ANTI-VARROA EFFICIENCY OF COUMAPHOS AND ITS INFLUENCE ON OXIDATIVE STRESS AND SURVIVAL OF HONEY BEES

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Apart from the efficiency of coumaphos against Varroa mites, its impact on the oxidative status and survival of the honey bee (Apis mellifera) was assessed. The research was conducted on hives from the same apiary, equalised regarding the number of bees, brood area and food storage. Based on Varroa infestation the hives were allotted to two groups: non-infested (N) and infested (I). Both groups were either treated (T) – NT and IT, or untreated (U) – NU and IU. The treatment of infested bees was controlled with a follow-up treatment with amitraz. The efficiency of coumaphos was 96-97%. This organophosphate had a negligible effect on bee survival, but it significantly affected their oxidative status: superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities, and the concentrations of malonyl dialdehyde (MDA). Coumaphos significantly (p<0.0001) decreased SOD activity in non-infested bees, but increased it in those infested. By contrast, both CAT and GST activities, as well as MDA concentrations significantly increased (from p<0.05 to p<0.0001) after treatment in all groups, with the exception of IT, where it declined. Coumaphos in non-infested hives caused oxidative stress per se, not unlike varroa in infested colonies. However, in infested colonies it decreased oxidative stress, owing to its efficacy against Varroa mites and contributed to the recovery of bee colonies. In spite of its certain downsides, coumaphos remains an effective anti-varroa substance, but should be used with precaution, not to add to the effects of environmental factors which may cause red-ox misbalance.

Key words: coumaphos, bee, Varroa, efficiency, oxidative stress

INTRODUCTION

Not only are honey bees cherished because of honey production, but also for their contribution to pollination in both their natural and managed habitats [1].

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Despite the efforts devoted to maintaining bee health, the honey bee is threatened by various pathogens, many of them parasites, dwelling inside the bees’ body or on its surface, such as *Varroa destructor*, the bee mite, which feeds primarily on the fat body and haemolymph, demolishing both the brood and adult inhabitants of the hive [2]. When *V. destructor* was first noticed, scientists could readily imagine that it would be a real nuisance causing a disaster to beekeeping [3]. Although some traits of the bee mite remained poorly known [4,5], later it has been recognised that besides causing a decline in the bee population in hives, it also serves as a vector to various bee pathogens [6-11]. However, recent research pointed to the fact that viruses are not decisive pathogens: it is highly probable that not they themselves, but *Varroa* mites are those which are of outstanding importance in producing bee pathology, given that they feed on the body fat [2], which is known to have hugely important functions: besides being a source of fat and proteins, it regulates metabolism and plays a pivotal role in the immunology of the honey bee [12,13]. The man can fight the mite by introducing a number of different measures, primarily involving acaricides, which may be either natural or synthetic. Unfortunately, the former proved to be less efficient, although could have the advantage of being nontoxic, without possible side-effects, and leaving no residues in bee products [14]. In addition, some synthetic acaricides may even have a genotoxic potential [15]. However, there is recent evidence on herbal acaricide formulations which are far more efficacious – even up to 80% [9]. In order to obtain safe bee products and prevent hive contamination with chemical substances, various means of combat against *V. destructor* have been tested, primarily based on hygienic and nursing behaviour of bee colonies [15-20].

Nevertheless, not much has been done in the recent past for the invention of new acaricides: some of them have been banned or their use has been hugely restricted, which is why we are to stick to some synthetic ‘hard’ acaricides traditionally used in apiculture. One of these is coumaphos. Apart from being used in veterinary medicine, in farm animals and dogs against ticks, lice and fleas [21,22], this organophosphorus acaricide is applied in apiculture for the control of *Varroa* infestation [23].

Although certain acaricides are known to produce oxidative stress in the honey bee [24,25], and coumaphos and imidacloprid applied simultaneously in cage experiments induced down-regulation of antioxidant genes, imidacloprid led to marked up-regulation of genes coding for enzymes involved in the response to oxidative stress [26], no corresponding data have been published for coumaphos in field experiments. It is known that oxidative stress in bees may be induced by various factors, even by their exposure to industrial habitats [27], or various chemicals applied in bee diet which can act synergistically with certain bee pathogens and deteriorate bee health [28].

These data render research into the parameters of oxidative stress in honey bees in various conditions justifiable. Thus, the following goals were set: to assess the efficacy of coumaphos in realistic conditions, that is in honey bee colonies in hives, to estimate the mortality of treated bees and to determine if oxidative stress is induced by the application of this synthetic acaricide.
**MATERIAL AND METHODS**

**Material**

The research was performed on winter bees originating from healthy and strong colonies of an apiary in Central Serbia (44°47′38″N 20°27′50″E). There were four experimental groups, each consisting of seven hives. The first two groups were considered non-infested with *Varroa* mites (the average number of fallen mites ≤0.5 per day, measured for 7 days before day 0), out of which one was untreated (NU), and the other treated with coumaphos (NT). The remaining two groups were infested with the bee mite: one remained untreated (IU), whilst the other (IT) was treated with coumaphos.

In addition, on day 49, 7 days after the treatment with coumaphos ended, there was a control, follow-up treatment with amitraz (Taktic®).

Coumaphos (CAS No. 56-72-4) was used in the form of CheckMite® (Bayer). Each strip for in-hive use measures 13.6 g and contains 1.36 g of coumaphos, carried by vehicles: titanium dioxide and polyvinyl chloride. The preparation was used in compliance with the manufacturer’s instructions (one strip was put into the brood chamber, where it remained for 42 days).

**Methods**

Bee colonies in the experiment were uniform regarding the number of frames, populations of adult bees and the quantity of stored food [29]. Throughout the experiment, the health of the colonies was checked on a regular basis, as recommended by OIE [30]. Only colonies free from bacterial, fungal and virus infections were included in the research.

A sticky board (a piece of plastic covered with vaseline) was put in every hive. To estimate the degree of infestation with mites, the bottom boards were left in place for 7 days. Groups were formed in accordance with the formula DI=N/7, where DI is the degree of infestation, and N the number of fallen *Varroa* mites, which was divided by the number of days of observation, that is seven [31].

In October, the colonies were classified as those with a low degree of infestation (≤0.5 fallen *Varroa* mites/day) and those with a high degree of infestation (≥10 fallen varroas/day) [31]. The bee colonies with low infestation were considered non-infested and 14 of them were chosen to form groups NU and NT, and the colonies with high infestation to form groups IU and IT.

Coumaphos preparation was put into the brood chambers of 14 chosen hives (experimental groups NT and IT, 7+7 hives). A sticky board was placed on the bottom board of each hive, where it was left for the collection of mites fallen during the experiment; each week a new sticky board was inserted and the numbers of ticks summed up to calculate their total number for the 42-day period.
**Efficacy of anti-varroa treatment.** The efficacy of coumaphos was calculated in accordance with the recommendations of EMA [32], using the following equation:

$$
MR = \frac{N_{tr}}{(N_{tr} + N_{fu})} \times 100, \text{ where}
$$

MR = mite reduction (%)

N_{tr} = \text{no. of mites in the test group killed by treatment (coumaphos)}

N_{fu} = \text{no. of mites killed in the test group AFTER follow-up treatment (amitraz)}

**Survival.** Simultaneously, the survival of bees was monitored with the use of a modified Todd trap for the collection of dead bees [33].

**Assessment of oxidative stress parameters.** In order to determine the parameters of oxidative stress bee sampling was first done at the beginning (day 0) and at the end of the experimental period (day 42). Whole bee samples were prepared, and CAT, SOD and GST activities, and MDA concentrations were analysed as described previously [34, 35]. For these analyses, 10 w/v homogenates of whole bees were prepared in a mortar containing tris-HCl buffer adjusted to pH 7.4 and liquid nitrogen. After centrifugation at 10,000 x g for 10 min, the supernatant was decanted and frozen at -20 °C until further processing. For each hive in the experimental groups the process was done with 3 x 10 bees and the analyses performed in triplicate. The specific activity of the enzymes was expressed in units of activity per milligram of protein (U/mg), and the concentration of MDA in nmol/mg of protein. All the analyses were done on UV/ VIS Spectrophotometer BK-36 S390 (Biobase).

The activity of superoxide dismutase (SOD) in bee homogenates was determined kinetically, as a change in the absorbance in time at wavelength of 480 nm. Adrenaline was added and the reaction mixture incubated at 30°C for 3 min. The unit of enzyme activity was defined as a quantity of enzyme necessary to decrease the rate of auto-oxidation by 50% in alkaline environment.

The analysis of catalase (CAT) activity in homogenates is based on the spectrophotometric monitoring of H\textsubscript{2}O\textsubscript{2} decomposition at 240 nm. The activity is monitored as absorbance decrease at the given wave length.

The substrate for the assessment of glutathione-S-transferase (GST) activity was 1-chloro-2,4-dinitrobenzene (CDNB). The method is based on the ability of CDNB to form a complex with glutathione, which is catalyzed by GST. The velocity of complex formation is calculated with the molar extinction coefficient for CDNB. The specific activity is defined as the number of nM of glutathione oxidized per minute [34].

The concentration of malondialdehyde (MDA) in homogenates was quantified according to Slater (1984), based on the principle that MDA, a specific product of lipid peroxidation reacts with thiobarbituric acid (TBA) and forms a colored MDA-TBARS complex with a maximum absorbance at 535 nm. The concentration is calculated with a molar extinction coefficient [34].
Statistical analyses. The data obtained were processed with GraphPad Prism (GraphPad Software), as described by Kojic et al. [36]. Owing to the homogeneity of the data (cv<30%), the groups were compared in a two-way repeated measures ANOVA, which was followed by Tukey’s or Sidak’s multiple comparisons test. The values are presented as means ± standard deviations. The significance of the differences was determined and presented; it ranged from p<0.05 to p<0.0001.

RESULTS

The efficacy of coumaphos

The results on the efficacy of coumaphos treatment are presented in Table 1 and Figures 1A and 1B.

Table 1. Numbers of mites fallen from bees treated/untreated with coumaphos

| Groups | Day 0            | Day 42         | Day 49         |
|--------|------------------|----------------|----------------|
| NU     | 4.14±1.07 b, A   | 18.14±3.63 b,D | 616.00±14.14 a,B |
| NT     | 4.43±1.27 b, A   | 611.57±18.50 a,C | 10.14±2.41 b,C |
| IU     | 79.29±2.87 c, A,B | 1268.57±45.92 b,B | 3209.43±116.20 a, A |
| IT     | 81.29±2.56 b, B  | 4308.14±135.86 a,A | 12.00±2.94 c,C |

Different lower-case letters in a row designate significant within-group differences. Cells of the same column which do not share the same upper-case letter differ significantly between groups.

At the beginning of the experiment, prior to the treatment (day 0), the average numbers of fallen mites in both non-infested groups of hives did not differ significantly (p>0.05, Table 1 and Figure 1A). The same was the difference between the two infested groups (p>0.05). In groups considered non-infested, the number of fallen mites increased after treatment: it was significantly higher (p<0.0001) in NT
compared to NU. The difference in the number of fallen mites was also noticeable between the treated and untreated groups of infested colonies. In these, the treatment led to significant (p<0.0001) increase in the number of mites which fell from their hosts. Thus, coumaphos treatment resulted in 96.0% efficacy against *Varroa* mites (day 42). The average numbers of mites fallen in a 7-day treatment with amitraz, which followed the trial with the organophosphate compound, resulted in an average of 3209.43±116.20 fallen mites in the infested untreated (IU) and 12.00±2.94 fallen mites in the infested treated hives (IT). This difference was significant (p<0.0001). Thus, control treatment with amitraz (day 49), revealed the 97.0% efficacy of coumaphos (Table 1 and Figures 1A).

The analysis of the numbers of fallen mites in each experimental group (Table 1 and Figure 1B) revealed certain significant differences. In the NU group, on day 49, the increase in the number of fallen mites was significant in comparison to both days 0 and 42 (p<0.0001), resulting from the control treatment with amitraz. In group NT the number of fallen mites was significantly (p<0.0001) higher in comparison to both days 0 and 49. In group IU the numbers of fallen mites differed significantly between all the timepoints of inspection (p<0.0001). The efficacy of coumaphos was most noticeable in group IT: the number of fallen mites following coumaphos treatment (day 42) was significantly (p<0.0001) higher in comparison to days 0 (prior to coumaphos treatment) and 49 (after follow-up treatment with amitraz, Table 1 and Figure 1B).

**Survival of treated bees**

The average numbers of dead bees at the beginning of the experiment did not differ significantly (p>0.05) and were even equal in the untreated groups (NU and IU, Table 2, Figures 2A and 2B). However, after treatment with coumaphos there were significant differences in the average numbers of dead bees both between the two non-infested groups, NU and NT (p<0.0001), and the two infested ones, IU and IT (p<0.0001), being higher in the latter pair (Figure 2A).

| Groups | Before     | After      |
|--------|------------|------------|
| NU     | 4.00±1.15a | 12.71±1.80d**** |
| NT     | 3.71±1.11a | 111.71±3.15b**** |
| IU     | 4.00±1.15a | 24.00±2.94c**** |
| IT     | 3.57±0.79a | 135.43±3.10a**** |

The asterisks designate significant within-group differences (**** p<0.0001)
Different letters in the same column designate significant between-group differences on each day

In the treated groups the mortality ranged from 111.71±3.15 dead bees (NT) to 135.43±3.10 (IT), which was significantly more that in untreated groups, but still negligible in comparison with the total number in the colonies (Figure 2B).
Oxidative stress following coumaphos treatment

Superoxide dismutase activity. SOD activity is shown in Table 3, Figures 3A and 3B. Before the administration of coumaphos strips into the hives (day 0) there were significant (p<0.0001) differences between the non-infested (NU and NT) and the infested bees (IU and IT). However, no differences (p>0.05) were noticeable between the two non-infested and between the two infested groups of hives. On day 42 all the differences between the groups were significant (p<0.05 to p<0.0001), with the exception of that between the two treated (p>0.05) with coumaphos – NT and IT (Figure 3A).

Table 3. SOD activities in bees before and after coumaphos treatment (U/mg of proteins)

| Groups | Day 0     | Day 42    |
|--------|-----------|-----------|
| NU     | 19.03±3.00a | 19.64±2.98a* |
| NT     | 18.92±3.07a | 14.15±2.47b**** |
| IU     | 11.53±1.69b | 8.08±1.57c**** |
| IT     | 11.53±1.91b | 16.15±2.29b**** |

The asterisks in a row designate significant within-group differences (* p<0.05, **** p<0.0001) Different letters in the same column designate significant between-group differences
The comparison of SOD activities within each group before and after treatment with coumaphos (Figure 3B) led to the following results: there was a significant (p<0.05) increase in the activity of SOD in non-infested bees which remained untreated (NU), and in bees infested with *Varroa* mites and treated with coumaphos (IT, p<0.0001). However, in the remaining two groups, the non-infested treated (NT) and infested but untreated (IU), the activity of SOD decreased significantly (p<0.0001 for both differences).

*Catalase activity.* At the beginning of the research, no significant (p>0.05) differences in the activities of CAT were detected either between the non-infested groups (NU and NT) or between those infested (IU and IT, Table 4, Figure 4A), but the differences were significant between either of the non-infested and either of the infested colony groups (p<0.0001). However, the differences proved significant (p<0.0001) on day 42 between all the groups, with the exception of that between the two treated ones (NT and IT, p>0.05).

**Table 4.** CAT activities in bees before and after coumaphos treatment (U/mg of proteins)

| Groups | Day 0     | Day 42     |
|--------|-----------|------------|
| NU     | 71.36±5.05a | 72.16±5.09c |
| NT     | 71.95±5.18a | 92.55±2.87b**** |
| IU     | 96.22±3.71b | 109.3±7.75a**** |
| IT     | 95.86±3.65b | 85.8±2.71b**** |

The asterisks in a row designate significant within-group differences (**** p<0.0001)
Different letters in the same column designate significant between-group differences

When analysed in each of the experimental groups, there were no significant (p>0.05) differences in CAT activity detected only in the non-infested untreated group (Table 4 and Figure 4B). In NT and IU groups a significant (p<0.0001) increase in the activity of this enzyme was recorded at the end of the research compared to that at its beginning, unlike in IT, where there was a significant decrease (p<0.0001).
Glutathione-S-transferase activity. At the beginning (day 0), the activity of GST was statistically similar (p>0.05) in both the non-infested (NU and NT) and in the infested groups (IU and IT, Table 5 and Figure 5A), but the differences were significant between either of the non-infested and either of the infested colony groups (p<0.0001). By contrast, after the treatment with coumaphos ended (day 42), there were significant (from p<0.01 to p<0.0001) differences between all the groups of tested colonies, the highest being in the infested but untreated one (IU) in comparison with all the others.

The changes in the GST within each group throughout the treatment period (Table 5 and Figure 5B) were as follows: no significant difference (p>0.05) was detected in the non-infested untreated group (NU), the activity significantly (p<0.0001) rose in both the other non-infested group (NT) and in the infested non-treated group (IU, p<0.0001), whilst, in the infested group treated with coumaphos (IT) a significant (p<0.0001) decrease in the enzyme activity was revealed.

Table 5. GST activities in bees before and after coumaphos treatment (U/mg of proteins)

| Groups   | Day 0         | Day 42        |
|----------|---------------|---------------|
| NU       | 55.32±1.99a   | 56.47±2.49d   |
| NT       | 54.44±1.36a   | 64.78±2.98b***|
| IU       | 72.07±1.75b   | 78.01±1.81a****|
| IT       | 72.33±1.47b   | 60.53±2.90c****|

The asterisks in a row designate significant within-group differences (**** p<0.0001)
Different letters in the same column designate significant between-group differences

Malonyldialdehyde concentrations. The concentrations of MDA before treatment with coumaphos did not differ significantly (p>0.05) either between the two non-infested (NU and NT) or between the two infested groups of colonies (IU and IT), but was significantly (p<0.0001) higher in either of those infested in comparison to any of those considered non-infested (Table 6, Figure 6A). After the treatment the highest MDA levels were measured in the infested untreated group (IU), significantly (p<0.0001)
higher than in all the others. In addition, the concentration of this lipid oxidation product was significantly (p<0.0001) higher in non-infested treated (NT) than in non-infested treatment-free bees (NU). In IT bees, MDA levels were significantly higher (p<0.05) than in NU, but significantly lower (p<0.01) than in NT.

**Table 6.** MDA concentrations in bees before and after coumaphos treatment (nmol/mg of protein)

| Groups | Day 0     | Day 42       |
|--------|-----------|--------------|
| NU     | 0.53±0.02a| 0.55±0.03a****|
| NT     | 0.53±0.02a| 0.76±0.09b****|
| IU     | 1.18±0.08b| 1.73±0.06c****|
| IT     | 1.18±0.06b| 0.64±0.06d****|

The asterisks in a row designate significant within-group differences (**** p<0.0001) Different letters in the same column designate significant between-group differences

**Figure 6A and B.** MDA concentrations: before and after coumaphos treatment (A) and comparison between groups (B)

Legend: NU=non-infested untreated bees, NT=non-infested treated, IU=infested untreated, IT=infested treated; Significance: *p<0.05, **p<0.01, ****p<0.0001

When compared within each hive group (Table 6, Figure 6B), in the non-infested untreated group (NU) the average concentrations of MDA remained without significant differences (p>0.05). However, a significant rise in MDA levels was detected in the non-infested treated bees (NT, p<0.0001) and in infested which remained untreated (IU, p<0.0001). Only in infested bees treated with coumaphos (IT), a significant (p<0.0001) fall in the concentrations of the lipid peroxidation product was confirmed.

**DISCUSSION**

In the present work the treatment with coumaphos proved highly effective against *V. destructor* - 96.0%, assessed by the number of fallen mites in the IT group of hives in comparison with the number of fallen mites in IU, and 97.0% if the number is compared to the total number of fallen mites in IT after the follow-up treatment with amitraz. These results are in accordance with those previously published [26,37].
The high effectiveness of coumaphos was confirmed by Gregorc and Planinc [37], when the effects of various acaricides in colonies moderately infested with varroa mites were tested. It has been proven that during the brood-rearing season treatment with thymol (Apiguard or Thymovar) or oxalic acid is not completely satisfactory: the use of coumaphos strips led to significantly higher varroa mortality ($p<0.001$). It was suggested that soft acaricides may be completely satisfactory in organic beekeeping only when moderate infestations are to be dealt with. However, a high efficacy of coumaphos (98.03%) was confirmed both in laboratory conditions and in queen-right colonies [26]. Somewhat lower values for the anti-varroa efficacy of coumaphos in our research can be explained by differences in the experimental design or, possibly, by the development of resistance to coumaphos by the mites. The first data on the resistance of varroa towards coumaphos were published following the research performed by Spreacifico et al. [38], who in Lombardy in a field study detected lower coumaphos (Perizin) efficacy, 46% on average (from 28 to 88%), against varroa mites. Soon, Elzen and Westervelt [39] confirmed the resistance of mites to coumaphos in laboratory conditions, having detected lower efficacy of CheckMite+ in the control of the mite in Southwest Florida. However, there are data which suggest that the resistance may reverse after the cessation of coumaphos administration [40,41].

Apart from high efficacy in mite control, preparations based on coumaphos show a degree of negative influence on adult bees and their brood, when used alone or in combination with some other pesticides [42-52].

The average numbers of dead bees at the beginning of our experiment did not differ significantly ($p>0.05$) between experimental groups. However, after treatment with coumaphos there were significant differences in the average mortality both between the two non-infested groups, NU and NT ($p<0.0001$), and the two infested ones, IU and IT ($p<0.0001$), which suffered a higher loss.

In Slovenia, for example, a case of coumaphos intoxication of honey bees was described in 2012 [45], when the insertion of CheckMite stripes resulted in high mortality. However, in the two following months, the population increased and reached levels similar to other hives in the apiary.

Recently it was proven that bees fed coumaphos at concentrations 185,200 and 92,600 ppb suffered significantly higher mortality than those treated with lower concentrations (46,300; 23,150 and 11,500 ppb) or the control. The three lower concentrations proved relatively non-toxic [26].

Given the widespread use of chemicals in all human areas of practice, the bees are not safe from exposure to various types of agropesticides [11]. In North America, for instance, hives have been found to be contaminated by 120 pesticides and their metabolites [44], the most common one being coumaphos. In vitro testing revealed that coumaphos in a concentration of 25 mg/L of diet decreased the survival of bee larvae [52], whilst those similar to maximum residues in pollen and honey/nectar (1.8, 6.0 and 8.0 mg/L) had no impact on their development and survival. It was suggested...
that the tested concentrations of coumaphos, possibly present as residues, are unlikely to influence larval survival in real conditions, but that some sublethal effects or synergism cannot be excluded. When assessing the toxicity of certain substances to bees, one must have in mind the possibility of synergism: for example, the fungicide prochloraz may increase the toxicity of coumaphos, supposedly through the inhibition of the detoxifying activity of cytochrome P450 monooxygenase [48]. In addition, prochloraz in combination with coumaphos can even alter immune gene expression in the honey bee [50]. Further, it has been estimated that the risks of acaricides to bee larvae are below 1% when chemicals are used individually, but may increase substantially when synergistic mixtures are used, for example when tau-fluvalinate is combined with coumaphos or amitraz [51]. It is interesting that queen bees can survive high doses of coumaphos (2.7 mg/g) and are at least 11-times as tolerant of coumaphos as worker bees [46]. However, coumaphos topical treatment in doses of 0.3 to 3.0 µg per bee did not influence significantly the reproductive capacity of drones [47]. Coumaphos can increase the levels of apoptosis in worker bees, but resulted in less extended necrosis than imidacloprid [43], which suggested the influence of both pesticides on the reduced size of hypopharyngeal glands and cell death. Finally, long-lasting exposure to coumaphos or imidacloprid alone, or the combination of these, in concentrations present in the environment may negatively affect olfactory learning and memory in honey bees [49]. This may contribute to depopulation of bee colonies, as a consequence of a failure in orientation and behaviour patterns essential for foraging.

ROS (reactive oxygen species) are inevitably involved in the biology of honey bees. For example, they are the honey bees’ effective weapons against pathogens [35], but are prone to variations [36]. Their levels were found to be lower in wintering bees, which may imply the reason for higher susceptibility to diseases [35].

Our research imply that coumaphos used as in our experiment may induce changes in parameters of oxidative stress (CAT, SOD and GST activities, and MDA concentration, Tables 3-6, and Figures 3A,B-6A,B). Although no data were published on the oxidative-stress-inducing capabilities of coumaphos in the honey bee, it is known that some other insecticides exert such influence. For instance, imidacloprid can increase glutathione peroxidase and CAT activities as well as the concentrations of MDA [53]. In addition, coumaphos may downregulate the gene coding for CAT, which has not been confirmed for SOD gene [26].

In the current work coumaphos treatment significantly (p<0.0001) decreased SOD activity in non-infested bees, but increased it in those infested. After coumaphos treatment (day 42) the lowest SOD activity was recorded in infested bees which remained untreated, significantly lower in comparison to all the other groups (from p<0.001 to p<0.0001).

However, it is interesting that in NU bees after coumaphos treatment (day 42) SOD activity significantly (p<0.05) increased, but in NT group it decreased (Figure 3B), which may be the contribution of coumaphos. The decline is even sharper in IU,
possibly due to the effects of *V. destructor*. Further, in IT group SOD activity rose, which may be justified by the positive effect of coumaphos, which declines *Varroa* infestation, leading not only to direct benefit, but also aiding the recovery of SOD activity in comparison to IU bees.

Given the lack of published research into the combined influence of varroa mites and coumaphos on oxidative stress in bees, our results on SOD activity can be discussed as an individual or combines influence of causative agents and formulations used for their control. When inspecting the influence of fumagillin, thymol, Beewell AminoPlus (a vitamin-amino acid preparation), Medenko forte (a herbal supplement rich in oak bark extract, absinthe and sage) and the extract of *Agaricus blazei* (rich in polysaccharides) on bee colonies infected with *Nosema ceranae* [28], a similar decline in SOD activity in IU group in the present work was probably the consequence of *V. destructor* infestation, analogous to the effect of *Nosema* in the research conducted by Glavinic [28].

Contrary to SOD, both CAT and GST activities, as well as MDA concentrations significantly increased (from *p*<0.05 to *p*<0.0001) after treatment in all groups, with the exception of IT, where it decreased (Figures 4A-6A). When CAT activities were compared on finishing the treatment, the infestation seemed not to have any influence on CAT activity, since in both treated groups, NT and IT, it was statistically similar (*p*<0.05) regardless of the mite infestation, but was still significantly higher in IU group than in all the others. After anti-varroa treatment GST activity was significantly lower (*p*<0.01) in IT than in non-infested bees which received treatment. In the current research the analysis of variations in enzyme activities and MDA concentrations between groups detected even more pronounced changes (Figures 4B-6B). The levels of MDA prior to coumaphos administration were significantly (*p*<0.0001) higher in infested colonies (IN and IT) than in those non-infested (NU and NT), but declined in the infested ones (IT) after treatment, whilst the non-infested bees (NT) produced higher concentrations of MDA than those infested. After coumaphos treatment, the average MDA concentration was highest in IN group and differed significantly (*p*<0.0001) in comparison with all the others. Our results suggest that the ectoparasite *V. destructor* and coumaphos each per se may alter the parameters of oxidative stress, but that coumaphos in IT hives contributes to the recovery in bees owing to decrease in *Varroa* infestation, increased SOD activity, that is decrease of CAT and GST, and MDA concentrations. This is similar to the findings by Glavinic [28], who noticed the variations in oxidative stress parameters due to pathogens (*N. ceranae*). He discovered that all the preparations per se, as well as the investigated pathogen led to oxidative stress in bees, but that the preparations decreased the values of oxidative stress parameters (CAT and GST activities, and MDA concentrations), similarly to coumaphos applied in our work, which contributed to the recovery of bee colonies alongside declined infection.

However, it has been known that some parameters of oxidative stress, (CAT and GST activities, and MDA concentrations) may be influenced by numerous factors, such as living in greenhouses [54] or industrial areas [27], migratory beekeeping [55], seasonality
[35,36], flying activities [36,56], aging [36,56] and brood rearing [36]. Although radio frequency electromagnetic field of certain strength can influence oxidative stress, it is not likely to happen in realistic conditions [57]. A study of oxidative stress in honey bee drones suggests that survival of oxidative stress is due to tolerance of oxidative damage to lipids, rather than to its prevention or repair [58]. Given the connection between oxidative stress and aging, and the fact that drones which lived longer exhibited higher levels of lipid peroxidation, these authors claim that bees may represent suitable model for research into oxidative stress and/or aging.

Although coumaphos possesses some downsides, namely, there is a possibility of its leaving residues [59] and resistance development in mites [38,39], it still remains an effective anti-varroa substance. However, for these reasons, it is necessary to use it following the manufacturers’ instructions carefully. Given that oxidative stress in bees can be initiated by a range of factors including the type of environment and beekeeping, possibly present pesticides both in hives and on pastures etc., attention should be paid not to add to the burden which is hardly controllable. Based on the activities of enzymes of antioxidative defence and the concentrations of MDA, in the current research it has been shown that coumaphos contributed to lowering the oxidative stress which developed in Varroa-infested honeybees. On the other hand, it certainly did increase the parameters of oxidative damages in bees considered non-infested.

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Authors’ contributions

ZB carried out the experiment, contributed to laboratory work, contributed to the draft of the manuscript. AN conceived of the study, contributed to the composition of the manuscript, gave her final approval of the manuscript. RM contributed to the field experiment and the molecular analysis. GU conceived the study, contributed to laboratory analyses. VB performed the statistical analyses and data interpretation. KI contributed to the field experiment. SZ conceived the study and coordinated the experimental work, contributed to drafting the manuscript and gave his final approval.

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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EFIKASNOST KUMAFOSA PROTIV VAROE I NJEGOV UTICAJ NA OKSIDATIVNI STRES I PREŽIVLJAVANJE MEDONOSNE PČELE

ZIKIC Biljana, ALEKSIC Nevenka, RISTANIC Marko, GLAVINIC Uros, VEJNOVIC Branislav, KRNJAIĆ Igor, STANIMIROVIC Zoran

Procenjivana je efikasnost kumafosa protiv varoe, kao i njegov uticaj na oksidativni status i preživljanje medonosne pčele (Apis mellifera). Ispitivanje je obavljeno na košnicama iz istog pčelinjaka, ujednačenim po broju pčela, površini legla i količini uskladištene hrane. Na osnovu infestacije varoom, košnice su podeljene u dve grupe: neinfestirane (N) i infestirane (I). Obe grupe su bile ili tretirane (T) – NT i IT, ili netretirane (U) – NU i IU. Tretman infestiranih društava je kontrolisan naknadnom primenom amitraza. Efikasnost kumafosa iznosila je 96-97%. Ovaj organofosfat imao
je zanemarljiv efekat na preživljanje пчела, али je značajno uticao na njihov oksi-
dativni status: aktivnosti superoksid-dismutaze (SOD), katalaze (CAT) и glutation S-
transferaze (GST), i koncentracije malonil-dialdehida (MDA). Kumafos je značajno
(p<0,0001) smanjio aktivnost SOD kod neinfestiranih пчела, али ju je povećao kod infe-
stiranih. Za razliku od toga, aktivnosti CAT i GST, kao i koncentracije MDA značajno
su se povećale (od p<0,05 до p<0,0001) posle tretmana u svim grupama, sa izuzetkom
IT, u kojoj su opale. Kumafos je sam po sebi u neinfestiranim društvima prouzrokovao
oksidativni stres kod пчела, slično kao Varroa u infestiranim. Međutim, u infestiranim
košnicama on je smanjio oksidativni stress zahvaljujući efikasnosti protiv varoe, чиме
je doprinelo oporavku društava. Uprkos određenim nedostacima, kumafos ostaje ef-
ikasna supstanca u borbi protiv varoe, али ga treba koristiti sa oprezom, da bi se izbeglo
dodatno opterećenje пчела prouzrokovano faktorima sredine koji mogu da izazovu
red-oks neravnotežu.