INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 16, No. 1, p. 126-138, 2020

RESEARCH PAPER OPEN ACCESS

Comparative field efficiency of the extracts of plant materials for controlling *Varroa destructor* in relation to brood development in honey bee (*Apis mellifera*) colonies

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Key words: Honey bee, *Apis mellifera*, Ethanol extracts of plant, Mortality of mites, Pakistan.

http://dx.doi.org/10.12692/ijb/16.1.126-138 Article published on January 15, 2020

Abstract

Plant extracts are non-chemical compounds which play a vital role in ectoparasitic mite control and are considered safe to human being and bees. In this regard, a research experiment was laid out at Honeybee Research Institute, National Agricultural Research Centre, Islamabad, Pakistan in field conditions for evaluating comparative effectiveness of ethanolic plant extracts (basil, garlic, lemon, lemongrass and thyme) at three concentrations (500, 400 and 200 ppm) to reduce varroa damaging bee colonies (*Apis mellifera*). Data was recorded on reduction per cent infestation of *V. destructor* on adult bees and brood, dead fallen varroa per colony, mean percentage of mite mortality and sealed worker brood area after four treatment applications in experimental bee colonies. Results showed that all plant extracts at 500 ppm concentrations performed better against varroa mite as compared to control. The extract of these plants were found also safe to *A. mellifera*. The highest (143 ± 1.20) dead fallen varrroa and the mean 82.11% mortality of mite was recorded with the extract of lemongrass after four treatments application and was found significant from all other treatments. Similarly, maximum brood area of worker (1207.4 ± 19.63 cm^2) at 500 ppm concentration of lemongrass was recorded in *V. destructor* infested colonies. Ethanol extracts of lemongrass and thyme proved the best and can be efficiently applied against varroa to increase honey production in field conditions.

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Introduction

Honey bees health play an important role in honey production and crop pollination throughout the world. European honey bee *Apis mellifera* L. health is on risk by various pests and diseases. *Varroa destructor* (Acari: Varroidae) is one of most serious ectoparasitic mite and the major limiting factor in production of high quality honey and by products damaging honey bee colonies resulting in huge monetary loss to the beekeeping industry and agriculture worldwide as well as in Pakistan (Guzmán-Novoa *et al*., 2010; Aziz *et al.,* 2015). Varroa feeds on adult bee's haemolymph and on bee brood and this feeding activity can result in weakness, colony disorder; severe deformations of the wings, loss of 25% adult body weight and reduced longevity of workers and drones (Wilfert *et al*., 2016). Parasitism of bee colonies by varroa decreased the population of worker bees significantly and thus may be died eventually if mites are not controlled (Murilhas, 2002). *V. destructor* also acts as vectors lethal honey bee viruses (Gisder *et al*., 2009) which affect honey bee immunity due to increased pathogenicity and virulence making them susceptible to various pathogens (Yang and Cox-Foster, 2005). For reducing varroa population under economic injury level honey bees should be treated one or two times in a year to control varroa for their survival and higher honey production.

Different acaricides, methods and techniques have been applied for controlling ectoparasitic mite varroa in recent years but none of them are effective, safe and easy to use in *A. mellifera* colonies. Chemicals used for mites control categorized as hard and soft chemicals (Rosenkranz *et al*., 2010). Hard chemicals include products such as fluvalinate, coumaphos, flumethrin and amitraz which were used widely with variable efficacy in the last decades. They exhibit harmful effects on honey bees health by reducing the ability of a colony for producing the bee queens and the males bees are not capable for producing the cells of sperms (Burley *et al.,* 2008; Mullin *et al*., 2010) and acaricides residue in hive products (Wallner, 1999) with potential health risk to consumers (Radakovic *et al*., 2013). Moreover, extensive use of chemicals may lead to the development of resistance against flumethrin, coumaphos, fluvalinate and amitraz in the population of *V. destructor* (Skerl *et al.,* 2011). Therefore, due to the increased resistance in synthetic acaricides to *V. destructor* nowadays researchers have driven considerable interest in the use of natural soft chemicals such as plant extracts and their components in controlling parasitic mites of honey bee. Besides, when compared with synthetic acaricides plant extracts have low toxicity to mammals and non-target organisms, safe, low environmental impact, no residues are left in hive products and volatized rapidly (Damiani *et al*., 2011). Different research studies have been conducted on the use of plant extracts and their acaricidal efficacy were observed by many researchers in different countries of the world. Thyme extract has been observed to be highly effective in causing mortality of mites in treated bee colonies. Most common sub-lethal effects against *V. destructor* by using natural products recorded were repellent, inhibition in reproduction and the most common is narcosis (Rahimi *et. al.,* 2017). A natural substance which changes the behavior of feeding, mite reproduction and development in a colony of bee is helpful to control varroa. These natural acaricides exhibit lower risk in hive products contamination (Floris *et al*., 2004; Bogdanov, 2006) and lesser chance of mite resistance induced due to repeated treatment applications. Little research conducted on the usage of botanical extracts for controlling varroa mite in *A. mellifera* in field conditions in Pakistan and their results have not been conclusive. Our study objective was to evaluate the miticidal efficacy of *Cymbopagon citrate, Thymus linearus, Ocimum basilicum, Citrus limon* and *Allium sativum* extracts to control *V. destructor* in apiary of European honey (*A. mellifera*) colonies.

Material and methods

Selection of honey bee, Apis mellifera colonies

Research study was carried out on fifty four infested colonies of *A. mellifera* with varroa mites in a single body standard Langstroth hives having removable

bottom boards at Honeybee Research Institute (NARC) Islamabad, Pakistan from December 2013-May 2014. Experimental bee colonies were equalized on the basis of adult worker bee population (7-9 frames with bees), sealed worker brood frames (3-4) and quantity of food stored (1-2 honey frames) according to the method of Delaplane *et al.*, (2013) and the mite infestation levels. Tested bee colonies were divided into six grouping, five groups for treatments and one group for control. Three colonies were used for each concentration of plant extracts with three replications to every treatment. Three colonies of bees were considered for (untreated) control (Abou Elenain *et al.,* 2014). During experimental duration, honeybee colonies were also checked regularly for food, or any detrimental effect of plant extracts on honeybees, performance and development. All the experimental colonies were given sugar supplemental feeding @ 1:1 at weekly interval in frame feeder from December, 2013 to January 2014 due to the non-availability of food.

Plant materials and their parts used for plant extracts

Miticidal efficiency of lemon, garlic, lemongrass, basil and thyme ethanol plant extracts at three concentrations of 200, 400 and 500 ppm with three colonies for each treatment was investigated in this research study under field conditions. The following parts of five plants were used in preparation of plant extracts:

Method used for plant extraction

Various components of five plants *viz.*, lemongrass, thyme, basil, lemon and garlic were dried in shade and make powdered. Extract of plants were prepared using ethanol and distilled water from each plant material were placed in a 2 L capacity. In Erlenmeyer flask of 2 liter, 200 gram powdered of each plant were combined with 1000 ml of 50% ethanol. All samples were shaked for 24 hours on 120 rpm. Extracts were filtered and concentrated using rotary evaporator under reduced pressure. The resulting residues were re-dissolved in appropriate solvents. Solutions of the ethanolic extracts were placed on 4°C till further testing, while water extracts were freeze.

Plant extracts field application

Plant extracts 100 ml sprayed on adult workers and worker sealed brood with the help of 500 ml hand held sprayer. Each treatment was applied at seven day intervals four times. In control treatment ethanol (2 ml) only was used. All treatments were assigned randomly in infested bee colonies with varroa.

Adult worker bee's infestation

Mite infestation on adult bees was determined by 70% alcohol wash method. From each honey bee colonies 100 bees were randomly collected from worker brood frames in jar having 70% alcohol and kept overnight for 24 hours. For one minute the bees were then hand shaken in 70 per cent alcohol solution and rinsed with tape water and then passing the bees and alcohol through a 3 mm sieve. The infestation % was calculated as follows (Alloui *et al.*, 2002):

$$\text{Infestation \%} = \frac{\text{No. of mites}}{\text{No. bees}} \times 100$$

Degree of infestation of worker brood

Varroa infestation in brood was determined at pupal stage by examine 50 infested cells of brood randomly selected after treatments for mite infestation level (Satta *et al.*, 2005). From the number of varroa mites observed in brood cells, the degree of infestation observed was worked out by the formula given by Ritter (1980).

$$\text{Infestation of brood (\%)} = \frac{\text{Number of mites in the brood cell examined}}{\text{Number of brood cell examined}} \times 100$$

Varroa mites fall on screened bottom board

For recording varroa mites from treated with plant extracts and control colonies were counted after each treatment on white formica sheet under mesh screen at the bottom of each hive (Gregoric and Planinc, 2005). After application of each treatment, dead fallen varroa mites were counted after one week interval from the mite collection trays that were placed inside the bottom boards. Effectiveness of each treatment was calculated by the formula Allam *et al.*, (2003):

$$\text{Efficacy (\%)} = \frac{\text{Number of dead fallen mites}}{\text{Total No. of fallen mites}} \times 100$$

Measurement of brood area in cm²

After one month treatment application of plant extracts sealed worker brood area in square inches was measured with a scale and changed into cm² with a formula given by Ismail *et al.*, 2006.

Data analysis

Data regarding degree infestation of varroa on honey bee brood and adult bees, mites fall on formica sheet and brood area were subjected to Completely Randomized Design (CRD) in a two factor factorial with Statistix software version 8.1. For separation of means (LSD) Least Significant Difference at $p \leq 0.01$was used.

Results and discussion

Reduction in (%) infestation of adult bees

Percentage adult bee's infestation of varroa mites with ethanol plant extract was reduced from the first week to the last week. Data showed 13.0 ± 0.69 to 5.2 ± 0.23%, 15.6 ± 0.52 to 7.8 ± 0.35%, 18.2 ± 0.64 to 10.5 ± 0.17%, 20.8 ± 0.40 to 13.0 ± 0.29% and 23.5 ± 0.46 to 15.7 ± 0.32% lemongrass, thyme, garlic, lemon and basil, respectively. Similarly, at concentration of 400 ppm of lemongrass infestation rate of varroa mites was decreased from 10.5 ± 0.35 to 2.9 ± 0.32%, (13.2 ± 0.49) and (5.3 ± 0.29%) thyme, (15.7 ± 0.46) and (7.8 ± 0.26%) garlic, (18.4 ± 0.58) and (10.5 ± 0.35%) lemon and (21.0 ± 0.64) and (13.1 ± 0.52%) basil respectively. Percent rate of *V. destructor* infestation reduced against 500 ppm from 7.9 ± 0.29 to 0.5 ± 0.06%, 10.5 ± 0.35 to 2.6 ± 0.17%, 13.0 ± 0.69 to 4.7 ± 0.46%, 15.7 ± 0.64 to 6.9 ± 0.40% and 18.3 ± 0.52 to 8.9 ± 0.35% against lemongrass, thyme, garlic, lemon and basil extracts from applying the extracts from the first to the fourth week of treatment, respectively (Figs 1, 2, 3 and 4). While in untreated colonies varroa infestation increased (26.2 ± 0.75%) to (33.5 ± 0.58%) on adult bees during experiment time. Data indicated that there was a significant difference among the colonies treated with other plant extract treatments. Our results are in agreement with those obtained by Razavi *et al.*, (2015) who reported that 500 ppm methanolic extracts of *L. latifolium* and *Z. multiflora* decreased *V. destructor* mites infestation (0.0%) and (13.74%) on adult bees after four plant extract treatment applications under field conditions.

Table 1. Different plants and their parts used for extracts

S. No.	Common Name	Botanical Name	Parts used
1	Lemongrass	*Cymbopagon citrate*	Leaves
2	Thyme	*Thymus linearus*	Whole plant
3	Basil	*Ocimum basilicum*	Leaves and flowers
4	Lemon	*Citrus limon*	Whole fruits
5	Garlic	*Allium sativum*	Bulb

Reduction in infestation of worker brood

Field application results of extracts on mortality percentage of varroa reduction in brood of workers are shown in Fig. 5 to 8. Mean data shows that after treatment with 200 ppm concentration of different plant extracts varroa mite infestation reduced slowly on brood after 4th treatment. Results indicated that at 200 ppm concentration lemongrass extract mite infestation (17.0 ± 0.53) decreased significantly to (9.0 ± 0.38%), thyme (20.0 ± 0.90) and (11.5 ± 0.40%), garlic (23.3 ± 0.69) and (14.5 0.35 %,) lemon (26.6 ± 0.46) and (17.7 ± 0.23%) and basil (29.5 ± 0.35) and (20.7± 0.46%) 1st to 4th treatment. Similarly, with lemongrass extract at 400 ppm treated bee colonies the highest percent reduction rate (14.5 ± 0.52) and (6.3 ± 0.46%) recorded in mite on brood of worker. A minimal rate of reduction in infestation percentage recorded with the extract of basil was 27.0 ± 0.64 to 18.0 ± 0.59%.

The results obtained showed significantly maximum 12.0 ± 0.25 to 3.0 ± 0.29% mite reduction was

observed at 500 ppm of lemongrass after first to 4th treatment. Whereas the minimum 24.2 ± 0.52 to 12.3 ± 0.64% infestation reduction of mite was noticed in honey bee colonies treated with 500 ppm basil extract. This percentage was increased from 32.3 ± 0.92 to 40.0 ± 0.87% in untreated (control) colonies during the period of experiment.

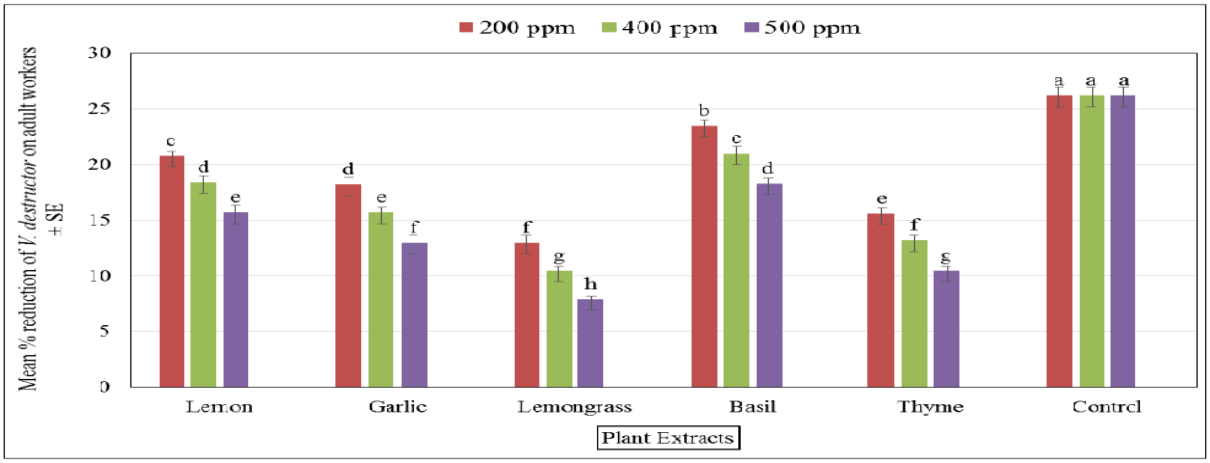

Fig. 1. Mean % reduction of infestation of *Varroa destructor* on adult workers after first.

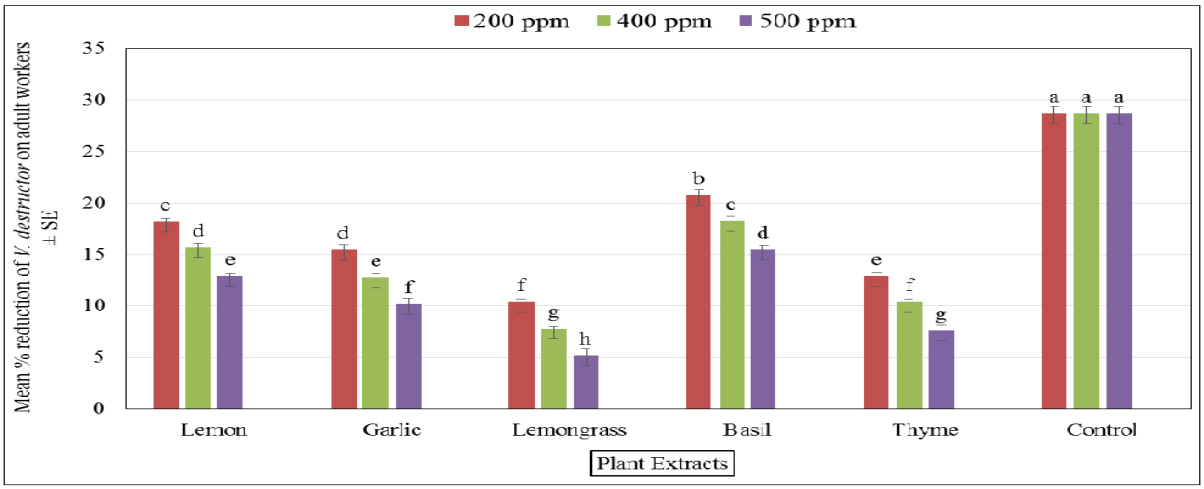

Fig. 2. Mean % reduction of infestation of *Varroa destructor* on adult workers after second treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

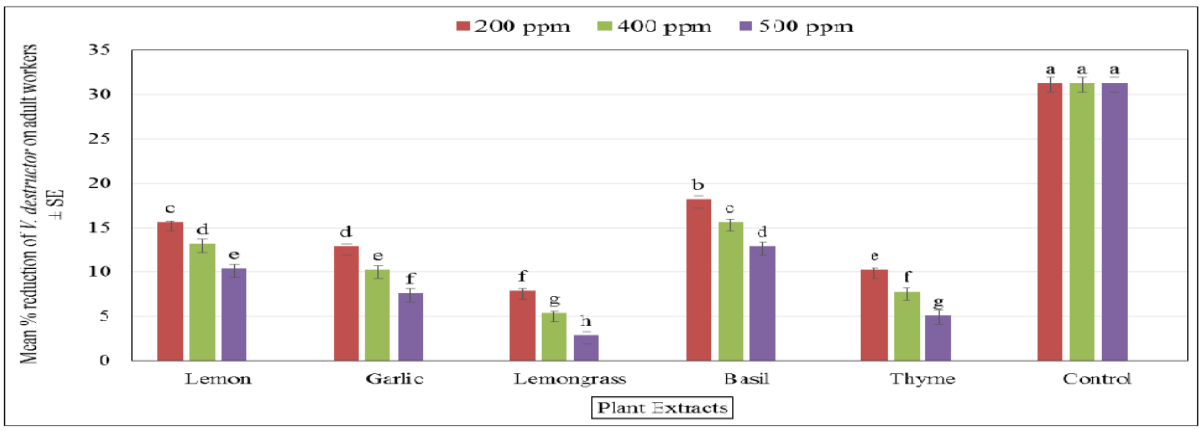

Fig. 3. Mean % reduction of infestation of *Varroa destructor* on adult workers after third treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

Lemongrass and thyme extracts were found best in reducing *V. destructor* population in brood cells in comparison to other plant extracts tested colonies (Figure 5, 6, 7, and 8). Our findings are similar with Satta *et al.*, (2008) and Abdel-Rahman (2008) who observed that medicinal plants have acaricidal efficacy for mites and can be used for safely and effectively against varroa in honey bee colonies.

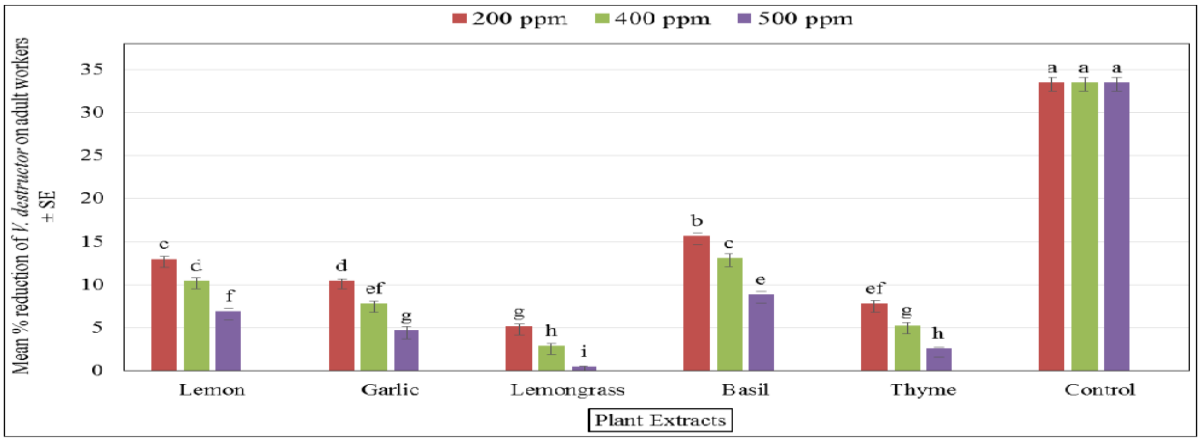

Fig. 4. Mean % reduction of infestation of *Varroa destructor* on adult workers after fourth treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

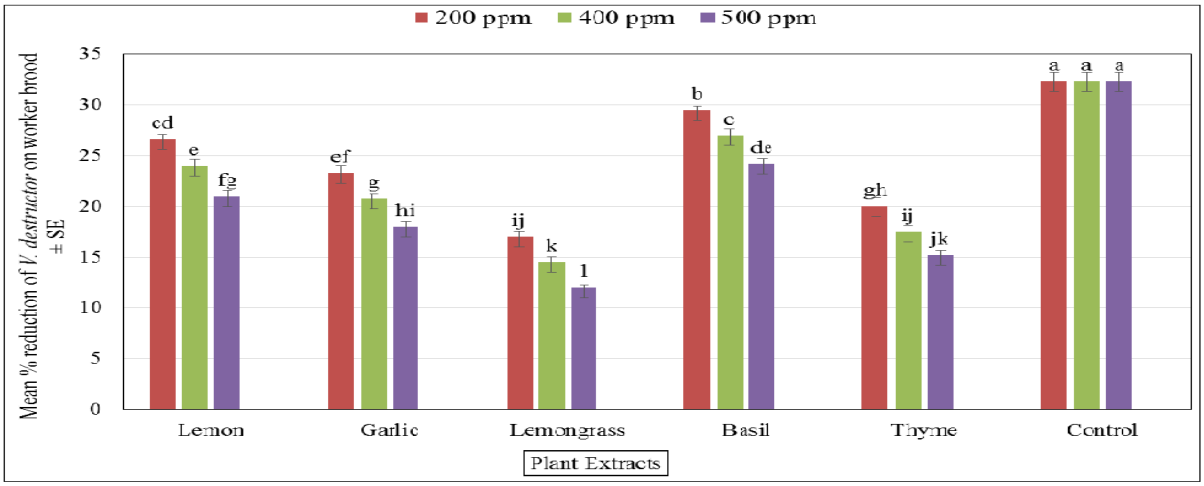

Fig. 5. Mean reduction in infestation percentage of *Varroa destructor* in worker brood cells after first treatment with different concentration of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

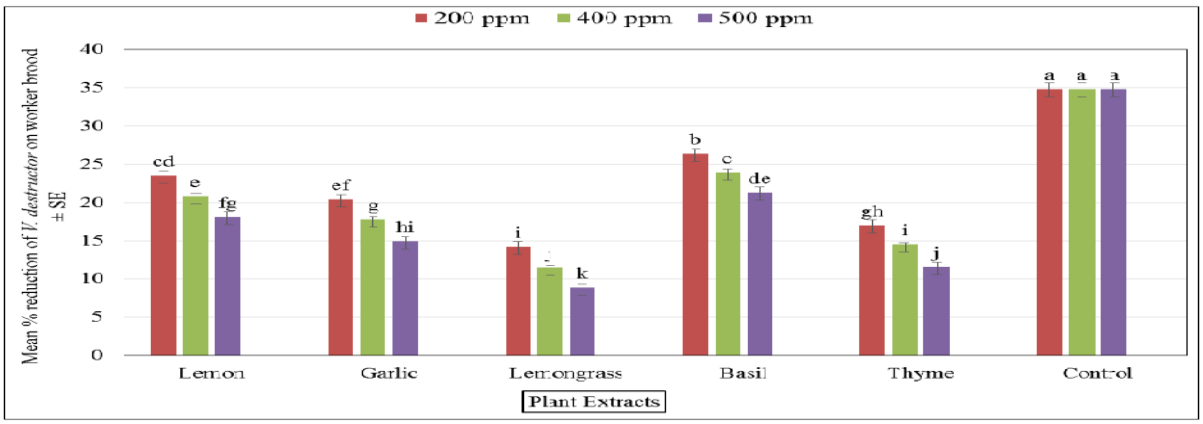

Fig. 6. Mean reduction in infestation percentage of *Varroa destructor* in worker brood cells after second treatment with different concentration of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

Number of dead varroa mites

Mites fall after each plant extract treatments (1st to 4th) are shown in Fig. 9, 10, 11 and 12. When different plant extracts were compared for *V. destructor* mites, statistical differences were observed for dead mites.

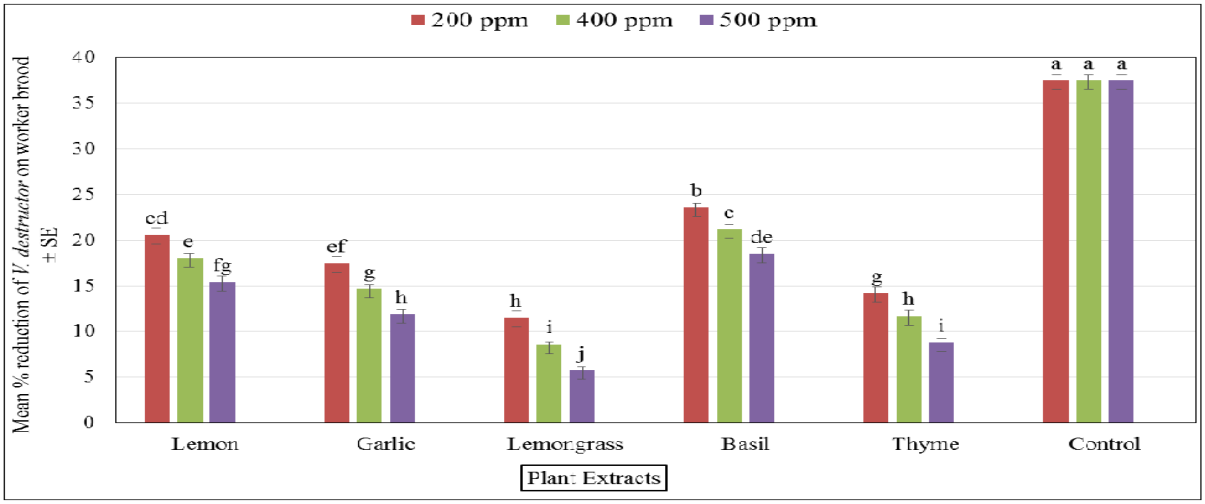

Fig. 7. Mean reduction in infestation percentage of *Varroa destructor* in worker brood cells after third treatment with different concentration of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

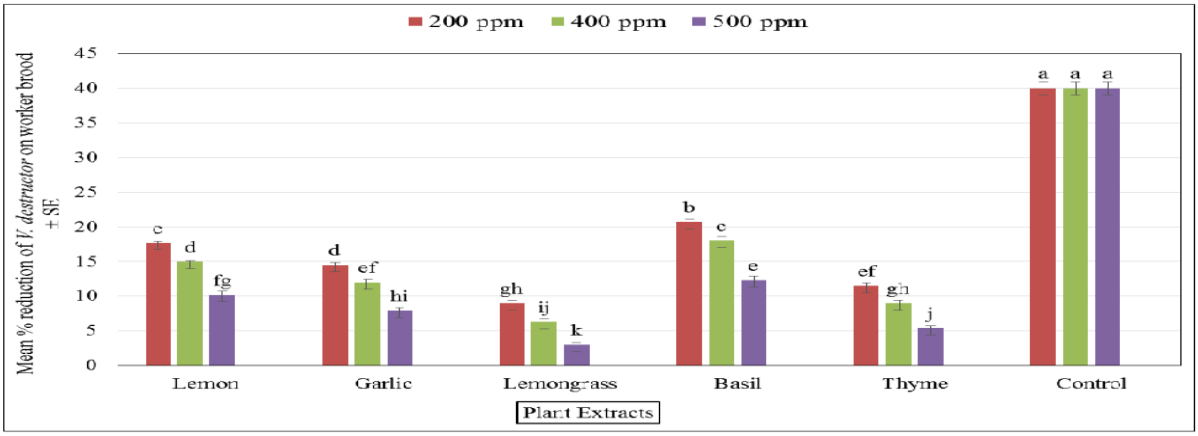

Fig. 8. Mean reduction in infestation percentage of *Varroa destructor* in worker brood cells after fourth treatment with different concentration of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

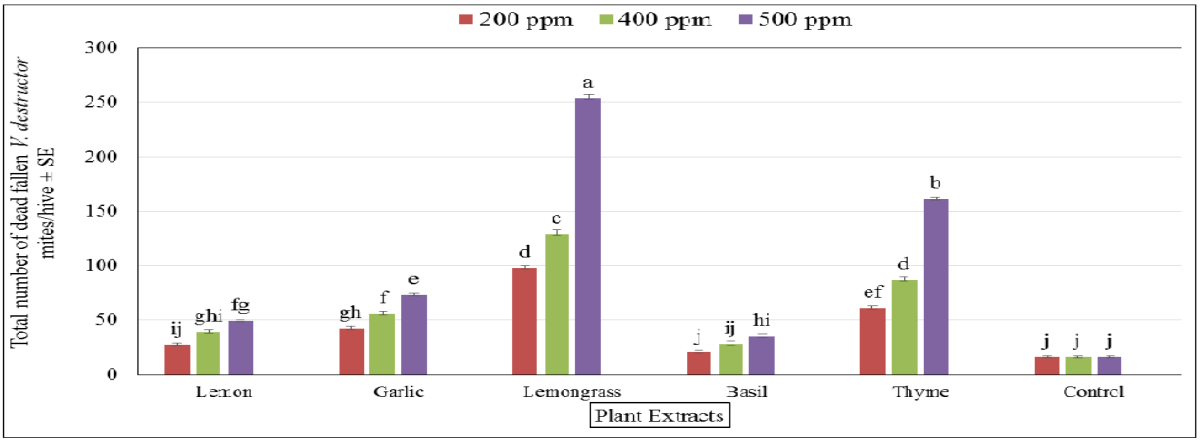

Fig. 9. Total number of fallen dead *Varroa destructor* mites/hive after first treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

Data analysis indicated that each plant extracts indicated that highest total number of 98 ± 2.31, 62 ± 1.73, 56 ± 2.08 and 29 ± 1.73 dead fallen *V. destructor* mites was recorded with lemongrass, 61 ± 2.08, 44 ± 2.31, 39 ± 0.58 and 23 ± 1.15 with thyme, 42 ± 2.52, 31 ± 2.08, 24 ± 1.73 and 23 ± 0.58 with garlic, 27 ± 1.73, 20 ± 1.15, 16 ± 1.00 and 10 ± 0.58 with lemon and 21 ± 1.53, 15 ± 0.58, 8 ± 1.15 and 2 ± 1.00 with basil at 200 ppm treatment respectively.

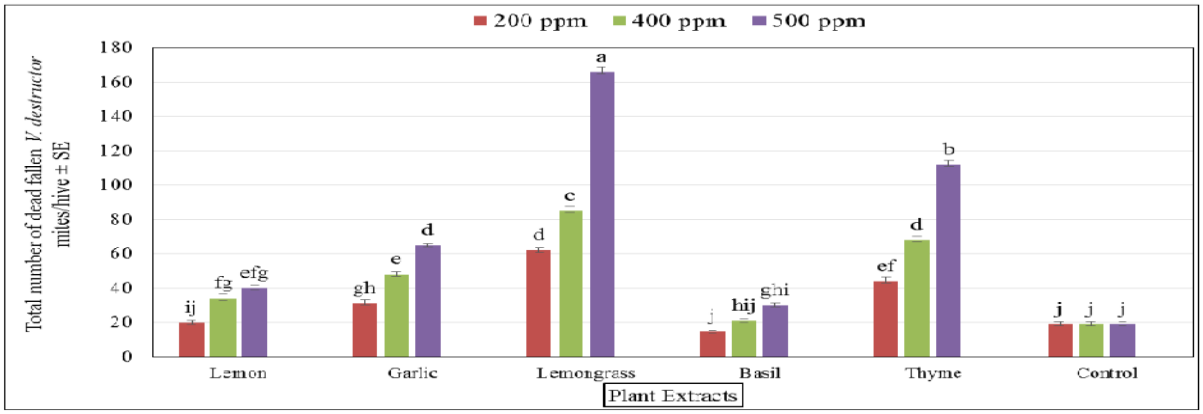

Fig. 10. Total number of fallen dead *V, destructor* mites/hive after second treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

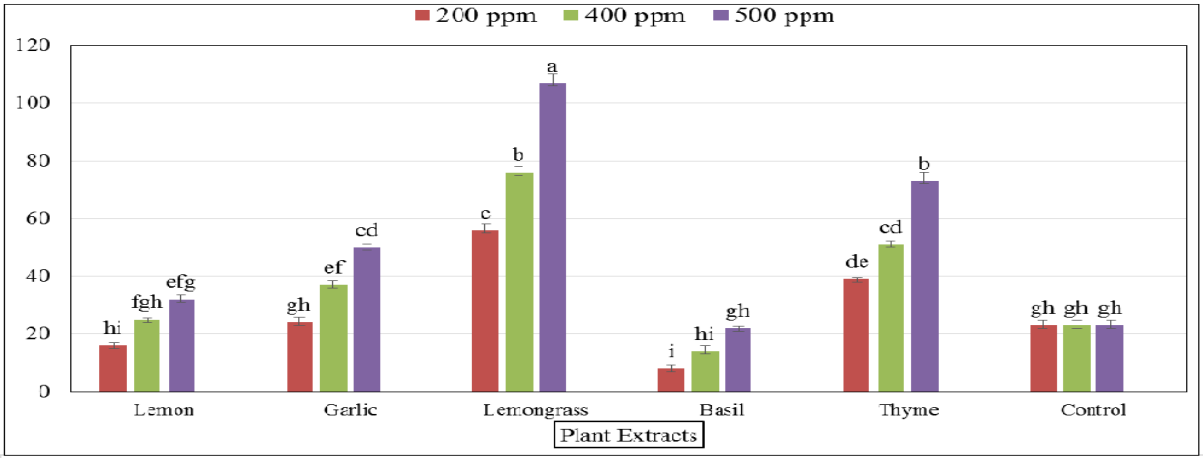

Fig. 11. Total number of fallen dead *Varroa destructor* mites/hive after third treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

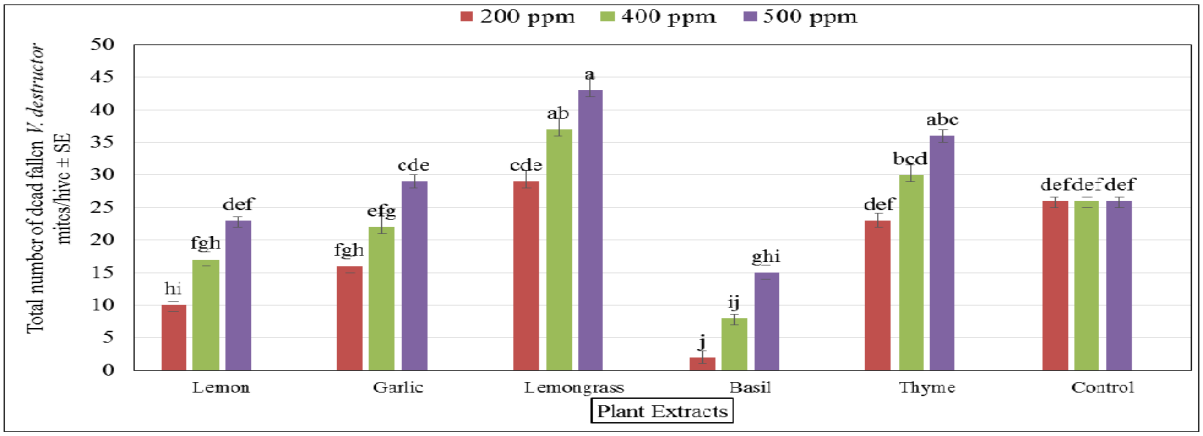

Fig. 12. Total number of fallen dead *Varroa destructor* mites/hive after fourth treatment with different concentrations of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

At 400 ppm concentration of colonies treated with different plant extracts highest fallen mites were 129 ± 3.79, 85 ± 2.65, 76 ± 2.08 and 37 ± 1.73 (lemongrass), 87 ± 2.52, 68 ± 2.08, 51 ± 1.15 and 30 ± 1.53 (thyme), 56 ± 2.31, 48 ± 1.73, 37 ± 1.53 and 22 ± 2.08 (garlic), 39 ± 2.08, 34 ± 2.65, 25 ± 0.58 and 17 ± 1.15 (lemon) and 28 ± 2.65, 21 ± 1.00, 14 ± 2.08 and 8 ± 0.58 (basil) respectively.

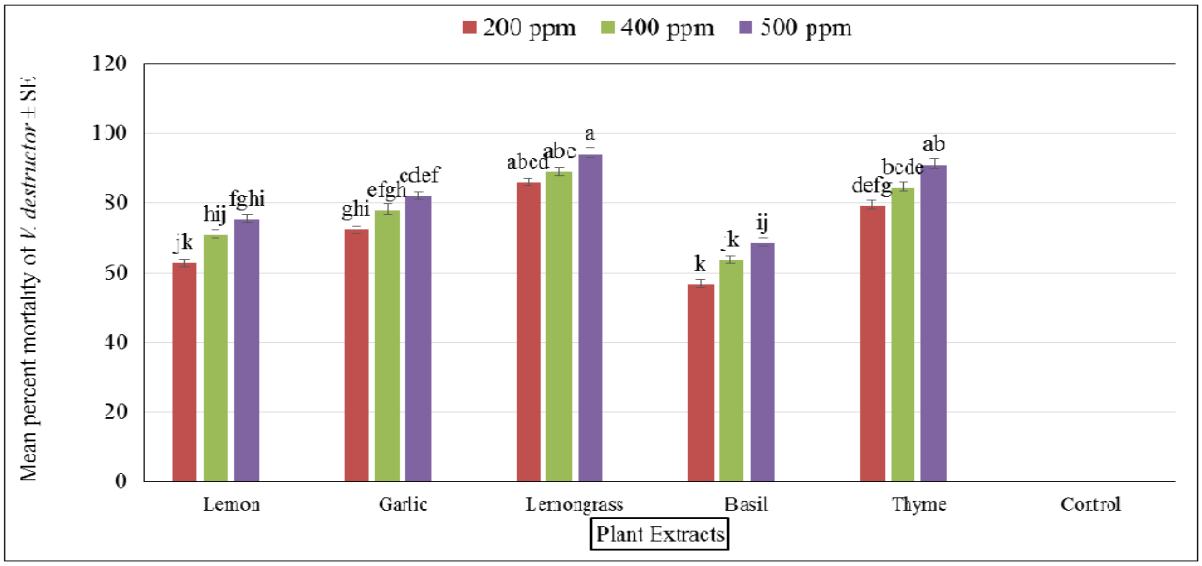

Fig. 13. Efficacy of plant extracts against *Varroa destructor* after first treatment based on percent mortality in *Apis mellifera* colonies (Two way ANOVA using LSD test at p value ≤ 0.01).

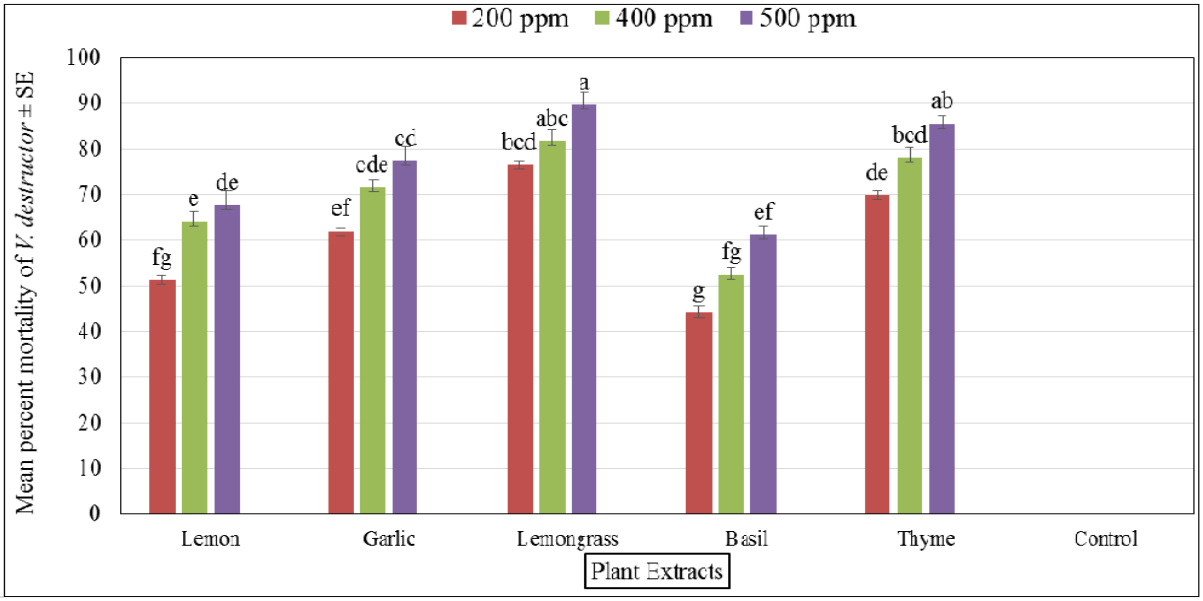

Fig. 14. Efficacy of plant extracts against *Varroa destructor* after second treatment based on percent mortality in *Apis mellifera* colonies (Two way ANOVA using LSD test at p value ≤ 0.01).

Maximum total dead fallen *V. destructor* per hive recorded during 1st to 4th treatment with concentration of 400 ppm of lemongrass extract were (254 ± 3.21, 166 ± 2.89, 107 ± 3.21and 43 ± 2.08) followed by thyme (161 ± 1.53, 112 ± 2.31, 73 ± 2.89 and 36 ± 1.0), garlic (73 ± 1.73, 65 ± 1.0, 50 ± 1.15 and 29 ± 1.0), lemon (49 ± 1.53, 40 ± 1.73, 32 ± 1.53 and 23 ± 0.58) and basil (35 ± 2.31, 30 ± 1.15, 22 ± 0.58 and 15 ± 1.15) respectively.

Statistical data analysis showed that at highest (500 ppm) concentration of extracts observed the

maximum dead fallen varroa mites in comparison to untreated colonies in all the treated colonies than the control during 1st three weeks of treatments. These results are in confirmation with Stangellini and Raybold (2004) and Elbassiouny *et al.*, (2006).

Mortality (%) of Varroa destructor

Highest mortality % of *V. destructor* observed after first treatment applications (200, 400 and 500 ppm) with lemon grass, thyme garlic, lemon and basil was (85.96 ± 1.14, 88.97 ± 1.32, 94.07 ± 1.86%), (79.22 ± 1.64, 84.47 ± 1.47, 90.96 ± 1.84%), (72.41 ± 0.95, 77.78 ± 2.07, 82.02 ± 1.30%), (62.79 ± 0.90, 70.91 ± 1.36, 75.38 ± 1.24%) and (56.76 ± 1.45, 63.64 ± 1.19, 68.63 ± 1.46%), respectively.

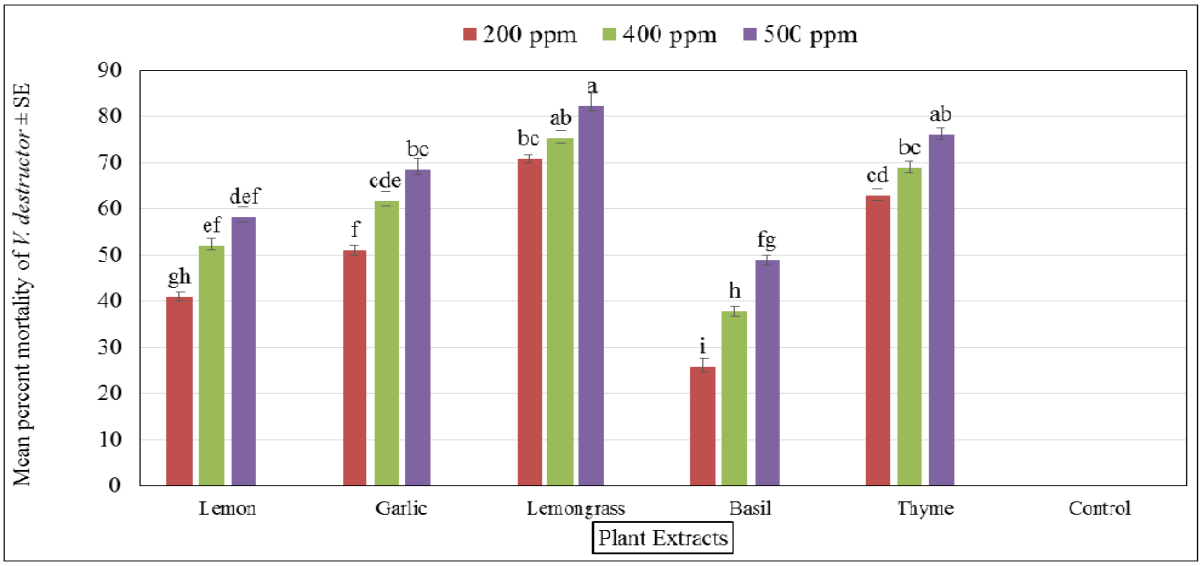

Fig. 15. Efficacy of plant extracts against *Varroa destructor* after third treatment based on percent mortality in *Apis mellifera* colonies (Two way ANOVA using LSD test at p value ≤ 0.01).

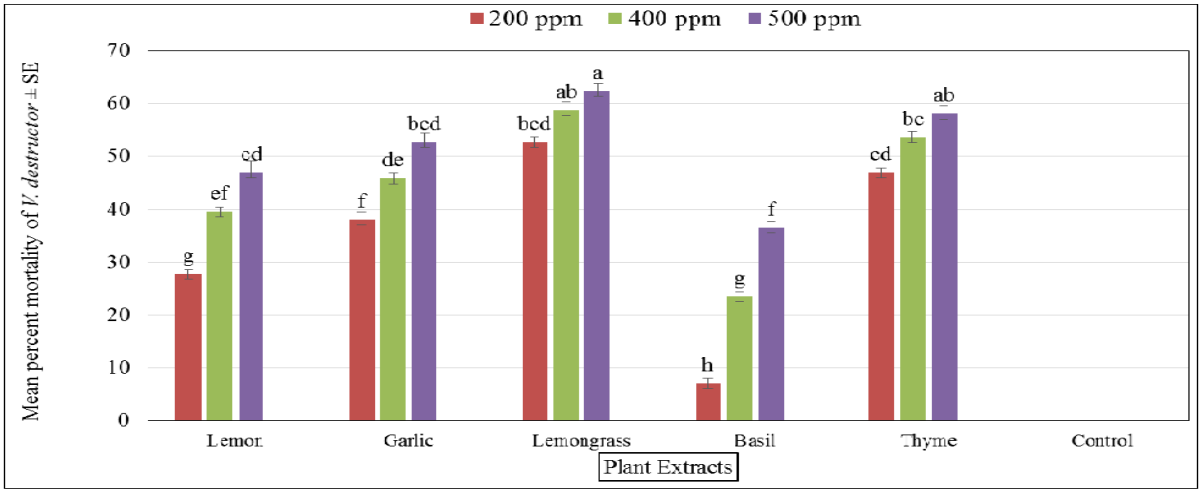

Fig. 16. Efficacy of plant extracts against *Varroa destructor* after fourth treatment based on percent mortality in *Apis mellifera* colonies (Two way ANOVA using LSD test at p value ≤ 0.01).

Plant extracts 2nd treatment showed that maximum mite % mortality was recorded at 200, 400 and 500 ppm found was 76.54 ± 0.74, 81.73 ± 2.40 and 89.73 ± 2.65% by lemongrass, 69.84 ± 1.06, 78.16 ± 2.21 and 85.50 ± 1.75% by thyme, 62.00 ± 0.66, 71.64 ± 1.59 and 77.38 ± 3.09% by garlic, 51.28 ± 0.99, 64.15 ± 2.23 and 67.80 ± 3.04% by lemon and 44.12 ± 1.55, 52.50 ± 1.46 and 61.22 ± 1.80% respectively (Fig. 13). In the third week and fourth week similar mite mortality trend was observed as in 1st and 2nd

treatments. Data showed higher mortality of mites at 500, 400 and 200 ppm of lemongrass, thyme, garlic, lemon and basil (82.31± 2.81, 75.27 ± 1.78 and 70.89 ± 0.90), (76.04 ± 1.41, 68.92 ± 1.46 and 62.90 ± 1.54), (68.49 ± 2.45, 61.67 ± 2.10 and 51.06 ± 1.02), (58.18 ± 2.29, 52.08 ± 1.61 and 41.03 ± 0.98) and (48.89 ± 1.00, 37.84 ± 1.07 and 25.81 ± 1.89) after third treatment, respectively. During the fourth week data trend for lemongrass, thyme, garlic, lemon and basil at 500, 400 and 200 ppm was (62.32 ± 1.48, 58.73 ± 1.51 and52.73 ± 0.95), (58.06 ± 1.43, 53.57 ± 1.17 and 46.94 ± 0.77), (52.73 ± 1.66, 45.83 ± 1.02 and 38.10 ± 1.42), (46.94 ± 2.16, 39.53 ± 0.80 and 27.78 ± 0.89) and (36.59 ± 1.15, 23.53 ± 0.86 and7.14 ± 0.89) after fourth treatment, respectively (Fig. 14, 15 and 16). Razavi *et al*., (2015) used *Lepidium latifolium* and *Zataria multiflora* leaf extracts at 500 ppm in controlling varroa mites which resulted in 100% and 86.26% mite mortality, respectively.

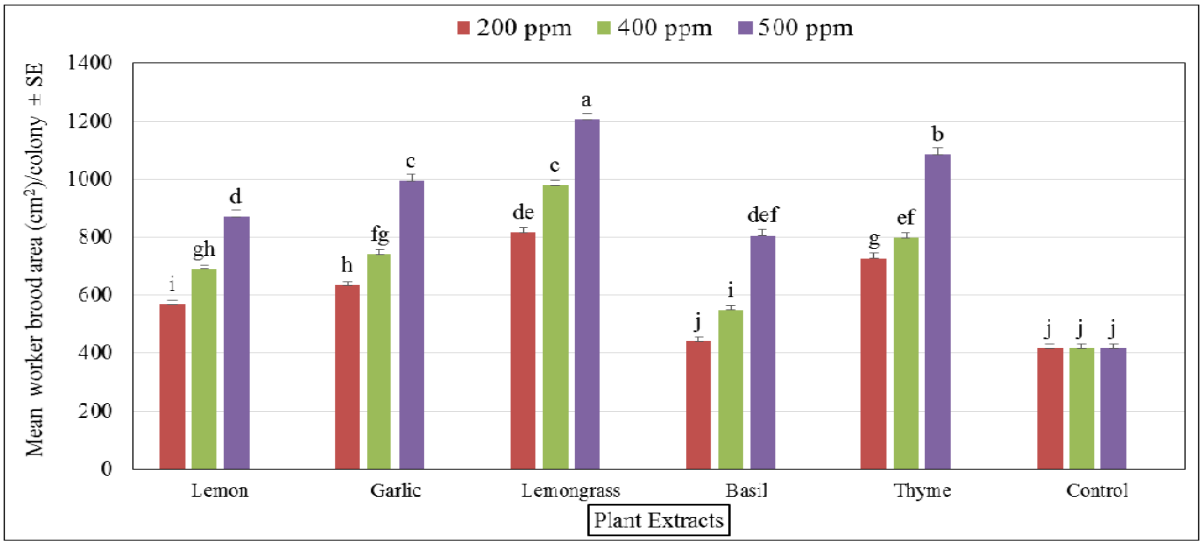

Fig. 17. Mean worker brood area (cm²) per colony after treatment against *Varroa destructor* with three concentration of plant extracts (Two way ANOVA using LSD test at p value ≤ 0.01).

Change in sealed brood area (worker) cm²

Results indicated that treated colonies showed significant difference in brood area. Data clearly showed that in treated colonies sealed worker brood area increased largely. Whereas small brood area was observed in control untreated (treated) colonies. Higher concentration of extracts caused increased area of brood 1207.4 ± 19.63 cm² especially lemongrass (Fig. 17). This finding coincides with Elbassiouny *et al* (2006).

Conclusion

It is concluded from the present study that lemongrass was found very useful in *V. destructor* mite control which indicated in ascending order as thyme > garlic >lemon and >basil. Data also indicated that infestation of varroa was reduced by increasing the concentrations of all plant extracts used in this experiment. Extracts of lemongrass showed highest reduction in varroa mites in sealed worker brood, fallen dead mite on bottom board sheet, percent mortality of mite and large brood area in treated bee colonies.

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