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Management of *Varroa destructor* by essential oils and formic acid in *Apis Mellifera* Linn. Colonies

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Abstract
Four essential oils of Thyme (*Thymus linearis*), Lemon grass (*Cymbopogon citratus*), Rosemary (*Rosmarinus officinalis*), Mint (*Mentha longifolia*) and Formic Acid (65%) used at three concentrations (25, 50 and 100%) against *Varroa destructor* damaging honey bee *Apis mellifera* colonies in Honeybee Research Institute, National Agricultural Research Centre, Islamabad, Pakistan. The percentage of infestation by *V. destructor* on worker brood and adult workers, number of dead fallen *Varroa* on the sheet, per cent mite mortality and their honey yield (kg) was determined before and after the treatments in the experimental colonies. The results revealed that formic acid and the highest concentrations (100%) of tested essential oils caused effective control of *Varroa* mites, whereas the infestation reduction percentage with formic acid, lemon grass, thyme, mint and rosemary oils was recorded more than 96% after the end of treatments on both worker brood and adult workers. The highest total number of dead *Varroa* mites (323) fallen on the sheet was recorded by formic acid followed by lemon grass (306), thyme (263), mint (204) and rosemary (188) respectively. The hives treated with essential oils and formic acid also showed good persistence with mite mortality ranging from 46.97 to 63.41% up to fourth treatment. High mean value for honey yield (14.2 kg) was recorded by formic acid, followed by thyme (13.7 kg), lemon grass, mint (13.0 kg) and rosemary (12.4 kg) per colony as compared with control colonies (6.5 kg) per colony.

Keywords: *Apis mellifera*, essential oils, formic acid, mite mortality, *Varroa destructor*

1. Introduction
The parasitic bee mite *Varroa destructor* [1] is the most devastating pest of honeybee and causes high economic losses in beekeeping industry worldwide [2]. This mite can be found on adult bees, on the brood and in hive debris. In case of a heavy infestation, pupae do not develop into adult bees. Severe infestations cause considerable damage to honey bee populations [3] resulting in the reduction of quality of honey production as well as pollination [4]. *V. destructor* mites feed on the haemolymph of pupa and adult bees, causing severe physiological disorders resulting in a loss of up to 25% of adult weight, reduced longevity of workers and drone honey bees and deformation of wings [5]. The parasitic mite destroys the mechanical protective barriers of the integument and also impairs the immune system of honey bees [6]. It reduces the ability of honeybees to pollinate plants [7]. It also serves as a vector of diseases, which may cause up to 100% mortality of honeybees [5]. The *Varroa* mite has been a threat to beekeeping industry worldwide and has become a potential threat to apiculture industry of Pakistan as well [8].
V. destructor are generally kept under control by using synthetic acaricides such as amitraz, coumaphos and pyrethroids including fluvalinate and flumethrin [1, 10]. However, the regular use of these insecticides has resulted in the development of resistance in mites in many areas of the world [11-15] as a result of which, the efficacy of these insecticides for controlling *Varroa* mite has decreased significantly [11]. In recent years, the problem of resistance to acaricides has evolved rapidly. Increased tolerance has been observed to the most widely used synthetic acaricides. In addition, contamination of hive products with pesticide residues has also been reported, especially in honey, beeswax [16, 17] and propolis [18, 19]. This situation has resulted in an increased concern about the bee products contamination with synthetics used against *Varroa* [20]. All these problems have negatively affected the beekeeping industry.

There is an urgent need to find a sustainable solution to combat the threat of *Varroa* mites for the economic sustainability of agriculture and apiculture, as well as for the health and conservation of honey bees for ecosystem services. The use of acaricides should also be minimized in the beekeeping industry because of their residues and breakdown products in honey and wax [19].

Therefore, alternative control strategies including the use of natural products such as organic acids, plant extracts and essential oils against *V. destructor* have been developed. In the last decade, several studies throughout the world have confirmed the use of formic acid [21], oxalic acid [22- 24], thymol and eucalyptol [25, 26] in *Varroa* control. Organic acids have a status of generally recognized as safe (GRAS) [27] and essential oils are safe to use as they pose lower health hazards to both humans as well as honeybees [16]. There is an increasing interest in using natural pesticides, derived from plants and microorganisms [28] because they are generally safer than the synthetic insecticides. Recently, many studies have been conducted worldwide on the use of essential oils for *Varroa* management. The acaricidal effect of essential oils has been studied extensively [29, 30] has reported the biological activity of thyme oil (*Thymus vulgaris* L.), hyssop (*Hyssopus officinalis* L.) and salvia oil (*Saliva officinalis*) on *V. destructor*. The efficiency of some essential plant oils such as eucalyptus, thyme, neem, sage and grape fruit against *Varroa* has also been reported by many researchers [31-35]. [36] Reported that citronella oil had a toxic effect on the mites. [37] reported that essential oils from *Tagetes minuta* L. *Heterodera latifolia*, Buckey and Eucalyptus sp. are effective in reducing the *V. destructor* infestation. In Pakistan, little work has been done on the use of essential oils for controlling *V. destructor* on *Apis mellifera* L. colonies. [38] reported that clove oil + tobacco extract was the best combination for controlling *V. destructor* and their efficacy was found to be 96.48%. Moreover, maximum honey yield (20.5 kg) was obtained from the colonies treated with clove oil + tobacco extract.

Keeping in view the serious threat of *Varroa* mite in the beekeeping and several constraints for its control, the present investigation was carried out to develop safe and effective management option for the mite population control. The present study aimed to determine the effective control of *Varroa* mites by using essential oils of rosemary, lemongrass, thyme, and mint along with widely used chemical, formic acid as a positive control in the hives of *A. mellifera* colonies.

2. Materials and Methods

The experiments were conducted at apiary of Honeybee Research Institute, National Agricultural Research Centre (NARC), Islamabad during December 2014 – March 2015. Fifty four honeybee *A. mellifera* L. colonies in Langstroth beehives naturally infested by *V. destructor* mites were used for this study. Three colonies were used for each concentration of essential oils and Formic acid and three untreated colonies were left as control. Each colony consisted of 8-9 full depth combs of worker bees and had 3-4 brood frames. The hives were placed at a distance of 5 meters from each other.

For conducting these experiment four natural plant oils of *Cymbopogon citratus* (lemon grass), *Thymus linearis* (Thyme), *Rosmarinus officinalis* (Rosemary) and *Mentha longifolia* (Mint) were used at concentrations of 25%, 50% and 100% for each of the tested oil along with Formic acid (65%). Lemon grass, Thyme and Rosemary were collected from the Extraction and Analysis of Essential Oils from Rose, Jasmine and Aromatic Herbs, Plant Genetic Resources Institute, NARC, Islamabad whereas Mint was collected from Pakistan Forest Institute, KPK, Peshawar. The plants were identified from the National Herbarium Programme, NARC, Islamabad. Collected samples were shadow dried at room temperature and chopped into small pieces using a mill with rotary knives. Dried leaves were taken in separate plastic bags inside a refrigerator until the time for oil extraction.

The oils were extracted by hydrodistillation using a Cleavenger-type European Pharmacopoeias apparatus [39] for 2 hours, an average of 100 g of plant material was used each time and several distillations were made until obtaining an appropriate volume for all trials. The oils were dried over a hydrous sodium sulphate and stored in screw-capped dark glass vials at 5-8 ^{0}C until further analysis. Five ml of each essential oil and formic acid was applied on one stripe of cotton (2 × 20 cm) and held in one comb between the brood nest of each tested colony. Each tested colony was treated four times at 12 days intervals from the beginning of experiment. Before treatment all the cracks and crevices in the hive were plugged with mud.

The reduction percentage of *V. destructor* infestation in the tested colonies during the experimental period was determined in sealed worker brood cells and adult workers. For worker brood 50 sealed worker cells were randomly selected from the brood frames in each colony and the pre-pupae or pupae inside it were carefully examined and adult female mites on it were counted. The walls of the cells and removed caps were also examined as the mite frequently hides there. The frames were returned to the colonies immediately after being examined. In adult workers, the infestation percentage was determined in 100 living worker bees taken directly from the combs of each colony [40]. To have a representative sample about 4 to 6 frames were chosen both from the brood chamber and the honey super. The percentage of infestation in worker brood cells and adult bees was determined two times at 3 days intervals before the treatments and while during the treatments they were determined at 12, 24, 36 and 48 days post treatment. The reduction percentages of *Varroa* infestation were calculated from the collected data according to Henderson and Tilton (1955) equation [41]:

$$\% \text{ Reduction of infestation} = 1-\{(T_a \times C_b) / (T_b \times C_a)\} \times 100$$

Where T = % infestation of treated mites and C = % infestation of untreated mites (a = after; b = before treatment).

To check the mite mortality mite collection trays having white formica sheet covered by a wire screen to avoid the bees to contact were kept in the bottom board of each hive. Dead *Varroa* mites fallen on the bottom board trays were collected and counted in all tested colonies an interval of 12 days before treatments and 12, 24, 36 and 48 days post treatment [42].

% mortality in bee colonies was calculated by using formula [43]:

$$\textbf{Percent mite mortality} = \frac{\text{Mite mortality in treatment} \times 100}{\text{Mite mortality in treatment} + \text{Mite mortality in control}}$$

At the end of experiment the production of honey (*Acacia modesta*) was measured in kg by harvesting with the help of manually centrifugal honey extracting machine to compare honey yield of treated and untreated honey bee colonies. No queen loss was observed during the whole experiment and there was no adult honeybee mortality in any of the experimental colonies.

2.1 Statistical Analysis
Analysis of variance (ANOVA) was carried out by using F-test and least significant difference (LSD) test at 1% probability level was used to compare between the different treatments [44, 45].

3. Results and Discussion
A range of organic compounds that occur naturally in honey can be used to control parasitic mites. Few of them including formic acid and plant essential oils have shown potential against *V. destructor* mites, without any negative effect on the development of honeybee colonies [26, 46]

The efficacy of experimental essential oils and formic acid (65%) against infestation with *V. destructor* mites is shown in Table 1 and 2. Data indicated that the percentage of *Varroa* mite infestation on sealed brood and adult workers clearly reduced at the end of treatments (fourth treatment) in all the treatments. After the second and following treatments a significant differences were observed in the mean percentage of infested worker brood and adult workers between the treated colonies. In the colonies treated with essential oils and Formic acid, the percentage of infestation with *Varroa* mites on the worker brood and adult workers reduced gradually from the first to the fourth week after the treatments. It was observed that the highest concentration (100%) of the tested essential oils and Formic acid 65% were highly effective in controlling the *Varroa* mites, as the infestation reduction percentages of formic acid, lemon grass, thyme, mint and rosemary oils at concentration 100% recorded as (99.03 and 100%), (98.82 and 98.24%), (98.11 and 97.73%), (97.93 and 96.76%), (96.96 and 95.37%) after the fourth treatment on the worker brood and adult workers, respectively.

The obtained results are in agreement with the findings of [47] and [48] who found that formic acid caused 91.7% mortality of the *Varroa* mites. [42] observed that, the mean percentage of Varroa infestation on the worker brood and adult workers reduced to 100% after the fourth week of treatment with *C. aurantium* L. (Sour Orange) and *C. flexuosus* (lemon grass). According to the investigations of [49] Thyme oils spray resulted in 65.9% *Varroa* mite mortality and it has potential under Egyptian conditions. [50] also observed high mite mortality, when the bee hives were fumigated with seed extracts of Anise (*Pimpinella anisum*). According to the studies [51] during the late fall and early winter the *Varroa* mite is very susceptible to control by essential oils, due to the formation of a cluster and the lack of brood. By treating hives with the essential oils, the *Varroa* mites will have no place to hide and all can be killed.

Results obtained (Table 3) indicated that the highest total number of dead *Varroa* fallen mites/hive/treatment on the sheet was recorded after the first and second treatment particularly at 100% concentration of all tested essential oils in comparison with control colonies. Formic acid recorded the highest total number of *Varroa* fallen on the sheet followed by lemon grass, thyme, mint and rosemary represented by 323, 306, 263, 204 and 188 as compared to control 113 dead mites respectively.

The increasing mean number of *Varroa* mite fallen on the sheet in tested honey bee colonies treated with essential oils may be due to the activation of the defense behavior mechanisms of honeybee workers by these plant oils against *Varroa* mite. [52] found that the honeybee colonies, which were fed on neem extract showed the highest mean number of *Varroa* mite fallen on the sheet. They suggested that these extracts caused changes in the haemolymph of honeybee workers that resulted in increased of *Varroa* mite fallen on the sheet. [53] stated that some defense behavior mechanisms against *Varroa* mite were detected in some races and hybrid of honeybees. These mechanisms resulted in increasing the number of *Varroa* mite fallen on the bottom board of bee hive.

The results (Table 4) regarding % mite mortality showed that among six treatments applied for *Varroa* mite control lemon grass and formic acid treatments were significantly better. Formic acid treatment gave highest mite mortality (81.07%) after first treatment which decreased to 71.85% after second treatment, 56.89% after third treatment and then to 43.84% after fourth treatment giving an overall mean mortality of 63.41%. Similarly lemon grass oil treatment recorded 80.12% mortality after first treatment which decreased to 70.02% after second treatment, 52.62% after third treatment and further to 35.85% after fourth treatment with an overall mean mortality of 59.65%. It can be concluded that the tested essential oils were more effective against *Varroa* mites' infestation particularly at the highest concentration (100%).

Generally, the honeybee activities increased gradually after the treatments as a result of curing the colonies from infestation by using the experimental essential oils. In the cases, control of *Varroa* mite using naturally plant products are more recommended than other chemical acaricides to keep the social life of honey bee away from any harmful effects [31, 54, 32]. Furthermore, the effectiveness of these compounds seems to be greatly dependent on temperature, time of year, colony strength and brood area.

Concerning the results of the honey yield data (Table 3), the honey bee colonies treated with essential oils and Formic acid recorded high honey yield in comparison with control colonies during experimental period. In control (untreated) colonies 6.5 kg honey per hive was extracted/harvested which shows the increased mite infestation on bees causing low honey yield. Formic acid gave high honey yield with a mean value of 14.2 kg per hive followed by thyme 13. 7 kg, lemon grass and mint 13.0 kg and rosemary 12.4 kg/honey per hive. [55, 56] found out that formic acid treated colonies produced more honey as compared to the essential oils treated and control colonies.

From the above results it can be concluded that the tested essential oils are more effective against *V. destructor* mite infestation. The effectiveness of lemon grass oil can be an alternative to formic acid, a chemical treatment widely being used in *A. mellifera* colonies against *Varroa* mite. The use of essential oil may fit well into Integrated Pest Management (IPM) Programme for alternative use with other control measures for the management of *Varroa* mite and other pests in honey bee colonies although they enhance chance for colony survival and ensure residue free hive products.

Table 1: The reduction percentages of *V. destructor* infestation on worker brood in honeybee colonies treated with different concentrations of essential oils and formic acid

Tested essential oils and formic acid	Mean percentage of *Varroa* infestation on worker brood					
	Concentration of tested oil and formic acid					
	25%	% of reduction	50%	% of reduction	100%	% of reduction
Mint Before treatment	30.5		34.3		31.2	
First treatment	19.9 a	41.49	15.8 b	58.69	13.6 b	60.91
Second treatment	12.3 b	66.39	8.4 b	79.59	5.5 b	85.31
Third treatment	7.5 b	81.13	5.4 b	87.91	2.3 b	94.34
Fourth treatment	2.4 b	94.93	1.6 b	96.99	1.0 b	97.93
Thyme Before treatment	22.5		15.4		20.5	
First treatment	11.7 b	53.37	7.6 cd	55.75	6.2 c	72.87
Second treatment	8.6 c	68.15	5.7 c	69.16	4.1 bc	83.34
Third treatment	3.7 c	87.38	2.4 c	88.04	1.1 c	95.88
Fourth treatment	2.5 b	92.84	1.8 b	92.47	0.6 b	98.11
Lemon grass Before treatment	20.3		23.5		21.8	
First treatment	9.5 c	58.03	7.5 cd	71.38	6.4 c	73.67
Second treatment	5.6 d	77.01	4.2 cd	85.12	2.5 d	90.44
Third treatment	3.8 c	85,63	2.3 c	92.49	1.2 c	95.78
Fourth treatment	1.2 c	96.32	0.8 bc	97.88	0.4 b	98.85
Rosemary Before treatment	20.0		22.3		21.2	
First treatment	10.4 bc	53.37	8.5 c	65.82	6.9 c	70.81
Second treatment	6.6 d	72.50	5.1 c	80.94	3.6 cd	85.85
Third treatment	3.8 c	85.42	3.0 c	89.68	1.8 bc	93.48
Fourth treatment	1.5 bc	95.33	1.2 bc	96.65	1.0 b	97.06
Formic acid Before treatment	23.0		22.0		20.0	
(65%) First treatment	9.3 c	63.74	6.2 d	74.73	5.4 c	75.78
Second treatment	5.7 d	79.35	3.1 d	88.26	2.2 d	90.83
Third treatment	3.2 c	89.32	2.0 c	93.02	1.0 c	96.16
Fourth treatment	0.9 c	97.56	0.5 c	98.58	0.3 b	99.06
Control Before treatment	16.5		16.5		16.5	
First treatment	18.4 a		18.4 a		18.4 a	
Second treatment	19.8 a		19.8 a		19.8 a	
Third treatment	21.5 a		21.5 a		21.5 a	
Fourth treatment	25.6 a		25.6 a		25.6 a	
LSD First treatment	2.00		1.71		2.05	
Second treatment	1.34		1.64		1.57	
Third treatment	1.37		1.18		0.94	
Fourth treatment	1.13		1.08		1.05	

Mean followed with the same letter (s) in a single treatment are not significantly different at 1% level of probability.

Table 2: The reduction percentages of *V. destructor* infestation on adult workers in honeybee colonies treated with different concentrations of essential oils and formic acid

Tested essential oils and formic acid	Mean percentage of *Varroa* infestation on adult workers					
	Concentration of tested oils and formic acid					
	25%	% of reduction	50%	% of reduction	100%	% of reduction
Mint Before treatment	19.5		16.2		18.5	
First treatment	10.9 b	49.94	8.8 b	51.35	6.1 b	70.47
Second treatment	7.5 b	68.81	4.8 bc	75.98	3.2 b	85.96
Third treatment	4.7 b	81.34	2.9 bc	86.14	1.8 b	92.47
Fourth treatment	2.1 b	91.92	1.2 b	94.44	0.0 b	100
Thyme Before treatment	12.5		10.8		16.5	
First treatment	8.3 cd	40.54	6.2 cd	48.59	5.5 bc	70.15
Second treatment	5.5 cd	64.32	3.7 cd	72.22	2.4 bc	88.21
Third treatment	3.3 c	79.56	2.0 c	85.66	1.0 bc	95.31
Fourth treatment	1.5 bc	91.00	0.6 bc	95.83	0.0 b	100
Lemon grass Before treatment	15.0		13.5		17.0	
First treatment	6.8 e	59.40	5.6 cd	62.85	4.8 c	74.71
Second treatment	4.5 de	75.68	2.9 d	82.58	1.5 cd	92.85
Third treatment	2.0 d	89.68	1.0 d	94.27	0.3 c	98.63
Fourth treatment	1.0 cd	95.0	0.2 c	98.89	0.0 b	100
Rosemary Before treatment	14.4		11.7		12.8	
First treatment	9.5 c	40.92	7.1 c	45.66	5.6 bc	60.82
Second treatment	6.7 bc	62.27	5.6 b	61.19	2.9 b	81.63
Third treatment	5.2 b	72.04	3.4 b	77.50	2.1 b	87.30
Fourth treatment	1.9 bc	90.10	1.0 b	93.59	0.5 b	97.07
Formic acid Before treatment	13.5		15.5		14.0	
(65%) First treatment	7.2 de	52.24	5.1 d	70.53	4.5 c	71.22

Second treatment	3.8 e	77.18	2.1 d	89.01	1.1 d	93.63
Third treatment	1.8 d	89.68	0.9 d	95.50	0.0 c	100
Fourth treatment	0.3 d	98.33	0.0 c	100	0.0 b	100
Control Before treatment	12.0		12.0		12.0	
First treatment	13.4 a		13.4 a		13.4 a	
Second treatment	14.8 a		14.8 a		14.8 a	
Third treatment	15.5 a		15.5 a		15.5 a	
Fourth treatment	16.0 a		16.0 a		16.0 a	
LSD First treatment	1.28		1.59		1.28	
Second treatment	1.61		1.70		1.23	
Third treatment	1.20		1.02		1.13	
Fourth treatment	1.05		0.67		0.58	

Mean followed by the same letter (s) in a single treatment are not significantly different at 1% level of probability

Table 3: The effect of different concentrations of essential oils and formic acid on the number of dead *Varroa* mites fallen on the sheet and honey yield

Tested essential oils and formic acid	**Average number of dead/fallen mite and honey yield (kg) per hive**						
	Concentration of tested oil, formic acid						
	25%	50%	100%	25%	50%	100%	Mean value of honey (kg)
Mint Before treatment	11	12	14	12.5 a	12.9 b	13.6 bc	13.0
First treatment	41 d	62 d	88 d				
Second treatment	25 c	49 d	67 d				
Third treatment	13 d	23 d	34 d				
Fourth treatment	4 d	10 e	15 e				
Total	83	144	204				
Thyme Before treatment	13	15	10	12.7 a	13.5 ab	14.9 ab	13.7
First treatment	53 c	70 c	102 c				
Second treatment	35 b	57 c	89 c				
Third treatment	18 c	31 c	47 c				
Fourth treatment	7 c	16 d	25 d				
Total	113	174	263				
Lemon grass Before treatment	12	10	14	12.3 a	12.9 b	13.8 abc	13.0
First treatment	61 b	81 b	112 b				
Second treatment	37 ab	69 b	96 b				
Third treatment	22 bc	35 b	68 a				
Fourth treatment	11 b	21 c	30 c				
Total	131	206	306				
Rosemary Before treatment	13	11	15	11.8 a	12.5 b	12.9 c	12.4
First treatment	39 d	56 e	80 e				
Second treatment	20 d	35 e	64 d				
Third treatment	11d	20 d	30 e				
Fourth treatment	3 d	8 e	14 e				
Total	73	119	188				
Formic acid Before treatment	12	10	11	12.7 a	14.5 a	15.3 a	14.2
(65%) First treatment	66 a	85 a	118 a				
Second treatment	40 a	79 a	102 a				
Third treatment	25 b	40 a	64 b				
Fourth treatment	13 b	25 b	39 a				
Total	144	229	323				
Control Before treatment	14	14	14	6.5 b	6.5 c	6.5 d	6.5
First treatment	20 e	20 f	20 f				
Second treatment	26 c	26 f	26 e				
Third treatment	30 a	30 c	30 e				
Fourth treatment	35 a	35 a	35 b				
Total	113	113	113				
LSD First treatment	4.67	2.88	3.67	1.67	1.53	1.54	
Second treatment	3.53	2.49	4.55				
Third treatment	4.32	3.81	2.49				
Fourth treatment	2.49	2.88	2.88				

Mean followed by the same letter (s) in a single treatment are not significantly different at 1% level of probability

Table 4: Efficacy of essential oils and formic acid against *V. destructor* in *A. mellifera* colonies based on percent mortality

Tested essential oils and formic acid	% mite mortality			
	Concentration of tested oil and formic acid			
	25%	50%	100%	Mean
Mint First treatment	67.21	75.61	81.48	73.1
Second treatment	49.02	65.33	72.04	62.13
Third treatment	30.23	43.40	53.13	42.25
Fourth treatment	10.25	22.22	30.0	20.82
Over all mean value	39.18	51.64	59.16	49.58
Thyme First treatment	72.60	78.26	83.61	78.16
Second treatment	53.38	86.67	77.39	72.48
Third treatment	37.50	50.82	61.04	49.79
Fourth treatment	18.92	31.37	41.67	30.65
Over all mean value	45.60	61.78	65.93	57.77
Lemon grass First treatment	75.31	80.20	84.85	80.12
Second treatment	58.73	72.63	78.69	70.02
Third treatment	42.31	46.15	69.39	52.62
Fourth treatment	23.91	37.50	46.15	35.85
Over all mean value	50.07	59.12	69.77	59.65
Rosemary First treatment	66.10	73.68	80.0	73.26
Second treatment	43.48	57.38	71.11	57.32
Third treatment	26.83	40.0	50.0	38.94
Fourth treatment	7.89	18.60	28.57	18.35
Over all mean value	36.08	47.42	57.42	46.97
Formic acid First treatment	76.74	80.95	85.51	81.07
(65%) Second treatment	60.61	75.24	79.69	71.85
Third treatment	45.45	57.14	68.09	56.89
Fourth treatment	37.14	41.67	52.70	43.84
Over all mean value	54.99	59.25	71.50	63.42
Control First treatment	0.0	0.0	0.0	0.0
Second treatment	0.0	0.0	0.0	0.0
Third treatment	0.0	0.0	0.0	0.0
Fourth treatment	0.0	0.0	0.0	0.0
Over all mean value	0.0	0.0	0.0	0.0

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