±0.95
			80% (3.30)	18 ºC+	8.86+	0.3*	16.0+	228±0.65	58±0.45	56±0.54	0	71.6±0.63(9-18)	45.6±0.56	18.9±0.65	0
January 2012	Control	-	-	10 ºC	8.56	0.6	19.0	1432±0.52	462±0.85	142±0.52	182±0.93	100(7-10)	100	100	100
	Eu+Se+Ag	11.13	40% (4.45)	10 ºC*	8.18+	0.4*	19.0*	840±0.96	80±0.32	28±0.65	92±0.85	86.5±0.58(9-12)	56.5±0.65	34.2±0.85	14.6±0.93
			80% (8.90)	10 ºC+	8.09+	0.4*	18.0+	720±0.74	103±0.63	92±0.56	26±0.65	68.7±0.45(10-15)	45.2±0.74	23.5±0.45	12.6±0.65
February 2012	Control	-	-	21 ºC	9.64	0.7	18.0	1468±0.48	242±0.56	48±0.65	142±0.63	100(8-10)	100	100	100
	Eu+Se+Ag	8.92	40% (3.56)	21 ºC*	9.64+	0.5*	18.0*	1032±0.74	214±0.45	54±0.36	0	74.6±0.65(9-12)	68.8±0.75	35.8±0.93	19.7±0.71
			80% (7.12)	21 ºC+	9.68+	0.5*	17.0+	983±0.84	128±0.98	26±0.56	0	63.5±0.65(11-14)	36.5±0.56	18.6±0.95	0
March 2012	Control	-	-	22 ºC	9.63	0.6	18.0	739±0.78	234±0.83	132±0.98	84±0.71	100(7-11)	100	100	100
	Eu+Se+Ag	9.30	40% (3.72)	22 ºC*	9.62+	0.6*	17.0*	638±0.85	176±0.32	83±0.54	52±0.65	86.4±0.96(8-12)	62.5±0.65	39.8±0.65	27.4±0.84
			80% (7.44)	22 ºC+	9.53+	0.5*	15.0+	563±0.25	206±0.65	162±0.45	88±0.78	84.3±0.65(9-15)	32.1±0.85	14.6±0.65	0
April 2012	Control	-	-	26 ºC	9.86	0.6	19.0	1452±0.78	232±0.85	189±0.65	64±0.86	100(7-9)	100	100	100
	Eu+Se+Ag	8.56	40% (3.42)	26 ºC*	9.73+	0.7*	19.0*	1063±0.32	190±0.56	143±0.65	0	76.2±0.98(8-10)	56.8±0.45	36.5±0.65	23.1±0.81
			80% (6.84)	26 ºC+	9.65+	0.5*	15.0+	940±0.96	285±0.96	86±0.32	51±0.87	62.5±0.78(9-11)	32.5±0.32	13.5±0.96	0
May 2012	Control	-	-	28 ºC	9.65	0.6	18.0	2631±0.96	282±0.62	94±0.63	0	100(7-9)	100	100	100
	Eu+Se+Ag	7.55	40% (3.02)	28 ºC*	9.41+	0.5*	17.0*	1537±0.69	124±0.63	76±0.25	34±0.95	85.4±0.65(8-10)	65.4±0.62	32.8±0.84	22.6±0.92
			80% (6.04)	28 ºC+	9.36+	0.5*	17.0+	847±0.98	243±0.45	68±0.96	0	82.6±0.65(10-16)	36.8±0.54	23.4±0.86	14.9±0.96
June 2012	Control	-	-	31 ºC	9.65	0.6	18.0	1650±0.91	168±0.53	148±0.65	76±0.85	100(8-10)	100	100	100
	Eu+Se+Ag	3.18	40% (1.27)	31 ºC*	9.28+	0.6*	18.0*	1593±0.85	173±0.32	85±0.96	0	75.6±0.98(9-12)	52.6±0.63	37.4±0.65	21.6±0.68
			80% (2.54)	31 ºC+	9.24+	0.3*	17.0+	859±0.85	168±0.85	39±0.36	0	65.2±0.85(3-16)	36.5±0.65	14.6±0.84	0
July 2012	Control	-	-	32 ºC	9.62	0.6	14.0	1526±0.65	238±0.51	92±0.95	46±0.56	100(7-10)	100	100	100
	Eu+Se+Ag	3.41	40% (1.36)	32 ºC*	9.56+	0.6*	12.0*	1248±0.65	73±0.74	62±0.74	42±0.98	86.5±0.65(8-11)	67.5±0.69	41.3±0.73	26.8±0.65
			80% (2.72)	32 ºC+	9.32+	0.4*	12.0+	854±0.32	88±0.45	72±0.78	36±0.65	74.1±0.78(10-15)	47.4±0.56	21.5±0.56	8.6±0.54
August 2012	Control	-	-	34 ºC	9.82	0.8	14.0	1354±0.86	263±0.74	158±0.74	65±0.63	100(7-9)	100	100	100
	Eu+Se+Ag	4.15	40% (1.66)	34 ºC*	9.81+	0.7*	14.0*	924±0.65	132±0.45	83±0.54	38±0.65	86.4±0.54(9-11)	74.6±0.52	34.6±0.52	22.9±0.65
			80% (3.32)	34 ºC+	9.62+	0.5*	14.0+	611±0.32	212±0.23	54±0.52	44±0.65	71.2±0.54(9-13)	58.2±0.95	32.6±0.65	14.6±0.86
September 2012	Control	-	-	31 ºC	9.89	0.7	18.0	1265±0.98	235±0.56	148±0.85	96±0.63	100(7-9)	100	100	100
	Eu+Se+Ag	5.90	40% (2.36)	31 ºC*	9.89+	0.6*	18.0*	932±0.65	186±0.65	124±0.45	53±0.96	65.4±0.65(8-10)	74.5±0.93	35.9±0.36	15.8±0.95
			80% (4.72)	31 ºC+	9.82+	0.5*	15.0+	445±0.32	134±0.65	54±0.74	0	58.6±0.63(9-13)	41.2±0.98	23.5±0.52	0
October 2012	Control	-	-	26 ºC	9.63	0.8	18.0	1256±0.96	169±0.85	126±0.96	76±0.63	100(7-9)	100	100	100
	Eu+Se+Ag	5.75	40% (2.30)	26 ºC*	9.56+	0.6*	18.0*	865±0.52	73±0.32	49±0.63	0	82.5±0.50(9-12)	63.1±0.63	32.8±0.65	15.6±0.65
			80% (4.60)	26 ºC+	9.52+	0.6*	16.0+	324±0.52	68±0.56	56±0.69	0	76.3±0.41(10-16)	39.0±0.65	14.6±0.65	0

Each experiment was replicated six times and the value of temperature, pH, Dissolved oxygen and free CO2, fecundity, hatchability and survival is the mean of six replicate. Product movement correlation coefficient in between the fecundity and a biotic factor indicate significant (P<0.05) (+) positive / (*) negative correlation. Abbreviation: Eu=Eugenol, Se=Serine, Ag=Agar

Table 2
Effect of sub-lethal concentration (40% and 80% of 24 h LC50) of eugenol + starch + agar bait formulation on the reproduction of snail L. acuminata.

Month	Treatment	24h LC_{50}	Sub-lethal dose of 24h LC_{50} mg/l	Abiotic Factor				Fecundity/20 snails/Day				Hatchability (%)	Survival (%)		
				Temp	pH	DO ppm	CO_2 ppm	24h	48h	72h	96h		24h	48h	72h
November 2011	Control	-	-	24 ºC*	9.34	0.7	18.0	1646±0.85	284±0.92	254±0.81	196±0.84	100(7-9)	100	100	100
	Eu+St+Ag	3.03	40% (1.21)	24 ºC+	9.35*	0.7+	18.0+	760±0.98	153±0.25	93±0.75	52±0.81	86.2±0.45(8-9)	56.3±0.23	32±0.52	18±0.65
			80% (2.42)	24 ºC*	8.69*	0.5+	16.0+	614±0.69	145±0.65	124±0.63	76±0.58	71.6±0.87(10-12	44.2±0.86	26.5±0.89	0
December 2011	Control	-	-	18 ºC	8.91	0.5	18.0	1234±0.85	164±0.96	168±0.74	93±0.85	100(7-10)	100	100	100
	Eu+St+Ag	4.14	40% (1.65)	18 ºC+	8.82*	0.6+	18.0+	314±0.71	108±0.78	156±0.63	81±0.53	84.9±0.58(7-16)	56.4±0.87	32.1±0.85	21.4±0.58
			80% (3.30)	18 ºC*	8.75*	0.5+	13.0+	241±0.95	132±0.96	98±0.93	0	68.7±0.65(8-17)	68.0±0.45	41.6±0.79	35.6±0.65
January 2012	Control	-	-	10 ºC	8.56	0.6	19.0	1432±0.52	462±0.85	142±0.52	182±0.93	100(7-10)	100	100	100
	Eu+St+Ag	11.13	40% (4.45)	10 ºC+	8.76*	0.5+	17.0+	926±0.83	312±0.83	36±0.85	0	78.9±0.96(9-12)	67.4±0.96	41.6±0.56	18.7±0.65
			80% (8.90)	10 ºC*	8.79*	0.3+	14.0+	812±0.54	96±0.58	54±0.48	47±0.56	71.0±0.74(10-15)	46.8±0.89	24.7±0.78	0
February 2012	Control	-	-	21 ºC	9.61	0.7	18.0	1468±0.48	242±0.56	48±0.65	142±0.63	100(8-10)	100	100	100
	Eu+St+Ag	8.92	40% (3.56)	21 ºC+	9.63*	0.4+	16.0+	1232±0.65	242±0.79	32±0.74	18±0.96	62.1±0.85(9-12)	66.5±0.65	32.5±0.45	14.6±0.98
			80% (7.12)	21 ºC*	9.62*	0.4+	16.0+	893±0.79	172±0.86	123±0.81	49±0.74	86.2±0.75(11-14)	35.8±0.54	14.1±0.96	0
March 2012	Control	-	-	22 ºC	9.63	0.6	18.0	739±0.78	234±0.83	132±0.98	84±0.71	100(7-11)	100	100	100
	Eu+St+Ag	9.30	40% (3.72)	22 ºC+	9.62*	0.5+	15.0+	632±0.96	352±0.81	79±0.76	33±0.68	68.8±0.91(8-12)	62.4±0.74	48.7±0.89	26.9±0.56
			80% (7.44)	22 ºC*	9.58*	0.3+	12.0+	462±0.85	363±0.51	132±0.95	74±0.69	62.7±0.83(9-15)	53.1±0.95	32.5±0.65	17.6±0.52
April 2012	Control	-	-	26 ºC	9.86	0.6	19.0	1452±0.78	232±0.85	189±0.65	64±0.86	100(7-9)	100	100	100
	Eu+St+Ag	8.56	40% (3.42)	26 ºC+	9.69*	0.6+	18.0+	931±0.76	314±0.62	92±0.56	64±0.49	74.3±0.84(8-10)	75.4±0.65	53.6±0.78	36.5±0.45
			80% (6.84)	26 ºC*	9.65*	0.5+	16.0+	864±0.38	235±0.34	136±0.57	94±0.69	71.6±0.78(9-11)	52.1±0.54	29.6±0.54	12.4±0.96
May 2012	Control	-	-	28 ºC	9.65	0.6	18.0	2631±0.96	282±0.62	94±0.63	0	100(7-9)	100	100	100
	Eu+St+Ag	7.55	40% (3.02)	28 ºC+	9.74*	0.7+	19.0+	1644±0.74	203±0.65	48±0.96	19±0.58	88.4±0.92(8-10)	67.8±0.96	59.1±0.69	35.4±0.78
			80% (6.04)	28 ºC*	9.14*	0.5+	18.0+	1432±0.81	183±0.74	63±0.95	0±0.78	79.2±0.74(10-16)	41.2±0.84	30.1±0.78	14.6±0.65
June 2012	Control	-	-	31 ºC	9.65	0.6	18.0	1650±0.91	168±0.53	148±0.65	76±0.85	100(7-9)	100	100	100
	Eu+St+Ag	3.18	40% (1.27)	31 ºC+	9.24*	0.5+	15.0+	1624±0.65	93±0.69	65±0.63	57±0.74	90.3±0.73(7-9)	52.5±0.71	32.6±0.96	16.5±0.78
			80% (2.54)	31 ºC*	9.62*	0.3+	12.0+	1386±0.93	186±0.74	95±0.45	91±0.74	74.6±0.62(9-16)	36.8±0.82	15.7±0.92	0
July 2012	Control	-	-	32 ºC	9.62	0.6	14.0	1526±0.65	238±0.51	92±0.95	46±0.56	100(7-10)	100	100	100
	Eu+St+Ag	3.41	40% (1.36)	32 ºC+	9.63*	0.5+	16.0+	1383±0.76	230±0.52	53±0.48	0	65.4±0.53(8-11)	61.2±0.58	36.4±0.69	24.7±0.78
			80% (2.72)	32 ºC*	9.41*	0.5+	15.0+	1244±0.65	189±0.85	48±0.54	0	67.5±0.85(10-15)	56.8±0.74	31.4±0.98	12.3±0.98
August 2012	Control	-	-	34 ºC	9.82	0.8	14.0	1354±0.86	263±0.74	158±0.74	65±0.63	100(7-9)	100	100	100
	Eu+St+Ag	4.15	40% (1.66)	34 ºC+	9.83*	0.6+	16.0+	1210±0.45	124±0.65	96±0.78	84±0.63	71.0±0.69(9-11)	74.6±0.75	46.5±0.93	26.8±0.65
			80% (3.32)	34 ºC*	9.62*	0.4+	14.0+	853±0.95	88±0.96	73±0.56	0	63.1±0.86(10-13)	64.6±0.65	34.7±0.98	23.8±0.85
September 2012	Control	-	-	31 ºC	9.89	0.7	18.0	1265±0.98	235±0.56	148±0.85	96±0.65	100(7-9)	100	100	100
	Eu+St+Ag	5.90	40% (2.36)	31 ºC+	9.46*	0.6+	16.0+	845±0.91	92±0.75	62±0.74	0	85.6±0.39(8-10)	56.8±0.85	29.6±0.35	13.5±0.58
			80% (4.72)	31 ºC*	9.32*	0.6+	14.0+	343±0.65	143±0.84	46±0.63	0	59.8±0.45(9-13)	42.1±0.69	18.7±0.48	0
October 2012	Control	-	-	26 ºC	9.63	0.8	18.0	1256±0.96	169±0.85	126±0.96	76±0.63	100(7-9)	100	100	100
	Eu+St+Ag	5.75	40% (2.30)	26 ºC+	9.62*	0.7+	18.0+	836±0.76	96±0.58	58±0.84	37±0.52	81.4±0.45(9-10)	47.6±0.75	27.6±0.87	18.4±0.37
			80% (4.60)	26 ºC*	9.51*	0.5+	15.0+	521±0.63	82±0.65	73±0.87	42±0.75	76.3±0.48(10-16)	36.8±0.54	14.6±0.65	0

Each experiment was replicated six times and the value of temperature, pH, Dissolved oxygen and free CO2, fecundity, hatchability and survival is the mean of six replicate. Product movement correlation coefficient in between the fecundity and a biotic factor indicate significant (P<0.05) (+) positive / (*) negative correlation. Abbreviation: Eu=Eugenol, St=Starch, Ag=Agar.

Table 3
Effect of sub-lethal concentration (80% of 24 h LC50) of eugenol + serine + agar bait formulation on the biochemical changes in ovotestis and AChE action in the nervous tissue of L. acuminate.

Month	Treatment	24h LC_{50}	80%of 24h LC_{50} mg/l	Protein	Amino Acid	DNA	RNA	AChE
November 2011	Control	-	-	84.12±0.89 (100)	62.45±0.84 (100)	89.54±0.85 (100)	96.21±0.96 (100)	1.65±0.06 (100)
	Eu+Se+Ag	3.03	2.42	56.34±0.23 (66.97)	23.56±0.56 (36.82)	72.05±0.65 (80.46)	74.55±0.36 (77.48)	0.72±0.05 (43.63)
December 2011	Control	-	-	82.91±0.81 (100)	62.47±0.88 (100)	90.84±0.85 (100)	95.31±0.98 (100)	1.63±0.06 (100)
	Eu+Se+Ag	4.14	3.31	53.51±0.52 (64.53)	20.90±0.85 (33.45)	73.53±0.65 (80.94)	55.51±0.85 (58.24)	0.62±0.06 (38.03)
January 2012	Control	-	-	83.96±0.80 (100)	65.45±0.64 (100)	81.84±0.95 (100)	95.58±0.86 (100)	1.62±0.06 (100)
	Eu+Se+Ag	11.13	8.90	55.21±0.74 (65.75)	21.34±0.52 (32.60)	53.86±0.52 (65.81)	13.12±0.52 (13.72)	1.18±0.06 (72.83)
February 2012	Control	-	-	82.90±0.82 (100)	66.45±0.74 (100)	88.34±0.89 (100)	94.61±0.86 (100)	1.64±0.06 (100)
	Eu+Se+Ag	8.92	7.13	62.16±0.56 (74.98)	15.94±0.53 (23.98)	66.28±0.41 (75.02)	15.82±0.54 (16.72)	1.12±0.05 (68.29)
March 2012	Control	-	-	85.97±0.81 (100)	67.15±0.74 (100)	88.58±0.95 (100)	96.78±0.76 (100)	1.63±0.04 (100)
	Eu+Se+Ag	9.30	7.44	58.22±0.65 (67.72)	31.23±0.74 (39.88)	73.05±0.32 (82.46)	60.16±0.64 (62.16)	0.99±0.04 (60.73)
April 2012	Control	-	-	85.38±0.83 (100)	65.35±0.94 (100)	87.72±0.95 (100)	95.21±0.96 (100)	1.64±0.03 (100)
	Eu+Se+Ag	8.56	6.85	61.32±0.65 (71.82)	26.78±0.65 (40.97)	51.12±0.85 (58.27)	44.74±0.53 (46.99)	1.21±0.03 (73.78)
May 2012	Control	-	-	86.91±0.84 (100)	64.65±0.84 (100)	87.98±0.75 (100)	98.68±0.98 (100)	1.66±0.06 (100)
	Eu+Se+Ag	7.55	6.04	58.32±0.42 (67.10)	13.16±0.15 (20.35)	29.67±.75 (33.72)	66.86±0.87 (67.75)	1.10±0.06 (66.26)
June 2012	Control	-	-	87.95±0.83 (100)	67.65±0.74 (100)	86.81±0.95 (100)	96.76±0.88 (100)	1.66±0.03 (100)
	Eu+Se+Ag	3.18	2.54	52.29±0.32 (59.45)	8.05±0.23 (11.89)	32.09±0.65 (36.96)	21.24±0.95 (21.95)	1.12±0.06 (67.46)
July 2012	Control	-	-	81.91±0.82 (100)	66.55±0.54 (100)	86.34±0.75 (100)	93.41±0.86 (100)	1.67±0.06 (100)
	Eu+Se+Ag	3.41	2.72	46.36±0.52 (56.59)	11.33±0.65 (17.02)	18.70±0.52 (21.65)	31.20±0.65 (33.40)	1.14±0.06 (68.26)
August 2012	Control	-	-	82.98±0.85 (100)	68.46±0.89 (100)	87.56±0.85 (100)	96.25±0.99 (100)	1.67±0.06 (100)
	Eu+Se+Ag	4.15	3.32	41.47±0.74 (49.97)	23.63±0.32 (34.51)	35.52±0.74 (40.56)	46.87±0.36 (48.69)	1.03±0.04 (61.67)
September 2012	Control	-	-	81.47±0.89 (100)	68.42±0.88 (100)	87.54±0.85 (100)	96.21±0.96 (100)	1.68±0.06 (100)
	Eu+Se+Ag	5.90	4.72	49.41±0.71 (60.64)	25.47±0.47 (37.22)	42.13±0.52 (48.12)	54.63±0.85 (56.78)	0.78±0.06 (46.42)
October 2012	Control	-	-	83.93±0.81 (100)	67.15±0.84 (100)	86.73±0.97 (100)	97.64±0.86 (100)	1.68±0.04 (100)
	Eu+Se+Ag	5.75	4.60	47.52±0.41 (56.61)	35.89±0.74 (53.44)	74.65±0.54 (86.07)	62.14±0.65 (63.64)	0.69±0.03 (41.07)

Each experiment was replicated six times and the value of protein, amino acid, DNA, RNA and AChE is the mean of six replicate. t-test between biochemical parameters indicate significant ($P<0.05$) test. Protein, Amino acid, DNA and RNA levels, µg/mg. Acetylcholinesterase activity, µmole 'SH' hydrolyzed/ min/ mg protein. Abbreviation: Eu=Eugenol, Se=Serine, Ag=Ag

Table 4
Effect of sub-lethal concentration (80% of 24 h LC50) of eugenol + starch + agar bait formulation on the biochemical changes in ovotestis and AChE action in the nervous tissue of L. acuminate.

Month	Treatment	24h LC50	80%of 24h LC50 mg/l	Protein	Amino Acid	DNA	RNA	AChE
November 2011	Control	-	-	84.12±0.89 (100)	62.45±0.84 (100)	89.54±0.85 (100)	96.21±0.96 (100)	1.65±0.06 (100)
	Eu+St+Ag	3.03	2.42	66.84±0.23 (79.45)	24.10±0.56 (38.59)	83.50±0.93 (93.25)	62.53±0.65 (64.99)	0.70±0.06 (42.42)
December 2011	Control	-	-	82.91±0.81 (100)	62.47±0.88 (100)	90.84±0.85 (100)	95.31±0.98 (100)	1.63±0.06 (100)
	Eu+St+Ag	4.14	3.31	63.58±0.62 (76.68)	19.93±0.98 (31.90)	65.28±0.91 (71.86)	59.82±0.65 (62.76)	0.59±0.04 (36.19)
January 2012	Control	-	-	83.96±0.80 (100)	65.45±0.64 (100)	81.84±0.95 (100)	95.58±0.86 (100)	1.62±0.06 (100)
	Eu+St+Ag	11.13	8.90	58.28±0.84 (69.41)	18.06±0.74 (27.59)	49.59±0.98 (60.59)	37.77±0.78 (39.51)	1.22±0.04 (75.30)
February 2012	Control	-	-	82.90±0.82 (100)	66.45±0.74 (100)	88.34±0.89 (100)	94.61±0.86 (100)	1.64±0.06 (100)
	Eu+St+Ag	8.92	7.13	65.36±0.86 (78.84)	18.73±0.65 (28.18)	29.67±0.85 (33.58)	45.00±0.52 (47.56)	1.15±0.06 (70.12)
March 2012	Control	-	-	85.97±0.81 (100)	67.15±0.74 (100)	88.58±0.95 (100)	96.78±0.76 (100)	1.63±0.04 (100)
	Eu+St+Ag	9.30	7.44	68.62±0.95 (79.81)	18.25±0.65 (27.17)	62.87±0.76 (70.97)	60.65±0.32 (68.86)	1.03±0.05 (63.19)
April 2012	Control	-	-	85.38±0.83 (100)	65.35±0.94 (100)	87.72±0.95 (100)	95.21±0.96 (100)	1.64±0.03 (100)
	Eu+St+Ag	8.56	6.85	66.52±0.75 (77.91)	13.42±0.54 (20.53)	51.12±0.65 (58.27)	51.41±0.45 (53.99)	1.13±0.03 (68.90)
May 2012	Control	-	-	86.91±0.84 (100)	64.65±0.84 (100)	87.98±0.75 (100)	98.68±0.98 (100)	1.66±0.06 (100)
	Eu+St+Ag	7.55	6.04	68.39±0.44 (78.69)	12.14±0.64 (18.77)	76.00±0.36 (86.38)	55.16±0.32 (55.89)	1.53±0.06 (92.16)
June 2012	Control	-	-	87.95±0.83 (100)	67.65±0.74 (100)	86.81±0.95 (100)	96.76±0.88 (100)	1.66±0.03 (100)
	Eu+St+Ag	3.18	2.54	62.51±0.82 (71.07)	9.41±0.98 (13.90)	34.91±0.45 (40.21)	18.28±0.74 (18.89)	1.13±0.07 (68.07)
July 2012	Control	-	-	81.91±0.82 (100)	66.55±0.54 (100)	86.34±0.75 (100)	93.41±0.86 (100)	1.67±0.06 (100)
	Eu+St+Ag	3.41	2.72	54.46±0.32 (66.48)	12.05±0.93 (18.10)	26.68±0.58 (30.90)	34.86±0.45 (37.31)	0.71±0.05 (42.51)
August 2012	Control	-	-	82.98±0.85 (100)	68.46±0.89 (100)	87.56±0.85 (100)	96.25±0.99 (100)	1.67±0.06 (100)
	Eu+St+Ag	4.15	3.32	47.87±0.54 (57.68)	12.63±0.84 (18.44)	41.63±0.52 (47.54)	47.42±0.45 (49.26)	1.08±0.04 (64.67)
September 2012	Control	-	-	81.47±0.89 (100)	68.42±0.88 (100)	87.54±0.85 (100)	96.21±0.96 (100)	1.68±0.06 (100)
	Eu+St+Ag	5.90	4.72	42.47±0.44 (52.12)	12.98±0.65 (18.97)	74.00±0.96 (84.53)	56.87±0.98 (59.11)	1.02±0.05 (60.71)
October 2012	Control	-	-	83.93±0.81 (100)	67.15±0.84 (100)	86.73±0.97 (100)	97.64±0.86 (100)	1.68±0.04 (100)
	Eu+St+Ag	5.75	4.60	57.63±0.48 (68.66)	16.71±0.65 (24.88)	52.63±0.82 (60.68)	54.68±0.65 (56.00)	0.69±0.04 (41.07)

Each experiment was replicated six times and the value of protein, amino acid, DNA, RNA and AChE is the mean of six replicate. t-test between biochemical parameters indicate significant ($P<0.05$) test. Protein, Amino acid, DNA and RNA levels, µg/mg. Acetylcholinesterase activity, µmole 'SH' hydrolyzed/ min/ mg protein. Abbreviation: Eu=Eugenol, Se=Starch, Ag=Agar

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