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Development Of Essential Oil Based Fipronil Nanoemulsions For The Treatment Of Ectoparasite Infestation In Animals.

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ABSTRACT

Fipronil is an effective drug to control fleas, ticks, biting lice and other mites in domestic animals. However, its use is limited due to its toxic effects on the animals. The objective of this research was to provide a fipronil nanoemulsion using citronella as an oil phase to improve its safety and efficacy. Citronella oil based fipronil nanoemulsions were prepared using tween 80 and PEG 400 as surfactants. The prepared nanoemulsion was characterized by the reported methods and the stability testing, and the drug release studies were also performed. The insecticidal activity of fipronil, citronella oil and the prepared nanoemulsion was performed by *in vitro* coated-vial bioassay. The irritation potential of the prepared nanoemulsions was also carried out. It was observed that encapsulation of fipronil and citronella into o/w nanoemulsion decreased the irritation potential of fipronil and citronella resulted in the development of a relatively safe formulation for the veterinary application. It has been concluded that the developed nanoemulsions demonstrated low irritation potential and enhanced insecticidal efficacy.

Keywords: Fipronil, Citronella, Nanoemulsion, Insecticidal Activity, Irritation Potential.

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INTRODUCTION

Infestation by ectoparasites including fleas, ticks, and lice causes considerable economic losses due to loss of productivity, mortality, and skin diseases in animals [1-3]. Ectoparasites cause a wide range of health problems in pet animals such as irritation, inflammation, hypersensitivity, mechanical tissue damage, weight loss, lameness, and in severe cases death of the infested animals [1-3]. Furthermore, ectoparasites are known to have zoonotic importance and be capable of transmitting several types of disease pathogens from animals to animals and from animals to human due to their blood-sucking habit [4].

Fipronil has been a leader on the ectoparasite market products for pets since 1994 [5, 6]. First available as 0.25% spray, it was then marketed as spot-on formulation. Fipronil, a second-generation phenylpyrazole insecticide, is an effective drug to control fleas, ticks, biting lice and other mites in domestic animals. It acts by inhibiting GABA receptors and has high selectivity for GABA receptor of insects as compared to that of animals making it a good insecticide for veterinary use. Fipronil has high efficacy; however, its use is limited by its toxic effects on the animals. Alopecia, irritation, dermatitis, pruritus, erythema, and cutaneous skin reactions appear to be the most common adverse drug effects reported for fipronil in dogs and cats [7]. Decreasing the dose or formulating a suitable formulation which prevents direct contact of fipronil to animal skin will improve the safety of fipronil.

Recently, the nanoemulsion strategy has emerged as a promising alternative for dermal formulations [8-10]. The oil phase in nanoemulsion serves multiple purposes. The oil phase not only acts as a drug carrier but also acts as a barrier to rapid drug release leading to the sustained release of the drug. Reports also suggested that essential oils, such as citronella oil, possess insecticidal or acaricidal efficacy in a variety of pests [11-13]. In addition to the ovicidal effect, these oils also reduce the occurrence of resistance. However, peculiar smell and short lasting action of these oils limit their use as an antiparasitic agent [14]. Few reports also suggest that dilution of these oils with water improves the insecticidal activity of these oils [15]. Based on the considerations mentioned above, the present research aimed to develop fipronil nanoemulsions using citronella as oil phase (FC nanoemulsions). Simultaneous use of fipronil with essential oil by formulating into a nanoemulsion formulation which has good dispersibility and penetrability due to nanosized globules will improve efficacy and safety of fipronil, and the developed FC nanoemulsion formulation will be a useful product for control of ectoparasites in animals.

MATERIALS AND METHODS

Materials

Fipronil was received from Parijat Industries India Pvt. Ltd. Different grades of the tween, span, PEG, propylene glycol, glycerine, and carbitol were obtained from CDH. Cremophor EL was purchased from Sigma, and the triacetin, carbitol, and labrafa were purchased from Gattefosse. All analytical grade solvents were purchased from the Merck.

Solubility study of Fipronil

Fipronil solubility in the citronella oil, various surfactants and co-surfactants was determined. A specified amount of fipronil was added to 2 mL of the medium in stoppered vials (5 mL), and mixing was carried out using a magnetic stirrer for about 10 minutes. The vial was kept in the mechanical bath shaker for about 72 hours at about 37 ± 0.5ºC. The sample was then centrifuged at 10000 rpm for 5 minutes; then the supernatant was separated, filtered and after the appropriate dilution with ethanol, solubility was determined by UV spectroscopic method at 287 nm.

Preparation of FC Nanoemulsions

Citronella oil based fipronil nanoemulsions (0.25%) were prepared using PEG 400 as co-surfactant and tween 80 as the surfactant. Briefly, 250 mg of fipronil was dissolved in 5.0 ml mixture of an appropriate concentration of citronella oil, tween 80 and PEG 400 (nanoemulsion oily mixture, NMO) under constant stirring at 500 rpm for 5 minutes. The NOM was then dispersed in up to 100 ml of the aqueous phase to form spontaneous nanoemulsion. The concentration of the surfactant, oil, and co-surfactant was optimized using pseudo-ternary phase diagrams.

Characterization of FC Nanoemulsion

Dispersion test: The formulation was diluted about 20 times with DW at about 37°C, with constant stirring at about 50 rpm and it was observed for formation of the stable nanoemulsions. The nanoemulsions obtained were observed visually for the phase clarity and the self-emulsification time.

Droplet Size and Size Distribution: It was obtained by the Zetasizer (Nano ZS, Malvern Instruments, UK). The light scattering of the nanoemulsion was monitored at about 25°C at about 90° angle. Each value was the average of the three measurements. The polydispersity index (PI) was obtained as the ratio of the standard deviation to the mean droplet size of the prepared formulation.

Transmission Electron Microscopy (TEM): It was done using TEM (Morgagni 268D SEI, USA) operating at 200 KV. Nanoemulsions were negatively stained using 1% uranyl acetate dye, dropped on the holey film grid, dried and observed using increasing magnification bright field imaging by TEM. Data acquisition was made on the AMT Image Capture Engine.

Viscosity and pH: It was performed by Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) using spindle # CPE40 at about 25 ± 0.5 °C. Average and standard deviation (SD) of the three data of the single point viscosity at a shear rate of 120.0 s^{-1} were reported. The pH of the formulation was determined by a pH meter (AccumentAB 15, Fisher Scientific, U.S.A.) in triplicate at about 25±1ºC.

Spray pattern: Spray pattern of FC nanoemulsion was compared to marketed fipronil spray (Bugnix Spray, Parijat Industries, New Delhi, India). To determine the spray pattern, both the samples were filled in separate spray bottles to which very minute quantity of rhodamine B dye was added. The solutions were then sprayed by pressing the valve once, ensuring that they deliver an equal quantity of liquid over a piece of paper. Spray area was then marked and diameter calculated to determine spray area.

Thermodynamic Stability of FC Nanoemulsions: To determine the thermodynamic stability of the nanoemulsion, the clarity, the phase separation, the droplet size, and the drug content was evaluated, before and after subjecting to the following stress tests:

• Heating-cooling cycle: Nanoemulsions were subjected to 6 cycles between the refrigerator temperature (about 4°C) and at about 45°C (storage not less than 48 hours at each temperature). The stable nanoemulsions were then subjected to the centrifugation test.
• Centrifugation: Nanoemulsions were centrifuged at about 3500 rpm for about 30 minutes and those nanoemulsions that did not show any phase separation, were selected for the freeze-thaw stress test.
• Freeze-thaw cycle: Nanoemulsions were subjected to three freeze-thaw cycles between about 21°C and about +25 °C (storage not less than 48 hours at each temperature).

Drug Release from FC Nanoemulsion: Release profile of fipronil from FC nanoemulsion was studied using in vitro dialysis method. Briefly, FC nanoemulsion was filled in a dialysis bag (pore size 12kD, Sigma, India) was suspended in 500 ml of dissolution medium (water containing 10% ethanol). The releases of fipronil from the FC nanoemulsion to dissolution medium was carried out at about 50 rpm at room temperature for up to 12 hours.

The aliquots were taken at different time intervals, replaced with the fresh medium and were centrifuged to obtain clear supernatants, which were then analyzed using an UV method.

Irritation Potential of FC Nanoemulsion: The fertile hen's eggs were incubated at about 37±0.5°C and about 40±5% relative humidity for about ten days. The eggs were turned each day during the incubation period but were left in a horizontal position for about 10 minutes to assure the proper position of the embryo. On the 10th day of the incubation, a portion of the egg-shell above the air space was removed, and the CAM was exposed carefully using forceps for the application of the formulation. Eggs which did not show live embryo and intact yolk were discarded. The nanoemulsion was tested by gently placing 200 µL onto the CAM. Fipronil solution (0.25%) and citronella oil were tested to determine the irritant effect of the drug and the oil. Sodium hydroxide (0.1 M) and normal saline were used as +ve and –ve controls, respectively. Ten-minute post application, the blood vessels, and the capillary system were analyzed for the irritant effects of hemorrhage, clotting, hyperaemia, and/or coagulation. The sum of numerical scores for all three responses provided a single numerical value indicating the irritation potential of the nanoemulsion.

Insecticidal activity of FC Nanoemulsion: The insecticidal activity of fipronil, citronella oil and FC nanoemulsion was evaluated by the *in vitro* coated-vial bioassay. The desired titration ranges were prepared by suitable dilution of stock solutions with distilled water. Microcentrifuge vials were separately treated with different concentrations of fipronil, citronella oil and FC nanoemulsion. Insecticidal solutions were drained off from the vials and vials were allowed to dry for four h. Solvent only and untreated vials served as control. Ten newly emerged (0 to about seven days old) unfed adult fleas were added to every vial. Vials were capped, and the flea susceptibility was checked at 48 hours post-exposure by evaluating the mortality. Those showing the normal movement and/or the jumping ability were considered live, and those showing no movement after tapping in the vials were considered as dead. The EC_{50} and the combination index (CI) values were calculated to determine the potential for synergistic activity. The CI was calculated as follows:

$$\mathrm{CI} = \frac{\mathrm{EC}_{50} \text{ of fipronil nanoemulsion}}{\mathrm{EC}_{50} \text{ of fipronil alone}} + \frac{\mathrm{EC}_{50} \text{ of fipronil nanoemulsion}}{\mathrm{EC}_{50} \text{ of citronella oil alone}}$$

A CI value of approximately 1 indicated that the efficacy of the compounds was simply additive, a CI < 1 was interpreted as synergistic and a CI > 1 as antagonistic.

RESULTS AND DISCUSSION

Development of FC Nanoemulsion

The Fipronil is an insecticide. However, its use is limited due to the dose-dependent dermal toxicity and adverse drug effects. Recent studies suggested that essential oils such as citronella oil possess good insecticidal properties. They not only had an ovicidal effect but also reduces the occurrence of resistance. However, peculiar smell and short lasting action of these oils limit their use. Therefore, the objective of this study was to formulate citronella oil-based fipronil nanoemulsion formulation. The proposed nanoemulsion would overcome the limitations of both fipronil and citronella oil and would result in a formulation with enhanced efficacy. The solubility of fipronil in citronella oil was conducted as it is decisive in the selection of appropriate surfactant(s) and co-surfactant(s) for fipronil nanoemulsion. The solubility of fipronil in citronella oil was found to be 38.2±0.8 mg/ml (Fig. 1). Solubility studies revealed that 6.5 ml of citronella oil must emulsify in 100 ml of water to obtain the required dose (0.25%) of fipronil. The high solubility of fipronil was also observed in PEG 400 (58.7±1.4 mg/ml) and glycerine (51.9±2.0 mg/ml), and thus they were selected as co-surfactants for the present study (Fig. 1). Further, selection of surfactants was made by their emulsification efficiency. The emulsification studies indicated that the cremophor EL and the tween-80 had a good ability to emulsify the citronella oil. Thus, the cremophor EL and the tween-80 were selected as the surfactants and the PEG 400 and glycerine were selected as co-surfactants for further investigation.

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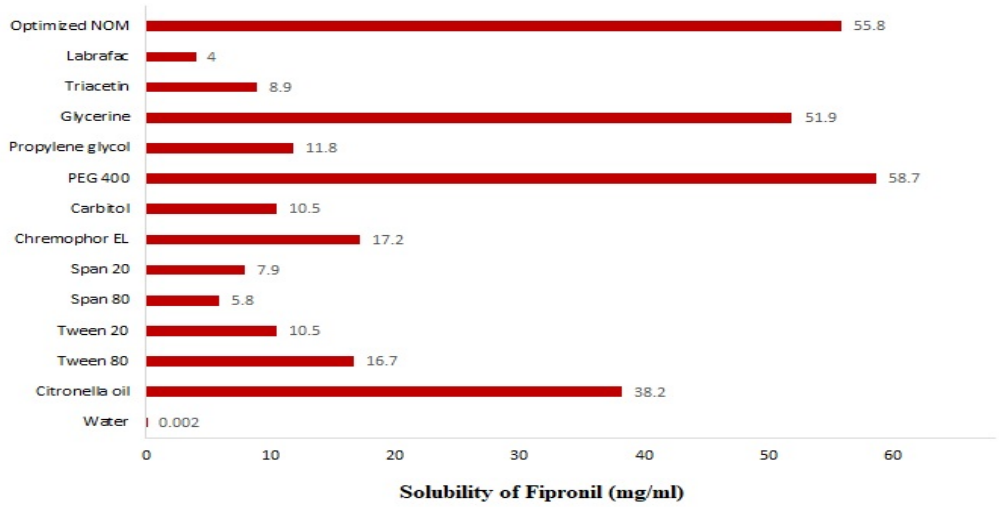

Figure 1: Solubility of fipronil in various solvents

Results of pseudo-ternary phase diagram study are shown in Fig. 2. The mixture (oil phase, surfactants and co-surfactant) (1 ml, NOM) were diluted with distilled water (20.0 ml), and regions of nanoemulsion appeared as transparent dispersions were identified (purple regions). The remaining region on the phase diagram represents the turbid and conventional emulsions based on the visual observation. Due to the larger nanoemulsion region and the greater capacity for incorporation of oil phase, a citronella oil, tween 80 and PEG 400 system was selected. The optimized NOM was citronella oil, tween 80 and PEG 400 in a ratio of 2:5:3. The solubility of fipronil in optimized NOM was found to be 55.8±1.0 mg/ml. The prepared NOM was colorless and transparent. Upon dilution of 1.0 ml of NOM up to 20 ml with distilled water, a fine bluish-white nanoemulsion was formed. The dispersion results suggested that FC nanoemulsion with 0.25% fipronil could be formed by the optimized NOM and its dispersion in water. FC nanoemulsion had a pH value of 6.8, favorable for topical application.

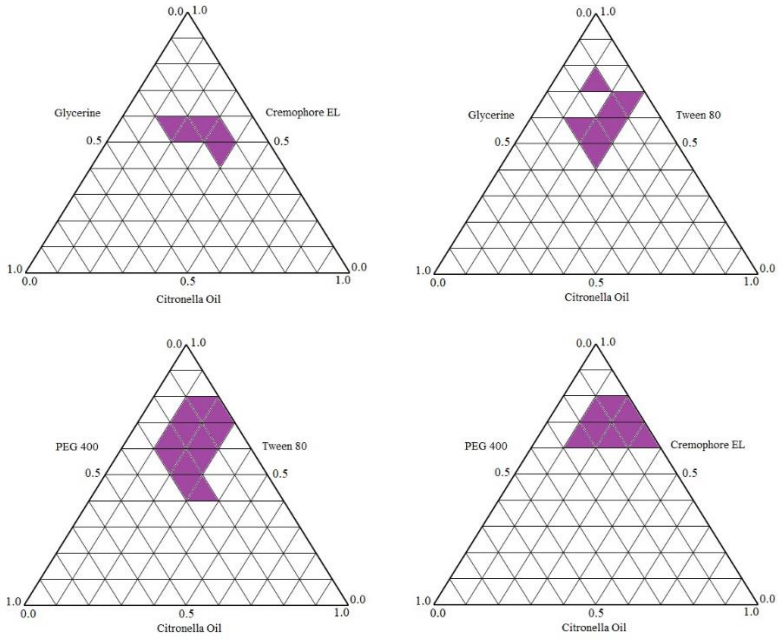

Figure 2: Pseudo-ternary phase diagram for nanoemulsion preparation

Size and Morphology of FC Nanoemulsion

Droplet size and the size distribution is a critical parameter while developing a nanoemulsion system. It is well reported that the droplet size has an important effect on drug penetration and the smaller sized nanoemulsion has shown better drug penetration properties owing to the larger interfacial surface area. Results of DLS study revealed mean droplet size of the optimized FC nanoemulsion (5.0 ml NOM diluted up to 100 ml (v/v) with water) was in the nano-size range (78.5±8.4 nm). TEM results (~80 nm) are in agreement with the results of DLS analysis. Further, nanoemulsion droplets were observed to be spherical as can be seen in the TEM image (Fig. 3). Values of PDI (the measure of the uniformity of the droplet size within the formulation) were also calculated. The prepared nanoemulsion exhibited a narrow size distribution as is evident by the low PDI value of 0.14.

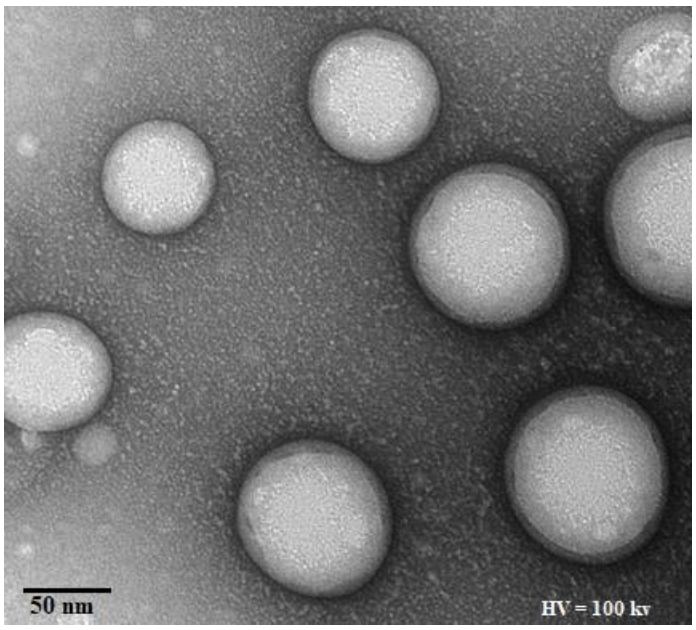

Figure 3: TEM image of FC nanoemulsion

The viscosity of FC Nanoemulsion

The viscosity of FC nanoemulsion was determined to observe whether the formed nanoemulsion could be sprayed or not. The viscosity of the FC nanoemulsion was low (32.5 ± 2.4 mP), as per the expectations for the o/w emulsion. The lower viscosity of the FC nanoemulsion is probably due to the low amount (20-fold less compared to water) of NOM in the final formulation, thus having very little impact on viscosity. The low viscosity of FC nanoemulsion allows it to be sprayed easily and effectively.

The spray pattern of FC Nanoemulsion

The spray pattern of FC nanoemulsion was compared to Bugnix spray. No difference in the spray pattern was observed between Bugnix and FC nanoemulsion (Fig. 4). Nevertheless, FC nanoemulsion spray demonstrated higher spray area (38.47 cm^2) compared to Bugnix (33.17 cm^2). The higher area observed for FC nanoemulsion could be due to its better spreadability owing to the presence of oil. From the results, it could be interpreted that FC nanoemulsion requires less application dose and would possibly be more effective.

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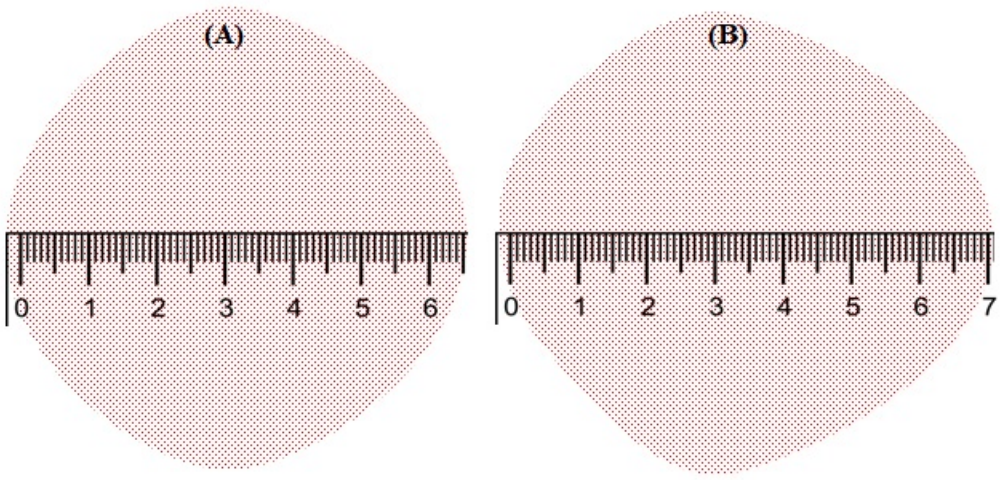

Figure 4: Spray pattern of (A) Marketed spray and (B) FC nanoemulsion

Thermodynamic stability of FC Nanoemulsion

Stress test, including the heating-cooling cycle test, the centrifugation test, and the freeze-thaw cycles tests showed that the prepared nanoemulsion had a good level of physical stability. After about three months, fipronil was found to be stable with a recovery of more than 98%. No appreciable change in the mean droplet size was seen during three months. Therefore, it can be concluded that the prepared nanoemulsion was thermodynamically stable.

Drug release from FC Nanoemulsion

The release profile of fipronil in vitro from FC nanoemulsion is shown in Fig. 5. Results showed that nanoemulsion resulted in the sustained release of fipronil for 12 h with the release of about 32.5% drug at one h, followed by 51.0%, 70.8% and 86.4% and 98.9% release at 2, 4, 8 and 12 h, respectively.

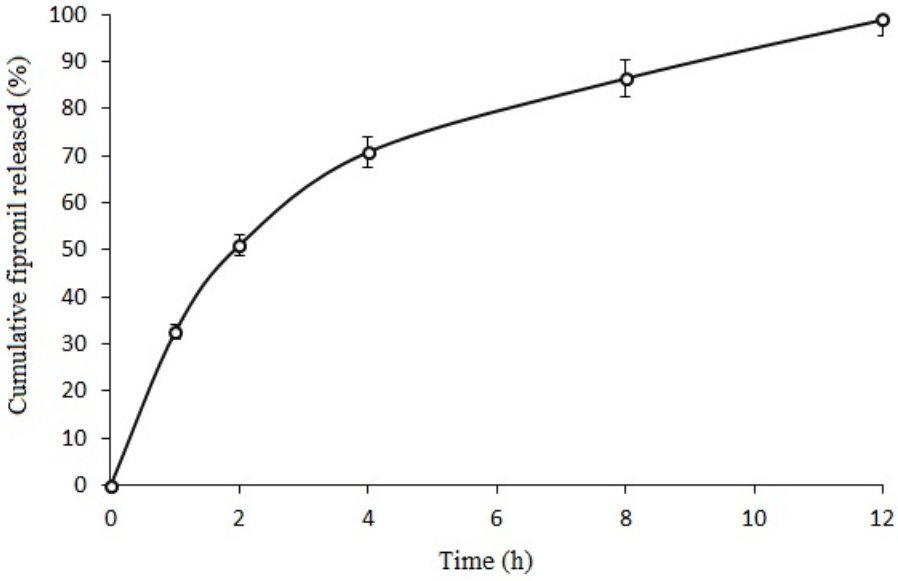

Figure 5: Release profile of fipronil from FC nanoemulsion

Irritation Potential of FC Nanoemulsion

The irritation potential of the FC nanoemulsion was evaluated by *in vitro* HET-CAM studies. The saline solution (negative control), displayed no signs of the vascular response classifying them as practically non-irritant (Score 0; N = 4) (Fig. 6). All the three signs of the vascular response were observed after application of 0.1 M NaOH (positive control), grading it a very strong irritant (Score > 9.0; n = 4). Similar result was also observed for fipronil solution and citronella oil. Interestingly, FC nanoemulsion has a score of 2.6 (n=4), grading it as slightly irritant. From the result, it is evident that encapsulation of fipronil and citronella into o/w nanoemulsion decreases the irritation potential of fipronil and citronella resulted in the development of a relatively safe formulation for the veterinary application.

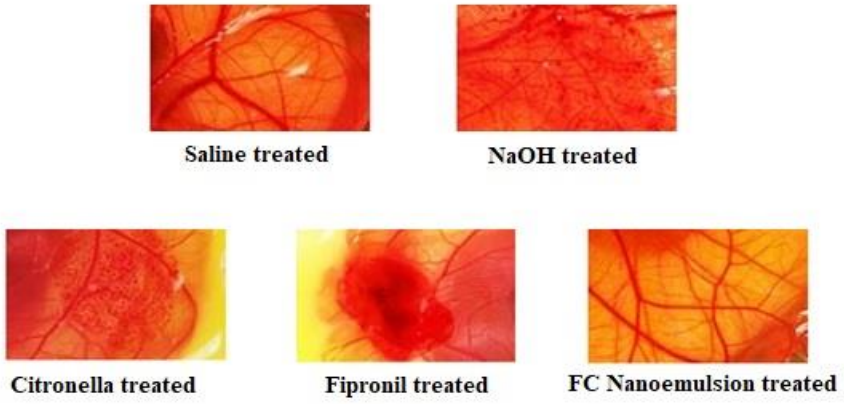

Figure 6: HET CAM images of control and test samples

Insecticidal activity of FC Nanoemulsion

There was no mortality in the solvent and the untreated vials. Therefore, there was no need to perform the mortality correction to calculate the EC_{50} values. Results showed that the activity against *C. felis* was dose-dependent for fipronil as well as for the citronella. Fipronil (EC_{50} = 5.2 ppm) displayed more potent than citronella oil (EC_{50} = 100 ppm) against the fleas. It was observed that the FC nanoemulsion was more effective against the fleas than either compound alone, with a CI of 0.42, indicating a very strong synergistic effect. The FC nanoemulsion significantly shifted the dose-response curve to the left and also significantly reduced the IC_{50} values of the fipronil and the citronella.

CONCLUSION

The fipronil-citronella nanoemulsion was successfully developed by spontaneous emulsification. The developed FC nanoemulsions demonstrated low irritation potential and enhanced insecticidal efficacy.

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REFERENCES

[1] Clark, N.J., Seddon, J.M., Šlapeta, J., Wells, K. Parasite spread at the domestic animal - wildlife interface: anthropogenic habitat use, phylogeny and body mass drive risk of cat and dog flea (Ctenocephalides spp.) infestation in wild mammals, Parasit Vectors, 2018, 11(1), 8. doi: 10.1186/s13071-017-2564-z.

[2] Dobler, G., Pfeffer, M., Fleas as parasites of the family Canidae. Parasites Vectors, 2011, 4, 139. doi: 10.1186/1756-3305-4-139.

[3] Blagburn, B.L., Dryden, M.W., Biology, treatment, and control of flea and tick infestations. Vet. Clin. North. Am. Small. Anim. Pract., 2009, 39, 1173-1200.
[4] Linardi, P.M., Fleas and diseases. In: Marcondes, C.B., editor. Arthropod Borne Diseases. Springer International; Cham, Switzerland: 2017. pp. 517-536.
[5] Tomlin, C.D.S., The pesticide manual. 12th Ed., The British Crop prot Council, Surrey, UK, 2000, 413-415.
[6] Cole, L.M., Nicholson, R.A., Casida, J.E. Action of phenylpyrazole insecticides at the GABA-gated chloride channel, Pestic. Biochem. Physiol. 1993, 46(1), 47–54.
[7] Tingle, C.C., Rother, J.A., Dewhurst, C.F., Lauer, S., King, W.J. Fipronil: environmental fate, ecotoxicology, and human health concerns. Rev. Environ. Contam. Toxicol., 2003, 176, 1-66.
[8] Iqbal, B., Ali, J., Baboota, S. Recent advances and development in epidermal and dermal drug deposition enhancement technology. Int. J. Dermatol., 2018, 57(6), 646-660.
[9] Rai, V.K., Mishra, N., Yadav, K.S., Yadav, N.P. Nanoemulsion as pharmaceutical carrier for dermal and transdermal drug delivery: Formulation development, stability issues, basic considerations and applications. J. Control. Release, 2018, 270, 203-225.
[10] Guan, W., Tang, L., Wang, Y., Cui, H. Fabrication of an Effective Avermectin Nanoemulsion Using a Cleavable Succinic Ester Emulsifier. J. Agric. Food. Chem., 2018, 66(29), 7568-7576.
[11] Castillo, R.M., Stashenko, E., Duque, J.E. Insecticidal and Repellent Activity of Several Plant-Derived Essential Oils Against Aedes aegypti. J. Am. Mosq. Control Assoc., 2017, 33(1), 25-35.
[12] Mishra, P., Tyagi, B.K., Chandrasekaran, N., Mukherjee, A. Biological nanopesticides: a greener approach towards the mosquito vector control. Environ. Sci. Pollut. Res. Int., 2018, 25(11), 10151-10163.
[13] Sakulku, U., Nuchuchua, O., Uawongyart, N., Puttipipatkhachorn, S., Soottitantawat, A., Ruktanonchai, U. Characterization and mosquito repellent activity of citronella oil nanoemulsion. Int. J. Pharm., 2009, 372(1-2), 105-111.
[14] Maia, M.F., Moore, S.J. Plant-based insect repellents: a review of their efficacy, development and testing. Malar. J., 2011, 10(Suppl 1), S11.
[15] Agrawal, N., Maddikeri, G.L., Pandit, A.B. Sustained release formulations of citronella oil nanoemulsion using cavitational techniques. Ultrason. Sonochem., 2017, 36, 367-374.