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ORIGINAL RESEARCH ARTICLE

# Evaluation of the toxicity of a propolis extract on *Varroa destructor* (Acari: Varroidae) and *Apis mellifera* (Hymenoptera: Apidae)

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## Summary

The effects of a propolis extract on *Varroa destructor* and *Apis mellifera* were evaluated by three different application methods: topical, spraying and oral. A propolis sample was extracted and its organoleptic and physic-chemically traits characterized. These analyses showed that it was a typical propolis from the Pampean region in Argentina, with elevated contents of biologically active compounds. Topical application was carried out by subjecting mites to contact with various propolis concentrations for different periods of time, which resulted in mortality and narcosis. Acaricidal effects were stronger with increasing concentrations of the propolis extracts. Spraying infested bees with a 10% propolis solution was harmless for bees but killed 78% of mites. Feeding infested bees with propolis extract in sugar syrup was not toxic to the mites but caused the death of bees treated with the highest concentration. Our results suggest that the propolis extracts from the Pampean Region could be incorporated into bee colonies by spraying, although the appropriate doses and concentrations to be administered, and the mechanism of action of the extracts on the mites are still to be elucidated.

# Evaluación de la toxicidad de un extracto alcohólico de propóleos sobre *Varroa destructor* (Acari: Varroidae) y *Apis mellifera* (Hymenoptera: Apidae)

### Resumen

Los efectos de un extracto de propóleos sobre *Varroa destructor* y *Apis mellifera* fueron evaluados por medio de tres métodos de aplicación diferentes: tópica, pulverizado y oral. Una muestra de propóleos fue extractada y caracterizada organoléptica y físico-químicamente. Las características de la muestra resultaron coincidir con las de un propóleos típico de la región pampeana argentina, con un elevado contenido de compuestos biológicamente activos. La aplicación tópica fue realizada manteniendo a los ácaros en contacto con diferentes concentraciones de propóleos durante ciertos períodos, resultando en mortalidad y narcosis de los ácaros expuestos. Los efectos acaricidas se incrementaron a medida que lo hicieron las concentraciones de los extractos de propóleos aplicados. Las abejas infestadas que fueron pulverizadas con una solución de propóleos al 10% no resultaron afectadas pero el 78% de los ácaros que las parasitaban resultaron muertos. La alimentación de las abejas con extractos de propóleos en jarabe de azúcar no mostró efectos tóxicos para los ácaros pero causó la muerte de las abejas tratadas con la concentración más elevada. Nuestros resultados sugieren que los extractos de propóleos de la región pampeana podrían ser incorporados en las colonias de abejas en forma pulverizada, aunque las dosis y las concentraciones adecuadas a administrar y, el mecanismo de acción de los extractos sobre los ácaros, aún deben ser elucidados.

**Keywords:** *Varroa destructor*; *Apis mellifera*; propolis extract

# Introduction

The parasitic mite, *Varroa destructor* (Anderson and Trueman, 2000), is considered to be one of the most serious pests of the honey bee, *Apis mellifera*, causing great economic losses to the beekeeping industry (de Jong *et al.*, 1982). Female mites parasitize both adult and brood bees by feeding on their haemolymph, but only reproduce inside the capped brood cells (Ifantidis, 1983). Efforts to control this pest have often focused on applying synthetic acaricides. Although these compounds provide favourable results, the development of acaricide resistance in *V. destructor* populations (Milani, 1999) and the contamination of hive products (Wallner, 1999) indicate that new treatment strategies that minimize the above hazards should be developed (Ritter, 1992). Natural acaricides offer a highly desirable alternative to the synthetic products. Most research has focused on the use of organic acids and essential oils, because they naturally occur in bee colonies and possess significant acaricidal activities (Imdorf *et al.*, 1999; Eguaras *et al.*, 2003). Another natural product that can be taken into account is propolis.

Propolis is composed of resins collected from plants, which are masticated by the bees, mixed with their salivary enzymes and beeswax, and applied to combs and walls of the hive (Burdock, 1998), thereby insulating and reinforcing the hive as well as giving antiseptic proprieties to the nest environment (Bankova *et al.*, 2000). The biological activity of propolis is due to its high resin content, mainly phenolic compounds (Bankova *et al.*, 1983). Numerous studies have shown its versatile pharmacological activities: antibacterial; antifungal; antiviral; anti-inflammatory; antioxidant; antitumour, etc. (Banskota *et al.*, 2001). Previous tests of the use of propolis as acaricides or insecticides have, however, been very limited. Garedew *et al.,* (2004) have shown that topical application of propolis reduced the duration of the pupal period of the greater wax moth, *Galleria mellonella,* and was toxic to its larval instars. More significantly, Garedew *et al.,* (2002) demonstrated that *V. destructor* was sensitive to a propolis extract applied as a topical solution in alcohol. The effect that propolis extracts could have on the honey bees has not, however, been investigated. The aim of this work was to evaluate the toxicity of a propolis alcoholic extract on *V. destructor* and on *A. mellifera* by different means of administration.

# Materials and methods

## Extraction and analysis of the propolis sample

A sample of raw propolis was obtained from a beekeeper's apiary placed in Camet station, Mar del Plata, Buenos Aires province, Argentina (37º53'S; 57º36'W). The sample was weighed, frozen, ground with a mortar, and then stored at 4ºC until use. The propolis was organoleptic and physic-chemically characterized in the Agroindustries Laboratory, Famaillá Agricultural Experimental Station, National Institute of Agricultural Technology, Tucumán province. The organoleptic properties assessed were: appearance, consistency, visible impurities, aroma, flavour and colour. The physicochemical properties analyzed were: content of water, ash and wax, mechanical impurities, total resins, total phenols and total flavonoids (expressed as quercetine dihydrate), according to the protocol of IRAM-INTA norms (IRAM-INTA 15935-1 Norms, 2008).

For the experiments, a propolis extract in alcohol was prepared. The propolis powder was dissolved in 70% ethanol at a ratio 1:9 (w/v) (Cunha *et al.*, 2004). It was then extracted at 60°C for 2 h in constant shaking, cooled at room temperature and filtered by suction. After filtration the solution, free of wax and impurities, was dried at 40°C, in order to obtain a soft extract, due to evaporating the alcohol. The humidity content of the soft extract was determined by drying an aliquot of this extract to 105ºC, until constant weight (IRAM-INTA 15935-1 Norms, 2008). The relation between wet weight and dry weight was used to prepare different concentrations of propolis solutions for the experiments.

As the presence of acaricidal residues in the propolis sample, remnants of previous treatments, may introduce false results, the propolis sample used in our experiments was collected from hives which had not been treated with any synthetic acaricides. The apiary whence the sample was obtained had been managed by alternating formic acid and thymol applications for at least two year prior to collecting the propolis.

## Biological material

*Apis mellifera* colonies were used. The hives were placed in an experimental apiary of the University of Mar del Plata, near Mar del Plata, Argentina (38º10'06''S; 57º38'10'W). All colonies had been left untreated for *V. detructor* for the preceding 12-24 months. Adult worker bees and capped brood combs were taken from the colonies according to each assay (see below).

## Topical and spraying application methods

The soft extract was dissolved in 55% ethanol in order to reduce the effect of strong ethanol solution on the experimental organisms. The solutions were prepared considering the humidity content of the soft extract. The concentrations used in the treatment were 1.25, 2.5, 5, 7.5 and 10% (w/v).

Adult female mites were collected from capped healthy brood by opening and inspecting individual cells. In order to avoid starvation, mites were kept on bee larvae or pupae in Petri dishes during the collection process. Mites that seemed newly moulted, weak or abnormal were discarded, because they may have different responses.

For the topical application, methodology adapted from Garedew *et al.,* (2002) was used. For each treatment, 200 µl of a specified concentration of propolis were applied to six mites placed on a piece

of filter paper (3 x 3 cm) in a Petri dish. Each treatment was terminated after the allowed contact time (15, 30, 45, 60, 75 and 90 s) by removing the mites from the filter paper, and transferring them into a clean Petri dish (90 x 15 mm). Five replicates for each experimental unit were run. Controls (four replicates) were made by treating mites with a 55% ethanol solution for similar contact times. All treatments were carried out at room temperature (22-24°C) and the treated mites were incubated at 28°C and 60% R.H. Mite activity was observed under a dissecting microscope at 10 min, 30 min, 60 min and then after one hour for the next seven hours (total = 10 observation times). Each individual mite was classified as mobile or inactive; it was considered inactive when no leg movement or movement of any body part was seen when gently prodded with a paintbrush. If a mite remained inactive after 8 h from the beginning of the treatments, it was considered to be dead.

The proportion of inactive mites was calculated for each treatment concentration (tested at different contact times) at each observation time. In the statistical model, the effects of treatment concentration, contact time (and its interaction) and repetition were included, whereas the observation time was considered as repeated measures in time periods. Least-squares means were compared using Tukey-Kramer test. A 5% level of significance was used, unless otherwise stated. Data analysis was conducted using PROC MIXED (SAS Institute, 2007).

For the spraying application method, Petri dishes (150 x 20 mm) padded with absorbent filter paper on the inner bottom and with an extra lid of metallic mesh, were used. Ten adult female mites and ten adult worker bees (free of mites) were placed in every dish. Once the mites were attached to the body of the bees in each experimental unit, 3.4 ml of 10% propolis solution were sprayed on the bees through the metallic lid, using a hand sprayer. The spraying volume was equivalent to the volume used in the preceding experiment (the topical method), considering the surface of the filter paper (22 µl/cm$^2$, see above). A device with candy and water was placed inside each unit as food for the bees. Ten bees and ten mites in modified Petri dishes sprayed with 55% alcohol were included as controls. Five replicates for each experimental group were run. The dishes were placed in incubators at 28ºC and 70% RH. Death of bees and mites was assessed after 24, 48, and 72 h. Mortality was evaluated by gently prodding each mite with a narrow paintbrush; lack of response to consecutive stimulus over 1 min was considered an indication of death. All bees (dead or survivor bees) were visually inspected for the presence of mites.

The proportion of dead mites and dead bees was calculated for each observation time. Data were analysed using GLIMMIX Procedures (SAS Institute, 2007) with logit as the link function. Statistical significance of pairwise differences between treatments per observation time was evaluated using the PDIFF option in conjunction with the LSMEANS statement.

### Oral administration method

For this method, an average of 340.8 ± SE 59 adult worker bees with no age differentiation, coming from colonies (n = 24) with high *V. destructor* infestation, were placed in individual cages (16 x 12 x 6 cm) according to Maggi *et al.,* (2010). The bees remained in the cages with no food for 4 h and, the propolis solutions were then administered in 10 ml of 2:1 syrup (sugar diluted in 70% alcohol) as nutrient. Concentrations tested were: 5, 10, 15 and 20%. Each treatment was replicated five times. Bees in cages with 10 ml of 2:1 syrup (as above but without propolis) were included as controls (n = 4). All cages were maintained at room temperature (22-24ºC and 65% RH) and a synthetic queen pheromone (Agroindustries Laboratory, INTA Famaillá Agricultural Experimental Station) was included. After 24 h all bees were fed only with the 2:1 syrup, depending on demand. Dead bees and mites were recorded after 24, 48 and 72 h. After the experiment was terminated, the bees were killed by immersing the cages in 70% alcohol. The final number of mites and bees from each cage was recorded.

The proportion of dead mites and bees at each observation period was calculated. Arcsine square-root transformed proportions were used. For each observation time, data were analyzed using a model where the dose effects and total number of mites were included as co -variables for the proportion of dead mites, and the total number of bees as co-variable for the proportion of dead bees. For least-squares means comparison, the Tukey-Kramer test was utilized with a 5% level of significance, unless otherwise stated. Data analyses were conducted using PROC MIXED (SAS Institute, 2007).

## Results

### Propolis analysis

The propolis sample was opaque with shiny irregular fragments of soft consistency. Visible impurities were found, especially remains of plants, wood and other materials, but vestiges of paint, paper or cardboard were not found. The aroma of the propolis was aromatic resinous; the flavour was sweet and the colour greenish-yellowish brown. The contents of wax, ash and water of the sample were 15.06%, 3.65% and 0.82%, respectively. Only 5.89% mechanical impurities were detected. The total amounts of resins, phenols and flavonoids were 77.45%, 21.74% and 9.18%, respectively.

### Topical and spraying methods

The effects of repetition ($F_{[4,134]}$ = 2.96, $P$ = 0.0220), treatment concentration ($F_{[5,134]}$ = 116.62, $P$ < 0.0001), contact time ($F_{[5,134]}$ = 4.11, $P$ = 0.0017) and observation time ($F_{[9,1467]}$ = 198.46, $P$ < 0.0001) had a significant effect on mite activity; moreover, a significant interaction between the effects of treatment concentration and observation time was found ($F_{[45,1467]}$ = 19.28, $P$ < 0.0001; Fig. 1).

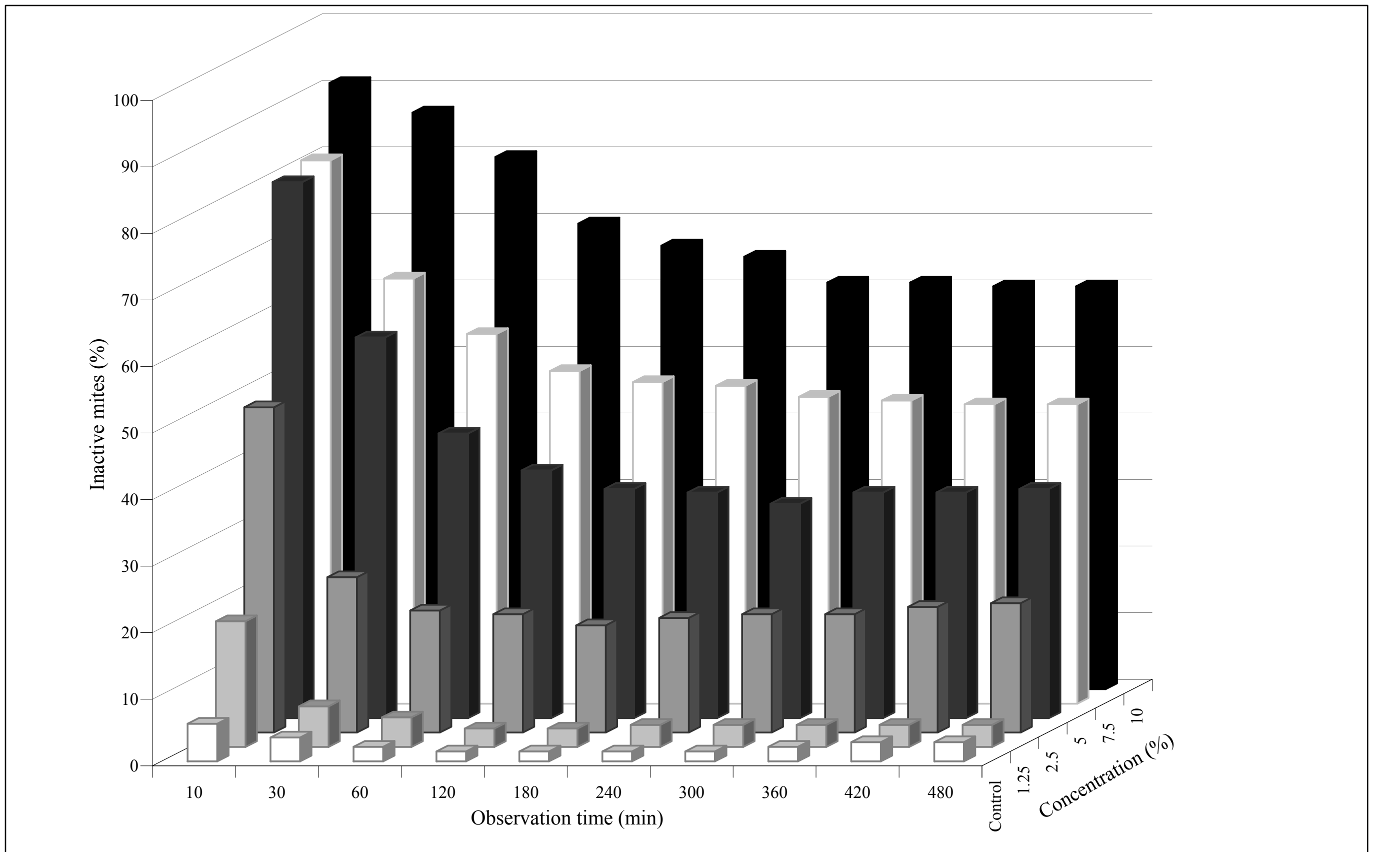


***Fig. 1.*** Effect of treatments with different propolis extract concentrations by the topical application method on the activity of *V. destructor* during 8 hours of observation. Least-squares means values of inactive mites after treatments (expressed in percentage) are presented here (n = 174, 10 observation times). The values obtained for the different contact times tested in each treatment concentration were grouped.

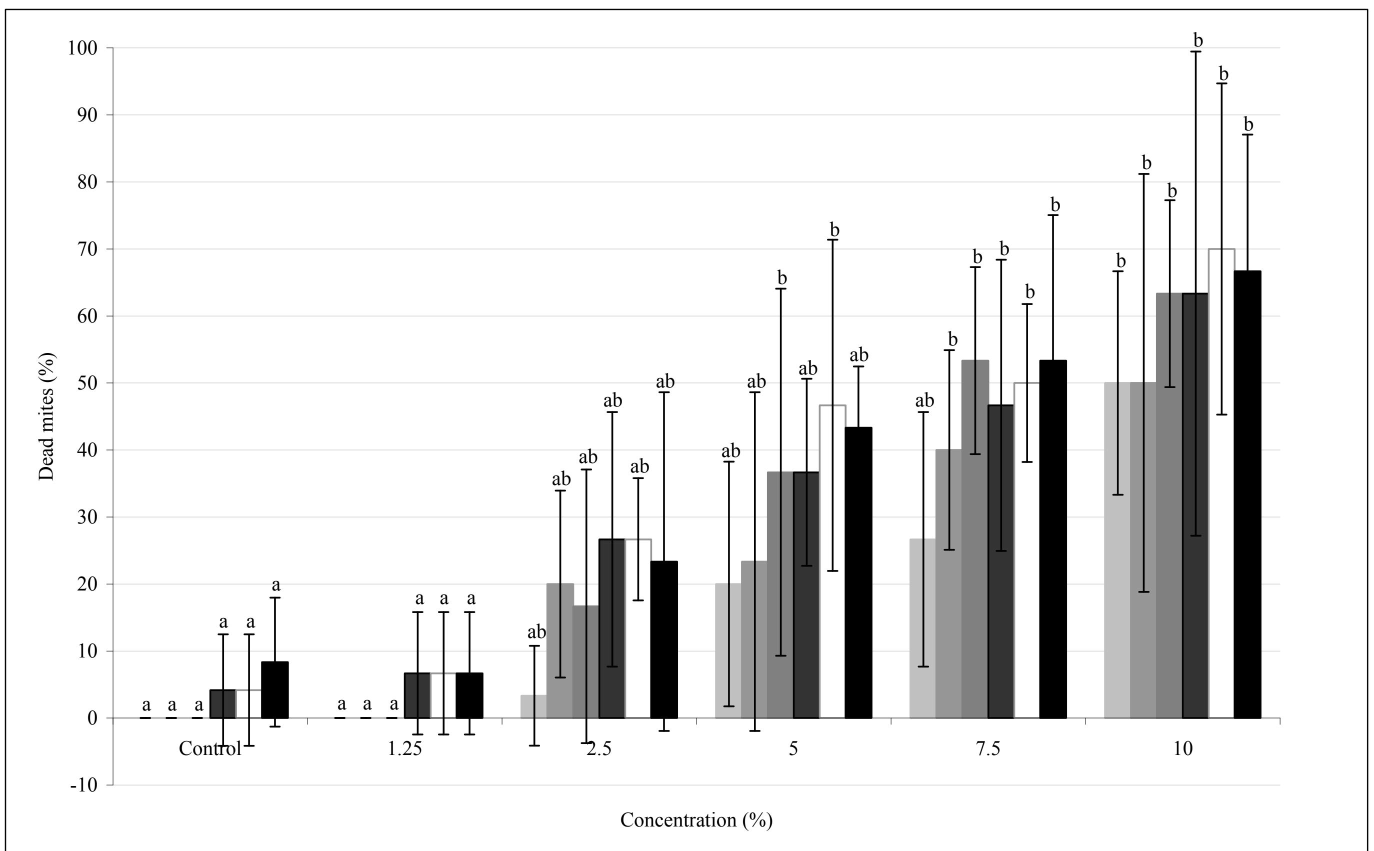


***Fig. 2.*** Mean percentages of dead mites after treatment during different contact times with diverse propolis extract concentrations by the topical application method (after 8 hours from the beginning of treatments). Least-squares means + SE values of dead mites are presented here (n = 174). Means with at least one letter in common do not differ (P > 0.05). Contact times: 15s, 30s, 45s, 60s, 75s, 90s

***Table 1.*** Mean percentages of dead mites +Standard Error (SE) and dead bees +SE at 24, 48 and 72 h after treatment of infested bees with the 10% propolis solution by the spraying application method. *Original analyses were conduced using proportions. Depicted values are back-transformed least means from the appropriate model. Ten bees and 10 mites per experimental unit (n = 10). Means with different letters indicate statistically significant differences (P < 0.01) between treatments within groups.

| | | Observation times | | |
|---|---|---|---|---|
| | | **24 h** | **48 h** | **72 h** |
| **Mites** | **Control** | 2 (2) a | 6 (3) a | 8 (4) a |
| | **Treated** | 48 (7) b | 68 (6) b | 78 (6) b |
| **Bees** | **Control** | 6 (3) a | 8 (4) a | 10 (4) a |
| | **Treated** | 6 (3) a | 8 (4) a | 10 (4) a |

***Table 2.*** Mean percentage of dead mites +Standard Error (SE) at 24 h, 48 h and 72 h after treatment of naturally infested bees in cages fed with different concentrations of propolis solution by the oral administration method. *Original analyses were conduced using arcsine square-root transformed proportions. Depicted values are back-transformed least means from the appropriate model (n = 24).

| Propolis | Observation times | | |
|---|---|---|---|
| **Concentration** | **24 h** | **48 h** | **72 h** |
| **Control** | 6.35 (4) | 6.35(4) | 6.35 (4) |
| **5 %** | 1.54 (1) | 1.54 (1) | 1.54 (1) |
| **10 %** | 0.00 (0) | 0.00 (0) | 0.00 (0) |
| **15 %** | 1.82 (1) | 4.32 (3) | 4.32 (3) |
| **20 %** | 4.58 (3) | 6.86 (4) | 12.97 (7) |

***Table 3.*** Mean percentage of dead bees +Standard Error (SE) at 24 h, 48 h and 72 h after treating naturally infested bees in cages with different concentrations of propolis solutions by the oral administration method. *Original analyses were conduced using arcsine square-root transformed proportions. Depicted values are back-transformed least means from the appropriate model (n = 24). Means followed by the same letter are not significantly different ($P > 0.05$).

| Propolis | Observation times | | |
|---|---|---|---|
| **Concentration** | **24 h** | **48 h** | **72 h** |
| **Control** | 7.87 (1) ac | 9.01 (2) a | 9.30 (2) a |
| **5 %** | 4.78 (2) ab | 7.71 (3) ab | 8.76 (3) ab |
| **10 %** | 2.45 (0.66) b | 2.94 (0.72) b | 3.35 (0.86) b |
| **15 %** | 3.16 (0.36) b | 5.75 (0.62) ab | 6.60 (0.97) ab |
| **20 %** | 12.02 (1) c | 22.60 (2) c | 25.23 (3) c |

When the interaction effects between contact time and treatment concentration, including all observation times, were analyzed, they were not significant for the effect on *V. destructor* ($F_{[25,134]} = 0.48$, $P = 0.9828$). When the final observation time (8 h) and a contact time greater than 30 seconds were considered for the test, the proportions of dead mites in each treatment concentration were similar (*all* $P > 0.05$; Fig. 2). Eight hours after the beginning of treatment, any mites that remained inactive were considered dead. From that point on, the acaricidal effect increased along with increasing concentrations of the propolis extracts. At the end of the assay, the result of the treatment with the lowest concentration of propolis extract (1.25%) was not different from the control ($P = 0.9996$). However, mites treated topically with 10% extract showed greater mortality than 60%, indicating high toxicity even after short contact periods (Fig. 2).

In addition to the mortality effect, the propolis treatment induced a narcosis effect in *V. destructor*. This was noticed when the mites that remained inactive during the firsts hours after treatment initiation, recovered their activities, regardless of treatment

concentration and contact time with the solution. This effect was more intense with increasing propolis concentration (Fig. 1). In treatments with higher concentrations (7.5% and 10%) a significant proportion of mites remained in narcosis during the first two hours (*all* $P < 0.05$), whereas mites exposed to lower treatment concentrations recovered from narcosis during the first hour post-treatment (*all* $P < 0.05$). However, not all mites recovered from the narcosis, and those that did not become fully active during the first 8 h post-treatments were considered dead. This narcotic effect was not observed in the control group (*all* $P > 0.05$; Fig. 1).

The proportion of dead mites differed significantly from the controls after 24 h ($F_{[1,8]} = 13.20$, $P = 0.0067$), 48 h ($F_{[1,8]} = 27.52$, $P = 0.0008$) and 72 h ($F_{[1,8]} = 35.41$, $P = 0.0003$) when sprayed with the 10% propolis solution. In contrast, the proportion of dead bees after treatment did not differ significantly from the control bees ($F_{[1,8]} = 0$, $P = 1$). Spraying the mites with the 10% propolis extract thus resulted in 48, 68 and 78% mortality after 24, 48 and 72 h, respectively (Table 1).

### Oral administration method

The prevalence of *V. destructor* found in the cages was 4.49 ± SE 2 (n = 24). When propolis extracts were administered by the oral method in cages with infested bees, the proportion of dead mites after 24, 48 and 72 h was not significant among all concentrations ($F_{[4,18]} = 0.96$, $P = 0.4516$) and the co-variable ($F_{[1,18]} = 1.78$, $P = 0.1992$; Table 2). The effect of the different propolis concentrations, however, differed significantly for the bees during the observed period ($F_{[4,18]} = 10.87$ at 24 h; $F_{[4,18]} = 31.95$ at 48 h and $F_{[4,18]} = 23.09$ at 72 h; all $P < 0.05$). The proportion of dead bees at each observation time was not different within the first three propolis concentrations administered (all $P > 0.05$); in the treatment with the highest concentration this proportion was significantly different from the control and from the other treatments after 48 and 72 h ($P < 0.05$; Table 3).

## Discussion

The effect of propolis extracts on several micro-organisms has been demonstrated (Burdock, 1998) and, when tested against some parasites, it showed amoebicidal and antigiardial properties, and affected *Trypanosoma cruzi* (Higashi and De Castro, 1994; Freitas *et al*., 2006; Topalkara *et al*., 2007). Recent research has shown its biological effect on causal agents of certain bee pathogens and pests such as American Foulbrood, caused by *Paenibacillus larvae* (Gende *et al*., 2007; Antúnez *et al*., 2008), the greater wax moth *G. mellonella* (Garedew *et al*., 2004) and *V. destructor* (Garedew *et al*., 2002).

The most biologically active components may be obtained when propolis is extracted in 70% ethanol (Cunha *et al*., 2004). The major bioactive compounds are found in the resinous fraction of propolis, and these resins are mainly soluble in alcoholic solutions (Medana *et al*., 2008). For this reason, parasites of honey bees are not affected by the propolis produced in the hive by the bees.

The values of the organoleptic and physicochemical properties in the propolis sample used in this study were in accordance with data obtained from other propolis samples from the Pampean region (Bedascarrasbure *et al*., 2006). Due to the high concentration of these biologically active components, such as phenols and flavonoids, propolis collected from that zone has the best quality in Argentina.

With regard to the effect of propolis extracts on *V. destructor*, a previous study showed that mites are highly susceptible to propolis (Garedew *et al*., 2002). In that research, the propolis in alcoholic solution was applied topically on mites, the treatment with 10% solution resulting in 100% mortality, regardless of contact time. However, in the present study, only 60% of the mites were dead after a 30 second contact with the 10% propolis solution. These different results could have been due to a different geographical origin of the propolis samples, a variable that determines its phenolic fraction composition. The acaricidal activity of the propolis extracts is probably due to the presence of bioactive components in this fraction; no analyses of the propolis sample were made in the previously cited research. During the present studies mites that had remained in contact with the propolis showed a narcosis effect, which was more evident with the higher propolis concentrations. This effect was also observed by Garedew *et al.* (2002). When the highest treatment concentration was sprayed on bees and mites, 48, 68 and 78% of the mites fell, respectively, 24, 48 and 72 h after the treatment. In contrast, this treatment was harmless for bees. The narcosis and lethal effects seen when the mites were maintained with the propolis solutions clearly indicated the potential of spraying bees with propolis extracts to control *V. destructor*.

Feeding infested bees with the propolis extract in sugar syrup had little effect on *V. destructor*, but the highest propolis concentration caused 25% bee mortality. For this reason, if propolis is to be applied in hives, either alone or in combination with other natural substances, it should be used in concentrations under 20% in order to avoid the death of bees. No data are available about the oral administration of propolis extract in hives, although in practical beekeeping in Argentina syrup with unknown concentrations of propolis is commonly added to feed bees. The propolis may increase honey bee immunity; enhancement of their defensive response by propolis could also be important for the control of honey bee diseases (Evans *et al*., 2006).

The results obtained suggest that propolis extracts from our geographical zone could be incorporated into honey bee colonies by spraying, although it is still necessary to adjust the doses and concentrations to be administered, and the mode of action of the propolis on mites should be studied. Garedew *et al.,* (2002) suggested that contact with the propolis solution could lead to a weakening of the mite cuticle, which would facilitate the entry of active compounds

that are present in propolis. In the present research, oral administration of propolis had little effect on the mites, even at concentrations that were toxic for bees. Although propolis, by itself, may not be useful for mite control, this compound could have an indirect effect due to stimulating the bees' immune system when orally applied. Further investigations are required to obtain a better understanding about the effects of alcoholic propolis extracts on *V. destructor*. The variability in the propolis chemical composition, according to its phytogeographical origin and the various modes of application in the hives, are the main factors to be considered in planning future research. Such data would facilitate developing an integrated management programme for *V. destructor* that would reduce the amount of synthetic acaricides applied in the hives.

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