



Evaluation of the tickcide, genotoxic, and mutagenic effects of the *Ruta graveolens* L. (Rutaceae)

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ABSTRACT. Current analysis investigated the tickcide effects of the aqueous extract and chloroform fractions of *Ruta graveolens* L. (rue) on engorged females of *Rhipicephalus microplus*, as well as their genotoxic and mutagenic effects on human leukocytes. The best tickcide activity (non-dependent dose) and genotoxic / mutagenic effects (dependent-dose) were observed on exposure to chloroform fractions. Results suggest that extract fractions of *R. graveolens* L are efficient against *R. microplus*, although the fraction and the tested concentrations show genotoxic and mutagenic potential for human leukocytes.

Keywords: acaricide, genotoxicity, rue.

Avaliação dos efeitos carrapaticida, genotóxico e mutagênico da *Ruta graveolens* L. (Rutaceae)

RESUMO. O efeito carrapaticida do extrato aquoso e frações da *Ruta graveolens* L. (arruda) sobre teleóginas de *Rhipicephalus microplus*, bem como seu potencial genotóxico sobre leucócitos humanos foram investigados neste trabalho. A melhor atividade carrapaticida (dose não dependente) e efeito genotóxico / mutagênico (dose dependente) foram observados nas frações clorofórmicas. Os resultados encontrados sugerem que frações clorofórmicas do extrato de *Ruta graveolens* L. são eficazes contra *R. microplus*, embora a fração e as concentrações testadas apresentem potencial genotóxico e mutagênico para células leucocitárias humanas.

Palavras-chave: acaricida, genotoxicidade, arruda.

Introduction

Rhipicephalus microplus is one of main parasites which cause financial liability in livestock. The parasite has toxins that cause lack of appetite in animals and vectors such pathogenic agents as *Anaplasma* spp. and *Babesia* spp. (GOMES, 1998).

Further, *R. microplus* has resisted conventional acaricides through mutations in active site of acetylcholinesterase, GABA receptor, and sodium channels (MUTERO et al., 1994; HEMINGWAY, 2000; VULULE et al., 1999; HE et al., 1999).

Ruta graveolens L., popularly known as the common rue, is a plant of the Rutaceae family. It is reported in the literature as an easily cultivated medicinal plant with important fungicide (OLIVA et al., 2003), anti-parasite and anthelmintic effects (YAMASHITA et al., 2009). In addition, it also affects *Pediculus humanus* and *Leishmania* sp (MEJRI et al., 2010).

Current analysis demonstrates the acaricide effects of chloroform fraction from *R. graveolens* L. against *R. microplus* and its cytotoxic and genotoxic potential.

Material and methods

Botanical material

Samples of *R. graveolens* L. were acquired commercially in Uruguaiana RS Brazil and cultivated in a greenhouse at the Universidade Federal do Pampa (Unipampa), Uruguaiana, Rio Grande do Sul State, Brazil (29° 45' 23" S; 57° 5' 37" W). The plant was identified by a botanist and the samples were deposited in the Bruno Irgang Herbarium (Unipampa) under register number HBEI 169.

Preparation of crude extract and fractions

The plant was dried in a desiccation stove at 30°C and monitored daily until the leaves had constant weight. An aqueous extract (AE) (200 g of dry mass to 1000 mL of distilled and deionized water) was prepared by macerating the mixture in a dark room at 25°C, and daily stirring for seven days. Extract volumes were divided into aliquots and percolated to obtain the following fractions:

methanol:water (MF) (70:30), acetonitrile:acetone (AF) (70:30), hexane:acetone (HF) (75:25) and chloroform:acetone (CF) (75:25). Further, 100 mL of AE and 150 mL of each combined solvents were added to the percolation to obtain the respective fractions. AE and fractions were later submitted to a rotary evaporator to obtain dry residues which were kept in a hermetically closed amber glass bottle and stored at -20°C until use. The following yields were obtained from 0.5 g AE or fractions at 105°C until constant weight: AE 10.73%; MF 10.22%; AF 13.51%; HF 15.81%; CF 20.26%.

Phytochemical analysis

The analysis of the polyphenols, flavonoids, and alkaloids from *Ruta graveolens* L. were performed in triplicate through specific colorimetric reactions using Folin-Ciocalteu (CHANDRA; GONZALEZ, 2004), aluminum chloride (ZHISHEN et al., 1999) and bismuth nitrate (OLIVEIRA et al., 2006), respectively.

Acaricide assessment

Acaricide *R. graveolens* L. (AE and fractions) was assessed by using engorged female ticks kindly donated by the Merial Saúde Animal Ltda (Uruguiana, Rio Grande do Sul State, Brazil). Five groups, each constituted by 20 engorged females, were used. Amitraz 0.2% was the positive control and water the negative control. Test groups were represented by aqueous extract and fractions cited above. Acaricide assessment was carried out in quadruplicate and the effect on the engorged females was evaluated according to Frazzon et al. (2000).

Tested concentrations were based on studies by Borges et al. (2003). Initially, AE and fractions residues were dissolved in distilled water at final concentration of 6%. The groups were submerged in 10 mL of respective suspensions during 60 seconds. The ticks were quickly dried and stored on petri dishes for later incubation at 25°C for 14 days. The females were evaluated daily to calculate survivors and the egg laying. At the end, survival index (Is) and egg laying capacity (Cp) were calculated, as described by Frazzon et al. (2000). Dimethyl sulfoxide (DMSO) was employed at a final concentration of 0.5% to dissolve HF and CF.

Toxicological Evaluation – Cytotoxicity and Genotoxicity

The experimental protocols of current study were approved by the Committee for Ethics in Research of the Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul State, Brazil, under register number 0089.0.243.000-07. All

toxicological and genotoxic tests were performed with human leukocyte cells, in triplicate, taking into account their rapid responses and sensitiveness to assess the exposure of cells to xenobiotics.

The toxicological evaluations were carried out on two concentrations (5 µg mL⁻¹ and 20 µg mL⁻¹) from AE and CF. The selected followed results from tickcide assessment, the general directives to the comet assay (TICE et al., 2000), and the polarities diametrically opposed of these preparations. Further, the researchers wanted to test whether the lower concentrations were capable of inducing genotoxicity and /or mutagenesis as a possible damage via induction on ticks. Moreover, levels of cellular viability must be over 70% to perform the comet assay. In this case, concentrations 40 µg/mL and 60 µg/mL were tested and the two demonstrated levels of cellular viability around 60%, i.e., 67 and 59%, respectively.

RPMI 1640 culture media were used for genotoxic and cytotoxic protocols. For all samples, 0.5 mL of venous blood from a 20-year-old young adult was added in the culture media and incubated at 37°C for 72 hours. H₂O₂ 4 mM was added to the positive group.

The cytotoxicity of AE and CF was assessed by analyzing the leukocyte membrane integrity with Trypan Blue (BUROW et al., 1998) and results were expressed by average percentage obtained from the unviable cells within a total of 300 cells per slide.

Alkaline comet assay was carried out following Montagner et al. (2010). The analysis was performed by microscope, magnification 400X, and 100 nucleoids per slide were counted and classified according to cell damage, ranging between 0 (no damage) to 4 (maximum damage). The average obtained for each treatment was the respective damage index, taking into account the DNA damage between level 0 (100 cells x 0) and 400 (100 x 4) to calculate effects.

Micronucleus technique was performed to evaluate the mutagenic effect of AE and CF. Slides were prepared from smears of cultures previously described and stained by the Panoptic Method, whilst 1000 cells were observed per slide under a microscope, at a magnification 1000 X, taking note of the number of micronuclei.

Antioxidant activity – DPPH

Antioxidant activity was performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (BRAND-WILLIAMS et al., 1995). Absorbance rates from samples were converted into percentage of antioxidant activity (AA), with the following equation:

$$AA \% = \{ [Abscontrol - Abssample] \times 100 \} Abscontrol^{-1},$$

where:

Abscontrol is the initial absorbance from DPPH methanolic solution and

Abssample is the absorbance from the reaction mixture (DPPH plus sample).

Data Analysis

Data from survival index evaluation, egg laying capacity and percentage of engorged females mortality were subjected to one-way variance analysis (ANOVA) and complemented by the Bonferroni's multiple comparison test, at $p < 0.05$; data from toxicological assessments were analyzed by one-way ANOVA and complemented by Newman-Keuls's multiple comparison test at $p < 0.05$.

Results and discussion

Phytochemical analysis

Phenolic compounds are widely distributed in plants, constituting the most abundant secondary metabolites in plants (KOMALI et al., 1999). Flavonoid compounds and alkaloids occur naturally in plants. Table 1 shows the quantity of alkaloids, polyphenols and flavonoids in $mg\ g^{-1}$ of dry weight

from different preparations of *R. graveolens* L of fresh plants.

Table 1. Alkaloids, polyphenols and flavonoids in $mg\ g^{-1}$ of dry weight fresh plant from different preparations from *R. graveolens* L.

Extract / Fractions	Alkaloids $mg\ g^{-1}$	Polyphenols $mg\ g^{-1}$	Flavonoids $mg\ g^{-1}$
Aqueous	4.66	84.06	72.12
Methanolic	2.36	115.12	85.75
Ethyl Acetate	0.15	9.25	0.41
Hexane	1.21	256.87	77.86
Chloroform	15.32	38.33	7.34
	$y = 0,0404x + 0,002$	$y = 40.112x + 0,0581$	$y = 0.891x + 0,0149$
	$R^2 = 0,9997$	$R^2 = 0.994$	$R^2 = 0.9723$

Acaricide assessment

Figure 1 A shows the survival index (SI) of engorged females in different groups under analysis. Groups exposed to CF had a survival index significantly lower than negative control ($p < 0.05$), but not different from positive control.

Survival index (SI) is a parameter used to evaluate the tickcide effect of plant preparations, drugs or isolated compounds. Low indexes express the incapacity of egg laying and decreasing in tick population. Reports in the literature reveal bioactive compounds in chloroform fractions from *R.*

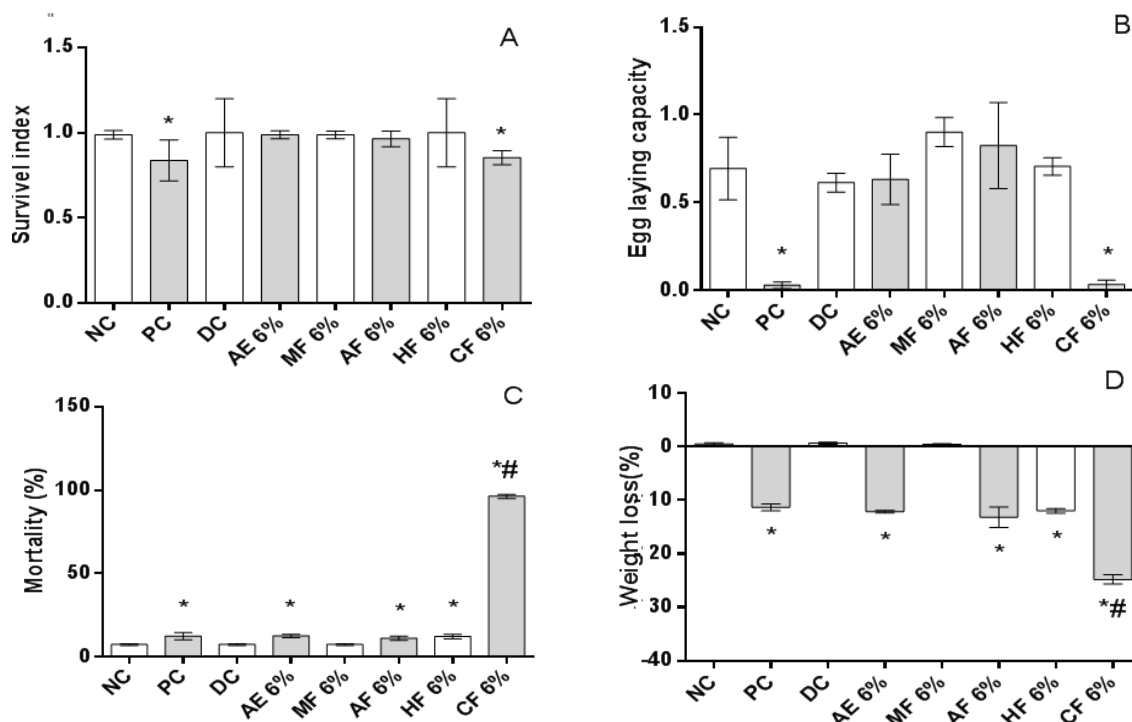


Figure 1. Survivor index of engorged females A; engorged females egg laying capacity B; mortality of engorged females C, percentage of weight lost D exposed to different *Ruta graveolens* L. preparations. The data are expressed in average \pm standard deviation, $n = 4$. *Significant statistic difference to the negative* or positive# controls, $p < 0.05$ or $p < 0.001$. NC = negative control; PC = positive control; DC = DMSO control; AE = aqueous extract; MF = methanolic fraction; AF = acetonitrile fraction; HF = hexane fraction; CF = chloroform fraction.

graveolens, such as alkaloids, in particular rutaline and rubalidine, with allelochemical activity (NEBO et al., 2014). Moreover, other alkaloids from this plant, such as dictamine, gamma-fagarine, ptelein, and kokusagine, had a strong mutagenic effect on *Salmonella typhimurium* strain TA98 without S9 mix, and moderate mutagenic effects with and without S9 mix on TA100 strain (PAULINI et al., 1987). When these data are taken into account, it is reasonable to suggest that alkaloids in CF of current study may be involved in the effect, due to their cell cycle interference and mutagenic potential.

Figure 1B demonstrates the significant inhibition in engorged females' egg laying capacity for CF 6%, when compared to negative control ($p < 0.001$), albeit not different from positive control.

Engorged female's egg laying capacity is a determinant factor for the perpetuation of the species. Similarly, egg laying inhibition is one of the desirable parameters in compounds or drugs with therapeutics prospection to the combat ticks. In fact, tested CF seems to contain active agents against the ingurgitated females' egg laying.

Figure 1C shows the mortality percentage of the engorged females from the different groups assayed, when the positive control (Amitraz 0.2%), AE, AF, HF and CF were more capable of inducing the death in engorged females when compared to negative control ($p < 0.05$). Nevertheless, the highest mortality induction occurred in CF (96.25%), with more efficiency than Amitraz 0.2%, or rather, a 12.5% mortality percentage.

Preparations of *R. graveolens* L. have been tested as an option in antiparasite therapy. Crude extract and fractions have proved to be effective against *Ctenocephalides canis* (LEITE et al., 2006), *Acanthoscelides obtectus* (MAZZONETTO; VENDRAMIM, 2003) and *Trypanosoma cruzi* (AMBROZIN et al., 2004). On the other hand, the plant has not yet been tested against ticks, at least to our knowledge.

Although acetonitrile and hexane fractions induced death on engorged females, CF revealed to be a better tickcide in current study. This fact suggests that chloroform was more lethal than other solvents in extract compounds, with more effectiveness against ticks.

Figure 1D shows the loss of weight of engorged females by the end of the treatment. There were a loss of weight in PC, AE 6, AF 6 and CF 6%. However, CF exposure caused the most significant loss when compared to NC ($p > 0.001$), perhaps associated with mortality rate or egg laying capacity of the engorged females. In this case, there was a

decrease in egg laying capacity and an increase in mortality of engorged females subjected to CF.

Figure 2 shows the amount of laid eggs from engorged females exposed to CF as well as to the negative and positive controls at the end of the experimental period. The visual difference in the amount of laid eggs between the groups, particularly between CF (6%) and negative control should be enhanced. Moreover, there is a minor amount of laid eggs in CF 6% than in the positive control.

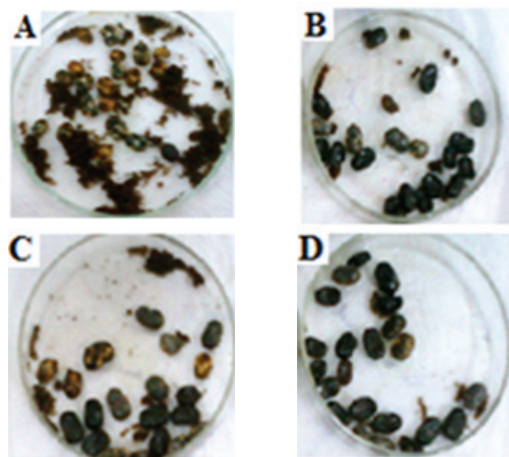


Figure 2. Engorged females in the end of the experiment, exposed to different concentrations of *Ruta graveolens* L. chloroform extract, in comparison with the negative A and positive B controls; Aqueous extract 6% C; Chloroform fraction 6% D.

Genotoxic Assays

Cytotoxicity is an important toxicological parameter and may be assessed by Trypan Blue technique. The method identifies dead or unviable cells which show up stained with blue. According to Collins (2004), high cell viability is necessary as a previous condition for the development of the comet assay; at the same time, it shows the membrane cytotoxicity of substances or compounds. Figure 3A reveals acceptable levels of cellular unviability for the groups assayed, where positive control ($p < 0.001$) and CF 6%, at $5 \mu\text{g mL}^{-1}$ and $20 \mu\text{g mL}^{-1}$ concentration ($p < 0.001$), demonstrated higher levels than those of negative control.

The comet assay is a useful test for drugs and compounds in a pre-clinical phase to detect simple and double breaks in DNA strands, which represent DNA damage and non-possibility of cell repair (FAUST et al., 2004; MALUF; ERDTMANN, 2000). In current study, the damage indexes are shown in Figure 3B, where the positive control ($p < 0.001$) and CF 6% at $5 \mu\text{g mL}^{-1}$ and $20 \mu\text{g mL}^{-1}$ concentration ($p < 0.001$) reported higher indexes than negative control, but similar to positive control.

CF shows DNA damage indexes in a dependent-concentration manner and significantly different from negative control. The higher concentration of this fraction induced damage similar to that of positive control. On the other hand, the two preparations of aqueous extract show DNA damage indexes similar to that of negative control.

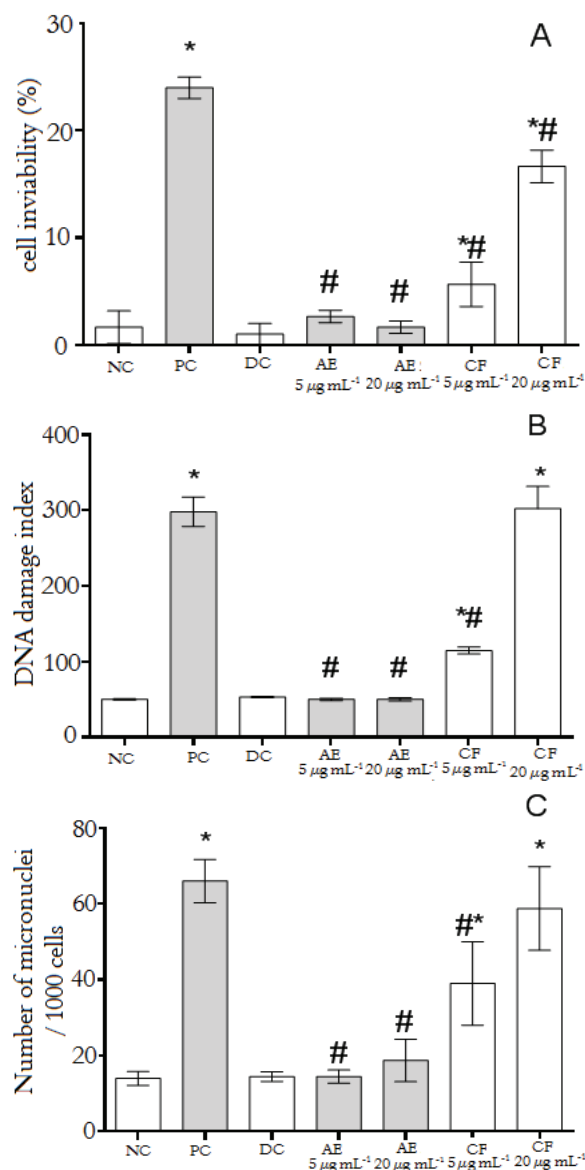


Figure 3. Percentage of cellular inviability A, DNA damage indexes B, and number of micronuclei C in human leukocytes exposed to different concentrations of the AE and CF ($5 \mu\text{g mL}^{-1}$ e $20 \mu\text{g mL}^{-1}$) from *Ruta graveolens* L. The data are expressed in percentage of the obtained average in the treatments; $n = 4$. *Statistic difference when compared to the negative control; #Statistic difference when compared to the positive control. NC = negative control; PC = positive control; DC = DMSO control; AE = aqueous extract; CF = chloroform fraction.

Consequently, data report a genotoxic effect of chloroform fractions from *R. graveolens* on human

leukocytes, considering the concentration and experimental conditions assayed, since this fraction was able to induce breaks in DNA strand, which were visualized by a non-homogeneous migration of the leukocyte nucleoids in the electrophoresis.

Indeed, the literature reports that the aerial parts of *R. graveolens* have such compounds as chapelin and chalepsin that perform cross-reactions with either purine and pyridine bases on DNA molecule, inducing transcriptional mistakes and mutagenesis (GÜNEYDİN; SAVCI, 2005).

The micronucleus test is a useful and recommended tool for studies that assess the drugs or compounds capacity of breaking chromosome structures, which is associated to an aneugenic effect and an intrinsic toxic-mutagenic potential (FENECH, 2000; RIBEIRO et al., 2003).

Figure 3C shows the number of micronuclei in human leucocytes exposed to different concentrations of the aqueous extract and chloroform fraction 6% of *R. graveolens* L., where positive control ($p < 0.001$) and CF ($5 \mu\text{g mL}^{-1}$ and $20 \mu\text{g mL}^{-1}$) ($p < 0.001$) reveal a higher number of micronuclei than negative control.

As may be observed, similar to the comet assay, CF demonstrates a number of micronuclei concentration-dependent manners, in which there was no difference between positive control and CF at $20 \mu\text{g mL}^{-1}$ concentration. On the other hand, the number of counted micronuclei for the two preparation of AE (5 e $20 \mu\text{g mL}^{-1}$) did not differ from the number found in the negative control.

The micronuclei test is a cytogenetic tool used for in vivo or in vitro chromosome damage assessment (VON LEDEBUR; SCHMID, 1973; HAYASHI et al., 1998). This test is based on the observation of cells that suffer alterations in chromatid distribution (fuse effect) or chromatid break (SCHMID, 1975), leading to the non-incorporation of the entire or acentric chromosomes condensed to the daughter-cell during cellular division, evidenced as a small rounded dark structure, identical in appearance and coloration to the cellular nucleus (AL-SABTI; METCALFE, 1995). It is thus plausible to suggest that chromatid alterations may be linked to a minor egg-laying capacity of engorged females' ticks, as current results demonstrate (Figure 1B).

Antioxidant activity – DPPH

Table 2 reveals oxidative effects on different concentrations or dilutions to corroborate results reported in the cytotoxic and genotoxic assays from CF of *R. graveolens* L.

Table 2. Profile oxidative from CF of *R. graveolens* L. by DPPH assay.

Concentrations	Sample $\mu\text{g mL}^{-1}$	% Oxidation	% Oxidative inhibition
dw	7,800	42.862	57.138
dw 10^{-1}	780	67.302	32.698
dw 100^{-1}	78	96.891	3.109
dw $1,000^{-1}$	7.8	98.261	1.739
dw $10,000^{-1}$	0.78	99.432	0.568

$Y = 41.011x + 0.6437$; $R^2 = 0.9984$; $IC_{50} = 1.203 \text{ mg mL}^{-1}$; dw = dry weight

When the concentrations of CF of *R. graveolens* L. used in comet ($20 \mu\text{g mL}^{-1}$) and in DPPH assays are taken into consideration, the concentration range is not able to counteract the oxidative status induced by CF. This fact corroborates DNA damage induction observed when the leukocytes are exposed to CF. Moreover, the main results of current study derive from CF, which make the authors suggest the possible involvement of alkaloids compounds, since CF differs from other fractions and aqueous extract due to a higher presence of alkaloids than all other fractions.

Conclusion

Data from CF of *R. graveolens* L., at the concentrations and experimental conditions tested, were efficient against *R. microplus*, although the fraction shows genotoxic and mutagenic potential for human leukocytes. However, although CF is somewhat toxic, this fact does not discourage the use of the plant as an acaricide. It rather enhances the need for further research involving isolation and identification of its active compounds and alternatives in the field of pharmaceutical technology to establish limits to systemic absorption.

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