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Histological alterations in the salivary gland of semi engorged females of *Ornithodoros savignyi* (Acari: Argasidae) induced by Eucalyptus oil nanoemulsion

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Abstract

The present study aimed to demonstrate the effects of eucalyptus oil nanoemulsion on the *Ornithodoros savignyi* semi engorged females. For this purpose, the semi engorged females of *O. savignyi* were exposed to eight serial dilutions of oil nanoemulsion (5, 10, 15, 20, 25, 30, 35, and 40%) using a “dipping method” in vitro. The semi engorged ticks were immersed in different dilutions (5 replicates per dilution) for 2 min then each replicate was incubated in separate vial at 27°C±1, 75% relative humidity. The mortality percentage for adult ticks exposed to different dilutions of eucalyptus oil nanoemulsion showed a concentration-dependent effect. It was however not significant only for the 5% when compared to the non-treated ticks ($P < 0.05$). While, it was significant for other dilutions ($P < 0.05$). The highest mortality was reported for ticks treated with 40%. The LC₅₀ obtained from bioassay test was 25%. Salivary glands were dissected and evaluated through morphological techniques using light and scanning microscopy. Results showed that eucalyptus oil nanoemulsion is an effective substance that acts morpho-physiologically on the tick glandular tissue, causing changes in the acini shape, vacuolation in acinar cells, and disruption of the tissue by cell death process with subsequent formation of apoptotic bodies, thus preventing the accurate identification of different types of acini. These results confirmed the potential of eucalyptus oil nanoemulsion as an alternative method for controlling *Ornithodoros savignyi* ticks, instead of synthetic acaricides.

Keywords: *Ornithodoros savignyi*; salivary gland; eucalyptus oil nanoemulsion

1. Introduction

The sand tampan (*Ornithodoros savignyi*) is grouped within the family Argasidae (soft ticks). They occur all over the northwestern parts of South Africa and is also found in the semi-desert areas of both North and East Africa, India, the Middle East and Ceylon [1]. Tampans were found to cause the death of domestic animals through excessive blood-loss in addition to the toxic component in the tick salivary gland secretions [2]. According to Moorhouse and Tatchell [3] saliva produced by the salivary glands is considered the primary agent through which the micro-organisms enter the host's bloodstream.

Salivary gland structure and biology of ticks have been described in several reviews [4-8]. The histological, histochemical and ultrastructural studies of argasid salivary glands showed that at least three granular cell types existed [9, 10]. The granules of these cell types varied in electron density and submicroscopic appearance. Cell types ‘a’ and ‘b’ contain granules of variable electron density, while cell type ‘c’ contains granules with an electron compact core. Ultrastructural studies on *Ornithodoros moubata* showed similar granule types [11]. The description of El Shoura [11] will be used in this study, as *O. moubata* is the relatively closest to *Ornithodoros savignyi* and no equivalent have been found for the ‘c’ cell granules of *O. moubata* in Argas ticks.

Chemical acaricides have been broadly used and still the main method to control ticks. However, the development of tick resistance to the different active ingredients of these chemicals is mainly due to the unwise use of chemicals [12]. Furthermore, the products are of high cost, chemical residues in the environment; contamination of the soils, rivers and animals, all affecting human health [13-17]. The use of bio acaricides, such as plant-borne products containing acaricidal compounds, have been suggested by Castrejon [18] and Pavela *et al.* [19], as

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an alternative to eliminate the environmental and economic impacts of synthetic acaricides.

Biopesticides as essential oils (EO), appear to be a balancing method for integrated pest management [20-23]. EO contain mixtures of bioactive compounds, such as alcohols, aldehydes, ketones, esters, aromatic phenols, and lactones also monoterpenes and sesquiterpenes [24]. Despite EO promising properties, difficulties related with EO volatility, their poor solubility in water, and tendency to oxidation should be solved before they can be used as an alternative in pest control system [25].

Nanoformulation of the EO may resolve these problems, protecting EO from degradation and losses by evaporation, achieving a controlled release of EO and easy handling [26]. A nano insecticide is a formulation that designedly consists of elements that are in a nanometer size range and gain new characteristics related with this small size range [27]. Some advantages of nanoformulations are: improvement in the efficiency due to increasing the exposed surface area, higher solubility in water, inducement of systemic activity due to smaller particle size, higher mobility and lower toxicity due to the elimination of organic solvents compared to conventional pesticides, and their formulations [27, 28]. Nanotechnology was utilized to develop new nanopesticides hires nanoparticles (NP) which claim one or more dimensions in the order 10 – 1,000 nm [29].

Therefore, this study gets benefit from the current knowledge about herbal preparations and nanoparticles to control the soft tick, *Ornithodoros savignyi*. Here we focused on the effect of eucalyptus oil nanoemulsion on the morphology and histology of salivary gland tissue of semi engorged female *Ornithodoros savignyi*.

2. Materials and Methods

2.1 Ticks rearing

The soft tick, *Ornithodoros savignyi* (Audouin, Argasidae), were collected from sand near cattle rearing places in Dahshore, Giza governorate, Egypt. To establish a laboratory colony, ticks were maintained at 27 ± 1 °C, 75% RH and 16 hrs day light. The ticks were held in transparent polyethylene tubes (25 × 100 or 13 × 100 mm) which were sealed at one end by a mixture of gypsum and graphite at a ratio of 1:5. The tubes were covered at the other end with muslin cloth securely held by rubber bands [30]. Ticks colony was maintained in an insectary provided by Department of Entomology, Ain Shams University. New Zealand white rabbits from the commercial breeder in Cairo were kept in an animal room provided by Research and Training Center on Vectors of Diseases (RTC), for feeding the ticks. The basic principle for animal experimentation is considered and the study is organized and operated according to the CIOMS (Council for International Organizations of Medical Sciences) and ICLAS (International Council for Laboratory Animal Science).

2.2 Preparation of eucalyptus oil nanoemulsion

The nanoemulsion was formulated using eucalyptus oil, water and non-ionic surfactant (tween 80). At first, coarse emulsion was set up by adding water to organic phase containing oil and surfactant using a magnetic stirrer, which was then subjected to 20 kHz Sonicator for ultrasonic emulsification [31]. Energy input was given through sonotrode containing a piezoelectric crystal with a probe diameter of 13mm. Coarse emulsion is converted to nanoemulsion by the sonicator probe which generates disruptive forces that reduce the droplet

diameter to form the stock solution. Different concentrations were prepared from the stock solution

2.3 Characterization of eucalyptus oil nanoemulsion

The nanoemulsion formulation was centrifuged at 3000 rpm for 30 min and observed for phase separation. Samples were further analyzed.

a) Heating-cooling cycle

The formulated nanoemulsion was kept between 40 °C and 4 °C, alternating each temperature for 48h. The cycle was repeated three times. The stability of nanoemulsion at varying temperature was checked.

b) Freeze-thaw stress

The formulated nanoemulsion was kept between - 21°C and 25 °C for 48h alternatively at each temperature. This cycle was repeated two times. The formulations which passed the thermodynamic stress tests were chosen for additional characterization studies.

2.3.1 Droplet size distribution and polydispersity index

The droplet size distribution and polydispersity index of eucalyptus oil nanoemulsion formulation was determined using particle size analyzer. Polydispersity index values below 0.2 indicate a narrow size distribution and thus ensure long-term stability of the formulated nanoemulsion.

2.3.2 Morphology of emulsion droplets

The shape and morphology of the formulated eucalyptus oil nanoemulsions were visualized using Transmission electron microscopy (TEM). A drop of the emulsion was stained negatively with phosphotungstic acid and was positioned on a copper grid. The TEM micrographs were acquired using a transmission electron microscope with a tungsten source and operating at 80kV.

2.4 Bioassay test

Semi engorged females of *Ornithodoros savignyi* were subjected to different eucalyptus oil nanoemulsion concentrations through adult immersion test. Briefly, the semi engorged females were washed in a small sieve with tap water then were dried on soft absorbent paper. After that, 200 females were divided into eight groups (25 females each). They were immersed in Petri dishes containing different concentrations (10, 15, 20, 25, 30, 35, and 40%) of eucalyptus oil nanoemulsion for 2 min (each concentration had five replicates). The control group was also consisted of 25 semi engorged females that had been dipped in distilled water only for 2 min. Ticks were dried in the absorbent paper then were placed in an incubator at 27 °C and 75% relative humidity. The observations of oils effects on tick mortality were recorded 24 h post-application. Death of ticks was confirmed by observing loss of mobility. The number of dead females was recorded and the percentage of mortality of females was estimated in the average of the five replicates. Mortality data was used to estimate probit regression line and calculate LC₅₀.

2.5 Scanning Electron Microscopy

The salivary glands of semi- engorged females of *Ornithodoros savignyi* from normal and treated group were removed and fixed at 3% glutaraldehyde for 24h and dehydrated in a graded 70-100% acetone series. The material was processed by critical point drying, sputtered with gold and examined by Jeol100cx at 25KV.

2.6 Histological study

Semi-engorged females from normal and treated groups were dissected on Petri dishes containing phosphate buffered saline-PBS (NaCl 0.13M, Na₂HPO₄ 0.017M, KH₂PO₄ 0.02M, PH 7.2), and salivary glands removed immersed in 3% glutaraldehyde then transferred to initial fixative. They were fixed overnight and given 3 brief rinses in a sucrose-cacodylate buffer (pH 7.2); post fixed 1 hr in 1% osmium tetroxide in 0.1M cacodylate buffer, rinsed 3 times in distilled water, and dehydrated through a graded series of ethanol. Then specimens were infiltrated with Spurr's epoxy resin in a graded series of absolute alcohol-Spurr's resin mixtures, then embedded in freshly prepared Spurr's resin at 70°C for 72 hrs. Semithin sections 5-6µm were stained with toluidine blue. Specimens were examined and photographed by equipped Leica light microscope with a camera using objective lens 40X with 50 µm scales.

2.7 Statistical Analysis

The obtained data were subjected to analysis of variance (ANOVA) followed by post-hoc analysis (Tukey's HSD test) with the help of SPSS version19 for Windows, in which the equation of the standard deviation, standard errors, and probabilities (p) were used. The level of significance was expressed as significant ($P < 0.05$) and non – significant ($p > 0.05$).

3. Results

3.1 Characterization of eucalyptus oil nanoemulsion

The formulated nanoemulsion was prepared using eucalyptus oil and Tween 80 (organic phase) and water (aqueous phase). The formulated nanoemulsion was noticed to be stable after being exposed to centrifugation for 30 minutes at 3,000 rpm. Also, it was more stable when stored at different temperatures (4 °C, -21 °C, 25 °C and 40 °C). The average diameter of nanoemulsions is ranged from 10 to 12.2 nm. The polydispersity index of the nanoemulsions is 0.060 which indicates that it is uniform. The particles are presented in a transmission electron microscope image. According to TEM evaluation, the average particle size is 10.63 ± 0.53 nm and the droplets are spherical in shape (Figure 1).

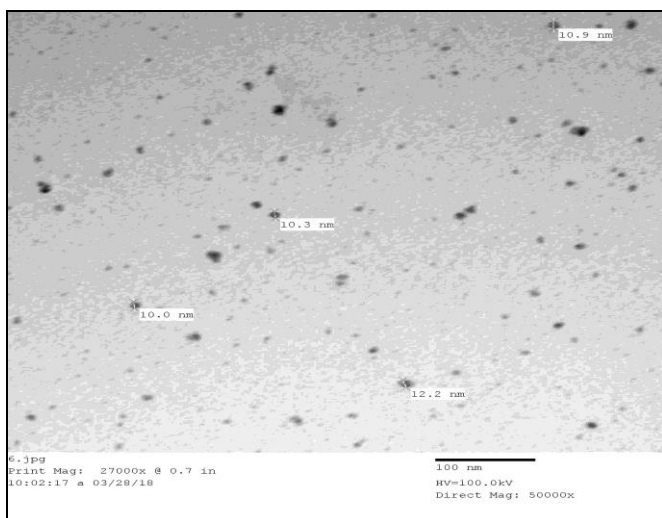


Fig 1: Transmission electronic microscopy image of eucalyptus oil nanoemulsion

3.2 Bioassay test

The results of the bioassay experiment show no mortality and no changes in behaviour of *Ornithodoros savignyi* adult females of the control group. However, at the concentration of 5%, mortality is observed soon after the onset of treatment of individuals. At the concentrations from 10 to 40, a progressive increase in mortality is observed throughout the observation period as shown in Table 1.

Table 1: Percent mortality of *Ornithodoros savignyi* adult females exposed to different concentrations of eucalyptus oil nanoemulsion.

Eucalyptus oil nanoemulsion%	Average Mortality% ± SE
Control (water)	0 ± 0 ^a
5	4 ± 4 ^a
10	12 ± 4.89 ^b
15	20 ± 0 ^c
20	28 ± 4.89 ^d
25	36 ± 8 ^d
30	52 ± 4.89 ^e
35	76 ± 7.48 ^f
40	96 ± 4 ^g

*Data are presented as mean ± SE

*Means bearing different letters within column are significantly different ($P < 0.05$) ANOVA, Tukey's HSD test.

These data were then subjected to Probit analysis, a mathematical model that determined that the LC₅₀ of eucalyptus oil nanoemulsion for semi-engorged females of the *Ornithodoros savignyi* is 24.966% ~ 25%.

Table 2: Results obtained by Probit analysis—Bioassay test.

LC ₅₀ : 24.966% limits = 18.5913 - 35.4991%
Slope: 3.49
Chi-value: calculated= 15.0457 tabulated= 12.6
r-value: calculated = 0.8914 tabulated = 0.707

3.3 Scanning Electron Microscopy (SEM)

The salivary glands of *Ornithodoros savignyi* consist of a pair of grape-like clusters of acini (alveoli), surrounded by a myo-epithelial sheath (Figure 2A). They comprise 2 major types of acini the agranular and granular acini. The salivary glands surround a pair of salivary ducts, extending along the basis capituli and opening into the salivarium. Away from this main structure, intermediate or secondary branches of smaller size and diameter ducts, canaliculi or acinar ducts, which are distributed and extended along the length of the gland. Saliva flows through these ducts into the host's bloodstream allowing successful feeding (Figure 2A,C). In female *Ornithodoros savignyi* treated with LC₅₀ of 25% eucalyptus oil nanoemulsion, salivary glands lost their regular shape and compatibility with myo-epithelial sheath. Acini became smaller in size, loose and irregular, great parts of acini were detached from their sheath, in other parts acini and acini sheath were ruptured making clear holes in the epithelial sheath and some parts of the gland completely disappeared or deleted leaving an empty sac-like structure. Salivary ducts become narrower and showed gradual decrease in diameter along the same duct, some ducts appeared with no distinct ends, others were also disappeared. (Figure 2D,F).

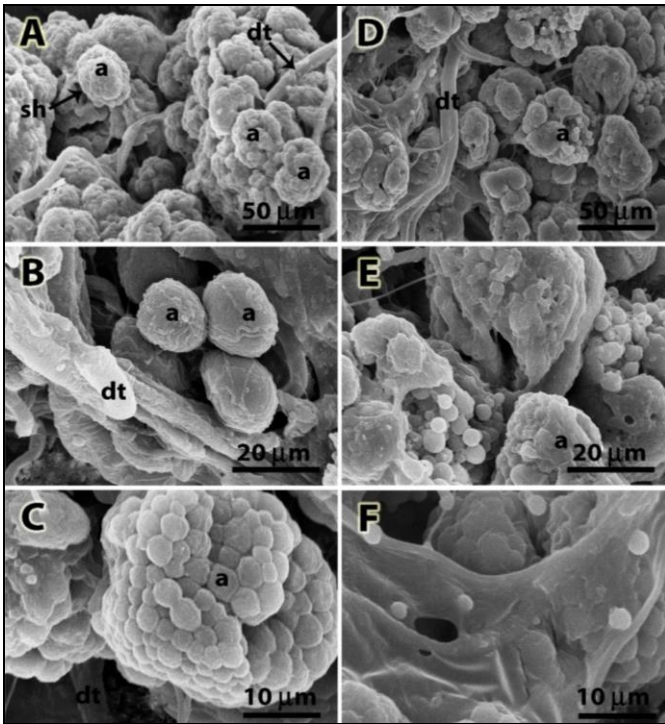


Fig 2: Scanning Electron Microscopy (SEM) of salivary glands of *Ornithodoros savignyi* semi-engorged female of the control group (A, B and C) & treated group exposed to eucalyptus oil nanoemulsion (D, E and F). A. General view showing acini (a), duct system (dt) and myo-epithelial sheath (sh). B. magnification of duct system (dt) and glandular acini (a) showing wrinkled surface of acini. C. magnification of single acini (a). D. General view of the irregular shape of acini (a) and ducts (dt). E. details of irregular acini (a) with loss of turgidity or completely shriveled (shrunken) and irregular in shape and size. Note that acini seem to have withered, thus modifying their initial morphology. F. myo-epithelial sheath (sh) surrounds soft tick salivary glands is ruptured and individual acini (a) can be observed where the sheath is torn. Bars: A, D= 50µm; B, E= 20µm; C, F= 10µm.

3.4 Histology

Histological examination of salivary glands in control group revealed that it consists of two types of acini: Acini I, which are agranular, with large central cell and strongly stained nucleus (Figure 3B). Acini II (granular) are oval to spherical and are formed of a, b and c cells (Figure 3A, C). Cell type 'a' contains dense core granules these cells are released during feeding. Type 'b' cells contain homogenous granules. Type 'c' cells contain smaller granules. The different cell types of salivary gland acini (I, II) of *Ornithodoros savignyi* semi-engorged females were already described by El shoura^[11] in *Ornithodoros moubata*. In the present study, individuals of the control group were used only as reference to demonstrate the changes caused in the salivary glands by tested compound.

Female ticks exposed to LC50 of 25% eucalyptus oil nanoemulsion, showed acini with great histological alterations. Acini I showed dilated lumen (Figure 3E). Acini II showed irregular shape of granular secretion, change in outline of the acini, intense vacuolation, acini identification still impossible (indeterminate) (Figure 3D, F). Secretion granules were rarely observed in indeterminate acini cells or have irregular shape (Figure 3D, F). Numerous apoptotic bodies is formed due to degeneration process in salivary glands (Figure 3D).

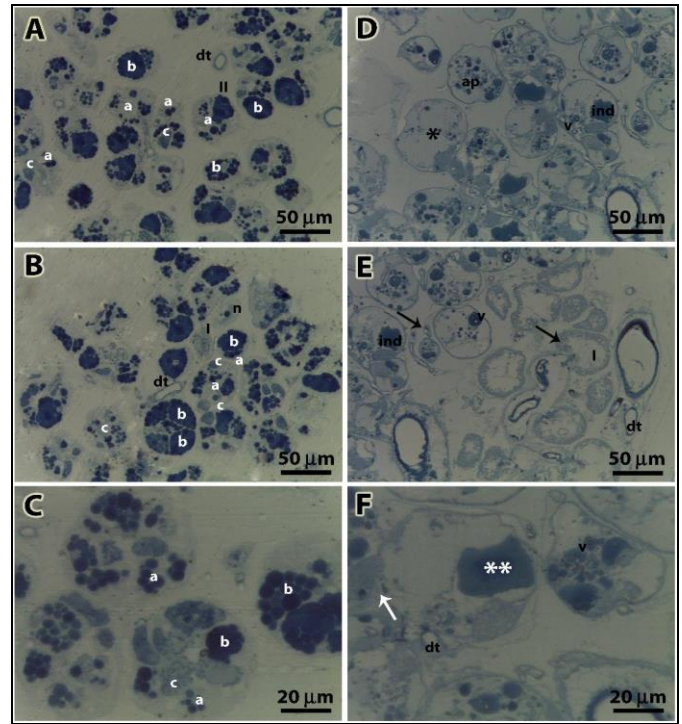


Fig 3: Histological sections of salivary glands of *Ornithodoros savignyi* semi-engorged female of the control group (A, B and C) showing acini I and acini II. dt (duct), n (nucleus), a (a cell) b (b cell), c (c cell) & treated group exposed to 25% of eucalyptus oil nanoemulsion (D, E and F) showing appearance of vacuoles (v), duct (dt), lysis of secretion (*), irregular shape of granular secretion (**), appearance of indeterminate acini cells (ind), irregular appearance of acini outline (black arrow), ruptured acini (white arrow). Note in D apoptotic bodies (ap). Bars: A, D, B, E = 50µm; C, F= 20µm.

4. Discussion

Ticks are promise hematophagous living beings that control the hemostatic and immune systems of hosts they attacked, by emitting bioactive components during feeding^[32]. These components are put away in salivary gland granules until a stimulus prompts secretion. The salivary glands are additionally the main transmission route for pathogenic agents^[7]. The present study is targeting the female soft tick, *Ornithodoros savignyi*. It is known that when female ticks are incapable of completing their feeding process, vitellogenesis is corrupted, so that, the egg-laying processes depend indirectly on salivary gland activity^[33].

In the previous years, the best technique to control ticks was utilization of manufactured acaricides^[34, 35]. However, these chemicals prompted the improvement of resistance in targeted vector and increase residues in the environment. Moreover, these acaricides are hurtful to animals and humans^[36, 37], thus, instilling the need for finding alternative ways to control ticks. The plant-based pesticides are in great demand, because, they are sustainable in the environment; they are pleasant to use and are also safe^[38]. The utilization of essential oils is being progressively considered for pest control as they are generally seen to be less toxic to humans, animals and the environment than manufactured neurotoxic insecticides^[39]. An issue that emerges with the utilization of essential oils as pesticides is that their effect is short enduring^[40]. It additionally has technological obstacles, such as, reactivity, volatility, and hydrophobicity of the bioactive constituent part of the essential oils^[41]. Other issues related to their instability, poor

water solubility and aptitude for oxidation have to be settled before they are utilized as an alternative pest control management [25]. The EO compounds may enter through the insect cuticle, in a comparable way to ordinary insecticides, yet because of their exceedingly lipophilic composition, passage into the hemolymph may be moderate and constrained [42]. Isman *et al.* [43] pointed out that a principal disadvantage of EO used as pesticides is their lack of persistence, which requires two or more applications to exert a satisfactory administration of pests.

These issues may be overwhelmed by formulating Essential oils (EO) into nanoemulsion [44]. The emulsions that have droplets which are very small with size extending from 10 to 200 nm are called nanoemulsions. The technique of preparation determines the stability of the formulated nanoemulsion. Eucalyptus oil nanoemulsion was prepared with Tween 80 as a surfactant and water as the aqueous phase. The nanoemulsions are formed when the oil phase is scattered in an aqueous phase and it is stabilized due to the presence of surfactant that forms a layer at the interface of the droplet, in this manner, isolating the oil from aqueous phase owing to the stability of emulsion [45-47].

Eucalyptus oil nanoemulsion was characterized for size and stability, and the droplet size ranged from 10 to 17 nm, which is proved to be potent larvicidal and antibacterial activity [31, 48]. The stabilization of nanoemulsion would be due to the non-ionic surfactant, which reduces interfacial free energy and provides a steric barrier against coalescence [49]. The resultant nanoemulsion was homogenous, optically transparent and exhibited no birefringence and shows a hydrophilic nature. The formulated nanoemulsion was noticed to be stable after being exposed to centrifugation and when stored at different temperatures. The attractive forces among the droplets when its size is in the nano range are weak, accordingly, preventing particle aggregation and helps in making the nanoemulsions extra stable [45, 46]. At present, the nanoformulation pesticide aims toward estimated arrivals of vital and adequate measures of these products for a period of time to achieve the fullest biological efficacy [50]. Nanoformulation improved the EO interaction toxicity against pests. Anjali *et al.* [51] revealed that the insecticidal activity of neem oil was increased in nanoemulsion formulation. This effect can be because of the smallest droplet size of EO nanoemulsion (31.03 nm). Also Yang *et al.* [52] demonstrated that stacked nanoparticles with garlic EO are active to control *Tribolium castaneum*. Werđin Gonzalez *et al.* [53] suggested that the novel system of nanoformulations of EO could be developed as control agents against *Blattella germanica* (German cockroaches).

This is the first time that a nanoemulsion formulation using eucalyptus oil with a small droplet size, of 10 nm diameter, has been reported against *Ornithodoros savignyi*. The mortality percentage for semi engorged female ticks exposed to different dilutions of eucalyptus oil nanoemulsion showed a concentration-dependent effect. It was significant for serial dilutions from 10% to 40% when compared to the non-treated ticks ($P < 0.05$). The effect of oil based on particles size was previously demonstrated in a study proceeded by Anjali *et al.* [54], the neem oil nanoemulsion showed higher mortality rate against *Cx. quinquefasciatus* larvae which was based on the droplet size and its distribution. The nano form increases the surface area of the droplets, therefore, the reactivity is increased; thus, making nanoemulsions become more

effective than its bulk counterpart. The insecticidal activity of the nanoemulsion is expected due to the presence of eucalyptol, which is the major component of the eucalyptus oil. Several researchers have reported that the insecticidal and antimicrobial activity of an essential oil is related to its chemical composition [55-57].

In the present study individuals of the control group showed salivary glands with the characteristics described by El Shoura [11] in *ornithodoros moubata* which is closest relative to *Ornithodoros savignyi*. Salivary glands in semi-engorged females subjected to LC₅₀ of Eucalyptus oil nanoemulsion undergo morphological and histological destruction of the acini I such as irregular shape and dilated lumen compared to control group. This was also reported by Nodari *et al.* [33] after treatment of semi- engorged females of *Rhipicephalus sanguineus* with permethrin and by Pereira *et al.* [58] after treatment of same species with Fipronil. These authors suggested that acini I would be osmoregulators and through the saliva, the tick remove toxic compound from the haemolymph as suggested here by increase in lumen diameter of acini I in order to reduce harmful effect of Eucalyptus oil nanoemulsion on tick physiology. Acini II showed many vacuoles which function as agent in degradation of cell organelles damaged by Eucalyptus oil nanoemulsion. Other acini II could no longer identified beside the presence of apoptotic bodies and numerous nuclei with fragmented parts and loss of secretory cell membrane integrity or presence of few secretory granules in the cell [58, 59]. Furquim *et al.* [59] showed that involvement of ecdysone hormone that could trigger tissue degeneration [60]. The synthesis and release of this hormone would happen at beginning of the feeding period with highest production soon after detachment of ectoparasite from its host [61]. The present study revealed that tested compound increased salivary gland degeneration that would normally occur after full female engorgement. Early gland degeneration probably results in less blood loss of the host, and the transmission of pathogens through these parasites through saliva. Also when semi engorged females were subjected to feed on the host after two weeks of treatment with sub-lethal concentration, it is clear that oil nanoemulsion interrupt the engorgement process of female ticks by stopping the active feeding process. Thus, salivary gland degeneration caused by the nanoemulsion would cause not as much of blood loss in the hosts, as well as a reduced rate of pathogen into the host organism transmission through salivary glands. Therefore, further work is needed to evaluate the acaricidal effect of the EO nanoemulsions and their possible application in veterinary and agricultural fields.

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