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EFFECT OF CLOVE OIL ON THE BIOCHEMICAL PROFILE OF OVOTESTIS AND HEPATOPANCREAS OF *Achatina fulica*

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ABSTRACT

The giant African snail *Achatina fulica* is widespread across the subtropical and tropical countries and has picked up the global pest status. Its voracious feeding capacity and protandrous reproduction makes it almost invincible once established. Measures to combat its menace have for long focussed on chemical molluscicides. However the rising environmental sensitivity in the past few decades has attracted the use of natural compounds to target this pest. The current study was aimed to evaluate the impact of clove oil on the biochemical profile of its ovotestis and hepatopancreas. The snails were randomly divided into three groups i.e., control, vehicle treated group (1% Tween 80) and clove oil treated group. Snails in the clove oil treated group were subjected to subacute doses of clove oil (20% and 60% of LD₅₀ value/24 hrs) by topical application. The activity levels of vital enzymes namely acetylcholinesterase, acid phosphatase, alkaline phosphatase, protease and lactic dehydrogenase were pertinently altered in the ovotestis and hepatopancreas of the clove oil treated snails as compared to the control. Contents of important biomolecules like DNA, RNA, protein, and phospholipids were reduced, the level of lipid peroxidation was significantly enhanced with a concomitant decrease in glutathione content in clove oil treated groups vis-a-vis control in both the tissues studied, highlighting the toxic effect of clove oil to the snail. Thus it is suggested that clove oil can be used in controlling the population of this harmful pest.

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1 Introduction

The giant African snail *Achatina fulica* has been in the spotlight for the damages it has inflicted on the agri-horticultural fields globally. It is known to be amongst the world's worst hundred invasive species as stated by the International Union for Conservation of Nature and Natural resources (IUCN) and has spread out globally from its native home in East Africa (Lowe et al., 2000). In India, it has dispersed in large numbers since its introduction in Calcutta almost 140 years ago (Naggs, 1997), due to its high reproductive capacity (lays 250- 300 eggs in a year per pair) and generalist feeding pattern (feeds on more than 500 species of plants) (Raut & Ghose, 1984; Sajeev, 2011).

A. fulica induces threefold damages to tropical agriculture. First, there is a substantial loss of agricultural productivity due to herbivory on crop plants, this damage may also be caused through the transmission of plant pathogens. Secondly, there is a huge cost of labour and materials involved in managing the pest population in such situations. Thirdly, enforced changes in agricultural practices have to be induced such as limiting the crop species to be grown in the region to those resistant to *A. fulica* resulting in loss of production of a conventional crop (Raut & Barker, 2002). Unfortunately, the snail spurt continues, recently it was found invading and causing major damages in the urban areas of the Chandrapur district, Maharashtra (Chatap et al., 2020). In many Asian, Pacific, and American communities, it transmits human parasites and pathogens in slime trails or when infested snails are eaten raw or undercooked (Nelson, 2012). One such pathogen is rat lungworm –*Angiostrongylus cantonensis*, which causes angiostrongyliasis and Eosinophilic meningoencephalitis in humans (Vitta et al., 2011). The faeces of this snail mediates the spread of the black pod disease of plants caused by *Phytophthora palmivora* (Raut & Barker, 2002). An array of literature pertaining to its management is available both at the global level and in the Indian context (Jayashankar et al., 2013). For a long time, the control measures for combating this snail heavily relied on chemical molluscicides. Some of the commonly used chemical molluscicides are metaldehyde (Basavaraju et al., 2001; Vanitha et al., 2008; Ciomperlik et al., 2013); carbamates (Shevale & Bedse, 2009; Ciomperlik et al., 2013), and organophosphates (Saxena & Mahendra, 2000; Justin et al., 2008). Although these chemical molluscicides are effective they cause enormous damage to the environment and non - target species (Kashyap et al., 2019). Hence these days natural compounds are preferred for snail control. Essential oils derived from plants help in controlling the snail population by their selective action, and little or no harmful effects on the non- target organisms and the environment (Isman, 1999). Also, these essential oils are biodegradable and the chances of molluscs acquiring resistance to them is very low (Kashyap et al., 2019). The toxic effect of clove oil is well elucidated on various

organisms such as bacteria, human beings (Kumar et al., 2011), insects (Afonso et al., 2012), fish species viz., *Danio rerio* and *Poecilia reticulata* (Dolezelova et al., 2011), and albino rats (Shalaby et al., 2011). The negative impact of clove oil on the survival and histology of various tissues of *A. fulica* was comprehended by Parvate & Thayil (2017). Previous studies have also revealed that the reproductive efficiency of *A. fulica* is severely compromised on treatment with clove oil (Parvate et al., 2016). Thus an attempt was made to investigate the biocidal potential of clove oil as a molluscicide on the biochemical profile of ovary and hepatopancreas of *A. fulica* as these are the major organs supporting its prodigious reproduction and voracious feeding habit.

2 Materials and Methods

2.1. Test chemicals and test solution

Clove oil and Tween 80 were purchased from Merck and Himedia respectively. 1% Tween 80 solution was utilized as a vehicle compound. The test solutions i.e., required concentrations of clove oil dissolved in 1% Tween 80 solution, were freshly prepared just before use for all the experiments performed.

2.2. Collection and maintenance of snails

Healthy adult snails of *A. fulica* were collected from local gardens of Navi Mumbai and Jijamata Udyan, Mumbai, India. These were maintained at a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in transparent plastic boxes measuring (14'' x 10.5'' x 10''). A 4 cm layer of the garden soil was laid down at the bottom of the boxes. The open ends of these boxes were covered with muslin cloths which were tied to the boxes to prevent the animals from escaping and to facilitate the free flow of air. Snails were fed on fresh cabbage daily and appropriate humidity was maintained by sprinkling water on the soil layer at regular intervals.

2.3. Experimental design

The snails were randomly divided into three groups i.e. Control, vehicle-treated, and clove oil-treated group with 24 snails in each group. The snails in the control group were maintained at optimum conditions without any treatment, while the vehicle-treated group was given 1 ml of 1% Tween 80 solution, and clove treated groups were subjected to 20% and 60% value of ($\text{LD}_{50}/24$ hrs) of clove oil prepared in 1% Tween 80 solution (v/v). The technique of topical application was utilized for the administration of the test compound as well as the vehicle. The snails from each group were dissected after 24 hours of treatment and their tissues were excised, cleaned, weighed, and were stored at -80°C until further analysis. The assays were performed in triplicates for each parameter ($N = 6$).

2.4. Biochemical assays

2.4.1. Estimation of DNA and RNA

DNA and RNA contents were estimated by the method of Schneider (1957). For both the parameters 50 mg each of ovotestis and 100 mg each of hepatopancreas were homogenized in 5% TCA at 90°C. The homogenates were centrifuged at 10,000 r.p.m for 10 min at 4°C and the resulting supernatants were used as samples for DNA and RNA estimations respectively.

2.4.2. Estimation of Protein, Phospholipid, Lipid peroxidation (LPx) and Glutathione content (GSH)

For the aforementioned estimations, 50 mg each of ovotestis and 100 mg each of hepatopancreas were homogenized in 0.01 M Tris-HCl buffer at pH 7.4 for 5 min in an ice bath. The homogenates were centrifuged at 10,000 r.p.m for 40 min for protein estimation and 10 min each for the other estimations, at 4°C. The resulting supernatants were used as samples for the respective estimations.

The protein concentration was detected by adopting the method of Lowry et al. (1951) while, the estimation of phospholipid was carried out by Fiske & Subbarow (1925) method. For analyzing the level of lipid peroxidation, the method of Okhawa et al. (1979) was used and GSH content was assessed as per the method described by Moron et al. (1979).

2.4.3. Enzyme assays - Acetylcholinesterase (AChE), Acid phosphatase (ACP), Alkaline phosphatase (ALP), Protease and Lactate dehydrogenase (LDH) assays

For enzyme assays, 50 mg each of ovotestis and 50 mg each of hepatopancreas (100 mg for protease and LDH) were homogenized in appropriate buffers (0.1M phosphate buffer at pH 8.0 for AChE, 50 mM citrate buffer at pH 5.3 for ACP, 50 mM glycine NaOH buffer at pH 10.4 for ALP, 50 mM Tris HCl buffer at pH 8.0 for protease and 0.1 M phosphate buffer at pH 8.0 for LDH) for 5 min in an ice bath and centrifuged at 10,000 r.p.m for 10 min at 4°C. The respective supernatants were then utilized as the enzyme source for the assays.

Acetylcholinesterase activity was analyzed by adopting the method of Ellman et al. (1961) while, acid phosphatase and alkaline phosphatase activities were measured by following the method of Bergmeyer et al. (1974). Protease assay was performed according to the method of Windle & Kelleher (1997). The method described by King (1965) was followed for the LDH assay.

2.5 Statistical Analysis

Data were expressed as mean $\pm$ Standard error of the mean (S.E.M.), analyzed by one-way analysis of variance (ANOVA), and the means separated by p values: (*p < 0.05), (**p < 0.01), (***)p < 0.001).

3 Results

In ovotestis the nucleic acids (DNA and RNA) and the protein contents of both the treated groups (20% and 60% of LD₅₀/24 hrs) of clove oil were significantly lowered as compared to the control group. The content of phospholipids which are important constituents of the cell membrane were found reduced to an extremely significant extent in both the clove oil-treated groups. Also, the treatment with clove oil led to significant elevation in the levels of lipid peroxidation in both the groups vis-à-vis control with a concomitant reduction in the glutathione content. However, this reduction was not significantly different from the glutathione content of the control. The vehicle (1% Tween 80) treated group had values similar to the control group for most of the parameters studied. The only exceptions were the DNA content and level of lipid peroxidation where the deviation was significant as compared to the control group (Table 1).

In hepatopancreas the amount of DNA and RNA was reduced in the groups treated with both lower and higher subacute doses of clove oil. However, the reduction in DNA content was non-significant while in the case of RNA content, an extremely significant difference was reported from the control group. The protein and phospholipid contents revealed a significant decline in both the treated groups. Elevation in the lipid peroxidation level was extremely significant in both the clove oil treated groups with a concurrent decline in the glutathione content. However significant decline in GSH content was noticed in the group subjected to a higher subacute dose of clove oil only. The results of the vehicle control group were similar to the control group except for its RNA content which demonstrated significant reduction vis-à-vis control (Table 2).

In ovotestis the activity levels of the enzymes acetylcholinesterase, lysosomal marker enzyme acid phosphatase, and protease were found to be lowered on treatment with clove oil, with significant impact observed in the groups exposed to (60% of LD₅₀/24 hrs) of clove oil. Clove oil enhanced the level of activity of the enzyme lactate dehydrogenase significantly in the group exposed to its lower subacute dose and extremely significantly in the group exposed to its higher subacute dose. The activity of the enzyme alkaline phosphatase was altered non significantly. The vehicle (1% Tween 80) treated group exhibited activity level of all the enzymes analogous to the control group, except the activity level of lactate dehydrogenase which exhibited a significant increase as compared to the control group (Table 3). In hepatopancreas the activity level of all the five vital enzymes namely acetylcholinesterase, acid phosphatase, alkaline phosphatase, protease, and lactic dehydrogenase was altered at both exposures of clove oil (20% and 60% of LD₅₀ value/24 hrs) but this was non-significant in relation to the control. The vehicle control group exhibited the activity level of all the enzymes close to the control group (Table 4).

Table 1 Effect of clove oil on the biochemical parameters of ovotestis of *A. fulica*

Parameter	Control	Vehicle Control	20% of LD ₅₀ /24hrs	60% of LD ₅₀ /24 hrs
DNA (µg/mg. tissue)	1.669 ± 0.049	1.502 ± 0.041*	1.457 ± 0.042**	1.325 ± 0.041***
RNA (µg/mg. tissue)	1.621 ± 0.027	1.57 ± 0.045 ^{NS}	1.443 ± 0.020***	1.383 ± 0.046**
Protein mg.tissue	0.042 ± 0.001	0.041 ± 0.001 ^{NS}	0.040 ± 0.001*	0.039 ± 0.001*
Phospholipid (10 ⁻³) (µM/mg. tissue)	1.967 ± 0.119	1.865 ± 0.134 ^{NS}	1.345 ± 0.057***	1.27 ± 0.080***
LPx (µg/mg. tissue)	0.102 ± 0.001	0.105 ± 0.001**	0.191 ± 0.001***	0.130 ± 0.001***
GSH (µg/mg. tissue)	0.543 ± 0.017	0.547 ± 0.015 ^{NS}	0.523 ± 0.006 ^{NS}	0.545 ± 0.008 ^{NS}

Values are expressed as Mean ± SEM for six animals in each group (One way ANOVA); NS-Non significant (*p < 0.05), (**p < 0.01), (**p < 0.001).

Table 2 Effect of clove oil on the biochemical parameters of hepatopancreas of *A. fulica*

Parameter	Control	Vehicle control	20% of LD ₅₀ /24 hrs	60% of LD ₅₀ /24 hrs
DNA (µg/mg. tissue)	1.322 ± 0.14	1.204 ± 0.12 ^{NS}	1.042 ± 0.064 ^{NS}	0.978 ± 0.095 ^{NS}
RNA (µg/mg. tissue)	5.57 ± 0.433	4.26 ± 0.328*	1.81 ± 0.158***	1.365 ± 0.1***
Protein mg.tissue	0.104 ± 0.01	0.081 ± 0.01 ^{NS}	0.070 ± 0.003**	0.067 ± 0.002**
Phospholipid (10 ⁻³) (µM/mg. tissue)	5.15 ± 0.289	4.91 ± 0.323 ^{NS}	3.88 ± 0.334*	3.325 ± 0.431**
LPx(µg/mg. tissue)	0.040 ± 0.002	0.038 ± 0.003 ^{NS}	0.080 ± 0.005***	0.098 ± 0.003***
GSH (µg/mg. tissue)	1.60 ± 0.137	1.61 ± 0.213 ^{NS}	1.38 ± 0.157 ^{NS}	1.07 ± 0.161*

Values are expressed as Mean ± SEM for six animals in each group (One way ANOVA); NS-Non significant (*p < 0.05), (**p < 0.01), (**p < 0.001).

Table 3 Effect of clove oil on the activity levels of vital enzymes of ovotestis of *A. fulica*

Parameter	Control	Vehicle control	20% of LD ₅₀ /24 hrs	60% of LD ₅₀ /24 hrs
Acetylcholinesterase(µM of SH hyd/min/mg.protein)	0.16 ± 0.02	0.18 ± 0.006 ^{NS}	0.161 ± 0.01 ^{NS}	0.099 ± 0.01*
ACP (µM of PNP/min/mg.protein)	2.67 ± 0.175	2.58 ± 0.10 ^{NS}	2.22 ± 0.20 ^{NS}	1.83 ± 0.171**
ALP (µM of PNP/min/mg.protein)	1.54 ± 0.09	1.63 ± 0.06 ^{NS}	1.71 ± 0.14 ^{NS}	1.57 ± 0.15 ^{NS}
Protease (10 ⁻²) µM tyr.eq.rel/mg.protein	0.89 ± 0.06	0.88 ± 0.04 ^{NS}	0.79 ± 0.06 ^{NS}	0.68 ± 0.02**
LDH (I.U/mg protein)	30.09 ± 3.70	43.31 ± 4.46*	46.89 ± 4.44*	62.79 ± 2.84***

Values are expressed as Mean ± SEM for six animals in each group (One way ANOVA). NS- Non significant, (*p < 0.05), (**p < 0.01), (**p < 0.001)

Table 4 Effect of clove oil on the activity levels of vital enzymes of hepatopancreas of *A. fulica*

Parameter	Control	Vehicle control	20% of LD ₅₀ /24 hrs	60% of LD ₅₀ /24 hrs
Acetylcholinesterase (µM of SH hyd/min/mg.protein)	4.158 ± 0.969	3.66 ± 0.547 ^{NS}	3.55 ± 0.539 ^{NS}	3.515 ± 0.412 ^{NS}
ACP (µM of PNP/min/mg.protein)	1.41 ± 0.137	1.42 ± 0.22 ^{NS}	1.43 ± 0.11 ^{NS}	1.75 ± 0.30 ^{NS}
ALP (µM of PNP/min/mg.protein)	5.07 ± 1.28	5.25 ± 1.44 ^{NS}	6.68 ± 1.71 ^{NS}	7.145 ± 1.27 ^{NS}
Protease (10 ⁻²) µM tyr.eq.rel/mg.protein	0.58 ± 0.11	0.58 ± 0.12 ^{NS}	0.41 ± 0.02 ^{NS}	0.45 ± 0.05 ^{NS}
LDH (I.U/mg protein)	63.10 ± 8.97	52.08 ± 4.75 ^{NS}	50.08 ± 10.37 ^{NS}	43.31 ± 5.86 ^{NS}

Values are expressed as Mean ± SEM for six animals in each group (One way ANOVA). NS- Non significant.

4 Discussion

The major active components of clove oil are eugenol (88.58%), eugenol acetate (5.62%), and β -caryophyllene (1.38%) (Chaieb et al, 2007). The results of the current study revealed that clove oil exerted its toxic effects on both ovotestis and hepatopancreas on its subacute exposure mediated through its aforesaid components. The reduction in the concentrations of important biomolecules like DNA, RNA, proteins, phospholipids, an elevation in the level of lipid peroxidation with the corresponding decline in GSH content was pertinent in both ovotestis and hepatopancreas. However, the activity levels of the vital enzymes namely AChE, ACP, ALP, protease, and LDH were affected to a greater extent in ovotestis as compared to the hepatopancreas.

The DNA, RNA, and protein levels of both the tissues were significantly lowered on subacute treatment with clove oil in contrast to the controls. The reduced DNA content had an inhibitory effect on the transcription activity which lead to reduced synthesis of RNA. This is in concordance to the results obtained by Atwa & Bakry (2019) who revealed the genotoxic effect of the drug mefloquine on the soft tissues of the snail *Lymnaea natalensis* and suggested that the drug acted as a potent inhibitor of DNA synthesis resulting in the lowering of RNA levels. Since RNA plays an important role in protein synthesis, the inhibition of RNA synthesis at the transcription level lowers the protein content (Singh & Singh, 2010). Compromised uptake of amino acids in the polypeptide chain during protein synthesis due to the action of the chemical administered can also lower the protein content (Singh et al., 2020). These reasons well explain the diminished protein content of the clove oil treated tissues observed in the current study. Similar results in terms of decline in nucleic acids and protein contents were reported by Singh et al. (2010) on exposing the snail *L. acuminata* to sublethal doses of deltamethrin.

In the present study, the level of lipid peroxidation was enhanced and the glutathione content was compromised on sub-lethal exposure to clove oil. Lipid peroxidation is one of the primary causes of cellular damage and the increase in lipid peroxides results in oxidative stress (Ming et al., 2010). Malondialdehyde (MDA) serves as the biomarker of lipid peroxidation and its quantification directly reveals the level of oxidative stress (Tao et al., 2013). Thus the elevated MDA content of the treated tissues in the present study suggested that clove oil might have induced oxidative stress in the snails. Significant depletion in the phospholipid level was observed in the treated tissues of ovotestis and hepatopancreas. This is probably the manifestation of oxidative degradation of membrane phospholipids which further confirms the lipid peroxidation ability of clove oil. Results of the current study are supported by those obtained by Rao & Singh (2000) who reported a significant decline in the phospholipid level and an elevation in the MDA levels in ovotestis of the snail *A. fulica* exposed to plant derived molluscicides.

Glutathione acts as a reducing agent and protects cells against peroxidative attack by scavenging free radicals (Verma et al., 2007, Zitka et al., 2012). Thus the reduction in GSH content in all the clove oil treated tissues could have been probably due to its utilization in scavenging of reactive oxygen species i.e. lipid peroxides generated on treatment. A drastic decrease in the GSH content with an enhanced level of lipid peroxidation was observed by Bakry et al. (2013) in the snails *Biomphalaria alexandrina* exposed to the pesticides diazinon and profenfos. Results of the current study are following the observations of Khalil (2015) who demonstrated that sublethal exposure of Chlorpyrifos induced oxidative stress in *Lanistes carinatus* which lead to elevated MDA level and depleted GSH content in its hepatopancreas and nervous tissue.

Acetylcholinesterase (AChE) enzyme is vital for normal behavior and muscular function as it regulates nervous transmission by reducing the concentration of acetylcholine (ACh) in the junction through AChE - catalyzed hydrolysis of acetylcholine to acetate and choline (Kopecka & Pempkowiak, 2004). Earlier studies by Yadav & Singh (2016) have revealed a reduction in AChE activity in *L. acuminata* treated with stem bark extracts of *Croton tiglium* and *Codiaeum variegatum*. However, the treatment of snails with clove oil in the present study caused significant depletion in AChE activity only in the ovotestis exposed to 60% of LD₅₀/24 hrs.

Enzymes ACP and ALP are known to get influenced by molluscicidal treatment, they catalyze the breakdown of ester bonds in the orthophosphate esters under acidic and alkaline conditions, respectively (Daihan, 2008). In the current study, the activity levels of both ACP and ALP were altered in the treated tissues, however, the alteration was significant only in the activity of ACP in ovotestis exposed to a higher sub-acute dose of clove oil. A significant alteration in the activity of ACP and a non - significant change in the activity level of ALP was observed by Wang et al. (2018) on treating the snail *B. straminea* with synthetic derivatives of pyridylphenyl ureas which supports the findings of this study.

Proteases are enzymes catalyzing proteolysis which is directly or indirectly involved in most of the cellular processes (Schaller, 2004). Proteolysis is essential to supply the pool of amino acids essential for synthesizing new proteins as well as for the removal of damaged proteins when under stress (Palma et al., 2002). A reduction in protease activity was noted by Mahmoud et al. (2020) in the gut of Zebra fish (*Danio rerio*) treated with Fenvalerate which they associated with its altered substrate specificity. The reduction in the protease activity in the clove oil treated tissues of ovotestis and hepatopancreas in the current study may also be attributed to the same.

In the present study elevation in lactate dehydrogenase (LDH) activity was observed in the treated tissues which probably suggests a bias towards the anaerobic glycolytic pathway, as LDH is associated with cellular metabolic activity and is a pivotal

enzyme between the glycolysis and citric acid cycle (Singh et al., 2013). Previous studies by Gohary et al. (2011) on two land snails – *Monacha cantiana* and *Eobania vermiculata* exposed to three molluscicidal baits also presented results similar to the ones obtained in the current study. All the baits used in their study resulted in increased LDH level in the treated tissues.

Conclusion

This study indicates that clove oil induced toxicity in both ovotestis and hepatopancreas of the snail *A. fulica* probably mediated through the oxidative stress mechanism. However, the impact was more profound on ovotestis as compared to hepatopancreas. The disturbed biochemical profile of ovotestis (the organ supporting prodigious reproduction of this snail) implies that clove oil inflicted an assault on its structural integrity leading to its malfunctioning. This substantiates the fact that the molluscicides act by either directly hindering metabolic pathways of the snails or by controlling their rate of proliferation. Based on the results of the current study it is proposed that clove oil can act as a prospective lead compound in controlling the population of this snail.

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Conflict of interest

The authors declare that they have no conflict of interest.

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