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The secondary effect of Metronidazole and Thymol Crystals application in- hive on some biological, histological, and biochemical activities of honeybee workers (*Apis mellifera* L.)

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ABSTRACT
Several factors have been linked to honeybee colonies' decline, but many inhive therapies have been frequently used and little is known about their effects on honeybees. This study is aimed at evaluating some biological, histological, and biochemical changes in honeybees (*Apis mellifera* L.) workers exposed during development to the antibiotic Flagyl (metronidazole), commonly used as nosemosis therapy, compared to the natural substance thymol. This work was conducted in early spring, and the medicated sugar syrup was administered by feeding it to the honeybee colonies. Results revealed a significant reduction in the mean worker brood area and honey yield in colonies that received flagyl compared to control and thymol colonies ($P<0.05$). The weight of newly emerged workers in control colonies tended to be heavier than those in medicated colonies, though the differences were statistically insignificant. The histological analysis of the midgut confirmed severe changes in the cell structure of bees that received flagyl during development. It induced clear alterations in the epithelial cells, peritrophic membrane, and muscular layer. A significant reduction in protein content, as well as a decline in basic digestive enzymatic activity, were accompanied by changes in the midgut tissue as a result of flagyl application. Overall, these results are valuable in highlighting the validity of midgut histology and enzyme activities as indicators for evaluating the toxicity of used inhive therapy.
Keywords: *Apis mellifera*, Metronidazole, brood area, honey yield, midgut.

INTRODUCTION
Honeybees are economically valuable insects worldwide due to their important role in crop pollination and, in turn, responsibility for much of the world's agricultural production and the conservation of biodiversity (Gallai *et al*., 2009; Cirkovic *et al.*, 2018). In addition, production of honey and other vital products: royal jelly, pollen, propolis, bee venom, wax, queens and bee packages (Ullah *et al*., 2021). Managed honeybee colonies are exposed to several pressures in the modern world. Stressors have been related to pesticide exposure, environmental stress, and nutrition, whilst parasites and pathogens directly affect individual bees and entire colonies, as well as contributing to a decrease in hive products and colony mortality (Refaei and El-Naggar, 2008; Goulson *et al.,* 2015; Crenna *et al.*, 2020). Great efforts are made to replace the dead colonies to keep the bees' population steady or increase. Nosema, a microsporidian fungus, is considered an important pathogen for honeybees. It attacks the mid-gut epithelium of adult honey bees, causing digestive disorders, physiological starvation, and subsequently damaging queen activity, reducing the quantity of brood reared and shortening the lives of honeybee workers (Kauko *et al*, 2003; El-Shemy *et al*., 2012).

Most studies have been focused on therapeutic methods for nosemosis while neglecting the side effects of these treatments on honeybees. The antibiotic Metronidazole, sold under the common name Flagyl, is used for treating anaerobic bacteria and certain parasites, including Nosema bee infections of the small intestine, where it proved to be very effective against Nosema spores (Gisder *et al*., 2015; Sweelam *et al.*, 2019). Despite it being considered a cheap alternative to fumagillin and becoming commonly used by beekeepers to treat nosema infections, its side effects can only be explained in medical reports and little information has been reported about its effect on honeybees. A substance of natural origin has been previously reported to inhibit Nosema disease and reduce mortality in infected bees (Yücel and Dogaroglu, 2005; Maistrello *et al*.; 2008; Costa *et al*., 2010; Van den Heever *et al*., 2016) and is commonly used in beekeeping for varroa mite control (Glavan *et al*., 2020).

The current study aimed to evaluate the impact of inhive therapy with Metronidazole (Flagyl) and Thymol on some biological, histological, and biochemical parameters of *A. mellifera* L. workers who received medicated sugar syrup during their development.

MATERIAL AND METHODS

Experimental Colonies:

The experiment was carried out at the apiary of the Apiculture Department, Plant Protection Research Institute, Dokki, Cairo, Egypt. The experimental period extended from early spring until clover honey yield. A total of 9 colonies headed by sister queens and showing a homogeneous development were used. Colonies were examined to ensure that they were free from diseases, and no other treatments were used before or during the current study. Selected colonies were nearly identical in strength, brood areas, and food stocks. Bees were allowed to free-fly to collect any available pollen and nectar from the surrounding areas. Colonies were divided randomly into three groups of three colonies each; two were treated, and one was left as a control. Each colony was provided with an empty honeycomb to obtain bees of a uniform age. Treatments were applied just before egg-laying. The bees were collected at the adult stages of new emergence.

Experimental Treatments:

- Flagyl syrup (Metronidazole): Flagyl was given as 2ml/ colony in 250 ml 1:1 sugar syrup and repeated five times at four days intervals.

- Thymol crystal: (a natural product of El-Gomhoria Chemicals Co., Egypt). Thymol was used at a concentration of 0.44 mM crystallised thymol (equal to 0.25 g/gallon of syrup). 12.5g of thymol dissolved in 88 ml of 91% isopropyl and 2ml of premix were then slowly poured into a gallon of sugar syrup. For 4 successive weeks, 250 ml of the mixture was applied once a week to the bee colonies.

- Control group: was offered only pure 1:1 sugar syrup solution.

Pure and medicated sugar syrup was poured directly into marked top empty brood frames in honeybee colonies to ensure obtaining the therapeutic dose (Adams *et al*., 2007).

Biological assay

Weight of new emergence worker bees

A sample of 30 newly emerged Worker bees for each treatment was collected directly from their capped cells. Workers were weighed individually using an electronic balance (precision: 0.0001).

Brood area measurement:

Sealed worker brood areas of the experimental colonies were conducted till the end of the clover flow period. Brood measurements were made every 12 days using a transparent plastic grid sheet divided into square inches. For easier and safer measuring with lower bee kill, bees covering each brood comb were first shaken off, and the number of squares with sealed broods was then counted. The total sealed brood area per colony was then calculated (Kasim *et al.*, 2017).

- Honey yield:

At the end of clover flow period by the first week of June, honey yield was determined for each experimental colony individually. The surplus honeycombs were weighed before and after honey extraction. Thereafter, honey yield was estimated in (kg/colony) by calculating the difference between the weight of honeycombs before and after honey extraction, separately for each colony (Erdogan *et al*., 2009).

-Histological assay:

Histological investigations were carried out using newly emerged bees selected from each group after the termination of the treatment experiment. Bees were anaesthetized, and the midgut was removed. Then, midguts were fixed in formalin (10%) for 24 hours, dehydrated in ethyl alcohol, cleared and embedded in paraffin. Paraffin sections of 5 µm thick were then stained with hematoxylin and eosine, and visualised using the light microscope.(NLCD-120, PN14003, LAMPS-LEDW1, China) (Pokora and Szilman, 1991).

Biochemical assay:

Samples Preparation of bees for biochemical assay:

Workers at emergence were sampled, weighed, and frozen for the estimation of total soluble protein and enzyme activities. The frozen samples were homogenised in 0.5 ml of distilled water using a chilled glass Teflon homogenizer (MPW-309 Mechanic-Preczyina, Poland) and centrifuged at 5000 r.p.m. for 10 min at 5°C. The supernatant was then used for protein content and enzyme activity estimation.

Total soluble protein (TSP) :

Total soluble protein was calorimetrically measured according to the method mentioned by Eldidamony et al., (2020). A volume of 0.2 ml of homogenate was added to 5ml of Biuret reagent and incubated for 30 min at 20 – 25 °C. The absorbance of the sample against a blank Biuret was measured at 546 nm wavelength.

Amylase and invertase enzyme activity:

The method used to determine the digestion of starch and sucrose by amylase and invertase respectively was carried out according to the method mentioned by Ishaaya and Swiriski (1976). The enzymes activity was estimated calorimetrically at 550 nm. and expressed as µg glucose released /g body weight/min.

Protease enzyme activity:

Protease activity was estimated according to the method explained by Tatchell *et al*. (1972) where the increase in free amino acids split from substrate protein (albumin) was measured during one-hour incubation at 30 ºC.

Lipase enzyme activity:

The lipase activity was assessed using methyl resorufin method according to Panteghini *et al.* (2001).

Statistical analysis:

The means of newly emerged workers weight and enzymes activities were compared by one-way analysis of variance (ANOVA), Tukey honestly significant difference (HSD) (Tukey-HSD) tests with least significant difference at p≤ 0.05 using computer statistical software according to El-Sharabasy et al. (2015).

RESULTS

Biological assay:

New emergence workers body weight:

The average body weight of emergent workers was recorded at 0.095, 0.098, and 0.101 mg for flagyl, thymol, and control groups, respectively (Table 1). Analysis of variance showed no significant differences between treated and control groups (P > 0.05). Nonetheless, treatments displayed some decrease in body weight in comparison to control.

Sealed brood area:

The average number of sealed worker brood at the beginning of the experiment was nearly equalized colonies groups. Sealed worker brood area (inch²) noted at 12 to 13 days intervals in medicated and control bee colonies are presented in Table (1). No significant difference in sealed brood cells was observed among treatments and control colonies in March. Whereas, there was a significant reduction (P<0.05) in the mean of worker brood area in colonies received flagyl (382.67,1367inch²) than control (456.33& 1454 inch²) and those which received thymol (431.33 and 1440 inch²) during April and May, respectively. As a whole, the maximum area of worker brood was observed in the control colonies during the time of experiment with no significant difference with thymol group.

Honey yields:

Clover honey yield was detected by calculating the difference in weights between honeycombs before and after honey extraction. The mean weights of clover honey yield recorded 6.21, 7.65 and 7.88 kg/colony for colonies received flagyl, thymol and the control, respectively (Table 1). Analysis of variance indicated that control and thymol groups produced significantly higher clover honey yield compared to that produced from the colonies that received flagyl (P=0.0011). Interestingly, control colonies produced more honey than medicated ones.

Table 1. Mean of worker body weight, sealed brood area and clover honey yield of medicated (Flagyl & thymol) and control colonies.

Treatments	Weight of emerged workers (mg)	Brood surfaces area (inch²)			Honey yield (kg/colony)
		March	April	May	
Flagyl	0 .095[a]	337[a]	382.67[b]	1367[b]	6.21[b]
Thymol	0.098[a]	333.3[a]	431.33[a]	1440[a]	7.65[a]
control	0.101[a]	347[a]	456.33[a]	1454[a]	7.88[a]
LSD (0.05)	0.00689	22.465	34.276	70.608	0.6139
P	0.296	0.367	0.0052**	0.0482*	0.0011**

Histological assay:

To detect any histological changes produced in the worker midgut as a result of using nosemacides, sections of the control and medicated worker midgut were examined. The midgut showed a normal appearance in control workers (Fig. 1 A-B). Its wall consisted of one layer of columnar epithelial cells, settled on the basement membrane with normal nuclei. Columnar cells possess microvilli that form a striated border consisting of fine parallel hairs arising from the epithelial cell surface towards the lumen. Another type of epithelium that could be observed is regenerative cells; their terminals did not reach the lumen. A well-structured peritrophic membrane appeared multi-layered, separated from the epithelial cells and extended in the lumen along the midgut length. The histological studies on the workers' midguts revealed severe structural changes after treatment with flagyl. Midgut epithelial cells were shown to be completely disorganised and partially ruptured (Fig. 1 C-D), as well as marked vacuolations in both columnar epithelial and striated borders (Fig. 1 E-F). Displacement and condensation of the nuclei were also apparent (Fig. 1C). There was clear fragmentation and rapture in the peritrophic membranes (Fig. 1D-E). Furthermore, separation of basement membrane (Fig.1 D-E), fragmentation and detachment of the muscle layer from the epithelium were also detected. On the other hand, histological sections in worker mid gut received thymol did not show any severe adverse effects compared to control. The peritrophic membrane appeared normal, multi-layered and nearly similar to that of control. Columnar cells exhibited slight orientation change. However; the basement membrane and the muscle layers remained intact (Fig. 1 G-H).

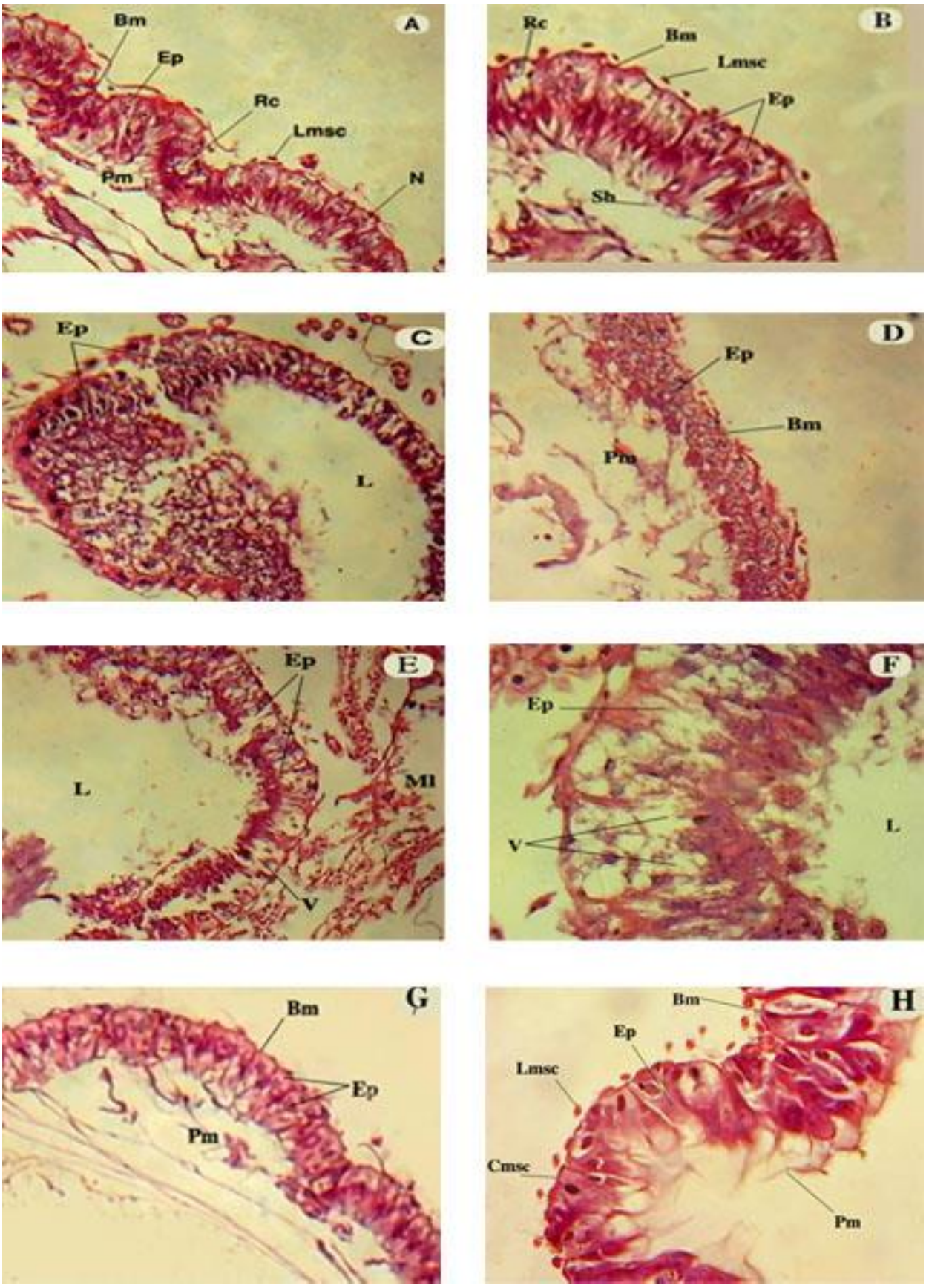

Figure 1. Light micrographs of the midgut in the newly emerged workers, B, F, H (X400) and A, C, D, G (X100). (A, B) represented the normal structure of the control group, (C, D, E, F) represented the midgut structure of the flagyl group, and (G, H) were for thymol. Basement membrane (Bm), epithelium cells (Ep), lumen (L), peritrophic membrane (Pm), regenerative cells (Rc), muscle layer (Ml), longitudinal muscles (Lmsc), circular muscles (Cmsc), striated border (Sb), vacuoles (V), and nucleus (N).

Biochemical studies:

Results in Table (2) expressed the total soluble protein and different enzymes activities related to digestion of newly emergent workers after treatment with flagyl and thymol compared to control.

Table 2. The biochemical response of newly emerged *A. mellifera* workers treated by flagyl and thymol during development.

	Total soluble protein (mg/g)	**Lipase** U/gm	**Protase** µg/ g.b.wt.	**Amylase** µg/g/min	**Invertase** µg/g/min
Flagyl	21.41^{b}	2.37^{a}	177.43^{b}	16.99^{b}	2.49^{b}
Thymol	30.69^{a}	2.48^{a}	226.119^{a}	19.24^{ab}	4.93^{a}
Control	29.36^{a}	2.68^{a}	222.96^{a}	22.20^{a}	5.29^{a}
LSD (0.05)	5.061	0.057	21.944	3.421	0.888
P	0.0084**	0.4455	0.0027**	0.027*	0.0005***

The result in Table (2) revealed that the total soluble protein recorded a highly significant reduction with the flagyl group ($p < 0.05$) in comparison to the control and thymol groups. In parallel, the protease, amylase, and invertase activities in the bodies of honeybee workers were sensitive to flagyl treatment. The enzyme activities were significantly reduced in the flagyl group compared to the control group ($p < 0.05$). Protease activity was 177.43 and 222.96 µg/g b wt., amylase activity was 16.99 and 22.20 µg/g/min, and invertase activity was 2.49 and 5.29 µg/g/min for flagyl and control groups, respectively. Meanwhile, there was no significant difference in lipase activity among groups or between thymol and control groups.

DISCUSSION

Nosemosis is a microsporidian fungal disease that can infect the intestinal tract of adult bees and cause harmful effects on queen performance, colony development, and production. The efficient control of this disease is carried out using antibiotics. Flagyl (metronidazole) is one of the most widely used antibiotics by beekeepers for nosema control. Nevertheless, it has no available scientific reports about its effects on honeybee colony health. Therefore, this study focused on its effect on honeybee colonies incomparable to that of thymol, a substance of natural origin also used for nosemosis combat (Costa *et al*., 2010; Van den Heever *et al*., 2016) and well known by beekeepers due to its suppressive effects against the parasitic mite *Varroa destructor* (Chiesa and D'agaro, 1991, Glavan *et al*., 2020).

The current study found that the antibiotic flagyl, used in apiculture, influences rearing brood, honey production, mid gut cells as well as bee physiology represented in digestive enzyme activity that corresponds to the mid gut. Sealed brood and honey yield were used as criteria for measuring the colonies' development and strength. The brood amount in the honeybee colony reveals its state and can be used to expect the honey production at the end of the season (Taha and Al-Kahtani, 2013) as there is a positive correlation between the amount of produced honey and the area of reared brood (Bhusal and Thapa, 200; Jevtić *et al.,* 2009; Taha and Kahtani, 2020). The obtained results showed that during April and May flagyl treatment caused a significant drop ($p<0.05$) in sealed brood area than control and thymol groups.

Meanwhile, during March, the brood areas had increased in all groups, with no significant difference. Concerning the honey yield at the end of the clover flow season, the flagyl group exhibited a significant decrease in honey yield compared to the thymol or controlled groups. Subsequently, measuring brood area and honey production of a bee colony can be used as an indicator for measuring the colony strength where forager bees originated from strong colonies can make longer flights and bring significantly more nectar than those from weak colonies (Eckert *et al.,* 1994). Concerning the newly emerged workers, treatments displayed some decrease in the average body weight than the control workers. Body weight is an important indicator for the individual bee quality (Cheng *et al.*, 2008) and low weight may harm honey production and other beehive products (Yousif- Khalil *et al*., 2009).

Otherwise, the digestive system is an important organ for honeybee health and is considered the site of contact with pathogens and xenobiotics (Han *et al*., 2012 and Johnson *et al*., 2009). The epithelial cells in the midgut are responsible for the detoxification of the ingested xenobiotics (Higes *et al*., 2013) and represent an important interface between the insect and environment (Forkpah *et al*., 2014). Subsequently, the midgut is considered as one important organ for toxicity analysis that is responsible for digestion and absorption of ingested food.

In the present study, the midgut epithelium of the bee group fed pure sugar solution showed a normal appearance with one-layer normal columnar cells resting on a basement membrane, a striated border at the apex, a normal nucleus, and an intact cell boundary. With the presence of a multi-layered and intact peritrophic membrane, this result is also compatible with that of Kakamand et al. (2008). Similarly, honeybee workers who received thymol showed an intact and normal peritrophic membrane. The midgut epithelial tissue of the worker bees that received thymol appeared to be slightly affected but remained stabilised at the basement membrane. The cell membrane was not affected and appeared to remain intact.

presence of regenerative cells, which are responsible for the replacement of any affected by new intact cells (Kakamand, *et al*., 2008).

In contrast, severe histological alterations in the midgut tissues of worker bees in the flagyl group were observed. Among the most noticeable changes was the partial rupture in columnar cells, which may contribute to the rapture of the peritrophic membrane that acts as a food envelope and protective barrier against various physical, chemical, and microbial food components (Terra, 1996; Diniz *et al*., 2020). In addition, Spread of cytoplasmic vacuoles in epithelium columnar and separation of muscle layer. Damage in the midgut tissue could lead to starvation, microbial infections and worker bee loss (Vidau *et al*., 2011) and therefore, not only affect the individual fitness but the whole colony as well (Grella *et al*., 2019).

Flagyl also induced a highly significant reduction in protein levels in the bodies of newly emerged workers in comparison to control and thymol workers. Protein is a major biochemical component required for organism development, growth, and the performance of many vital activities. Protein has been shown to influence important individual associated traits such as body size, growth rate, and fecundity (Fagan *et al al.*, 2002). The decrease of total protein in treated insects may also reflect the decrease in the activity of various enzymes (El-Kordy *et al*., 1995). In connection, the activity of digestive enzymes like proteases, amylases, and invertase appeared to significantly decline after flagyl syrup feeding compared to control and thymol groups. These changes affect mainly insect physiology where these enzymes convert complex food materials into micro molecules, producing energy and metabolites necessary for growth, development, and many vital functions.

The digestion process of honeybee workers depends mainly on the activity of the enzyme present in secretions of the salivary, hypopharyngeal glands and the midgut epithelial cells (Standifer, 1980) and the in vivo digestive enzymes in the bee intestine include lipase, protease, amylase and invertase (Sudd, 1985). Protease and lipase digest bee bread into amino acids and lipids (Howell, 1985). whereas, amylase aids in carbohydrate digestion and the conversion of nectar and pollen into honey, and thus has a direct impact on bee health and quality, honey production, and brood-rearing activity (Kumar, 2017). Subsequently, the activities of digestive enzymes can reflect the bee's ability to digest and absorb nutrients that affect colony strength and production.

CONCLUSION

In summary, the antibiotic flagyl revealed harmful side effects on brood rearing, honey yield, midgut cells, and digestive enzymes, whereas thymol exhibited the best results in all characteristics. Therefore, it is very necessary to evaluate the impact of in-hive treatments on internal target organs and vital functions, which are a suitable indicator of their toxicity on a honeybee. On the other hand, regular observation and early detection of nosema infection in honeybee colonies enable beekeepers to know when necessary that the bee colonies should be medicated, which could reduce the application of drugs to bee colonies. This study suggests the usage of natural origin products inside beehives as a saving alternative biochemical product for honeybee health. They also suggest their importance for clean and organic bee products and for avoiding any resistance that could occur with antibiotics.

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التأثير الثانوي لاستخدام مركب المترونيدازول و بلورات الثيمول في الخلايا على بعض الأنشطة البيولوجية والنسيجية والبيوكيميائية لشغالات نحل العسل

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الملخص

يعتبر التعرض المتكرر لعلاجات النحل المستخدمة داخل الخلية هى واحدة من العوامل التى ساهمت فى انخفاض عدد طوائف النحل .لذلك كان الهدف من هذه الدراسة هو تقييم بعض التغيرات البيولوجية والنسيجية والبيوكيميائية لشغالات نحل العسل (*Apis mellifera* L.) نتيجة التعرض اثناء فترة النمو للمضاد الحيوي فلاجيل (Metronidazole) الذى يشيع استخدامه كعلاج لمرض النوزيما مقارنة بالثيمول كمركب علاجى طبيعى يستخدم داخل خلايا النحل. تم إجراء هذا العمل في أوائل فصل الربيع ، من خلال امداد طوائف النحل بالمحلول السكرى العلاجي عن طريق التغذية. أظهرت النتائج انخفاضًا معنويًا واضحًا في متوسط مساحة حضنة الشغالات خاصا خلال شهرى ابريل ومايو وإنتاجية العسل في الطوائف التى تلقت الفلاجيل مقارنةً بالمجموعة الضابطة والثيمول. كما سجلت الشغالات حديثة الخروج فى الطوائف المعاملة وزنا اقل من الكنترول. وادت المعاملة بالفلاجيل الى حدوث تغيرات شديدة فى انسجة المعى الاوسط خاصا الخلايا الطلائية العمودية والغشاء المحيط بالغذاء والطبقة العضلية بالاضافة الى خفض كبير فى محتوى البروتين وتثبيطا فى انشطة الانزيمات المرتبطة بالهضم . وبشكل عام ، تعد هذه النتائج ذات قيمة في تسليط الضوء على صلاحية أنسجة الأمعاء الاوسط وأنشطة الإنزيمات كمؤشرات لتقييم سمية العلاجات المستخدمة فى التغذية لطوائف نحل العسل.

الكلمات المفتاحية: المترونيدازول، شغالات نحل العسل، انتاج العسل، المعى الاوسط ،انشطة الانزيمات