ORIGINAL RESEARCH ARTICLE

Oral toxicity of essential oils and organic acids fed to honey bees (*Apis mellifera*).

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Summary

Natural plant products have been studied for potential use as in-hive fumigants for suppression of parasitic mites and other pests. A more direct application through direct feeding of bees would avoid problems with fumigant volatility in cold climates and provide a more systemic route of exposure for the target pest. However, there must be a balance between toxicity to hive pests and toxicity (safety) to the bees. We focused on adult bee toxicity when testing ten products: cineole, clove oil, formic acid, marjoram oil, menthol, oregano oil, oxalic acid, sage oil, thymol, and wintergreen. Each product was tested at several concentrations in a sugar syrup fed to bees over several days, and dead bees were counted daily. Oxalic acid was the most toxic of the products tested. Menthol and cineole had mortality levels no different from controls fed plain syrup after 8 days of treatment. At 14 days of treatment, wintergreen was the least toxic, but neither menthol nor cineole were a part of the testing that went to 14 days. Our results indicate that the tested products could all be used safely for treating bees orally if dose is carefully managed in the hive.

Toxicidad oral de aceites esenciales y ácidos orgánicos en la alimentación de la abeja de la miel (*Apis mellifera*)

Los productos naturales de plantas han sido estudiados para su uso potencial como agentes fumigantes de represión de ácaros parásitos y otras plagas. Una aplicación más directa a través de la alimentación de las abejas evitaría problemas como la volatilidad de los fumigantes en climas fríos y proporcionaría una vía más sistémica de exposición para las plagas. Sin embargo, debe haber un equilibrio entre la toxicidad para las plagas y la toxicidad (seguridad) para las abejas. Nosotros nos hemos centrado en la toxicidad sobre abejas adultas de diez productos: eucaliptol, aceite de clavo, ácido fórmico, aceite de mejorana, mentol, aceite de orégano, ácido oxálico, aceite de salvia, timol y aceite esencial de wintergreen (salicilato de metilo). Cada producto fue probado con diferentes concentraciones en un jarabe de glucosa que alimentó a las abejas durante varios días, las abejas muertas fueron contadas diariamente. El ácido oxálico fue el producto más tóxico de todos los analizados. El mentol y el eucaliptol presentaron niveles de mortalidad similares a los controles, que fueron alimentados únicamente con jarabe después de 8 días de tratamiento. Tras 14 días de tratamiento, el aceite esencial wintergreen fue el menos tóxico, pero ni el mentol ni el eucaliptol se incluyeron en el análisis a los 14 días. Nuestros resultados indican que todos los productos testados pueden ser utilizados con seguridad por vía oral para el tratamiento de las abejas si la dosis es administrada cuidadosamente en la colmena.

Keywords: Medicaments, oral toxicity, natural plant products, mortality, miticides, protectants

Introduction

Various essential oils and organic acids have been evaluated as materials to manage mite populations afflicting honey bees. In general, these products are proposed to be used as in-hive fumigants (Imdorf *et al*. 1999) or as contact treatments (Amrine *et al*. 1996). In-hive fumigants have the disadvantage of needing warmth for sublimation or evaporation, and therefore they are less effective in colder weather (Scott-Dupree & Otis 1992). Nevertheless, organic acids like formic acid, and essential oils like thymol, have been found to be effective in management against mite pests in honey bee hives (Imdorf *et al*. 1999). Oral application circumvents the problems of fumigation in cold weather, but this application strategy has been mostly neglected. Menthol can be administered orally to honey bees, in microencapsulated formulation, with beneficial effects in suppressing population growth of tracheal mites (*Acarapis woodi*) (Kevan *et al*. 1997;2003). If essential oils and organic acids are to be considered as potential medicaments, rather than fumigants, one must be sure that the target animals (e.g. honey bees) are not poisoned. As with the research on menthol, we investigated oral toxicity of various essential oils and organic acids with the aim of assessing the potential problem of poisoning the patients with the active ingredient of the medicine (Kevan *et al*. 1999). As a control, we also used a plant compound, amygdalin, known to be poisonous to honey bees (Kevan & Ebert 2005), and reassessed menthol as a compound known to be innocuous to honey bees (Kevan *et al*. 1999). Menthol provides a positive control that is useful in evaluating the toxicity of other medicaments.

This research was on the toxicity of various natural plant compounds to honey bees with the intent to use these compounds to treat hives for various hive pest problems. It is likely that such treatments will involve exposing the colony to the compound for weeks or months. It is therefore necessary to assess how both the dose and the length of exposure influence mortality.

Materials and Methods

Honey bees (*Apis mellifera ligustica*) were obtained from hives at the Ohio Agricultural Research and Development Center (OARDC) Honey bee Lab, and placed in cages similar to those used by Kulencevic & Rothenbuhler (1973), and previously described in Kevan & Ebert (2005). Each cage had an average of 48 bees (+/-15 S. D.; range 20–134). Fed bees were given sugar syrup (69% sucrose), or a 69% sucrose syrup spiked with one of 10 possible natural plant products: cineole (CAS 470-82-6), clove oil, formic acid (CAS 64-18-6), marjoram, L-menthol (CAS 2216-51-5), DL-menthol (CAS 1490-04-6), oregano oil, oxalic acid (CAS 144-62-7), sage oil, thymol (CAS 89-83-8), or natural wintergreen oil (CAS 119-36-8). The clove oil was a commercial extract from *Eugenia caryophyllata* Thunb. (Myrtaceae). The main chemical components of clove oil are eugenol, eugenol acetate, iso-eugenol and caryophyllene (http://www.essentialoils.co.za/essential-oils/clove.htm). The oregano oil was a commercial extract from *Origanum vulgare* L. (Lamiaceae). The main chemical components are carvacrol, p-cymene, y-terpinene, and b-caryophyllene (Chorianopoulos *et al*. 2004). The marjoram oil was a commercial extract from *Origanum marjorana* L. (Lamiaceae). The main chemical constituents are sabinene, a-terpinene, y-terpinene, p-cymene, terpinolene, linalool, cis-sabinene hydrate, linalyl acetate, terpinen-4-ol and y-terpineol (http://www.essentialoils.co.za/essential-oils/marjoram.htm). The sage oil was a commercial extract from *Salvia sclarea* L. (Lamiaceae). The main chemical components of sage oil are linalool, a-terpenol, linalyl acetate, neryl acetate, and sclareol (Pitarokill *et al*. 2002). All solutions, including the control, had 5ml ethanol added to bring the total syrup volume to 100ml. Insoluble potential medicaments were dissolved in the ethanol first, then mixed with the syrup to bring the volume up to 100ml. In addition to these treatments, we included an unfed control, with no food or water. The starvation treatment was necessary because it was rumored that some of these medicaments would reduce feeding. We needed a starvation treatment to differentiate between toxicity and death due to starvation or dehydration.

Bees were fed *ad libitum* for the duration of the experiments. No additional water was provided, except what they could get by feeding on the syrup solution. We will call this days of treatment (DOT), since the bees are continually exposed for the entire duration. Each treatment was replicated four times. Mortality was checked daily, and dead bees were removed. Bees were considered dead when they would no longer move in response to poking with forceps. At the end of the experiment, the live bees were frozen and then counted. Cages were kept in open laboratory conditions that ranged from 17 to 25°C with a R.H. (Relative Humidity) from 18 to 37%.

We present the results from two tests. The first test evaluated the toxicity of all potential medicaments every day over an 8 day period at concentrations of 100 and 1000 ppm. The second test evaluated the toxicity of clove oil, formic acid, oxalic acid, oregano oil, and sage oil along with a fed and a starved control over a 14 day period. Potential medicament concentrations in the second test were at 100, 500, 1000, 5000, 10000, and 100000 ppm. Note; the 100000 ppm solutions for all potential medicaments tended to separate, or crystals developed in the solution. It is likely that the bees never experienced a potential medicament at 100000 ppm, even though we tried mixing the potential medicament back into the sugar syrup by shaking up the bottles once per day. Also note that we tested both D and DL menthol because sometimes a particular isomer is more toxic than others. However, we could not find any evidence of such a difference in the toxicity of these products. Therefore, our discussion of menthol will be the results from the combined data of the L-menthol and DL-menthol treatments.

Data were analyzed in SAS using a time-dose-mortality analyses and probit analyses. The time-dose-mortality model was a complimentary log-log model using a SAS program that was written as an implementation of the work by Priesler & Robinson (1989). However, the Hosmer-Lemeshow goodness-of-fit test (Nowierski *et al*. 1996) was highly significant for all models. We suggest that this lack-of-fit was caused by long tails in the data. When individual time intervals were of special interest, we used

Proc Probit to estimate the LD_{50} values for that day. Although the Proc Probit corrected for control mortality, the results from the time-dose-mortality analysis did not correct for control mortality. For this reason, the time-dose-mortality analysis overestimates the toxicity of these products.

Results

We have included the results from the amygdalin (known to be toxic to honey bees) trials for comparison (Kevan & Ebert 2003). Relative to amygdalin, all of the materials we tested were innocuous. Although oxalic acid is quite toxic relative to the other materials (*Table 1*), *Table 2* shows it to be somewhat less than half as toxic as amygdalin on a molecule per molecule basis.

Starved bees do not live long, with 40% dying in the first 24 H, and all the bees dead within four days (*Table 1*). No other treatment had higher first day mortality rates. High concentrations of oxalic acid were the most toxic treatment, and differed from the starvation treatment by having relatively low first day mortality. Mortality was 100% at the highest oxalic acid concentration, but bees at the lowest concentration only had about 60% mortality after 14 days (there was 40% mortality in the controls by this time). The second most toxic was formic acid, for which the last survivors died eight days post treatment at the highest concentration tested. However, mortality was only 30% after 14 days at the lowest concentration. A few other potential medicaments also showed high mortality levels at the maximum concentration tested. In contrast to these products, we conclude that cineole, menthol, marjoram, and thymol are non-toxic to bees because their mortality levels never exceeded background mortality at any concentration tested (*Table 2*).

All products tested were much less toxic than amygdalin on a molecular basis. Although the LT_{50} for oxalic acid and amygdalin are similar, it takes over twice the number of molecules of oxalic acid to achieve an equivalent level of toxicity (*Table 2*). However, when comparing LD_{50}s at 8 days of treatment (DOT), oxalic acid appears more toxic (*Table 3*). The reason the model is non-significant ($\alpha \leq 0.01$) in this case is because mortality in all treatments was too high and too variable (average mortality in lowest dose was 50% +/- 40% S. D.). The reason the model for sage is also non-significant ($\alpha \leq 0.01$) is because, even at the highest dose, mortality was only 60% +/- 40% S. D.

The critical feature in the potential utility of all these products is their long term effects on bee health. This was assessed by keeping bees in the cages as long as possible. Treatment with sage oil resulted in highly variable mortality, for which we have no explanation. Oxalic acid, with the lowest LD_{50} value, was most toxic and wintergreen was the least (*Table 4*).

All LD_{50} levels decline over time. However, most show a rapid decline in LD_{50} values within the first few days, followed by a leveling off. In part this trend reflects ever increasing levels of background mortality. However, most cages of bees had a few individuals that lived many days longer than their sisters. This made the tails of the mortality distribution long, and probably accounted for most of the significance in the lack-of-fit tests.

Table 1. Twenty four hour mortality and average mortality at 8 days and 14 days exposure (DOT) for various essential oils and organic acids fed to honey bees, together with the total number of bees tested (sum of all replicates and dosages).

Potential	**Day 1 Average**	**Average % mortality**		**Number of Bees**
		8 DOT	14DOT	
Medicament	% mortality	1000 ppm	100,000 ppm*	Tested
Amygdalin	0	79**		221
Cineole	2	11		214
Clove oil	3	28	96	927
Control – fed	1	10	40	1189
Control – unfed	46	100***	100***	495
Formic acid	1	33	100	1438
Marjoram oil	2	34		308
Menthol DL	1	25		256
Menthol L	1	21		239
Oregano oil	1	41	92	953
Oxalic acid	6	96	100	1217
Sage oil	3	21	87	1289
Thymol	8	43		201
Wintergreen	1	24	99	1308

* Cells with missing data are left blank. ** Dosage for amygdalin was 2250 ppm. *** All bees were dead in 4 days.

Table 2. Estimated LT_{50} for potential medicaments at 1000 ppm. In this analysis, data from tests 1 and 2 were combined to estimate the LT_{50}. Except as noted, all terms in the models were significant ($\alpha \leq 0.01$), and all lack-of-fit tests were also significant ($\alpha \leq 0.01$).

Material	Molarity*	LT_{50} in days	Lower 95% Fudicial Limit	Upper 95% Fudicial Limit
Amygdalin**	0.0049	4.6	3.0	6.3
Cineole	0.0065	NS		
Clove Oil		11.2	10.2	12.8
Controls – Fed		17.0	15.9	18.7
Controls – unfed		1.9	1.7	2.1
Formic Acid	0.0217	11.8	8.1	30.3
Marjoram Oil		27.0	15.0	3935.0
Menthol	0.0064	NS		
Oregano Oil		10.8	9.9	12.1
Oxalic Acid	0.0111	4.8	2.6	5.9
Sage Oil		11.5	10.6	12.7
Thymol	0.0067	NS		
Wintergreen	0.0029	14.4	12.6	17.7

NS = no significant model. Mortality in these tests did not reach 50%, so accurate estimates cannot be made.
* These plant extracts are blends of several compounds so it is not possible to calculate the molarity of each component.
** Amygdalin concentration was 2,250 ppm, the lowest concentration tested by Kevan and Ebert (2005).

Table 3. Estimated LD_{50} for the tested essential oils and organic acids at 8 Days exposure (DOT).

Material	LD_{50} in ppm	Lower 95% Fudicial Limit	Upper 95% Fudicial Limit
Amygdalin	1600	1300	1800
Clove Oil	7800	2300	18100
Formic Acid	5600	3500	8100
Oregano Oil	14900	0	43400
Oxalic Acid	NS		
Sage Oil	NS		
Wintergreen	13500	8500	21700

NS = model non-significant ($\alpha \leq 0.01$). Oxalic acid was too toxic and mortality by Sage Oil was too variable.

Table 4. LD_{50} values 14 days exposure (DOT).

Material	LD_{50} in ppm	Standard Error of Log_{10} (dose)	Lower 95% Fudicial Limit	Upper95% Fudicial Limit
Clove Oil	240	0.1483	80	710
Formic Acid	450	0.0653	280	720
Oregano Oil	600	0.1327	230	1620
Oxalic Acid	80	0.0635	50	130
Sage Oil*	NS			
Wintergreen	800	0.0632	500	1270

* dose was not a significant variable in the time-dose-mortality model.

Discussion

Looking at mortality in *Table 1*, we conclude that wintergreen, menthol, sage oil, and cineole all are relatively innocuous, and that marjoram oil is quite benign. At the other extreme, oxalic acid and, as expected, amygdalin were the most toxic, and has the potential to cause high levels of mortality if the dosage is high.

These results also demonstrate that development of hive treatment protocols require balancing the exposure time and the dose given to the hive. Our results are sufficient for dosing bees for up to 14 days, but we expect that dosage would have to be more limited if the exposure period is lengthened. If continuous dosing is the best treatment option, then additional research would be needed to assess toxicity during the over-wintering period and the effects these products may have on egg laying viability of the queen, and development of larvae. However, all of these products have a low enough toxicity that they could be used as ingested medicines (rather than fumigants) with minimal effects on the hive. Clearly, the next phase is to determine if safe doses of these products can be fed to bees to effectively manage hive pests, and whether it is better to shock the hive with a short term massive dose, or to try long term exposures at low dosages.

We would like to caution readers that these results are most relevant to adult worker health. It is possible that the adult workers could feed the medicaments to larvae, and that the larvae may be more sensitive. It is also possible that the medicaments fed to the queen or drones could affect their reproductive capacity. However, exposure of these individuals is buffered through the workers. Unless the only source of food for the entire hive is the treated sugar water, there will be a dilution effect where the treated sugar water is mixed in the hive with nectar from outside sources. A queen that gets one drop of treated syrup from one worker followed by a droplet of nectar from outside the hive is effectively consuming a nectar at half the dosage of the treated syrup. Consequently, workers will be exposed to greater dosages of these medicaments than will other members of the hive. Therefore, testing the toxicity of the medicaments to the workers is a natural first step, and it may be the only necessary step unless other problems occur as these products are developed.

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