# Effect of Tagetes minuta essential oil on the central nervous system (synganglion) of unfed Rhipicephalus sanguineus sensu lato ticks

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# Abstract

The *Rhipicephalus sanguineus sensu lato* tick, (Acari Ixodidae) is considered as sanitary concern due to its role as a vector. Tick strain resistant to synthetic acaricides has caused difficulties in its control, besides synthetic acaricides are harmful to the environment and the health of other animals. The use of plants with acaricidal and repellent properties has shown promising results under laboratory conditions with characteristics that resemble an ideal acaricide/insecticide. The genus *Tagetes* spp. excels for its use as traditional pest control in households and plantations and also for its potential as acaricide/insecticide in laboratory conditions against *R. sanguineus*. The first aim of this research was to evaluate the effect of different doses of *Tagetes minuta* essential oil (TMEO) on the central nervous system (synganglio) in unfeed *R. sanguineus* adults. The synganglion histologic analysis showed a remarkable effect with signs of cell damage including volume increase, loss of shape, and vacuolization, in addition to chromatin alterations such as condensation, margination, and fragmentation. TMEO were analyzed by gas chromatography coupled with mass spectrometry showing the presence of 21 compounds that according to its chemical structure are classified as terpenoids, of these compounds (Z)-β-ocimene, o-cimene, (Z)-tagetone, and verbenona were found in major quantities. The Gas chromatography analysis showed a composition variability related to the incidence of biotic and abiotic factors.

# 1. Introduction

The Brown dog tick *Rhipicephalus sanguineus sensu lato* (ACARI: IXODIDAE), is a tick widely distributed around the world, commonly parasites dogs but also it can be found on a diverse range of wild and domestic animals, including humans (Walker et al., 2000; Dantas-Torres, 2008). This tick species can transmit several pathogens (*e.g. Coxiella burnetii, Ehrlichia canis, Rickettsia conorii e Rickettsia rickettsii*) of sanitary concern (Straube, 2011; Bechara, 2013).

The tick control efforts present some difficulties due to the complex biological cycle, high reproductive capacity, and the number of hosts required to complete its life cycle (De la Fuente et al., 2015). In general, tick control methods include chemical products, vaccines, phytotherapeutics, genetic selection of the host, biological control, among others. Each method has advantages and disadvantages, although they could be more effective if used in a integrated management way, which considered the ideal approach (Quadros et al., 2020). Dogs can be treated with a wide range of veterinary products such as spot-on, impregnated necklaces, shampoos, sprays, baths, and powders (Garris, 1991). The main compounds with proven anti-tick activity *on R. sanguineus* are coumaphos, deltamethrin, Amitraz, Cypermethrin, Fipronil, among others (Bicalho et al., 2001, Burridge et al 2004). The prolonged use and misuse of these acaricides is harmful for the environment and can lead to resistance in some populations of ticks (Labarthe, 1994). *R. sanguineus* resistant strains related to a wide range of chemical compounds has been described (Quadros et al., 2020). Resistance to pyrethroids and fipronil has been reported on tick lineages in North America (Eiden et al., 2015), In Mexico, resistance to Cypermethrin and Amitraz was demonstrated in several tick populations on the Yucatan Peninsula (Rodríguez Vivas et al., 2017). Recently, the resistance to Ivermectin in *R. sanguineus* was documented for the first time (Rodriguez Vivas et al., 2017). *R. sanguineus* strains resistant to Cypermethrin, Deltamethrin and Coumaphos were identified in Goiânia state, Brazil (Borges et al., 2007). Different investigations have been performed using extracts of several plant species, around the world. The repellent or acaricide properties of about 200 species of plants have been tested (Adenubi et al., 2016; Quadros et al 2020).

*Tagetes spp.* are native from Mexico and other regions of warm and tempered climate of the American continent, and are traditionally used with ectoparasite control purposes (Nates et al., 1996). This genus has been studied broadly, in the treatment of trypanosomiasis, and also in the control of different agricultural pests (Baldeon, 2011, Priyanka et al., 2013). Some studies assessed the acaricidal properties of these plants on *R. sanguineus* and *R. microplus*, among other tick species, with promising results (García et al., 2012, Nchu et al., 2012). *Tagetes spp.* essential oil has volatile constituents, such as monoterpenoids, sesquiterpenes, and aromatic compounds, but different plants of the same genus but different species have different chemical composition (Green et al., 1991, Martínez, 2002). Furthermore, different studies have shown an important acaricidal effect of TMEO in *R. sanguineus* and *R. microplus*, with notorious rates of mortality both in vitro and in vivo conditions. The authors also suggested that the tick octopaminergic system would be a possible monoterpenes target (Garcia et al., 2012; Gil et al., 2013; Silva et al., 2016).

Research efforts have been addressed to determine the effects of conventional acaricides, plant extracts and plant EO with toxic potential against *Rhipicephalus spp*; (Roma et al., 2013; Remedio et al., 2015; Pereira et al., 2017, Matos et al., 2018) Similarly, the effect of different substances on the tick nervous tissue have arisen interest due that the tick synganglion is a key structure where resides the control of all physiological and behavioral functions of ticks for that reason the study of this structure and in particular the way, several substances affect its morphophysiology becomes so valuable. (Binnington, 1982; Lees and Bowman, 2007; Simo et al., 2013; Matos et al., 2018).

Considering the economic, environmental, and health impact of *R. sanguineus*, and the emergence of resistant strains to acaricide substances. This study aimed to assess the effect of different concentrations of TMEO on synganglion of unfed *R. sanguineus sensu lato* (ACARI: Ixodidae) adult by histological analysis. Besides that, TMEO composition was evaluated by Gas Chromatography analysis coupled to mass spectrometry (GC-MS) to establish the EO composition and compared it with other studies to better understand the effect of such substances in the *R. sanguineus* synganglio morphology.

# 2. Materials And Methods

## 2.1 Obtention of *Tagetes minuta* Essential Oils

Leaves and stems of *T. minuta* were collected in the peri-urban area of the city of Foz do Iguaçu, Paraná, Brasil. The vegetable material was identifided in the botanical laboratory located in ITAIPU Technological Park, Foz do Iguaçu. The plant samples were incorporated into the collection of the herbarium Evaldo Buttura at Universidade Federal da Integração Latino-Americana with the code EVB 2359. Images and data are available online in Consulta Pública del Herbário Virtual and + speciesLink network websites. One set of samples was collected in august 2018 in a rural property close the Vila "C" Nova neighborhood with coordinates Latitude 25 ° 27'25.35"S, Longitude 54 ° 33'40.99"W. The second collection ocurred in March and April of 2019 in a rural property near the Ecomuseum Latitude 25 ° 27'25.01"S, Longitude 54 ° 35'7.06"W, both places are located in the municipality of Foz do Iguaçu – PR, Brazil.

The plant material was selected and separated into leaves and flowers, then dried in an oven during four days at 40°C. The dry material was sent to the Chemical Laboratory of the Universidade Tecnológica Federal do Paraná - UTFPR Campus Santa Helena – Paraná, Brazil for the extraction process. Five (5) samples were processed which were labeld as PD2 (flowers) and PD3, PD6, PD7 and PD8 (leaves). The EO were obtained by hydrodestillation method using a Clevenger type apparatus, which lasted around 3 hours. As a result, were obeined hydrolates and EO, which were seperated usying ethyl ether. The obteined organic phase were dried with sodium sulfate Na2SO4 and rotary evaporated at reduced pressure and temperature. The TMEO obtained were stored in flasks and hermetically sealed.

## 2.2 *T. minuta* Essential Oil composition

The essential oil analysis was carried out in the Biotechnology Laboratory of Natural and Synthetic Products at the Universidade Caixas do Sul, Caixas do Sul city, Rio Grande do Sul, Brazil using gas choromatography coupled to mass spectrometry (GC/MS). The GC/MS was performed in a gas chromatograph coupled to a Hewlett Packard 6890 / MSD5973 mass selective detector, equipped with HP Chemstation software and Wiley 275 spectrophot library. A fused silica capillary column was used. HP-Innowax (30 mx 250 mm) 0.50 mm film thickness (Hewlett Packard, Palo Alto, USA). The temperature program used was the same one used in GC-FID: interface 280 ° C; division ratio 1:20; carrier gas He (56 kPa); flow rate: 1.0 mL / min .; ionization energy 70 eV; injected volume 1 μL diluted in hexane (1:10). The EO constituents were identified by comparison of their mass spectra with those of the Wiley library (GC/MS) and by comparison of the practical linear retention index with data from the literature (Nist). The linear retention index was calculated through the Van den Dool and Krats equation, using a standard solution of C7 to C30 hydrocarbons.

## 2.3 Adult Immersion Test

The *R. sanguineus* unfed adults were supplied by The Laboratory of Ixodology of Universidade Federal Uberlandia, Uberlandia, MG, Brazil. The ticks were received in nymph stage and maintained under controlled conditions (28 ± 1°C, 80% relative humidity and 12 h photoperiod) in a Biological Oxygen Demand at the Universidade Federal da Integração Latino-Americana - UNILA, in Foz do Iguazu, Paraná, Brazil. Once developed into adults the unfed *R. sanguineus* were distributed into seven groups according to the TMEO sample PD7 concentration and other substances to test as follows: group I (1.25% TMEO), group II (2.5% TMEO), group III (5% TMEO), group IV (10% TMEO), and group V (20% TMEO). Tween 20 at 2% which was used as a emulsifier was chosed too as a negative control group and amitraz at 12.5% (half the dose recommended by the manufacturer) was used as positive control. For each treatment group was prepared an amount of 1 ml as shown in Table 1. The bioassay was performed using a variation of the Adult Immersion Test or Drummond Test (Drummond, 1973). Each treatment group consists of 10 *R. sanguineus* unfeed adult individuals, the experiment was done in duplicate, totaling 140 individuals used. Each tick was submerged in 1 mL of each solution for 5 seconds. After that ticks were placed in groups of 10 in small Petri dishes labeled and equipped with filter paper to dry the treatment substance excess. In this way the Petri dishes containing the ticks were placed again in the incubator with the same controlled conditions of oxygen, humidity and photoperiod as mentioned above.

Table 1
*T. minuta essential oil dilution* PD7 sample

| Concentration (%) | Essential Oil μL | Tween 20 μL | H2O destilled μL |
|---|---|---|---|
| 1.25 | 12.5 | 20 | 967,5 |
| 2.5 | 25 | 20 | 955 |
| 5 | 50 | 20 | 930 |
| 10 | 100 | 20 | 880 |
| 20 | 200 | 20 | 780 |

## 2.4 Histology

Twelve (12) hours after treatment, ticks were exposed to carbon dioxide (expired air) and gently touched with the tip of a paintbrush to stimulate movement. Those ticks that showed no movement were considered dead and were discarded. Individuals who were found alive after 24 hours were subjected to dissection to remove the synganglion. The tick groups exposed to 1.25%, 2.5%, 5% of TMEO, and 2% Tween 20 were dissected in a time frame of 24 to 48 hours after exposure to the treatments. In the case of amitraz some individuals were dissected from 24 hours after treatment and up to 72 hours. Each tick was put on a petri dish containing phosphate-saline buffer solution (PBS: NaCl 0.13M, Na2HPO4 0.017M, KH2PO4 0.02m, pH 7.2), and dissected under a Leica EZ4 stereomicroscope using small optometry tweezers and scissors type. The synganglion was fixed in 4% Paraformaldehyde for 48 hours. After that, the material was transferred to sodium phosphate buffer solution where it remained for 24 hours. After that, the material was dehydrated in crescent series of ethanol to 70%, 80%, 90%, 95% and 100% (baths of 30 minutes). Subsequently the material was embedded in resin (Leica Hystoresin) included in plastic molds containing polymerizer resin for 24 hours at room temperature. The blocks were sent to the Histology Laboratory in Universidade Estadual Paulista Julio de Mezquita Filho (UNESP) Biosciences department, Rio Claro city, São Paulo state, Brazil, to be sectioned in microtome Leica RM2265 (Leica®) at 3-μm thickness. Once mounted on glass slides, the material was rehydrated in distilled water for 1 minute and stained with Harris hematoxylin for 8 minutes. Then, the material was washed in running tap water for 3 minutes, stained with aqueous eosin for 5 minutes and washed in running tap water again (Junqueira & Junqueira,1983). After drying at room temperature, the samples were, covered with rapid mounting medium for microscopy and a coverslip.

### 3. Results

# 1.1 Histological analysis

## 1.1.1 Control group Tween 20 at 2%

The Tween 20 at 2% control group treatment showed the normal *R. sanguineus* synganglion typical characteristics, previously described by Roma et al., (2012) (Fig. 1A-C). In general, the *R. sanguineus* synganglion consists of a merged mass of nervous tissue. This structure has no external segmentation, consisting of a simple structure covered by an acellular neural sheet or neurilema, next below is the perineurium, made of glial cells (Fig. 1A-C). Internally, the synganglion is divided into a cortical region that contains the body or soma of the neurons.

The synganglion morphology of the ticks exposed to Tween 20 at 2% was almost intact, it was possible to observe some signs of cellular injury circumscribed to cellular bodies whit slight chromatin alteration; the cortical synganglion region was well preserved (Fig 1. A - C). The synganglion central region or neuropile showed no alterations, presenting a normal morphology, its structure, as also described by Roma et al., (2012) consist of a fibrous nucleus which is formed by a set of the neuron fibers or axons related to the neurons bodies in the cortical region. This region comprises a set of bilateral symmetric lymph nodes. The cortex and neuropile are subdivided by the sub-perineurium conformed by glial cells which were found in normal conditions in this study (FIG 1. A - C).

## 3.1.2 Amitraz at 12.5% /2 (control group)

The *R. sanguineus* individuals exposed to amitraz at 12.5% (50%) showed different alterations almost in all the different regions of the synganglion (Fig. 2. A-D). The neurilemma was intact, however, at the perineurium level was possible to identify some cells with signs of chromatin condensation (pyknosis) (Fig. 2. B-D). In the cortex underlying the perineurium some areas of vacuolization were present. There was a discontinuity between the perineurium and the contiguous cortical region. It was possible to visualize cells with loss of shape and vacuolization in the surrounding area, as well as cells with fragmentation or chromatin condensation. These alterations were spread in the cortex (Fig. 2. A-D). At the level of the sub-perineurium region contiguous to the neuropile was a significant loss of the tissue morphology with wide vacuolization areas (Fig. 2. C). A loss of continuity at the neuropile level and broad areas of structural disorganization are evident (Fig. 2. A-D).

## 3.1.3 Grupo I (*T. minuta* essential oil at 1.25%)

It was possible to observe important alterations on the synganglion in individuals exposed to 1.25% TMEO, although in general, the tissue maintains its normal morphology (Fig. 3. A - C). The neurilemma showed no alterations, however, at the perineurium level was a clear formation of vacuoles distributed intermitenttly. In the cortex region occurred cells with loss of shape and increased cytoplasmic volume, it was also possible to find neuronal nucleus with chromatic alteration like signs of picnosis or initial chromatin marginalization stage; cells with increased size, loss of shape and vacuolization areas circumscribed to cells tumefaction or hydropic degeneration stage were also observed (Fig. 3. B-C). Vacuolization areas were observed in the neuropile underlying the subperineurium, in general the neuropile showed a pattern of widespread disorganization (Fig. 3. B-C).

## 3.1.4. Grupo II (*T. minuta* essential at 2.5%)

The ticks exposed to 2.5% TMEO showed some irregularities at the neurilema. There were vacuolization signs at the subperineurium level with a clear detachment of the cortex (Fig. 3. D-F). Some cortical cells presented nuclear alteration, cells bodies with volume increase and shape loss, presence of cells with chromatin condensation between the cortical region and neuropile, there was an area of vacuolar degeneration between both structures (Fig. 3. D-F). Although the general structure of the synganglion is still preserved, signs of cellular injury similar to those observed at concentrations of 1.25% are evident.

## 3.1.5. Group III (*T. minuta* essential oil at 5%)

Ticks exposed to 5% TMEO showed at neurilemma level folds and irregular appearance; there was a detachment of the perineurium in some sections (Fig. 3. G-I); The perineurium showed a considerable degree of detachment related to the cortex, apparently this injury is a continuation of the neurilemma detachment. An important degree of alteration at the cortex is evident; significant number of cells with shape disorganization, increased size; vacuolization was observed, there were cells with sign of condensation, marginalization, and fragmentation of the chromatin (Fig. 3G – I). At the subperineurium level some cells showed increased volume and chromatin alteration as well as vacuoles presence between the sub-perineurium and the neuropil; a considerable degree of detachment between the sub-perineurium and neuropile is evident. The neuropil shows structural disorganization (Fig. 3G-I).

## 3.1.6. Group IV (10% and 20% *T. minuta* e.o)

The *R. sanguineus* groups exposed to TMEO 10% and 20% caused death of all the ticks in less than 12 hours, included the duplicated test, due to the tissue fast degradation was not possible to extract the synganglion, thus the histological analysis was not performed.

## 3.2. *T. minuta* essential oil chemical composition

Twenty one compunds were identified in *T. minuta* essential oil, which was dominated by (Z)-β-ocimeno (33.05%), o-cimeno (19.14%), (Z)-tagetona (13..24%) and Verbenona (7.24%) (Table 1).

Table 2
Relative proportion for each TMEO compound according with the Cromatograpy analysis. All the compounds found in TMEO in this study are listed below with each compound percentage, not all the samples analysed presented the same composition, the major compounds are highlighted.

| Composition | Percentaje relative (%) | | | | |
|---|---|---|---|---|---|
| | PD2 (flower) | PD3 (leaves) | PD6 (leaves) | PD7 (leaves) | PD8 (leaves) |
| β-pinene | 0,21 | - | - | 0,22 | - |
| β-phellandrene | 0,23 | - | - | - | - |
| Eucalyptol | 0,64 | 0,57 | 0,37 | 0,29 | 0,17 |
| **(Z)-β-ocimene** | **33,05** | **6,63** | **15,64** | **15,66** | **8,56** |
| (E)-β-ocimene | 0,32 | - | - | - | - |
| **o-cimene** | **19,14** | **50,99** | **34,14** | **45,49** | **28,8** |
| Allo-ocimene | 0,37 | - | - | - | 0,99 |
| (E)-tagetone | 0,97 | 0,9 | 0,96 | 1,02 | 2,04 |
| **(Z)-tagetone** | **13,24** | **15,69** | **11,4** | **11,94** | **9,18** |
| β-cubebene | 0,04 | - | - | - | - |
| (Z)-caryophyllene | 1,27 | 0,15 | 1,8 | 1,19 | 1,55 |
| α-humulene | 0,05 | - | - | - | - |
| (L)-α-terpineol | 0,11 | - | - | - | - |
| germacrene D | 0,06 | 0,09 | - | - | - |
| **Verbenone** | **7,24** | **3,39** | **6,57** | **5,31** | **2,83** |
| δ-cadinene | 0,06 | - | 0,08 | 0,14 | 0,49 |
| Spathulenol | 0,62 | 0,22 | 0,37 | 0,16 | 0,83 |
| trans-β-ionone | - | - | 0,15 | 0,07 | 0,33 |
| Caryophyllene oxide | - | - | 0,34 | 0,3 | 0,11 |
| **Eugenol** | - | - | 0,13 | 0,07 | 0,28 |
| α-cadinol | - | - | 0,01 | - | 0,11 |

## 4. Discussion

Different plant essential oils have been studied due to their properties as efficient alternatives for tick control. The toxicity of these compounds has been demonstrated by immersion test (Drummond test) or contact tests with impregnated surfaces, as well as vapor exposure to essential oils. Some experiments have determined through microscopy techniques the effect of synthetic substances and plant extracts on different *R. sanguineus* tissues, important alterations on the morphology of the tissues exposed to the assessed substances have been found. ( Roma et al., 2010; Araujo., 2012; Roma et al., 2012; Roma et al., 2013a; Roma et al., 2013b; Remedio et al., 2015; Pereira et al., 2017; Matos et al., 2018). TMEO has shown acaricidal activity against different tick species, being a potential opportunity for the development of new acaricides, (García et al., 2012; Da Silva et al., 2016; França et al., 2017).

The results found in the CG / MS analysis showed some variations in the TMEO samples analyzed; a total of 21 compounds were identified, of which Eucaliptol, (Z)-β-ocimene, o-cimene, (E)- tagetona, (Z)-tagetona, (Z)-cariofileno, Verbenona, Espatulenol were present in the TMEO samples extracted from leaves and flowers PD3, PD6, PD7, PD8 and PD2 respectively. Regardless of the type of plant material, place, and date of collection, the major compounds found were always (Z)-β-ocimeno, ocimeno, Z)-tagetona and Verbenona. Andreotti et al., (2013) found as TMEO major compounds dyhidrotagetone (54.10%), Limonene (6.68%), tagetona (6.73%) and (e)-β-ocimene (5.11%) which presented significant acaricide activity against larvae, nymphs and adults of *R. sanguineus*. García et al., (2012) described the presence of limonene (6.96%), β-Ocimene (5.11%), dihydrotagetone (54.21%), and Tagetona (6.73%) as major compounds, the same authors found that the TMEO is highly effective against several tick species including *R. sanguineus*. Kaul et al. (2005) assessed the TMEO composition finding a total of 24 compounds which represent 93.7% of the analyzed EO, the major compounds were limonene y beta-felandreno (4.7%), (Z)-β-ocimeno (36.8%), dihidrotagetona, (E)-β-ocimeno (15.5%), (Z)-tagetona (17.1%), (Z)-tagetenona (3.0%) and (E)-tagetenona (7.5%) the authors identified compounds not reported here in both TMEO flowers and leaves. The chemical composition variability of the same species EO could be the result of different chemotypes related to biological variations linked to factors like soil, temperature, climate conditions, illuminance, etc (Gakuubi et al., 2016). Therefore, there is a clear variation of the chemical profile on the TMEO when compared with the results found in the present study. Different factors may have influenced these findings, some variations in the EO composition can be due to the vegetable material used in the extraction either leaves or flowers, also the local and collection date could influence the composition of the different samples, Morais (2009) suggests the effect of abiotic factors like temperature, light intensity, seasonal effects among others in the EO composition. There was a mild difference in the concentration of the major and minor compounds related to the geographical location, that sort of variation was described by Salehi et al., (2018), meanwhile Gobbo-Neto & Lopes (2007) suggest that factors like time of collection, seasonality and development phase, temperature and ultraviolet radiation could affect the EO composition.

The compounds found in TMEO are classified as terpenes, whose structure is formed of isoprene units, which are hydrocarbons of 5 carbons. Monoterpenoides are those compounds formed by 2 isoprene units and a molecular structure of 10 carbon atoms (Perveen, 2018), as in compounds like (Z)-β-ocimene, (e)-tagetone, β-pinene, eucalyptol, (E)-β-ocimene, o-cimene, (Z)-tagetone, eugenol, (L)-α-terpineol, that correspond to most of the found compounds. Terpenes cover a wide variety of substances of vegetable origin and their ecological importance as defensives is well studied (Viegas, 2003).

The component β-cariophylene present in *Piper aduncun* EO and also present in TMEO when evaluated individually against *Tetranycgys urticae* presented important acaricidal activity (Araujo et al., 2012), could have influenced the tissue alterations in the present study. Ayoub et al., (2018) evaluated the gender *Thymus* species EO which caused high mortality on *Sitophilus oryzae* those EO present some constituents also found in TMEO such as β-pinene, o-cymene, eucalyptol, α-humulene, e-β-ocezen, Germacreno D, cariophilene oxide. *Artemisia campestris* EO which chemical profile is similar to TMEO include β-pinene, α-pinene, mircene, germacreno D, (Z)-β-ocemine showed toxicity against *Culex quinquasciatus* and *Musca domestica*. Similarly, *Arabic pulicaria* and *Saccocalyx satureioides* EO which contains compounds like epi-α-Cadinol, Δ-Cadinene, α-Cadinol and Germacrene D-4-OL and Timol, α-terpineol, Borneol and P-Cimen respectively, some of them also present in TMEO were active against the *Spodoptera littoralis* agricultural moth (Ammar et al., 2020). Huang et al., (2002) mentioned the contact toxic effect of Eugenol, isoeugenol, and methyleugenol against *Sitophilus zeamais*. Furthermore, a recent study found that eugenol in association with thymol and carvacrol had a synergistic acaricidal effect in *Amblyomma sculptum*, therefore suggesting that different compounds show a synergistic effect when acting simultaneously (Vale et al., 2021). The proven toxicity of Eugenol in insects and tick may have contributed to the toxic effect reported in this study.

*Murraya microphylla* EO which chemical profil have similarities to the found in TMEO, showed insecticide activity and repellent effect against tobacco beetle *Lasioderma serricorne*, that suggest a possible synergistic effect of the components of the TMEO (You et al., 2015). The insecticidal effect of (Z)-β-ocimene has also been analyzed individually agaisnt *Anopheles spp*, *Aedes spp, Culex spp* (Govindarajan & Benelli., 2016). Hüe et al., (2014) assessed the (Z)-β-ocimene acaricidal effect at 2% concentration on *R. microplus* without remarkable effects. Though the EO compounds toxicity have been recognized, their mechanism of action has not been totally understood, the evidence indicates that most of the compounds differs in their mode of action (Gross et al., 2017; Viega, 2003; Quadros et al., 2020). Monoterpenoids compounds mode of action may not be represented by a single mechanism (Grodnitzky & Coats, 2002). Cardoso et al., (2020) noted that the inhibition of ACHE may be the main mechanism associated with the effect of some terpenoids on *R. microplus*. Meanwhile, Ali et al., (2014) reported the cytotoxic effect of TMEO on MCF-7 line mammary tumor cells, showing moderate cytotoxic activity, the authors raise a possible synergistic effect between the different compounds. According to Gross et al., (2014), the mixture of the different components present in an EO could competitively be coupled to octopamine and tyramine receptors associated with G protein. Another proposed mode of action is the inhibition of the monoxidase enzyme (MAO) causing a neurotoxic effect associated with the increase of cyclic monophosphate adenosin (cAMP); others could be the possible TMEO mechanism of action, as in the case of Timol and Carvacrol which interact by inhibiting ACHE in *R. (B.) microplus* (Cardoso et al., 2020). The hydrophobic nature of EO could simultaneously exert a mechanical effect on the parasite well obstructing the area of spiracles entailed to death by water stress or suffocation as suggested by Burgess (2009) could be considered another mechanism of action involved.

The histological findings suggest a toxic causal effect over the tissues exposed to the different TMEO concentrations. *R. sanguineus* ticks when exposed to Tween 20 at 2% showed no considerable alterations in the synganglion morphology. These findings correspond to the *R. sanguineus* synganglion normal structure described by Roma et al. (2012) as well as by other authors who confirmed similar findings (Pereira et al., 2017, Matos et al., 2018). However, the ticks exposed to amitraz at 12.5% (50% sublethal doses) and the TMEO different concentrations, the synganglion morphology showed gradual alterations, mainly in the cortex and neuropile, these alterations were related to the EO concentration. The ticks exposed to amitraz presented alterations almost all over the synganglion. Although the neurilemma was intact, the perineurium presented vacuoles and cells with cytoplasm alterations, as well as significant signs of nuclear alteration like condensation, marginalization, and fragmentation of the chromatin were present, such alterations at the nuclear level are suggestive of irreversible cell injury (Damjanov, 2009). The cortex cells showed some morphological alterations like swelling, shape loss, and vacuolization, there were also vacuolization at the extracellular matrix; similar alterations were reported in *R. sanguineus* synganglion exposed to permethrin (Roma et al., 2013). It is considered that vacuolization related to autophagy is a cell mechanism to eliminate possible toxic substances or damaged structures, if the injuring effect is higher than the mechanisms of detoxification and repair, then the toxic substances will lead to irreversible lesions and the consequent cell death (Roma et al., 2013a; Elmore et al., 2016; Aki et al., 2012). Occasionally, the vacuolation process in response to injury is reversible, while in other circumstances, continuous exposure to levels of a certain substance, entails cell death, in a likely scenario the vacuole will end tearing up, causing the rupture of the cell membrane (Henics & Wheatley, 1999).

There are similar studies where ticks were exposed to amitraz, showing cell injuries similar than the observed in the present study. Kanapadinchareveethyl et al., (2019) suggest that Amitraz could cause vacuolization in the *R. annulatus* oocytes phase II cytoplasm; Likewise, Mangia et al., 2018 found that cell cultures of *Ixodes ricinus* exposed to different doses of Amitraz, Permethrin, and Fipronil, showed signs of cellular injury like increase in extracellular waste and loss of cell shape, which are evidence of death in addition to a reduction in the total count of cells; the authors suggested that toxic effects in vitro are due to oxidative stress which is a trigger of the apoptosis process. Some signs of structural disorganization in the neuropile are evident, presumably due to the amitraz effect in the cortex cells whose nerve fibers are projected into the neuropile; these alterations are more evident in the region underlying the subperineurium that shows widespread vacuolization. Roma et al., (2013) described a similar pattern in the neuropile in ticks exposed to permethrin. Although the amitraz mechanism of action is not fully understood, it is known that the effects of this substance in insects at a molecular level are related to an increase in the tyramine and octopamine levels; the amitraz is generally classified as an agonist receptor of octopamine and enzyme Oxidase inhibitor (Evans & Gee, 1980, Davenport et al., 1985 & Kita et al., 2016). Formamidines, such as the Clordimeform (CDM) and Amitraz, are considered prodrugs that are metabolically converted into agonists of octopamine receptors (OAR), DimethylClordimeform (DCDM) and N2- (2,4-dimethylphenyl) -n1-methyformamidine (DPMF) respectively; It has been shown that DPMF increases the intracellular levels of CA2 + in the Silkworm *Bombyx mori* although both amitraz and DPMF potently act in the octopamine receptors BMOAR1 and BMAAR2 of *R (B.) microplus*, increasing CA2 + levels and cAMP respectively (Kita et al., 2016).

The histological alterations observed in synganglion of *R. sanguineus* exposed to Amitraz are compatible with the type of cellular injury associated with acute cell death (Elmore et al., 2016). The agonistic effect of amitraz on octopamine receptors increases the calcium levels (Ca2+) and cAMP Cytosolic, in turn the receptors coupled to G protein are activated, like the case of the octopaminergic receptor (Roeder, 1999), Besides that, this will lead to the production of inositol triphosphate (IP3), which releases CA2 + from the endoplasmic reticulum (Blenau et al., 2012). The increase in CA2 + affects the cell through the predominant role of this element in the cell signaling, the cell injuries can also be a product of the direct effect of the CA2 + which induces the activation of hydrolases, the alterations at the cellular level induced by CA2 + are generally acute, rapid and in some circumstances irreversible; in the CA2 + case, this mechanism of cell injury would likely lead to cell death (Orrenius et al., 2011, Dong et al., 2006). Secondly, it could be possible that the continued increase of cAMP entails depletion of the ATP cytosolic reserves, which could trigger the cell death process. It has been evidenced that calcium ionophores induce apoptosis under ATP supply conditions, but in conditions of exhaustion of ATP, this can trigger cell death by necrosis (Eguchi et al., 1997). Thus amitraz could act at cellular level through these two mechanisms, more studies are required to elucidate the action of amitraz in cell components of *R. sanguineus*.

The histological slides of the ticks exposed to TMEO concentrations at 1.25% and 2.5% showed similar patterns of alterations compared with the control and the amitraz treatment, as well as in other studies where the synganglion of tick exposed to different substances was analyzed. The neurilemma did not show evident alterations but at the perineurium were vacuolization signs not being possible to identify any cellular structure, similar finds were described by Roma et al., (2013) in ticks treated with permethrin. Remedio et al., (2015) described similar tissue injuries in synganglion of tick exposed to Nim *Azaridacta indica.* Besides the cortex cells showed signs of volume increase and shape loss, condensation, and chromatin marginalization, suggesting a progressive cellular injury, those signs would correspond to a reversible cell lesion (Miller & Zachary, 2017). The persistence of the damaging agent can cause an irreversible injury that would result in cells death; additionally, an irregular distribution of the extracellular matrix is evident in the cortex region with the presence of vacuolization, similar findingss were already reported (Roma et al., 2013; Pereira et al., 2017, Matos et al., 2018). The increase in cytoplasmic and vacuoles volume may suggest that the cell membrane function was altered due to the effects of the TMEO constituents. Some cells of the cortex were in advanced state of degradation, which suggests that the mechanisms of detoxification (vacuolization) cannot reverse the effects of toxic compounds or eliminating them.

Ticks exposed to TMEO at 5% showed alterations similar to those described at the 1.25% and 2.5% concentrations, but was possible to observe more drastic effects. The neurilemma is intact but at the perineurium level there is an evident detachment of this structure next to the cortex, with a space between both structures, even in some areas the perineurium is no longer visible or shows structural alterations, suggesting that TMEO components affect the permeability of the neurilemma which would allow the contact of the TMEO with the interior of the synganglion. The perineurium composed mostly by glial cells showed degradation signs such as vacuolization and nuclear alterations. Once in contact with outer substances the cells would put into function detoxifying mechanisms, if fails, the cell would enter into an irreversible process, the empty areas that were found here probably corresponded to cells degrades. Remedio et al., (2015) described similar alteration in *R. sanguine*us synganglion exposed to *Azadirachta indica* EO, due that perineurium cells make first contact with exogenous substances, these cells show effects similar than the observed with the TMEO small concentrations, there is a remarkable alteration of a large number of cortex cells when compared to other treatments, with different degree of cell injury, some with an increase in volume or hydropic degeneration suggesting a reversible cell lesion. Other type of biochemical alterations associated with an eventual depletion of ATP and increase in CA + + intracellular levels may be involved in this case triggering irreversible alterations at the nuclear level such as picnosis, cariorrexis and karyolysis which are irreversible cell damage signs, (Miller & Zachary, 2017) then according to these findings is possible to suggest that TMEO components could affect the cell homeostasis mechanisms.

Similarly, to the ticks exposed to Amitraz a loss of continuity between the cortex and the sub-perineurium was observed at the neuropil, there is a considerable structural disorganization with signs of vacuolization, however, not as intense as observed in the Amitraz group or the reported in synganglion exposed to permethrin by Pereira et al., (2017). The structural alteration observed in neuropil can be linked to the injuries observed at the level of the cortex, like mentioned above the neuropil corresponds to nerve fibers from the cells located in the Cortex region.

## Conclusion

TMEO is constituted mostly by compounds of the terpenoids family, especially monoterpenoids and sesquiterpenoids.

There are important variations in the essential oil composition, qualitatively and quantitative, which are related to different biotic and abiotic factors; additionally, the type of vegetable material like leaves or flowers are related to variations.

The different TMEO concentrations showed notorious toxic effects on the *R. sanguineus* nervous tissue, several signs of cellular injury like shape loss, volume increase (swelling), chromatin alterations, and vacuolization in perineurium, cortex, subperineurium, and neuropile.

There were vacuolization areas distributed in the different synganglia regions; those alterations are possibly related to the essential oil concentration. Such findings become more notorious in higher doses. the 1.25% and the 2.5% doses showed mild to moderate signs of cell alterations, the 5% TMEO concentration doses showed a pattern of severe injury similar to that observed in the control treatment group with amitraz which caused alterations similar to different synthetic acaricides described in other authors. The tween 20 at 2% caused no considerable alterations in cells.

It is necessary to address other studies to elucidate and widen the knowledge about the effect of terpenoids to better understand the effect of those compounds on *R. sanguineus* central nervous system and other tick tissues

## Declarations

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### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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## Figures

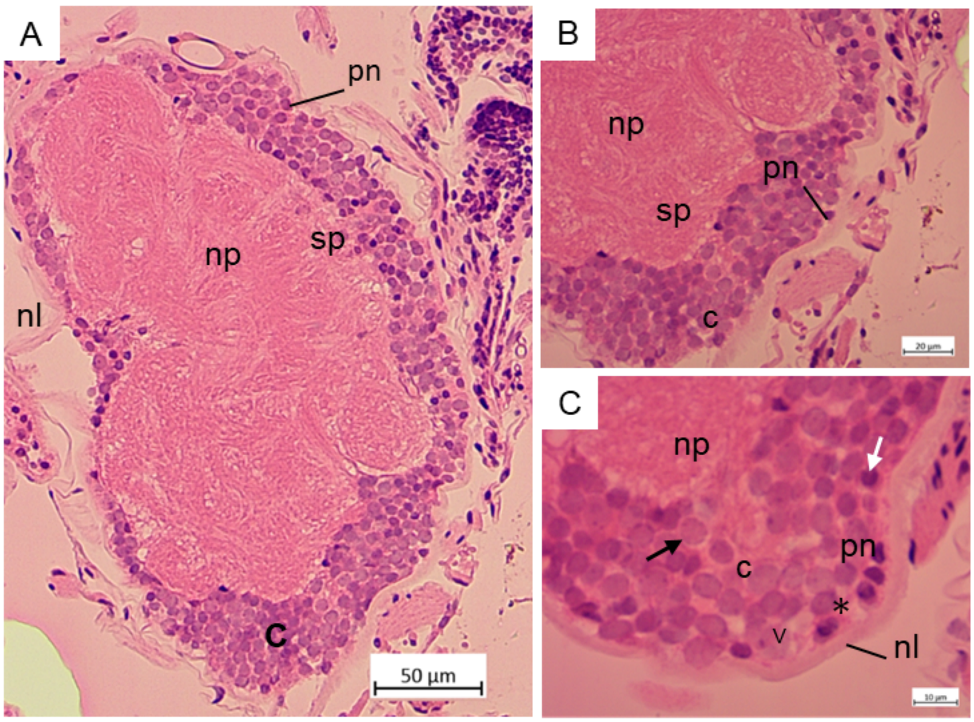


**Figure 1**

Histological sections of unfed *R. sanguineus*synganglion stained with Hematoxyline-Eosin. Group Tween 20 at 2 %. A General view. B y C detail. neurilema (*nl*); perineurum (*pn*); córtex (*c*); neuropilo (*np*). Karyorrhexis (*). Vacuolization, (*v*); celular swelling (*Black arrow*). Chromatin condensation (white *arrow*).

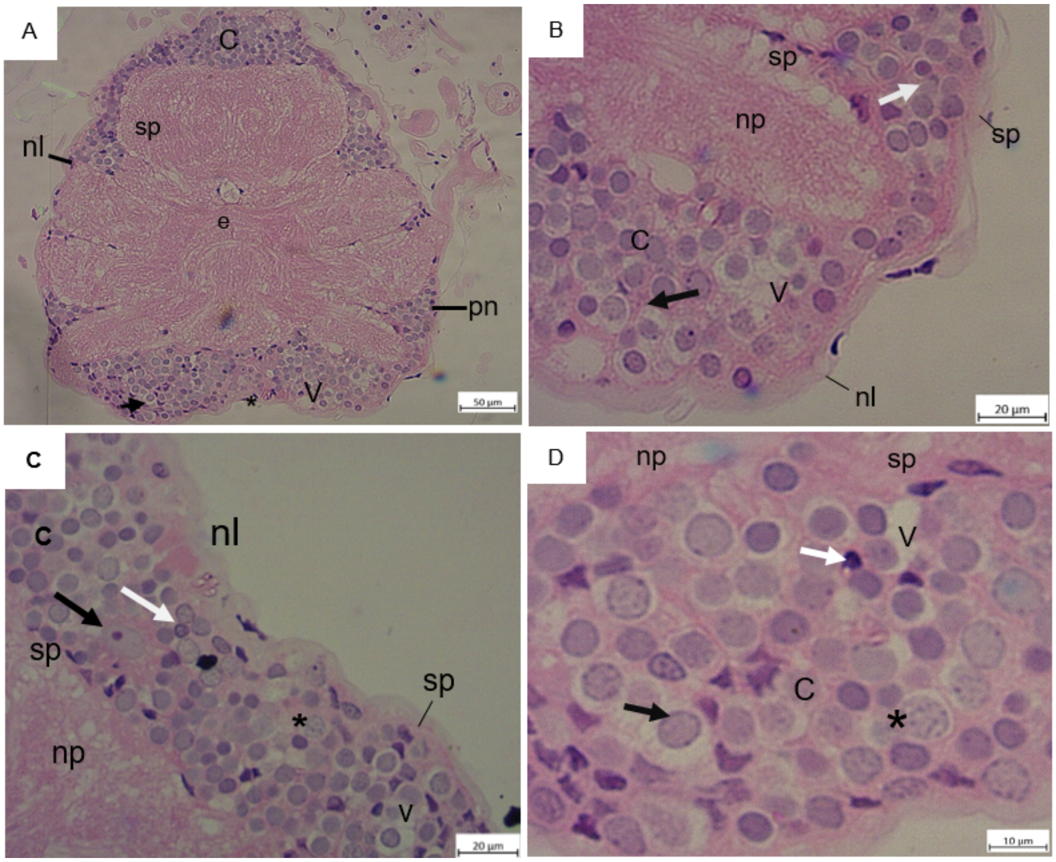


**Figure 2**

Histological sections of unfed *R. sanguineus*synganglia stained with Hematoxyline-Eosin. Group I – amitraz at 12.5 %. A, B, C and D General view. An irregular pattern of cells undergoing apoptosis. lamella neural (*nl*); perineurum (*pn*); córtex (*c*); neuropile (*np*). Karyorrhexis (*). Vacuolization, (*v*); celular swelling (*Black arrow*). chromatin condensation (white *arrow*).

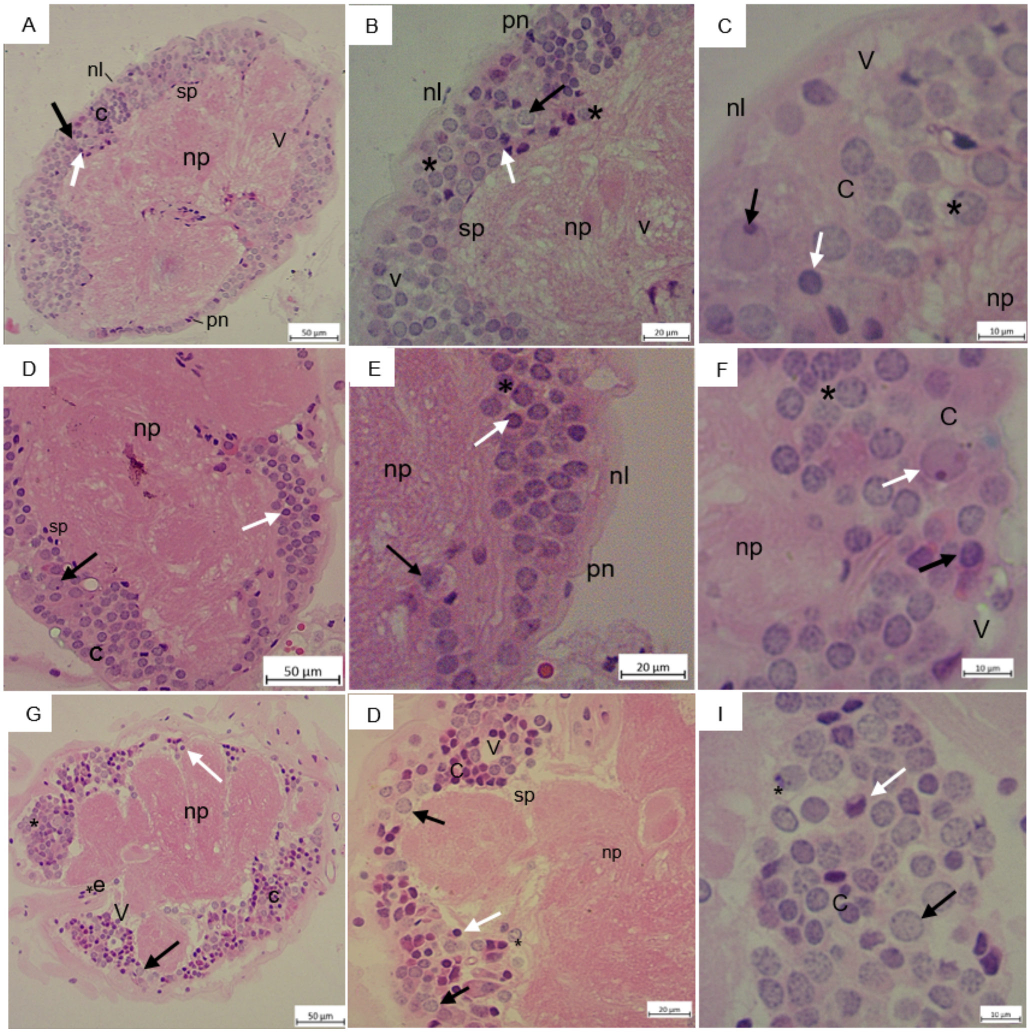


**Figure 3**

Histological sections of unfed *R. sanguineus* synganglia stained with Hematoxyline-Eosin. Group I A-C – *T. minuta* EO 1.25%. Group II D-F – *T. minuta* EO 2.5%. Group III G-I – *T. minuta* 5%. Neural lamella (*nl*); perineurium (*pn*); córtex (*c*); neuropile (*np*); vacuolization (*V*); Cellular swelling (Black arrow); chromatin condensation (white arrow); Karyorrhexis (*).