Evaluation of natural origin products for the control of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) on cattle artificially infested

Romo-Martínez\textsuperscript{a}, M. Fernández-Ruvalcaba\textsuperscript{b}, V. M. Hernández-Velázquez\textsuperscript{c}, G. Peña-Chora\textsuperscript{d}, L. P. Lina-García\textsuperscript{e}, J. Osorio-Miranda\textsuperscript{e}

\textsuperscript{a}Facultad de Ciencias Agropecuarias-Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Colonia Chamilpa, Cuernavaca, Morelos, México, C.P. 62209, Mexico
\textsuperscript{b}CENID-Parasitología Veterinaria, INIFAP-SAGARPA, Carretera Federal Cuernavaca-Cuautla, no. 8534, Colonia Progreso, Jiutepec, Morelos, C.P. 62550, México
\textsuperscript{c}CEIB-Universidad Autónoma del Estado de Morelos. Av. Universidad 1001, Colonia Chamilpa, Cuernavaca, Morelos, México, C.P. 62209, México
\textsuperscript{d}CIB, Universidad Autónoma del Estado de Morelos. Av. Universidad 101, Colonia Chamilpa-Cuernavaca, Morelos, México, C.P. 62209, Mexico

\textsuperscript{*}Corresponding author email: rdez51@yahoo.com

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The aim of the present study was to evaluate under stable conditions, the biological efficacy of three natural products *Metarhizium anisopliae*, *Bacillus thuringiensis* and *Solanum verbascifolium*, sprayed on cattle artificially infested for the biological control of *R. (Boophilus) microplus*. The results demonstrated by the number of engorged female ticks collected on 7\textsuperscript{th} day post-treatment, show that the combinations of *S. verbascifolium + M. anisopliae*, *M. anisopliae* and *B. thuringiensis* produced the lower tick numbers with 40.50, 52.25 and 52.75 respectively. At 14 days post-treatment, the treatment with *B. thuringiensis*, *M. anisopliae* and the combination of *S. verbascifolium + M. anisopliae* produced the lowest engorged female numbers with 1.5, 6.25 and 6.25 respectively. At the 21 days post-treatment with *B. thuringiensis*, *M. anisopliae*, the combination of *S. verbascifolium + M. anisopliae* and *S. verbascifolium* had the lower engorged female numbers with 0.50, 0.75, 3.5 and 3.5 respectively. The treatments that produced engorged female with lower weights at the 7th day post-treatment corresponded to the combinations of *S. verbascifolium + M. anisopliae*, *M. anisopliae* and *B. thuringiensis* with 8.34 g, 11.13 g and 15.05 g respectively. At the 14 days post-treatment, the treatment that produced female with lower weights were *B. thuringiensis*, the combinations of *S. verbascifolium + M. anisopliae* and *M. anisopliae* with 0.25 g, 1.36 g and 1.61 g. At the 21 days post-treatment, the treatment with *B. thuringiensis* produced females with lower weight, and the combination of *S. verbascifolium + M. anisopliae* and *S. verbascifolium* produced 0.14 g, 0.27 g, 0.81 g and 1.01 g respectively. At the 7th day post-treatment, the application of *S. verbascifolium + M. anisopliae* and *M. anisopliae* produced the lower egg mass weight with 0.81 g and 0.94 g respectively. At the 14th day post-treatment, with *B. thuringiensis*, the combination of *S. verbascifolium + M. anisopliae* and *M. anisopliae* were the treatments that achieved the lowest egg mass weights with 0.04 g, 0.12 g and 0.14 g respectively. At the 21 days post-treatment, *B. thuringiensis*, *M. anisopliae*, *S. verbascifolium + M. anisopliae* and *S. verbascifolium* with 0.08 g, 0.09 g, 0.33 g and 0.33 g respectively. At the 7th days post-treatment, the treatment that showed the lower egg hatch percentage were *S. verbascifolium + M. anisopliae* and *M. anisopliae* with 22.42 and 26.40% respectively. At the 14 days post-treatment the *B. thuringiensis*, *M. anisopliae* and *S. verbascifolium + M. anisopliae* treatments showed the lower egg hatch percentages of 0, 2.74, 7.55 and 12.40% respectively. Regarding the 21 days post-treatment, *B. thuringiensis*, *M. anisopliae*, the combination of *S. verbascifolium + M. anisopliae* and *S. verbascifolium* showed the lowest percentage of 0.44%, 2.90%, 8.75% and 19.98% respectively. Values of percentage...
control or reproductive potential inhibition higher than 80% were achieved. For the *R. (Boophilus) microplus* larvae stage treated with *Bacillus thuringiensis*, *M. anisopliae*, *S. verbascifolium* and *M. anisopliae*, the percentage of control on the reproductive potential was 99.99%, 99.46%, 98.93% and 97.84%. For the nymph stage, we found that *B. thuringiensis*, *S. verbascifolium* + *M. anisopliae* and *M. anisopliae* produced a percentage of control of 99.99, 99.91 and 99.73% respectively. For the adult stage we observed that the treatment with *S. verbascifolium* + *M. anisopliae* and *M. anisopliae*, achieved a control of 99.28 and 98.17% respectively.

**Keywords:** Cattle ticks, Biological control, Botanical extracts, *In vivo* tests

**INTRODUCTION**

In the tropical and subtropical regions of the world, where cattle are raised, the ectoparasites with the major economical impact are ticks. In America, from the northern frontier with the United States of America to Argentina *Rhipicephalus* spp. is the predominant genus due to the direct and indirect damage caused to livestock (Jonsson., et al. 1998). The control strategies for tick populations worldwide, has rely on the use of chemical acaricides treatments, some are not more in use due to the several adverse effects (George, 1990; Shelton and Karns, 1988). The indiscriminate use of these products has led to the development of acaricide resistance on tick populations, a worldwide problem of difficult solution. In México, the tick specie predominant on bovine livestock is *R. (Boophilus) microplus*, with reports of tick populations that had develop acaricide-resistance against major families of ixodicides (Soberanes et al., 2002). The losses due to ticks and tick-borne diseases has been estimated to be of approximately of 19,000 mdd annually (De Castro, 1997) with severe economic losses for the need of chemicals to control the ticks and drugs to treat the animals that develop tick borne-diseases anaplasmosis and babesiosis. (De Castro, 1997). More over, there are other costs not easily measured related with the development of chemical resistance, since conventional acaricides are toxic products not selective and of very low degradation, that harm benefic species (López-Carrillo, 1993; Shelton and Karns, 1988) and non-target organisms, including humans.

When chemicals are use on fields or farm animals, soil receives a great amount of these products that accumulate on water and contaminate streams and reservoirs. Some of these products can incorporate on the air and travel great distances, others are absorbed by plants that will be ingested by animals and humans (López-Carrillo, 1993).

Different approaches to the chemical control against *R. (Boophilus) microplus* had been recommended in an attempt to decrease the problem, among these are some cultural practices, focused on the exposure of the ticks different stages of development, to extreme conditions of temperature and humidity, the removal of brush, grassland burning, planting of forage species, the use of grass with anti-tick effect, high vegetation pruning in and around the area surrounding pastures. There are other alternatives, as the use of natural products (Fernández, 2003; Zahid et al., 2006), the breeding and selection of cattle resistant to ticks, and the use of vaccines (Suárez et al., 2007). However, these strategies had not been enough for a sustainable control against *R. (Boophilus) microplus* in México. Some of the alternatives for tick control that are gaining importance today are the use of entomopathogenic fungus and some bacteria as well plants extracts with acaricide properties, that can be used in an effective integrated pest management with low environmental impact (Ginsberg et al., 2002). The entomopathogenous fungus is of extreme importance for the ectoparasites control, almost all the ectoparasites are susceptible to fungus diseases and there are 700 species of entomopathogenic fungi and about 100 genera (Monzón, 2001). Among these, the most important against ticks are *Metarhizium anisopliae*, *Beauveria bassiana* and *Leacanicillium lecanii*. Samsinakova et al., in 1974, were the first to report the efficacy of the entomophagous fungi against the tick *Ixodes ricinus*, with the presence of mycelia of *Beauveria* spp. In México, Assam (1993) found five fungus associated to *Boophilus* spp. ticks in two zones from the municipality of Tecomán Colima; in studies from august 28 to November 20 in 1988. Among these the genus *Beauveria* spp., *Verticillium* spp., *Fusarium* spp., *Entomophthora* spp. and *Aspergillus* spp. In 1989 Lezama and Hernández reported the *in vitro* sensibility of *Boophilus* spp. to the fungus *M. anisopliae* in the larval stage. In 1999 Samish and Rehacek found that: entomopathogenic fungi are the best ticks pathogens in nature due to their high virulence, wide distribution and ability to penetrate the host via cuticle. *In vitro* studies with *M. anisopliae* and *B. bassiana*, have shown high mortalities ranging from 85 to 100% on several developmental stages of *R. microplus*, *R. appendiculatus*, *R. sanguineus* and *Ixodes scapularis*. (Bittencourt et al., 1997, 1999b; Frazzon et al., 2000; Onofre et al., 2001). Lately, in México, Fernández et al., in 2005, by an *in vitro* study with engorged females,
compared the infectivity of *M. anisopliae* against two *R. microplus* strains susceptible and resistant to organophosphates and found that the resistant strain was more susceptible to the fungi effect than the susceptible strain. On other in vitro assay with a multiple resistant strain they found the same results. Alonso-Díaz et al., (2007), reported an efficient control of *R. microplus* in cattle naturally infested, of 80 to 90% with the *M. anisopliae* Ma34 strain based on engorged female account, after 7 days of treatment at a concentration of (10^8 spores per milliliter) 1x10^8 conidias/ml. Angel-Sahagún et al., (2010), in Colima, México, after a laboratory evaluation of the efficacy of 53 Mexican isolates of entomophagous fungus against *R. microplus* larvae; 33 of *Metarhizium anisopliae* and 20 of *Paecilomyces fumosorosea*, and selected the isolated 14 of *M. anisopliae* based upon its higher virulence. Lately, this isolated plots under field conditions, demonstrated a reduction in the larval population between 61 and 94.2% and conclude that the nature of the vehicles and emulsifier have a great impact in the efficacy of the conidial formulations.

México, has a great diversity of plants with medical and insecticidal properties, among these *Solanum verbascifolium*, is used in traditional medicine to cure several diseases (Argüeta et al., 1994), besides its insecticidal properties, due to the presence of glicoalcaloids as the solasolidina and solanina, it has become an interesting candidate as a tick control method of *R. microplus*.

*Bacillus thuringiensis*, is a gram-positive bacteria, aerobic strict, and its life cycle has two phases: vegetative growth, when the bacteria duplicate by bipartition and the sporulation. *B. thuringiensis*, is considered an ubiquitous bacteria since it has been isolated from several parts of the word and from diverse systems, like soil, water, plants leaves, insects bodies, spider webs, and others. *B. thuringiensis* is characterized by producing a body pore parasporal body known as crystal during its sporulation phase, of proteinic nature known by its insecticide properties. The crystal protein is an α-endotoxin also known as proteins Cry and Cyt, with insect activity against several orders within the: Lepidoptera, Coleoptera, Diptera and Hymenoptera, as well for acari and nematodes, flat worms and protozoa (Soberón y Bravo, 2008). Hassanain et al., (1997), performed an experiment in order to evaluate the effect of the three subspecies of *B. thuringiensis* (kurstaki, israeliensis and thuringiensis) against two tick species. They found that the specie *Argas persicus* (soft ticks) was the most affected by *B. thuringiensis* than the *Hyalomma dromedarii* specie (hard ticks). In the assays with soft ticks, at a dose of 1250 μg/ml of exotoxins of *B. thuringiensis* var. kurstaki, the achieved mortality was of 100% in a period of 36 hours at 5 days. Studies by Zihoua et al., (1999), exposed *Ixodes capelinus* to a *B. thuringiensis* varietie kurstaki, by the adult immersion test during 30 seconds in a spore suspension achieving a patogenicity with a CL<sub>50</sub> of 1x10^2 spores/ml.

In Fernández et al., (2006a) assays, to evaluate the effect of four *B. thuringiensis* strains against *R. microplus* resistant to ixodicides, by the adult immersion test (AIT) using a dose of 1 mg/ml during a period of 20 days, found that the *B. thuringiensis* GP138 strain achieved the higher mortality of 95%, followed by the GP123 strain with a 91%. The strains that achieved the lowest mortality were the GP140 strain with a 85%, followed by the GP139 strain with a 79%. When they evaluate the efficacy or effect of this strains upon oviposition, they found that the GP138 strain inhibited the 92%, followed by the GP140 strain with a 80%. The lowest percentage of inhibition on egg laying of *R. microplus* by effect of *B. thuringiensis* corresponded to the GP123 strain that inhibited the 70%. The GP138 strain was the only that showed a reduction on oviposition and egg hatching of *R. microplus* in parallel.

The specie *Solanum verbascifolium* (Solanaceae) is a shrub 2 to 6 m of high with high stem and branches that have trichomes woolly-star. The leaves are oval and lanceolate, acute, green colored. The flowers are White and its fruits are globose and yellow (Argüeta et al., 1994). *S. verbascifolium* is distributed in different regions of Mexico: Veracruz, Chiapas, Guerrero, Sonora, Oaxaca, Nuevo León, San Luis Potosí, Hidalgo, Sinaloa, Morelos and Yucatán, except in Baja California Sur. And it can be found in some Latinamerican countries like: Belice, Salvador, Guatemala, Honduras, Nicaragua, Chile, Venezuela, Puerto Rico, also in the United States, China and South Africa (Argüeta et al., 1994; Ockers et al., 2002; http://www.discoverlife.org/mp/20m?kind=Solanum+verbascifolium). This plant is used in traditional medicine to treat diabetes, acne, metrorrhagia (bleeding of the uterus), eczema, tooth pain, dermatitis, soothing, healing, athletes foot and vaginal candidiasis. (Zhou and Ding 2002; Lans et al., 2001; Pesewu et al., 2008). Besides, in the district of Samburu in Kenia, África; the leaves and fruits are use to treat some ectoparasits and gastroenteritis (Nanyingi et al., 2008). Among the different compound that have been isolated from *S. Verbascifolium* are two glycoalkaloids steroidal such as solavarina, I, II, III, and the sapogenoles solaverol A and B, the alkaloïds and steroids, solasodina, tomatidenol, diosgenina, vespertilina, solatriosido, chacotriosido, solanidina, solaverina, and solasodina one of the mayor components. There have isolated a cinnamic derivative, ethyl p-hydroxyphenyl, p-cumaramida and vanillic acid (Yamashita et al., 1990; Weissenberg, 2002; Zhou and Ding, 2002). The genus *Solanum* has solasodina and solanidina, steroidal alkaloïds which play...
an important role protecting the plant from pathogens and herbivores (Lee et al., 2007). Mosquera et al., (2004), and Niño et al. (2006), in an in vitro experiment demonstrated that methanol and dichloromethane extracts from the species Solanum witheringia, S. coccolohoides, Solanum sp, S. deflexiflorum and S. leucocarpum, solasodine and solanidine are the main inhibitors of acetylcholinesterasa (AChE), acting in a similar way to the ixicidices organophosphates and carbamates, that inhibit the acetylcholinestena complex, that hidrolized acetylolinia. Fernández et al., (2007), performed an experiment to evaluate the effect of aqueous and methanolic extracts from leaves and stems of S. verbascifolium on R. (Boophilus) microplus engorged females. They found that the methanolic extract from the stem at a concentration of 40 000 ppm was the one with the highest mortality on day 5th with a 43.7% on R. (Boophilus) microplus. Gómez (2009), evaluated the effectivity of aqueous and methanolic extracts from leaves and stems of S. verbascifolium against R. (Boophilus) microplus. Besides, he determine the lethal concentrations 50 (CL50) and 90 (CL90) from the same extracts, using the adult immersion test. He obtained a mortality of 57.5% at a concentration of 20mg/ml 10 days post-treatment with the stem dry extract 60% at a concentration of 60mg/ml, of 67% at a concentration of 70mg/ml and of 87% at a concentration of 80ml/ml for the dry extract from the stem dry methanolic. He also, obtained a 92% at a concentration of 70mg/ml and 95% at a concentration of 80mg/ml for the methanolic dry leaf extract. The evaluated extracts had a lethal concentration of 50 (CL50) the leaf extract had the highest toxicity (CL50=38.19mg/ml) followed by the stem extract (CL50=54.47 mg/ml).

The objective of the present study was to find an option to use in an Integrated Pest Management. With the aim to use natural products to gradually reduce the use of conventional chemicals. With the use of the entomopathogenic fungi, Metarhizium anisopliae and Bacillus thuringiensis, and extracts from plants that have shown biocidal or biostatic activity that can be used for the control of livestock pests of economic importance like the R. (Boophilus) microplus tick. The main objective of this study was to determine by stable test conditions the biological efficacy of natural products (Solanum verbascifolium, Metarhizium anisopliae and Bacillus thuringiensis), sprayed on cattle artificially infested for the control of R. (Boophilus) microplus.

**MATERIALS AND METHODS**

**Obtaining natural products**

The extraction of the different natural products was done at the Laboratory of the Investigation Centre in Biotechnology of the University of the State of Morelos.

**Obtaining the solanum verbascifolium extract**

The flowers, leaves, stems and fruits from Solanum verbascifolium were collected in the municipality of Villa de Ayala, state of Morelos. The fresh stems were separated and cut in small pieces. One kilogram of this material was introduced in a glass containers in a solution of 20 L of methanol/kg during 72 h. Lately, the macerated was filtered and concentrated in a rotary evaporator to take to complete dryness and placed in a dehydrator. The dry extract was weighed and dissolved with distilled water to obtain the corresponding dilutions for the test.

**Bacillus thuringiensis**

The Bacillus thuringiensis GP543 used in this study is a native strain from the collection of the Laboratory of Plant Parasitology from the Center of Biological Investigations of the University of Morelos. The pathogenicity of this strain was previously demonstrated by in vitro bioassays and adult immersion test on R. (Boophilus) microplus engorged females (unpublished data). The GP543 strain was cultivated in a Luria Bertoni broth (LB) with 10g of peptone, 5g of yeast extract, 10g of sodium chloride and 15g of agar. After 72 h the sterile water was recovered (a layer of 5 ml of sterile water) and added a serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 100 mM/ml of water. The total protein concentration was quantified by the Bradford technique (Bradford, 1976).

**Metarhizium anisopliae Inoculum preparation**

Metarhizium anisopliae strain (Ma379) belong to the collection of the National Reference Center of Biological Control (SAGARPA-DGVS) at Tecomán, state of Colima, México, isolated from Aenolamia postica (Walker), maintained and reproduced at the Laboratory of Biological Control from the Center of Investigation in Biotechnology of the Laboratory of Biological Control at the University of Morelos and at the National Center of Disciplinary Research in Veterinary Parasitology (CENID-PAVET). The M. anisopliae conidial production was obtained by the solid fermentation system of Berlanga and Hernández (1997). The Ma379 strain was grown on Saboraud dextrose agar (SDA), on agar slant tubes. With a inoculation loop the spore sample was inoculated on
the medium by streaking an incubated at 28ºC ± 1ºC and 70 RH% for 10 days, until fungi sporulation.

Substrate preparation

For the solid fermentation phase 4 k of rice grain was used as substrate, previously washed tree times with top water and left to stand in tetracycline at 130 ppm in a hot solution (75ºC ± 5ºC) during 30 min. The water was drained out and the rice was left for 20 min.

After that, the rice was introduced in (polypropylene) polyethylene bags of high density in aliquots of 300 g/bag, sealed and grapped with a rubber band. Bags were sterilized at 120ºC for 20 min (120 Kg/ cm²) in an autoclave. After cooling during 24 h at room temperature, the bags with the substrate were inoculated with the fungus.

Inoculation of the polipapel bags

To recover the inoculum from the test tube we added 20 ml of sterile distilled water with dispersant 1%Tween 20, and with an inoculation loop an inoculum of the conidia was removed. The conidia suspension was transferred into a flask with 500 ml of the same solution (water + dispersant), and homogenized by agitation. To inoculate the rice, the rubber from the bag was removed and added 10 ml of the inoculum in each bag. Bags were agitated manually to facilitate impregnate the suspension in all the grains, and labeled indicating the date, batch number and pathogen. All the process was performed under a laminar flow hood.

The bags with the sustrate and inoculum were incubated in a growing room at 28 °C ± 1 °C with a photoperiod of 14:10 h light/dark, for 16 ± 2 days, to achieve the highest sporulation percentage.

During this time was necessary to move the bags by hand every four days, in order to increase the sporulation surface, and thereby the amount of spores/bag.

Conidial concentration

To obtain a suspension, 0.5 g of the final product were added in 100 ml of water with a drop of dispersant (Tween 20 at 1%). The conidial concentration in the suspension was determinate in a Neubauer haemocytometer. The number of conidial/ml was multiply by the volume of the dilution to achieve the number of conidia/g.

Conidial viability

A small amount of conidium were suspended in 20 ml of sterile water with a drop of dispersant. We took a drop from this suspension and placed in a plastic Petri dish (100 mm) with Sabouraud dextrose agar (SDA) and extended in all the medium surface, with the aid of a sterile glass rod. The medium with the conidium was incubated at 28ºC and 70%RH during 16 h (Moore and Higgins, 1997). The conidia germination was assessed and quantified under a contrast phase microscope at 40X, the number of conidia germinated and not germinated was counted in each Petri dish until the number achieved 300 conidial (Hedgecock et al., 1995; Moore et al., 1995). The criteria to determinate the conidia germination was that the germ tube was equal to at least half of the long axis of the conidium (Moore and Langewald, 1997). The percentage of conidium germination was calculated only to the samples that achieved a 90% viability, using the next formula:

\[
\frac{a}{a+b} \times 100\%
\]

Were:

\(a\) = conidia germinated.
\(b\) = conidia not germinated.

Chemical product

The chemical product was used as a control group positive to amidina (amitraz, Taktic) the dose was prepared following instructions in the label (1ml of amitraz/10 L of water).

Stable test with infestation chambers

This experiment was carried out at the National Center of Services of Constatation on Animal Health (CENAPA), located at Km 11.5 Federal road Cuernavaca-Cuautla in Progreso, municipality of Jiutepec, Morelos, México. To determinate the biological activity of the different products of natural origin, on the parasitic phase of *R. (Boophilus) microplus*, by the stable test with infestation chambers proposed by Dowin et al., (1977) was selected as a feasibility study.

To avoid a host-parasite response variation, expected when several animals are tested, and the impact on the results, the following methodology was used, isolate pre-stablished areas on the animal body, with the objective to test the several products by repeated infestations in order
to have at the same time ticks of several development stages, including the moulting stages, before treatment was applied (Osorio, 1989).

Besides, with the collected ticks during the post-treatment period were made observations in vitro to compare with the following infestations.

Seven steers Aberdeen Angus (approximately 6-8 months old) weighing 200 to 300 kg each were use in this experiment, due to their low resistance to ticks. Throughout the study each animal were stalled individually and provided by water and food ad libitum, daily monitoring of the infestation chambers was performed to avoid their detachment.

The infestation areas selected were on the back of the animals, with a total of four areas, two on each side of the animal, were the infestation chambers were attached. The area of attachment of the chambers was shaved with 4-5 cm wide to make a circle 20 cm diameter, the fabric of the infestation chamber measure 15 x 30 cm attached to the shaved skin of the animal with a contact glue one day before the first infestation. The open side of the infestation chamber was closed with a rubber band to allow the daily observations and collection of ticks, and to avoid their scape. Each day the chambers were monitored to avoid their detachment.

The infestations begin one day after the chambers were attached, with R. (Boophilus) microplus larvae susceptible to acaricides a strain named “Meda Joya”. Each animal was infested with 15 mg of larvae in each infestation chamber equivalent 555 larvae (2-week-old larvae) additional larval infestation were performed on ten separate occasions, each third day, during a period of 21 days between the first and the last infestations. This infestation regime allows the collection of engorged females before the application of the first treatment, being necessary to calculate the percentage of control.

The pretreatment collection of ticks from the chambers began when engorged female ticks were detected. And it was carried out during three days, to assure the infestations were done correctly. Engorged female ticks were collected daily and taken to the laboratory a selected group of 20 females, weighed and placed in plastic Petri dishes (9 cm diameter, 1.5 cm high), labeled with the day of collection and incubated at 27 ± 1°C, 80-90% RH. This was done daily during the pre-treatment and post-treatment periods. The four treatments were applied 23 days after the first infestation with larvae of R. (Boophilus) microplus susceptible strain.

The treatments tested in this study were: G1 A mixture of Solanum verbascifolium at 20,000 ppm + Metarhizium anisopliae strain at a concentration of 379 x10^6 conidia/ml, G2 emulsion of Metarhizium anisopliae strain 379 at a concentration of 1x10^6 conidia/ml, G3 extracts of Solanum verbascifolium methanolic at 70,000 ppm, G4 The GP543 Bacillus thuringiensis strain, G5 Positive control. Chemical product (Amitraz), G6 Negative Control, emulsion mineral oil, G7 negative control. (water + dispersant). The distribution of the different treatments on the animal body during the experiment are shown in Table 1.

The animals for the study were randomly selected, to receive the treatment according to the experimental design for infestations. In all cases, the treated group and the control group received, four repetitions, for each chamber.

The application of all treatments was made with a hand sprayer using 250 ml/each chamber with the aim to facilitate the impregnation of the area with the solution. Animals in the control group were treated with a control solution consisting of water and mineral oil plus a dispersant without conidia. This was used as negative controls, and the group sprayed with the commercial product (amitraz), was used as positive control. All treatments received the same volume.

The days following the application of the treatments, were considered as post-treatment period. The engorged female tick collection and counting inside the infestation chambers of the treated and control groups, was done daily during 21 days. The number of engorged female ticks recovered on each animal/chamber/treatment, was recorded and registered.

The total of semi engorged and engorged female ticks (ticks of 4.5-8.0 mm) (Wharton and Utech, 1970) collected

### Table 1. Distribution of Dowing chambers for 7 treatments in a stable experimental test in bovine.

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each day/chamber on the control and treated groups, were counted, weighed, and recorded. From this group 20 ticks were selected and placed in plastic Petri dish, with a label of the day of collection. The selected biological material was incubated at 28°C and 80-90% of RH during 14 days to allow oviposition. Later, the egg mass collected from each group, were weighed and transferred into glass vials (5 ml) closed with a cotton lid and incubated for 14 days until larval hatching. The vials were incubated under the same conditions as the adult females.

At 26 days of egg incubation (time necessary for egg hatching) vials are removed from incubators and determine the hatch percentage (data). The vials with larvae were introduced in an oven to kill the larvae by heat at 45°C during 20 minutes.

To calculate the daily percentage egg hatch for the control and treated groups, the egg and larvae of each vial was thoroughly mixed, and visually estimated the proportion of larvae to the proportion of unhatched eggs under a stereo microscope.

The analysis of all data and results were made to establish differences due to the treatments in the experimental groups, the following biological parameters were determined by the following equations. For each variable we use an analysis of variance a non parametric test for multiple ranges Wilcoxon, by the SAS statistical analysis software version 9.1.

Variables evaluated

Percent effectiveness upon repletion (%E/R).

This variables allow us to determine the number of ticks that reach engorgement after treatment. To be able to obtain it is necessary to know the number of engorged female tick collected daily during the pre and post-treatment periods, in all the groups (control and treated):

\[
\text{Efficacy } \% = 1 - \frac{(A \times D)}{(B \times C)} \times 100
\]

Where:
- A = Mean tick numbers on the control group before treatment.
- B = Mean tick numbers of the control group by day, post-treatment.
- C = Mean tick numbers on treated group before treatment.
- D = Mean tick numbers on treated group by day, post-treatment.

Percentage inhibition of oviposition (% I.O.).

This variable allow us to know, the percentage of egg laid by the females after the treatment. After 14 days of incubation of the engorged female, egg mass was collected, weighed, recorded and incubated under the conditions mentioned above. The effect of the products on egg laying was calculated by the relation of the number of eggs / weight of engorged females by group and batch:

\[
\frac{\text{weight of eggs laid (g) (20,000)}}{\text{weight of females (g)}}
\]

This parameter was calculated, for treated and control groups as follows:

\[
\frac{(\text{OP/T} - \text{OP/t})}{\text{OP/T}} \times 100
\]

Where:
- O/PT = Estimated reproduction of the control group.
- O/PT = Estimated reproduction of the treated group.

Inhibition of reproductive potential (IRP)

These variables allow us to know the control of the product applied on tick at the moment of completion of the life cycle. by the following equation:

\[
\% \text{IRP} = 1 - \frac{(A \times (B \times C \times D))}{(B \times C \times D)} \times 100
\]

Where:
- A, B, C and D are the same data mentioned to calculate the effectiveness on the repletion.
- #XL = Mean number of viable larvae, obtained by the following:

\[
\# \text{XL} = (\text{Phg} \times (\text{X} \times 100)) \\
\text{Phg} = \text{weight of eggs (g)}
\]

0,000 = Constant, mean number of larvae in one gram.
- F.C. % Ecl. = Fraction centesimal of the percentage of larval hatching.

This calculations were performed per day and globally. The data obtained allowed to analyze the treatment effect under controlled conditions.

RESULTS

Effect of the natural origin products on R. (Boophilus) microplus

Engorged female ticks Numbers

Once detected the presence of engorged female ticks
inside the infestation chambers, a pre-treatment collection begin during three days, with the aim to detect their presence inside each infestation chambers. In this part of the experiment, one animal achieved a low number of engorged females and was selected as positive control (amitraz). Table 2.

Post-treatments, daily collection and counts of engorged females were performed. For the statistical analysis of the treatments, collections were made in groups from 7, 14 and 21 days, for each different stage of development (adult, nymph and larvae).

The numbers of engorged female of R. (Boophilus) microplus collected seven days post-treatment are given in Table 3. The treatment was applied on adult engorged females. When performing an analysis of variance for this variable, we found significant statistical differences (P< 0.001) between the treatments. On comparing the media for this variable, we can observe that the treatment with amitraz achieved the best control of engorged females, since only 9.50 engorged females were recovered from the chambers, followed by the combination of S. verbascifolium + M. anisopliae with 40.50, M. anisopliae with 52.25 and B. thuringiensis with 52.75, being these two last treatments statistically similar. The treatments with a lower control and a high number of engorged ticks after the treatment application were S. verbascifolium plus water and a dispersant with 211.25 and 267 respectively, being these two statistically similar to each. However, the treatment with the highest number of engorged females collected from the infestation chambers was the mineral oil with a total of 499.25, being this treatment statistically different to the others.

The results of engorged female of R. (Boophilus) microplus collected at the 14 days post-treatment against ticks that were nymph at the time of the treatment are shown on Table 3. The analysis of variance for the variable number of engorged female collected at 14 days showed a significant statistical difference (P< 0.001) between treatments. Once the analysis for comparison of means, we can see that the treatment with amitraz presented the best tick control, since no engorged female was recovered from the chambers, followed by the B. thuringiensis with 1.25 engorged females recovered, M. anisopliae and S. verbascifolium + M. anisopliae with 6.25 each one, being all of these three treatments statistically similar between them. Treatment with S. verbascifolium show a un lower control with 76.25 engorged tick recovered. However, it was statistically similar to previous treatments. However, the treatment with the highest number of engorged females collected from the chambers, was the mineral oil with 290, being this treatment different to the others.

The number of engorged females of R. (Boophilus) microplus collected at the 21 days post-treatment against ticks that were larvae at the time of the treatment are shown in Table 3. The statistical analysis of this variable show significant statistical differences (P< 0.001) between treatments. In the analysis for comparison of means, most of the treatments are statistically similar, being the best treatment amitraz, since no engorged females were recovered from the infestation chambers, followed by the B. thuringiensis with 0.50, M. anisopliae with 0.75, S. verbascifolium + M. anisopliae with 3.5, S. verbascifolium with 3.5 plus water plus a dispersant with 16.5 engorged females collected from the chambers, being this treatments statistically similar. However, the only treatment that show the highest number of engorged females collected, was the mineral oil with 63.5, being this statistically different to the others. Table 3.

### Table 2. Mean of engorged females collected from the infestation chambers, on each animal, 3 days after treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>X</th>
<th>(SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral oil emulsion</td>
<td>232.00</td>
<td>±16.87</td>
</tr>
<tr>
<td>S. verbascifolium</td>
<td>164.33</td>
<td>±20.68</td>
</tr>
<tr>
<td>S. verbascifolium + M. anisopliae</td>
<td>108.33</td>
<td>±34.51</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>91.00</td>
<td>±19.41</td>
</tr>
<tr>
<td>Water+dispersant</td>
<td>78.66</td>
<td>±28.02</td>
</tr>
<tr>
<td>Bacillus thuringiensis</td>
<td>74.00</td>
<td>±13.63</td>
</tr>
<tr>
<td>Amitraz</td>
<td>3.33</td>
<td>±0.96</td>
</tr>
</tbody>
</table>

Weight of engorged female of R. (Boophilus) microplus

The total weight of engorged females of R. (Boophilus) microplus are given in Table 4 at seven days post-treatment. The analysis of variance shows significant statistical differences (P< 0.001) between treatments. The media comparison shows that the treatment with amitraz applied to ticks was the one that achieved the lowest weight on females collected from the chambers with 0.42 g, followed by S. verbascifolium + M. anisopliae with 8.34 g, M. anisopliae with 11.13 g and B. thuringiensis with 15.05g, being these treatments statistically similar. The treatments with S. verbascifolium plus water plus a dispersant weighted 62.92 and 90.06 g
respectively, with an increase in the weight of the engorged females recovered, being statistically similar to each other. However, the treatment with the highest weight of females recovered from the infestation chambers, was the mineral oil with 135.85 g, being this treatment statistically different to the others.

The weight of engorged females of R. (Boophilus) microplus at 14 days post-treatment and that at the time of the application ticks were in the nymph stage, the analysis of variance show significant statistical differences (P< 0.001) between treatments. When comparing the medias (Table 4) we can see that the treatment with amitraz provided a reduction in the weight of the females collected with 0 g, followed by B. thuringiensis with 0.25 g, S. verbascifolium with 1.36 g and M. anisopliae with 1.61 g, being statistically similar. The treatments that showed a highest weight in the females recovered post-treatment inside the chambers were S. verbascifolium with 29.49 g plus water and a dispersant with 41.43 g which are statistically similar. The treatment with the highest engorged female weight, recovered from the infestation chambers, was the mineral oil with 81.23 g, being this treatment statistically different to the others.

With respect to the variable weight of engorged female of R. (Boophilus) microplus at 21 days post-treatment and at the time of treatment the ticks were in the larvae stage (Table 4). The analysis of variance show significant statistical differences (P< 0.001) between treatments. When comparing the medias between treatments, we can see that the treatment with amitraz was the one that gave a higher reduction on female weight, M. anisopliae with 0.81 g, S. verbascifolium with 1.01 g and water plus a dispersant with 4.44 g being statistically similar. However, the treatment with mineral oil was the one with the lowest reduction upon female weight with 16.16 g, being statistically different to the other treatments. Table 4.

### Table 3. Mean number of engorged females from infestation chambers, grouped by 7, 14 and 21 days, each of different stage (adult, nymph and larvae) at the time of the treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>At 7 days</th>
<th>At 14 days</th>
<th>At 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X (EE)</td>
<td>X (EE)</td>
<td>X (EE)</td>
</tr>
<tr>
<td>Mineral oil emulsion</td>
<td>499.25 ±68.66 a</td>
<td>290.00 ±52.37 a</td>
<td>63.5 ±15.41 a</td>
</tr>
<tr>
<td>Water+dispersant</td>
<td>267.00 ±20.42 b</td>
<td>135.25 ±23.84 b</td>
<td>16.5 ±5.56 b</td>
</tr>
<tr>
<td>S. verbascifolium</td>
<td>211.25 ±34.20 b</td>
<td>76.25 ±12.33 bc</td>
<td>3.5 ±1.71 b</td>
</tr>
<tr>
<td>B. thuringiensis.</td>
<td>52.75 ±14.08 c</td>
<td>1.25 ±0.75 c</td>
<td>0.50 ±0.50 b</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>52.25 ±19.09 c</td>
<td>6.25 ±2.53 c</td>
<td>0.75 ±0.48 b</td>
</tr>
<tr>
<td>S.v.+ M.a.</td>
<td>40.50 ±26.17 c</td>
<td>6.25 ±3.35 c</td>
<td>3.5 ±1.44 b</td>
</tr>
<tr>
<td>Amitraz.</td>
<td>9.50 ±1.94  c</td>
<td>0.00 ±0.00  c</td>
<td>0.00 ±0.00 b</td>
</tr>
</tbody>
</table>

* means with the same letter are not significantly different a0.05.
EE= Standard Error

### Egg mass weight produced by R. (Boophilus) microplus.

With respect to the effect of the treatment on the adult female R. (Boophilus) microplus upon egg mass weight produced on day 7th post-treatment, the variance analysis show significant statistical differences (P< 0.001) among treatments. When comparing the media, the treatment with amitraz achieved the lowest egg production followed by S. verbascifolium + M. anisopliae with 0.81 g, and M. anisopliae with 0.94 g, being this treatments statistically similar. The treatment with B. thuringiensis produced an egg mass of 6.71 g, being statistically different to the previous treatments and to the treatments with mineral oil with 16.41 g, water plus dispersant with 17.14 g and S. verbascifolium with 17.51 g, being statistically similar (Table 5).

The egg weight in grams produced by the engorged females at the 14 days post-treatment when the ticks were in the stage of nymph, the analysis of variance show a significant statistical differences (P< 0.001) between treatments. In the media comparison analysis (Table 5) we can see that the product amitraz was the treatment with a lower egg mass production with a weight of the recovered females of 0 g, followed by B. thuringiensis with 0.04 g, S. verbascifolium + M. anisopliae with 0.12 g, M. anisopliae with 0.14 g, being this statistically similar. The treatment with S. verbascifolium obtained 6.11 g of egg, 6.11 g, being statistically different to previous treatments and to the treatment with water plus a dispersant with 12.19 g and mineral oil with 13.71 g, with a greater quantity of egg mass with respect to the previous with a higher egg mass production with respect to the previous, being this statistically similar.

The eggs mass weight in grams produced by engorged females of R. (Boophilus) microplus at 21 days post-treatment applied when the ticks were in the larvae stage,
Table 4. Mean weight of engorged females collected from chambers, from groups of 7, 14 and 21 days each corresponding to a stage of development (adult, nymph and larvae).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>At 7 days</th>
<th>At 14 days</th>
<th>At 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X (g.) (EE)</td>
<td>X (g.) (EE)</td>
<td>X (g.) (EE)</td>
</tr>
<tr>
<td>Mineral oil emulsion</td>
<td>153.85 (±23.38)</td>
<td>81.23 (±18.02)</td>
<td>16.16 (±4.05)</td>
</tr>
<tr>
<td>Water+dispersant</td>
<td>90.06 (±8.46)</td>
<td>41.43 (±8.47)</td>
<td>4.44 (±1.48)</td>
</tr>
<tr>
<td>S. verbascifolium</td>
<td>62.92 (±12.95)</td>
<td>20.49 (±4.27)</td>
<td>1.01 (±0.41)</td>
</tr>
<tr>
<td>B. thuringiensis.</td>
<td>15.05 (±4.09)</td>
<td>0.25 (±0.15)</td>
<td>0.14 (±0.15)</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>11.13 (±5.00)</td>
<td>1.61 (±0.67)</td>
<td>0.81 (±0.19)</td>
</tr>
<tr>
<td>S.v.+ M.a.</td>
<td>8.34 (±6.21)</td>
<td>1.36 (±0.15)</td>
<td>0.27 (±0.47)</td>
</tr>
<tr>
<td>Amitraz.</td>
<td>0.42 (±0.14)</td>
<td>0.00 (±0.00)</td>
<td>0.00 (±0.00)</td>
</tr>
</tbody>
</table>

*Means with the same letter are not significantly different α0.05.
EE= Standard Error

Table 5. Mean weight of eggs produced by engorged females collected by chamber at 7, 14 and 21 days each covering a different parasitic life stage (adult, nymph and larvae).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>At 7 days</th>
<th>At 14 days</th>
<th>At 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X (g.) (EE)</td>
<td>X (g.) (EE)</td>
<td>X (g.) (EE)</td>
</tr>
<tr>
<td>Mineral oil emulsion</td>
<td>16.41 (±0.79)</td>
<td>13.71 (±1.39)</td>
<td>5.29 (±1.38)</td>
</tr>
<tr>
<td>Water+dispersant</td>
<td>20.49 (±1.30)</td>
<td>12.19 (±1.11)</td>
<td>2.23 (±0.76)</td>
</tr>
<tr>
<td>S. verbascifolium</td>
<td>17.14 (±1.10)</td>
<td>6.11 (±1.29)</td>
<td>0.33 (±0.15)</td>
</tr>
<tr>
<td>B. thuringiensis.</td>
<td>15.05 (±4.09)</td>
<td>0.04 (±0.04)</td>
<td>0.08 (±0.08)</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>0.94 (±0.57)</td>
<td>0.14 (±0.07)</td>
<td>0.09 (±0.06)</td>
</tr>
<tr>
<td>S.v.+ M.a.</td>
<td>0.81 (±0.52)</td>
<td>0.12 (±0.09)</td>
<td>0.33 (±0.15)</td>
</tr>
<tr>
<td>Amitraz.</td>
<td>0.00 (±0.00)</td>
<td>0.00 (±0.00)</td>
<td>0.00 (±0.00)</td>
</tr>
</tbody>
</table>

*Means with the same letter are not significantly different α0.05.
EE= Standard Error

the analysis of variance shown significant statistical differences (P< 0.001) between treatments. When comparing medias (Table 5), the treatment with amitraz achieved the lower egg mass laying of 0 g, followed by B. thuringiensis with 0.08 g, M. anisopliae with 0.09 g, S. verbascifolium + M. anisopliae with 0.33 g, S. verbascifolium with 0.33 g and water plus a dispersant with 2.23 g being statistically similar. The treatment with mineral oil produced 5.29 g of eggs being statistically different to the others. Table 5.

Egg hatching percentage of R. (Boophilus) microplus.

The hatching percentage of engorged females collected 7 days post-treatment and at the time of the treatment the tick was in the adult stage the analysis of variance shows significant statistical differences (P< 0.001) between treatments. When comparing the medias between treatments (Table 6) in the treatment with amitraz no hatching was achieved being this statistically different to the other treatments. Treatments with S. verbascifolium + M. anisopliae and M. anisopliae achieved a considerable reduction on hatching percentage of 22.42 and 26.40% respectively, being statistical similar. Treatments with S. verbascifolium had a 50.41%, mineral oil a 51.21%, water plus dispersant with 65.02% and B. thuringiensis with 66.61%, no significant reduction on the hatching percentage with respect to the others treatments, being statistically similar.

The hatching percentages of engorged females collected at 14th days post-treatment, and at the time of the treatment the tick was on the nymph stage, the analysis of variance demonstrated a significant statistical difference (P< 0.001) between treatments. When media comparison (Table 6), treatments with Amitraz, B. thuringiensis, M. anisopliae and S. verbascifolium + M. anisopliae shown a marked reduction on hatching percentage of 0, 2.74, 7.55 and 12.40% respectively, being these treatments statistically similar. The treatments with mineral oil achieved a hatching
Table 6. Hatching percentages at 7, 14 and 21 days per stage of development (adult, nymph and larvae).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>At 7 days</th>
<th>At 14 days</th>
<th>At 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (EE)</td>
<td>% (EE)</td>
<td>% (EE)</td>
</tr>
<tr>
<td>Mineral oil emulsion</td>
<td>51.21 (±2.30) a</td>
<td>45.41 (±1.86) a</td>
<td>40.60 (±5.44) ab</td>
</tr>
<tr>
<td>Water+dispersante</td>
<td>65.02 (±1.37) a</td>
<td>50.67 (±0.99) a</td>
<td>53.79 (±7.73) a</td>
</tr>
<tr>
<td>S. verbascifolium</td>
<td>50.41 (±3.63) a</td>
<td>47.21 (±2.73) a</td>
<td>19.98 (±10.99) bc</td>
</tr>
<tr>
<td>B. thuringiensis.</td>
<td>66.61 (±3.03) a</td>
<td>2.54 (±2.37) b</td>
<td>0.44 (±0.45) c</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>26.40 (±9.86) b</td>
<td>7.55 (±5.19) b</td>
<td>2.90 (±2.91) c</td>
</tr>
<tr>
<td>S.v.+ M.a.</td>
<td>22.42 (±4.42) b</td>
<td>12.40 (±7.43) b</td>
<td>8.75 (±1.41) c</td>
</tr>
<tr>
<td>Amitraz.</td>
<td>0.00 (±0.00) c</td>
<td>0.00 (±0.00) b</td>
<td>0.00 (±0.00) b</td>
</tr>
</tbody>
</table>

X² = 24.73.

**Means with the same letter are not significantly different α0.05.
EE= Standard Error.

percentage of 45.41%, S. verbascifolium with 47.21% plus water plus a dispersant of 50.67%, not presented a marked reduction on hatching, being statistically similar each other.

The hatching percentages of engorged females collected 21 days post-treatment and at the time of the treatment the tick was in the larvae stage, the analysis of variance show significant statistical differences (P<0.001) between treatments. When comparison of media between treatments (Table 6), the chemical product amitraz achieved a hatching percentage of 0%, followed by B. thuringiensis with 0.44%, M. anisopliae with 2.90%, S. verbascifolium + M. anisopliae with 8.75% and S. verbascifolium with 19.98%, being this treatments statistically similar. The treatment with mineral oil plus water and a dispersant were not efficacies upon hatching reduction since they show a 40.60% and 53.79% respectively, being statistically similar. Table 6.

The results obtained by the stable test with the infestation chambers, challenging the biological activity of different natural products for the control R. (Boophilus) microplus, formulated to be applied by aspersion on cattle artificially infested, show on Table 7, the control percentages or inhibition of the reproductive potential (%I.P.R.) reached values higher than 80%, with the exception of the treatment with mineral oil, that achieved lower percentages of 60% and was used as positive control with amitraz.

Results obtained from larvae of R. (Boophilus) microplus, treated with Bacillus thuringiensis, M. anisopliae, S. verbascifolium + M. anisopliae and S. verbascifolium, achieved a control percentage upon the reproductive potential of 99.99%, 99.46%, 98.93% and 97.84% respectively, very close to the control achieved with the commercial product Amitraz of 100%.

From the results obtained with the nymph stage of R. (Boophilus) microplus, we can observed that treatments with the highest control upon the reproductive potential are B. thuringiensis, S. verbascifolium + M. anisopliae and M. anisopliae, with 99.99, 99.91 and 99.73% respectively, also very close to the 100% obtained for this stage with the commercial product.

The assays with the adult parasitic phase R. (Boophilus) microplus demonstrated that treatments with the highest control percentage over the reproductive potential with values more close to the use of the chemical product Amitraz with 100%, are treatments with S. verbascifolium + M. anisopliae, with a 99.28 and 98.17% of control respectively. Table 7.

**DISCUSSION.**

Cattle ticks have being traditionally controlled with the use of chemical acaricides, however this had, adverse effects on the non-target organisms and the environment. The intensive use of treatments with chemical products during long periods used for the control of R. (Boophilus) microplus has selected for resistance rendering these products ineffective. Due to these inconvenience has emerged the need for alternative control methods including the enthomopathogenic fungi M. anisopliae, considered one of the most promising agents for biological control of ticks, and also but in lesser extent, the enthomopathogenic bacteria B. thuringiensis (Kaaya and Hassan, 2000; Gindin et al., 2001; Leemon et al., 2008). The products of natural origin among them plant extracts are promising alternatives strategies since these have active molecules that can be used as acaricide control (bioregulators) and are degraded in the environment, with the target insect developing resistance more slowly than for the chemical acaricides (Fernandes and Freitas, 2007).

In this study, the variable engorged female numbers collected, was decreasing according to the collection date (7, 14 and 21), being 21 days where a better control was achieved by the treatments, in relation with the time of the treatment the ticks were in the larvae stage. The
treatment with the chemical product amitraz achieved the best control, since no engorged females were recovered. However, it is the treatment that causes environmental pollution and produces tick populations resistant to the chemical.

An important point in this study is that among the alternatatives evaluated the bacteria *B. thuringiensis* shows the reduction of adult female as collection day were increasing and it was day 21 when the best effect was found comparing with the control group, which agree with Hassanain *et al.*, 1997 findings with *H. dromedarii* had higher mortality at 10 days after treatment.

Similar results of Fernández *et al.* (2006a), found that mortality increases significantly from 15 and 20 days post-treatment with the same bacteria by the method immersion adult females.

However, regarding the female numbers recovered from the treatment with *Solanum verbascifolium*, at the 7 days when this variable was evaluated no difference was found in relation with the control group with water + dispersant, not being the same for the 14th and 21 days post-treatment were we can observed a reduction in the engorged females numbers of *R. (Boophilus) microplus* recovered. These results are according with the obtained by Gómez (2009), were he found that the methanolic extracts from dry stems and leaves of *S. verbascifolium* by the adult immersion test of *R. (Boophilus) microplus*, were the most lethal at 5 and 10 days post-assay. Similar results were obtained by Alvarez *et al.*, (2008), with the hydroalcoholic extract of *Cinnamomum zeylanicum* and the root of *Morus alba* achieving a mortality of 100% and 68% respectively after 10 days of exposure. The specie *S. verbascifolium*, synthesized compounds as saponins glicoalcaloideas and solasolidina which act as inhibitors of the acetylcholinesterase enzyme *in vitro*, causing hyperactivity of the insect nervous systems (Gómez, 2009).

Alonso-Diaz *et al.*, (2007) with cattle artificially infested found by engorged female records an efficient control of *R. (Boophilus) microplus* from 80 to 90% with the *M. anisopliae* Ma34 strain after 7 days of treatment at a concentration of $1 \times 10^8$ conidias/ml, which agrees with the results obtained in this study, showing that after 14 days post-treatment there is a considerable control, which reaches a 98% in all the female stages with relation to the control group. In the same study, the Ma34 strain show an greater efficacy than 45% from the second treatment until the end of the experiment agreeing with De Castro *et al.*, (1997) *in vivo* studies with a different strain of *M. anisopliae* found a reduction lower than 50% on the population of *Boophilus microplus* treated. However, in the study by Alonso-Diaz *et al.* (2007), no engorged females were collected and monitoring in laboratory to determinate the effect on the reproductive parameters.

The mixture of *M. anisopliae* and *S. verbascifolium*, produced a notorious reduction on the number of females collected from the application of the treatment, being the nymph and larvae stages, at the 14 and 21 days respectively post-treatment when we can observe a lower number of recovered engorged females of *R. (Boophilus) microplus*.

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**Table 7.** Control percentages on each parasitic stage of *R. (Boophilus) microplus* for the reproductive parameters Efectivity upon engorgement (%E/R), Inhibition of the Oviposition (%I.O) and Inhibition of the reproductive potential (%IRP)

| Stages | Treatment | %.E|R | %I.O | %IRP |
|--------|-----------|------|------|-------|
| Larvae | *S. verbascifolium*+ *M. anisopliae* | 89.07 | 33.18 | 98.93 |
|        | *M. anisopliae* | 94.28 | 29.54 | 99.46 |
|        | *S. verbascifolium* | 89.97 | 17.12 | 97.84 |
|        | Amitraz. | 100 | 100 | 100 |
|        | *Bacillus thuringiensis* | 98.02 | 0 | 99.99 |
|        | Mineral oil emulsion | 0 | 26.52 | 40.42 |
| Nymph  | *S. verbascifolium*+ *M. anisopliae* | 97.42 | 75.11 | 99.91 |
|        | *M. anisopliae* | 95.44 | 75.36 | 99.73 |
|        | *S. verbascifolium* | 74.27 | 11.1 | 84.24 |
|        | Amitraz. | 100 | 100 | 100 |
|        | *Bacillus thuringiensis* | 98.99 | 53.98 | 99.99 |
|        | Mineral oil emulsion | 30.72 | 5.13 | 44.8 |
| Adult  | *S. verbascifolium*+ *M. anisopliae* | 85.59 | 69.03 | 99.28 |
|        | *M. anisopliae* | 81.48 | 70.39 | 98.17 |
|        | *S. verbascifolium* | 60.24 | 2.91 | 76.88 |
|        | Amitraz. | 0 | 99.27 | 100 |
|        | *Bacillus thuringiensis* | 80.07 | 0 | 86.42 |
|        | Mineral oil emulsion | 35.11 | 19.03 | 60.46 |
No difference was observed on the egg mass weight produced by engorged female tick treated with *S. verbascifolium* at 7 days post-treatment, with relation to the control groups. However, in the following data we can observe that on the nymph and larvae from the 14th and 21 days post-treatment there is a reduction on egg weight obtained from the adult females, in comparison with the control group, this could be due to the metabolites with insecticide activity, that alter the metabolism and development of several species of insects and cause physiological dysfunction that can lead to inhibition of reproduction or even death of the organism, due to interference with food intake and development (Schmutterer, 1990).

Evaluating this same variable, but with *B. thuringiensis* GP138 strain, Fernández et al. (2006a), found a reduction in eggs mass weight of 87%. However, in this experiment using the GP543 strain, the results were different, obtaining a reduction of 98% in the weight of eggs at the 21 days post-treatment.

With the formulations of the *M. anisopliae* M379 strain, we found a reduction of 98% in egg mass weight on the 21 day post-treatment. This results were also obtained by Ojeda, (2007), using the Ma34 strain he achieved a 100% of efficacy in the weight of *R. (Boophilus) microplus* eggs and adults recovered, using a concentration of $1 \times 10^8$ conidia/ml.

Regarding to the mixture of *M. anisopliae* and *S. verbascifolium* used in this study, we found that for the different female development stages of *R. (Boophilus) microplus*, this treatment had a marked effect on the reduction of egg mass weight produced by the recovered engorged females of 99 and of 93% for the nymph and larvae stages respectively, and of 95% for the adult stage, being the nymph stage the most susceptible to this treatment.

The methanolic extracts of the dry material of *S. verbascifolium* evaluated by Gómez (2009), affected significantly the egg hatch of *R. (Boophilus) microplus*, with an egg hatching of 6.45% produced by the engorged female ticks treated with the methanolic extract of the dry leaf, being a superior effect to the one found in this test, since we reach a 19.98% at the 21 days post-treatment.

Martins (2006), assays with *Cymbopogon winterianus Jowitt* oil at a concentration of 10, 8.33 and 7.14%, achieved a 0% of hatching from eggs of the females treated.

In the evaluation of *B. thuringiensis* upon this same variable, Fernández et al. (2006a), reported that with the GP138, GP139 and GP140 strains they achieved a 30% of egg hatching, reaching a 47% with the GP123 strain. However, in this experiment using the GP543 strain we obtained an egg hatching of 0.44% for the group of engorged female collected at the 21 days post-treatment. The great effect shown by *B. thuringiensis* upon hatching, is because in this stage the external layer of the egg is more thin and is more easier to penetrate by microorganisms (Hassanain et al., 1997).

Studies on the evaluation of *M. anisopliae* upon the variable hatch percentage, by Arguedas et al., (2008), obtained a 78% of hatching on eggs exposed to the action *M. anisopliae* at a concentration of $2.25 \times 10^8$ conidia/ml. The same results were obtained by Bittencourt et al., (1994), describing the effects of *M. anisopliae* upon *R. (Boophilus) microplus* eggs, achieving hatching percentages of 9.33% at a concentration of $1 \times 10^6$ conidia/ml. However, in this experiment the M379 strain achieved a 2.90% for the female group collected at 21 days post-treatment, demonstrating a better effect upon hatch on eggs treated with this strain.

When we combine *M. anisopliae* and *S. verbascifolium*, the hatching percentage found on the female group collected at 21 days post-treatment, show a better effect than the control group, with a 8.75% hatch on the treated eggs.

Fernández et al., (2006a), found high inhibitions on oviposition by *in vitro* assays with the GP138, GP139, GP140 and GP123 strains with values from 70-92% in ticks that were in the adult stage at the time of the treatment application. Considering that in this test the GP543 strain achieved an oviposition inhibition of 53.98% on ticks that were in the nymph stage at the time of the treatment, not being the same for the adult and larvae stages in which no oviposition inhibition was obtained with this treatment.

The treatment with *S. verbascifolium*, Gómez (2009) achieved a 63.70% inhibition of the oviposition, using a methanolic dry leaf extract by the adult immersion test *in vitro* (Drummond et al., 1973). In other study, Fernández et al. (2006c) reported an oviposition inhibition of 84% with *Heliopsis longipes* and of 88% with the extract of *Azadirachta indica* seed by the Adult Immersion Test *in vitro*. Ribeiro et al., (2007), achieved a 19.20% in inhibition of oviposition with an hexane extract of *Hypericum polyanthemum* and of 12.80% when used a methanolic extract from the same plant in a Adult Immersion Test *in vitro*. This results are superior to those obtained in this test with the *S. verbascifolium* extract from fresh stem, with a 2-17%, applied on the three stages of the development stage of *R. (Boophilus) microplus*.

Regarding the treatment with *M. anisopliae*, Arguedas et al., (2008), reported inhibition of the oviposition of 77.29% at a concentration of $2.25 \times 10^8$ conidia/ml *in vitro*, in other *in vitro* study with engorged females of *R. (Boophilus) microplus*. Fernández et al., (2006c), using the *M. anisopliae* MaX strain, plus corn oil, motor oil and petroleum reported a 100, 97 and 100% respectively, attributed this high percentages to the oils used in the test. However, for the M379 strain used in this test they
The percentage of inhibition of oviposition of the treatment with M. anisopliae + S. verbascifolium, in this test we obtained a 99.46, 99.73 and 98.17% for the larvae, nymph and adult stages respectively. In comparison with the results obtained by Fernández et al. (2006b) on cattle artificially infested, treated by spray bath with the M. anisopliae MaX strain with a concentration of 1x10^6 conidia/ml for each animal, achieved a control of 80.86% on adult females. In other study, Arguedas et al. (2008) treated engorged female of R. (Boophilus) microplus with M. anisopliae and obtained a 78.79% of control, which is more than the reached with the M. anisopliae M379 strain used in this study, demonstrating that this strain has a better control on R. (Boophilus) microplus.

The control of the treatment with S. verbascifolium achieved a 97.84, 84.24 and 76.88% of control for the larvae, nymph and adult respectively. In comparison with the results of Álvarez et al. (2008), the extracts of Piper nigrum, Echinacea angustifolia, Glicricidin sepium, Poligonum punctatum and Morus alba achieved a control percentage of 76-100% on R. (Boophilus) microplus engorged females and the extracts of Arlocarpus altillus, Adenium obesum, Melia azedarach, Dahlstedia pentaphylla and Piper aduncum a 50, 75.10, 46, 79.73 and 61, 81%, respectively, on R. (Boophilus) microplus engorged females. The results of this study, found that the stage more susceptible to control is the larvae stage (Williams 1993; Mongojkwe and Okoye 2001; Borges et al., 2003; Pereira y Famadas 2004; Silva et al., 2009).

The percentage of control of the treatment with B. thuringiensis, show that the better control of engorged females of R. (Boophilus) microplus, was achieved at 14 and 21 days post-treatment, including the nymph and larvae stages, with a 99.99% of control for both development stages, being this stages more susceptible to microorganisms.

The percentage of control of M. anisopliae and S. verbascifolium, was of 99% of control for the different development stages of R. (Boophilus) microplus, with a protection period of 21 days, demonstrating a biological performance for the control of R. (Boophilus) microplus, similar to the achieved by the chemical ixodicides use to control this tick.

According to the results obtained by the stable test with infestation chambers, using different formulations of natural products, sprayed on cattle artificially infested with R. (Boophilus) microplus, the treatments with S. verbascifolium + M. anisopliae 1x10^6 conidia/ml, M. anisopliae 1x10^6 conidia/ml and B. thuringiensis, achieved an acaricide effect of 80-98%, when applied on the different development stages of R. (Boophilus) microplus, upon the number of engorged females recovered, being B. thuringiensis applied on nymphs the one who achieved the best effect of 98%.

For the variable inhibition of oviposition, the treatment with M. anisopliae 1x10^6 conidia/ml, achieved the highest inhibition of 79%. However, the application of S. verbascifolium + M. anisopliae 1x10^6 conidia/ml and M. anisopliae 1x10^6 conidia/ml, on the nymph stage, achieved an inhibition of 75%, being good candidates as factors in the integrated pest management.

The treatments with S. verbascifolium + M. anisopliae 1x10^6 conidia/ml, M. anisopliae 1x10^6 conidia/ml and B. thuringiensis achieved the lowest hatch percentages in the study, when applied in the larvae stage, with a wide control spectrum of this parasite.

The percentage control obtained by this test demonstrated that the natural products used achieved a percentage above 90% and in some cases the treatments with S. verbascifolium + M. anisopliae 1x10^6 conidia/ml, M. anisopliae 1x10^6 conidia/ml, obtained a 98-99%, being this similar to those obtained by the chemical commercial product mitrazin.

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