

# Calendar for the Prevalence of Honey Bee Diseases, with Studying the Role of Some Materials to Control *Nosema*

Hossam F. Abou-Shaara\*

Department of Plant Protection, Faculty of Agriculture, Damanshour University, Damanshour 22516, Egypt

## 몇 가지 꿀벌 노제마병 방제물질의 평가와 꿀벌 질병의 발생시기 조사

호산 에프 아보-샤라\*

다마누루대학 농업학부 식물보호과, 다마누루시 22516, 이집트

**ABSTRACT:** *Nosema* spp. (NS) causes severe problems to honey bee colonies including the death of the highly infected ones. Searching for effective materials to control this parasite is very important. The objectives of this study were to identify the calendar for the prevalence of NS and other bee diseases, and to test the efficacy of three materials: diluted honey mixed with lemon juice (M1), chamomile extract mixed with sugar syrup (M2) and sutrivet mixed with sugar syrup (M3) against *Nosema*. To realize these objectives, diseases of brood and adult honey bees were surveyed over one year. Also, the efficacy of M1, M2 and M3 against *Nosema* was evaluated under field and laboratory conditions. The results showed that few diseases for immature and mature stages of honey bees were recorded. NS was detected during winter and spring in link with low temperature and high relative humidity. Under field conditions, M2 reduced the infection by 36.66% while M3 by 23.33% and finally M1 by 13.33%. In the laboratory, the highest efficacy was to M2 followed by M1 and finally M3. The three materials impacted the percentage of survived bees significantly higher than infected bees without any treatments over the experimental period. The study suggests the potential role of chamomile as a natural material to control NS.

**Key words:** Parasites, Control, Chamomile, Infection rates, beekeeper

**초록:** 미포강충류(*Nosema* spp. (NS))는 양봉꿀벌에 심각한 문제를 야기시키는 기생충으로 효과적인 방제물질의 선발이 무엇보다 중요하다. 본 연구는 노제마병과 기타 꿀벌의 발생유행시기의 구명과 더불어 3가지 노제마병 방제물질(M1 = 벌꿀희석의 레몬주스; M2 = 설탕시럽 혼합의 카모마일 추출물; M3 = 설탕시럽 혼합의 향생물질 스트리베트)을 평가하고자 수행하였다. 꿀벌 성충과 유충집단의 질병 유행시기를 년간 조사하였으며, 야외 및 실험실 조건에서 노제마병에 대한 M1, M2, M3의 효과를 평가하였다. 조사결과 극소수의 꿀벌 성충과 유충 질병이 발견되었다. 노제마병은 겨울과 봄 기간 저온과 고습조건에서 검출되었다. 포장실험에서 M2는 36.66%까지 발병억제 능력을 보였으며, 반면 M3는 23.33%, M1는 13.33%의 억제효과를 보였다. 실내실험에서 M2가 방제효과가 가장 좋았고, 그 다음 M1와 M3 이었다. 3가지 방제물질은 병에 감염된 꿀벌성충의 생존력을 크게 높이는 것으로 나타났다. 본 연구는 노제마병 방제를 위한 천연물질로 카모마일의 잠재적 방제효과를 제시하고 있다.

**검색어:** 기생자, 방제, 카모마일, 감염율, 양봉

*Nosema* spp. (NS) as an internal parasite to honey bees, *Apis mellifera*, is considered as one of the major threats to beekeeping. There are two species of *Nosema* infesting honey bees *Nosema apis* and *Nosema ceranae* (Fries et al., 2013). Infected colonies

with NS are badly impacted in a number of ways leading at the end to the death of the colonies. The epithelial layer of the ventriculus is greatly impacted causing digestion problems (García-Palencia et al., 2010) and reducing yeast number in the infected bees (Borsuk et al., 2013). The hypopharyngeal glands of the infected bees are passively impacted (Wang and Moeller, 1969; Wang and Moeller, 1971). The infection can alter the production of the primer pheromone ethyl oleate of worker

\*Corresponding author: [hossam.farag@agr.dmu.edu.eg](mailto:hossam.farag@agr.dmu.edu.eg)

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bees (Dussaubat et al., 2010). Also, the flight ability of infected bees is less than healthy ones (Kralj and Fuchs, 2010). Moreover, queen replacement is induced when queen is infected (Alaux et al., 2011). *Nosema* spores can be existed in bees and honey as well (Giersch et al., 2009). Currently, NS is existed in various countries. For example, *N. cerana* has been detected in some Asian countries including Japan (Yoshiyama and Kimura, 2011), Jordan (Haddad, 2014), and Saudi Arabia (Ansari et al., 2017), some European countries (Higes et al., 2006 and Paxton et al., 2007), and has been detected in Egypt (El-Shemy et al., 2012).

So far, fumagillin (Fumidil B) is the most common treatment to NS. It is the only treatment recommended by the World Organization for Animal Health for this disease as mentioned by Botías et al. (2013). This treatment has shown efficacy against NS (Furgala and Boch, 1970), and has effects on the spore membrane (Liu, 1973). However, this treatment can enhance bee health only for a short-term (Mladan et al., 2000) and the infection can be repeated after the end of the treatment period (Higes et al., 2011). Also, this treatment is considered expensive in some countries and is not available in other countries including Egypt and some other Arabian countries. Searching for effective alternatives to this treatment is essential. Some natural materials including herbal preparation or acids have been reported as potential control materials to NS (Gajger et al., 2009; Porrini et al., 2011; Gajger et al., 2013). In this study, the prevalence of honey bee diseases including NS over a year was investigated. Also, three materials were tested under field and laboratory conditions to assess their potential abilities to control NS. Two of them were natural materials (diluted honey mixed with lemon juice and chamomile extract mixed with sugar syrup) beside one chemical material (sutrivet mixed with sugar syrup).

## Materials and Methods

### Calendar for diseases prevalence

Ten honey bee colonies at an apiary at Damanshour city, El-Behera governorate, Egypt were marked and inspected regularly on weekly basis started from November 2016 until October 2017 to identify the calendar for diseases prevalence.

No chemical treatments were used with these beehives. The colonies were under normal beekeeping practices (e.g. inspection and feeding). The presence of any diseases for adults or immature stages was recorded. The identification of infection with diseases was performed using key infection symptoms; irregular pattern of sealed brood and flaccid dead larvae inside cells for European Foulbrood (EFB) (Forsgren et al., 2013), uncapped sealed brood containing discolored, spoilage and ropy brood for American Foulbrood (AFB) (e.g. Spivak and Reuter, 2001), also the common test (rope test) was used to distinguish EFB than AFB, removed cell cappings due to tunnels of wax moth larvae for bald brood (BB) (Ellis et al., 2013), the sac shape of infected larvae for sacbrood virus (SBV) (Freiberg et al., 2013), fecal marks on combs or hives, beside abnormal guts of infected bees for *Nosema* infection (Fries et al., 2013), and deformation of wings of adult bees for deformed wing virus (DWV) (De Miranda and Genersch, 2010). The monthly distribution of diseases was then compared and discussed. Also, values of average temperature and relative humidity (RH) over the study period at the study region were obtained from world weather online website ([www.worldweatheronline.com](http://www.worldweatheronline.com)).

### Role of some materials to control *Nosema*

Sugar syrup (1 sugar : 1 water w/w) was firstly prepared to be mixed with the tested materials. These materials, M1) diluted honey (3 honey : 1 sugar syrup, w : w) mixed with lemon juice (1 ml lemon juice per 20 ml diluted honey per hive), M2) dried chamomile (*Matricaria* sp.) flowers was boiled for 5 minutes (9 gm in 100 ml water) and the extract was mixed with sugar syrup as 1 ml per 20 ml syrup per hive, and M3) sugar syrup mixed with sutrivet (1 ml per 20 ml syrup per hive), were used. Sutrivet is a veterinarian treatment contains sulphamethoxazole 40 g/200 ml and trimethoprim 8 g/200 ml produced by Memphis company, Cairo, Egypt. The amounts used were adopted according to the practical experience, especially that 1 ml of natural extracts or other materials mixed with syrup per hive is the amount used by beekeepers for treating *Nosema* with repeating the treatments three times with 4 days interval, and 1 ml mixed with syrup is used at each treatment time per hive.

### Effects on *Nosema* infection rates under field conditions

Each treatment was replicated three times (three hives) beside three hives remained untreated (control colonies). All the hives contained hybrids of Carniolan honey bees with only three combs covered with bees. The experiment was done during winter 2016/2017. The infection rates with NS were firstly determined before treatment application. Ten worker bees were collected from each hive from the lateral combs, and then their guts were pulled out using forceps and were mixed individually with 0.5 ml of clean water in Eppendorf tube. The solution of each bee was examined under microscope using 400X separately to detect the presence of NS spores (Fig. 1). The infection rate was calculated by dividing the number of infected workers on 10 and multiplied by 100. The treatments were sprayed over the bees, and the treatments were repeated three times with interval of 4 days. The infestation rates were assessed again at the end of the treatment period by 4 days. The gut solutions of the infected bees with NS were mixed together to be used in the laboratory experiment.

### Effects on *Nosema* infection rates and survival of bee workers in the laboratory

Five groups were prepared and each group contained 4 plastic jars with perforated covers. In each jar, 15 forager Carniolan bee workers were placed. The bees were collected from the lateral combs of NS free hives. The caged bees were left without food for 24 hours. After that the bees were provided with sugar syrup 50% mixed with filtered gut solution of infected bees with NS (1 : 1) over 24 hours prior treatment application to ensure that all the bees were infected with NS. This solution was full of spores as the microscopic analysis

showed. The control group (healthy bees without NS infection) was provided only with sugar syrup.

The treatments were applied as M1, M2 and M3 for group 1, 2, and 3, respectively while group 4 was infected with NS without any treatments, and group 5 was considered as the control group without infection. The treatments were prepared as mentioned previously but each cage received only 300  $\mu$ l at each treatment time. The treatment was repeated three times with one day interval to ensure that all the bees received the test materials. After the onset of the treatments, the bees were provided with sugar syrup 50% on daily basis. Group 4 and 5 were provided only with sugar syrup over the experimental period. The daily number of survived bees over the 6 days after treatment application was recorded. The percentage of survived bees was calculated as  $([\text{number of survived bees}/15] \times 100)$ . After the end of the experiment, gut solutions of 10 live or recently dead bees from each group were inspected under microscope using 400X to detect the presence of any spores of NS. Bees without any noticeable spores were considered healthy while those with any spore detected were considered as infected bees. Then, percentage of infected bees was calculated  $([\text{number of bees with detected spores}/10] \times 100)$ . The best treatment in regard to NS control was then identified.

### Statistical analysis

The means were presented with their standard errors (SE). The treatments were considered as the independent factors while the percentages of studied parameters as the dependent factor. The percentages were transferred into degrees using



**Fig. 1.** *Nosema* spp. detected in gut solutions of honey bee samples.

arcsine transformation before the analysis. Means of infection percentages before and after the treatments were compared using t-test while Duncan test<sub>0.05</sub> was used for the other comparisons after ANOVA. The analysis was performed using SAS 9.1.3 (SAS Institute, Cary, NC, USA, 2004). Moreover, the correlations (Pearson<sub>0.05</sub>) between temperature, RH and percentages of detected diseases were calculated.

## Results

### Calendar for diseases prevalence

Diseases distributed over the year for immature and mature stages of honey bees (Table 1 and Fig. 2). Three diseases for immature stages were recorded; bald brood from August to

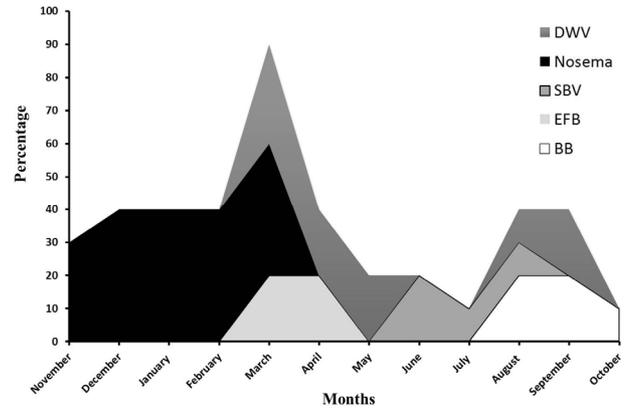


Fig. 2. Percentages of detected diseases over the study months.

October, European Foul Brood (EFB) during March and April while Sacbrood virus (SBV) was detected from June to August. Few colonies (about 20%) were infected with these

**Table 1.** Calendar for diseases prevalence of honey bees (brood and adults) over one year. Number of colonies is denoted with + while \* means that crawling was observed on the apiary ground. BB: Bald Brood, EFB: European Foul Brood, SBV: Sacbrood Virus, DWV: Deformed Wing Virus. Percentages of infection with detected diseases are presented. Also, average temperature (Tem.) and relative humidity (RH) are presented

Month/year (Tem./RH)	Brood			Adults		
	BB	EFB	SBV	<i>Nosema</i>	DWV	Crawling
November/2016 (24°C/60%)	-	-	-	+++ 30	-	*
December/2016 (17°C/67%)	-	-	-	++++ 40	-	*
January/2017 (16°C/64%)	-	-	-	++++ 40	-	*
February/2017 (19°C/67%)	-	-	-	++++ 40	-	*
March/2017 (22°C/63%)	-	++ 20	-	++++ 40	+++ 30	*
April/2017 (26°C/56%)	-	++ 20	-	-	++ 20	-
May/2017 (31°C/53%)	-	-	-	-	++ 20	-
June/2017 (34°C/52%)	-	-	++ 20	-	-	-
July/2017 (36°C/57%)	-	-	+ 10	-	-	*
August/2017 (34°C/61%)	++ 20	-	+ 10	-	+ 10	*
September/2017 (31°C/62%)	++ 20	-	-	-	++ 20	-
October/2017 (27°C/61%)	+ 20	-	-	-	-	-

diseases while American Foul Brood (AFB) was not detected. Concerning adults, *Varroa* mites were not included in Table 1 because *Varroa* is a common parasite to bee colonies. NS was detected from November until March (30 to 40% of the colonies) while bees with deformed wings from March to May and during August and September for 10 to 30% of the colonies. Bees were observed crawling on the ground at various times except from April to June and September to October. Average temperature correlated significantly with *Nosema* and SBV ( $r = -0.89$ ,  $P = 0.00$ ;  $r = 0.65$ ,  $P = 0.02$ , respectively), and insignificantly with BB, EFB, and DWV ( $r = 0.37$ ,  $P = 0.23$ ;  $r = -0.16$ ,  $P = 0.61$ ;  $r = 0.13$ ,  $P = 0.66$ , respectively). Relative humidity correlated significantly only with *Nosema* infection ( $r = 0.74$ ,  $P = 0.00$ ), and insignificantly with BB, EFB, SBV, and DWV ( $r = 0.13$ ,  $P = 0.68$ ;  $r = -0.07$ ,  $P = 0.82$ ;  $r = -0.53$ ,  $P = 0.07$ ;  $r = -0.17$ ,  $P = 0.58$ , respectively).

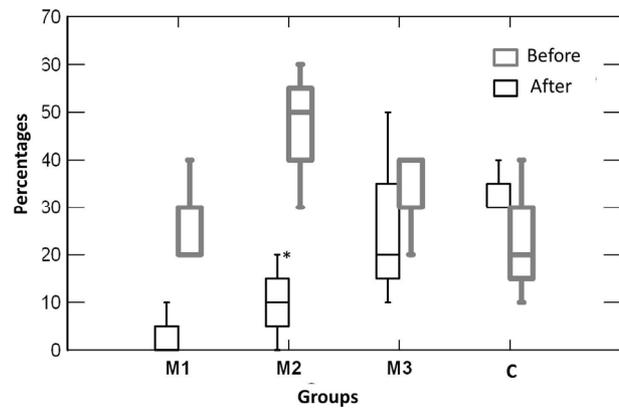
#### Role of some materials to control *Nosema*

##### Effects on *Nosema* infection rates under field conditions

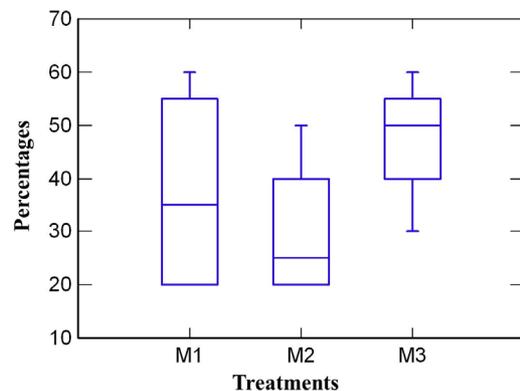
The infection percentages of the colonies before the treatments were not significantly different ( $DF = 3$ ,  $F = 1.71$ ,  $P = 0.24 > 0.05$ ). The infection percentages after applying the treatments differed significantly among treatment groups ( $DF = 3$ ,  $F = 4.34$ ,  $P = 0.04 < 0.05$ ). The percentages of infected bees with NS in the colonies reduced after applying the treatments. According to t-test, the comparisons between infection percentages before and after applying the treatments were not significant to all the groups except M2 which caused significant reduction. The highest reduction in infection percentages was significantly ( $P < 0.05$ ) to M2 while M3 occupied the second rank after M2 but without significant ( $P > 0.05$ ) differences than M1 and control colonies (Table 2 and Fig. 3).

**Table 2.** Percentages (means  $\pm$  S.E.) of infected bees with *Nosema* before and after applying the treatments. M1: diluted honey mixed with lemon juice, M2: chamomile, M3: sutrivet, and control: bees without treatment. Means in the same row with different letters denote significance according to Duncan test while \* denotes the significance according to t-test

Treatments	M1	M2	M3	Control
Infection % (before)	33.33 $\pm$ 6.66a	46.66 $\pm$ 8.81a	26.66 $\pm$ 6.66a	23.33 $\pm$ 8.81a
Infection % (after)	26.66 $\pm$ 12.01a	10.00 $\pm$ 5.77ab	3.33 $\pm$ 3.33b	33.33 $\pm$ 3.33a
t-statistics	0.903	10.381	2.729	1.081
P-value	0.46	0.009*	0.11	0.39
Difference (=before - after)	13.33 $\pm$ 3.33b	36.66 $\pm$ 3.33a	23.33 $\pm$ 8.81ab	-16.66 $\pm$ 3.33b



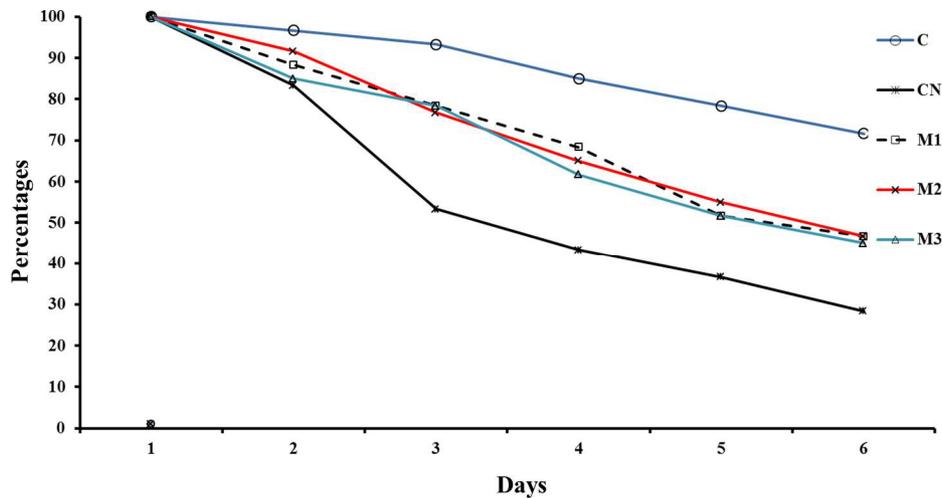
**Fig. 3.** Median and interquartile range of infected bee percentages before and after the treatments. M1: diluted honey mixed with lemon juice, M2: chamomile, M3: sutrivet, and C: control. \*: only significant differences were detected between percentages before and after the treatment with M2 according to t-test.



**Fig. 4.** Distribution of infected bee percentages in replicates of studied treatments. M1: diluted honey mixed with lemon juice, M2: chamomile, and M3: sutrivet. Median and interquartile range are shown.

##### Effects on *Nosema* infection rates and survival of bee workers in the laboratory

No *Nosema* spores were detected in all the investigated bees from the control group (without infection) while spores were detected in all bees from the group 4 (infected bees without any



**Fig. 5.** Percentage means of survived bees during the experimental period. C: bees without infection, CN: bees infected with *Nosema* without treatment, M1: diluted honey mixed with lemon juice, M2: chamomile, and M3: sutrivet.

treatments). The highest percentage mean of infected bees at the end of the experiment was to M3 ( $47.50 \pm 6.29\%$ ) followed by M1 ( $37.50 \pm 10.30\%$ ), and finally M2 ( $30.00 \pm 7.07\%$ ) (Fig. 4). The variations between the treatments were not significant ( $DF = 2, F = 1.18, P = 0.35 > 0.05$ ). The difference between the highest mean and the lowest one was 17.5%.

As shown in Fig. 5, the percentage of survived bees declined over days in all the groups until the end of the experiment. At day 6, the means were  $71.66 \pm 3.19$ ,  $28.33 \pm 3.19$ ,  $46.66 \pm 7.20$ ,  $46.66 \pm 2.72$ , and  $45.00 \pm 4.19\%$  for control group, bees infected with NS without treatment, M1, M2, and M3, respectively. The highest decline and the highest survival rates were significantly different ( $DF = 4, F = 3.90, P = 0.0052 < 0.05$ ) to infected bees without any treatments and the control group than the treatments, respectively. The three treatments impacted the percentage of survived bees in a similar way without significant differences ( $DF = 2, F = 0.20, P = 0.81 > 0.05$ ).

## Discussion

### Calendar for diseases prevalence

EFB was detected in few colonies during March and April. This disease appeared at the beginning of the active season while bald brood (BB) appeared during August to October (towards the end of the active season). BB happens due to the

infection of colonies with wax moths (Ellis et al., 2013), and the colonies during this period are expected to be more susceptible to the infection with wax moths. After clover season (during June), SBV was detected in few colonies. Average temperature showed significant correlation with SBV (65%). This indicates potential impacts of temperature on SBV. In fact, the infection with brood diseases was not high.

The infection of adult bees with NS was found in 30 to 40% of the colonies from November until March. Similarly, heavy infection with NS was found during January, February and March at Qena Government, Egypt (Aly et al., 2012). The infection with NS is through the year (García-Palencia et al., 2010) but the highest levels are during spring (Traver et al., 2012). Severe infections in autumn are correlated positively with spring infection (Fries, 1988). In Taiwan, high infection with NS was found during winter (Chen et al., 2012). Bees during winter live longer and do huge efforts in the thermoregulation of the colonies. Hence, NS can easily infect such older bees, especially they are more susceptible to infection compared to younger bees (Fries et al., 2013). Another reason for the presence of NS during this period could be the impacts of low temperature. Especially the results showed the presence of high negative and significant correlation between average temperature and NS (-89%), and positive significant correlation with RH (74%). This means that the high NS occurs at low temperatures and high RH. Similarly, the highest NS was recorded at about  $15^{\circ}\text{C}$  while high temperature showed

negative effects (Chen et al., 2012). Also, high temperature of 40°, 45°, or 49°C negatively impacted the viability of spores after short period (Malone et al., 2001). Moreover, the intensity of colonies infection with NS positively impacted with low ambient temperature (Retschnig et al., 2017).

Bees with deformed wings are indication to the infection of bees with Deformed Wing Virus (DWV) which can be transmitted by *Varroa* mites (De Miranda and Genersch, 2010). Fortunately, this disease was found in few colonies, and DWV has been recorded in Egypt previously (Abd-El-Samie et al., 2017). The crawling of bees can be explained by the presence of NS and DWV in some colonies. Bees infected with NS or have malformations in their wings can not fly normally and are mostly crawling. Insignificant correlations were detected between average temperature and BB, EFB, and DWV, and between RH and BB, EFB, SBV, and DWV. This indicates the low impacts of temperature and RH on these diseases.

## Role of some materials to control *Nosema*

### Effects on *Nosema* infection rates under field and laboratory conditions

In the field experiment, M2 (chamomile) showed the highest efficacy than M3 (sutrivet) and M1 (diluted honey with lemon). This suggests the potential ability of chamomile extract to suppress the development of NS spores with higher degree than M1 and M3. The laboratory experiment supported the role of M2 and showed that M1 was better than M3. The study by Michalczyk et al. (2016) supported the role of herbal extracts in NS control. They have found that a compound contained extract from *Artemisia absinthium* (similar to chamomile) mixed with acetylsalicylic acid has shown efficacy in reducing the infection by 63.36% in a field trail. But on the contrary, Porrini et al. (2011) found no reduction in spore numbers when plant extract of *Artemisia absinthium* was tested. The variations in experimental conditions may be impacted the results of *Artemisia* in the previous studies. Herbal preparation has shown ability to protect the gut layers from *Nosema* spores (Gajger et al., 2011). Also, natural material (thymol) has shown efficacy against NS infection (Costa et al., 2010). The efficacy of M1 may be attributed to its impacts on gut acidity (reducing the acidity) due to the addition

of lemon juice. A mixture of oxalic acid and sugar syrup reduced significantly spore numbers and infection prevalence in laboratory and field trails, respectively (Nanetti et al., 2015). This supports the role of using materials with low acidity in reducing the infection rates. However, the alterations of bee gut pH using acetic acid or benzoic acid did not influence the infestation rate or even the disease development (Forsgren and Fries, 2005). Perhaps the type of the acid material (e.g. oxalic, benzoic or others) has also effect on the infection beside pH, thus dissimilar results were found. Concerning M3, this compound impacted *Nosema* but these impacts were not very high, especially its components are effective mainly against bacteria.

### Percentage of survived bees in the laboratory

The healthy bees provided only with sugar syrup were able to survive significantly more than all the other groups. This finding suggests the role of NS in reducing the survival of bees. Treated bees were able to survive in a similar way significantly more than infected bees without treatment. This point highlights that leaving bees without any treatment shortened their survival ability in a rapid way. In a similar way, infected bees and treated with thymol lived significantly longer than those infected without any treatment (Costa et al., 2010). The high death rates in infected groups can be understood by the passive impacts of spores on the gut and the immune system (Antúnez et al., 2009).

## Conclusion

Few diseases of brood and adult bees were detected over the study period, suggesting the high adaptability of local honey bees to these diseases. NS was detected from end of autumn until spring. Thus, beekeepers are advised to carefully examine their colonies during this period to detect any infection with NS and to treat their colonies. The study showed the potential use of chamomile to control NS more than diluted honey mixed with lemon juice, and sutrivet. Further studies to identify exactly the effective ingredients of chamomile and its mode of action to control NS are advisable. For beekeepers, the use of chamomile can be considered as a natural treatment to the infected colonies.

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