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PRELIMINARY BIOASSAY FOR NEEM OIL TO BE IMMOBILIZED INTO POLYMERIC BIOMATERIAL

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Summary. *In recent years several studies have been performed in search of new products such as insecticides of natural origin. Azadirachta indica has been extensively described by its properties of different insecticides and insect repellent, which is due to azadirachtin, an active principle contained mainly in the seeds of this plant from which oil is extracted and used for such purposes, especially in agriculture. This is a product of low toxicity, biodegradable and economically feasible. This work aimed to evaluate the behavior of the flea Ctenocephalides felis felis, common in dogs and cats in the presence of neem oil in order to develop devices repellent ectoparasites by means of encapsulation of the oil in the encapsulating agent suitable for incorporation in the polymeric matrix from PVA. Adult fleas were used and grown in the laboratory results demonstrate the absence of repellency for the fleas, however a moderate insecticidal effectiveness.*

Keywords: Neem oil, Ctenocephalides felis felis, bioassay, Azadirachta indica, PVA.

1. INTRODUCTION

Azadirachta indica A. Juss 1830 popularly known as Neem or Nim is a vegetal specie from Indian, belonging to the family Meliaceae, which was introduced in Brazil in 1986 by the Agronomic Institute of Paraná - IAPAR and today is widely cultivated for presenting various bioactive properties that can be used in medicine, agriculture, veterinary and other areas (MORGAN, 2009; PINTO and SPEARS, 2010, KIKUCHI et al., 2011).

In different parts of the plant there is a large amount of active ingredients with different properties such as antiinflammatory, antiarrhythmic, antibacterial, antifungal, diuretic, and hypoglycemic and others (BISWAS et al., 2002).

It is also considered an insecticide indirect to act as antifeedant, growth regulator and interfere in the reproduction, besides acting as a repellent for various insects. Among the compounds found, act as repellents or insecticides indirect, azadirachtin, salanina, meliantriol and nimbin, but the azadirachtin shows the most promising repellent than others and therefore has received the most attention from researchers (SCHMUTTERER, 1990; SUMAN, 2010).

Azadirachtin is a secondary metabolite, triterpenoid class of limonoids, non-toxic to the environment and vertebrates. It is found in most of the *A. indica* seeds, and the concentrations may vary depending on the plant in each region of the cultivation, season and means of extraction, and the maximum quantity of azadirachtin is obtained in fruits ripening and storing (MORGAN, 2009).

Some studies have shown positive results in the control of *Ctenocephalides felis felis* (Bouche, 1835), one of the most important ectoparasite infestations in cats and dogs, because

the bite may promote transmission of pathogens, or even cause allergic dermatitis flea arising from existing components in the saliva of this insect (RUST, 2005, CHIN et al., 2010).

The flea life cycle comprises four stages: egg, larva, pupa and adult. Its onset is when the eggs are deposited among the hairs of their hosts. After oviposition, they fall to the ground, and get together in large quantities in places frequented by hosts. After an interval of one to ten days, the larvae hatch and the mean development of the larvae is five to eleven days. At the end of its development, the larva stops eating and empties its digestive tract, initiating the production of thin silk viscose for the formation of the pupa, which will adhere to any environmental contamination.

The birth of adult fleas occurs in about five to nine days after the onset of pupation, reaching a length of one hundred and forty days. Shortly after birth, fleas begin the blood meal and oviposition occurs in a maximum time of thirty-six to forty-eight hours.

The pulicideos adults are only 5% of the parasites on, the remaining 95% being distributed among the other life stages and demonstrated, therefore, that most of the cycle of *Ctenocephalides felis felis* goes outside the host (DRYDEN and RUST 1994).

Ctenocephalides felis felis can act as intermediate host of *Dipylidium caninum*, a common cestode in dogs, cats and occasionally children, *Dipetolonema reconditum*, a heartworm of dogs and can even be a vector of cat scratch disease and *Bartonella henselae* transmit some rickettsiae species (AVELAR et al., 2007; REIF and MACALUSO, 2009).

In 1998, Guerrini and Kriticos published a study that demonstrated the correlation of dose-dependent action of azadirachtin on *Ctenocephalides felis*. The results revealed significant reductions in the flea population in both animals and the environment using extracts *A. indica* combined with DEET and citronella oil, sprayed on dogs.

Ribeiro, et al., (2008) in his work "Activity of Neem extract on the embryonic development of *Ctenocephalides felis felis* (Bouche, 1835) (Siphonapeta: pulicidae)" used an aqueous solution of 10% neem, spray, infested with fleas in dogs and obtained results showed that significant amounts in relation to inhibition of embryo development of the fleas, and 95,78% inhibition on day assessment of the treated group and 82,20% inhibition on the fourteenth day.

According to the properties studied until now, *A. indica* products can be an alternative to currently used pesticides, most synthetic, such as pyrethroids, organophosphates, carbamates, fenilprazoles, nitroguanidinas, neonicotinoids, macrocyclic lactone and growth regulating substances for the control *C. felis felis* to be obtained from renewable products, fastly degradable, economically feasible and low environmental impact. Moreover, because it is a combination of different active principles, upon the occurrence of tolerance or resistance by the insects, it occurs more slowly (KUMAR et al., 2005, RIBEIRO et al., 2008; LANDAU et al., 2009).

Devices for the controlled release systems can be an alternative for use in controlling fleas found in domestic animals. Polymeric materials are widely used in these systems is that the polymer of choice will always have a relationship with the active principle intended to be used, the type of release desired and the physicochemical properties of this polymer (UHRICH et al., 1999).

Poly (vinyl alcohol) (PVA) is a polymer commonly used in delivery systems because it is a material easy to obtain and biodegradable. Its hydrogel form allows the dispersion of microcapsules containing active ingredients that are compatible with aqueous solution such as those present in oil so that there is an interaction between the components without interfering with the release.

The microencapsulation to be used to protect the active ingredient to be dispersed in the PVA solution must be compatible with both oil and aqueous solution, and allows the release of these in time and quantity desired.

One method of crosslinking of the PVA solution is cycles of freezing and thawing. This is a physical crosslinking which allows the formation of crystallites in each cycle depending on the time and temperature that does not promote changes in the active principles contained in the device. The properties of the product formed depend on the molecular weight of the polymer, the aqueous solution concentration, temperature and frozen storage and thawing (STAUFFER and PEPPAS, 1992).

The present study aimed to develop a controlled release system properly after repellent bioassay to assess the behavior of fleas *Ctenocephalides felis felis* in the presence of neem oil, which will be incorporated into the polymer matrix to form a device for use in dogs and cats.

2. MATERIALS AND METHODS

2.1. Material

Poly (vinyl alcohol) (PVA) Celvol (325 Mw = 85 000, degree of hydrolysis 98.4%), manioc starch Lorenz™, Aerosil™ R805, available from Evonik, Kollidon™ SR supplied by BASF, modified starch and maltodextrin RD545™ Globe A1920™ both provided by Corn Products.

2.1.1 Bioassay with fleas

2.1.1.1 The experimental site

The bioassay was performed at the Laboratory of Animal Parasitology, Center for Research and Development of Animal Health of the Biological Institute in Sao Paulo.

2.1.1.2 Collection of adult fleas

The fleas used in the bioassay were grown in the laboratory under artificial infestation of a cat breed allocated in a cage suspended over a bench.

Days prior to performing the bioassay bench and cage were wiped with a brush to harvest flea eggs with residues of dust and sand, placed in Petri dishes, apart from possible impurities in bottles and kept in a greenhouse with temperature 27 ± 1° C and relative humidity of 63% where it remained until the stage of adult fleas. (FIG. 1).

Figure 1 - obtaining adult fleas for bioassay. A) Containing on the workbench cage flea eggs. B) Material collected from the cage and bench. C) Oven for storing eggs. D) Storage Bottles egg to adulthood.

2.1.1.3 Evaluation of the action of neem oil on the behavior of *Ctenocephalides felis felis*

To evaluate the behavior of fleas in the presence of neem oil were used adult fleas with approximately three days after birth. In test tubes of 10 ml were placed strips of filter paper measuring 1,5 x 8 cm, and in ten tubes papers were impregnated with 20µL of mineral oil (control group) and other ten tubes papers were impregnated with 20µL of neem oil (treated group). After this period, the fleas were analyzed visually.

In each tube were added ten fleas and the tubes were sealed with rubber stoppers and allowed to rest in an air conditioned room with temperature of 27° C for a period of 24h. (FIG. 2).

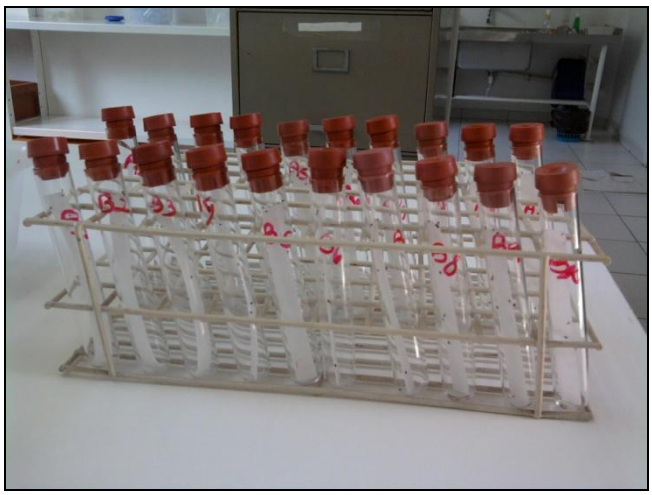

Figure 2 - tubes containing adult fleas in the presence of neem oil. Control group and treated group.

2.2 Preparation of devices

2.2.1 Preparation of PVA solution

The aqueous PVA was prepared in a concentration of 10% by autoclaving for 15 minutes.

2.2.2 Preparation of microcapsules

The microcapsules were made through a physical mixture of vegetable oil in cassava starch, Kollidon™ SR, Aerosil™ R805, Aerosil™ R972, modified starch RD545™ and maltodextrin Globe™ A1920.

2.2.3 Incorporation of microcapsules in PVA solution

After its formation, the microcapsules were mixed in 10% PVA solution prepared as described above.

2.2.4 Preparation of devices of PVA containing microcapsules

The prepared microcapsules were added to the PVA solution poured into molds and taken to the freezer at -20º C. Part of the samples were crosslinked for two hours of thermal cycles of freezing and thawing 2 hours and another part in a 12 hour cycles of freezing and thawing same period.

3. Results and discussion

3.1. Bioassay with fleas

Moments after the placement of fleas in the test tubes was not observed any adverse behavior of them so that the fleas remained on the filter paper impregnated with oil, it was realized then that this had no repellent effect.

Passing the period of 12 hours the fleas remained in tubes containing paper impregnated with neem oil was made a visual analysis. The criterion used to evaluate the death or insect life was motility. All fleas that presented a minimum of motility were considered alive. It was observed that part of fleas had died (TABLE 1). Thus, the dead fleas were counted and the result was 40% of mortality and the survivors had some behavioral changes such as reduction of the movements.

The evaluation of the effect of neem in *Ctenocephalides felis felis* in vitro was determined through statistical treatment of the Tukey test and confirmed by the value P.

Table 1 - Fleas mortality results in the in vitro neem oil repellency or insecticide bioassay

Control group (tubes)	Living	Dead	Total
1	0	10	10
2	0	10	10
3	0	10	10
4	0	9	9
5	0	11	11
6	1	9	10

Treated group (tubes)	Living	Dead	Total
1	4	8	12
2	5	5	10
3	1	9	10
4	3	7	10
5	5	5	10
6	9	1	10

7	0	10	10
8	0	11	11
9	0	10	10
10	0	10	10
Total	**1**	**100**	**101**

7	9	1	10
8	10	1	11
9	9	1	10
10	7	3	10
Total	**62**	**41**	**103**

3.2. Obtainment of devices with encapsulated oil

For cassava starch, Kollidon™ SR, RD545™ modified starch and maltodextrin, were necessary encapsulating agent 3 parts to 2 parts oil and the two types of Aerosil was necessary part of the encapsulating agent 0,5 to 2 parts oil.

The devices prepared by 12 hour cycles of freezing and thawing of 12 hours had sensory characteristics more suitable for the intended use.

4. CONCLUSIONS

The results of the bioassay leads us to conclude that the neem oil doesn't act as a repellent to *Ctenocephalides felis felis*, however, can be considered an insecticide with moderate efficacy, showing a mortality rate of 40% compared to control.

Therefore, the neem oil can be incorporated into polymeric matrix for use as collars or pendants in domestic animals, since besides the fleas tested present mortality rate of 40%, the survivors showed lethargic behavior, which facilitates their exclusion from the animal.

The encapsulation of the oil in various agents demonstrated compatibility of the PVA solution and the oil, allowing a good dispersion of microcapsules in the device.

The use of Aerosil allows use of smaller amount of encapsulating agent when compared to the other, however, is still necessary to release tests to verify the most suitable for use in question.

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