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INHIBITORY EFFECT OF SOME ESSENTIAL OILS ON OVARIES DEVELOPMENT OF HONEY BEE WORKERS UNDER QUEENLESS CONDITIONS

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Abstract: Appearance of egg-laying workers in honey bee colonies, under queenless condition, is considered to be one of the most important problems confronting the beekeepers. The present study aimed to prevent the appearance of the egg-laying workers by using certain essential oils. The tested oils were spearmint, eucalyptus, fennel, citronella, coriander, geranium, anise and thyme oils. Significant differences in worker ovarian development index were found between most of essential oils and control. The lowest value of ovarian development index (the highest inhibitory effect) was recorded by using spearmint and fennel oils, inducing 80 and 64% ovarian inhibition as compared to control. Whereas the highest value of ovarian index (the lowest inhibitory effect) was recorded by using thyme oil, resulting only in 24% ovarian inhibition. However, using of anise oil gave a converse result, resulting 4% activation of ovarian development. According to results the essential oil types could be classified into four categories depending on their inhibition or activation effects on ovarian development of bee workers as follows: the first category (strong inhibition), the percentage of inhibitory effect was more than 60%, which included spearmint and fennel oils. The second category (considerable inhibition), the inhibitory value ranged from 50-60%, included eucalyptus, citronella and geranium oils. The third one (slight inhibition), the inhibitory value ranged from 0<50%, included coriander and thyme oils. While the fourth (activation), the value was less than 0%, included anise oil. The appearance of egg-laying workers was recorded only by using thyme, anise oils and control (without oils).

Key words: honey bee, laying workers, essential oils, ovarian development, inhibitory effect.

Introduction

It is well known that the presence of the queen in a bee colony inhibits the ovaries develop-ment of bee workers (Velthuis, 1970). The mated queens give more inhibitory effect than virgin ones (Butler and Fairey, 1963; Khodairy, 1990 and Khodairy, 2008). The development of worker ovaries was also inhibited by the presence of unsealed brood (Jay, 1968; 1972). The presence of unsealed brood provides stronger inhibitory effect than the queen (Kropakova and Haslbachova, 1970), and unsealed brood extracts showed inhibitory effects on worker ovaries (Kubisova and Haslbachova , 1978). In case of absence of both unsealed brood and queen in a colony, laying workers appear, but they can lay only male-producing eggs. In queenright colonies, worker ovaries develop-ment are inhibited by queen-produced pheromones (Pain,. 1961; Winston and Slessor, 1992). The inhibitory effect of a queen only occurs when workers have been in direct contact with other workers which contacted the queen (Free, 1987). The queen's mandibular gland pheromone is well known for its releaser action on retinue behaviour, on delaying queen rearing and thus swarming (Slessor *et al.*, 1988; Winston *et al.*, 1990; 1991). Some studies suggest that queen mandibular gland phero-mone , and particularly its main compound, 9-ODA (9-OXO-2-decenoic acid), has a primer effect as well by inhibiting the develop-ment of worker ovaries (De Groot and Voogd, 1954; Butler and Fairey, 1963). The inhibition of worker ovaries by brood occurs when workers have been in contact with, or within a close distance to the brood (Jay, 1972). This suggests that brood could release low volatile pheromones which elicit the inhibition of ovaries development.

Honey bee brood pheromones have been identified. The first is the glyceryl-1,2-dioleate-3-palmitate which is secreted by worker, drone and queen pupae, and elicits the clustering of workers on dummy queen cells (Koeniger and Veith, 1983; 1984). The second is a mixture of four fatty acid esters (methyl palmitate, methyl linoleate, methyl linolenate and methyl oleate) which singly or mixed elicit sealing of cells by workers (Le Conte *et al.*, 1990). Recent results show that a mixture of ten fatty acid esters (methyl and ethyl esters of palmitic, linoleic, linolenic, stearic and oleic acids) which present naturally in the larval cuticle (Le Conte *et al.*, 1989) induce a strong inhibitory effect on ovary devel-opment of caged bees in controlled conditions. This inhib-ition occurs by contact, diffusion, or ingestion of the blend (Arnold *et al.*, 1994). Ethyl palmitate and methyl lino-lenate are involved in inhibition of worker ovaries under natural conditions (Mohammadi *et al.*, 1998). The virgin queen tergal gland extracts of honey bee inhibited ovarian development of workers (Wossler and Crewe, 1999). The anti-juvenile hormone, precocene II caused more inhibitory effect on ovarian development of bee workers (Khodairy and Tawfik, 2003). The absence or removal of stored pollen (bee bread) induces an inhibitory effect on ovaries develop-pment of caged bee workers under controlled conditions (Khodairy, 1990 and Khodairy and Moustafa, 2006). The natural productions of essential oils including citronella, peppermint, eucalyptus, marjoram, fennel, coriander and thyme oils were used to control insects and mites especially, the bee mite, *Varroa destructor* (Nemec *et al.*, 1990 and Khodairy, 1998).

Appearance of egg-laying workers in the honey bee colonies is considered one of the important problems confronting the beekeepers, especially after colonies had been dequeened because it is very difficult to introduce a queen into queenless colony with egg-laying workers. The present study focused to investigate the response of bee workers to some essential oils in laboratory to possibly use them or their mixture for inhibition of the ovarian development of bee workers and prevent appearance of egg-laying workers in bee colony, especially, in case of the absence of queen until introducing a new queen.

Materials and Methods

The experiments were carried out in Faculty of Agriculture, Assiut University apiary during active season of 2008.

Preparation of the cages with bees:

The first hybrid of Carniolan honey bee, *Apis mellifera* L. workers were used in the present study. Sealed brood combs, containing hatching brood, were taken from queenright colony, then incubated at 32°C±1 and 60% RH., and the brood were observed until adult emergence. Five thousand and four hundreds workers, less than 12 hours old, were placed inside twenty-seven wooden cages (12x12 x5 cm). The cages were provided with a vial of tap water, and a vial of sucrose solution (50% aqueous sugar), and a piece of comb with enough bee bread, attached to the cage top. The cages were continuously provided with water and sugar solution. Three cages (replicates) were used for each treatment (eight types of essential oils) and control (without oil). The cages for each treatment and control were held in a separation dark incubator at 32°C±1 and 60% RH.

Bioassay of essential oils:

The essential (volatile) oils used were as follows:

- Spearmint oil (*Mentha spicata*).
- Eucalyptus oil (*Eucalyptus citriodora*).
- Fennel oil (*Foeniculum vulgare*).
- Citronella oil (*Cymbopogon* sp.)
- Coriander oil (*Coriandrum sativum*).
- Geranium oil (*Pelargonium* sp.).
- Anise oil (*Pimpinella anisum*).
- Thyme oil (*Thymus vulgaris*).

Pure forms of the essential oils were used as vaporous in the experiments because they have high vapour pressures. A filter paper, 10 mm in diameter, contained with a droplet of 10 µl of test substance was placed inside each cage and the tested substance was renewed every three days, whereas the control was without essential oils.

Determination of ovarian development:

To study the effect of the different types of essential oils on ovarian development under queenless condition, twenty bee workers were removed from each cage every three days. This procedure was repeated seven times at three days interval. Each worker was dissected under stereo-microscope (40 times magnification force) to determine the ovaries development by using the classify-cation of developmental stages as given by Sakagami and Akahira (1958). According to this method, the degree of development was classified as O, undeveloped (rudimentary), I, slightly developed (commencement of swelling and constriction) and II, well developed (distinct ova).

Also, the ovarian development index was calculated according to Jay and Jay (1976), as an indication of ovarian development for all stages determined in the bee worker samples. The means of various scores when multiplied by the number of bees whose ovaries fall with each ovary development category were 0, undeveloped (score = 1); I, slightly developed (score = 2); II, well developed (score = 3). The index value 1.0 means that all workers with undeveloped ovaries, whereas the value 3.0 means that all workers with well developed ovaries. The inhibitory effect of the different types of essential oils on ovarian development of bee workers was calculated using the following suggested equation:

$$\textbf{Inhibition of ovarian development (\%)} = \frac{\textbf{IOI of control} - \textbf{IOI of tested oil}}{\textbf{IOI of control (without oil)}} \times 100$$

IOI, increase in ovarian development index more than score one (undeveloped ovary).

Statistical analyses:

The statistical analyses were conducted using the SAS general linear models procedure. Differences among means were determined by L.S.D. significant differences at P<0.05 (SAS Institute, 1990).

Results and Discussion

The ovarian development of bee workers treated with different types of essential oils are shown in Table (1). The results showed significant differences in the ovarian develop-pment index of bee workers among the control and the most of essential oils.

Table(1): Ovarian development index and inhibitory effect of some essential oils in honey bee workers, at a period of 21 days.

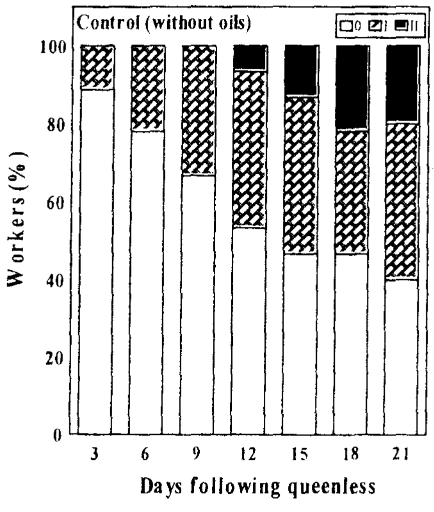
Days following queenless	Ovarian development index (Mean±SD) and inhibition (%)								
	Spearmint	Fennel	Eucalyptus	Citronella	Geranium	Coriander	Thyme	Anise	Control
3	1.11 ±0.04	1.13 ±0.0	1.11 ±0.04	1.15 ±0.04	1.11 ±0.04	1.18 ±0.04	1.15 ±0.04	1.29 ±0.03	1.11 ±0.04
6	1.13 ±0.07	1.15 ±0.04	1.15 ±0.04	1.18 ±0.04	1.20 ±0.07	1.20 ±0.07	1.18 ±0.04	1.38 ±0.04	1.22 ±0.04
9	1.11 ±0.04	1.15 ±0.04	1.18 ±0.04	1.25 ±0.04	1.20 ±0.07	1.27 ±0.07	1.31 ±0.03	1.49 ±0.03	1.33 ±0.07
12	1.13 ±0.07	1.18 ±0.04	1.15 ±0.04	1.18 ±0.04	1.27 ±0.07	1.31 ±0.03	1.31 ±0.03	1.49 ±0.03	1.53 ±0.07
15	1.08 ±0.04	1.18 ±0.04	1.27 ±0.07	1.15 ±0.04	1.22 ±0.04	1.31 ±0.03	1.33 ±0.07	1.67 ±0.14	1.67 ±0.67
18	1.08 ±0.04	1.20 ±0.07	1.31 ±0.03	1.27 ±0.07	1.33 ±0.07	1.31 ±0.03	1.49 ±0.08	1.73 ±0.06	1.82 ±0.08
21	1.08 ±0.04	1.27 ±0.07	1.29 ±0.03	1.31 ±0.03	1.40 ±0.07	1.33 ±0.07	1.91 ±0.10	1.62 ±0.04	1.80 ±0.13
Grand mean±SD	1.10^{D} ±0.02	1.18^{CD} ±0.05	1.21^{CD} ±0.08	1.21^{CD} ±0.06	1.24^{BCD} ±0.09	1.27^{BC} ±0.06	1.38^{AB} ±0.26	1.52^{A} ±0.15	1.50^{A} ±0.28
Ovarian inhibition (%)	80.0	64.0	58.0	58.0	52.0	46.0	24.0	-4.0	-

Means have the same letter(s) are not significantly different at 0.05 level of probability.

The general means of ovarian development index were 1.10, 1.18, 1.21, 1.21, 1.24, 1.27, 1.38, 1.52 and 1.50 for spearmint, fennel, eucalyptus, citronella, geranium, coriander, thyme, anise and control (without oils), respectively. The lowest values (highest inhibitory effect) of ovarian development index (1.10 and 1.18) were recorded by using the spearmint and fennel oils, inducing 80 and 64% ovarian inhibition as compared with un-treated control. Using of eucalyptus, citronella and geranium oils induced considerable inhibitory effect in ovarian development of bee workers. The percentages of ovarian inhibitory effect were 58, 58 and 52%, respectively as compared with control. Whereas the highest value of ovarian development (lowest inhibitory effect) index (1.38) was recorded by using thyme oils, resulting in only 24% ovarian inhibition. In contrast using of anise oil gave a converse result, it gave 4% activation of ovarian devel-opment. The appear-ance of stage II of ovaries devel-opment, well develop-ed (egg-laying workers) was record-ed only on the 18th day (2%) for thyme oil, then increased to reach maximum value on the 21st day (3.3%).Also, the laying workers appeared clearly on the 15th day (13.3%) for bees treated with anise oil and for control bee workers (6.7%) on the 12th day. The percentage of general mean of egg-laying workers was 8.7, 6 and 3.3% for control, anise oil and thyme oils, respectively (Figs. 1, 2 and 3).

Fig. 1. Ovarian development of bee workers in queenless condition , at a period of 21 days.

Degree of ovary development:
0, undeveloped
I, slightly developed
II, well developed



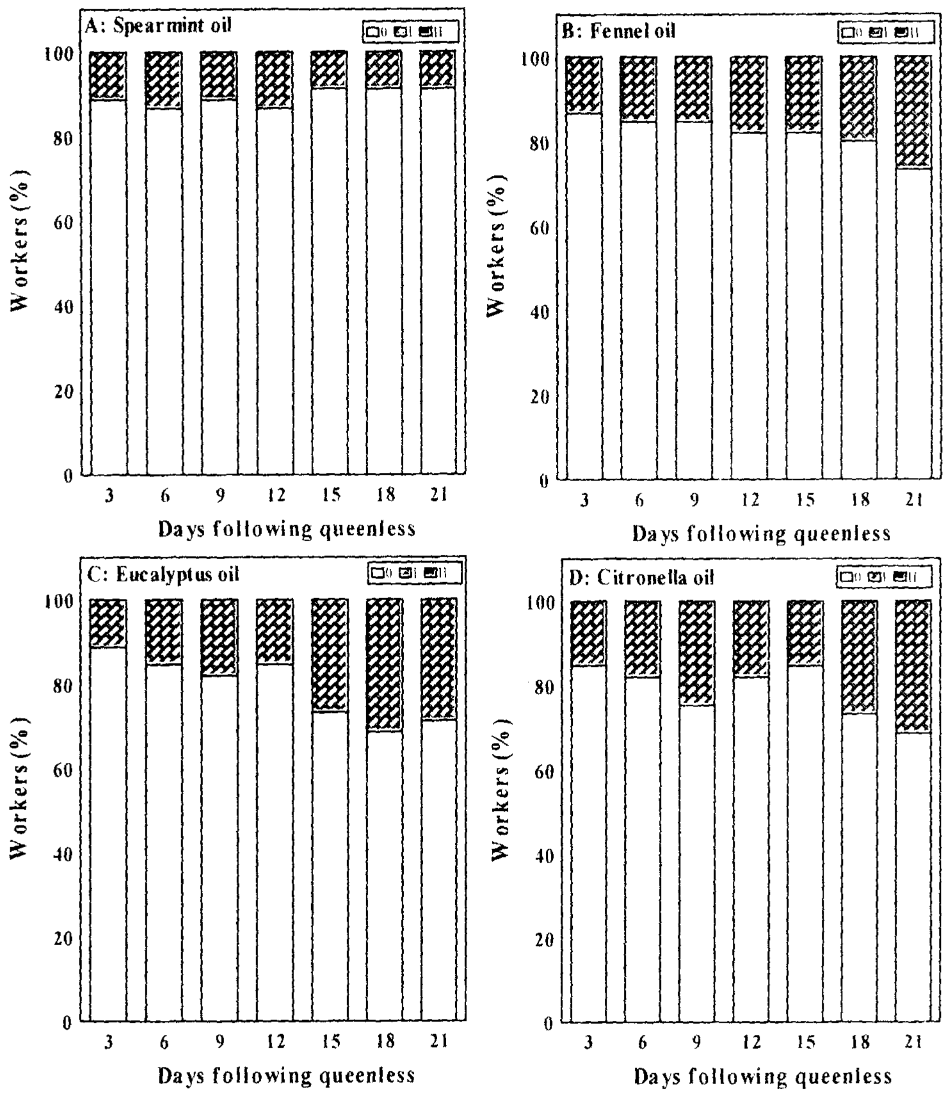


Fig. 2. Ovarian development of bee workers in queenless condition treated with different essntial oils, at a period of 21 days.

Degree of ovary development:
0, undeveloped **I, slightly developed** **II, well developed**

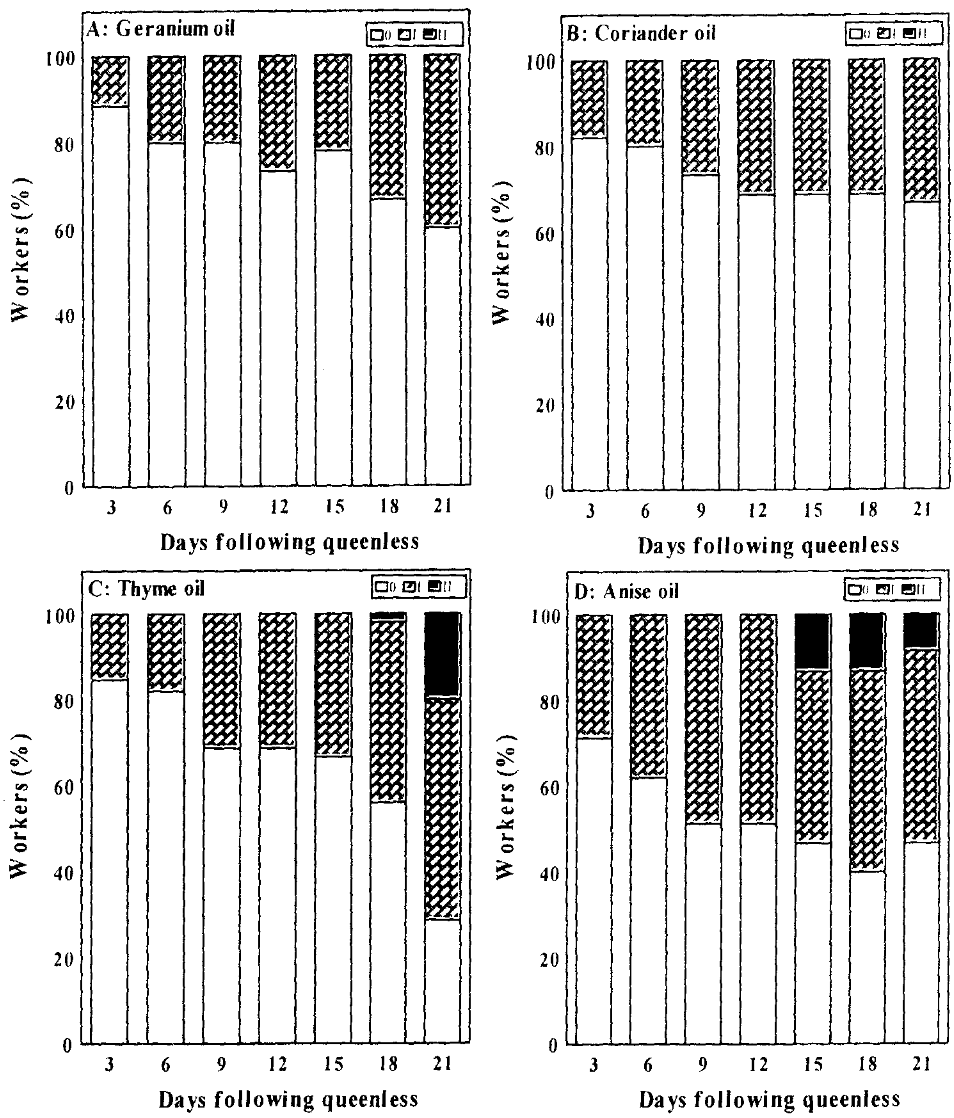


Fig. 3. Ovarian development of bee workers in queenless condition treated with different essntial oils, at a period of 21 days.

Degree of ovary development:
0, undeveloped **I, slightly developed** **II, well developed**

According to the present results, the types of essential oils could suggested to classify to four categories, depending on their inhibition or activation effects on ovarian development of bee worker as follows: the first category (strong inhibition), the percentage of inhibitory effect was more than 60%. This category included spearmint and fennel oils. The second category (considerable inhibition), the inhibitory value ranged from 50 to 60%, included eucalyptus, citronella and geranium oils. The third category (slight inhibition), the inhibitory value ranged from 0 to <50%, included coriander and thyme oils. While, the fourth category (activation), the value was less than 0% and included anise oil.

The findings of the present investigation indicate strong inhibitory effect of ovarian development of bee workers in the essential oils of spearmint and fennel, followed by the oils of eucalyptus, citronella and geranium which considerable inhibitory effect. Whereas coriander and thyme oils had less inhibitory effect. The effectiveness of spearmint oil to inhibit ovarian development and prevent appearance of laying workers may be due to the presence each of carvone (55-70%), which is known as ovicidal, limonene and esters in this oil. The chemical group of spearmint oil is ketone (Balbaa, 1980). Also fennel oil contains anethole (60%) and ketone (20%) as the active ingredients (ovicidal), the chemical group was ethers. The important constituents of eucalyptus oil (aldehydes) were citronellol (70%) and terpenes, in geranium oil (alcohols) were citronellol, geraniol and esters, in coriander oil (alcohols) were linalol and terpenes, in thyme oil (phenols) was thymol in a percentage of 20-40% (Balbaa, 1981). The present results are confirmed by Rice and Coats (1994) who showed that the ketone compounds proved to be more insecticidal than structurally similar alcohol as an ovicide to the house fly. Essential oil of citronella disrupted the growth and development of some insects (Marimuth *et al.*, 1997). The components of essential oils such as, gernaiol, thymol, linalool, terpenes, citronellol and carvone were ovicidal activity. The present results are confirmed by Saxena and Basit (1982), who used eucalyptus and coriander oils, and citronellol, gernaiol and farnesol for inhibition of oviposition of leafhopper, *Amrasca devastans*. Farag *et al.* (1994) used some essential oils such as thyme and their basic components, thymol and eugenol, they indicated that these oils reduced the egg production, caused abnormallities in the ovaries of treated insects, and reduction in both fecundity and fertility (Hashem *et al.*, 1994). Recently, many authors have reported that many essential oils such as thyme, eucalyptus and citronella and their components have a high acaricidal activity against varroa mite (Omar *et al*., 2001; Baggio *et al*, 2004 and El-Zemity *et al*., 2006). Plant essential oils in general have been recognized as an important natural resource of insecticides (Adebayo *et al*., 1999; Gbolade *et al*., 2000). Their lipophilic nature facilitates them to interfere with basic metabolic, biochemical, physiology-ical and behavioural functions of insects (Nishimura, 2001). They have the potential of being acute ovicidal, fumigant, insect growth regulator and insecticidal against various insect speices (Tsao *et al*., 1995). Generally, they are safe to human and other mammals (Tripathi *et al*., 2002). The pure compounds like thymol, carvacrol and α-pienene have been documented for larvicidal activities towards *Culex* sp. (Traboulsi *et al*., 2002).

As well known, that the corpora allata (CA) secretes the juvenile hormones (JH). The activity of bee worker CA is influenced by several factors, such as nutritional factors (Laere, 1972), queen pheromones (Gast, 1967), anti-juvenile horm-ones, precocenes (Bowers *et al*., 1976; Khodairy and Tawfik, 2003) and neural stimuli and exposure to allatostatins and allatotropins (Stay and Woodhead, 1993; Stay *et al*., 1997). In queenless, the treatment with anti-juvenile, precocene II caused a decrease in the CA volumes and induced more inhi-bitory effect on worker ovarian development(Khodairy and Tawfik, 2003). The change in the CA volumes is joined to several physiological processes like oog-enesis. The juvenile hormones (JH) are essential alone or with others for regulation of many physiological and behaviour pro-cesses. It plays an important and diverse role in the oogenesis and egg-laying behaviour in many insect species by regulating vitellogenesis in the fat bodies, vitellogenin (Vg), reproductive protein, uptake by the ovaries (Tobe and Stay, 1985; Nijhout, 1994; Davey, 1996), and oviposition behaviour (Cayre *et al*., 1994). Many studies suggested that JH is involved in the regulation of ovarian development in primitively social Hymenoptera such as, polistine wasps (Röseler, 1991), halictine bees (Bell, 1973) and bumble bees (Röseler *et al*., 1981). In bumble bees, oogenesis is dependent on JH induction, in queenless workers, vitellogenesis was correlated with increased JH synthesis. Workers have well developed ovaries have higher rates of JH synthesis and higher JH haemolymph titers than similarly aged queenright workers. An increased Vg synthesis was also observed in queens after JHI treatment. Even queenright workers, which have a low JH titer, could develop to lay eggs when treated with JHI (Röseler, 1977). The effectiveness of essential oils may affect CA activity and/or interrupt vitellogenin synthesis then prevents the appearance of vitellogenin in haemolymph and causes inhibition in ovarian development, hence prevents appearance of laying workers of honey bees.

Other hormones, ecdusteroid also affect with JH many physiological and behaviour processes. Often interactions between JH and ecdysteroid play a pivotal role in the regulation of a particular process, such as vitellogenesis (Hagedorn, 1983). In bumble and honey bees, the ecdysteroid titres were low in both nurses and foragers, high in egg-laying workers and functional queens had a higher titers than virgin queens (Kaatz, 1987; Robinson *et al*., 1991; Bloch *et al*., 2000). They all suggested that ecdysteroid hormone may be involved in the regulation of honey bee reproduction.

On the other hand, there are several factors that affect bee reproduction, i.e., nutritional factor, as absence of bee bread induced an inhibitory effect on ovarian development (Khodairy, 1990). Also, an inhibitory effect of queen pheromones on JH synthesis has been shown in honey bee workers by Kaatz *et al*. (1992). These results indicate that regulation of JH synthesis in honey bees is mediated via queen pheromone and neurosecretory cells. Moreover, serotonine and octopamine regulate JH production in the CA by stimulating JH synthesis (Kaatz *et al*., 1994; Rachinsky, 1994). The brain levels of dopamine, N-acetyldopamine and norepinephrine were positively correlated with ovaries development of honey bee workers (Sasaki and Nagao, 2001). They suggested that these metabolites may be involved in the change of reproductive states of workers. A mixture of ten fatty acid esters, methyl and ethyl esters of palmitic, linoleic, linolenic, stearic and oleic acids, present naturally on the larval cuticle (LeConte *et al*., 1989) induce a strong inhibitory effect on ovarian development of caged workers, in controlled conditions. Ethyl palmitate or and methyl linolenate are involved in inhibition of ovarian development of workers under natural conditions (Mohammadi *et al*., 1998). They suggested that both esters can interact synergistically with each other or with the other esters. The aim of the present study was to control the laying workers of honey bee, that means prevent ovarian development by essential oils. These results showed an inhibitory effect of the ovaries and may decrease the CA volume and were in agreement with the previous studies.

The present investigation, indicated that the essential oils may be like the mode of action of precocenes, that was suggested by Bowers *et al*. (1976). It stopped and reversed allatal development, caused a slow regression in the CA volume and caused oocyte resorption and ovarian atrophy. It is clear, that the essential oils may act directly by causing corpora allatal regression and stop JH secretion which results, in ovaries atrophy. It acts indirectly in the CA, as may influence brain control of allatal development by inhibition of the secretion of an allatotropin. Also, may interference with JH biosynthesis in the CA, disruption of brain regulation of CA, induction of enzymes responsible for metabolism of JH or a biosynthetic precursor and disruption of vitellogenesis, then Vg uptake by the ovaries decrease afterword, the ovaries become undeveloped, preventing appearance the egg-laying workers.

In conclusion, the findings of the present study indicate that the essential oils, especially spearmint, fennel, eucalyptus, citronella and geranium oils are promising as safe natural products for control (prevent) the appearance of laying workers of honey bee, especially, in case of the absence of colony's queen until introducing a new queen. Also, these materials proved to be harmless to the bees and quite safe to the environment. These essential oils especially spearmint and fennel can be useful for using as replacements the queens and synthetic queen substances during preparing and transferring the package-bees. Further studies on synergistic combinations and isolation of bioactive fraction constituent which may provide futuristic lead products for apiary application of laying workers control.

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التأثير التثبيطى لبعض الزيوت العطرية على تطور مبايض شغالات نحل العسل تحت ظروف عدم وجود ملكة

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يعتبر ظهور الشغالات الواضعة للبيض (الأمهات الكاذبة) فى طوائف نحل العسل واحده من أهم المشاكل التى تواجه مربى النحل . ولقد أجريت هذه الدراسة لمنع ظهور الشغالات الواضعة للبيض فى حالة عدم وجود ملكات وذلك باستخدام الزيوت العطرية للنباتات التالية : النعناع والشمر والكافور وحشيشة الليمون والعتر والكزبرة والزعتر والينسون. وقد أوضحت النتائج إختلافات معنوية لدليل تطور المبايض بين معظم المعاملات بالزيوت العطرية والكنترول (بدون زيوت عطرية) . وقد أعطت المعاملة بزيت النعناع وزيت الشمر أعلى تأثير تثبيطى للمبايض (أقل قيمة لدليل تطور المبايض) . وكانت نسبة التثبيط 80% لزيت النعناع و 64% لزيت الشمر وذلك بالمقارنة بالكنترول . بينما سجلت المعاملة بزيت الزعتر أقل تأثير تثبيطى للمبايض (أعلى قيمة لدليل تطور المبايض) وكانت نسبة التثبيط 24% فقط مقارنة بالكنترول . وقد لوحظ باستخدام زيت الينسون أنه أعطى نتيجة عكسية (تنشيطية للمبايض) وكانت نسبة تنشيط المبايض 4% مقارنة بالكنترول. ومن خلال النتائج أمكن تقسيم أنواع الزيوت العطرية المستخدمة إلى أربعة مجموعات بناءاً على تأثيرها التثبيطى أو التنشيطى لتطور المبايض فى شغالات نحل العسل وهى : المجموعة الأولى وهى أكثر تثبيطاً للمبايض وفيها تكون نسبة التأثير التثبيطى أكثر من 60% وهذه المجموعة تشتمل على زيت النعناع وزيت الشمر . والمجموعة الثانية وهى ذات تأثير تثبيطى متوسط وفيها تكون نسبة التأثير التثبيطى ما بين 50–60% وهذه المجموعة تشتمل على زيت الكافور وحشيشة الليمون والعتر. والمجموعة الثالثة وهى ذات تأثير تثبيطى طفيف وفيها تكون نسبة التأثير التثبيطى ما بين صفر – <50% وتشتمل على زيت الكزبرة وزيت الزعتر . بينما المجموعة الرابعة كانت ذات تأثير تنشيطى وفيها تكون النسبة أقل من الصفر أى منشطة وتشتمل على زيت الينسون . وقد سجل ظهور للشغالات الواضعة للبيض فقط فى حالة استخدام زيت كل من الزعتر والينسون وأيضاً الكنترول .