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PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF SOME ESSENTIAL OILS ON THE GRANARY WEEVIL, *Sitophilus granarius* (L.) (COLEOPTERA: CURCULIONIDAE)

ABSTRACT:
The effects of sub lethal doses of three plant essential oils extracted from *Pimpinella anisum* (anise), *Eugenia aromatica* (clove), and *Ocimum basilicum* (basil) on the physiological and biochemical parameters of the granary weevil, *Sitophilus granarius* adults were investigated. The total carbohydrates, protein and lipid contents as well as enzymatic activities of acetylcholine esterase, glutamate pyruvate transaminase and glutamic-oxaloacetic transaminase were measured. The LC50 values were estimated to be 0.391, 0.615, and 0.642 ml/kg grain for anise, clove and basil respectively after 7 days. The protein contents of clove oil-treated weevils was significantly increased (0.067 ± 0.004 mg/ml), basil (0.81 ± 0.28 mg/ml) and anise (0.51 ± 0.018 mg/ml) treated insects as compared with control. Lipid contents were significantly increased in anise (3.92 ± 0.6 nM/mg) and basil (3.01 ± 0.77 nM/mg) treated weevils whereas they were significantly decreased in clove treated insects (0.22 ± 0.008 nM/mg) as compared to control. Significant increase in the amount of carbohydrates was observed in anise (120.495 ±7.1 nM/mg) and clove (113.77 ± 9.817 nM/mg) -treated weevils but not in basil (89.33 ± 1.897 nM/mg) -treated weevils. Basil oil increased the activity of GOT 18.04 ± 6.3 U/l. In contrast, anise and clove oils did not cause significant change in GOT activity (5.53 ± 1.47 and 5.24 ± 1.55 U/l), respectively. GPT activity increased in clove and basil oil-treated weevils 95.74 ± 28.6 and 26.48 ± 8.3 U/l, respectively. Anise and basil significantly inhibited the activity of acetylcholinesterase (AChE) (0.0022 ± 0.0016 and 0.0047 ± 0.0014 μmole/ mg/ min), respectively. Meanwhile, clove oil did not cause significant changes in AChE activity (0.005 ± 0.0017 μmole/ mg/ min).

KEY WORDS:

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INTRODUCTION:
The granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) is considered as one of the primary pests of stored grains in developing countries, causing considerable loss to stored cereal grains affecting their quantity and quality (Phillips et al., 2010). The control of stored-grain insect pests using chemical insecticides and fumigants (methyl bromide, phosphine) is an essential tool for preventing or minimizing insect damage to stored grain. However, the use of synthetic pesticides in stored grain protection resulted in potential hazards for mammals, none-target organisms and had led to development of pest resistance to pesticides (Prakash and Rao, 1986 & 1987). These problems had necessitated the development of new classes of safer insect control agents that would be biodegradable, cheap, readily available, and non-toxic to consumers and environmental friendly. Plant extracts and phytochemicals have long gained attention as alternatives to conventional insecticides with reduced health and environmental impacts as they are non-persistent in the environment and relatively
safe to natural enemies, non-target organisms and human beings.

Several plants that used locally for medicinal purposes had proven to be efficient in pest control (Yildirim et al., 2011; Olotuah, 2014). Pesticides based on aromatic plant essential oils or their constituents have demonstrated efficacy against a range of stored product pests. Botanical pesticides possess an array of properties including insecticidal activity, repellency to pests, antifeedant and insect growth regulation (Prakash and Rao, 1986 & 1987; Prakash et al., 1987 & 1990).

To assess the effects of a toxic material on insects, responses to sublethal level of this toxicant should be studied rather than performing acute toxicity tests. Sublethal concentrations of toxic compounds may cause direct sublethal effect including development, growth, reproduction, morphological and genetic changes (Takada et al., 2001; Willrich and Boethel, 2001) and indirectly through biochemical and physiological effects (Crott, 1990; Sak et al., 2006; Saleem et al., 2013; Ali et al., 2014). In insects, carbohydrate, protein and lipid are major components of the body and play an important role in the body construction and energy metabolism. The relative use of protein, lipid and carbohydrate as energy source can be influenced by external factors specially pesticides (Jabakumar and Jayaraman, 1988). Sublethal concentrations of insecticides affect insects by targeting different biochemical and regulatory molecules in different metabolic pathways. Pesticides interfere with carbohydrate metabolism in different species (Babu et al., 1988).

Clove, Eugenia aromatica (Myrtaceae), is an herb. People use its oil, dried flower buds, leaves, and stems to make medicine. Anise, Pimpinella anisum (Umbelliferae) oil obtained from extraction of the seeds found application in many traditional medicines. Basil Ocimum basilicum (Lamiaceae) leaves hold many notable plants from which chemical compounds are derived. They are known to have disease preventing and health promoting properties. Basil herb contains many polyphenolic flavonoids like orientin and vicenin. Basil leaves are composed of several health benefiting essential oils such as eugenol, citronellol, linalool, citral, limonene, and terpineol. These compounds are known to have anti-inflammatory and anti-bacterial properties.

The sublethal effect of plant essential oils on the biochemical parameters of the stored grain pests has not yet been adequately studied. Therefore, the aim of this study was conducted to investigate the physiological and biochemical responses of Sitophilus granarius adults to sublethal concentrations of anise, clove, and basil essential oils in order to evaluate their efficiency in preserving wheat grains against these insect pests.

**MATERIAL AND METHODS:**

**Weevil culture and its maintenance:**

The stock culture of the granary weevil, Sitophilus granarius was continuously reared free of any insecticidal contamination for several years at the Department of Stored Product Pests, Plant Protection Research institute, Sakha Agriculture Research Station. Approximately, 200-400 adults were collected from the stock culture and introduced into glass bottles (850 ml) containing 400 g of whole wheat grains (Giza 172) with a muslin cloth screen cover. Wheat grains were stored at -18ºC for 2 weeks before use to avoid any possible infestation. The moisture content of the grains was about 14%. The culture was maintained at 28 ± 2ºC with 60 ± 5% relative humidity. All experiments were conducted with 7 to 14 days - old adults.

**Essential plant oils:**

Three commercial essential plant oils purchased from the local Egyptian market were used in the present study. They were obtained from basil, Ocimum basilicum (Lamiaceae) leaves, clove, Eugenia aromatica (Myrtaceae) whole plant parts and anise, Pimpinella anisum (Umbelliferae) seeds.

**Estimation of LC90:**

To determine LC90, serial concentrations of essential oils (0.5, 1, 2, 3, and 4 ml/kg grains) were prepared. These concentrations were uniformly applied separately to twenty grams of wheat grains in glass jars (5 cm diameter × 7.9 cm height) covered with muslin cloth fixed by rubber bands and kept for 72 h at room temperature for homogenous oil distribution. Then, 10 weevils aged from 1 to 2 weeks were placed in each jar and covered. Three replicates were used for each concentration and all jars were kept at 26 ± 1ºC and 60 ± 5% RH. Another three replicates contained untreated grains were used as control. Percentage mortality was estimated 1, 3, 5, 7, and 14 days post-treatment and the values were corrected by Abbott's formula (Abbott, 1925). LC90 values were statistically computed using probit analysis.

**Exposure of weevils to sublethal doses of essential oils:**

The sublethal dose equivalent to LC90 was used to determine the effects of essential oils as at these doses because the mortality was low and the physiological and biochemical responses are significant enough to understand their mode of action. After determination of LC90, healthy weevils were exposed separately to sublethal concentration of essential oils along with their respective controls in triplicate for seven days. Alive insects from each treatment were randomly
selected then weighed and used for the estimation of total protein, lipids, carbohydrates as well as the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and acetylcholinesterase (AChE). The biochemical studies were carried out on the whole body homogenate.

Preparation of whole body homogenates for biochemical analysis:

Adult weevils, 7 days old after treatment with essential oils at concentrations of LC50, were freeze killed at -20°C. The insects from each treatment were weighed and the whole body was homogenized in 10 times volumes (w/v) of phosphate buffer (pH 7.2) and then centrifuged for 10 min in 4000 rpm. The supernatant was transferred to new tubes and preserved at -20°C until onset of experiments.

Assay of total protein contents:

The whole body total protein content was estimated by the method of Lowry et al. (1961). The blue color product was measured spectrophotometrically after 30 minutes at 750 nm against the blank. Bovine serum albumin was used as standard. Protein concentration was calculated and the protein concentrations were expressed as mg/ml wet tissue.

Assay of total carbohydrate:

The whole body total carbohydrate content was measured according to Kemp and Van Heijningen (1954). The absorbance was determined spectrophotometrically at 546 nm against blank. The concentration of carbohydrates was expressed as mg/ml wet tissue.

Assay of total Lipid peroxidation (LPO) assay:

Determination of total lipid peroxidation was achieved according to Nair and Turner (1984) method. The concentration of malondialdehyde level was measured spectrophotometrically at 532 nm, and the concentration of lipid was expressed as nM/mg wet tissue.

Acetylcholinesterase activity (AChE):

AChE activity was assayed according to Ellman et al. (1961) method using acetylthiocholine iodide and S-butylthiocholine, propionylcholine as substrates. In a 5 ml test tube, 2.9 ml of sodium phosphate buffer (0.1 mM, pH 8) and 20 ml aliquot of whole body extract were added and incubated at room temperature for 5 min. To this mixture, 20 µl of the substrate acetyl thiocholine followed by 20 acetylthiocholine iodide (75 mM) as substrate were added. After 10 min, the optical density of the developed yellow color product was recorded at 412 nm on a spectrophotometer against blank. The enzyme activity was expressed as µmol / mg protein / min.

Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activity:

GPT and GOT activity was measured according to the method of Gello et al. (1985). The activities of enzymes were assayed spectrophotometrically at 340 nm against blank. The enzyme activity was expressed as U.L-1.

Statistical analysis:

Data were subjected to one-way analysis of variance (ANOVA) (SPSS, 12) software to determine the main effects of essential oils. Means were separated using SNK method (Steel and Torrie, 1980) and the results were considered statistically significant when P < 0.05.

RESULTS:

Estimation of LC50:

The LC50 and LC99 values at confidence limit 95% 7 days post exposure of the granary weevil to essential oils are depicted in Table 1. The LC50 value of anise was significantly lower than those of clove and basil oils however at LC99 basil oil showed higher value than those of clove and anise.

Table 1. The LC50 and LC99 values 7 days post exposure of the granary weevil, Sitophilus granarius to anise, Pimpinella anisum, clove, Eugenia aromatica and basil, Ocimum basilicum essential oils. Numbers in column followed by the same letter are not significantly different (P > 0.05; Steel and Torrie (1980) test).

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>LC50 (ml/kg)</th>
<th>LC99 (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anise</td>
<td>0.391c</td>
<td>3.451bc</td>
</tr>
<tr>
<td>Basil</td>
<td>0.642a</td>
<td>8.908*</td>
</tr>
<tr>
<td>Clove</td>
<td>0.615b</td>
<td>2.151b</td>
</tr>
</tbody>
</table>

Total protein, carbohydrates and lipid contents:

Table 2 shows the effects of sublethal doses (LC50) of anise, clove, and basil essential oils on the granary weevil total levels of protein, carbohydrates and lipids. Significant reduction (P < 0.05) of total body protein was observed in clove oil treated weevils as compared to the control weevils. On the other hand, basil and anise oil treatments significantly (P < 0.05) increased the total body proteins as compared to control.

The level of total lipids significantly (P < 0.05) increased after anise and basil treatments and decreased in clove oil treated weevils as compared to control.

Carbohydrate contents of anise and clove essential oil-treated weevils were significantly (P < 0.05) higher than those of the control, whereas there was no significant (P > 0.05) difference between carbohydrate content of the weevils treated with basil and control.
Table 2. Effect of sublethal concentrations of anise, *Pimpinella anisum*, clove, *Eugenia aromatica* and basil, *Ocimum basilicum*, essential oils on the total protein, lipid and carbohydrate levels of the of the granary weevil, *Sitophilus granarius* 7 days after exposure

<table>
<thead>
<tr>
<th>Essential oils</th>
<th>Mean (± SD) total protein (mg/ml)</th>
<th>Mean (± SD) total lipid (mM. mg⁻¹)</th>
<th>Mean (± SD) total carbohydrate (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.38 ± 0.092</td>
<td>0.45 ± 0.051</td>
<td>89.22 ± 16.59</td>
</tr>
<tr>
<td>Anise</td>
<td>0.51 ± 0.018</td>
<td>3.92 ± 0.594</td>
<td>120.495 ± 7.1</td>
</tr>
<tr>
<td>Basil</td>
<td>0.81 ± 0.28</td>
<td>3.01 ± 0.77</td>
<td>89.33 ± 1.89</td>
</tr>
<tr>
<td>Clove</td>
<td>0.06 ± 0.004</td>
<td>0.22 ± 0.008</td>
<td>113.77 ± 9.817</td>
</tr>
</tbody>
</table>

Numbers in column followed by the same letter are not significantly different, P > 0.05.

**Enzyme quantifications:**

Sublethal concentration of essential oils showed significant alterations in the biological activity of *S. granarius* metabolic enzymes (Table 3). Sub lethal concentration of basil oil significantly (P < 0.05) increased the activity of GOT. Whereas, anise and clove sublethal concentrations did not cause significant (P > 0.05) change in GOT activity. Meanwhile, clove and basil oils significantly (P < 0.05) increased GPT activity as compared to control. The LC₅₀ concentrations of anise and basil significantly (P < 0.05) inhibited the activity of AChE compared with control (Table 4). However, sublethal concentration of clove did not cause significant changes in AChE activity.

Table 3. Effect of sublethal concentrations of anise, *Pimpinella anisum*, clove, *Eugenia aromatica* and basil, *Ocimum basilicum* essential oils on the activities of glutamate oxalotransaminase, and glutamate pyruvic transaminase of the of the granary weevil, *Sitophilus granarius* 7 days after exposure

<table>
<thead>
<tr>
<th>Essential oils</th>
<th>Mean (± SD) GOT activity (U/l)</th>
<th>Mean (± SD) GPT activity (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.98 ± 2.3¹</td>
<td>1.76 ± 0.0009³</td>
</tr>
<tr>
<td>Anise</td>
<td>5.53 ± 1.47⁵</td>
<td>5.24 ± 1.9⁹</td>
</tr>
<tr>
<td>Basil</td>
<td>18.04 ± 6.2⁸</td>
<td>26.48 ± 8.3³</td>
</tr>
<tr>
<td>Clove</td>
<td>5.24 ± 1.55⁵</td>
<td>95.74 ± 28.6³</td>
</tr>
</tbody>
</table>

Numbers in column followed by the same letter are not significantly different, P > 0.05.

Table 4. Effect of sublethal doses of anise, *Pimpinella anisum*, clove, *Eugenia aromatica* and basil, *Ocimum basilicum* essential oils on the activity of acetycholinesterase enzyme of the granary weevil, *Sitophilus granarius* 7 days after exposure

<table>
<thead>
<tr>
<th>Oils</th>
<th>Mean (± SD) specific activity of AChE (µmole. mg⁻¹ . min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(0.0058 ± 0.0023)¹</td>
</tr>
<tr>
<td>Anise</td>
<td>(0.0022 ± 0.0016)²</td>
</tr>
<tr>
<td>Basil</td>
<td>(0.0047 ± 0.0014)³</td>
</tr>
<tr>
<td>Clove</td>
<td>(0.005 ± 0.0017)⁴</td>
</tr>
</tbody>
</table>

Values that have a different superscripts (a, b, & c) differ in significance (P < 0.05). Means with same letters are not significantly different.

**DISCUSSION:**

The results obtained from this study showed that all essential oils of basil, clove, and anise were highly toxic to *S. granarius* as indicated by the low LC₅₀ values. However, essential oil of anise was the most toxic to one.
to highly significant decrease in protein content of the surface grasshopper, *Chortoicetes trachypterus* (Shakeet and Bakshi, 2010). Spinosad depleted total protein contents in *Tribolium castaneum* (Hussain et al., 2009) and *S. oryzae* (Hamza et al., 2014). Botanical extracts reduced total protein in the black cutworm, *Agrotis ipsilon* (Abou El-Ghar et al., 1996; El-Shiekh, 2002), in the cotton leaf worm, *Spodoptera littoralis* (Abd El-Wahab, 2000) and in *Helicoverpa armigera* (Padmaja and Rao, 2000). Similarly, a reduction in the protein content in the haemolymph of silkworm larvae was reported due to pyriproxyfen residue (Etebari et al., 2007).

The obtained data reveal that there was no significant difference in the total body carbohydrates in basil-treated weevils while in anise and clove treated insects there was a significant increase. Anise and clove treated weevils might be unable to assimilate the food thereby increasing the level of carbohydrate in their tissues. The stress induced by anise and clove oils might have enhanced glycogenolysis leading to the hyperglycemia (Sharma et al., 2011). Several investigations on the effect of different pesticides revealed that pesticides interfere with carbohydrate metabolism in different species. Some authors have reported elevated carbohydrate contents in some insects, while others have noticed either the opposite or no change. Moghamad et al. (2011) revealed that Chlorfluazuron and pyriproxyfen did not affect total carbohydrate contents in the white leaf borer, *O. terebinthina*. Parveen and Miyata (2000) found that Chlorfluazuron as insect growth regulator had no effect on total carbohydrate in *Spodoptera litura*, Chitagar et al. (2014) observed no significant changes in the carbohydrate contents in *Andrallus spinidens* following sublethal exposure to diazinon, fenitrothion, and chlorpyrifos. On the other hand, pyriproxyfen treatments of *Schistocerca gregaria* nymphs resulted in reduced carbohydrate contents in the fat body (Taneja et al., 2012). Treatment with oils extracted from the seeds of *Trigonella foenumgraecum, Rumex dentatus*, leaves of *Acacia nilotica*, volatile oil extracted from seeds of *Piper cubeba* and leaves and stems of *Salvia officinalis* significantly decreased the total carbohydrates level in *Plodia interpunctella* (Shoukry et al., 2003). Controversy, sublethal concentrations of hexaflumuron increased the content of total carbohydrate and trehalose in *S. litura* (Zhu et al., 2012).

Transaminase enzymes (GOT and GPT) are mitochondrial enzymes which transfer an amino group from amino acid to keto acid Ali et al. (2013). They are released in the haemolymph of insects only when the cells are damaged or destroyed. In the present study sublethal concentrations of basil and clove oils significantly increased GPT activity while basil oil increased the activity of GOT indicating of increased synthesis of both enzymes. This result might suggest that essential oil application enhanced transaminase enzyme activity due to toxic stress. Increased transaminase activity might have been required by weevils to metabolize amino acids to obtain energy under stress. In fact the varying effect of plant extracts on GOT and GPT activities might be due to the effect on the synthesis or functional levels of these enzymes directly or indirectly by altering the cytormorphology of the cells (Nath, 2000). This interpretation corroborates the observation that basil oil increased the total body proteins of weevils. This finding agreed with some other findings on the effect plant oils on GOT and GPT activates (Tabassum et al., 1994; Arshad et al., 1999; Hassan, 2002; Abdel-Latif and Al Moajel, 2004).

Acetylcholinesterase is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine in the nervous system (Ryan and Byrne, 1988; Lopez and Pascual-Villalobos 2010). It has been widely accepted that inhibition of AChE in cholinergic synapses of the nervous system is the primary mechanism of acute toxicity of insecticides. In the present study the LC50 concentrations of anise and basil significantly inhibited the activity of AChE while clove oil did not cause significant change. Anise and basil essential oil might act by interfering with the passage of impulses in the insect nervous system. The inability of AChE to hydrolyze acetylcholine, the buildup of concentration of the acetylcholine in the synapse and excessive neuro excitation are the results of prolonged binding of ACh to its postsynaptic receptor (Lopez and Pascual-Villalobos, 2010; Rajashekar et al., 2014). Previous works indicated that monoterpenoids in most plant essential oils cause insect mortality by inhibiting acetylcholinesterase enzyme (Gracza, 1985; Grundy and Still, 1985; Lopez and Pascual-Villalobos, 2010). Picollo et al. (2008) reported that the monoterpenoid, 1, 8-cineole was a potent AChE inhibitor. The action of anise and basil oils as fumigants on *S. granarius* could be neurotoxic. Evidently, *S. granarius* may be suitable candidate as toxicity indicator insect using AChE inhibition as parameter of toxicity.

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