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MITICIDAL ACTIVITY OF CLOVE ESSENTIAL OIL AND ITS MAJOR COMPOUND AS POTENTIAL CONTROL AGAINST *Varroa jacobsoni* MITES IN HONEY BEE.

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ABSTRACT

The varroa mite, *Varroa jacobsoni* is the most serious ectoparasitic pest affecting honeybee *Apis mellifera* L. on world wide basis and causes damage to the apiaries. Control with synthetic acaricides has been used extensively for varroa control in many different parts of the world but causes some problems such as residues in hive products and acaricide resistant mites. Thus essential oils offer an attractive alternative to synthetic acaricides for the control of varroa. They are generally inexpensive and most pose few health risks. In the present study essential oil of dried clove buds was extracted by steam distillation and analysed by Gas Chromatography-Mass spectroscopy (GC-MS) then investigated the activity against honey bee parasite *Varroa jacobsoni* by two methods of application spraying and evaporating trails in field tests after lab bioassay with essential oil and its major compound eugenol. Essential oil yield was 12.7 ± 0.07g/100g from dried clove buds sample. Thiry four components were identified by GC-MS and the major constituent was eugenol (*cis*-eugenol 18.02% and *trans*-eugenol 3.86%). The other main compounds were chavicol (6.87%), α-pinene (5.77%), junipene (5.46%), fenchone (4.94%), anethole (4.76%), limonene (4.65%) β-caryophyllene (4.16%) and carvone (3.99%). Additions to 12 compounds were recorded as minor constituents and 13 compounds as trace constituents (less than 1%).The results of biological experiments on varroa mites indicated that lab bioassay average mortality in the controls were ranged between 10.2 and 21. Average mortality in the eugenol treatments were ranged from 11.4 to 23.6 but in treatments of whole essential oil ranged from 19.20 to 70.50. In field experiments essential oil has significant differed from control in its effect as a miticide and evaporating technique is more effective on varroa mites comparing with spraying technique. The average mortality in mites was 50.38 and 71.42 for evaporation method and 12.12 and 20.1 for spraying method compared with control. Also the honey, sensory tests didn't give any evidence of clove after essential oil application.

Keywords: *Varroa jacobsoni*, Varroa mites, honey bees, *Apis mellifera*, Clove buds, essential oil composition, control.

INTRODUCTION

Varroa jacobsoni (oudemans) is parasitic mite of honey bee *Apis mellifera* (L.), that has had a catastrophic effect on the population of both managed and feral hony bee colonies, beekeepers and the beekeeping industry as a whole in 1995-1996 the U.S reported epidemic losses of managed bee colonies ranging from 25 to 80% (Finely *et al.*, 1996).

In addition, feral bee colonies, hidden in crevices, trees and houses are virtually gone. Also varroa damage immature and adult bees by feeding on bee hemolymph, reproduction rate are higher than bees and also transmitting harmful viruses. Left uncontrolled, varroa population can kill an entire bee colony in approximately two years (Eizen *et al.*, 2001).

Chemotherapy with synthetic acaricides has been used extensively for varroa control in many different parts in the world (Elzen *et al.,* 1999). Treatment option for varroa by synthetic acaricides is a potentially serious problems because of the recent report of synthetic acaricides resistant mites sin Italy (Loglio and Plebani, 1992 and Milani, 1995) , in France (Colin *et al.,* 1997), Israel (Gerson *et al.*, 1991) and in some states of America (Eizen *et al.,* 2001). Also these synthetic acaricides can contaminate hive products (Ruffinengo *et al.*, 2002). Thus, there is much interest by the beekeeping industry in the development of new control compounds for varroa.

It is know that the different essential oils contain volatile acetylene, monoterpene and sesquiterpenes components has a varity of biological properties including antifeedant, antimicrobial, antioxidant, insecticidal activity and acaricidal (Regnault-Roger and Hamaroui, 1994; Zhu *et al.,* 2001; Eun-Hee *et al.*, 2003 and Cairns *et al.*, 2003). In addition these compounds were easily biodegradable, non-phototoxic, more environment friendly and safe (Jurd and Manners, 1980; Asthana *et al.*, 1989, Charoenkul *et al.*, 2003).

Recently, several studies evaluated the effect of some natural products extracted from different plant species on varroa parasite. Colin *et al.* (1997) showed that aerosol application of thymus and salvia oils to honey bee colonies killed 95.4% of mites. Ibrahim and Shoreit (1999) used certain parts of some plants (coriander, caraway, chamomile, neem and rosemary) for studying the varroa infested colonies. The using of these plants increased the number of dead varroa found on the bottom board compared with the control. Also Sammataro *et al.* (1998);Al-Abbadi and Nazer (2003), and Rice *et al.* (2002) recorded the positive significant effect of some volatile oils of (origanum, thyme eucalypyus, tea tree, clove, lavender, peppermint and sage) as potential control agents for varroa mites in honey bee colonies.

The aim of this work was evaluate the miticidal activity of dried clove buds essential oil and its major component eugenol as potential control against varroa mites in honeybee colonies.

MATERIALS AND METHODS

Source of samples:

Dried clove buds (*Syzygium aromaticum)* was obtained from local markets in Egypt during 2002.

Extraction of the essential oil:

Essential oil was extracted from dried clove buds using steam distillation apparatus for 3hr then yield oil collected over sodium sulphate anhydrous for drying.

Chemicals:

Eugenol (99%) was purchased from Sigma Chemical Co. (St.Louis, MO). The other chemicals used throughout the present work were purchased from different companies.

GC-MS analysis of essential oil:

Gas Chromatography-Mass Spectroscopy was used for identification of components of essential oil according to (Adams, 1995). Essential oil of dried clove buds analyzed as described by (Mona Al-Shalaby and Hanaa Ali, 2001). Analytical GC/MS was carried out on a HP spectroscopy 6890 series with HP selective detector 5973, under the control of a HP chemstation version A02.12 data system. A carbowax capillary column, 50m × 0.53mm I.D., 1.5 m thickness (HP company, U.S.A) was used with helium as carrier gas (flow rate 1.5ml/min). Sample was injected using the split sampling technique, ratio 1 : 50 with sample amount 1µl. Injection port temperature 280°C. Column temperature was held at 40°C for 5min and then programmed at 3°C/min to 280°C and held these for 20min. Detector temperature: 300°C. Mass spectroscopy operating parameters were: electron ionization at 70 eV, accelerating voltage 10kV and scan M/z range 30-650. The identification of constituents was carried out by comparing retention time with those of authentic reference compounds, or peak-matching library research using the standard mass library (NIST Standard Mass Library).

Laboratory bioassy:

Essential oil from clove buds and its major constituent eugenol was first screened for efficacy as miticides by exposing adult varroa to the volatile fumes of oil and eugenol according to Sammataro *et al.* (1998) methods as follows:

Adult mites were removed from infected bee colonies at bee research station, Faculty of Agriculture, Cairo University, and placed in glass petri dishes (10 mites per dish). All dishes contained a bottom layer of damp tissue paper, covered with a sheet of laboratory parafilm. Small hols were punched in the film to allow evaporation of the water from tissues paper below. A piece of filter paper equal in size to the diameter of the dish was placed on the top of the parafilm sheet. 10, 20, 50, 100 and 200 microliterss of a 50% emulsion solution of each essential oil and eugenol diluted in 50% sucrose solution as food source were placed on a second piece of filter paper attached to the lid of the Petri dish. Control dishes contained only 50% sucrose solution on the filter paper. (Triton-20 was added to the solution as a emulsifier). Each treatment for essential oil, eugenol and control were replicated two times for each concentration in petri dishes as described above (five dishes for each concentration).

All petri dishes were placed in an incubator set at 35±1°C for 6hr. The number of live mites at each 2hr interval was counted. A t-test was performed to determine if mortality in dishes with the treatment differed from control.

Field Experiments:

The results for lab bioassay indicated that (eugenol) was not effective enough to control varroa under all conditions, thus whole essential oil was tested in spring season during two consecutive years 2002, 2003 in bee research station, Faculty of Agriculture, Cairo University as follows:
Forty infected colonies were treated by two methods of application, spraying and evaporating trails. In addition, ten colonies were used as control.

Spraying trails:

Spraying trails were done according to method described by Fathy and Fouly (1997) as follows: six infected colonies (for each treatment) of natural hybrid between Carniolan and Egyptian bees were chosen randomly in the apiary. For each treatment three replicates were used. Two concentrations of emulsion solution 5 and 10% (w/v) of essential oil were prepared. The measured weight was dissolved in a 50% (w/v) of sucrose and Triton-20 was added to the solution as an emulsifier. For spraying trail, a hand sprayer was used and adjusted to yield 5 and 10g for each face of the comb, and so each comb in each treated colony get 10g of prepared solution with first concentration and 20g of the second prepared solution.

Evaporating trails:

A piece of carton 5×3cm was soaked in the two concentrations of emulsion solution (5 and 10% w/v) of essential oil for approximately one hour, then put on the top of the combs in the hives.

For spraying and evaporating trails colonies were split to form one deep super hives, provided with a new queen and managed normally and easy to count varroa mites before and after treatments. Teatments were repeated four times with four-day intervals. The control of all treatments was 50% sucrose as food source. Sticky plastic sheets were placed on the bottom boards of the chosen hives and collected on 15 June and replaced with new ones. Boards were recovered and replaced on 20, 25 June and 1 and 7 July. Mites counts on sticky plastic sheets board after the essential oil treatments application were divided by the number of mites counted prior to the treatment (mites after treatment/mites before treatment = mites ratio). Mites' ratio values greater than one indicate a greater numbers of mites counted on the sticky plastic sheets board after applying the essential oil.

The number of dead mites was recorded through out the last week experiment.

Sensory evaluation:

`All the honey samples after every year were subjected to a panel testing, using 20 trained members from the faculty staff and staff of bee research station, different age, sex groups. Samples were taken random then panelists evaluated for odor and taste.

Statistical analysis:

Statistical analysis of data was achieved using the analysis of variance (t-test) within groups and between groups as described by Knapp and Miller (1992).

RESULTS AND DISCUSSION

Chemical composition of clove buds essential oil was analyzed by GC-MS and the results are summarized in table (1). The essential oil yield in the dried buds was 12.7±0.07g/100g sample after steam distillation. Thirty-four volatile components were identified (95.89%) and eight components were unknown (4.11%). Particularly, the major constituent was eugenol (*cis-*

eugenol 18.02% and *trans*-eugenol 3.86%). The other main compounds were chavicol (6.87%), α-pinene (5.77%), junipene (5.46%), fenchone (4.94%), anethole (4.76%), limonene (4.65%) β-caryophyllene (4.16%) and carvone (3.99%). Addition to tewleve compounds were recorded as minor constituents (less than 4%) i.e, α-copene (3.45%), 1,8 cineole (3.24%), β-pinene (3.23%), humulone (3.22%), octaborane (2.76%), 8-dodecenal (2.76%), *p*-cymene (2.67%), *cis*-fernesol (1.99%), γ-terpinene (1.84%), β-cubebene (1.32%), heptadecene (1.11%) and sabinene (1.00%). Thirty compounds were identified as traces constituents (less than 1%) such as acetaldehyde (0.77%), heptanone (.07%), octyl acetate (0.26%), δ-guaiene (0.19%), azetidinone (0.30%), cyclo dodecanol (0.65%), butanoic (0.36%) norbornanone (0.12%) nonenol (0.71%), β-selinene 0.67%, pentadecane (.04%), cedranone (0.67%) and 8-dodecanal (0.65%).

In general these results is agree with previously studies (Masada, 1976, Gopalarishnan *et al.*, 1988, Sangwan *et al.*, 1990, Al-Shalaby Mona and Ali, 2001 and El-Ghoraby and El- Massry, 2003). The percentages of the major and minor components are differed with our previously analysis (Al-Shalaby Mona and Ali, 2001). These differences may be due to the climatic and storage conditions of clove buds which could widely influence qualitative composition of the oil.

Biological experiments:

In lab bioassy, which tested the activity of essential oil of clove and its major compound eugenol on varroa mite mortality. The average mortality in the controls ranged between 10.2 and 21 percent, this mortality was to be expected because these parasites out of this natural habitat and placed in an artificial environment. The results of lab bioaasy are summarizedin table (2). The concentrations of clove essential oil caused significantly more varroa mortality than the corresponding control. The average ratios were 19.20, 25.1, 44.06, 54.12 anad 70.50 with 10, 20, 50, 100, 200 microliters respectively. Eugenol caused non-significant effect until 200 microliters. The average ratios were 14.3, 13.1, 11.4, 23.6 and 19.2 with 10, 20, 50, 100, 200 microliters respectively. This due to the major essential oil constituent eugenol alone may not be selective enough to kill varroa mites under lab conditions. The high percentage of oxygenated compounds (61.68%) in this oil leads us to believe that this oil could possess a useful acaricidal effect on honeybee colonies (Lindberg *et al.*, 2000).

Filed experiments:

After 4 treatment with essential oil at spring season during two consecutive years 2002, 2003 because in colder weather the effectiveness of some oils may not be enough to kill or repel mites (Sammataro *et al.*, 1998). The results obtained from lab bioassay showed that eugenol was not effective against varroa mites compared with whole essential oil, thus essential oil was used in filed tests by two methods spraying and evaporation. Data in Table (3) showed that essential oil has significant different from control in its effects as a miticide in evaporating trail with concentration 5% and 10%. The average ratio of varroa counted on sticky boards in a colony after exposure to essential was 50.38 and 71.42 respectively. In spraying trail

with 5% and 10% (w/v) the average ratio in varroa mite mortality were 12.12, 20.1%, respectively. The direct contact to the essential and mite showed significant activity in causing varroa to dislodge from adult honey bees. These due to essential oil may repel the mites or narcotized them so that they could not longer cling to their hosts (Sammataro *et al.*, 1998). Compared with corresponding control and the average ratio of varroa mites mortality in control ranged from 10.3 to 22.1% may be due to the exposure of varroa to any sprayed solution (distilled water) may caused some varroa to detach (Eizen *et al.* 2001). Also honeybee mortality was low or absent in all experiment with clove oil.

Table (1): GC-MS analysis of dry clove buds essential oil.

Compound	M+(m/z)	Base peak	Rt	Percentage %
Acetaldehyde	44	29	3.98	0.77
P-cymene	134	91	8.98	2.67
Heptanone	112	54	10.07	0.07
β-pinene	136	93	11.74	3.23
Octyl acetate	172	48	12.07	0.26
δ-Guaiene	204	105	12.33	0.19
Sabinene	138	98	13.29	1.00
α-pinene	136	93	13.45	5.77
Heptadecene	238	57	13.86	1.11
Azetidinone	116	56	14.09	0.30
limonene	136	93	15.46	4.65
1,8 cineole	154	81	15.74	3.24
α-copene	204	161	16.10	3.45
γ-terpinene	136	121	16.65	1.84
Cyclo dodecanol	166	55	16.82	0.65
Butanoic	88	60	17.31	0.36
Octaborane	98	93	17.66	2.76
Fenchone	152	81	17.96	4.94
Norbornanone	113	68	18.81	0.12
Nonenol	142	57	21.80	0.71
trans-eugenol	164	103	22.28	3.87
Carvone	150	82	23.77	3.99
β-cubebene	204	119	25.54	1.32
β-selinene	208	109	29.91	0.67
β-caryophyllene	204	93	30.29	4.16
Junipene	204	161	31.25	5.46
Pentadecane	206	80	31.57	0.04
Anethole	148	77	32.11	4.76
Chavicol	134	91	33.10	6.87
8-dodecenal	182	41	34.09	2.76
cis-eugenol	164	131	35.97	18.02
Cedranone	220	205	36.60	0.67
Humulone	362	182	37.43	3.22
Cis-fernesol	222	81	38.98	1.99
Unknowns				4.11
Total				100

Table (2): Average percent mortality± S.E. Varroa mite mortality from exposure to the volatiles of clone essential oil and eugenol for 6 hours in the lab assay.

Concentration (microliters)	Control	Treatment
I) Clove essential oil		
10	13.1±1.8	19.20±2.6
20	12.5±2.9	25.1±2.1
50	10.2±1.1	44.06±4.1
100	20.2±4.1	54.12±2.1
200	18.5±1.9	70.50±2.3
II) Eugenol		
10	12.1±1.6	14.3±1.4
20	10.5±2.1	13.1±2.5
50	11.2±1.3	11.4±1.0
100	21.0±1.3	23.6±4.5
200	17.9±2.3	19.2±1.9

Also the high amount of essential oil may cause an antifeedant effect on wide species of mites (Moretti *et al.*, 1998). Addition to essential oil of clove contains mono-terpene and sesqui-terpenes components (34.41%) have an antifeedant activity (Wallner, 1995 and Regnauit-Roger and Hamaroui, 1994).

Table (3): Average ratio of varroa counted on sticky boards in a colony after exposure to essential oil at spring season during 2002 and 2003.

Essential oil treatment	Control	Treatment
Spraying with 5% (w/v)	10.1±1.8	12.12±1.6
Spraying with 10% (w/v)	12.5±1.3	20.1±2.1
Evaporating with 5% (w/v)	10.3±3.1	50.38±1.1
Evaporating with 10% (w/v)	22.1±2.1	71.42±3.1

These results are accordance in with that of Calderone (1999) who evaluated the acricidal activity of blend of thymol, eucalptus oil, menthol, comphor and linalool in field trails against *Varroa jacobsonii*. Average of mite mortality was 96.7% in the colonies receiving thymol based blend and 27.5% in the colonies receiving linalool. Also Sammataro *et al.* (1998) claimed that the essential oils of bay, bee calm, cineole, cinnamon, clove, origanum, patchouli, tea tree and thymol were effective miticides against varroa mites in the lab bioassay. Average precent mortality of varroa mites from exposure to the volatilels of essential oils for 6 hours were 75.9%, 32.6, 29.9%, 29.8%, 87.29%, 100%, 83%, 59.4% and 95.7%, respectively. EunHee *et al.* (2003) reported that essential oils of bay, citronell, java, clove buds, clove leaf, lemon grass, nutmeg, oregano, pimento berry and thyme white have acricidal

activity against *Tyrophagus putrescentiae* compared with benzoate and N, N-diethyl-n-toluamide (deet). Over 80% mortilty was observed with oils.

At the same time Calderone and Nasr (1999) found that evaporating methods with essential oil were effective more than spraying method in killing varroa. Also Ritter and Matheson (1993) showed that application methods to kill *Varroa jacobsoni* in hony bee colonies include smoking, atomization by aerosol, spraying dusting, dripping (of aqueous solutions), feeding, evaporation from absorbent materials application via plastic carrier strips.

Similar results were obtained by Mahmoud *et al.* (2002) using essential oil of *Artemisia judaica* to control honeybee parasite *varroa jacobsonii* by two methods of application, spraying and evaporating trails with 5% and 10%. The average ratios of mortality were 13.22 and 22.1 percent in spruing trail and 54.76 and 69.42 percent in evaporating trail with 5% and 10% respectively.

According to food legislation, foreign odours and tastes are not allowed in honey for these reasons it is important to know the perception threshold as clove in honey. In sensory testing (smelling and tasting) by staff of faculty and bee research station it was found that honey didn't give any evidence of clove essential oil application. Whereas artimisia essential oil residue was found in honey (Mahmoud *et al.*, 2002) because of concern regarding honey quality the use of artimisia essential oil can't be recommended for practical application while clove essential oil could be recommended for control honeybee parasite.

Chemical miticides are the first choice of beekeepers because they are easy to use and effectiveness. In this respect Calderone (1999) evaluated the acricidal activity of apistan as chemical acricide and and thymol based blend of natural product and formic acid against *varroa jacobsonii.* Mite mortality averaged 99% in colonies receiving apistan , 70% in the those receiving the thymol blend, 51% in those receiving formic acid and 33% in control. According to prolonged and indiscriminate use of chemical acricides have resulted in honey and wax contamination and the appearance of resistant mites (Thomas *et al.*, 1997). High toxicity of mammalian and serious environment problem of chemical control also natural acricides offer desirable alternative to synthetic acricides (Mourad *et al.*, 2000). After honey analyzed by sensory testing (smelling and tasting) by staff of faculty and bee research station it was found that honey didn't give any evidence of clove after essential oil application. Therefore clove essential oil is recommended for parasite mite control which is safer to honeybee, human and cheaper than acricide apistan (Mourad *et al.*, 2000).

CONCLUSION

As a result of this study clove essential oil decreased the overall population of varroa mites in a colony and the practical results of this study indicate that spraying treatment with essential oil may be useful in lowering varroa population in filed but evaporation treatment indicated that control of mites would probably be best than spraying due to direct contact and after different treatments clove residue in the honey samples is unavoidable due to

their high volatility and according to the world health organization(WHO) clove residues in food are without danger to the consumer. Therefore it could be concluded that clove oil has acarcide effect against varroa mites in honeybee and evaporation technique is more effect than spraying technique in filed experiments in controlling of varroa mites.
Therefore it could be concluded that clove oil could be recommended for mites control, as they are safer and cheaper than acracides apistan.

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نشاط الزيت الطيار للقرنفل كمضاد للاكاروسات واستخدامه فى مقاومة الفاروا فى طوائف نحل العسل

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يعتبر مرض الفاروا من أخطر الأمراض التي تصيب نحل العسل في مصر ويؤدى إلى قلة إنتاجه بشكل واضح وهو عبارة عن مرض اكاروسى مسبب بواسطة طفيل خارجي اسمه الفاروا *Varroa jacobsoni* وهو عبارة عن اكاروس Mite يتبع عائلة Dermanyssidse وهو يتطفل على النحل البالغ والحضنة المقفولة وقد انتشر هذا الاكاروس فى جميع أنحاء العالم فيما عدا استراليا ونيوزيلندا حتى عام ١٩٩٩ وترجع خطورة هذا الطفيل انة يتكاثر بمعدل نمواكبر من العائل ويعمل على تدمير النحل البالغ والغير بالغ وذلك بالتغذية على hemolymph الخاص بالنحل وينقل العديد من الفيروسات الممرضة للنحل وترك هذه الاكاروسات بدون مقاومة تعمل على القضاء على طوائف النحل المختلفة فى مدة زمنية لا تزيد عن سنتان لذلك لابد من إجراء العديد من طرق المقاومة المختلفة منها المقاومة الكيماوية باستخدام المواد العضوية مثل حامض الفورميك والاكساليك والمبيدات الاكاروسية المختلفة وهى اما تستخدم رشا أو تعفيرامثل السينيكار أو مواد تتطاير داخل الخلية مثل الفاروستان ولكن يعاب على استخدام هذه المواد ظهور سلالات مقاومة من الاكاروس لهذه المبيدات علاوة على تلويثها لمنتجات النحل من عسل وحبوب لقاح وغذاء ملكات وبروبوليس لذلك كان لابد من إيجاد وسيلة أخرى للمقاومة باستخدام مواد طبيعية لها تأثيرات عالية على مقاومة الاكاروس وفى نفس الوقت لا تؤثر على منتجات المناحل و بالتالي صحة المستهلك ومن أهم هذه المواد الزيوت الطيارة وفى هذا البحث تم اخذ قرون القرنفل الجافة التي يحتوى على ١٢,٧ % زيت طيار واجرى له تحليل لمكوناته باستخدام جهاز GC/MS فوجد أن الزيت يحتوى على ٣٤ مركب منهم حوالي ثمانية مركبات موجودين بنسب عالية من أهمهم الايجنول وتم إجراء تجربة معملية لمعرفة نشاط الزيت الطيار واهم مركباته الايجنول ضد الاكاروس قي فصل الربيع لسنة ٢٠٠٢ - ٢٠٠٣. وقد وجد أن الزيت الطيار له تأثير واضح في قتل الاكاروسات بعكس الايجنول الذي كان له تأثير مقارب للكنترول لذلك أجريت تجربة حقلية في وحدة النحل بكلية الزراعة-جامعة القاهرة باستخدام تركيزات مختلفة من الزيت الطيار وطريقتين من المقاومة هم الرش والتبخير وقد وجد ان معاملة التبخير أعطيت نتائج أفضل فى مقاومة الفاروا من معاملة الرش ويرجع ذلك الى عملية الاتصال المباشر بين الزيت الطيار والاكاروسات وأيضا تم إجراء اختبارات حسية على العسل الناتج فوجد انه لا يحتوى على اى آثار من زيت القرنفل نظرا لتطايره لذلك يوصى باستخدام زيت القرنفل فى مقاومة الفاروا نظرا لأنه رخيص الثمن وليس له آثار جانبية على منتجات المناحل والنحل وصحة المستهلك.