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Evaluation of different controlling agents alone and in combination against *Tropilaelaps clareae* in relation to honey production in *Apis mellifera* colonies

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Key words: *Apis mellifera,* Formic acid, Thymol, Queen caging, *Tropilaelaps clareae*, Mortality of mites, Pakistan

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Abstract

Effectiveness of Formic acid (T_1), Thymol (T_2) and Queen caging (T_3) alone and Formic acid + queen caging + thymol (T_4), Formic acid + thymol (T_5) in combination and Control (T_6) was under Randomized Complete Design were investigated against honeybee ectoparasitic mite, *Tropilaelaps clareae* at Honeybee Research Institute (HBRI), National Agricultural Research Centre (NARC), Islamabad, Pakistan in 2021. Formic acid (15 ml/colony), Thymol (4g/colony), Queen caging (21 days), Formic acid (10 ml/colony) + queen caging (14 days) + thymol (4 g/colony), Formic acid (10 ml/colony) + thymol (4 g/colony) were used in the honeybee, *Apis mellifera* colonies. *Tropilaelaps clareae* mites' infestation was recorded 24 hours before and after 7, 14, and 21 days in worker brood, with total dead fallen mites/hive on screened bottom board having white Formica sheet and Sidr honey production in kg per colony. Results of data analysis showed that all treatments performed well against *Tropilaelaps clareae* mites as compared to untreated control. Formic acid + queen caging + thymol (T_4) used in combination was found the best in mean (98.29%) reducing *Tropilaelaps clareae* worker brood infestation, highest mean total dead fallen (410 mites/colony) and higher differed significantly Sidr (7.6 kg honey/colony) as compared to other treatments and control. Therefore, T_4 was the most effective treatment in reducing the population of *T. clareae* mites. Formic acid + thymol (T_5) in combination was found the second most effective treatment against *T. clareae* mites in this study.

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Introduction

Beekeeping is an important agricultural subsidiary and profitable business in Pakistan. Honeybee, *Apis mellifera* plays an important role in economic, nutritional, and ecological security. These are the most beneficial insects in the world and produce valuable commodities like bee wax, pollen, honey, bee colonies, queen, royal jelly, propolis, and bee venom used in medicine and cosmetics (Tiwari *et al.,* 2014; Qadir *et al.,* 2021). In addition, honeybees also play a key role as a pollinator of crops and entomophilous species all over the world (Hung *et al.,* 2018; Gallai *et al.,* 2009; Klein *et al.,* 2007). However, the population of honeybee (*A. mellifera*) colonies is declining globally as well as in Pakistan and the average honey production per colony/annum is very low which is of great concern due to their role in global food production (Seitz *et al.,* 2016; Steinhauer *et al.,* 2018; Topal *et al.,* 2019; vanEngelsdorp and Meixner, 2010). This decline in bee population is not due to a single factor but more than one factor combines to devastate bee health.

Managed honeybee colonies are exposed to different types of diseases and pests. Currently, ectoparasitic *Tropilaelaps clareae* and *Varroa destructor* are the key threatening issue for the beekeeping industry in Pakistan (Alam *et al.,* 2022; Islam *et al.,* 2020; Islam *et al.,* 2017; Mahmood *et al.* 2014a; Qadir *et al.,* 2021) as well as in the world (Guzman-Novoa *et al.,* 2010; McMenamin and Genersch 2015; Jack and Ellis 2021). *T. clareae* and *V. destructor* have damaged thousands of bee colonies resulting in billions of dollars of huge economic loss worldwide (Lattorff *et al.,* 2015; Welsh, 2012). In Pakistan, *T. clareae* was recorded for the first time in 1981. After that these mites extend in 1991 to *A. mellifera* by migratory beekeepers throughout the country. For harvesting different types of honey where the best honey flora is available beekeepers are shifting their colonies on that flora. Due to the highest infestation of *T. clareae* heavy losses of colonies of *A. mellifera* and has been reported lower production of honey by neighboring countries such as China (Youguan *et al.*, 2000), Afghanistan (Woyke, 1984), Nepal (Neupane, 2009), India (Atwal and Goyal, 1971) and Pakistan (Camphor *et al.,* 2005; Mahmood *et al.* 2014a) which need suitable control measures. Heavy infestation of *T. clareae* causes an irregular pattern of sealed and unsealed brood as found with all brood diseases. Camphor *et al.* (2005) reported *T. clareae* peak infestation in February, March, and April and started declining in population in the summer period (May to August). Bee researchers forecasted that *T. clareae* would play a key part by damaging *A. mellifera* colonies far more than varroa and *Acarapis woodi* which also affect honey production in the future. Bee colonies infested with *T. clareae* have observed *A. mellifera* colony loss of 30-70% and also the production of honey was reduced (Woo and Lee, 1997). Anderson and Morgan (2007) observed that brood mortality is caused by parasitism and colony decline. Furthermore, *T. clareae* also acts as a vector of deformed wing virus (DWV) disease and by interacting with varroa may cause further decline (Dainat *et al.* 2009; Khongphinitbunjong *et al.,* 2015; Sanpa and Chantawannakul 2009). *T. clareae* are ectoparasites of honeybees that feed on brood. Brood mortality and colony decline occurred due to these mites' parasitism (Ritter, 2008). *T. clareae* mites have a shorter life cycle and higher reproductive rate as compared to *V. destructor* when both species of mites exist in the same colony. *T. clareae* is a very dangerous pest worldwide for the rearing of honey bees due to this recent geographic spread and rapid reproduction (Ritter, 2008; Sammataro *et al.,* 2000). Regular monitoring and timely treatment of honeybee *A. mellifera* colonies for *T. clareae* are necessary to maintain the population of mites below an economic injury level otherwise the infested bee colonies would collapse in two to three years.'

Still, no particular synthetic acaricides are available in the market for controlling *T. clarea* in honeybee *A. mellifera* colonies in the country. Beekeepers are using acaricides that are effective against *V. destructor* such as Checkmite+® (coumaphos), Apistan® (fluvalinate), and Bayvarol® (flumethrin) (Camphor *et al.,* 2005; Kongpitak *et al.,* 2008). In addition, the effectiveness of many commercial

synthetic acaricides was determined for the control of *T. clareae* which are used against *V. destructor* in field conditions. Experimental results showed that formic acid (MiteAway Quick Strips®) was the only effective product that significantly reduced the *T. clareae* population without any harmful effect on honeybees after 8 weeks of treatment. Pettis *et al.* (2017) reported that these acaricides are not always effective for controlling *T. clareae* mites which are used against *V. destructor* in honeybee colonies. They further stated that the efficacy of Apivar® (amitraz) was also investigated against *T. clareae* but it had no or little effect on the population of mites in colonies of *A. mellifera* (Pettis *et al.,* 2017). Furthermore, the performance of the queen, viability of sperm in drones, and colony growth impact were also observed after the treatment of several acaricides in honeybee colonies (Burley *et al.,* 2008; Pettis *et al.,* 2017).

Commercial beekeepers in Pakistan are dependent on lower quality acaricides such as flumethrin strips, fluvalinate injection, and strips, amitraz, coumaphos, smokes of tobacco, sulphur, and formic acid for controlling *T. clareae* and *V. destructor* in their apiaries. These acaricides are imported from China in the country. Beekeepers neither observed the efficacy of these different acaricides against *T. clareae* in their bee colonies nor did use different control methods such as cultural, genetic, and biotechnological methods to bind the influence of these threats in their honeybee colonies. *T. clareae* mites reproduce in the sealed brood of *A. mellifera* hence well safe from the treatment of different types of acaricides treatment applications. Therefore, the control of *T. clareae* is difficult and a great challenge for scientists working as an apiculturist, acarologists, and insect pathologists. Camphor *et al.* (2005) reported that resistance to sulphur, tobacco, and naphthalene has developed by *T. clareae* due to the continuous use of these acaricides in honeybee *A. mellifera* colonies for the control of mites. The repeated use of synthetic acaricides may be avoided because ectoparasitic mites *V. destructor* and *T. clareae* are highly resistant to acaricides. Besides, the misuse of acaricides has led to residue accumulation in honey, propolis, and beeswax (Chaimanee *et al.,* 2019; Mullin *et al.,* 2010).

Therefore, it is very necessary to develop alternative control methods for *T. clareae* in honeybee *A. mellifera* colonies. Many organic acids and natural compounds have shown the potential to control pests and diseases in *A. mellifera* colonies. Pettis *et al.* (2017) have observed that both thymol and formic acid have been shown effective against *T. clareae*. Regular monitoring of honeybee diseases and ectoparasitic mites, *T. clareae,* and *V. destructor,* and their management on time is immediately essential to save the industry of beekeeping in Pakistan. Maximum research work has been carried out on control methods of varroa in honeybee colonies. Little research work was carried out on *T. clareae* which is also a threat to beekeeping in Pakistan as well as in Asian countries and is needed suitable control methods. Therefore, this study was designed to evaluate different controlling agents alone and in combination to develop environmentally profound control options for *T. clareae* mites in *Apis mellifera* colonies.

Material and methods

We start this research study from late 20th May 2021 to 20th October 2021 at a commercial private apiary placed in Islamabad nearby a maize field and Karak, Khyber Pakhtunkhwa Province, Pakistan on Ber *Zizyphus* spp. nectar flow from 1st week of September 2021 to 20th October 2021.

Experimental honeybee Apis mellifera colonies selection

In the apiary 150, honeybee *A. mellifera* colonies were examined for infestation of *T. clarea* mites before initiating the experiment. Fifty-four European honeybees of Italian stock, *A. mellifera* colonies established in single-story deep Langstroth hives naturally infested with *T. clarea* were used in this study. These experimental honeybee colonies were left untreated for mites control for six months before conducting this field trial. The infestation of mites was also made sure by providing frames infested with *T. clareae* to bee colonies which were allowed to

multiply during March- April. Each treatment was replicated three times including control in a Randomized Complete Design (RCD). Each bee hive had 10 worker bee frames and 4 sealed and unsealed worker brood frames, approximately two frames of honey, and half a frame of pollen. All bee hives had a laying queen. Uniform honey bee colonies were used in this experimental study. All selected bee colonies were fed sugar supplemental syrup @ 2:1 at weekly intervals and patties of pollen containing 1:1 (Bee collected pollen:50% sugar solution) in a dearth period from June to 25th July at the fortnightly interval to encourage development and rapid colony growth and to supplement the nutrition of the experimental bee colonies. Honey or super chambers were provided to a colony when the colony population increased. Subsequently, bee colonies were managed through standard apicultural techniques like removing and adding extra bee boxes such as robbing management, sugar supplemental feeding, and protecting honeybees from hornet attacks. Honeybee *A. mellifera* colonies were regularly inspected for any harmful effect of treatment alone and in combination on honeybees, queen performance, and availability of food during the dearth period.

Treatments used alone or in combination against Tropilaelaps clareae in Apis mellifera

All experimental honeybee colonies were divided randomly into 6 groups and each group consists of 3 honeybee colonies. Colonies of the first group (T_1) Formic acid (65%) @ 15 ml/colony, the second group colonies (T_2) treated with Thymol was applied @ 4 g/colony, the third group (T_3) received Queen caging for 21 days used alone, the fourth group (T_4) received Formic acid (10 ml/colony) + queen caging (14 days) + thymol (4 g/colony), the fifth group was treated (T_5) with Formic acid (10 ml/colony) + thymol (4 g/colony) used in combination and the sixth group (T_6) was used as control and these colonies received no treatment.

Treatments Application Methods

Formic acid was applied on a cardboard piece (7.5" × 5.5") by placing it inside the wire mesh of screened bottom board tray back side of the bee hive. Thymol finely ground was applied in 80 mm Petri dishes on the brood chamber top above the inner covers of the bee hive (Qadir *et al.,* 2021). Queens were caged for 21 days alone and 14 days in combination treatments to break in the life cycle of the *T. clareae* mites. All treatments used alone and in combination were applied to all experimental bee colonies at weekly intervals three times against *T. clareae.*

The effectiveness of different treatments used alone and in combination was found in the following ways:

Percent infestation

Percent infestation of tropilaelaps was observed before and after the application of treatments as below:

Colony Worker brood Infestation Rate of Tropilaelaps clareae

T. clarea percent infestation was recorded before and after treatments.in worker brood cells. The infestation was observed by selecting randomly 50 cells of worker brood/colony having one worker pre-pupa or pupa. Samples were taken from the center of the brood of three different frames to get a representative sampling. Samples were brought to the laboratory and examined under a stereomicroscope. Each cell was opened individually with a forceps and the removing larva, pre-pupa, or pupa was inspected for the presence of *T. clareae* eggs, larvae, protonymphps, deutonymphs, daughter/young and adult female and male mites and also on the surface of the larvae or pupae. Different immature stages of the mites were placed carefully with a camel hair brush in a petri dish. After examing each cell different stages of *T. clareae* were counted and noted. The observed honeybee frames for the presence of mites were immediately returned to the colonies. The degree of infestation was calculated by the formula of Anderson and Roberts (2013):

$$\text{Infestation rate (\%)} = \frac{\text{Total number of mites in the inspected worker brood cells}}{\text{Total number of brood cells inspected}} \times 100$$

Total Mite Fall

Mites screened bottom board tray with a white

Formica sheet in each bee hive was placed from the backside of the colony to record *T. clareae* mites along with hive debris (Mahmood *et al.,* 2011). Screened bottom board trays also increase the efficacy of the treatments aside from examination for checking economic injury levels of ectoparasitic mites. Natural fallen *T. clareae* mites were recorded before 24 hours of treatments while fallen dead mites were observed 7, 14, and 21 days after treatments with the screened bottom board tray by counting dead fallen mites. To knock down the remaining *T. clareae* mites after treatments Manhao™ (fluvalinate) China strips were used in each of the study colonies to calculate the efficacy of all the treatments. These strips remained for 28 days in each colony and were afterward removed and all the dead fallen/live *T. ckareae* mites were counted and recorded on the screened bottom board trays (Bakar *et al.,* 2018). The percent efficacy of treatments used alone and in combination in the experimental honeybee colonies was individually calculated with the following formula:

$$\text{Efficacy (\%)} = \frac{\text{(No. of mites fallen for each treatment)}}{\text{(Total number of fallen mites)}} \times 100$$

Honey yield in kg per colony

Ber, *Ziziphus mauritiana* honey yield was extracted from honeybee colonies treated with treatments alone and in combination with stainless steel centrifugal honey extractor machine at the end of the nectar flow season. Yield data of honey was calculated in kg/colony to match treated and control colonies' yield of honey to examine the impact of the different controlling agent's in combination and alone used in this study (Aziz *et al.,* 2015).

Statistical analysis

Data of treatments and control were subjected to analysis of variance (ANOVA) by Randomized Complete Design with Statistix 8.1 computer program (Analytical Software Statistix 8.1, 2003). Using Duncan s Multiple Range Test means were compared. Significant differences were expressed at the level of $p \leq 0.05$ to find out the difference between the treatments.

Results and discussion

Effectiveness of treatments applied alone and in combination in percent reduction of Tropilaelaps clareae in sealed worker brood

The worker brood rate of infestation in *A. mellifera* with *T. clareae* mites in tested colonies before treatments ranged from 26.5 to 29.0%. Worker brood infestation of *T. clareae* slowly decreased after 1st and 2nd weekly treatment in *A. mellifera* colonies and reached the lowest infestation rate after 3rd treatment application. Data analysis showed a 98.29% decreased percent reduction in brood with Formic acid + thymol + queen caging (T_4) in combination-treated colonies followed by Formic acid + thymol (T_5) in combination 93.98%. Formic acid 88.38% was used alone and Thymol (T_2) 78.53% was used alone.

Queen caging (T_3) reduced only 70.42% of mite infestation after the third week of treatments in worker brood (Fig. 1). Queen caging (T_3) was the least effective among the treatments used alone to control *T. clareae* mite infestation in honeybee colonies at commercial beekeeper apiary.

Statistical analysis showed that there were highly significant differences among the tested T_4 (Formic acid + thymol + queen caging), T_5 (Formic acid + thymol), T_1 (Formic acid), T_2 (Thymol), T_3 (Queen caging) and T_6 reduction percentage of *T. clareae* after treatment on worker brood. These observations are in confirmation of the findings of Ismail *et al.,* (2006) who observed 95.3% and 72.6% efficacy of formic acid + caging queen + geranium oil + basil oil and oxalic acid for *V. destructor* worker brood infestation reduction in *A. mellifera* colonies while geranium oil, basil oil, formic acid + geranium oil, oxalic acid + geranium oil, formic acid + basil oil, oxalic acid + basil oil, oxalic acid and formic acid presented a varying decrease in the range of 65.6-100% infestation for the brood of workers. Several investigators observed mites reduction by treating colonies with organic acids, and essential oils and recorded unlike control ranges (Abd El-Wahab and Ebada, 2006; Islam *et al.,* 2020; Islam *et al.,* 2017; Tiwari *et al.,* 2014).

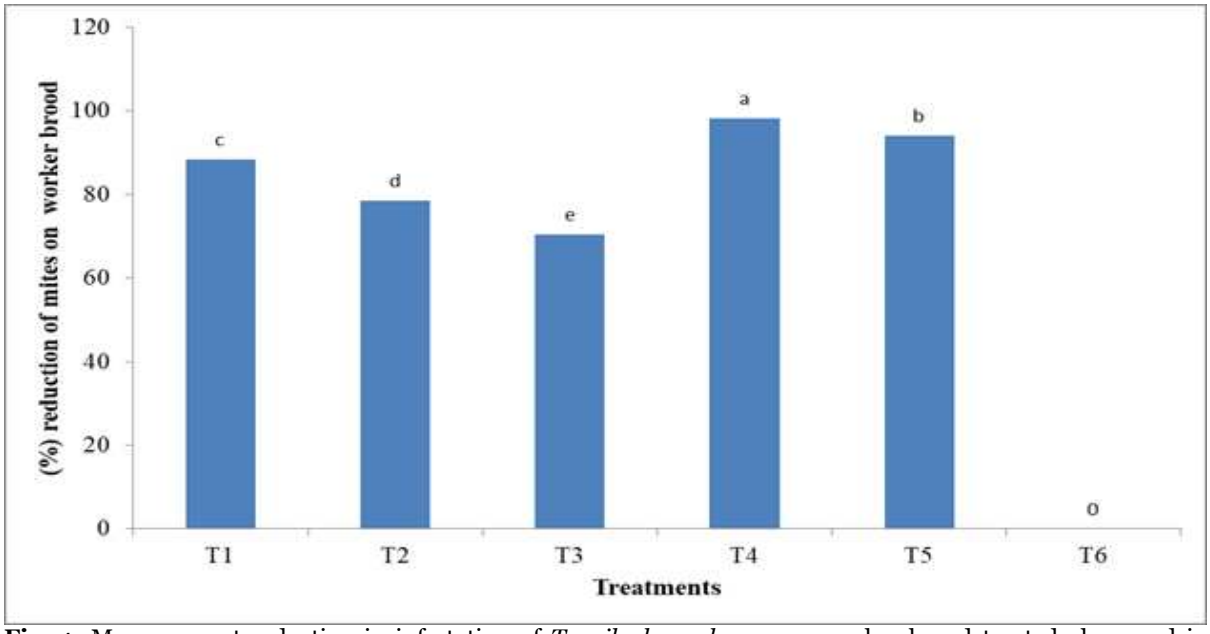

Fig. 1. Mean percent reduction in infestation of *Tropilaelaps clareae* on worker brood treated alone and in combination with different control agents (One-way ANOVA usingLSD test at *p*-value ≤ 0.01).

Total dead Tropilaelaps clareae Fallen Mites on Formica Sheet

The results showed that after 1st treatment dead fallen tropilaelaps per hive increased compared to control colonies. But the number of fallen dead mites slowly reduced from 2nd to 3rd treatment. After the first week of treatments, the highest (245) *T. clareae* per hive was observed in T4 and T5 (180), and T1 (169) respectively. Which were significantly different from T2 (125) and T3 (110) fallen dead mites per hive respectively in colonies treated alone with Thymol and queen caging as compared to (16) in T6 untreated colonies (Fig. 2).

Second-week treatment observation showed that a maximum (128) fallen dead *T. clareae* mites/hives were observed in T_4, 115 in T_5, 80 in T_1, 73 in T_2, 52 in T_3 while 19 in T_6 untreated control colonies (Fig.3).

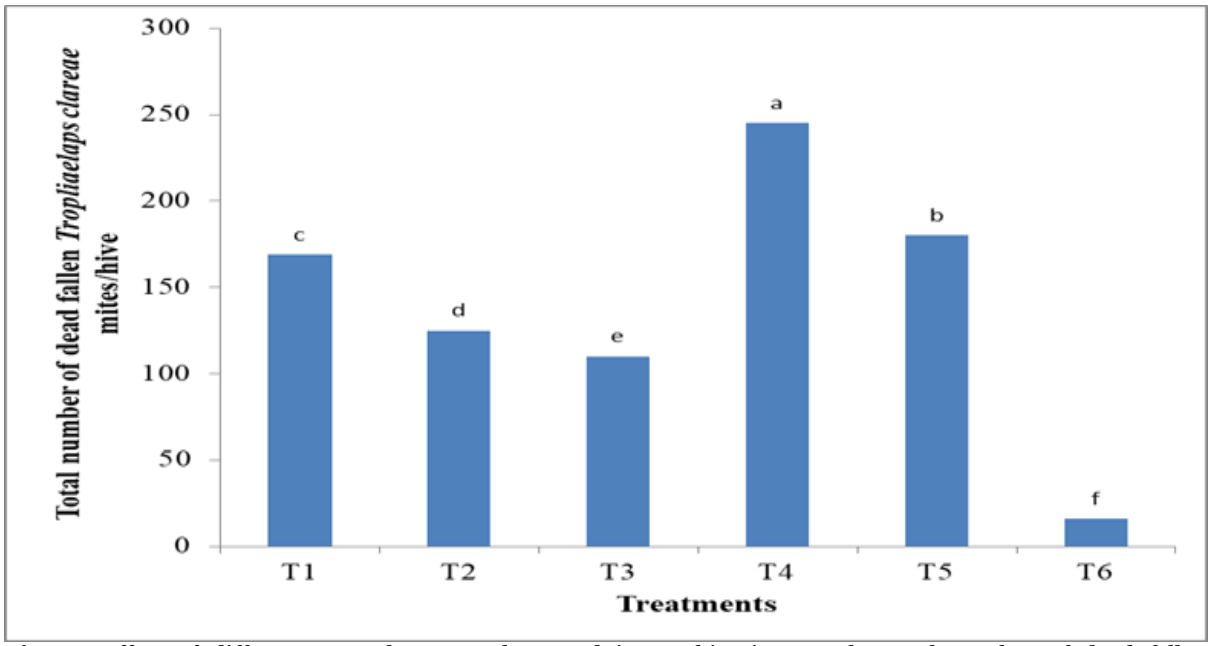

Fig. 2. Effect of different control agents alone and in combination on the total number of dead fallen *Tropliaelaps clareae* mites/hive after first treatment (One way ANOVA using LSD test at *p*-value ≤ 0.01).

A similar pattern was also observed after the third treatment maximum (37) fallen dead *T. clareae* mites/hive found in T_4 followed by T_5 (31), T_1 (20), and T_2 (16). While in T_3 the lowest (10) dead tropilaelaps fallen was recorded as compared to (25) in the untreated control colonies (Fig.4). These findings are in line with Islam *et al.,* (2020) and Alam *et al.,* (2022) recorded higher dead fallen *T. clareae* after first treatment which was reduced during 2nd and 3rd treatment.

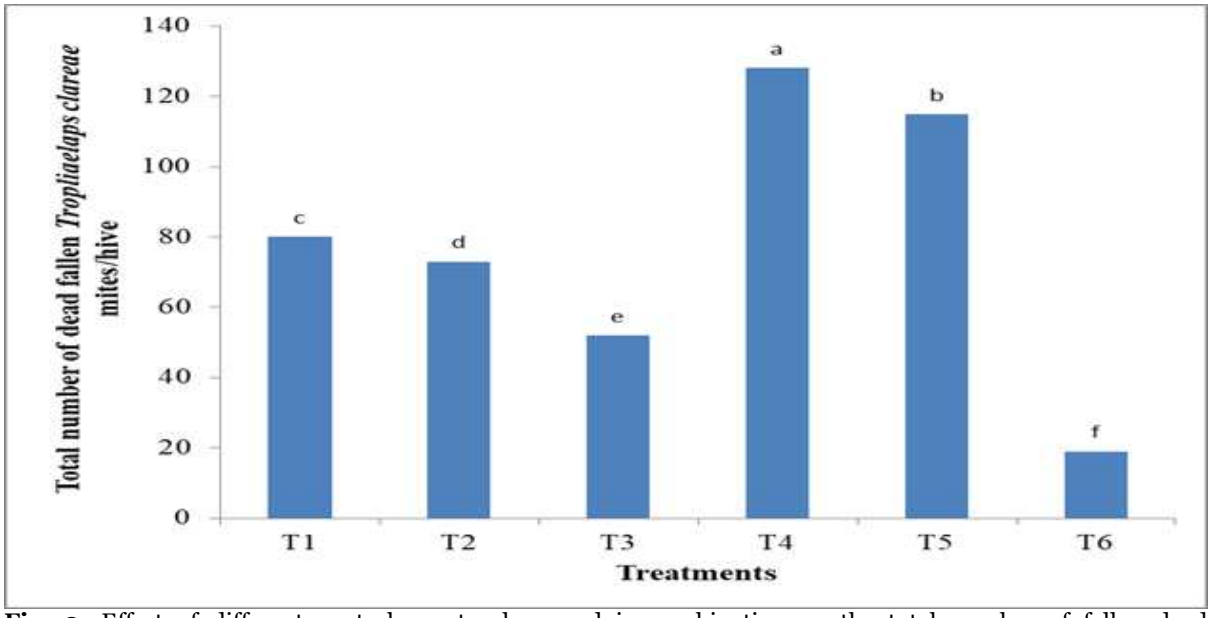

Fig. 3. Effect of different control agents alone and in combination on the total number of fallen dead *Tropilaelaps clareae* mites/hive after second treatment (One-way ANOVA using LSD test at *p*-value ≤ 0.01).

The mean total has fallen dead *T. clareae* mites per hive for six treatments including control after the first; second and third applications presented in Fig. 5). When different controlling agents alone and in combination were analyzed statistically for tropilaelaps highly significant differences for mean total dead fallen mites were found at $p < 0.01$ level of probability.

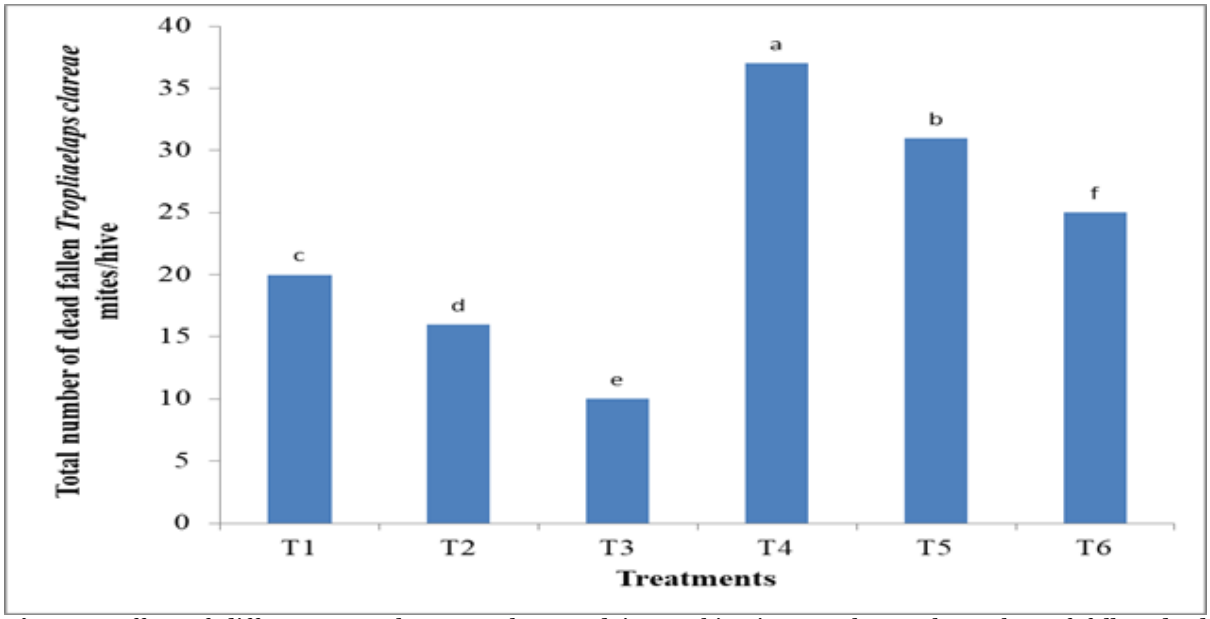

Fig. 4. Effect of different control agents alone and in combination on the total number of fallen dead *Tropilaelaps clareae* mites/hive after third treatment (One-way ANOVA using LSD test at *p*-value ≤ 0.01).

The results showed that a maximum (410) total fallen dead was recorded in T_4 after 1st, the 2nd and 3rd treatment applications *T. clareae* mites drop per hive was recorded in T_5 (326) and T_1 (269) followed by T_2 (214) and T_3 (172) after three treatment applications, which presents the lowest toxicity for the control of *T. clearage* and the control T_6 (60) (Fig. 5).

Different Treatments Percent Mortality of Tropilaelaps clareae

The results of the overall mean percent mortality of *T. clareae* of six treatments applied alone and in combination after 1st, the 2nd, and 3rd treatments application is indicated in Fig 6. Data showed that the overall mean percent mortality of *T. clareae* was significantly highly different at $p \leq 0.01$ between different treatments. The highest (95.35%) mean overall efficacy was recorded with Formic acid + thymol + queen caging (T_4) followed by Formic acid + thymol (T_5) (89.56%) and Formic acid (T_1) (82.26%).

While the lowest (76.43%) overall mean mite mortality with Thymol (T_2) and (69.35%) with Queen caging was observed as compared to control (20.55%) (Fig.6). Mahmood *et al.,* (2014b) compared different treatment (3.2% oxalic acid + 4 g thymol + 5% clove oil + tobacco extract + 65% formic acid) with (3.2% oxalic acid solution + 4 g thymol, tobacco extract 5% clove oil + formic acid) for controlling *T. clareae A. mellifera* colonies.

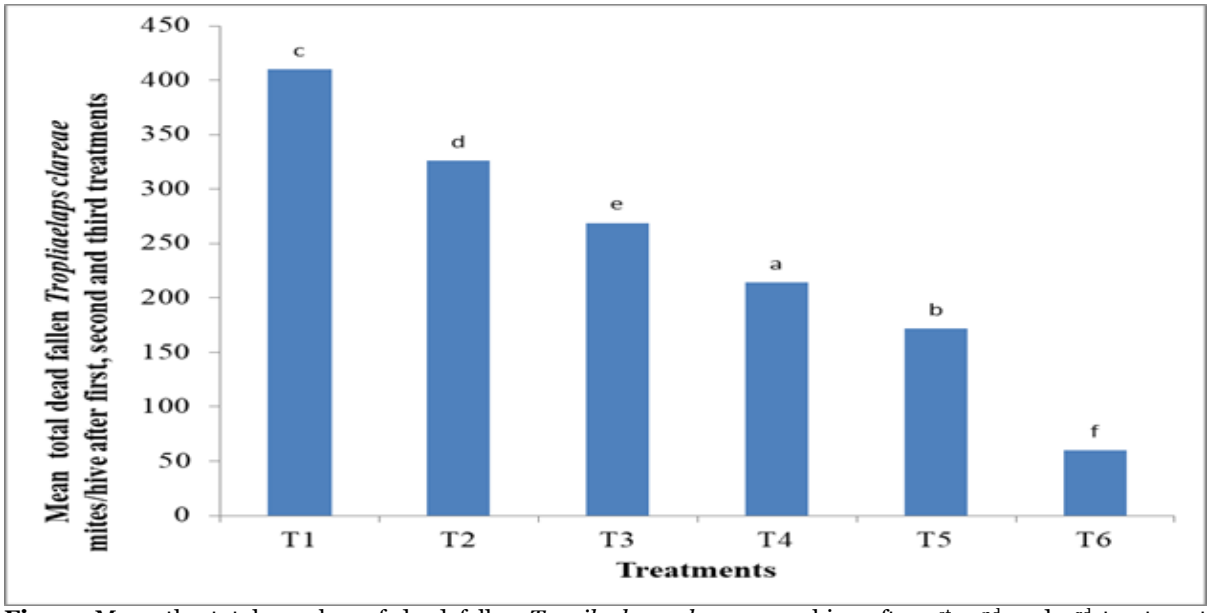

Fig. 5. Mean the total number of dead fallen *Tropilaelaps clareae* per hive after 1st, 2nd and 3rd treatment application (One-way ANOVA using LSD test at *p*-value ≤ 0.01).

They found that 5% clove oil + tobacco extract gave the highest mite mortality and percent efficacy for tropilaelaps.

These findings are in agreement with Mahmood *et al.* (2012) and Raffique *et al.* (2012) who observed that thymol, formic acid, and a combination of thymol and oxalic acid indicated maximum effectiveness for controlling *T. clareae* in honeybee (*A. mellifera*) colonies. Rana *et al.* (2010) stated that formic acid sponge pads inserted in summer gave 83-90% mortality of mites.

Honey Yield

Data showed that production of Sidr honey in kg per colony differed significantly in group T_1, T_4, and T_5 honeybee colonies treated in combinations and alone against *T. clareae*.

The collected sealed Sidr honey in each colony was extracted with 4 frame manual stainless steel centrifugal honey extracting machine and weighted. Data also showed that strong and healthy *A. mellifera* colonies produced higher honey yield in kg per colony as compared to the weak and control colonies.

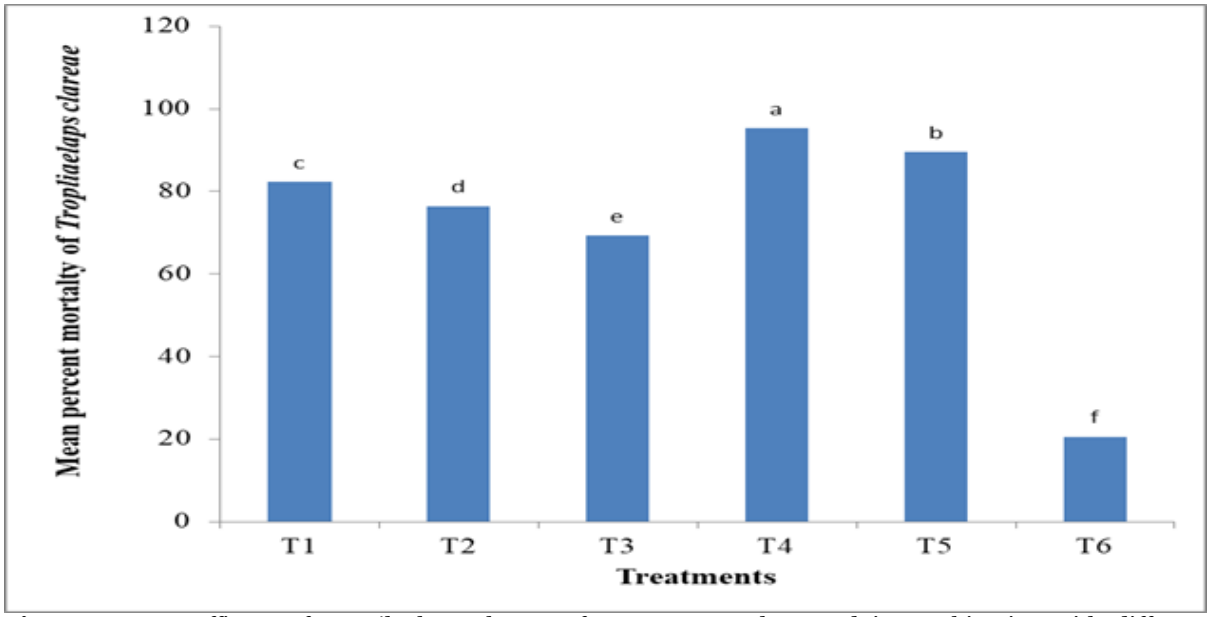

Fig. 6. Percent efficacy of *Tropilaelaps clareae* after treatment alone and in combination with different controlling agents (Two-way ANOVA using LSD test at *p*-value ≤ 0.01).

The maximum 7.6, 6.5, and 5.2 kg Sidr honey yield per colony was extracted from colonies treated with Formic acid + queen caging + thymol (T_4), Formic acid + thymol (T_5), and Formic acid (T_1) respectively. Whereas the lowest 4.0 and 3.0 kg Sidr honey yield per colony was recorded in colonies treated with Thymol (T_2) and Queen caging (T_3), respectively as compared to control (2) kg per colony (Fig. 7).

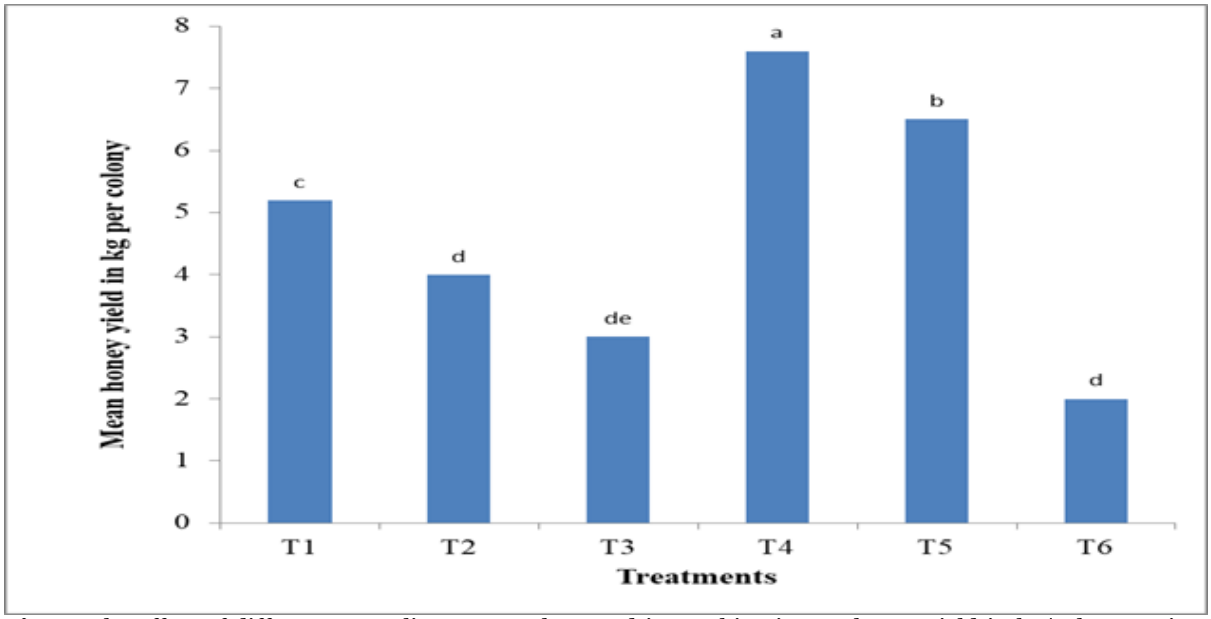

Fig. 7. The effect of different controling agents alone and in combination on honey yield in kg/colony against *Tropilaelaps clareae* mites (One-way ANOVA using LSD test at *p*-value ≤ 0.05).

Our results are in line with Mahmood *et al.* (2011) and Abd El-Wahab *et al.* (2012) recorded treated bee colonies with formic acid obtained highest honey yield in comparison to colonies used thymol and untreated group.

Conclusion

From the present study, it is concluded that Formic acid + thymol + queen caging (T_4) used in combination was found very effective in controlling *Tropilaelaps clareae* in honeybee (*Apis mellifera*)

colonies. The second most effective treatment as a combination against *T. clareae* control was Formic acid + thymol (T_5) followed by Formic acid (T_1) and Thymol (T_2) used alone for the reduction of tropilaelaps infestation. Formic acid + thymol + queen caging (T_4) used in combination indicated the highest percent reduction in infestation of *T. clareae* worker brood, dead fallen mites on Formica sheet, mortality percent of mites, and higher Sidr honey.

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