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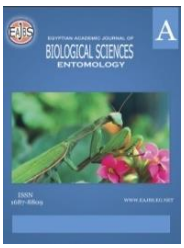

Effects of Acetylsalicylic Acid, Echinacea Purpurea Extract, and Vitamin C On Survival, Immunity and Performance of Honey Bees

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ABSTRACT

From late autumn until the end of winter conditions, bees are exposed to many stress factors including low air temperature and several pathogens. This study aimed to estimate the effects of three commercial materials (acetylsalicylic acid, *Echinacea purpurea* extract, and vitamin (C) mixed with sugar feeding on some parameters related to stress tolerance using laboratory and field tests. The laboratory tests supported the potential role of vitamin C and *Echinacea purpurea* extract to enhance the tolerance ability of bees to low temperatures. The most promising results for the recovery time of bees after exposure to low temperatures, survival rates after narcosis, and hemocyte count were recorded with these two materials. After winter the strength of bee colonies fed on sugar feeding mixed with vitamin C and *Echinacea purpurea* extract were approximately equal to the strength before winter. However, the contrary was obtained with acetylsalicylic acid and the control group. Thus, using vitamin C or *Echinacea purpurea* extract as safe additives can boost the survival of bee colonies during the winter period.

INTRODUCTION

The performance of honey bee colonies fluctuates over the year in response to seasons. The active seasons extend from mid-spring until early autumn in most countries including Egypt. Honey bee colonies can store a good amount of food during the active season (Crailsheim *et al.,* 1992), and can survive without the need for any artificial feeding. Otherwise, feeding has a good role in improving honey bee survival, immunity, and tolerance to harsh conditions as well as colony development (Babendreier *et al.,* 2004; DeGrandi-Hoffman *et al.,* 2016; Glavinic *et al.,* 2017). Therefore, beekeepers supply their colonies with alternatives to natural feeding including sugar feeding (Abou-Shaara 2017a), pollen alternatives (De Jong *et al.,* 2009; Saffari *et al.,* 2010; Aly *et al.,* 2014; Zaghloul *et al.*, 2017; Gamal Eldin *et al.,* 2018) under the shortage of natural resources availability. The period from autumn to early spring is considered the most challenging time for bee colonies due to the absence of natural food resources, harsh environmental conditions, and the

prevalence of bee diseases. Consequently, the loss of bee colonies was commonly recorded during this period, which may reach in some countries up to 40% (Brodschneider *et al.*, 2010; Gajger *et al.,* 2010; Topolska *et al.,* 2010; Brodschneider *et al.,* 2019). Therefore, specific actions should be done by beekeepers to protect their colonies.

Providing colonies with sugar feeding (carbohydrates) is an important practice during this period over the use of pollens (proteins) due to the low brood-rearing activity during winter time. On the other side, the longevity of winter bees is noticeably higher than those reared during active seasons (Free and Racey 1968; Mattila *et al.,* 2001; Behrends and Scheiner 2010). Such older bees are exposed to several stressors including cold air temperature, low food quality, and pathogens (Van Dooremalen *et al.,* 2012; D'alvise *et al.,* 2018; Feliciano-Cardona *et al.,* 2020). Hence, supplementing sugar syrup with specific materials can help improve the immunity and survival of such bees. Previous studies showed the role of vitamin C in improving brood rearing and the survival of honey bees (Herbert *et al.,* 1985; Andi and Ahmadi 2014; Farjan *et al.,* 2014). Indeed, available studies on vitamins are not sufficient but this specific vitamin has gained attention for a long time; however, it has not been tested with winter bees. Another important point is that Nosema spores are known to be more active during winter than other seasons (Abou-Shaara 2018a), and are affected by temperature (Malone *et al.,* 2001; Retschnig *et al.,* 2017). For this reason, treatments against Nosema are used during the period from autumn to spring (Goodman *et al.,* 1990; Williams *et al.,* 2011), and such treatments are added to sugar syrup. One of these treatments contains acetylsalicylic acid (Michalczyk *et al.,* 2016), but such material has not been tested with winter bees.

In addition to vitamin C and acetyl-salicylic acid, some herbal extracts can be added to sugar syrup to improve the health of bee colonies. Some herbal extracts have been previously tested including *Artemisia* sp., mint, and cinnamon (Pohorecka 2004; Abou-Shaara 2018a; Balamurugan *et al.,* 2020; Al-Ghamdi *et al.,* 2021). In this study, an herbal extract from *E. purpurea* was tested. This herb can enhance bee immunity (Gorzin *et al.,* 2017) but it has not been studied with winter bees. Specific parameters are usually used to judge the efficacy of specific feeding types or additives on honey bees. These parameters include colony performance (Amro *et al.,* 2016), survival (Škerl and Gregorc 2014; Abou-Shaara 2017a; Abou-Shaara 2018; Al-Ghamdi *et al.,* 2021), and some laboratory investigations such as body water content and hemolymph parameters (Amro *et al.,* 2016; Abou-Shaara *et al.,* 2017). Therefore, the objective of this study was to investigate the effects of three materials; *E. purpurea* extract, vitamin C, and acetylsalicylic acid on survival, some physiological variables, and colony performance. These materials were tested as additives to sugar syrup, and specific recommendations were presented based on the outcomes.

MATERIALS AND METHODS

Experiment Setup:

1. Test Materials:

Three materials were tested: 1) Acetylsalicylic acid (The Arab Drug Company, Egypt), 2) *E. purpurea* extract (in the form of Immulant syrup, 16.7 mg/ml) Arab Company for Pharmaceuticals and Medicinal Plants, Cairo, Egypt and 3) Vitamin C (Chemical Industries Development, Giza, Egypt). These materials were abbreviated hereafter as ACA, EE, and VC, respectively.

2. Concentrations:

The materials were used as 25 mg per each 100 ml of sugar syrup (1 sugar: 1 water for laboratory tests or 2 sugar: 1 water for apiary experiments w/l). The laboratory tests were conducted at two locations (Damanhour University and Assiut University) while field

experiments were performed at an apiary in Damanhour City.

Laboratory Experiments:

1. Honey Bee:

A local hybrid of Carniolan honey bee workers was used in these experiments during the winter season from December 2020 to February 2021.

2. Tolerance to Freezing Temperature:

Two experiments were done to evaluate the ability of the used materials to enhance the tolerance ability of bees to low temperatures according to Atmowidjojo et al. (1997), Abou-Shaara et al. (2012) and Abou-Shaara (2017a) with modifications. The first experiment recorded the recovery time after exposure to freezing temperature (-20°C), while the second experiment evaluated the survival of bees after this exposure. The details of these experiments are as follows:

3. Recovery Time:

The bees were placed individually in plastic queen cages and fed on the test materials for 12h. The bees were then placed in a freezer at about -20°C for 3 minutes until narcosis. After that, the bees were placed at room temperature (≈ 25°C) until full body movement was recorded. A total of 80 bees were used per treatment divided into 8 replicates each with 10 bees.

4. Survival After the Narcosis:

In this experiment, the bees fed on the test materials for two days. Then, the bees were placed in a freezer (-20°C) until narcosis. Subsequently, the bees were placed at room temperature without any food and the survival of bees was recorded every 6 hours until death. In this experiment, a group of 10 adult bees was placed in plastic cages with good ventilation (Abou-Shaara 2017b). For each treatment, 10 cages (replicates) were used with a total of 100 bees per material.

Differential Hemocyte Count (DHC):

In this experiment, newly emerged bees (i.e. one-day-old) were used. The bees were fed on the test materials for 20 days (3 cages per each treatment and in each cage 50 bees). Also, all cages were provided with mixed pollen loads as a protein source and then introduced inside an incubator at optimum conditions (34.5°C and 65 % RH), as described by Williams *et al.* (2013). On day 18, the hemocytes were counted (before exposure). On day 19, the bees were placed at a low temperature of 20°C for one day. Then, the hemocytes were counted again (after exposure). Fifteen workers from each group (5 from each replicate) before and after exposure to a low temperature were randomly chosen for the DHC. sample.

Hemolymph samples were collected from each adult worker by cutting the dorsal intersegmental membrane between segments 5 and 6 of the abdomen using a fine hypodermic needle (Wegener *et al.*, 2009). A drop of adult worker hemolymph was spread as a thin film on a clean glass slide using the edge of a cover, air-dried and fixed in absolute ethanol for 20 min. The slides were stained with Romanvsky – Giemsa stain for 12 hrs, washed with tap water, distilled water, and air dried, respectively. The prepared slides were examined under a light microscope (Olympus–CH20i BIMU) by the oil-immersion lens at a magnification of 100X. Five hemocyte types were identified in adult worker's hemolymph and classified as prohemocytes, plasmatocytes granular cells, spindle cells, and oenocytes. The type of hemocytes was identified using the key of Ribeiro and Brehélin (2006) and the numbers of each type were calculated from each treatment.

Colony Survival:

During autumn and winter very low brood rearing occurs; therefore, only areas covered with bees were considered to evaluate the effects of the used materials on colony survival. These areas were measured according to Jeffree (1958) using a wooden frame divided into units (square inches). The areas were then multiplied by 10 to calculate the

number of bees (Abou-Shaara *et al.*, 2013). The feeding mixed with test materials was presented to bee colonies weekly from the first week of November 2020 until the end of February 2021 (four colonies/ each material). The measurements were taken twice: the last week of December and the next February which covers the winter period. All colonies were equalized in their strength before the beginning of the experiment (4 combs covered with bees and headed with young hybrid Carniolan queens).

Statistical Analysis:

Parametric and non-parametric tests were performed on the data based on their normality, which was tested using the Shapiro-Wilk test. Data with normal distribution were subjected to ANOVA followed by the Tukey test (parametric tests) while those without normal distribution were subjected to Kruskal-Wallis (non-parametric tests). The survival data were analyzed using the Kaplan-Meier analysis (Breslow test), which is perfect for survival analysis (Abou-Shaara 2018b). The analysis of hemocyte count before and after exposure to a low temperature was performed using a t-test. SPSS (v. 16, Chicago, USA, 2007) as a statistical software was used in the analyses considering p value ≤0.05 as significant.

RESULTS

Laboratory Experiments:

1. Tolerance to Freezing Temperature:

a. Recovery Time:

The short recovery time was recorded with bees treated by the vitamin C group (9.32 min) followed by the *E. purpurea* extract group (9.36 min), then the control group (10 min), and finally acetylsalicylic acid group (10.11 min) (Fig. 1). The differences between bees treated with the vitamin C group and the other groups were 2.7, 40.79, and 47.45 seconds for *E. purpurea* extract, control, and acetylsalicylic acid, respectively. The Kruskal-Wallis test showed the presence of significant differences among the treatments (Chi-Square= 104.031, df=3, p<0.05). According to the Mann-Whitney test, significant differences between control and acetylsalicylic acid (P=0.039 <0.05) were recorded. There was no significant difference between vitamin C and *E. purpurea* extract (P=0.225>0.05) detected. Vitamin C and *E. purpurea* extract groups showed significant differences between the control and acetylsalicylic acid bee group (Kruskal Wallis Test: p<0.05).

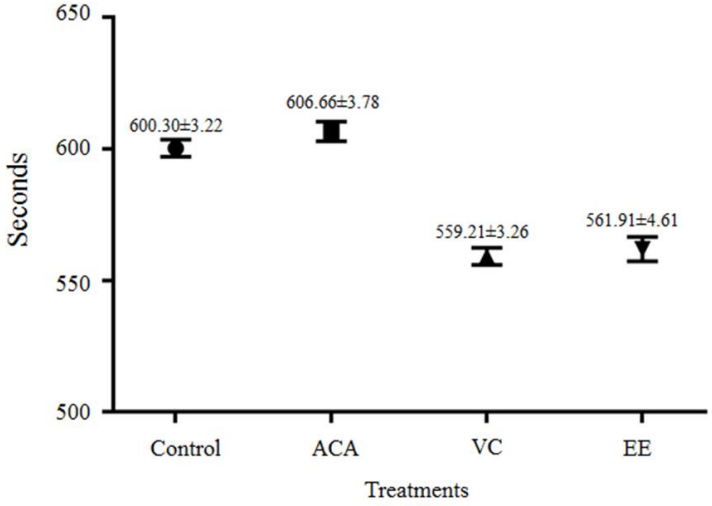

Fig. 1. Recovery time (seconds) of bee workers after exposure to low temperature. Control: Sugar syrup only, ACA: Sugar syrup and acetylsalicylic acid, VC: Sugar syrup and vitamin C, and EE: Sugar syrup and *E. purpurea* extract.

b. Survival after the narcosis

The bees in the *E. purpurea* group showed higher survival ability after narcosis than the other groups (Fig. 2). The second rank was occupied by the vitamin C group, then the acetylsalicylic acid group, and finally the control group. Significant variations were found between all groups (Mantel-Cox: Chi-Square=21.721, df=3, $p<0.05$) except between *E. purpurea* and vitamin C groups (Chi-Square=1.279, $p=0.258>0.05$) and between vitamin C and acetylsalicylic acid groups (Chi-Square=1.377, $p=0.241>0.05$). The half of bees (50%) died after 42±2.71h, 48±3.462h, 48±3.00h, and 54±3.00h for the control, acetylsalicylic acid, vitamin C, and *E. purpurea* group, respectively.

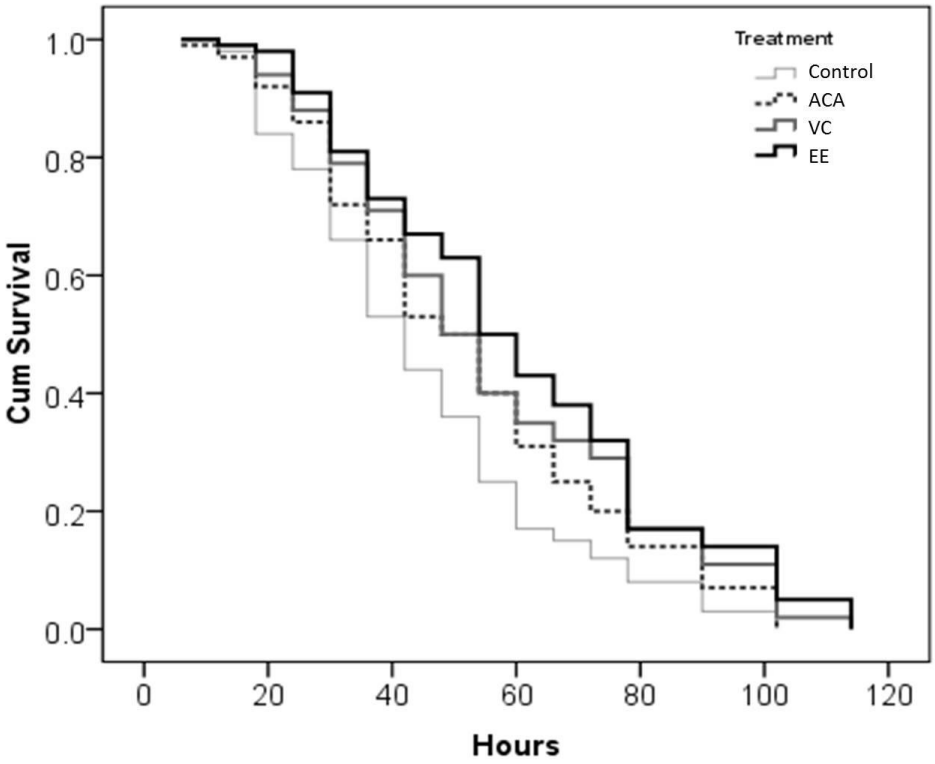

Fig. 2. Cumulative bee workers' survival after narcosis. Control: Sugar syrup only, ACA: Sugar syrup and acetylsalicylic acid, VC: Sugar syrup and vitamin C, and EE: Sugar syrup and *E. purpurea* extract.

2. Differential Hemocyte Count:

The bees fed only on sugar syrup without any additives (control group) had similar differential hemocyte counts compared with those fed on acetylsalicylic acid for most hemocyte types without significant differences. The hemolymph of bees fed on *E. purpurea* extract group showed a higher percentage of oenocytes and spindle cells, followed by bees from the vitamin C group (Table 1). After exposing bees to low temperatures, the counts of blood cells generally reduced in all groups except the vitamin C group. Such changes in numbers were significantly different in most cases. The counts of oenocytes and spindle cells generally increased in all groups after exposure to low temperatures. Bees from the vitamin C group occupied the first rank in counts of blood cells after exposure to low temperature than the other groups except in the case of spindle cells. The variations between groups tended to be significantly different ($p<0.05$).

Table 1. Statistical comparisons using t-tests between the number of different hemocyte types in control and test groups before and after exposure to low temperature. Means in the same row followed by the same letter are not significantly different according to the Tukey test.

Blood cell type	Time	Control	Acetylsalicylic acid	Vitamin C	*E. purpurea* extract
Prohemocytes	**Before**	23.40±0.927a	21.85±0.66ab	18.84±0.85bc	18.45±0.78c
	After	20.60±1.08a	17.18±0.70b	23.32±0.92a	14.85±0.74b
	t value	1.96	4.82	3.54	3.31
	p value	0.06	0.00	0.001	0.003
Plasmatocytes	**Before**	79.93±3.74a	70.82±2.24ab	56.81±3.12c	63.86±2.93bc
	After	102.20±3.73a	84.34±4.31b	108.62±2.66a	55.54±2.81c
	t value	4.21	2.78	12.62	2.04
	p value	0.00	0.01	0.00	0.05
Granular cells	**Before**	10.01±0.35a	8.28±0.31c	7.50±0.55d	9.89±0.44b
	After	7.34±0.39bc	8.61±0.44ab	9.88±0.50a	6.01±0.22c
	t value	5.03	0.61	3.16	7.77
	p value	0.00	0.054	0.004	0.00
Oenocytes	**Before**	1.01±0.10bc	0.74±0.07c	1.44±0.11b	2.34±0.16a
	After	1.32±0.11b	1.01±0.10b	2.74±0.31a	0.97±0.12b
	t value	1.99	2.11	3.91	6.72
	p value	0.05	0.04	0.001	0.00
Spindle cells	**Before**	4.57±0.17b	4.65±0.26b	5.24±0.31ab	5.98±0.33a
	After	10.06±0.72a	9.62±0.41a	6.90±0.26b	7.26±0.43b
	t value	7.35	10.12	4.08	2.33
	p value	0.00	0.00	0.00	0.027

2. Colony Survival

The results highlighted the positive effects of vitamin C and *E. purpurea* extract on the number of bees than acetylsalicylic acid and the control group after winter (Table 2). The number of bees before winter showed insignificant differences from that after winter in the case of vitamin C and *E. purpurea* extract while significant negative effects were observed in acetylsalicylic acid and control groups (Table 2).

Table 2. Effect of feeding three commercial materials on change of colony strength (number of bees) during the winter period. Statistical comparisons using t-test and Tukey between the number of bees for control and test groups before and after winter. Means±S.E. are presented. Means in the same row followed by the same letter are not significantly different according to Tukey test.

Time	Control	Acetylsalicylic acid	Vitamin C	*E. purpurea* extract
Before winter	5362.50±525.74a	4687.50±687.50b	7302.50±469.75a	6420.00±405.05a
After winter	4762.50±372.70b	4187.50±600.13b	7185.00±584.31a	6075.00±303.10ab
t value	3.307	4.714	0.790	2.253
p value	0.045	0.018	0.487	0.110

Each vitamin C and *E. purpurea* extract had a significantly higher number of bees after winter than the acetylsalicylic acid and control group. Figure 3 shows that vitamin C had the least variation in the number of bees before and after winter followed by *E. purpurea* extract while a clear decline in the number of bees after winter is observed in the acetylsalicylic acid and control group.

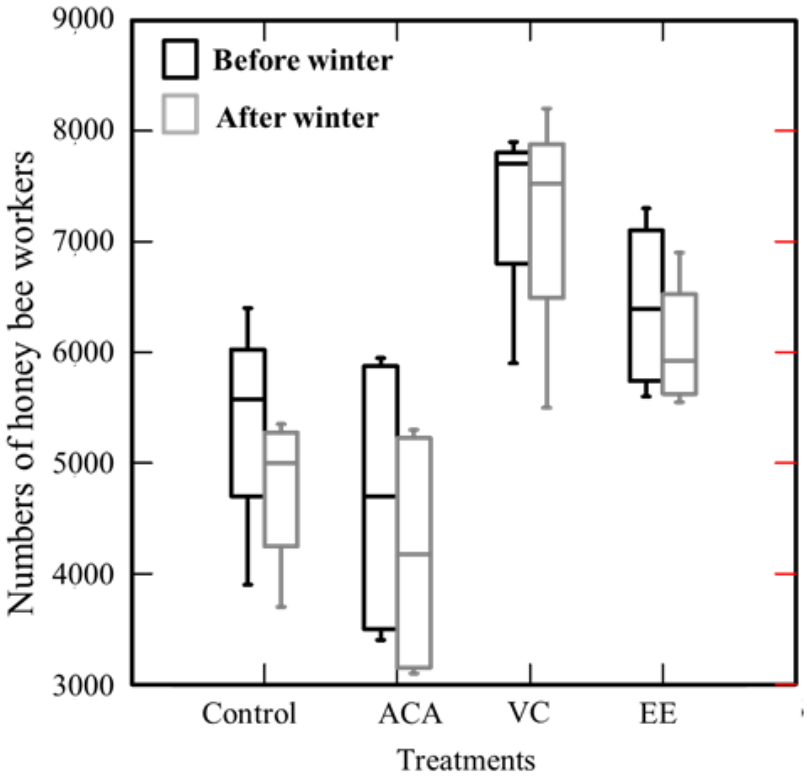

Fig. 3. Number of honey bee workers before and after winter in control and test groups. Control: Sugar syrup only, ACA: Sugar syrup and acetylsalicylic acid, VC: Sugar syrup and vitamin C, and EE: Sugar syrup and *E. purpurea* extract.

DISCUSSION

The laboratory studies showed the ability of vitamin C and *E. purpurea* extract to help bees recover faster after exposure to a low temperature. Moreover, these two materials showed the best results for the survival of bees after narcosis by improving bee survival during low-temperature stress. These results are also consistent with the field experiments. Bees exposed to low temperatures showed high counts of different blood cell types in the vitamin C group than the other groups (Zakaria 2007; Negri *et al.,* 2016). It is known that blood cells contribute to increasing the immunity of bees especially plasmatocytes which were high in the vitamin C group. This laboratory investigation suggests the role of vitamin C in enhancing the immunity of bees. It seems that the effects of *E. purpurea* extract on the blood cells of winter bees were not high although its ability to improve bee immunity (Gorzin *et al.*, 2017). Probably, this plant extract affects the immunity of bees using other mechanisms.

Using vitamin C and *E. purpurea* extract in bee feeding showed the ability to improve colonies' survival during winter. These two groups had the highest numbers of bees (i.e. colony strength) after winter. This outcome can be explained by their roles in boosting the immunity system of bees leading to positive effects on the whole colony. Plant extracts which are rich mostly in vitamins can help in boosting the health of honey bees and colony development. In line with this study, Al-Ghamdi *et al.* (2021) highlighted the role of *Matricaria chamomilla*, *Mentha Spicata*, and *Cinnamomum zeylanicum* in improving the development of the colonies. Also, the present findings are in line with previous studies (Herbert *et al.,* 1985; Andi and Ahmadi 2014; Farjan *et al.,* 2014) on using Vitamins C to

improve the strength and survival of bee colonies. Indeed, bee feeding could affect the development of bee colonies (Bodla *et al.,* 2009; Abou-Shaara 2017a). However, it is not expected that variations between groups are due to feeding type as bees were fed in the same way. So, the test materials are the main trigger for the noticeable variations between colonies.

On the other side, acetylsalicylic acid is perhaps useful in the control of some diseases such as Nosema but with less ability to improve bee health than vitamin C and *E. purpurea* extract. Some acids could be used as potential materials for the control of Nosema (Nanetti *et al.,* 2015). This supports the possible role of acetylsalicylic acid in improving the health of bees. However, it seems that natural plant extracts and vitamins are more effective. It has been found that using a mixture of natural materials that contain herbs and vitamins can improve bee health and aid in the control of Nosema (Gajger *et al.,* 2011; Gajger *et al.*, 2013; Michalczyk *et al.,* 2016; Abou-Shaara 2018a; Chaimanee *et al.,* 2021). This shades lighter on the benefits of plant extracts and vitamins for honey bees.

5. CONCLUSION

The study investigated the role of three materials (acetylsalicylic acid, vitamin C, and *E. purpurea* extract) in enhancing bee health and tolerance to low temperatures under laboratory and apiary conditions. The results supported the role of vitamin C and *E. purpurea* extract to enhance the survival of bee colonies during winter. Colony strength, tolerance to low temperature, and hemocyte count supported the roles of these two materials. For beekeepers, the use of the two materials as additives to bee feeding during autumn/winter could be helpful to reduce rates of winter colony losses. More investigations on the effects of *E. purpurea* extract on the immune system of honey bees are advised.

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