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TOXICITY AND PHYSIOLOGICAL EFFECT OF ESSENTIAL OIL OF *ARTEMISIA ANNUA* (LABIATAE) ON *AGRIOLIMAX AGRESTIS* L. (STYLOMMATOPHORA: LIMACIDAE)

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Abstract: Essential oil of *Artemisia annua* L. was investigated to find out its toxicity and physiological aspects on the slug *Agriolimax agrestis*, in controlled conditions (8±1°C, 75±5 RH and 14:10 LD). The slugs received different concentrations of essential oil treated radish leaves in methanol, while the control received methanol alone. LC_{10}, LC_{30}, LC_{50} and LC_{90} values were estimated at 4.67, 5.3, 5.81, 7.25%, respectively. The effect of the essential oil on some important enzymatic components like; cytochrome P450 monnooxygenase, acid phosphatase, alkaline phosphatase, lipase, amylase and protease were significantly increased compared to the control. These results indicate that the plant *Artemisia annua* L. not only shows toxicity but also shows some irreversible effect on some important biochemical components and deserves further investigation.

Key words: *Artemisia annua* L., *Agriolimax agrestis* L., physiological aspects

INTRODUCTION

Problems associated with the use of synthetic pesticides led researchers to look for natural plant protection compounds such as botanicals, insecticides, and antifeedants. Among the plant families studied, Meliaceae, Asteraceae, Labiateae, Piperaceae and Annonaceae are most promising (Isman 2006). Botanical products are useful tools in many pest management programs because they are effective and especially target the natural enemies of plants. Crops are affected by three main groups: the Limacidae – including the grey field slug (*Derocera reticulatum*) and its relatives; the Arionidae or round backed slugs – including garden slugs (*Arion bortensis* and *A. desstincus*); and the Milacidae or keeled slugs. The grey field slug is the species of greatest importance in arable crops, except for potatoes where damage is usually the result of the activities of keeled and garden slugs. In gardens, the garden slug is the most important species.

Slugs can cause significant damage in a wide range of agricultural and horticultural crops, as well as in gardens (Glen *et al*. 1991). *Agriolimax agrestris* L. (Stylommatophora: Limacidae), generally is the pest of cabbage, potato, alfalfa, clover, and some of the other crop plants. This pest is nocturnal and during the light period rests on the backs of leaves. *Agriolimax agrestris* Linne. are more active in the autumn and spring seasons when the weather is cool and moist. This pest eats different varieties of cabbages and causes severe damage in quality and quantity of crops (Khanjani 2005).

The genus *Artemisia* is a member of the large plant family Asteracea (Compositae) encompassing more than 300 different species of this diverse genus. The species *A. annua* known as sweet worm wood, grows wild in Europe and America and is planted widely in China, Turkey, Vietnam, Afghanistan and Australia (Bhakani *et al*. 2001). The plant also grows in northern parts of Iran around paddy fields. Several isolated compounds from this species have been shown to have antimalarial, antibacterial, anti-inflamatory, plant growth regulatory and cytotoxicity (antitomur) activities (Bhakani *et al*. 2001). Several studies have reported the insecticidal effects of sweet worm wood (Acher *et al*. 1993; Schmutterer *et al*. 1995; Rao *et al*. 1999; Jalali *et al*. 2002; Jalali *et al*. 2005; Isman 2006). There has been no report on its effects on *Agriolimax agrestis* (Limacidae). In the present study we have tried to elucidate the effect of the essential oil of *A. annua* on toxicity, and the possible mechanisms involved in its effect.

MATERIALS AND METHODS

Laboratory mass culture of *A. agrestis*

The slugs were collected in the vicinity of Rasht (Taremseshanbeh) in Guilan Province, Iran from radish fields. Only active animals of healthy appearance with no signs of stress or parasitical infection were used. Forty-five slugs which were 2 centimeters long were randomly selected and placed in 10x20 cm transparent container

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in which the lid had holes for aeration. These containers were maintained in the laboratory at 8±1°C; 14:10 LD; 75±5% RH.

Essential oil preparation

Plants were collected from paddy fields in northern Iran in mid May 2010. They were dried in the shade and made into a fine powder with the help of a hand mortar. Next, 50 g of dried herb powder was briefly mixed with 750 ml distilled water. After 24 h, this mixture was transferred to a Clevenger type apparatus. After distillation, which lasted about 2 h, the essential oil was obtained. The oil phase was isolated from the obtained solution. Sodium sulfate was used for dehydration.

Bioassays and treatment

Toxicity tests

After initial bracketing tests, three concentrations of 4, 6 and 8 percent, were selected for bioassay and evaluation of LC_{50} values, along with a control treated with methanol alone. In each experiment, 10 slugs were tested with 4 replicates per each concentration. Fresh leaves of radish, *Rhaphanus sativus* (Cruciferae), were dipped in the different concentrations of *A. annua* essential oil for 30 seconds and allowed to air dry. The control leaves were treated with methanol alone and dried in the same way. The slugs were starved 4 h prior to the experiments and then were allowed to feed on two leaves treated with the different concentrations of *A. annua* essential oil for each replication. The lethal and sub lethal doses were estimated after 48 h using Polo-Pc software (LeOra software 1987).

Biochemical analysis

After the treatment, slugs were randomly selected, and their guts were removed by dissection in ice-cold saline buffer (6 µmole/l NaCl). The gut was rinsed in-cold diluted buffer, placed in a pre-cooled homogenizer. The homogenates from preparations were separately transferred to 1.5 centrifuge tubes and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatants were pooled and stored at –20°C for subsequent analyses (Zibaee *et al*. 2008).

Digestive enzyme assays

The general protease amount of gut was determined using azocasein as a substrate (Elpidina *et al*. 2001). The reaction mixture was 30 µl of 2% azocasein in a solution in 100 µl of universal buffer and 30 µl proteases. The reaction mixture was incubated at 30°C for 60 min. Proteolysis was stopped by the addition of 150 µl of 10% trichloroacetic acid (TCA), and the reaction mixture was centrifuged at 13 000 rpm for 10 min. The 288 µl volume of 2 N NaOH was added to the mixture and the absorbance was read at 440 nm.

Lipase activity measurements were carried out as described by Tsujita *et al*. (1989). Ten µl of gut tissue extract, 100 µl of universal buffer solution (1M) (PH = 7.2), and 30 µl of p-nitrophenyl butyrate (27 mM) substrate, were incorporated, mixed thoroughly and incubated at 28°C for 10 min. Next, 100 µl NaOH was added to each tube (the control and experimental samples). After 5 min the absorbance was read at 450 (405) nm.

Amylase was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955) using 1% soluble starch (Merck, Darmstadt, Germany) as a substrate. Ten microliters of the enzyme were incubated for 30 min at 37°C with 100 µl universal buffer and 50 µl soluble starch. The reaction was stopped by the addition of 80 µl DNS and heated in boiling water for 10 min. DNS is a color reagent, hence the reducing groups released from starch by amylase were measured by the reduction of DNS. The boiling water stoped the amylase activity and catalyzes the reaction between DNS and the reducing groups of starch. Absorbance was then read at 540 nm.

Detoxifying enzymes

The method of Bessy *et al*. (1946) was used for measuring alkaline phosphatase (ALP) and acid phosphatase (ACP) activity. The reaction mixture was 100 µl of universal buffer, 30 µl of related substrate (acidic for ACP, alkalic for ALP) and 20 µl enzyme. This mixure was incubated for 30 min, after that 50 µl NaOH (1M) was added. Asorbance was read at 400 nm.

The P450 activities were determined according to the method reported by Hansen and Hodgson (1971). One hundred microliters of 2 mM p-nitroanisole solution and 90 ml enzyme stock solution were added to each well of a microplate and mixed. After incubation for 2 min at 271°C, the reaction was initiated by adding 10 ml of 9.6 mM NADPH. Then, the optical density at 405 nm was immediately recorded at intervals of 25 s for 10 min using the microplate reader.

Statistical analysis

The lethal concentration was estimated by POLO-PC software (LeOra software 1987). All data were analyzed in a completely randomized design using SAS software and Tukey's studentized test was used for comparisons of means (SAS 1997).

RESULTS

The LC_{90}, LC_{50}, LC_{30}, LC_{10} values, confidence limit (95%) and regression slope at 48 h exposure to plant essential oil are shown in figure 1. The LC_{50} value, confidence limit (95%) at 48 h exposure to plant essential oil was 5.81%. Results showed that the mortality was dose-dependent. As can be seen in table 1 and figure 1, increasing concentration of plant extract exacerbate mortality.

The effect of *A. annua* on biochemical and metabolic events in this slug, is shown on table 2 and 3. Differences in lipase, amylase, protease between the control and treated slugs are shown on table 2. The level of lipase in treated slugs was significantly enhanced 48 after treatment with *A. annua* and then was reduced 72 h. In treated slugs, amylase was reduced 24 h after application of the essential oil but increased 72 h later. These alternations were significant compared with the control. The protease was significantly increased in treated slugs 24 and 48 h after treatment.

Table 1. LC_{10}, LC_{30}, LC_{50} and LC_{90} of *A. annua* essential oil on slugs

Essential oil	LC_{10} [95% CL]	LC_{30} [95% CL]	LC_{50} [95% CL]	LC_{90} [95% CL]
A. annua	4.67	5.3	5.81	7.25

CL – confidence limit which has been calculated with 95% confidence

Table 2. Effects of *A. annua* essential oil in LC_{50} on some digestive enzymes of slugs (24, 48 and 72 h after treatment)

Treatments	Cytochrome P450 monooxygenase [IU/l]			Acid phosphatase [IU/l]			Alkaline phosphatase [IU/l]		
Hours after treatment	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Control	26,211.44± 0.010408 b	16,285.22± 0.025482 b	28,965.47± 0.039686 a	7743800± 0 a	987,112.3549± 0.016 b	120,5512.015± 1.57009E-16 a	683,2764.599± 9.06493E-17 b	101,7198.999± 0 b	101,7198.999± 9.06493E-17 b
A. annua	307,637.4± 0.048364 a	143,604.2± 0.018496 a	76,363.74± 0.041458 a	516,1365.008± 0.038 b	466,5463± 0 a	161,3922± 0.266252 a	469,5798.646± 0 a	560,3514.459± 1.28198E-16 a	407,5682± 0 a

Within columns, means (±SE) followed by a same letter do not differ significantly ($p \leq 0.05$); IU – international unit

Table 3. Effects of *A. annua* essential oil in LC_{50} on some detoxifying enzymes of slugs (24, 48, 72 h after treatment)

Treatments	Lipase [IU/l]			Amylase [IU/l]			Ptotease [IU/l]		
Hours after treatment	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Control	106,1149± 0.015177 a	491,80.86± 0.03238 b	185,54.68± 0.018774 a	521,34.8± 0.015762 a	6,345.763± 0.009292 a	10,257.53± 0.006333 b	102,1053± 0.002603 b	3,69332± 0.001856 b	21,74795± 0.004631 a
A. annua	907,051.7± 0.021385 a	765,868.7± 0.008686 a	349,228.9± 0.032936 b	106,68.57± 0.002404 b	63,161.31± 0.007535 a	174,057.9± 0.002603 a	777,5423± 0.019701 a	302,7624± 0.009387 a	853,2632± 0.067336 a

Within columns, means (±SE) followed by a same letter do not differ significantly ($p \leq 0.05$); IU – international unit

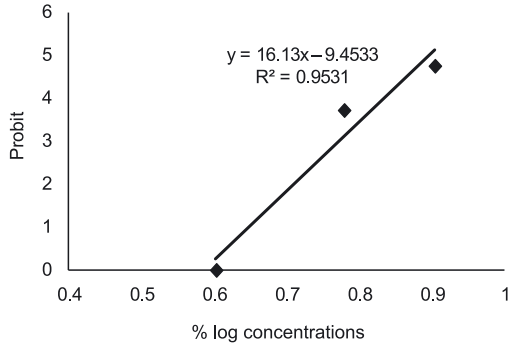

Fig. 1. The regression line of the mortality of *A. agrestis* by *A. annua* essential oil

Differences in detoxifying enzymes Cytochrome P450 monooxygenase, alkaline phosphatase (ALP), acid phosphatase (ACP) between the control and treated slugs are shown in table 3. In treated animals, Cytochrome P450 monooxygenase was increased after 24 and 48 h. Both ALP and ACP, were increased and were significant compared with the controls.

DISSCUSION

Essential oils are generally composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes. Examples include: 1, 8-cineole, the major constituent of oils from rosemary (*R. officinalis*) and eucalyptus (*Eucalyptus globus*); eugenol from clove oil (*Syzygium aromaticum*); thymol from garden thyme (*Thymus vulgaris*); and menthol from various species of mint (*Mentha* species) (Isman 2006).

The enzyme lipase breaks carboxyl ester in triacylglycerols, phospholipids and galactolipids. This group of enzymes has a role in storage and mobilization of lipids. Lipases are also the building blocks for many physiological processes such as; growth, reproduction and defense against pathogens. The increase after 24 h in lipase in the present investigation, may be due to the use of storage lipids. The metabolic enzymes have a role in metabolizing toxic materials. The metabolic processes include oxidation and hydroxylation. Hence, these enzymes are concerned with the mode of action of pesticides and resistance to pesticides. Alpha-amylase is an enzyme hydrolyzing starch to maltose and glycogen to glucose. This enzyme was reduced 24 h after treatment with essential oil. Proteases are a group of enzymes that hydrolyze peptide bonds in proteins and convert them into their respective amino acids. This enzyme increased after 24 and 48 h, significantly. We believe the increase in protease activity in the present study may be due to hydrolysis of stored proteins in this mollusk trying to compensate for the lack of proteins from food sources. Alkaline phosphatase, acid phosphatase are hydrolytic enzymes, which hydrolyse phosphomonoesters under acid or alkaline conditions, respectively. ALP is mainly found in the intestinal epithelium of animals and its primary function is to provide phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes. ALP is involved in the transphosphorylation reaction (Etebari *et al*. 2005). The present study showed that ACP activity decreased significantly after a 24 h post-treatment in all treatments, compared with the control. At the same time, the activity of this enzyme did not show a significant difference in slugs treated with essential oil or the control, 48 and 72 h after treatment. ALP activity after 24, 48 and 72 h was increased. Increased ALP and ACP activity level after treatment, compared to the control, may indicate the involvement of these enzymes in the detoxification processes. Acid phosphatase is a lysosomal enzyme dealing with digestion of foreign substances and bacteria inside the cells (Ham *et al*. 1979) and is involved in the defense mechanisms of both vertebrates and invertebrates (Cooper *et al*. 1976). In addition, the deterioration in the activity of alkaline phosphatese can occur in all types of liver diseases (Farkas *et al*. 2004).

Cytochrome P450 monooxygenase may have increased because of the detoxifying role of this enzyme. *A. annua* essential oil not only showed a toxicity effect but also had irreversible effects on biochemical metabolites, digestive and detoxifying enzymes. Thus, the biological processes of the slug were affected. The use of synthetic insecticides affects the environment and the food chain. While biological molluscicides having plant origins have little or no side effects on the environment and on the human food chain. Hence, it is worthwhile to search for new plants having environmently-friendly compounds.

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