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Original article

Efficacy assessment of soft and hard acaricides against *Varroa destructor* mite infesting honey bee (*Apis mellifera*) colonies, through sugar roll method

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ABSTRACT

The parasitic mite *Varroa destructor* is amongst the most serious problems of honey bees, *Apis mellifera* (Hymenoptera: Apidae) around the world including Pakistan. The present study estimates the mite density through powdered sugar roll method and evaluates the effectiveness of five miticides (fluvalinate, flumethrin, amitraz, formic acid, and oxalic acid) on *A. mellifera* colonies in German modified beehives. The results indicated that by treating the bees with one strip and two strips of fluvalinate per colony; the mite population remained below the economic threshold level (ETL) for 14 days and 25 days, respectively. Treatment of flumthrin @1 strip and @ 2 strips per colony resulted in mite population suppressed for 14 days and 39 days, respectively below ETL. Application of Amitraz @ 2 mL per 1.5 L water after every three days interval on sealed brood effectively controlled mites below ETL for 21 days. Formic acid @10 mL per colony applied through plastic applicator proved effective (below 3 mites per bee sample) for 24 days and oxalic acid applied through shop towel method resulted in mite population control for fifteen days. Use of powdered sugar roll method for easy sampling of *Varroa* mites and application of acaricides on precise economic threshold level during different seasons of the year for integrated management of *Varroa* mite is hereby advocated by current studies.

1. Introduction

Honey bees including, *Apis mellifera ligustica* are among supreme agricultural blessings, both for the production of hive products (honey, propolis, royal jelly, pollen, bee venom, beeswax) and conservation of biodiversity They also provide essential pollination assistance to hold up a wide range of crops, fruits, vegetables and wild plants (Hillier et al., 2013). Since the last decade, a population loss of honey bees due to colony health issues has become the cutting edge of research in apiculture. The term Colony Collapse Disorder (CCD) has been coined to describe complicated and diverse causes of alarming colony losses in different countries (Ellis, 2007). There are many theories behind spontaneous and sudden abandonment of workers from *A. mellifera* colonies; however, most of the researchers consider it as the combination of several factors

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like parasitic mites, diseases, diet, pesticides and inclement weather (Stankus, 2008). The acarine ectoparasite *Varroa destructor* is the primary reason for bee decline globally and results in heavy colony losses (Alattal et al., 2017; De Jong, 1990; Wilson et al., 1997).

Previously, it was considered that mites feed on the hemolymph from the adult bees as well as the pupae and larvae within the sealed brood bee cells (Ramsey et al., 2019), but Ramsey et al. (2019) recently have reported that *Varroa* mites primarily feed on honeybee fat bodies and kill them. Timely observation and estimation of mite infestation level is a fundamental component of *Varroa* management scheme. Various methods of mite sampling are reported in the literature but powdered sugar roll method has been very easy to operate and safer to bees. There are sticky pads on legs of *Varroa* mites which help them to grip bee bodies firmly, sugar particles remove the bond between bee body and mite legs and the mites drop from the bee, which is separated and counted afterward (Ellis and Acedo, 2001).

Applications of pyrethroid, amadine, and organic acids are frequently required to control *V. destructor* and maintain colony health. Acaricides such as fluvalinate, flumethrin, amitraz and organic acids such as oxalic acid and formic acid are amongst several medicated products available for the management of *Varroa* mites. Both fluvalinate and flumethrin are pyrethroid and act as contact and stomach poisons. The mites will get muscles spasm, movement disorder and death due to blockage of voltage-gated sodium channels (Davies et al., 2007). Amitraz as a contact poison acts on octopamine receptors. Its injections are mostly used for sealed brood. Due to volatile nature, it is unstable in honey and completely degrades in 10 days (Korta et al., 2001).

Oxalic acid effects the mitochondrial function of honey bees. Oxalic acid can effectively manage phoretic *Varroa* on the bodies of adult bees, but not those in brood cells (Planinc, 2004). Application of oxalic acid generally occurs in winter in brood less colonies by different methods. Formic acid is harmful to the respiratory system of mites. Formic acid is a natural constituent of honey and abolishes the mites both on the adult bees and the sealed cells (Islam et al., 2016). Formic acid (70%) evaporates at 18–25 °C temperature (Imdorf et al., 1990), penetrate the capped brood cells and eradicate the mites infesting bees developing inside them (Calis et al., 1998).

Pakistan has a diverse landscape with plenty of bee flora offering tremendous opportunities for the growth and expansion of sustainable beekeeping in the country (Khan et al., 2016). *Varroa* mite management is the biggest issue of beekeepers in the country. The available methods to check mite population are sticky board method, alcohol wash methods, and ether roll method. Although these methods have been used by scientists in some research experiments (Aziz et al., 2015; Mahmood et al., 2012), the beekeepers are not trained to use them in their beekeeping practices due to the complexity and technical issues. Therefore, the majority of our beekeepers do not learn how to assess the population of mites before after acaricides application and make an injudicious use of acaricides in the form of under-dosage or overdosage. Keeping in view this situation, there was a dire need to find out any simple and cheap method of mite assessment so that the beekeepers can easily use it and make timely decisions regarding the use of acaricides. The present study was conducted with the aim to evaluate: (1) the efficacy of different acaricides regarding duration (days) of effectiveness (2) efficacy of one strip vs. two strips per colony of most widely used acaricides i.e., flumethrin and fluvalinate, by using sugar roll method for the first time in Pakistan.

2. Materials and methods

The experimental work was conducted at Apiculture Research Farm Koont, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan. *A. mellifera* colonies naturally infested with *Varroa* mites during 2017.

2.1. Sugar roll method

The adult bee population of test colonies (42) were assessed for *Varroa* infestation by powdered sugar roll method before application of acaricides (Ellis and Acedo, 2001; Gregorc et al., 2017). To collect the sample a wide mouth plastic canning jar was taken containing two-piece lids with a fine mesh (8 per inch) to allows mites to pass through while bees remain retained. Approximately 300 bees were poured in the plastic jar avoiding the queen. For this purpose, a frame was selected and bees were shaken from the frame into a plastic container having a hole in the middle so that the bees entering in the container were poured in the jar. Three tablespoons of powdered sugar were added into a jar through the mesh. Bees were rolled gently for 2–3 min until all the bees were well coated with powdered sugar. The jar was kept still for about 2–3 min so that the bees could remove mites along with sugar from their bodies. The jar was inverted, rolled gently and sugar powder containing mites was shaken out on a white paper chart. A number of mites were counted in powdered sugar. Sampled bees were returned back into the top of the colony or at colony entrance. The mites collected in the white chart were counted. The number of mites divided by a number of bees. A standard economic threshold is 2% mites per 100 bees i.e., 6 *Varroa* mites per 300 adult bees during low population season (dearth period) and 9 *Varroa* mites per 300 bees during high population season (February-March).

2.2. Honey bee colonies

A. mellifera colonies of each group were standardized one week before the starting the experiments (10 frames bees per colony). Each treatment was applied on five colonies and one colony was kept as a control.

2.3. Acaricides treatments

There were seven treatments i.e. (i) fluvalinate (ManHao) @ 1strip per colony, (ii) fluvalinate @ 2 strips per colony, (iii) flumethrin (ManGing) @ 1strip per colony, (iv) flumethrin @ 2 strips per colony, (v) amitraz (Emulsion Amtrazi) @ 2 mL injection per 1.5-liter Water, (vi) 70% formic acid @ 10 mL (at alternate day) and (vii) oxalic acid 14.4 g per colony.

2.3.1. Fluvalinate and flumethrin

For fluvalinate and flumethrin one strip per colony treatments; half strip was applied between the 2nd and 3rd frame and a half between 8th and 9th frame of the colony. Similarly, for fluvalinate and flumethrin two strips per colony treatments; the first strip was applied between the 2nd and 3rd frame and second between 8th and 9th frames. Application of flumethrin and fluvalinate were done in the first week of March 2017 and were not repeated again to any avoid honey contamination.

2.3.2. Amitraz

Amitraz injection was added in 1.5-liter water and sprayed only on sealed brood comb with the help of water spray bottle after shaking the bees. Treatment was repeated after every three days' interval for four weeks during June 2017 in summer.

2.3.3. Formic acid

Formic acid was applied both for sealed and adult bees after Sidr (*Ziziphus mauritiana*) honey harvest during the last week of October 2017. For this purpose, 70% formic acid @10 mL per colony was applied on every alternate day through formic acid applicator up to 24 days.

2.3.4. Oxalic acid

Oxalic acid was applied especially for adult bees during the 2nd fortnight of December 2017. Oxalic acid was applied through the OA/glycerin shop towel method. Following ingredients were employed for the preparation: Oxalic acid: 240 gm, Water: 200 mL and vegetable glycerin: 260 mL, Oxalic acid were taken in a pan. 200 mL of water was added and warmed gently so that it dissolved. It was cooled and when the temperature reached at 43 °C, 260 mL glycerin was added and stirred. So that homogenous mixture was obtained and poured on 50 shop towels and left for 24 h. After one day these towels were ready for application. Three towel papers were applied per colony (Oliver, 2017).

2.4. Statistical analysis

All data collected during experiments were statistically analyzed through CO-STAT computer-based software (CoSTAT, Monterey, CA, USA). Analysis of Variance (ANOVA) was applied to test the significance of data, means were compared through Least Significance Difference test (LSD) at 5% probability.

3. Results

3.1. Mite population with the application of 1 strip fluvalinate per colony

ANOVA indicated significant difference ($F_{5,\ 23} = 125.86$; $p \leq 0.00$) between different dates of observation (Table 1). Means comparison of the data revealed significant differences between all the dates of observation (Fig. 1). Recording of data was stopped 18 days after treatment because the mite population had crossed ETL after these days. This experiment reflected the effectiveness of one strip of fluvalinate per colony to control the mites during March (an important population build-up season for bees) only for two weeks.

3.2. Assessment of mite population with the application of 2 strips fluvalinate

In this experiment, two strips of fluvalinate were used per colony; the first strip between the 2nd and 3rd frame and the second between 8th and 9th frame. ANOVA exhibited highly significant difference ($F_{10,\ 43} = 55.08$; $p \leq 0.00$) between dates of observation and mean comparison of date revealed pre-treatment data differed significantly in the mite population from that of 4th to 25th days after treatment (DAT), whereas there were non-significant differences between mite population of 28th, 31st and 35th days after treatment. The mite population exceeded ETL (9 mites/ 300 bees) after 28 days of fluvalinate treatment and reached to 12 mites per bee sample at 35th days after treatment (Fig. 2). It means that two strips of fluvalinate per 10 frame beehive remained effective only up to 25 days against mite population during March (an important population build-up season for bees).

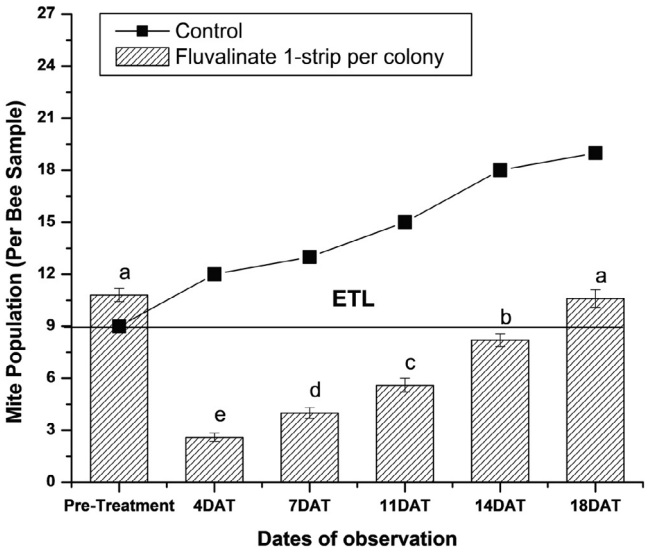

Fig. 1. *Varroa* mite population per 300 honey bees (*Apis mellifera*) sample at different dates of observation after application of fluvalinate @ 1 strip per colony. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAT = Days after treatment.

3.3. Assessment of mite population with the application of one strip of flumethrin

In this experiment, one strip of flumethrin was used per colony; half strip between 2nd and 3rd frames and the half between 8th and 9th frames. ANOVA showed a highly significant difference ($F_{5,\ 23} = 200.68$; $p \leq 0.001$) between dates of observation (Table 1). Means comparison of the data regarding the mite population revealed significant differences in the mite population up to 14th days after treatment (DAT). The mite population exceeded the ETL (9 mites per 300 bees) after the 14th day of application of treatment which reflected the ineffectiveness of one strip of flumethrin per colony to control the population of *Varroa* mite for a long time (Fig. 3). We stopped recording data after 18 days because the mite population was increased further with the passage of time.

Table 1
Analysis of variance of the data about *Varroa destructor* mite population infesting honey bee (*Apis mellifera ligustica*) colonies treated with different acaricides.

Sr. #	APC	df	SS	MS	F	P
1	Fluvalinate @ 1 strip	5, 23	295.767	59.153	125.858	0.000**
2	Fluvalinate @ 2 strips	10, 43	672.636	67.264	55.079	0.000**
3	Flumethrin @ 1 strip	5, 23	5211.833	42.267	200.684	0.000**
4	Flumethrin @ 2 strips	12, 64	616.062	51.338	74.639	0.000**
5	Amitraz @ 2 mL/1.5 L	7, 39	299.600	42.800	79.102	0.000**
6	Formic acid @ 10 mL	9, 49	651.220	72.358	182.415	0.000**
7	Oxalic acid @ 7.2 mL	5, 29	523.767	104.753	125.203	0.000**

APC = Acaricides per colony; df = Degree of freedom; SS = Sum of squares; MS = Mean squares; F = F-value, P = probability;
** P > 0.05.

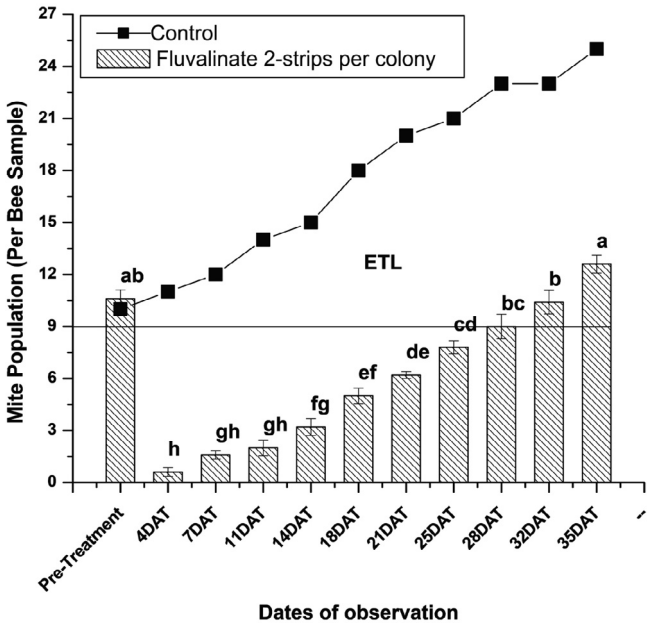

Fig. 2. *Varroa* mite population per 300 honey bees (*Apis mellifera*) sample at different dates of observation after application of fluvalinate @ 2 strips per colony. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAT = Days after treatment.

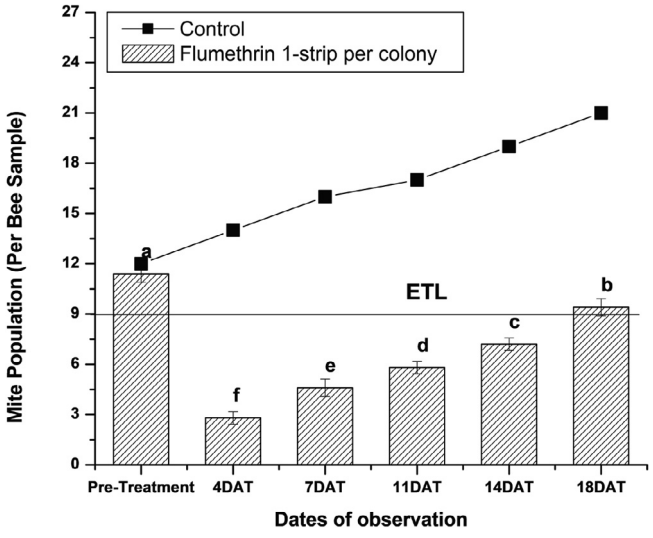

Fig. 3. *Varroa* mite population per 300 honey bees (*Apis mellifera*) sample at different dates of observation after application of Flumethrin @ 1 strip per colony. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAT = Days after treatment.

3.4. Assessment of mite population with the application of two strips of flumethrin per colony

In the colonies treated with two strips of flumethrin per 10 frame beehive, the data regarding the mite population were recorded twice a week up to 42 days. ANOVA revealed a highly significant difference ($F_{12,\ 64} = 76.64$; $p \leq 0.01$) between dates of observation. Mite population and exceeded ETL (9 mites per 300 bees) after 42nd days after treatment. (Fig. 4). This experiment reflected that two strips of flumethrin per colony were effective

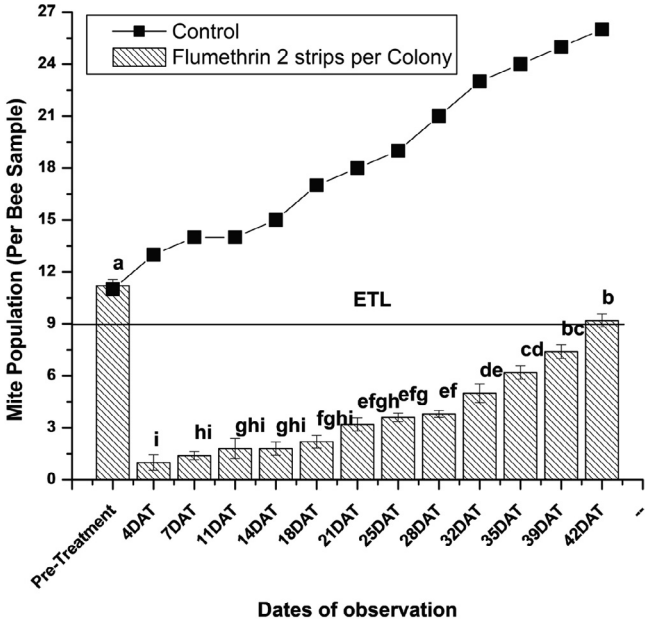

Fig. 4. *Varroa* mite population per 300 honey bees (*Apis mellifera*) sample at different dates of observation after application of flumethrin @ 2 strips per colony. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAT = Days after treatment.

and sufficient to keep the mite population below ETL up to 39 days during March and the first week of April (an important population build up season for bees).

3.5. Assessment of mite population with the application of amitraz injection per colony

The mite suppression was tested with amitraz injections for sealed brood during June 2017 and repeated after every three days' interval up to 24 days. ANOVA indicated a highly significant difference ($F_{7,\ 39} = 79.10$; $p \leq 0.00$) (Table 1). Mite population remained below ETL (6 mites per 300 bees) up to 21 days, then the mite started to increase exceeded the ETL (Fig. 5). The amitraz application was stopped after 15 days; to assess how long it may keep mites under check after final application. These results revealed that application of amitraz at three days' interval can successfully manage the mites for three weeks during the month of June, a difficult period for bees in which bee population becomes low and pollen and nectar availability reaches to a minimum (dearth period).

3.6. Assessment of mite population with the application of formic acid per colony

The mite population of the 6th experiment was tested with formic acid both for sealed brood and adult bees after Sidr (*Ziziphus mauritiana*) honey harvest during the last week of October 2017. ANOVA indicated highly significant difference ($_{F9,\ 49} = 182.41$; $p \leq 0.001$) between dates of observation (Table 1) and the means comparison of the data revealed pre-treatment data differed significantly in the mite population from of 4th to 21st day after treatment (DAT), whereas there were non-significant differences ($p = 0.05$) between mite population of 24th, 28th and 31th days after treatment. Formic acid kept mite population below ETL (6 mites per 300 bees) up to 31 days after start treatment in the month of October and November. (Fig. 6). This is again a period

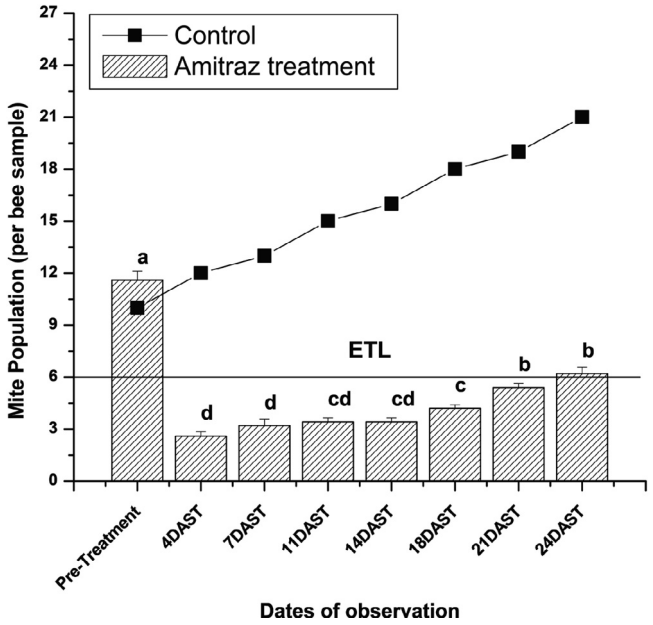

Fig. 5. *Varroa* mite population per 300 honey bees (*Apis mellifera*) sample at different dates of observation after application of Amitraz @ 2 mL per 1.5-liter water. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAST = Days after start of treatment.

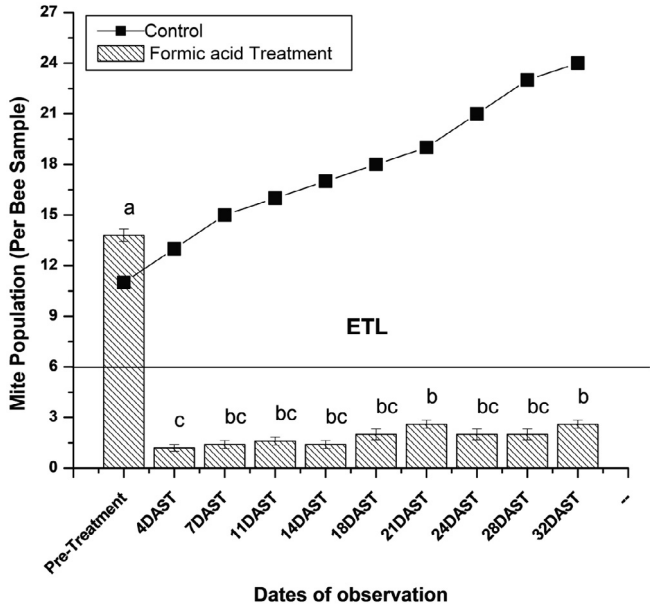

Fig. 6. *Varroa* mite population per sample of bees at different dates of observation after application of formic acid 70% @ 10 mL per honey bee (*Apis mellifera*) colony every 2nd day. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAST = Days after start of treatment.

of food shortage and the bee population becomes low after Sidr harvest in the country. This experiment reflected the effectiveness of formic acid 10 mL when used in plastic applicator applied every 2nd day per colony to control the mites during the last week of October (Low population density of bees).

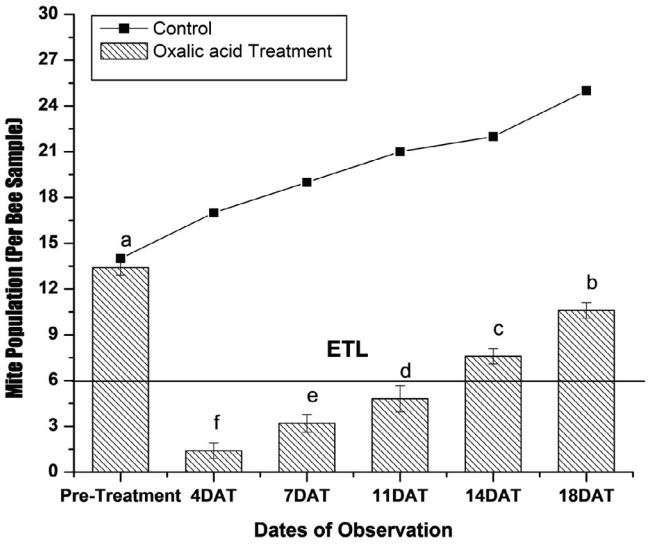

Fig. 7. *Varroa* mite population per sample of bees at different dates of observation after application of oxalic acid @ 7.2 mL per honey bee (*Apis mellifera*) colony by shop towel method. All the data are represented as mean ± standard error (indicated as error bars). Bars with different letters indicate significant ($p \leq 0.05$) difference among the observation dates. ETL = Economic Threshold level; DAT = - Days after treatment.

3.7. Assessment of mite population with the application of oxalic acid per colony

The mite reduction of the 7th experiment was evaluated with the medicated effects of oxalic acid dehydrate mixture in water for phoretic *Varroa* on adult bees during the 2nd fortnight of December 2017(winter). There was a significant difference ($_{F6, 34} = 142.36$; $p \leq 0.001$) between dates of observation and the means comparison of the data revealed pre-treatment data differed significantly in the mite population from that of 4th to 15th days after treatment, whereas there were non-significant differences between mite population of 18th and 22nd days after treatment. The mite population exceeded the ETL (6 mites per 300 bees) after 18th DAT and reached 10 mites/ bee sample on 22nd DAT (Fig. 7). This experiment reflected the effectiveness of oxalic acid through a shop towel method to control the mite population up to two weeks during the 2nd fortnight of December 2017 (low population density of bees).

4. Discussion

Experiments 1 and 3 were conducted to evaluate the effectiveness of 1 strip of fluvalinate and flumethrin per 10 frames hive, respectively, as these are the most frequently used acaricides treatment practice of our beekeepers. The results showed the effectiveness of these treatments only up to 14 days, after which the mite population exceeded ETL in the case of both experiments (Figs. 1 & 3). These findings indicate that application of 1 strip per colony is not sufficient to control the mite population in bee hives for a long period of time. Therefore, after two weeks sampling for mite population seems necessary to estimate mite population and further decide further management tactics. Our studies are in conformity with those of Mahmood et al. (2012) who mentioned the ineffectiveness of 1strip of /colony by using alcohol wash and bottom screen methods to monitor the mite population.

Experiments 2 and 4 were conducted to evaluate the effectiveness of fluvalinate and flumethrin 2 strips per colony. Fluvalinate was capable to control mites below ETL (3 mites/100 bee) up to

four weeks and flumethrin remained effective up to 5.5 weeks (Figs. 3 & 4). The difference in their effectiveness may be attributed to the fact that fluvalinate is being used against mites since last three decades, so due to a long duration of application exposure, the mites may have developed some resistance against this acaricides. The resistance against fluvalinate in *V. destructor* has also been observed by researchers like Harbo and Hoopingarner (1997), Sammataro et al. (2005), and Kanga et al. (2010). However, flumethrin, if used on the recommended dose, is still able to control the mites up to 5.5 weeks. We used these applications at crucial population build up month for bees both for colony division and honey production purposes. Gregorc and Škerl (2007) also reported high efficacy of fluvalinate and flumethrin in dealing with highly infested honey bee colonies with sealed brood.

Amitraz (formamidine) injection (2 mL per 1.5 L boiled water) when applied on bee hives during dearth period (June) kept mite population below ETL (6 mites per 300 bees) for 15 days (Fig. 5). Amitraz was applied on sealed brood instead of adult bees and open brood. We stopped the application of amitraz in July when bees started collecting maize pollens and queens begin to lay eggs again keeping in view the findings of Gregorc and Planinc (2012) who reported limited effectiveness of amitraz fumigation during brood periods.

Experiment 6 was conducted to evaluate the effectiveness of 70% formic acid. This treatment proved very effective to keep mites below ETL (6 mites per 300 bees) for 28 days (Fig. 6), which is very encouraging because as organic acid it provides an excellent alternative of hard acaricides (pyrethroid and organophosphates). The formic acid plastic applicator was used first time in the experiments because most of our beekeepers use cardboards for the application of formic acid which is dangerous both for applicator and honey bees. These applicators are designed to hang in the hive and hold acid effectively so that fumes of formic acid easily penetrate within sealed brood and adult bees to effectively control the mites, especially within sealed cells. Formic acid was applied during a period (last week of October) of food shortage when the bee population becomes low after Sidr harvest in the country. Giusti et al. (2017) reported the average efficacy was more than 95%, with a maximum level of 99% had no side effects on larvae, adult bees and queens recommended that medicine can be employed with brood throughout the season of the bee activity also added the compliment of product (medicine) the product is ready-to-use, safe for users and suitable for organic farming. Our results are infirmity with those of Mahmood et al. (2012) and Aziz et al. (2015) who reported the effectiveness of formic acid against *Varroa* mites.

Experiment 7 was conducted to evaluate the effectiveness of oxalic acid for phoretic *Varroa* during the period of low queen egg laying (2nd fortnight of December). Oxalic acid applied through shop towel method against *Varroa* mites during winter and was found effective to keep mites below ETL (6 mites per 300 bees) up to 14 days (Fig. 7). The bees eagerly assumed the paper towel containing oxalic acid. This method may be a promising alternate of sublimation and sugar syrup drip application of oxalic acid. The oxalic acid application was not repeated keeping in view that prolonged exposure of this acid may pose harmful effects to adult bees. Results of this study were in accordance with those of (Oliver, 2017).

Although powdered sugar has been recommended to beekeepers and researchers for practical sampling by Ellis and Macedo since 2001, however, it was not being used in Pakistan by beekeepers. In the present studies, this method was used successfully to monitor the mite population and evaluated the effectiveness of different acaricides. Sugar roll method is a simple substitute of sticky board method, alcohol wash methods, and ether roll method, which can be used to take mite data without harming bees and the beekeepers can use this method easily. The sample of 300 adult bees from the brood frame has been recommended to beekeepers (Lee et al., 2010). Studies do not encourage the use of 1 strip of flumethrin and fluvalinate per 10 frame colony for more than two weeks particularly in the population build-up period; as the mite population flares up at a fast pace at this time and causes heavy colony losses. Moreover, fluvalinate 2-strips per colony can be used only up to four weeks and colonies need resampling at this time to make a further decision regarding mite management. Flumethrin two strips per colony were effective to keep the mite population below ETL up to 5.5 weeks. Amitraz can be used be on sealed brood for 2 weeks during the dearth period. Formic acid is a promising organic acaricide which being soft chemical can be used successfully to keep the mites under control during the winter season. Oxalic acid shop towel treatment is a newly introduced method, we found it effective for 15 days, which is a promising and new method to control mites. It was concluded that an accurate sampling plan and the precise economic threshold level is necessary for the application of acaricides and organic acids and overcome resistance in mites against acaricides.

5. Conclusions

Sugar roll method can be used easily to monitor the mite population. Application of one strip flumethrin, one strip fluvalinate and oxalic acid per 10 frame hive can keep the mite population below economic threshold level up to 14 days. Application of two strips of fluvalinate and flumethrin were found effective up to 28 and 38 days, respectively. Amitraz treatment proved effective for up to 21 days. Application of 10 mL formic acid (70%) on alternate days remained effective throughout the period of application. Beekeepers are also suggested to avoid underdosing or/overdosing acaricide treatments, monitoring mite population regularly at least 15 days' interval and use of different chemicals and organic acid in the rotation to avoid excessive use of hard chemical acaricides.

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