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Effect of Sub-lethal Exposure of Essential Oils of Various Plant Origin Molluscicides on Different Biochemical Parameters of Vector Snail *Lymnaea acuminata*

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Abstract

The incidence of endemic disease fascioliasis can be reduced by effective snail control which is one of the most important tools. Essential oils and their constituents are gaining increasing interest for use as safe alternatives to pesticides for controlling various pests including gastropods. In the present study, some of the essential oils were demonstrated as potent molluscicides against the vector snail *Lymnaea acuminata*. The main objective of this research is to evaluate the molluscicidal activity of the essential oils of *Polianthes tuberosa* bulb, *Allium sativum* bulb, and their active molluscicidal components hecogenin and allicin against the snail *Lymnaea acuminata*. The effect of sub-lethal treatments (40% and 80% of 24h LC_{50}) of different plant-derived molluscicides on different enzyme activities in the nervous tissue of snail *Lymnaea acuminata* was studied. It is evident from the present results that *P. tuberosa* and *A. sativum* bulb essential oils exhibited as strong molluscicides of plant origin. The present study can help reduce the incidence of fascioliasis. These essential oils may offer an alternative tool for the control of fascioliasis in the cattle population of eastern Uttar Pradesh of India. Snails exposed to sub-lethal concentrations of essential oils of and compared with the active molluscicidal components a significant inhibition in acetylcholinesterase (AChE), acid/alkaline phosphatase (ACP/ALP), lactic dehydrogenase (LDH) and Na^+K^+ ATPase activities in the nervous tissue of *L. acuminata*. Essential oils and active compounds of both the plant origin molluscicides were highly toxic and show significant inhibition in different biochemical parameters to *L. acuminata* compared with their crude forms.

Keywords

Essential oils, Molluscicides, *Polianthes tuberosa*, *Allium sativum*, *Lymnaea acuminata*

1. Introduction

Fascioliasis is one of the important live-stock health problem in the eastern Uttar Pradesh state of India [1-8]. This disease is transmitted by the flukes *Fasciola hepatica* and *F. gigantica* through the vector snail *Lymnaea acuminata* [9]. Snail control with molluscicides has been one of the effective methods used for rapid and effective control of this disease [10-15]. An obvious solution to reduce the incidence of fascioliasis is to de-link the life cycle of fluke by destroying the vector snails. The development of a particular and safe molluscicide should always be a realistic aim. It must be effective at low attention and apply minimum adverse effect on the other biota participating in the same territory with the snail.

Essential oils are volatile and liquid aroma compounds from natural sources, usually plants. The odoriferous substances

(essential oils) themselves are formed in the chloroplast of the leaf, vesinogenou layer of the cell wall, or by the hydrolysis of certain glycosides. They may be found in different parts of the plant. Some could be in leaves (oregano), seed (almond), flower (jasmine), peel (bergamot), berries (juniper), rhizome (galangal ginger), root (angelica archangelica), bark (sassafras), wood (agar wood), resin (frankincense), petals (rose). Essential oils from different parts of the same plant may have completely different scents and properties. Geranium, for instance, yields oil both from the flowers and the leaves, and the oil from both parts differ in constituents, scents, and some other properties. The quantity of essential oil extracted from the plant is determined by many interrelated factors, including climatic, seasonal, and geographical conditions, harvest period, and extraction techniques [16]. The yield of oils from the plants can also be affected by the stages of the plant growth. Science regards essential oils in terms of functionality. They are considered" the chemical weapons" of the plant world as their compounds may deter insects, or protect the plant against bacterial or fungal attacks. They also act as "plant pheromones" in an effort to attract and seduce their pollinators. The oxygenated molecules of essential oils, which serve as chemical messengers to the cells bring life to the plants, destroying infestation, aiding growth, and stimulating healing. More poetically inclined souls regard them as the essence of the plant's soul, their ethereal nature concentrated as scents, through which plants communicate with their surrounding world. The therapeutic properties of essential oils have been reported by previous researchers [17, 18].

The naturally occurring essential oils are volatile and liquid aroma produced mainly by plants. These essential oils are manufactured in the chloroplast of the leaf, vesinogenou layer of cell wall or by hydrolysis of some glycosides. The interest in essential oils is widespread and has seen them proven effective against various pests including insects, mites, fungi, and nematodes [19-21]. The most poisonous oils overall feel to be thyme, oregano, basil, rosemary, and mint; still, testing of a wider range of oils on various pests will probably reveal particular exertion of certain oils against certain pests.

2. Materials and Methods

2.1 Isolation of Essential Oils

The essential oils of *Polianthes tuberosa* and *Allium sativum* were obtained by the hydrodistillation method described in British Pharmacopoeia [22]. The bioactive compound hecogenin of *Polinthes tuberosa* was purchased from Sigma Chemcals Company, USA and allicin of *Allium sativum* as synthesized in the laboratory by the method of Singh and Singh [23] modified by Tiwari and Singh [24].

2.2 Collection of Snails

Adult *Lymnaea acuminata* (2.25±0.20 cm in length) were collected locally from lakes and low-lying submerged fields in Gorakhpur. The snails were acclimatized for 72 hours in dechlorinated tap water at 25±10 C. The pH of the water was 7.1-7.3 and disappeared oxygen, free carbon dioxide, and bicarbonate alkalinity were set to6.5-7.2 mg/ l,5.2-6.3 mg/ l and102.0-105.0 mg/ l, independently.

2.3 Chemicals Used

Acetylthiocholine iodide (ATChI); 5,5- dithiobis-2-nitrobenzoate (DTNB); Ouabain (1β, 3β, 5β, 11α, 14, 19-hexahydroxycard-20[22] enolide 3-[6 deoxy α-L-mannopyranoside]; β, nicotinamideadeninedinucleotide (p NADH); sodium pyruvate; all these chemicals were supplied by Sigma Chemicals Co. USA. The snails were exposed to sub-lethal concentrations of 40% and 80% of 24h LC_{50} of essential oils of different plant-origin molluscicides and their bioactive compounds in the aquarium for 24h (Table 1).

Table 1. Sub-lethal concentrations of Essential oils of different plant-derived molluscicides were used in biochemical assay against the snail *L. acuminata*

Molluscicides	24h LC_{50}	40% of 24h LC_{50}	80% of 24h LC_{50}
Polianthes tuberosa Bulb	1.57	0.62	1.5
Allium sativum Bulb	1.35	0.54	1.0
Hecogenin	1.19	0.47	0.9
Allicin	1.25	0.5	1.0

2.4 Biochemical estimations

Snails were exposed to sublethal concentrations of 40% and 80% of 24h LC_{50} of essential oils and different molluscicides for 24 h (Table 1). Six batches were prepared for each concentration. The control aquarium contained pellets without molluscicicdes.

2.5 Enzyme assays in vivo

2.5.1 Acetylcholinesterase

Acetylcholinestease activity in the nervous tissue of *L. acuminata* was measured according to the method of Ellman et al. [25] as modified by Singh and Agarwal [26]. The nervous tissue was homogenized (50mg/ml) in 0.1 M phosphate buffer (pH 8.0) for 5 minutes in an ice bath and centrifuged at 1000xg for 30 minutes at 40 C. The clear supernatant was taken as enzyme source. The enzyme activity was measured in a 10 mm path length cuvette using an incubation mixture consisting of 0.1 ml of enzyme source, 2.9 ml of 0.1 M phosphate buffer (pH 8.0), 0.1 ml of chromogenic agent DTNB (5,5- dithiobis-2 nitro-benzoate) and 0.2 ml freshly prepared acetylthiocholine. The absorbance change in optical density at 412 nm was continuously observed on a spectrophotometer for 3 minutes at 25^0C.

2.5.2 Phosphatase Activity

The activities of phosphatases were measured by the method of Bergmeyer [27] as modified by Singh and Agarwal [28]. The nervous tissue was homogenized (2%w/v) in ice-cold 0.9% NaCl and centrifuged at 5000xg for 20 minutes at 40^0C. The supernatant was taken and used as an enzyme source. Standard curves were drawn with p-nitrophenol.

2.5.3 Alkaline phosphatase

For the assay of alkaline phosphatase, 0.1 ml of enzyme source supernatant was added in 1.0 ml alkaline buffer substrate solution (prepared by dissolving 375 mg glycine, 10 mg $MgCl_2$.6H2O and 165 mg p-nitrophenol phosphate sodium salt in 42 ml of 0.1 N NaOH and mixture was made up to 100 ml with double distilled water). The mixture was mixed thoroughly and incubated for 30 minutes at 37^0C. In the incubation mixture, 10 ml 0f 0.02 N NaOH was added. The reaction was stopped by the addition of an excess of NaOH solution. The activity of alkaline phosphatase was measured colorimetrically at 420 nm which is a measure of the yellow colour of nitrophenol produced by the hydrolysis of p-nitrophenyl phosphate buffer. The enzyme activity was expressed in µ moles substrate hydrolyzed/30min/mg protein.

2.5.4 Acid phosphatase

Acid phosphatase activity was determined by adding 0.2 ml of enzyme source containing supernatant, 1.0 ml of pre-incubated (10 min) acid buffer substrate solution (prepared by dissolving 0.41 gm of citric acid, 1.125 gm sodium citrate and 165 mg of disodium salt of p-nitrophenyl phosphate to 100 ml of double distilled water), the mixture was mixed thoroughly and incubated for 30 minutes at 370 C. Then 4.0 ml of 0.1 N NaOH was added to the incubation mixture. The yellow colour developed due to the formation of p-nitrophenol. The activity of acid phosphatase was measured at 420 nm. Enzyme activity is expressed as µ moles substrate hydrolyzed/30min/mg protein.

2.5.5 Na^+/K^+ ATPase

Activity of the enzyme $Na^{+/}K^+$ ATPase by the method of Svobodo Mossinger [29] as modified by Singh and Singh [30]. Fifty mg nervous tissue was homogenized in 1.0 ml of 0.32 M chilled sucrose solution for 5 min and centrifuged at 800 g for 10 min at 4°C. The supernatant obtained was used as an enzyme source. Mg^{++}–ATPase and Mg^{++}, Na^+/K^+ activated ATPase activities were simultaneously assayed. The difference in the enzyme activity between these two was considered as the Na^+/K^+ ATPase activity. The incubation medium for total ATPase contained 0.2 ml of supernatant, 0.25 ml Tris HCl buffer (50 mM, pH 7.5), 0.25 ml NaCl (100 mM), 0.25 ml KCl (20 mM) and 0.25 ml of $MgCl_2$ (4 mM). The incubation medium for Mg^{++}–ATPase was similar to the prior one except that it contained 120 mM NaCl, 2 × 10−4 M Ouabain to inhibit the ATPase, and no KCl. Both the mixtures were pre-incubated for 10 min, at 37°C before the addition of the substrate to start the enzyme reaction. The reaction mixture was incubated with the substrate for 15 min at 37°C. The reaction was stopped by adding 0.5 ml of 10% perchloroacetic acid (PCA) and kept in ice-cold water for 5 min. The inorganic phosphate (Pi) was liberated by the method of Fiske and Subbarow [15]. One milliliter of the reaction mixture (containing lipid layer) was pipetted out and 0.4 ml of 10% TCA was added to the reaction mixture and heated. After heating, 0.4 ml of 2.5% ammonium molybdate solution and 0.2 ml of amino napthosulphonic acid (ANSA reagent) were added and the reaction mixture was heated at 80°C for 15 min. The reaction mixture was cooled at room temperature

diluted with 4.0 ml of distilled water and was kept for 5 min. The absorbance was read at 640 nm against blank. The blank consisted of 1.0 ml distilled water, 0.4 ml of TCA, 0.4 ml ammonium molybdate solution, 0.2 ml of ANSA reagent and 4.0 ml of distilled water, but no tissue homogenate. The unit of the enzyme activity was expressed as µ mole Pi liberated mg^{-1} protein h^{-1}.

2.5.6 Lactic dehydrogenase:

The activity of LDH was measured according to Anon [31] as modified by Singh and Agarwal [32]. The tissue was homogenized (50 mg/ml) in 0.1 M phosphate buffer (pH-7.5) for 5 min and centrifuged (10000 g×30 min) at –4°C. To 0.01 ml of an enzyme source (supernatant), 0.5 ml of pyruvate substrate (10 mg NADH in 10 ml of 0.75 mM/l pyruvate buffer, pH-7.5) was added and kept for incubation for 45 min at 37°C. To this 0.5 ml of 2,4- dinitrophenyl hydrazine solution (0.2 g 2,4-dinitrophenyl hydrazine in 8.5 ml of concentrated HCl and volume made to 1 liter) was added and the mixture was left standing for 20 min at room temperature. Finally, 5.0 ml of 0.4 N NaOH was added to the mixture and left for 30 min at room temperature. LDH activity was measured by monitoring the decrease in absorbance at 540 nm. Values were converted into LDH units and expressed as pyruvate reduced/min/mg protein.

2.6 Estimation of Protein

Protein estimation was made according to Lowry et al. [33] using bovine serum albumin as a standard. Results have been expressed as mean ± SE of six replicates.

2.7 Statistical Analysis

Students' t-tests were applied between the control and the tested groups to locate the significant variations ($P < 0.05$) [34].

3. Results

In vivo 24h sublethal exposure of 40% and 80% of 24h LC_{50} of essential oils of plant origin molluscicides and their bioactive compounds caused a significant dose-dependent decrease in the AChE, ACP/ALP, LDH and Na^+K^+ATPase activity in the nervous tissue of snail *L. acuminata*. The AChE activity in the nervous tissue of *L. acuminata* was 0.087 -µ moles –SH hydrolyzed/min/mg protein in the control group of animals. Exposure to sub-lethal concentration of 40% and 80% of 24h LC_{50} *Polianthes tuberosa* bulb caused significant ($P<0.05$) when the snails were exposed with essential oils of 80% of 24h LC_{50} of *Allium sativum* bulb (63.86% of control). There was no significant inhibition in ALP activity in the nervous tissue of snails exposed to essential oil of 40% of 24h LC_{50} of all the plant derived molluscicides, except hecogenin and allicin (Table 3). The acid phosphatase (ACP) activity in the nervous tissue of the vector snail. *L. acuminata* was observed 2.05-µ moles/30 min/mg protein in the control group of animals. There was a significant inhibition in the acid phosphatase activity in the nervous tissue of *L. acuminata* after the exposure of 40% and 80% of 24h LC_{50} of the plant origin molluscicides containing essential oils of *Allium sativum* bulb. Maximum inhibition (43.9% of control) in ACP activity was observed in the nervous tissue of *L. acuminata* exposed with 80% of 24h LC_{50} of allicin. The lactic dehdrogenase (LDH) activity in the nervous tissue of the vector snails *L. acuminata* was 333.59 µ moles/30 min/mg protein in control group of animals. Maximum reduction in LDH activity (64.13% of control) was observed in the nervous tissue of *L. acuminata* exposed with essential oils with 80% of 24h LC_{50} of hecogenin (Table 5). The Na^+K^+ ATPase activity in the nervous tissue of the vector snail *L. acuminata* was 1.05 Pi liberated µ moles/30 min/mg protein in the control group of animals. Exposure of 40% and 80% of essential oils of plant origin molluscicides and their bioactive compounds caused significant reduction in the activity of Na^+ K^+ ATPase in the nervous tissue of *L. acuminata*. Maximum inhibition (20.95% of control) was observed in nervous tissue of *L. acuminata* exposed with 80% of 24h LC_{50} essential oils of *Polianthes tuberosa* bulb (Table 6).

4. Discussion

It is evident from the result section that snails exposed to sub-lethal concentrations i.e. 40% and 80% of 24h LC_{50} of different essential oils of plant origin molluscicides *Polianthes tuberosa* bulb, hecogenin, *Allium sativum* bulb and allicin caused a significant inhibition in acetylcholinesterase (AChE), acid/alkaline phosphatase (ACP/ALP), lactic dehydrogenase (LDH) and Na+ K+ ATPase activity in the nervous tissue of *L. acuminata*. Inhibition of AChE causes accumulation of ACh at the synapses, so that the post synaptic membrane is in a permanent stimulation which results in paralysis, ataxia,

general lack of co-ordination in the neuromuscular system and eventual death [35]. Alkaline phosphatase (ALP), which plays a critical role in protein synthesis, shell formation and other secretory activities its inhibition may result reduction in protein levels in gastropods. Acid phosphatase (ACP), a lysosomal enzyme that play an important role in catabolism, pathological necrosis, autolysis, and phagocytosis was also inhibited by these essential oils and bioactive compounds of plant-origin molluscicides [36]. There was a significant reduction in the activity of $Na^+ K^+$ ATPase when the snails were exposed to sub-lethal concentrations of essential oils of different plant-origin molluscicides. $Na^+ K^+$ ATPase is an essential enzyme of neurotransmitter process that maintains ion gradient, electric potential of membrane, and osmotic balance. The persistence of sodium channel activation stimulates sodium influx, which alters the activity of $Na^+ K^+$ ATPase to pump out sodium and evoke neurotransmitter release.

The disturbance in action potential during the exposure of AFP might be critical for snails. Inhibition of LDH activity by all the plant-derived molluscicides incorporated inside AFP indicates that they act on anaerobic metabolism in the snail body. The effect of all these plant-derived molluscicides taken in the present study when dissolved in aquarium water directly also caused significant alteration in different enzyme activities in the nervous tissue of vector snail *L. acuminata* [37]. The treatment of the essential oil of *Polianthes tuberosa* bulb and its bioactive compound hecogenin caused significant inhibition of AChE, ACP/ALP, LDH and $Na^+ K^+$ ATPase activities in the nervous tissue of snail *L. acuminata*. Essential oil of Allium sativum bulb and its bioactive compound allicin also caused a significant inhibition of AChE, ACP/ALP, LDH, and $Na^+ K^+$ ATPase activities in the nervous tissue of the snail *L. acuminata* [38]. It is clear from the present study that the essential oils of plant-origin molluscicides which were used earlier in aquarium water directly against harmful snails *L. acuminata*, when exposed was effective selectively in a similar manner in killing them [39, 40]. It has been observed previously that many of the essential oils extracted from different plants exhibit potent molluscicidal as well as repellent activities against snails of economic importance [41-44]. It is clear from the above discussion that essential oils have potent molluscicidal properties against the snail hosts of different flukes.

Table 2. Effect of *in vivo* 24h exposure of essential oils of 40% and 80% of 24h LC_{50} of different plant origin molluscicides and their bioactive compounds on acetylcholinesterase activity in the nervous tissue of *L. acuminata*

Treatments	AChE-µ moles-SH hydrolyzed/min/mg protein	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	1.08±0.06 (100)	1.08±0.06 (100)
Polianthes tuberosa Bulb	0.98±0.07 (90.74)	0.77±0.06 (71.29)*
Allium sativum Bulb	1.02±0.06 (94.44)	0.69±0.02 (63.86)*
Hecogenin	1.04±0.06 (96.29)	0.75±0.05 (69.44)*
Allicin	0.86±0.06 (79.62)	0.79±0.07 (73.14)*

Notes. Values are the mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant ($P<0.05$) when student's t-test was used for locating differences between experimental and control groups of animals.

Table 3. Effect of *in vivo* 24h exposure of essential oils of 40% and 80% of 24h LC_{50} of different plant origin molluscicides and their bioactive compounds on alkaline phosphatase activity in the nervous tissue of *L. acuminata*

Treatments	ALP-µ moles/30min/mg protein	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	1.28±0.06 (100)	1.28±0.06 (100)
Polianthes tuberosa Bulb	1.98±0.07 (90.74)	1.77±0.06 (71.29)*
Allium sativum Bulb	1.12±0.06 (94.44)	1.69±0.02 (63.86)*
Hecogenin	1.14±0.06 (96.29)	0.85±0.05 (69.44)*
Allicin	0.96±0.06 (79.62)	0.89±0.07 (73.14)*

Table 4. Effect of *in vivo* 24h exposure of essential oils of 40% and 80% of 24h LC_{50} of different plant origin molluscicides and their bioactive compounds on acid phosphatase activity in the nervous tissue of *L. acuminata*

Treatments	ACP- µ moles/30min/mg protein	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	2.05±0.05 (100)	2.05±0.05 (100)
Polianthes tuberosa Bulb	1.18±0.02 (57.56)*	0.99±0.02 (48.29)*
Allium sativum Bulb	1.53±0.02 (74.63)*	1.4±0.04 (68.29)*
Hecogenin	1.13±0.02 (55.12)*	0.93±0.02 (45.36)*
Allicin	1.04±0.03 (50.73)*	0.90±0.02 (43.9)*

Notes. Values are the mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between the experimental and control groups.

Table 5. Effect of *in vivo* 24h exposure of essential oils of 40% and 80% of 24h LC_{50} of different plant origin molluscicides and their bioactive compounds on lactic dehydrogenase activity in the nervous tissue of *L. acuminata*

Treatments	LDH- µ moles/30 min/mg protein	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	333.59±2.3 (100)	333.59±2.3 (100)
Polianthes tuberosa Bulb	266.38±0.83 (79.85)*	259.26±0.88 (77.71)*
Allium sativum Bulb	330.59±2.3 (99.1)	265.09±0.88 (79.46)*
Hecogenin	277.32±4.5 (83.13)*	213.96±1.3 (64.13)*
Allicin	306.4±1.04 (91.84)*	269.87±0.88 (80.89)*

Notes. Values are the mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between the experimental and control groups of animals

Table 6. Effect of *in vivo* 24h exposure of essential oils of 40% and 80% of 24h LC_{50} of different plant origin molluscicides and their bioactive compounds on Na^+/K^+ stimulated ATPase activity in the nervous tissue of *L. acuminata*

Treatments	Na^+/K^+ stimulated ATPase- moles/30min/mg protein	
	40% of 24h LC_{50}	80% of 24h LC_{50}
Control	1.05±0.003 (100)	1.05±0.003 (100)
Polianthes tuberosa Bulb	0.56±0.006 (53.33)*	0.22±0.01 (20.95)*
Allium sativum Bulb	1.01±0.01 (96.16)*	0.74±0.001 (70.47)*
Hecogenin	1.0±0.01 (95.23)*	0.94±0.001 (89.52)*
Allicin	0.9±0.008 (85.71)*	0.46±0.02 (43.8)*

Notes. Values are the mean SE of six replicates.
Values in parentheses indicate percent enzyme activity with control taken as 100%.
(*) Significant (P<0.05) when student's t-test was used for locating differences between the experimental and control groups of animals.

5. Conclusion

It can be concluded from the above study that essential oils have great potential to eradicate the major problem of fascioliasis by de-link the life cycle of the *Fasciola* species. In this context, the population of important vector snail *Lymnaea acuminata* can be controlled by applying these essential oils of plant origin. The essential oils are now considered as potent herbal molluscicides.

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