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Research article

IMPACT OF THE PLANT-BASED NATURAL SUPPLEMENT IMMUNOSTART HERB ON HONEY BEE COLONY PERFORMANCE

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Winter is the season that poses the greatest challenges for honey bee colonies. Therefore, the main approach in beekeeping practice is aimed mainly at providing sufficient quality food supplies for bee colonies in early autumn. We conducted the present study to test the influence of the natural plant extract IMMUNOSTART HERB on population strength, stored pollen area, capped worker brood area, and honey yield. The experimental groups were supplied with IMMUNOSTART HERB 4 times at 7-day intervals, whereas sugar syrup was given to the control groups. The obtained results showed that the applied supplemental diet affected all investigated biological parameters, with the most noticeable effect after the second application. In all measurements, the honey bee colony parameters in the treated groups showed higher values in comparison to the control groups. These results highlight the potential of herbal supplements to effectively improve bee colonies' development during the period of scarce bee forage, as well as to provide suitable conditions for successful overwintering.

Key words: *Apis* mellifera; herbal extract; colony strength; overwintering

INTRODUCTION

The honey bee (*Apis mellifera* Linnaeus, 1758) is known as the most effective pollinator among all insect species [1,2]. Honey bees produce various products, most of which are of great importance to mankind [3,4]. However, for over a decade, honey bees have been attacked by a large number of so-called biotic and abiotic drivers, which have an extremely negative effect on bee colonies [5-7]. The growth and development of honey bee colonies depend primarily on worker bees' foraging activity [8]. To collect nectar and pollen (primary food resources), honey bees fly different distances

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from the hive (up to 7-9 kilometers), depending on the location of the pasture, the quantity and quality of nectar released from the honey crops, the nature habitat, and the possibilities for orientation [9].

Determination of the type of supplemental nutrition for honey bees is crucial in beekeeping practice, as nutritional needs are highly variable, depending on the different subspecies and stages of life as well as seasonal variability in food availability [10]. During the seasons food resources in nature are scarce, some physiological changes are observed in bee colonies, e.g., decreased egg-laying, less availability of forager bees and an increase in bee rustling [11,12].

In Bulgaria, higher temperatures and dry climate conditions in the summer season in most parts of the country are the main factors of increased mortality of bee colonies during the winter. Heat stress leads to reduced bee forage (number of melliferous plants) and low availability of nectar and pollen, which constitute the main source of food for bees [13,14]. Thus, additional nutrition is required for maintaining colony health and for successful wintering. To meet the needs of bee colonies in the conditions of scarce bee forage, various artificial diets were formulated to replace natural honey and pollen [15-17].

In the last few years there has been an increase in interest regarding the application of plant extracts in order to enhance the reproductive performance and honey bee strength, disease resistance, and dietary consumption, by measuring the area of the worker broods or successful overwintering [18-21]. Usually, these products contain various biologically active substances, such as flavonoids, polyphenols, essential oils, terpenoids, mucus substances, amino acids, vitamins, minerals, etc. [22,23]. The plant extracts manifest low toxicity to bees, do not pollute the environment, and ensure safety for humans [24-26]. Due to these properties, they are widely used in beekeeping practice. On the one hand, they are applied to improve health and productivity of honey bee colonies as well as to increase resistance to pathogens and pesticides, to stimulate the egg-laying activity of the queen, brood rearing, etc. [27-29]. On the other hand, plant extracts can be successfully used for the control of a large number of bee diseases – bacterial, viral, parasitic, microsporidian, etc. [30-32]. The application of plant extracts against various diseases in bee colonies marks the beginning of a new approach in the battle against various pathogens. This holistic approach offers plenty advantages over the use of veterinary drugs, including lower cost of bee products, avoidance of toxic substances in bee products, which are potentially dangerous to human health, and adequate management of the emerging resistance to certain antibiotics.

In light of the increasing use of plant supplements in apiculture, the aim of the present study was to investigate the efficacy of a supplemental diet on different parameters of honey bee colony strength: including adult population, sealed worker brood area, stored pollen area and the amount of capped honey. Moreover, this study may help in determining some guidelines for beekeepers on how to work innovatively so as to

manage effectively the food scarcity problem of honey bees during times of pollen and honey shortage.

MATERIAL AND METHODS

Experimental design

All experiments were performed in Smolyan (Research Centre of Stockbreeding and Agriculture) (41°35'7.01" N, 24°41'30.98" E), a town in the south of Bulgaria, in the central part of the Rhodope Mountains. The bee colonies were kept in European Langstroth (LR) hives. According to our previous research, honey bees in this part of the country belong to the local Bulgarian *Apis mellifera rodopica* [33]. The current study was conducted from the end of July to September, 2019 (Fig. 1). Twenty colonies were randomly assigned to two equal experimental groups: 1. The IMMUNOSTART HERB® group (IG, n=10) – colonies treated 4 times at 7-day intervals (from 25 July, 2019 to 15 August, 2019) with the plant extract IMMUNOSTART HERB® (Extract Pharma, Sofia, Bulgaria) at a dose of 10 mL of the product, dissolved in 100 mL of sugar syrup (1:1, w/w), according to the manufacturer's instructions. The solution was sprayed with a syringe onto the bee combs in each experimental bee colony; 2. The control group (CG, n=10) – fed only with sugar syrup (1:1, w/w), at the same dose and time points as the IG. The biological parameters of each colony in the IG and the CG were evaluated a total of five times in 12 days – four times after each application of IMMUNOSTART HERB® (from 6 August, 2019 to 8 September, 2019). The last (fifth) measurement was performed 12 days after the penultimate (fourth) one, without treating the bee colonies meanwhile. Thus, we estimated the area of sealed worker brood, taking into account honey bee biology, i.e. 12 days passing from the laying of the egg to the appearance of the sealed brood. Furthermore, all bee colonies (IG and CG) received a total of 5 L water : sugar solution (1:1 w/w) during the experimental period, at intervals of 2 – 3 days, in order to provide enough food supply for the upcoming winter.

During the experimental period, all selected colonies (IG and CG) were regularly checked for both bee and brood pathology by a veterinary specialist, following the instructions of the "Office International des Epizooties" [34]. At the beginning of the experiment, the colonies were equalized regarding the following biological parameters – size population, the queen's age, areas of unsealed and sealed worker brood, pollen and honey reserves. All colonies from the experimental groups were managed according to beekeeping practices specific to this region.

Adult bee population

The honey bee population was measured by the mass of frames (kg) covered with bees, considering that one frame in a European (LR) hive contains approximately 200

g of bees [35]. The honey bee population was estimated a total of 5 times, at 12-day intervals after the first treatment in July, 2019 until the 8 September, 2019 (Fig. 1).

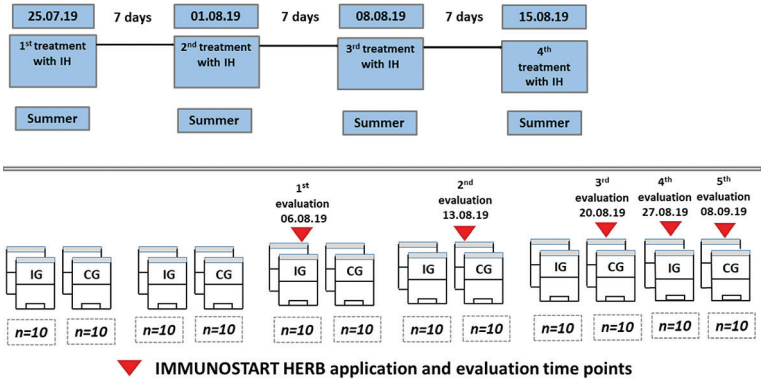

Figure 1. Experimental design.

Sealed worker brood area

The capped worker brood area was measured by using transparent grids with square areas sized 5 × 5 cm. The grid was placed over each side of a brood comb, and the number of squares with brood was recorded. After the measurements of a single hive, all frames with brood were summed for each colony. Sealed brood areas was expressed in cm^2, assuming that the area of 25 cm^2 corresponded to 100 worker brood cells [35]. The sealed worker brood area was assessed a total of 5 times, as the evaluation of the honey bee colony strength (Fig. 1).

Stored pollen area

The area of pollen reserves in the beehive was evaluated through direct surface measurements of the comb (cm^2), using a transparent grid with square areas sized 5 × 5 cm. The grid was laid over each side of every frame, and the number of squares covered with stored pollen were recorded [35]. The time points of measurement of the stored pollen area were the same as those for the evaluation of the sealed worker brood area (Fig. 1).

Amount of stored honey

Measuring the amount of capped honey in kg was performed by using frames sized 5×5 cm squares 8 squares contain 0.350 kg of honey [36]. This was carried out at the same time points and following the same manner as the measurement of the previous biological parameters (Fig. 1).

Statistical Analysis

Adult bee population, sealed worker brood and pollen areas, and the amount capped honey reserves data were compared after each treatment for a total of five times in the conducted study. The groups were compared, and graphs were created in two-way ANOVA, followed by a Dunnett's multiple comparisons test that was performed using GraphPad Prism version 9.3.1 for Windows (GraphPad, San Diego, CA, USA). The Tukey HSD post-hoc test was performed for multiple comparisons between groups and the levels of significance below 0.05 ($p < 0.05$) were considered significant. The results were expressed as (mean ± SD).

RESULTS

Colony strength parameters between groups

Adult bee population

The comparison between the two groups (IG and CG) during the experiment revealed differences in all the monitored biological parameters. After the first and the second measurement, the adult bee population in the IG was greater compared to the control CG, but the differences were not statistically significant ($F = 0.86$, $df = 6$, $p = 0.38$; $F = 3.42$, $df = 8$; $p = 0.10$, respectively) (Fig. 2). After the third treatment, at the beginning of autumn (20 August, 2019), adult bee population was significantly higher in the IG (1.70 ± 0.16 kg) than in the CG (1.40 ± 0.14 kg) (ANOVA with a Tukey's HSD post hoc test, $F = 13.50$, $df = 9$, $p = 0.006$, $p < 0.01$). These significant differences persisted during the next measurement period (27 August, 2019). Average adult bee population in the IG (1.60 ± 0.14 kg) exceeded with about 30 % that of the CG (1.24 ± 0.09 kg)

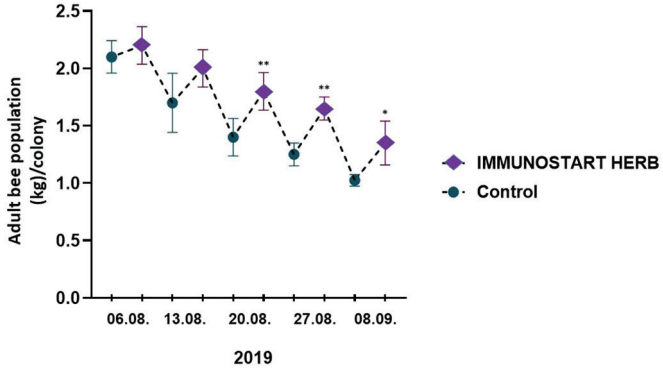

Figure 2. Comparison of parameter of colony strength (mean ± SD) between treatment and control group throughout the experiment. Asterisks indicated the level of significance as determined by an analysis of variance followed by post-hoc Tukey HSD multiple comparison test (*$p < 0.05$; **$p < 0.01$ compared to the control group).

($F = 23.14$, $df = 9$, $p = 0.001$, $p < 0.01$). This trend was maintained in the last measurement (8 September, 2019), when the adult bee population in the IG was with about 280 g bees more than in the CG ($F = 6.25$, $df = 9$, $p = 0.04$, $p < 0.05$).

Sealed worker brood area

The worker sealed brood area also differed between the IG and the CG (Fig. 3). After the first (6 August, 2019) and the second assessment (13 August, 2019), the values of this parameter were slightly higher in the IG compared to the CG. This parameter showed significantly higher values after the third treatment with IMMUNOSTART HERB®. Then the sealed worker brood area in the hives in the IG (5525 ± 28.68 cm^2) differed notably when compared to the CG (4400 ± 27.89 cm^2) ($F = 7.34$, $df = 7$, $p = 0.035$, $p < 0.05$). The most significant difference between the values of this biological parameter in the control and the experimental group was reported after the fourth application of the plant extract. The sealed worker brood area in the IG (3450 ± 30.28 cm^2) exceeded with 55 % that in the CG (2225 ± 26.72 cm^2) (ANOVA with Tukey's HSD post hoc test, $F = 7.77$, $df = 7$, $p = 0.031$, $p < 0.01$). During the last measurement (8 September, 2019), there was a sharp decline in the mean values of the sealed worker brood in both the control and the experimental groups, without a significant difference between the two groups ($F = 5.65$, $df = 7$, $p = 0.055$, $p > 0.0$).

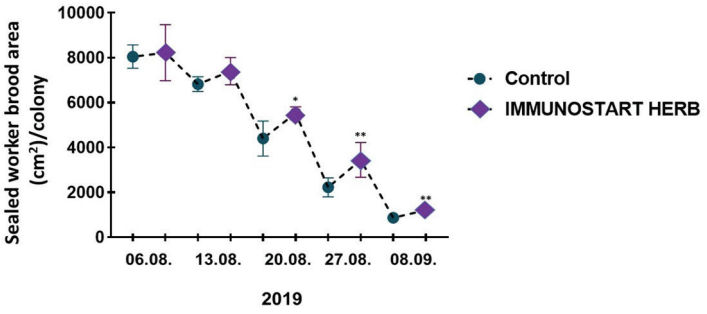

Figure 3. Comparison of parameters of sealed worker brood area (cm^2) (mean ± SD) between treatment and control group throughout the experiment. Asterisks indicated the level of significance as determined by an analysis of variance followed by post-hoc Tukey HSD multiple comparison test (*$p < 0.05$; **$p < 0.01$ compared to the control group).

Stored pollen area

Like the other studied biological parameters, the stored pollen area showed a significant difference between the two groups (IG and CG) after the third application of IMMUNOSTART HERB® (Fig. 4). Then the mean value of stored pollen in the IG (312.5 ± 31.3 cm^2) showed a significantly higher mean value compared to the CG (62.5 ± 30.8 cm^2) (ANOVA with Tukey's HSD post hoc test, $F = 15.4$ $df = 7$, $p = 0.001$, $p < 0.01$). The differences between the control and the experimental group

remained significant after the fourth treatment (15 August, 2019) with the plant extract (250.0 ± 32.5 cm^2; 43.7 ± 31.7 cm^2, $p < 0.01$). It is noteworthy that in the CG the stored pollen was completely exhausted at the end of the study, while in the IG it was observed, albeit in small quantities.

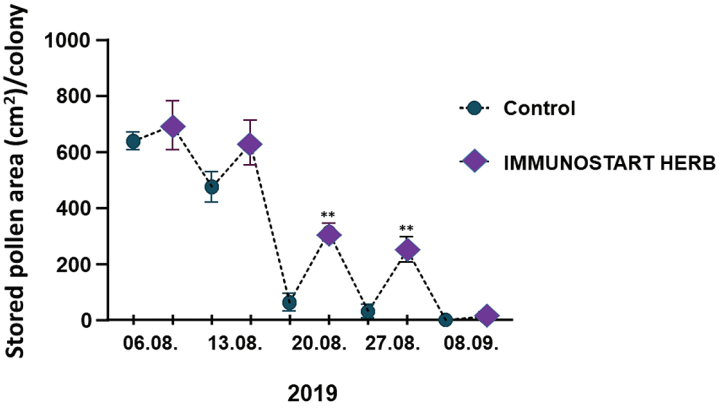

Figure 4. Comparison of parameter of stored pollen area (cm^2) (mean ± SD) between treatment and control group throughout the experiment. Asterisks indicated the level of significance as determined by an analysis of variance followed by post-hoc Tukey HSD multiple comparison test (**$p < 0.01$ compared to the control group). 474

Amount of capped honey

This parameter showed a very slight dissimilarity between the two groups (IG and CG) after the first and the second application of the plant extract (Fig. 5). A significant difference between the two groups was observed during the third assessment period (20

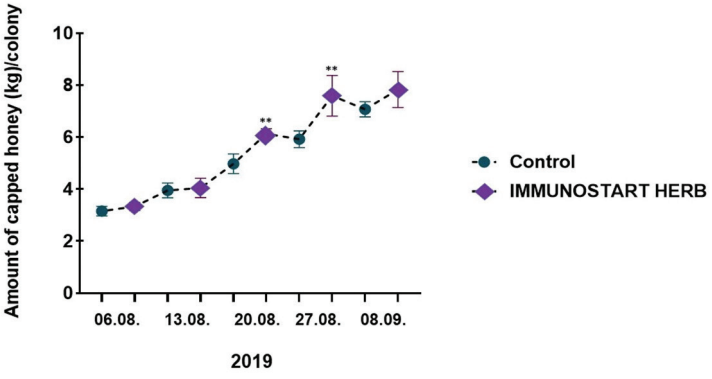

Figure 5. Comparison of parameters of amount of capped honey (kg)/colony (mean ± SD) between treatment and control group throughout the experiment. Asterisks indicated the level of significance as determined by an analysis of variance followed by post-hoc Tukey HSD multiple comparison test (**$p < 0.01$ compared to the control group).

August, 2019). Then the mean value of capped honey in IG (6.12 ± 0.19 kg) exceeded with about 23 % that in the CG (4.97 ± 0.37 kg) (ANOVA with Tukey's HSD post hoc test, $F = 29.7$ $df = 7$, $p = 0.002$, $p < 0.01$). This trend in both groups was maintained in the next recorded period (27 August, 2019). The mean value of capped honey in IG (7.58 ± 0.77 kg) was significantly different in that of the CG (5.91 ± 0.32 kg) ($F = 15.6$ $df = 7$, $p = 0.007$, $p < 0.01$). During the last assessment period (8 September, 2019) the amount of capped honey remained higher in the experimental group (7.83 ± 0.69 kg) compared to the control group (7.06 ± 0.28 kg), but the difference was statistically insignificant ($F = 4.12$ $df = 7$, $p = 0.088$, $p > 0.05$).

DISCUSSION

The use of medicinal plants by man has been in practice for thousands of years, as evidenced by the presence of various sources: written documents, preserved monuments, and even original herbal medicines [37]. It is assumed that the usage of medicinal plants started as a spontaneous process while searching for drugs against various diseases [38,39]. Due to the fact that in ancient times there was no information about the causes of diseases, nor about which plant and how it can be used as a medicine, people relied mainly on experience. Over time, sufficient experience has been gained regarding the use of specific medicinal plants for the treatment of certain diseases. Thus, the use of medicinal plants gradually abandoned the empirical framework and began to be based on explicatory facts [40,41].

In the present study, we have surveyed the effect of the herbal supplement IMMUNOSTART HERB® on honey bee colony strength parameters: adult bee population, areas of sealed worker brood, areas of pollen reserves and, amount of capped honey. The tested formulation is a patented herbal supplement and contains herbal extracts, vegetable glycerin, water, vitamin C (ascorbic acid), citric acid, and preservative potassium sorbate. The exact quantitative composition of this product is patent-protected and thereby not disclosed in this paper. According to the manufacturer (Extract Pharma, Sofia, Bulgaria), the extracts contain flavonoids, polyphenols, polysaccharides, mucous substances, amino acids, essential oils, vitamins, minerals, etc.

In the last few years, the increase in honey bee losses registered worldwide has posed a serious challenge to scientists. Most often, they are associated with the negative effects of stressors that are different in type and duration of impact, often interacting synergistically [5,42,43]. One of the commonly suspected stressors is poor nutrition. Given the negative effects on the health and protective mechanisms of bees as a result of inadequate diet, more and more attention is paid to the nutrition of bees [44]. The shortage of food sources in nature, most often observed at the end of summer, is compensated by beekeepers by feeding bees with sugar syrup to provide for a successful wintering. Sugar syrup is only an energy source without structural and bioregulatory feed components, necessary for the proper biological development of bee colonies.

An alternative solution to this problem is the use of nutritional supplements which can compensate for the lack of these substances in the diet of bees [45,46].

The results from our study showed that the application of the herbal supplement IMMUNOSTART HERB® has a positive effect on the honey bee colony strength (Fig. 2). Significant differences compared to the control group were observed after the third application of the preparation, thus indicating the benefits from the long-term use of the plant extract. These results are supported by previous research on the use of herbal supplements for increasing colony strength. For example, the administration of HiveAlive™ (Advance Science Ltd., Ireland) food supplement in candy or in syrup for a second successive year, before and after winter, increased considerably adult bee population of the supplement-treated group over the same period [47]. Another study investigated the effect of two *Laurus nobilis* L. extracts on colony strength [48], and the obtained results demonstrated that the hydroalcoholic extract from the aromatic herb clearly had a positive impact on honey bee colonies strength, presumably due to the antioxidant effect related to the phenolic compounds contained in the hydroalcoholic extract. Our previous research [49] has also shown the beneficial effect on honey bee colony strength from the application of two plant extracts (produced by Extract Pharma, Sofia, Bulgaria) – NOZEMAT HERB® (1.65 ± 0.05 kg) and NOZEMAT HERB PLUS® (1.63 ± 0.12 kg). It should be noted that the treatment with IMMUNOSTART HERB® resulted in an additional increase of about 0.5 kg in the mass of frames (kg) covered with bees, i.e. even higher values of this parameter after treatment (1.70 ± 0.16 kg) (Fig. 2).

Further, the application of IMMUNOSTART HERB® increased the sealed worker brood area, the amount of capped honey, and stored pollen area, compared to the control groups (Fig. 3, 4 and 5). Masry et al. [50] reported a similar result when the surface of the sealed worker brood area was increased after the treatment of honey bee colonies with *Jatropha curcas* oil. Similarly, Stevanovic et al. [51] presented the beneficial effect of a medicinal mushroom *Agaricus brasiliensis* extract (sugar syrup or candy) on brood rearing improvement and adult population growth in comparison to untreated bee colonies. Jovanovic et al. [29] revealed that plant-based supplement B + significantly increased the parameters of colony strength when compared to the control group (fed with plain sugar syrup). Similar results were obtained by Al-Ghamdi et al. (2019) when testing the effect of three plant extracts – chamomile flowers (*Matricaria chamomilla*), spearmint leaves (*Mentha spicata*), and cinnamon (*Cinnamomum zeylanicum*), whereby it was observed that chamomile had the greatest impact on honey bee colonies' development, while cinnamon accelerated wax comb building.

In this study, the amount of capped honey and surface of stored pollen area also increased significantly in the bee colonies fed on the supplemental diet with IMMUNOSTART HERB®, compared to the control colonies fed only with sugar syrup (Fig. 4 and 5). A similar result was by reported by Shehata [52], when applying various plant diets on Carniolan and Italian honey bee colonies. As a conclusion from the conducted study, it was noted that these diets helped maintaining the colony

strength during the dearth period, which resulted in attaining excellent pollen and honey reserves. The application of two *Laurus nobilis* L. extracts also showed a positive effect on collected pollen and capped honey, presumably due to the presence of some phenolic compounds, such as flavonoids, and the antioxidant capacity of the *Laurus nobilis* hydroalcoholic extract [48]. In the study of two herbal supplements by Shumkova et al. [49], considerable enhancement was found in the amount of capped honey and stored pollen area.

Generally, the application of IMMUNOSTART HERB® led to higher values of colony strength parameters as compared with the control group (fed only sugar syrup). At this stage, it is difficult to specify exactly which biologically active substances contained in the preparation cause the positive effects. Nevertheless, the study would undoubtedly be of benefit to beekeepers regarding the application of more appropriate nutritional supplements with a view to the successful overwintering of bee colonies.

CONCLUSIONS

In this study, herbal supplement IMMUNOSTART HERB® evinced a significant impact on colony strength parameters. Honey bee colonies have shown a significant increase in terms of stored pollen area, sealed worker brood area, adult bee population, and the amount of capped honey after the administration of this plant extract, with the most visible effect after the third and fourth administration, in comparison to the control group. The current study emphasizes the importance of adequate supplementary diets for honey bee colonies when amounts of nectar and pollen in nature are insufficient. The use of natural products for the treatment of bee colonies is in accordance with the requirements of the countries in the EU and worldwide, where the production of quality and safe (i.e. free of pollutants) bee products is imperative. In this regard, certain natural substances – essential oils, plant extracts, organic acids and more – are increasingly used as supplements to bee feeding. These substances, which are non-toxic to humans and bees and are contained in natural bee products, have a determined stimulating effect on the development of bee colonies; therefore, their use as alternatives should be further explored and encouraged.

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Authors' contributions

RS carried out the field experiment with natural plant extract (IMMUNOSTART HERB) and drafted the manuscript. RB and DS participated in the design of the study and performed the statistical analysis. PH conceived of the study, and participated in

its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UTICAJ BILJNOG PRIRODNOG SUPLEMENTA *IMMUNOSTART HERB* NA PERFORMANSE PČELINJE ZAJEDNICE

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Zimski period predstavlja najveći izazov za pčelinju zajednicu. Iz tog razloga, glavni zadatak pčelara je usmeren pre svega na obezbeđivanju dovoljne količine kvalitetne hrane za pčelinju zajednicu u toku rane jeseni. Cilj studije je bio da se ispita uticaj prirodnog biljnog ekstrakta *IMMUNOSTART HERB* na snagu populacije pčelinje zajedince, pohranjene zalihe polena, poklopljenog legla kao i uticaj na prinos meda. Eksperimentalnim grupama je davan *IMMUNOSTART HERB* četiri puta u razmacima od po 7 dana. Kontrolna grupa je primala šećerni rastvor. Dobijeni rezultati ukazuju da je primenjeni suplement u hrani uticao na sve ispitivane biološke parametre pri čemu je najznačajniji uticaj uočen posle druge aplikacije. U odnosu na sva merenja, parametri pčelinje zajednice u tretiranim grupama su pokazali veće vrednosti u poređenju sa kontrolnim grupama. Ovi rezultati ukazuju na potencijal koji biljni suplementi imaju u smislu efektivnog poboljšanja razvoja pčelinje zajednice tokom perioda kada je pčelinja paša oskudna uz obezbeđivanje pogodnih uslova za uspešno prezimljavanje.