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ORIGINAL PAPER

In vitro and field studies on the contact and fumigant toxicity of a neem-product (Mite-Stop®) against the developmental stages of the poultry red mite *Dermanyssus gallinae*

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Abstract The acaricidal activity of the neem product MiteStop® was investigated for its potential use as a botanical acaricide for the control of the poultry red mite *Dermanyssus gallinae*. This neem product is a special formulation of an extract of the seeds of the neem tree *Azadirachta indica* A. Juss. The efficacy was tested under laboratory conditions as well as in poultry houses. Four different methods of application were used in a filter paper bioassay to evaluate contact and vapour phase toxicity tests. The neem product proved to be already active in very small doses. In order to investigate the efficacy under field conditions, a poultry house was sprayed twice within a 7-day period using 1:33 and 1:50 diluted MiteStop®. Cardboard traps were used to assess the mite population before, during and after the treatment. The mite population could be reduced by 89%. In a second poultry house, the spraying of defined areas with a 1:30, 1:33 or 1:50 dilution of the acaricide proved to be highly efficacious against all mite stages. Three other field trials proved that MiteStop® is highly active against the red poultry mite. The most efficient dilution is 1:33 with tap water and spraying two or three times at intervals of 7 days.

Parts of this publication are included in the PhD-thesis of Doctor Nina Locher. This thesis has been accepted at the Free University of Berlin (Germany) to obtain the Doctor in Veterinary Medicine.

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Introduction

Parasites are a problem wherever poultry are raised and can lead to significant economic losses (Ruff 1999, Mul et al. 2009). The poultry red mite *Dermanyssus gallinae* (De Geer 1778) is the most important ectoparasite of farmed birds in Europe (Chauve 1998). This haematophagous mite is a nocturnal feeder that spends the day hidden in cracks and crevices of the chicken house. An infestation can be very stressful for the birds resulting in irritation, restlessness, feather pecking, and anaemia. Heavy infestations can decrease egg production, egg quality, weight gain in young birds, and it can even cause death (Chauve 1998, Kirkwood 1967, Pospischil 2001). It was found that mite-infested chicken need daily 15-20% more food than uninfested ones (Mul et al. 2009). Thus, the economic losses are not to be underestimated. Control and production losses in Europe have been estimated at €130 million per annum (van Emous 2006). Furthermore, *D. gallinae* can act as a vector and reservoir for several bacterial and viral pathogens such as *Salmonella* spp., *Erysipelothrix rhusiopathiae*, *Pasteurella multocida*, *Borrelia anserina*, *Coxiella burnetii*, chicken pox virus, Newcastle Disease virus and St. Louis encephalitis virus (Chirico et al. 2003, Lundh et al. 2005, Moro et al. 2007, Smith et al. 1945). Occasionally it also feeds on mammals including humans and can cause dermatitis (Auger

et al. 1979, Beck and Pfister 2006, Mignon and Losson 2007, Pritchard and Kruse 1982).

Control of the poultry red mite has been difficult, especially for food-producing poultry because there is currently no registered compound available on the German market.

The European legislation will ban traditional cage systems for poultry by 2012. Due to the peculiar life cycle of *D. gallinae*, less intensive farming systems like barns, free range, and organic farming show higher prevalence rates. This and the removal of acaricides from national markets due to the increase in acaricide resistance and welfare concerns will probably increase the problems caused by the poultry red mite *D. gallinae* (Sparangano et al. 2009). Therefore, the present situation calls for studies on the efficacy of alternative control methods such as the use of, i.e. plant-derived acaricides. The most important pesticides appear to be those derived from the seeds of the neem tree *Azadirachta indica* A. Juss. Several neem products proved to be efficacious against viruses, bacteria, endoparasites, and arthropods (Schmutterer 2002). Moreover, the complex mixture of several different, bioactive analogues in neem is thought to avoid the development of resistance (Mulla and Su 1999).

The aim of this study was to investigate the potential of a neem product (MiteStop®) as a botanical acaricide for the control of the poultry red mite *D. gallinae* in-vitro and in-vivo.

Material and methods

MiteStop®

The acaricide MiteStop® is a product of Alpha-Biocare GmbH, Düsseldorf (Germany). It is a special, patented formulation of the extract of the seeds of the neem tree *A. indica* A. Juss. It is sold as a concentrated product, which has to be diluted with tap water prior to use.

Mites

The mites for laboratory investigations were collected in different infested poultry houses in Germany. All investigations were carried out within 3 days after collection of the mites. The field investigations were done inside large stables in Europe containing egg laying chicken being kept either in cages or on the floor.

Life cycle parameters

In order to assess the ovicidal activity, it was necessary to collect some life cycle parameters of *D. gallinae* under specific conditions. All experiments were carried out at a temperature of 20°C (±0.5°C) and at a 40% relative humidity (RH). Engorged females, protonymphs and deutonymphs were placed in single wells of a 24-well plate (Nunclon™ surface, nunc™, Roskilde, Denmark). The wells were sealed with a special foil that allowed gas exchange. The ongoing development was observed every 24 h.

Filter paper bioassays

Four different methods of application were used to evaluate contact and vapour phase toxicity tests. The toxicity was tested on every developmental stage.

Method A (wetting of individual mites) was used to evaluate the toxicity of very small doses. About ten mites were placed on a filter paper (Rundfilter MN 615, Machery-Nagel) in a Petri dish (92×16 mm, Sarstedt). Each mite was treated with 0.5 µl of undiluted, 1:20, 1:40, 1:60, and 1:80 diluted acaricide. The control group was treated with water. Method B1 (contact with wet acaricide) and method B2 (contact with dried acaricide) were used to evaluate the toxicity of a treated area. An amount of 0.3 ml of undiluted and diluted (1:20, 1:40, 1:60, 1:80) acaricide was applied to filter papers. Control filter papers were treated with water (Fig. 1).

In method B1, the wet filter paper was placed in a Petri dish and groups of about 20-30 mites were placed on the filter paper. In method B2, the filter paper was left to dry at room temperature before putting it in a Petri dish. In order to prevent any mites from escaping, the Petri dishes were sealed with Parafilm®.

Method C was conducted to investigate the vapour phase toxicity. Groups of 20-30 mites were placed into an Eppendorf cup (2 ml). The lid of the cups had been cut off to seal the opening with a special foil allowing the entrance of vapours. The sealed cups were then placed in a glass container (20 ml). A folded filter paper treated like in method B1 and B2 was added and the container was sealed with a plastic lid. The Petri dishes and the glass containers were stored at room temperature. The number of dead mites

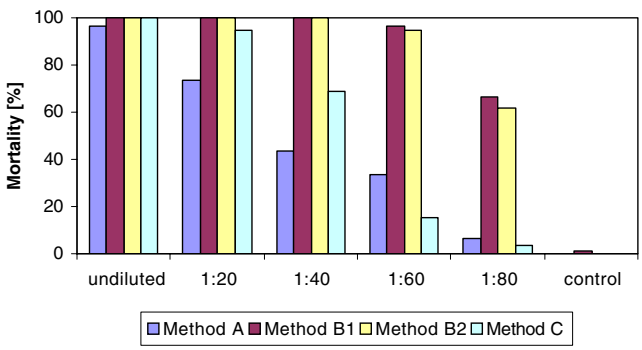

Fig. 1 Results of the filter paper bioassays for the efficacy of MiteStop®

was determined under a dissecting microscope. Mites were considered to be dead if no movement resulted upon pinching with a pair of fine tweezers. Every method was repeated four times.

The ovicidal activity was investigated by using the same four application methods. Due to the limited amount of available eggs, only a 1:50 dilution was tested. The eggs were observed for a period of 168 h.

Treatment inside poultry houses

Experiment 1

A small heavily infested poultry house of layers was sprayed twice within a 7-day period using 1:50 diluted MiteStop®. In order to assess the mite population, cardboard traps were used at distinct places before, during and after treatment. The traps were made of ordinary corrugated cardboard consisting of three layers. The outer layers were carefully separated from the inner layer by using a fine scalpel. A paper clip kept the layers together and allowed opening of the trap. The first monitoring covered 5 days prior to the first spraying. The second or third monitoring, respectively, started 2 days after each spraying and lasted 5 days. At the end of each monitoring period, the cardboard traps were collected, placed individually in freezer bags, and were put in a freezer at −18°C to kill the mites. The total number of the population was determined by calculating the dead mites of each trap.

Experiment 2

The second experiment was carried out in a scientific poultry house in Sinsteden (Germany). Defined areas were secured by using strong double-faced adhesive tape in order to prevent mites from leaving or entering the area. Two areas were sprayed with 1:30 and 1:50 diluted MiteStop®. Other areas were used as a control and remained untreated. Twenty four hours after the spraying all areas—treated or not—were cleaned thoroughly with a brush. The resulting material was put in plastic bags and brought to the laboratory in order to count the dead and the living mites.

Experiment 3

This experiment was done in a giant layer house with more than 50,000 hens close to Nancy in France. The chickens were kept at semi-night conditions in large metal cages each containing five chicken. The night situation made it possible that the mites stayed all the time on or close to the chicken leading to enormous amounts of mites. Spraying (1:40 dilution) was done four times at intervals of 7 days. However, the house construction allowed only the spraying of the front side of the cages.

Experiment 4

This experiment was done in four chicken houses in Egypt (Nile Valley). The chickens were kept on the floor inside 60-200 m^2 rooms inside farms. Spraying was done two times at an interval of 7 days using a MiteStop®-water dilution of 1:40 or 1:50. Two hours after spraying, soil was inspected for living and dead mites.

Experiment 5

This experiment was done in a stable close to Vechta (Germany), where about 7,500 chickens were kept on the floor. This experiment was planned to compare the activity of MiteStop® with another (phoxim containing) acarizide. Since the latter required due to its toxicity as organophosphorous compound the retraction of the food and water for 24 h, if it would be sprayed on the whole floor, the application was done in both cases only on the perch using a 1:33 dilution of the product with water. Controls were done in all five experiments at places that had not been sprayed or had been sprayed with pure water.

Data analysis

Acaricidal activity was classified as described by Kim et al. (2007): strong: mortality >80%; moderate: mortality 80-61%; weak: mortality 60-40%; little or no activity: mortality <40%. The LD_{50} values were calculated by using BioStat 2008.

Results

Life cycle parameters

Engorged females started to lay eggs after 24 h after their blood meal. The average time required for egg laying was 41.6 h. Within 72 h, all mite eggs had hatched. Almost all larvae moulted without feeding to protonymphs within 48 h. Moulting to deutonymphs occurred from 48 to 72 h. Most of the engorged deutonymphs moulted to adults within 72 h (Table 1).

Filter paper bioassays (Fig. 1)

The treatment of individual mites after method A showed a strong acaricidal activity for the undiluted neem product with an average mortality of 96.43%. The 1:20 dilution

Table 1 Developmental time of *D. gallinae* at 20°C and 40% relative humidity

Developmental stage	Time required (h)		Mode	Mean
	Minimum (%)	Maximum (%)	h (%)	h
Oviposition	24 (26.67)	48 (73.33)	48 (73.33)	41.60
Egg	48 (5.88)	72 (94.12)	72 (94.12)	70.59
Larva	24 (2.94)	48 (97.06)	48 (97.06)	47.29
Protonymph	48 (80.00)	72 (20.00)	48 (80.00)	52.80
Deutonymph	72 (80.00)	120 (6.67)	72 (80.00)	78.40

h hours

proved to be of moderate activity. The 1:40 diluted MiteStop® showed a weak activity. The 1:60 and 1:80 dilutions were of little acaricidal activity. The LD_{50} was 0.017 µl/cm² (0.019 mg/cm²). MiteStop® used after method B1 (contact with wet acaricide) proved to be highly efficacious to all developmental stages. It achieved a mortality of 100% at an undiluted status as well as when 1:20 and 1:40 diluted. The use of a 1:60 dilution showed also a strong acaricidal activity. The 1:80 dilution proved to be of moderate efficacy. The LD_{50} was 0.047 µl/cm² (0.052 mg/cm²). The lethal activity of dried acaricide (method B2) was also very strong. All dilutions up to 1:40 showed a mortality of 100%. The 1:60 dilution also proved to be very strong reaching an average mortality of 94.67%. The 1:80 dilution showed a moderate lethal activity. The LD_{50} was 0.051 µl/cm² (0.056 mg/cm²).

The vapour phase toxicity of undiluted and 1:20 diluted MiteStop® proved to be strong. The 1:40 dilution showed a moderate toxicity. The 1:60 and 1:80 dilution showed a weak acaricidal effect. The LD_{50} for method C was 0.117 µl/cm² (0.128 mg/cm²).

Application method A (wetting of individual eggs) and method C (vapour phase toxicity) had no ovicidal activity. All treated eggs had hatched within 72 h. The application methods B1 (contact with wet acaricide) and method B2 (contact with dried acaricide), however, had a strong ovicidal activity preventing all eggs from hatching.

Treatment of poultry houses

Experiment 1

In the first experiment, the mite population was reduced by 66.86% after the first spraying. After the second spraying, the total of the mite population had been reduced by 89%. Apparently, some mites had hatched from eggs and/or came from hidden unsprayed places (Fig. 2).

Experiment 2

In the second experiment in Sinsteden (Germany), defined areas infested with poultry red mites were treated with 1:30 or 1:50 diluted MiteStop®. The efficacy was 100% in both areas. Here, apparently all mites had been reached during spraying (Table 2).

Experiment 3

The size if this giant stable and the amount of mites in the chicken cages and their numbers around the channels and bands transporting food, faeces and eggs made it necessary to use an automatic spraying engine which was driven along the walk boards in front of the cages. This method implicated that only the front sides of the cages and of the food and water equipment was fully reached by the 1:40 water diluted neem extract. On occasion of the first inspection on day 0, the cages and the equipment were covered with layers of mites reaching diameters of several millimetres. Furthermore, numerous flies were able to transport mites from any region of the stable to another (e.g. the lamps at reduced light were covered with many mites, too). Furthermore, due to the darkness inside the stable, the mites did not hide anymore and stayed close to the chickens and were also found constantly on the chickens.

The four repeated treatment series (at intervals of 7 days) reduced the number of mites extremely. At the last inspection, the thick layers of mites had disappeared and mites were found only at hidden places. The

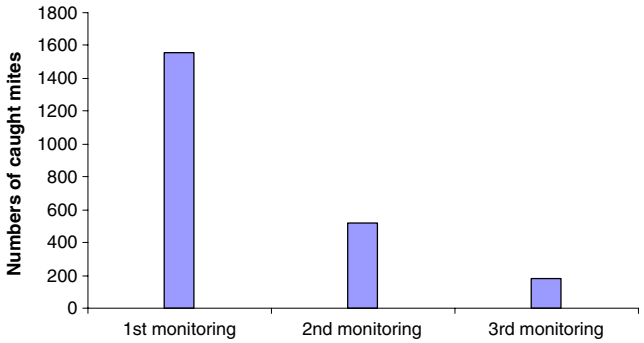

Fig. 2 Numbers of caught mites during the treatment of a poultry house (first experiment)

Table 2 Assessment of caught mites in the second experiment

Area	Number of living mites after 24h				Number of dead mites after 24h				Mortality
	Total	Adults	Nymphs	Larvae	Total	Adults	Nymphs	Larvae	%
MiteStop® 1:30	0	0	0	0	1,500	674	698	128	100.00
MiteStop® 1:50	0	0	0	0	1,013	437	489	87	100.00
Control A	1,994	824	912	258	328	167	134	27	16.45
Control B	469	145	236	88	64	23	41	0	13.65

reduction was calculated to be gone back to a level of about 10-15% of the initial value. This value has to be considered before the background that only the front of the chicken cages was reached by the automatic spraying machines and that behind the cages other mites had been hidden.

Experiment 4

The 1:40 or 1:50 water freshly diluted MiteStop® product was twice applied (on day 0 and day 7) onto the floor of the stables in Egypt, where the chickens lived. Samples of soil were taken 2 h after each spraying and studied by light microscopy. The soil samples were placed onto a sieve and a lamp was switched on to provide light and heat. Living mites are hygrotactic, thermotactic and phototactic. This introduced the movements of survivors downwards—away from the lamp. Table 3 shows the results obtained after the two treatments.

This experiment shows that a dose dependent reduction of the numbers of mites, which, however, appear still rather well protected in the soil dust. The 1:40 dilution revealed slightly better results, but the dust protected still some survivors.

Experiment 5

In the stable with 7,500 hens in Germany, 12 spots were selected to become sprayed with the 1:33 MiteStop®-water dilution. Previous to the first spraying, the 12 spots contained 878 living mites. After 1 week, these places contained only 102 living mites, i.e. corresponding to a reduction of more than 88%. Of course these mites could have immigrated from neighbouring places. After the second spraying, the reduction of living mites was another 10% so that it was concluded that the product prohibited reimmigration at a considerable degree. Otherwise, much more mites would have been expected to come from the surrounding non-sprayed places.

Discussion

Life cycle parameters

So far, there had been no data available for life cycle parameter at a temperature of 20°C and a relative humidity of 40%. Wisseman and Sulkin (1947) observed mites kept at room temperature and 70% RH. Tucci et al. (2008) observed mites at 20°C and a relative humidity ranging from 70% to 85%. Compared to Tucci et al. (2008) the average time needed for oviposition, larva, and deutonymph development are higher at 40% RH. However, the average time in which protonymphs moulted to deutonymphs was shorter. The prolonged developmental times might be due to the low relative humidity in our laboratory, while the poultry red mite favours high humidity.

Table 3 Example for mean numbers of counts of living mites found in soil of stables (mites per gram soil) 2 h after spraying of dilutions of the neem seed extract 1:50) on day 0

Mites	Day 0			Day 7		
D. gallinae (number per gram dust)	Control	1:40	1:50	Control	1:40	1:50
	0.300	0.180	0.240	0.350	0.070	0.140
	0.380	0.228	0.304	0.410	0.082	0.164
	0.420	0.252	0.336	0.481	0.096	0.192
	0.510	0.306	0.408	0.390	0.078	0.156
Total per 4 g	1.610	0.966	1.288	1.631	0.326	0.652

Filter paper bioassays

The filter paper bioassays showed that the neem product is already efficacious against the poultry red mite at a low dose. Compared to the commonly used pesticides, it has a stronger acaricidal activity than fipronil, phenothrin, permethrin, alphacypermethrin, furathiocarb, carbaryl, and fenotrothion which have an LD_{50} of >5.0 mg/cm². Diazinone (LD_{50}=0.25 mg/cm²), chloropyrifos-methyl (LD_{50}=0.15 mg/cm²), fenthion (LD_{50}=0.07 mg/cm²), and propoxur (LD_{50}=0.06 mg/cm²) are less efficacious as well. Only dichlorvos (LD_{50}=0.0004 mg/cm²), profenofos (LD_{50}=0.003 mg/cm²), prothiofos (LD_{50}=0.055 mg/cm²), and benfuracarb (LD_{50}=0.053 mg/cm²) reach a lower LD_{50} value for the same acaricidal activity. Compared to the 10 most toxic plant extracts for *D. gallinae* the neem product is more efficacious than *Glycyrrhiza glabra* (LD_{50}=0.14 mg/cm²), *Foeniculum vulgare* (LD_{50}=0.15 mg/cm²), *Illicium verum* (LD_{50}=0.09 mg/cm²), *Lysimachia davurica* (LD_{50}=0.09 mg/cm²), *Paeonia suffruticosa* (LD_{50}=0.11 mg/cm²) and *Schizonepeta tenuifolia* (LD_{50}=0.15 mg/cm²). Only *Mentha arvensis* var. *piperascens* (LD_{50}=0.0072 mg/cm²), *Eugenia caryophyllata* (LD_{50}=0.0069 mg/cm²), *Cinnamomum camphora* (LD_{50}=0.0051 mg/cm²) and *Asarum sieboldii* var. *seoulense* (LD_{50}=0.0063 mg/cm²) reach a stronger acaricidal efficacy (Kim et al. 2007). The vapour phase toxicity is less effective than the contact toxicity reaching an LD_{50} value of 0.117 µl/cm² or 0.128 mg/cm², respectively. A comparison of the LD_{50} value with other acaricides is not possible because there are no data available. Kim et al. (2007) published an efficacy of 100% for *A. sieboldii* var. *seoulense*, *Cinnamomorum camphora*, *E. caryophyllata*, *M. arvensis* var. *piperascens*, and the efficacy of dichlorvos at a dose of 0.28 mg/cm². MiteStop® yielded an average mortality of 94.64% at a dose of 0.25 mg/cm². The efficacy of theses acaricides is therefore comparable.

Ovicidal activity

An ovicidal activity was only obtained when using for method B1 (contact with wet acaricide) and method B2 (contact with dried acaricide). However, MiteStop® was only tested in a dilution of 1:50. It might therefore be possible that a higher concentration would lead to an ovicidal activity for the application methods A (wetting of individual mites) and C (vapour phase toxicity). A higher dosis may be necessary to penetrate the protective egg shell to kill the developing larva. It might also be possible that the neem product inhibits the hatching of larvae. A transmission electron microscope analysis might give some answers (Locher et al. 2010).

Poultry house experiments

Experiments 1 and 2

The spraying of the neem product in a low-grade infested poultry house was very successful. Already the first spraying, reduced the mite population by 66.86%. After the second spraying, the population had been reduced by 89%. These results are similar to those of Abdel-Ghaffar et al. (2008), who treated infested poultry in Egypt. The efficacy of a repeated spraying of MiteStop® is comparable to the efficacy of metrifonate (0.15%; Nordenfors and Hoeglund 2000) and ByeMite® (phoxim) (Meyer-Kühling 2007). The spraying of distinct areas with MiteStop® in another infested poultry house proved to be 100% efficacious (Table 2).

In conclusion, these two studies have shown that the neem-derived acaricide has a high acaricidal effect against all developmental stages of *D. gallinae*. Furthermore, it shows not only an efficacy at direct contact, but it also is of fumigant toxicity. Therefore, it has a great potential for the control of the poultry red mite, if it is used correctly.

Experiment 3

This experiment proved that a dilution of 1:40 is in principle able to kill the different stages of the red mite. However, from untreated places, amounts of mites will attack the chickens again (although in lower numbers). The short developmental cycle of 8-12 days will always deliver numerous newcomers from mite eggs that had been laid at untreated places. Thus, it is needed to spray as much mite-infested sites as possible.

Experiment 4

In this case, 1:40 and 1:50 dilutions were used being sprayed directly onto the mite-containing dust on the floors of the chicken houses. This dust protects several mites from the product, but nevertheless the reduction of the blood-sucking mites was considerable. Brush cleaning of the floors will increase the efficacy of the spraying.

Experiment 5

In this case, a dilution of 1:33 of the product was sprayed onto the sitting places of the chickens. Although there was the chance that the mites may migrate from unsprayed places to sprayed ones, the reduction of mites was nearly 90% of the number at the beginning of the experiment. This shows that the 1:33 dilution is very effective.

Conclusions

The in vitro and in vivo experiments show that the neem seed extract diluted at 1:33 with tap water kills all stages of the blood sucking mite *D. gallinae*. Lower concentrations or in cases when mites do not come into direct contact with the acaricidal plant compound some mites may survive. Such cases make it necessary, that treatment is repeated at least twice at an interval of 5-7 days. Since this biological biocide has also a high activity against other blood-sucking insects such as bed bugs, fleas, lice or different ticks, the product MiteStop® has become a very useful remedy against house inhabiting pests (Semmler et al. 2009, Schmahl et al. 2010, Abdel-Ghaffar et al. 2010).

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