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PHYSIOLOGICAL CHANGES AND CHEMICAL COMPONENTS OF HAEMOLYMPH OF THE MEDFLY *Ceratitis capitata* WIED. THIRD INSTAR LARVAE AFFECTED BY THREE DIFFERENT PREPARED BOTANICAL EXTRACTS

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ABSTRACT: The present study aimed to evaluate the physiological changes and chemical components of haemolymph affected by three botanical extracts, namely volatile garlic oil, volatile peppermint oil and Neemazal T/S formulation in the haemolymph of *C. capitata* 3rd instar larvae under constant laboratory conditions of 25 ± 1ºC, 75±5 RH % and 12/12 L/D at Plant Protection Department, Faculty of Agriculture, Zagazig University, Egypt. Artificial larval rearing medium was treated with two concentrations of 400 and 800 ppm for each botanical extract used as they are the most effective concentrations. Results showed that, in respect to carbohydrate, protein and lipid hydrolyzing enzymes, garlic oil recorded the highest enzyme activity valued 325.67, 297.33, 626.67 µg/ml, respectively; while Neemazal formulation recorded the lowest one of 112.67, 8.08, 44.33 µg/ml, respectively. In case of total free amino acids, Neemazal formulation recorded the highest level of 263.33 µg/ml, while garlic oil recorded the lowest one of 91.97 µg/ml. All botanical extracts reduced the total amount of mineral elements. However, Neemazal formulation was the most effective in reducing amount of sodium, potassium, nitrogen, phosphorus and calcium indicating 4.91, 29.00, 4.74, 1.82, 251.83 µg/ml, respectively. Results clearly revealed that all botanical extracts investigated reduced the proportion of enzymes in the haemolymph of the 3rd instar larvae than in control. The increasing of concentrations from 400 to 800 ppm increased the effects.

Key words: Medfly, physiology, haemolymph, organic and inorganic components.

INTRODUCTION

The Mediterranean fruit fly, *C. capitata*, is a pest of economic importance, which limits the development and expansion of agriculture in many locales. It occurs in areas with a great diversity of agriculture, ecological habitats and host plants. This species has a high biotic potential and a wide host range which includes more than 200 fruits, nuts and vegetables (**Harris, 1977; Hagen *et al*., 1981**).

Various control measures are available to combat the *C. capitata* population were first achieved with poison bait sprays (**Steiner, 1969**). The use of pesticides is often considered the most potent control measure for pests. However, chemical control alone has a number of limitations; yet most plant protection operations are still based on this unilateral approach. This is particularly true in developing countries, where knowledge and structure to develop and implement new pest control technologies, are generally lacking. Continuous or heavy use of some insecticides has created serious problems such as direct toxicity to parasites, predators, pollinators, fish and man (**Munakata, 1977**), pesticide resistance (**Georghiou and Taylor, 1977; Schmutterer, 1981; Waiss *et al.*, 1981**), susceptibility of crop plants to insect pests (**Pimentel, 1977**) and increased environmental and social costs

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(**Pimentel *et al.*, 1980**). Pesticide prices continuously increase over the years, and in developing countries this has led to a situation, where farmers can not longer afford to apply required amounts of chemicals on some crops. Even more important is the fact that current efforts to increase food production may be curtailed by these high prices.

Although synthetic pesticides will remain a primary measure for agricultural pest control during the predicted future, it is evident that society cannot tolerate the way of conventional chemicals are used (**Doutt and Smith, 1971**). This impasse can be avoided by the development of pest management systems based on the judicial application of insecticides and the improved use of new and alternative control methods (**Smith, 1974**). Implementation of less expensive and safer crop protection measures in corporation pesticides in a rational manner is necessary. The existence of naturally occurring insecticidal plant components has been known for centuries. They are less toxic and persistent than their synthetic counterparts and are in some instances a component of mammalian diets, are assumed to be environmentally more acceptable and less hazardous to humans. Interactions between phytophagous insects and plants over the ages have led to the evolution of numerous secondary plant chemicals which influence insect behavior, development, and physiology (**Thorsteinson, 1960; Fraenkel, 1969; Hedin, 1977**). These chemicals can be used to control specific pests in appropriately designed strategies. As naturally evolved ingredients of the biosphere, such plant derived products have a potential advantage over synthetic compounds in terms of ecological suitability. Their development as successful pest control agents can also be economically feasible, especially if the source materials are plants available in abundance (*eg*, common weeds, prolific herbs, shrubs and trees having a wide and rich distribution).

It is for the above reasons that the following study was conducted. The objectives of the study were to investigate the plant extract activity of three plant extracts (Neemazal formulation, garlic oil and peppermint oil) and evaluate the physiological changes of enzymes and amount of total amino acids and carbohydrate, protein, lipids and five minerals in the haemolymph of the 3rd larval instar of medfly.

MATERIALS AND METHODS

This study was carried out in Plant Protection Department, Faculty of Agriculture, Zagazig University, Egypt under laboratory conditions.

Rearing Method of the Tested Insect

The culture of *C. capitata* used in this research was supplied as pupae by Plant Protection Research Institute in Dokki, Giza, Egypt. Larvae were reared on wheat bran diet (330 g wheat bran, 82.5 g sugar, 82.5 g yeast, 4 g citric acid, 4 g sodium benzoate and 500 ml water) (**Leftwich *et al.*, 2017**). Diet is prepared with special mixers designed according to the maximum amount of daily preparation and components of diet. Ingredients are mixed dry at the beginning. Wheat bran, sugar and yeast were forming a homogenized mixture and then water was added. Larvae abandon the breeding container freely upon completing the third instar, jumping from the container into pupation substrate "sand" (**Vargas, 1989**). Pupae were maintained at 25 ± 1ºC and 75±5 RH (%) to complete maturation process (**Hernández *et al.*, 2010**).

Tested Prepared Botanical Extracts

Three commercial botanical extracts obtained from local and international companies as shown in Table 1. Garlic oil and peppermint oil were used in its pure form (100%) and Neemazal T/S formulation (75.5% neem oil, 3.5% azadirachtin). All botanical extracts were dissolved and diluted to two different concentrations (400 and 800 ppm) with 0.1% Tween 80 as an emulsifier.

Analytical Methods of Haemolymph Components

The required samples from 8-10 day-old of the 3rd instar larvae were treated with different concentrations 400 and 800^{ppm} by Neemazal formulation, peppermint oil and garlic oil.

Table 1. List of the tested plant extracts

Common name	Scientific name	Used part	Source
Neemazal T/S formulation	***Azadirachta indica* (A. Juss.)**	Formulation	T. STANE& COMPANY LIMITED, Tamil Nadu, India
Garlic oil	***Allium sativum* L.**	Leaves and fruits	El Captain Company CAP PHARM Al- Obour City, Cairo, Egypt
Peppermint oil	***Mentha piperita* L.**	Leaves	Elhawag Company Badr City, Egypt

All haemolymph components were determined in the Department of Pest Physiology, Plant Protection Research Institute, Dokki, Giza, Egypt. Amino acids were estimated in National Research Center, Dokki, Giza, Egypt.

Preparation of Insects for Analysis

The insects were prepared as described by **Amin, 1998**. They were homogenized in distilled water (50 mg/1 ml). Homogenates were centrifuged at 8000 rpm for 15 min at 2ºC in a refrigerated centrifuge. The deposits were discarded and the supernatant, which is referred as enzyme extract, can be stored at least one-week whithout appreciable loss of activity when stored at 50ºC.

Organic Components

Carbohydrate hydrolyzing enzymes

Determination of both trehalase and amylase activity

Digestive enzymes were determined according to the modifications of **Amin (1998)** to the method described by **Ishaaya and Swirski (1976)** using trehalose, sucrose, and soluble starch as substrates for trehalase, invertase and α-amylase, respectively. Amylase kit was obtained from Egyptian Company for Biotechnology (SAE), Al-Obour City, Industrial area, block 20008, piece 19A, Cairo, Egypt.

Determination of β-glucosidase activity

β-glucosidase activity was measured by assaying glucose liberated by enzymatic hydrolysis of salicin as described by **Lindorth, (1988).**

Protein hydrolyzing enzymes

Determination of chymotrypsin activity

Chymotrypsin was determined as described by **Broadway (1995)**. Enzyme activity expressed as μmol substrate hydrolyzed/min/g.b.wt.).

Determination of transaminases

Glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) were determined colorimetrically according to the method of **Reitman and Frankel (1957)**.

Lipids hydrolyzing enzymes

Determination of lipase activity

Lipase activity was determined by a slight modification of the procedure of **Tahoun and Abdel-Ghaffar (1986).** The method was based on the determination of the decrease in ester content of triolein as substrate.

Determination of non-specific esterases

Alpha-esterases (α-esterases) and beta-esterases (β-esterases) were determined according to **Van Asperen (1962)** using α-naphthyl acetate or β-naphthyl acetate as substrates, respectively.

Determination of total carbohydrates, total lipids, total proteins and total free amino acids

Determination of total carbohydrates

Total carbohydrates were estimated in acid extract of the sample by the phenol-sulphuric acid reaction (**Dubois *et al*., 1956)**.

Determination of total lipids

Total lipids were estimated by the method of **Knight *et al.* (1972)** using phosphovanillin reagent prepared by dissolving of 0.6 g pure vanillin in 10 ml ethanol and completed to 100

ml with distilled water. Then 400 ml of conc. phosphoric acid were added. Total lipids kit (Bio-diagnostic 29 Tahreer St., Dokki, Giza, Egypt) was purchased from High Lab Company.

Determination of total proteins

Total proteins was determined by the method of **Bradford (1976).**

Determination of total free amino acids

Total free amino acids was colorimetrically assayed by ninhydrin reagent according to the method described by **Lee and Takabashi (1966)**.

Inorganic Components

Determination of sodium and potassium

Ions measurements were made on a radiometer FLM3 falme photometer as described by **Amin and El-Halafawy (2002).**

Determination of nirogen

The nitrogen in protein is converted to ammonium sulphate by H_2SO_4 during digestion. This salt, on steam-distillation, liberates ammonia which is collected in boric acid solution and titerated against standard acid as described by **Sadasivam and Manickam (1991).**

Determination of phosphorus

The phosphate ion was detected using a commercial kit of Quimica clinica applicada SA (Spain) as described by **Amin and El-Halafawy (2002).**

Determination of calcium

Calcium ion was determined using bio-analytics kit described by **Sadasivam and Manickam (1991)**.

Statistical Analysis

Results were statistically analyzed using COSTAT programs, where analysis of variance, were achieved **(Fisher, 1954; Duncan, 1955)**. Results were recoded as mean ± standard error (SE). Simple correlation coefficient was calculated.

RESULTS AND DISCUSSION

Chemical Components of Haemolymph

Organic components

The qualitative and comparative quantitative presence of different enzymes activity in haemolymph of the third instar larvae of *C. capitata* reared diets mixed with the three different botanical extracts was as follows:

Carbohydrate hydrolysing enzymes in haemolymph

Trehalase enzymes

Results tabulated in Table 2 cleare that the trehalase enzyme activity differed between volatile plant oils and Neemzazl formulation as well as between concentrations used. The highest enzyme activity and the lowest one (325.67 µg/ml and 161.33 µg/ml) were recorded in case of garlic oil at 400 and 800 ppm for Neemazal formulation, respectively compared with 328 µg/ml in control. Generally, the tested botanical materials can be arranged descendingly according to their effects as follows: garlic oil (323 µg/ml), peppermint oil (284.83 µg/ml) and Neemazal formulation (218.16 µg/ml).

The obtained results are in harmony with those reported by **Sreekumar and Prabhu (1988**) who studied changes in digestive enzyme secretion during metamorphosis in *Oryctes rhinoceros* and trehalase enzyme present in larval, pupal and adult midgut.

Amylase enzymes

All plant extracts reduced amylase values than in control. Neemazal formulation comes in the first in reducing amylase enzyme (112.67 µg/ml) at 800 ppm concentration, while peppermint oil occupied the last category in reducing amylase amount, this opposed to control recording (247.00 µg/ml).

The obtained results are generally in harmoney with those mentioned by **Priya *et al.* (2010), Vatanparast and Hosseininaveh (2010), Omotoso and Adedire (2011), Riseh and Ghadamyari (2012) and Darvishzadeh and Bandani (2013)** who stated that amylase, being one of the most important carbohydrates in midgut and haemolymph of the larvae and adult stages. However, **Sridhar and Chetty (1989)** studied the effect of *Az. indica* and *Pongamia glabra* leaf extracts on digestive enzymes such as invertase, amylase and protease in *Euproctis fraternal* and found a reduction in activity of the three enzymes with admistration

Table 2. Effect of three prepared botanical extracts on carbohydrate hydrolyzing enzymes (µg/ml) activity in the haemolymph of *C. capitata* 3rd instar larvae under laboratory conditions

Enzyme		Trehalase			Amylase			β-glucosidase		
Botanical extract		400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean
Control		328.00±12.00			247.00±9.17			252.00± 7.21		
Neemazal formulation		275.00±10.15	161.33±13.80	218.16	223.00±15.39	112.67±5.86	167.83	229.33±6.51	196.67±4.93	213.00
Garlic oil		325.67±10.97	320.33±8.33	323.00	225.67±4.04	205.33±7.57	215.50	214.33±7.37	188.33±10.50	201.33
Peppermint oil		296.00±8.72	273.67±9.29	284.83	230.33±13.80	216.33±6.43	223.33	215.00±6.24	206.33±4.73	210.66
$LSD \leq_{0.05}$	Extract	14.37			11.92			6.75		
	Conc.	11.74			9.73			5.51		
	Extract × Conc.	14.56			12.07			6.83		

of increasing concentration of extracts. **Tatun *et al.* (2014)** tested the inhibitory effects of plant extracts (*Ricinus communis* and papaya) on α-amylase activity in the red flour beetle and decided that α- amylase activity was inhibited and glucose content was reduced in the larvae and adults of this insect. **Bezzar-Bendjazia *et al.* (2017) and Kilani-Morakchi *et al.* (2017)** cleared that azadirachtin reduced significantly the activity of larval α-amylase, chitinase and protease and increased the activity of lipase, in *Drosophila melanogaster* larvae.

β-glucosidase enzyme

As for, β-glucosidase enzyme, all extracts and formulation treatments reduced the activity and the amount of β-glucosidase than control. The amount of enzyme decreased by increasing treatment concentrations. Garlic oil was the most effective one and occupied the first category from the side of reducing β-glucosidase representing by (188.33 µg/ml) at 800 ppm concentration followed by Neemazal formulation (196.67 µg/ml) and finally peppermint oil (206.33 µg/ml) in the last category. Statistical analysis of data showed significant variations in β-glucosidase enzyme.

The obtained results are in harmony with those reported by **Yapi *et al.* (2007)** who purified a beta-glucosidase from the digestive juice of the palm weevil *Rhynchophorus palmarum* L. larvae; **Riseh *et al.* (2012)** indicated the presence of alpha and beta-glucosidaes and alpha and beta galactosidases in digestive system and haemolymph of *R. ferrugineus* and the mean specific activity of β-glucosidase was 0.067 Mmol/min/mg protein. **Taha *et al.* (2015)** mentioned the effect of certain extracts on enzymes activity of tomato leafminer. The enzyme activity reduced or increased significantly. Activity of alkaine phosphatase, α and β-esterases were higher in damasiea extract on *T. absoluta* in comparison to control.

Protein hydrolysing enzymes

Results given in Table 3 represent the changes in activities of chymotrypsin protease, AST (GOT) and ALT (GPT) enzymes in haemolymph of the 3rd instar larvae fed on three botanical extracts in diet.

Chymotrypsin protease

The effects of botanical extracts on chymotrypsin protease enzyme are shown in Table 3. After their treatment, Neemazal formulation gave the most effective treatment through the reduction in enzyme. This appear in the two concentrations of Neemazal formulation recording 18.50 and 16.17 µg/ml at 400 and 800 ppm concentrations and 17.33 µg/ml as the mean of the two concentrations, following by mean value 23.08 and 40.33 µg/ml in case of garlic oil and peppermint oil, respectively. Statistical analysis of data revealed significant variations in protein hydrolyzing enzymes.

The present results are generally in agreement with those reported by some authors (**Sridhar and Chetty, 1989; Ferreira *et al.*, 1990; Bedi and Gandhi, 1993)** who mentioned

Table 3. Effect of three prepared botanical extracts on protein hydrolyzing and transaminase enzymes (µg/ml) activity in the haemolymph of *C. capitata* 3rd instar larvae under laboratory conditions

Enzyme	Chymotrypsin			AST (GOT)			ALT (GPT)		
Botanical extract	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean
Control	46.77±2.89			8.40±0.43			383.00±10.44		
Neemazal formulation	18.50±1.31	16.17±1.53	17.33	8.07±0.38	5.37±0.25	6.72	286.33±11.85	192.33±23.16	239.33
Garlic oil	31.53±1.38	14.63±1.19	23.08	8.18±0.88	6.96±0.25	7.57	297.33±9.07	217.33±34.44	257.33
Peppermint oil	43.43±1.01	37.23±2.35	40.33	8.43±0.23	10.51±0.98	9.47	257.67±8.74	192.00±14.73	224.83
LSD ≤ 0.05 **Extracts**	1.36			0.81			25.37		
Conc.	1.11			0.66			20.71		
Extract×Conc.	1.37			0.82			25.69		

that there is an effect of *Az. indica* and *Pongamia glabra* leaf extract on digestive enzymes such as invertase, amylase and protease in *Euproctis fraterna*. A reduction in activity of these enzymes with admistration of increasing concentration of extracts. **Kaur *et al.* (2014)** determined the effect of plant extracts (*Acacia auriculiformis*) on activities of enzymes in *Bactrocera cucurbitae* (Coquillett) and showed altered activities of enzymes. **Bezzar-Bendjazia *et al.* (2017)** decided that azadirachtin reduced significantly the activity of larval α- amylase, chitinase and protease and increased the activity of lipase, in *Drosophila melanogaster* larvae.

AST (GOT) enzyme activity

Results given in Table 3 represent the changes in activities of AST (GOT) enzymes in haemolymph of the 3rd instar larvae. The activities of enzymes AST (GOT) were increased in the haemolymph of the 3rd instars larvae fed on peppermint oil (9.47 µg/ml) than control (8.40 µg/ml). The 400 ppm concentration gave highly significant increasing activity of GOT in peppermint oil where it was achieved (8.43 µg/ml). Generally, activity of GOT enzyme was less in both garlic oil and Neemazal formulation than in control especially in 800 ppm concentration, while peppermint oil recorded highly significant increasing activity to achieve (10.51 µg/ml).

ALT (GPT) enzyme activity

The same activity behavior of GPT was similar to that obtained in GOT enzyme, where the garlic oil (257.33 µg/ml) and Neemazal formulation (239.33 µg/ml) were less active than control (383.00 µg/ml) and the least activity was detected in peppermint oil (224.83 µg/ml).

Generally, the 800 ppm concentration decreased activity of GTP than the 400 ppm concentration, where it was reached the minimum activity in peppermint oil (224.83 µg/ml) and the highest one in garlic oil (257.33 µg/ml). **Sridhar and Chetty (1989)** mentioned the effect of *Az. indica* and *Pongamia glabra* leaf extracts on digestive enzymes such as invertase, amylase and protease in *Euproctis fraterna*. A reduction in activity of these enzymes with the admistration of increasing concentration of extracts. **Ferreira *et al.* (1990), Bedi and Gandhi (1993), Adedire and Balogun (1995), Alarcon *et al.* (2004), Elpidina and Goptar (2007), Wang *et al.* (2007) and ShengJun and Hui (2008)** reported that trypsin, protease, AST and ALT enzymes are dominant in larval and adult stages in different pest species. **Tatun *et al.* (2014)** decided that inhibitory effects of plant extracts (*Ricinus communis* and papaya) on α-amylase activity in the red flour beetle. α-amylase activity was inhibited and glucose content was reduced in the larvae and adults of this insect.

Lipid hydrolysing enzymes

Results in Table 4 show biological changes of lipase, α- esterase and β-esterase levels in the haemolymph of the 3rd instar larvae of *C. capitata* fed on three botanical extracts diets. α-esterase enzyme level activities increased, while

Table 4. Effect of three prepared botanical extracts on lipid hydrolyzing enzymes (µg/ml) activity in the haemolymph of *C. capitata* 3rd instar larvae under laboratory conditions

Enzyme / Botanical extract		Lipase			α -estrase			β-estrase		
		400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean
Control		110.33±10.12			421.67±17.04			113.33± 5.69		
Neemazal formulation		106.33±5.86	44.33±4.04	75.33	491.33±16.77	604.00±11.53	547.66	79.67±4.73	61.00±7.94	70.33
Garlic oil		105.67±7.02	95.33±4.51	100.50	451.00±7.55	626.67±12.58	538.83	126.33±5.51	157.67±6.66	142.00
Peppermint oil		88.67±3.21	86.33±4.73	87.50	434.67±6.66	585.67±12.90	510.17	110.67±3.21	79.00±3.61	94.83
$LSD \leq_{0.05}$	**Extract**	7.07			14.68			7.17		
	Conc.	5.77			11.98			5.85		
	Exttract×Conc.	7.16			14.86			7.26		

both lipase and β-estarase decreased in the 3rd instar larvae fed on the three plant extracts used with the exception of garlic oil.

Lipase enzyme

All botanical extracts reduced the activity and the amount of lipase than control. The amount of enzyme decreaed by increasing extract concentrations. Neemazal formulation was the most effective one from side of reducing lipase representing by 75.33 µg/ml, followed by peppermint oil (87.50 µg/ml) and finally garlic oil (100.50 µg/ml) in comparison with control (110.33 µg/ml). Generally, statistical analysis of data showed significant variations in different lipid hydrolyzing enzymes which affected significantly by extracts and proved to be less than control was detected.

Sridhar and Chetty (1989) mentioned the effect of *Az. indica* and *Pongamia glabra* leaf extracts on digestive enzymes such as invertase, amylase and protease in *Euproctis fraterna*. A reduction in activity of these enzymes with admistration of increasing concentration of extracts.

Alpha-estrases enzymes

The activity of alpha-esterases increased by increasing botanical extratcs concentrations than control (421.67 µg/ml), where it was reached in Neemazal formulation to the highest value (547.66 µg/ml). The 800 ppm concentration had the highest activity (626.67 µg/ml) in garlic oil, while the least activity occurred in peppermint oil 400 ppm concentration with (434.67 µg/ml).

Generally, the extracts effective activity on α-estrase especially in case of garlic oil and the least one was detected with peppermint oil. The 800 ppm concentration was more effective on α-estrases.

Beta-estrases enzymes

The activity of β-estrases enzymes had the similar behavior of α-estrases enzymes, where the activity is more in garlic extract (142.00 µg/ml) in comparison with control (113.33 µg/ml) with significant impact in peppermint oil and Neemazal formulation. The 800 ppm concentration has more effect on activity in garlic oil where it was reached to 157.67 µg/ml and the least one was in the same concentration (79.00 µg/ml) in peppermint oil.

Total proteins, total carbohydrates, total lipids in haemolymph

Total proteins

The concentration of total proteins in haemolymph of *C. capitata* 3rd instar larvae were studied. As clearly shown from the results compiled in Table 5, this organic component greatly affected by larval feeding diets. The content of total proteins differs according to diet treatment of larvae, which increased in control than all extract treatments, exhibiting 42.87 µg/ml as compared with other extracts. Peppermint oil showed the highest activity (39.53 µg/ml) while the lowest one was shown in Neemazal formulation (28.85 µg/ml). The 800 ppm concentration, peppermint oil recorded the highest activity enzyme (38.77 µg/ml) while the lowest one was recorded in Neemazal formulation (24.10 µg/ml). On the other hand,

Table 5. Effect of three prepared botanical extracts on the total proteins, total carbohydrates and total lipids (µg/ml) activity in the haemolymph of *C. capitata* 3rd instar larvae under laboratory conditions

Organic component		Total proteins			Total carbohydrate			Total lipids		
Botanical extract		400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean
Control		42.87±1.88			46.53±3.27			12.99± 0.13		
Neemazal formulation		33.60±0.87	24.10±1.65	28.85	34.53±1.38	26.13±1.20	30.33	11.09±0.11	10.45±0.18	10.77
Garlic oil		40.57±0.80	32.57±1.07	36.57	44.57±2.05	42.83±1.53	43.70	13.45±0.87	14.51±0.56	13.98
Peppermint oil		40.30±1.74	38.77±1.63	39.53	45.60±1.15	40.67±2.21	43.13	11.17±1.11	10.10±0.66	10.63
$LSD \leq_{0.05}$	Extract	1.79			2.30			0.94		
	Conc.	1.46			1.87			0.77		
	Extract*Conc.	1.82			2.32			0.96		

the diets of *C. capitata* with the three botanical materials feeding media affected on the total proteins content compared with the control.

These results are in harmony with trends given by some researchers (**Karnavar and Nair, 1969; Pant and Gupta, 1979; Rambabu and Rao, 1994; Sancho *et al*., 1998)** who all reported that total protein declined during larval activation and mean of total protein level was 22.53 mg/g fresh weight in eri silk worm. **Sharma *et al*. (2011)** investigated the impact of the most potent petroleum ether extract of *Artemisia annua* and *Az. indica* on total protein level of *Anopheles stephensi* and *Culex quinquefasciatus* larvae. Total protein levels were reduced to 63.13% and 92.62% in anopheline and to 32.39% and 42.12% in culicine larvae after treatment with *A. annua* and *Az. indica* extracts, successively. **Al Qahtani *et al*. (2012)** cleared that some dried plants named ginger (*Zingiber officinale*), shammar (*Foeniculum vulgare*) and hail (*Elettaria cardamomum*) against *Oryzaephilus surinamensis*, and decided that the ginger and shammar increased the insect proteins subfractions than normal; while hail reduced separated bands, specially proteins of moderate molecular weight. **Abdellaoui *et al*. (2013)** investigated the effect of gibberellic acid on biochemicals of *Locusta migratoria* and mentioned that GA3 induced significant quantitative variation of haemolymph metabolites, and significant decrease in total concentration of proteins. **Dos Santos Silva *et al*. (2016)** mentioned that citronella oil (*Cymbopogon winterianus* Jowittes Bor) was effective repellent and insecticide on *Spodoptera frugiperda* and a reduction in proteins was detected.

Total carbohydrates

Tabulated results in Table 5 show that the total carbohydrates decreased gradually from 46.53 µg/ml in control to 43.70 µg/ml in garlic oil and the least value (30.33 µg/ml) was recorded in Neemazal formulation. Generally, the 800 ppm concentration gave decreasing in activity of total carbohydrate without significant difference between garlic and peppermint oils. Statistical analysis gave evidence of significant differences in total carbohydrates. Also, peppermint oil proved to be the highest affective and Neemazal formulation was the lowest one.

Sharma *et al*. (2011) investigated the impact of the most potent petroleum ether extract of *Artemisia annua* and *Az. indica* on total carbohaydrates level of *Anopheles stephensi* and *Culex quinquefasciatus* larvae. Glucose levels were increased to 27.87% and 46.8%, respectively in anopheline larval tissues after treatment with petroleum ether extract of *A. annua* and methanolic extract of *A. indica*. In culicine larvae, glucose levels were reduced to 58.96% and 24.65%, consecutively. **Abdellaoui *et al*. (2013)** cleared the effect of gibberellic acid on biochemicals of *Locusta migratoria* and mentioned that GA3 induced significant quantitative variation in haemolymph metabolites, and significant decrease in total

concentration of carbohydrates. **Dos Santos Silva *et al.* (2016)** found that citronella oil (*Cymbopogon winterianus* Jowittes Bor) was an effective repellent and insecticide on *Spodoptera frugiperda* and a reduction in neutral carbohydrates was detected.

Total lipids

Total lipids were not affected by the botanical extracts used, where it was reached in larval haemolymph to 12.99 µg/ml in control. However, the 3rd instar larvae of *C. capitata* feeding on three botanical extracts diets of garlic oil, peppermint oil and Neemazal formulation recording nearly the same trend of 13.98,10.63, 10.77 µg/ml, respectively. The 800 ppm concentration was high affective (14.51 µg/ml) in garlic oil, peppermint oil (10.10 µg/ml) and Neemazal formulation (10.45 µg/ml), while 400 ppm concentration was the less affective in total lipids as shown in Table 5.

Wilps *et al.* (1992) mentioned that compounds of the neem seed inhibit the lipid-mobilizing system of *Shistocerca gregaria*. **Sharma *et al.* (2011)** investigated the impact of the most potent petroleum ether extracts of *Artemisia annua* and *Az. indica* on total lipids level of *Anopheles stephensi* and *Culex quinquefasciatus* larvae. After treatment with *A. annua* extract, lipid contents in anophline and culicine larvae decreased by 28.57% and 25%, respectively and increased by 14.29% and 50.00% in *Anopheles* and *Culex* larvae, successively after treatment with methanolic extract of *Az. indica.* **Abdellaoui *et al.* (2013)** studied the effect of gibberellic acid on biochemical contents of *Locusta migratoria* and mentioned that GA3 induced significant quantitative variation in haemolymph metabolites, significant increase in the total haemolymph lipids.

Total free amino acids

As shown in Table 6 the concentration of total sixteen free amino acids (glutamic, phenyl alanine, lysine, arginine, alanine, aspartic, leucine, valine, histidine, tyrosine, serine, threonine, glycine, proline, isoleucine and methionine) markedly decreased effect in larvae haemolymph in different extracts to 118.66, 123.33 and 249.33 µg/ml for peppermint oil, garlic oil and Neemazal formulation, respectively, while it was 263.33 µg/ml in control. The relatively low mean of total free amino acids was found in garlic oil as it was reached 123.33 µg/ml amino acids. The concentration of 800 ppm decreased the total free amino acids in different tested extracts as compared to 400 ppm concentration, while the least amounts in peppermint oil and garlic oil were detected with the former concentration, where they were reached 97.33 and 91.67 µg/ml, respectively. Generally, the amount of free amino acids was more in Neemazal formulation in both 400 and 800 ppm concentrations than in garlic and peppermint oils.

The obtained results are in accordance with those reported by **Kleiner and Peacock (1971)** who detected nineteen amino acids in the haemolymph of last instar larvae of *Scolytus*

Table 6. Effect of three prepared botanical extracts on the total amino acids (µg/ml) activity in the haemolymph of *C. capitata* 3rd instar larvae under laboratory conditions

Organic component		Total free amino acids		
Botanical extract		400 ppm	800 ppm	Mean
Control		263.33±9.87		
Neemazal formulation		258.67±24.19	240.00±10.00	249.33
Garlic oil		155.00±6.561	91.67±1.53	123.33
Peppermint oil		140.00±26.08	97.33±3.21	118.66
LSD ≤ 0.05	Extract	18.55		
	Conc.	15.15		
	Extract × Conc.	18.79		

multistriatus by thin- layer chromatography. In this concern, **Bradfisch and Punzo (1977)** analyzed the free amino acids composition in the hemolymph of *Tenebrio molitor* and mentioned that asparagine, phenylalanine, leucine and isoleucine were found in very small concentrations. Lysine, methionine, threonine and tyrosine were found in higher concentrations. Glycine, valine, alanine, histidine and arginine were the principle free amino acids of the hemolymph in this species. **Sharma *et al.* (2011)** investigated the impact of the most potent petroleum ether extract of *Artemisia annua* and *Az. indica* on total protein level of *Anopheles stephensi* and *Culex quinquefasciatus* larvae. Total protein levels were reduced to 63.13% and 92.62% in anopheline and to 32.39% and 42.12% in culicine larvae after treatment with *A. annua* and *Az. indica* extracts. **Al Qahtani *et al.* (2012)** used some dried plants named ginger (*Zingiber officinale*), shammar (*Foeniculum vulgare*) and hail (*Elettaria cardamomum*) against *Oryzaephilus surinamensis*, and indicated that the ginger and shammar increased the insect proteins subfractions than normal; while hail reduced separated bands, specially proteins of moderate molecular weight. On the other hand, **Yi *et al.* (2013)** estimated sixteen amino acid level in the haemolymph of three coleopteran species in comparison with soybean proteins. **Abdellaoui *et al.* (2013)** investigated the effect of gibberellic acid on biochemical content of *Locusta migratoria* and mentioned that GA3 induced significant quantitative variation in haemolymph metabolites, and significant decrease in total concentration of proteins haemolymph. From another point of view, **Qu *et al.* (2014)** mentioned that amino acids play important roles in insect development. In addition to their role in protein synthesis, they have additional functions related to the synthesis of phospholipids, detoxification, energy production, and neural transmission. **Dos Santos Silva *et al.* (2016)** mentioned that citronella oil (*Cymbopogon winterianus* Jowittes Bor) was an effective repellent and insecticide on *Spodoptera frugiperda* and a reduction in proteins was recorded. On the other hand, glycogen level was higher in larvae treated with citronella oil. Citronella oil demonstrated that oil has ability to interfere with the insect bio-chemistry and physiology.

Inorganic components

Sodium

The amount of total sodium was not affected by botanical extracts, while it reached to 7.67 µg/ml in control as shown in Table 7. However, larvae of *C. capitata* fed on three botanical extracts diets, sodium content decreased in all concentrations, where it was 7.35 µg/ml in peppermint oil at 400 ppm concentration. The lowest content was recorded in Neemazal formulation and garlic oil without any significant difference. The 800 ppm concentration had the lower effect than 400 ppm. Generally, sodium was high in peppermint oil, while the least ones were recoded in garlic oil and Neemazal formulation. The 800 ppm concentration was more effective on sodium than 400 ppm concentration.

Potassium

Potassium recorded in haemolymph of the 3rd instar larvae achieved the highest ratio in peppermint oil (60.83 µg/ml) comparing with control (67.33 µg/ml), while the lowest one was recorded in Neemazal formulation (29.00 µg/ml). The potassium was more found in haemolymph in 400 ppm concentration than 800 ppm, where it was reached the highest (66.33 µg/ml) in peppermint oil and the lowest in Neemazal formulation (24.67 µg/ml), respectively. Generally, potassium in haemolymph of the 3rd instar larvae was more affective with extract treatments especially in peppermint oil than Neemazal formulation.

Nitrogen

Nitrogen content in haemolymph of the 3rd instar larvae was 6.75 µg/ml in control, while it was almost simliar in peppermint and garlic oils showing 5.76 and 5.74 µg/ml, respectively and decreased in Neemazal formulation to 4.74 µg/ml. The highest effect was observed in 400 ppm concentration more than 800 ppm concentration especially in peppermint oil (6.27 µg/ml) and the lowest one in 800 ppm concentrations in Neemazal formulation (4.24 µg/ml), alternatively.

Phosphorus

Phosphorus ratio in haemolymph was the highest value in larvae fed on diet treated with peppermint oil (2.31µg/ml) comparing with control (2.47 µg/ml) and the lowest one was

Table 7. Effect of three prepared botanical extracts on the total sodium, total potassium, total nitrogen, total phosphorus and total calcium (µg/ml) activity in the haemolymph of *C. capitata* 3rd instar larvae under constant laboratory conditions

Inorganic component / Botanical extract	Sodium			Potassium			Nitrogen			Phosphorus			Calcium		
	400 ppm	800ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean	400 ppm	800 ppm	Mean
Control	7.67±0.42			67.33±3.06			6.75±0.23			2.47±0.15			319.00±20.95		
Neemazal formulation	6.20±0.26	3.63±0.25	4.91	33.33±2.08	24.67±3.06	29.00	5.24±0.22	4.24±0.17	4.74	1.99±0.19	1.66±0.15	1.82	312.00±11.14	191.67±15.28	251.83
Garlic oil	6.57±0.31	3.30±0.20	4.93	45.67±3.06	25.67±3.79	35.67	6.01±0.20	5.47±0.09	5.74	2.37±0.08	2.17±0.21	2.27	413.67±31.79	354.00±10.58	383.83
Peppermint oil	7.57±0.15	7.13±0.32	7.35	66.33±2.52	55.33±4.51	60.83	6.27±0.14	5.25±0.26	5.76	2.51±0.11	2.12±0.09	2.31	433.33±14.74	394.00±12.17	413.66
$LSD \leq_{0.05}$ Extract	0.34			4.47			0.26			0.20			24.71		
Conc.	0.27			3.65			0.21			0.16			20.17		
Extract×Conc.	0.34			4.53			0.26			0.20			25.02		

achieved in Neemazal formulation. The 400 ppm concentration was the best affective than 800 ppm concentration where it was reached to 2.37 and 2.17 µg/ml in case of garlic oil, respectively. The lowest content was achieved by Neemazal formulation showing 1.66 µg/ml in 800 ppm concentration. Generally, the phosphorus inorganic element was decreased by all tested botanical extracts and the 400 ppm concentration was the most effective than 800 ppm concentration.

Calcium

The calcium exceeded in haemolymph by using the tested botanical extracts, where it was the highest in peppermint oil (413.66 µg/ml), whereas in control it was 319.00 µg/ml and the lowest one was achieved in each of Neemazal formulation (251.83 µg/ml) and garlic oil (383.83 µg/ml). The 400 ppm concentration was the highest affective in peppermint oil (433. 33 µg/ml) and the lowest in Neemazal formulation (312.00 µg/ml), while the 800 ppm concentration was the lowest especially in Neemazal (191.67 µg/ml). Generally, calcium concentration increased by using peppermint oil and decreased by using neem formulation comparing with control.

Results recorded are generally in agreement with some researchers (**Studier and Sevick, 1992; Naidu, 2006; Amin, 2008)** who analyzed haemolymph from the red palm weevil 8th instar for cations (K^+, Na^+, Ca^{++} and Mg^{++}), anions (Cl^- and P_O^{4-}). they added that, the haemolymph plasma characterized by the presence of high level of Ca^{++} as the most abundant cation, while P was the highest among the anions studied. **Vatanparast and Hosseininaveh (2010), Parast *et al.* (2012), Darvishzadeh and Bandani (2013) and Tatli *et al.* (2013)** proved the importance of minerals content and relationship with activity of several enzymes in *R. ferrugineus*, leaf beetle *Xanthogalerucella luteola* and alfalfa weevil *Hypera postica*, such as NaCl, KCl, $MgCl_2$, $CaCl_2$ increased the activity of enzymes glucosidases and alpha-amylase. Ions K^+, Mg^{2+}, and Na^+ did not significantly affect the enzyme activity. On the contrary Ca^+ and Mg^{2+} significantly decreased α-glucosidase activity while, α-amylase activity was significantly decreased in the presence of Ca^{2+}, Mg^{2+} and sodium dodecyl sulfate.

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التغيرات الفسيولوجية والمكونات الكيميائية لهيموليمف يرقات العمر الثالث لذبابة فاكهة البحر الأبيض المتوسط *Ceratitis capitate* Wied. المتأثره بثلاثة مستخلصات نباتية مختلفة محضرة

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هدفت الدراسة الحالية إلى تقييم التغييرات الفسيولوجية والمكونات الكيميائية لليهيموليمف المتأثر بثلاثة مستخلصات نباتية المسماة زيت الثوم المتطاير، زيت النعناع المتطاير ومستحضر النيمازال في هيموليمف ذبابة الفاكهة *Ceratitis capitata* العمر اليرقي الثالث تحت ظروف المعمل الثابتة على درجة حرارة ٢٥±١°م ورطوبة نسبيه ٧٥±٥% و١٢ ساعة إضاءه/١٢ ساعة ظلام في قسم وقاية النبات، كلية الزراعة، جامعة الزقازيق، مصر. تم معاملة بيئة تربية اليرقات الصناعية بتركيزين هما ٤٠٠ و٨٠٠ جزء في المليون لكل مستخلص نباتي مُستخدم حيث أنهما أكثر التركيزات فعالية. أوضحت النتائج فيما يتعلق بالإنزيمات المحللة للكربوهيدرات والبروتينات والدهون، سجل زيت الثوم اعلى نشاط انزيمي بلغ ٣٢٥,٦٧، ٢٩٧,٣٣، ٦٢٦,٦٧ ميكروجرام/مل على التوالي، بينما سجل مستحضر النيمازال أدني مستوى ١١٢,٦٧، ٨,٠٨، ٤٤,٣٣ ميكروجرام/مل على التوالي، في حالة الاحماض الأمينية الحرة الكلية، سجل مستحضر النيمازال أعلى مستوى ٢٦٣,٣٣ ميكروجرام/مل بينما سجل زيت الثوم أدنى مستوى ٩١,٩٧ ميكروجرام/مل، جميع المستخلصات النباتية قللت الكمية الكلية للعناصر المعدنية، بينما كان مستحضر النيمازال الأكثر فعالية في خفض كمية الصوديوم، النيتروجين، البوتاسيوم، الفوسفور والكالسيوم إلى ٤,٩١، ٢٩,٠٠، ٤,٧٤، ١,٨٢، ٢٥١,٨٣ ميكروجرام/مل على التوالي، اظهرت النتائج بوضوح أن جميع المستخلصات النباتية خفضت من نسبة الانزيمات في هيموليمف يرقات العمر اليرقي الثالث عنها في الكنترو، وجد أن زيادة التركيزات من ٤٠٠ الى ٨٠٠ جزء في المليون زادت التأثيرات.

المحكمـــــون:

١- أ.د. عــزت فــرج الخياط أستاذ الحشرات الاقتصادية المتفرغ - كلية الزراعة بمشتهر - جامعة بنها.

٢- أ.د. رفعت مصطفى شريف أستاذ المبيدات المتفرغ - كلية الزراعة - جامعة الزقازيق.