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TOXIC EFFECT OF CLOVE OIL ON THE SURVIVAL AND HISTOLOGY OF VARIOUS TISSUES OF PESTIFEROUS LAND SNAIL *Achatina fulica* (BOWDICH, 1822)

Yadnya A. Parvate, Liji Thayil*

School of Biotechnology and Bioinformatics, D.Y. Patil University, Plot no.50,
Sector 15, CBD Belapur, Navi Mumbai - 400 614, India.

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Pragmatic approach

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Negative correlation

ABSTRACT

Achatina fulica is a global agricultural pest affecting subtropical and tropical areas including India. Nowadays using natural molluscicides for snail control is considered to be the most pragmatic approach. The aim of this study was to assess the molluscicidal potential of clove oil against this pestiferous land snail. Clove oil was topically administered to the snails and its toxicity was found to be time and concentration dependent up to three days of exposure exhibiting a significant negative correlation. Thus LD₅₀ values (%) decreased from 7.965 (24 hrs) to 5.157 (48 hrs) to 3.916 at (72 hrs) and remained the same i.e. 3.916 at (96hrs). The effects of sub lethal concentrations (20% and 60% of LD₅₀/24 hrs) were also observed on the histology of various tissues like ovotestis, hepatopancreas, gut represented by (Oesophagus, crop, stomach, and intestine) and foot. Control group snails exhibited normal histology of all the tissues, those in the vehicle treated group had histology close to normal, whereas those exposed to sublethal doses exhibited damages almost proportional to the dose administered, in all the tissues studied. Thus it can be concluded that clove oil obtained from plants is toxic to the snail and can help to control its population.

* Corresponding author

E-mail: lijithayil@gmail.com (Liji Thayil)

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1 Introduction

The giant African snail *Achatina fulica* which is classed as one of the world's worst hundred invasive species by the International Union for Conservation of Nature and Natural resources (IUCN), has been introduced around the globe from its native home in East Africa (Lowe et al., 2000). Its success as an introduced species is by virtue of its high reproductive capacity, voracious feeding habit (feeds on nearly 500 plant species), inadequate quarantine arrangements and also human aided dispersal (Fontanilla et al., 2007). The snail is also known to facilitate the spread of diseases such as angiostrongylosis and eosinophilic meningoencephalitis in humans due to its important role as a host in the life cycle of *Angiostrongylus cantonensis* (Vasconcellos & Pile, 2001; Santos Carvalho et al., 2003). The faeces of this snail is also responsible for the spread of the blackpod disease of plants caused by *Phytophthora palmivora* (Raut & Barker, 2002).

It is one of the most destructive pests known to affect subtropical and tropical areas. It causes large scale damages to farms, commercial plantations and domestic gardens. It can also be found on trees, decaying material and near garbage deposits (Vasconcellos & Pile, 2001). Sarma et al. (2015) have developed a model for predicting the invasion pattern of this snail in India based on variations in its climatic conditions which can also help in its management.

As such various control measures using non-specific molluscicides have been described for over a century for controlling the population of *A. fulica*. However nowadays more specific compounds are increasingly being used, in controlling these molluscs replacing toxic chemicals with natural agents that would be less harmful to the environment (Panigrahi & Raut, 1994). Lot of natural compounds have been explored and found to be effective in snails like *Lymnaea acuminata* (Chauhan et al., 2011), *Subulina octona* (Silva et al., 2012), *Indoplanorbis exustus* (Pandey & Singh, 2009), *Eobania vermiculata*, *Monacha cartusiana* (Aal & Hamed, 2010) etc.. However only few natural compounds have been tested against *A. fulica* (Panigrahi & Raut, 1994; Justin et al., 2008; Rao & Singh, 2002).

Essential oils from plants serve as excellent products of botanical origin due to their high bioactive potential, easy availability and economic viability (Lahlou, 2004). Essential oil of clove (clove oil) is one such example which has exhibited biological activity on a wide range of organisms ranging from microorganisms to humans (Kumar et al., 2011). 60% - 90% of clove oil is constituted by eugenol which is regarded to be the source of its antifungal, anaesthetic and antiseptic properties (Rani et al., 2012) and also its cytotoxicity (Watcharee et al., 2012). Clove oil is exempted from pesticide registration requirements and pesticide residue tolerance requirements (Hollingsworth et al., 2012).

Studies have shown potent ovicidal activity of clove oil against the eggs of *Cantareus apersus* and *Succinea* sp. at 1% while in *A. fulica* at a concentration as low as 0.05% (Hollingsworth et al., 2012). Hence this study was carried out to assess the molluscicidal activity of clove oil against the adult snails of *A. fulica* and to further evaluate its toxic effect on various tissues of the snail.

2. Materials and Methods

2.1 Collection and maintenance of snails

Adult snails of *A. fulica* were collected from local gardens and Jijamata Udyan, Mumbai, India. Healthy snails in the weight range of 65 gms $\pm$ 20 gms were used for the current studies. They were maintained at a temperature of 25°C $\pm$ 2°C in transparent plastic boxes (14'' x 10.5'' x 10''). These boxes were laden with garden soil at the bottom forming a 4 cm layer. The open ends of the boxes were fastened with muslin cloth to facilitate free flow of air. The snails were fed on cabbage leaves on a daily basis and appropriate humidity was maintained by sprinkling water on the soil layer intermittently.

2.2 Test chemicals and test solution

Clove oil and Tween 80 were procured from Merck and Himedia respectively. 1% tween 80 solution was used as a vehicle (Klopell et al., 2007). The test solutions i.e. required concentrations of clove oil dissolved in 1% tween 80 solution (v/v) were always prepared fresh before use.

2.3 Toxicity experiment

Toxicity experiments were performed according to the method of Hussein et al. (1994). In order to determine the LD₅₀ values of the test compound the animals were divided into three groups viz., control, vehicle treated group and clove oil treated group, with ten animals in each group. The clove oil treated animals were subjected to 1 ml of varying concentrations of clove oil (3%, 6%, 9%, 15%, 21% and 27%) prepared in 1% tween 80 solution (v/v). The test compound and the vehicle were administered topically to the animal. The mortality of animals was observed at a regular interval of 24 hrs of treatment till 96 hrs as per the WHO procedure (1965) by touching it with a stainless steel needle. Loss of response was considered as death of animal. After performing the experiments in quadruple the data obtained was subjected to Probit analysis and the values of LD₅₀ for 24 hrs, 48 hrs, 72 hrs and 96 hrs were determined.

2.4 Statistical analysis

The LD₅₀ values were computed using SPSS statistical software (version 14) for 24 hrs, 48hrs, 72 hrs as well as 96 hrs.

2.5 Histology studies

Table 1 Toxicity of clove oil (%) to *A. fulica*

Exposure time	LD ₅₀ value (LB - UB)
24hrs	7.965 (6.220 - 9.734)
48hrs	5.157 (3.506 - 6.651)
72hrs	3.916 (2.833 - 4.877)
96hrs	3.916 (2.833 - 4.877)

Four batches of 10 snails were exposed to different concentrations of clove oil. Mortality was determined every 24 h. No mortality was observed in the control snails. Regression coefficient showed that there was a significant ($P < 0.05$) negative correlation between exposure period and LD₅₀ values up to three days of exposure. LB – Lower Bound, UB – Upper Bound (95% Confidence limits).

For histological studies the snails were again divided in to three groups viz., control, vehicle treated group and clove oil treated group. The snails in control group were maintained at optimum conditions without any treatment, those in the vehicle treated group received 1 ml of 1% tween 80 solution, and those in the treatment group were exposed to 20% and 60% value of (LD₅₀ / 24 hrs) of clove oil prepared in 1% tween 80 solution (v/v). The technique of topical application was used for administration of the test compound as well as the vehicle.

After 24 hours the snails from each group were dissected and their tissues - Ovary, hepatopancreas, gut represented by four regions (oesophagus, crop, stomach and intestine) and foot were removed and transferred to 10% formalin solution. The tissues were kept in 10% formalin for 48 hours and then sectioned using Leica's semiautomatic microtome to obtain 4µm sections. The slides were stained with eosin and hematoxylin and observed under light microscope.

3 Results

3.1 Toxicity studies

Toxicity of clove oil was found to be time and concentration dependent up to three days of exposure period. LD₅₀ values (%) decreased from 7.965 (24 hrs) to 5.157 (48 hrs) to 3.916 at (72 hrs) and remained the same i.e., 3.916 at (96hrs). The LD₅₀ value of the fourth day was same as that of the third day (Table 1). A significant negative correlation was observed between LD₅₀ values of clove oil and the exposure period up to three days.

3.2 Histological analysis

3.2.1 Ovary

The snails in control group exhibited normal histology of ovary (Figure 1- A. i and ii). Ovary consists of three to four lobes with each lobe made up of numerous small follicles (acini, alveoli), separated from each other by a thin basement membrane. The follicles are lined with the follicular germinal epithelium which produces both the gametes. The spermatozoa in various developmental stages along with mature spermatozoa are present in large numbers within the follicles, while the ova which are large with prominent nuclei and nucleoli are very less in number and are rarely seen (Breckenridge & Fallil, 1973). The irregularly shaped sertoli cells are found lining the periphery of the follicles.

The histology of ovary of vehicle treated animals was almost similar to the control group (Figure 1- B. i and ii). The snails exposed to 20% of LD₅₀ /24hrs showed mild degenerative changes in the sperms along with a reduction in sperm density within the acini of ovary (Figure 1- C. i and ii), while severe degeneration of sperms was observed in snails treated with 60% of the LD₅₀/24hrs along with necrosis of sertoli cells at intervals (Figure 1- D. i and ii).

3.2.2 Hepatopancreas

The histology of hepatopancreas in the control group showed the presence of prominent hepatocytes (digestive cells) lining the hepatic tubules with basal nuclei. The hepatic tubules are lined by a mantle, enclose a lumen in the centre and are separated from each other by interlobular connective tissue. (Figure 2- A. i and ii). Vehicle treated snails exhibited histology close to normal barring slight degenerative changes observed in the hepatocytes at few places (Figure 2- B. i and ii). In contrast degenerative changes were seen in the cells of hepatic tubules, with distinct peripheral thickening of the mantle lining it, along with atrophy of surrounding connective tissue and clustering of leaked secretory material from the damaged hepatocytes in the snails treated with 20% of LD₅₀ / 24 hrs (Figures 2- C. i and ii). In snails treated with 60% of the LD₅₀ /24hrs excessive clustering of secretory material exuded from the hepatocytes due to damaged cell membranes along with loss/sloughing of cell layers of hepatocytes due to their necrosis was evident (Figures 2- D. i and ii) respectively.

3.2.3 Gut/Alimentary canal

The wall of alimentary canal is formed of several distinct layers which are more or less identical in structure along its entire length. The outermost layer is constituted of epithelium formed of single layer of flattened cells. However this epithelium is absent

Figure 1 Photomicrographs of sections through Ovotestis of *Achatina fulica* (A: Control, B: Vehicle control, C: Exposed to 20% of $LD_{50}/24$ hrs, D: Exposed to 60% of $LD_{50}/24$ hrs). (A – D): (i -10X and ii - 45X) AC: Acini; SP: Spermatozoa; SC: Sertoli cells; D: Degeneration; N: Necrosis

Figure 2 Photomicrographs of sections of hepatopancreas of *Achatina fulica* (A - D). (A: Control, B: Vehicle control, C: Exposed to 20% of $LD_{50}/24$ hrs, D: Exposed to 60% of $LD_{50}/24$ hrs). A and B: (i -10X and ii - 45X), C and D: (i and ii - 45X). HPT: Hepatopancreatic tubules; Lu: Lumen; D: degeneration; CL: Clustering ; PT: Periphrastic thickening; N: Necrosis

in crop and stomach regions. Below the epithelium lies a muscular layer of varying thickness divided into outer thick layer of circular muscle fibres and inner thin layer of longitudinal muscle fibres. This arrangement is reversed in stomach and intestine.

Underlying the muscular region is a layer of areolar tissue which is thrown in to folds and is often supported by circular, longitudinal and oblique muscle fibres. Found embedded in this layer are certain glands which pour their secretions in to the lumen of the alimentary canal via fine ducts. A thin layer of circular fibres is found below the areolar layer. The lumen of the alimentary canal is lined by a single layered epithelium which extends in to the ducts of the glands and is supported by a homogenous basement membrane. Some of the epithelial cells are modified to form unicellular mucous glands throughout the gut (Ghose, 1963).

In control snail the gut histology was found to be normal in all the parts studied i.e., oesophagus, crop, stomach and intestine (Figures 3 - A. i, 4 - A. i and ii, 5 - A. i and 6 - A. i and ii) respectively. The histology of the various parts of the gut of

vehicle treated group closely resembled that of control snails (Figure 3 - B. i, 4 - B. i and ii, 5-B. i) and (6 - B. i and ii).

The snails exposed to both 20% and 60% of LD₅₀ / 24 hrs showed

varying level of damages in the gut region. In case of animals treated with 20% of LD₅₀ / 24 hrs, the tissue architecture of oesophagus, crop and stomach exhibited degenerative changes. Infiltration of inflammatory cells was also observed (Figure 3 - C. i and ii; Figure 4 - C. i and ii; Figure 5 - C. i and ii) which was massive in the intestine (Figure 6 - C. i and ii).

Exposure to 60% of LD₅₀ / 24 hrs accrued severe damages in snails. There was a massive loss of epithelium almost throughout the oesophagus. Sloughing of muscular layers and areolar layer was observed at certain places, along with the necrotic degenerative changes throughout the tissue (3 - D.i, ii and iii). Tissue inflammation was also evident from the extensive infiltration of inflammatory cells (3 -D.ii and iii). In crop there was a massive loss of muscular layers, areolar layer and inner circular muscle layer. Severe degenerative changes along with partial sloughing was seen in the inner epithelium. (4 - D i and ii).

Stomach of these snails revealed extensive necrotic degeneration of various muscle layers, areolar layer along with their partial sloughing and infiltration of inflammatory cells surrounding the inner epithelial lining (5 - D. i and ii). Severe necrosis of cells

leading to major loss of almost all the layers were observed in the intestine (6 - D, i and ii).

3.2.4 Foot

The snails in control group exhibited normal histology of foot (Figure 7 - A. i and ii) with outermost cuticular layer as a protective layer of the foot. Inner to this lining there is a layer of tall columnar epithelium with unicellular glands which secrete mucous. Embedded in between are transversely inter woven muscle fibres, called as longitudinal muscle fibres. Major part of the foot muscles are represented by thickly arranged oblique muscle fibres (Nagare, 2013). The snails in vehicle treated group exhibited normal histology barring slight increase in the size of the empty spaces within the muscle fibres (Figure 7- B.i and ii). The snails exposed to 20% of LD₅₀ / 24 hrs exhibited moderate increase in the size of empty spaces within the muscle fibres (Figure 7- C.i and ii) whereas those which received 60% of the LD₅₀/24hrs exhibited substantial increase in the size of empty spaces within the meso-epithelial cells and muscle fibres. Infiltration of inflammatory cells in the empty spaces was also seen in the enlarged empty spaces between the muscle fibers (Figure 7- D. i and ii).

4 Discussion

Plant essential oils are potential candidates for snail control, due to their selective action, and little or no harmful effects on the non target organisms and the environment (Isman, 1999). Clove oil possesses strong bioactivity (Lahlou, 2004), and it has also exhibited ovicidal properties against eggs of several varieties of snails including *A. fulica* (Hollingworth et al., 2012). Thus this attempt was made to test its potential against the adult snails of *A. fulica*. On topical application the molluscicides primarily target the epithelial cells of skin including the mucus cells (Triebkorn et al., 1998). Infact one of the first responses of molluscs to stress is increased mucus production and secretion (Triebkorn & Ebert, 1989). Profuse mucus production was observed in snails exposed to both lower (20% of LD₅₀/24 hrs) as well as higher dose (60% of LD₅₀/24 hrs) of clove oil. This is in accordance to the observations of Desai et al (2015) who had exposed the snail *A. fulica* to various synthetic molluscicides. The mucus forms a protective barrier between the toxin and the surface of epithelial cells and helps in diluting the toxin (Triebkorn & Ebert, 1989).

The toxic effect of any compound on an organism can be evaluated by observing the histopathological changes caused by the compound. In the present study all the tissues from vehicle treated group were histologically similar to the control group barring mild degenerative changes observed in the hepatopancreas, however, treatment with clove oil resulted in significant dose dependent histopathological changes in all the

Figure 3 Photomicrographs of sections of oesophagus of *Achatina fulica* (A-D). (A: Control, B: Vehicle control, C: Exposed to 20% of $LD_{50}/24$ hrs, D: Exposed to 60% of $LD_{50}/24$ hrs). A and B: (i – 10 X), C (i – 10X, ii – 45X), D: (i – 10X, ii and iii – 45X). Mu: Mucosa; M: Muscular layer; D: Degeneration; I-Infiltration of inflammatory cells

Figure 4 Photomicrographs of sections of crop of *Achatina fulica* (A - D). (A: Control, B: Vehicle control, C: Exposed to 20% of $LD_{50}/24$ hrs, D: Exposed to 60% of $LD_{50}/24$ hrs). (A - D): (i - 10X and ii - 45X) Lu: Lumen of Crop; D: degenerative change; L: loss of Epithelium; I- Infiltration; Mu: Mucosa; CE: Columnar Epithelium, N: Necrosis

Figure 5 Photomicrographs of sections of stomach of *Achatina fulica* (A - D). (A: Control, B: Vehicle control, C: Exposed to 20% of $LD_{50}/24$ hrs, D: Exposed to 60% of $LD_{50}/24$ hrs). A and B: (i -10 X), C and D: (i -10X, ii - 45 X). Mu: Mucosa; CE: Columnar Epithelium; DC: degenerative Cells; Lu: lumen; I: infiltration; N: Necrosis

Figure 6 Photomicrographs of sections of intestine of *Achatina fulica* (A - D). (A: Control, B: Vehicle control, C: Exposed to 20% of LD_{50} /24 hrs, D: Exposed to 60% of LD_{50} /24 hrs). (A - D): (i - 10 X and ii - 45X) Lu: Lumen of intestine; D: degeneration; CE: Columnar epithelium; Mu: Mucosa; E: epithelium; I: Infiltration; L: Loss of layers

Figure 7 Photomicrographs of sections of foot of *Achatina fulica* (A - D). (A: Control, B: Vehicle control, C: Exposed to 20% of $LD_{50}/24$ hrs, D: Exposed to 60% of $LD_{50}/24$ hrs). A and B: (i - 10X and ii - 45 X), C and D (i and ii - 45 X). CL: Cuticular Layer; ES: Empty Space; LM: Longitudinal Layer; OM: oblique Muscles; I Infiltration; MSE: Mesoepithelium; ESI: Empty space infiltration.

tissues. Severe damages were seen in various organs of the gut, the hepatopancreas and the ovotestis, while the foot region showed comparatively less damage.

A. fulica is a hermaphrodite animal and its ovotestis produces both the gametes. Thus any structural damage to this vital organ and specially to the gametes shall adversely affect its reproductive efficiency. In the ovotestis of snails exposed to lower concentration of clove oil (20% of LD₅₀ / 24 hrs) the spermatozoa were degenerated to a mild extent while those of snails treated with higher concentration (60% of the LD₅₀/24hrs) were degenerated to a greater extent and also revealed necrosis of sertoli cells at intervals. Comparable results were obtained by Zhou et al. (1993) who recorded decrease in the number of spermatozoa along with their degeneration and also reduction in the number of acini in the snails *Biomphalaria glabrata* treated with niclosamide and extracts of *Eucalyptus camaldulensis*. The results are also in accord to those obtained by Amaeze et al. (2011) in *Archachatina marginata* where there was tissue necrosis observed in gonads exposed to higher dose of foliar fertilizer Boost Xtra.

Hepatopancreas (digestive gland) of molluscs is a multipurpose organ which is believed to perform functions like absorption of ingested food material, extracellular and intracellular digestion, secretion, excretion and osmoregulation (Owen, 1966) and it also plays a major role in detoxification (Beeby & Richmond, 1988). The digestive glands of molluscs are known as target organs for toxicants. Changes in the cytoarchitecture of digestive gland of snails is used as a biomarker of induced toxicity (Triebkorn & Kohler, 1996). Thus any structural damage to the digestive gland affects the animals in multiple ways. Previous studies by Zhou et al. (1993) on the snail *Biomphalaria glabrata* treated with niclosamide and extracts of *Eucalyptus camaldulensis*, showed irregularity and shrinkage in the digestive cells (hepatocytes) and calcium cells of hepatopancreas along with breakage of some digestive cells and leakage of its secretory material in to the lumen. Similar observations such as shrinking of lumen of digestive tubule, degeneration and necrosis of cells along with atrophy of the connective tissue of digestive gland were noted on exposure of *Lymnaea luteola* to Paraquat (Kanapala & Arasada, 2013). Irreversible necrotic changes were noticed in the digestive gland of *Lymnaea stagnalis* following exposure to thiodan (Unlu et al., 2005).

Comparable results were obtained in the current investigation wherein the digestive cells of the hepatopancreas were degenerated in snails exposed to both lower as well as higher concentration of clove oil roughly in proportion to the dose administered, along with peripheral thickening of hepatic tubules, and atrophy of connective tissue in snails exposed to lower subacute dose. Clustering of secretory material exuded from the

damaged hepatocytes was also observed which was massive in snails treated with higher sub acute dose of clove oil. This result is also in accordance with the data obtained in the studies on the snail *Archachatina marginata* exposed to sublethal concentrations of Cu salt wherein there was clogging of hepatocytes observed along with peripheral thickening of hepatic tubules (Otitoloju et al., 2009).

The alimentary canal along with the hepatopancreas is important for the digestion of food, and *A. fulica* being a voracious feeder, the alimentary canal is suitably modified for digesting the food (Dimitriadis, 2001). Studies have revealed that necrotic damage in the epithelial cells results in serious dysfunction of the tissue leading to deleterious effects at higher biological organization levels (Kanapala & Arasada, 2013). The epithelial lining of the gut of the snails treated with clove oil, in this study, was damaged severely, however the extent of damage in different zones was variable and dose dependent. This is in accordance with the results obtained by Manna & Ghose (1972) who studied the effect of endrin on the gut of *A. fulica*. The gut of the snail *Bithynia siamensis* exposed to niclosamide and crude extracts of camellia seeds and mangosteen pericarp also showed cellular damage and enlargement in the interlamellar space along with loss of texture (Aukkanimart et al., 2013). Severe necrosis and sloughing of the gut epithelium of *Archachatina marginata* treated with foliar fertilizer Boost Xtra further supports the results of our study (Amaeze et al., 2011).

The foot of snails usually forms a typical creeping sole, occupying the whole of the ventral surface. The foot of snails treated with both lower as well higher subacute dose showed increase in the size of empty spaces within the mesoepithelial cells and muscle fibres, which was more pronounced in higher subacute dose. Further at higher subacute dose the empty spaces within the muscle fibres were also infiltrated with inflamed cells. These observations are in congruence with the results of the earlier study using crude extracts of camellia seed and mangosteen pericarp, which affected cells in the foot by relaxing muscle fibres and creating gaps between epithelial cells and connective tissue, resulting in the derangement of the cilia in the snail *Bithynia siamensis* (Aukkanimart et al., 2013). Degenerative changes in the muscle fiber, protein and pigment cells of the foot were noticed in the snail *Lymnaea stagnalis* exposed to Thiodan in pesticide-free freshwater (Unlu et al., 2005).

These results indicate that clove oil is highly effective against the snail and the histopathological alterations caused were proportionate to the dose administered in almost all the tissues studied. Thus it can be concluded that clove oil can be used to control the population of pestiferous land snail *A. fulica*. Being eco-friendly and also being exempted from pesticide registration requirement and pesticide residue tolerance requirements

(Hollingsworth et al., 2012), it gets an edge over the other botanicals explored, to be used as a molluscicide.

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