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In vitro antimicrobial activity of baccharis, tamarind, cashew nut shell liquid, and clove oil against Gram-negative ruminal bacteria

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Abstract. This study aimed to evaluate the *in vitro* antibacterial activity of *Baccharis dracunculifolia* and *Tamarindus indica* L. aqueous extract, cashew nut shell liquid (CNSL) natural extracts, and clove essential oil (EO) against five species of Gram-negative ruminal bacteria. Cultures were grown in anaerobic media containing 0.1, 0.2, 0.5 and 1.0 mg mL^{-1} of the extracts or oils. Growth was evaluated by monitoring the optical density (OD 600 nm) at intervals of 0, 8, 12 and 24 hours of incubation at 39° C. The baccharis and tamarind aqueous extract, and CNSL natural extract inhibited the growth of *Prevotella albensis*, *Prevotella bryantii*, *Treponema saccharophilum* and *Succinivibrio dextrinosolvens*. For *Prevotella ruminicola* and *Succinivibrio dextrinosolvens*, the addition of the clove leaf EO of 1.0 mg mL^{-1} resulted in a greater impact on growth dynamics, with a reduction in optical density in all intervals of observations. The findings of this research establish the efficacy of natural additives aqueous extracts of baccharis and tamarind, CNSL, and clove essential oil, in antimicrobial activity *in vitro* against the Gram-negative ruminal bacteria analyzed.

Keywords: Antibacterial activity, natural extracts, essential oil, rumen bacteria

Atividade antimicrobiana in vitro de baccharis, tamarindo, líquido da casca da castanha de caju e óleo de cravo contra bactérias ruminais Gram-negativas

Resumo. Este estudo teve como objetivo avaliar a atividade antibacteriana *in vitro* do extrato aquoso de *Baccharis dracunculifolia* e *Tamarindus indica* L., dos extratos naturais líquidos da casca da castanha de caju (LCC) e do óleo essencial (OE) de cravo contra cinco espécies de bactérias ruminais Gram-negativas. As culturas foram cultivadas em meio anaeróbio contendo 0,1, 0,2, 0,5 e 1,0 mg mL^{-1} dos extratos ou óleos. O crescimento foi avaliado monitorando a densidade óptica (DO 600 nm) em intervalos de 0, 8, 12 e 24 horas de incubação a 39° C. O extrato aquoso de baccharis e tamarindo e o extrato natural de LCC inibiram o crescimento de *Prevotella albensis*, *Prevotella bryantii*, *Treponema saccharophilum* e *Succinivibrio dextrinosolvens*. Para *Prevotella ruminicola* e *Succinivibrio dextrinosolvens*, a adição de 1,0 mg mL^{-1} de OE de folhas de cravo resultou em maior impacto na dinâmica de crescimento, com redução na densidade óptica em todos os intervalos de observações. Os resultados desta pesquisa estabelecem a

eficácia dos aditivos naturais extratos aquosos de baccharis e tamarindo, LCC e óleo essencial de cravo, na atividade antimicrobiana *in vitro* contra as bactérias ruminais Gram-negativas analisadas.

Palavras-chave: Atividade antibacteriana, extratos naturais, óleo essencial, bactérias ruminais

Introduction

Ruminant nutritionists are studying the effects of natural additives in the diets of animals to reduce the preventable losses during the fermentation process (Benchaar & Greathead, 2011; Monteschio et al., 2017; Ornaghi et al., 2017; Souza et al., 2019; Wallace et al., 2002). These compounds are potential rumen fermentation modulators, and without causing risk to human and animal health.

Tropical plants are rich in secondary metabolites, such as phenolic and flavonoids compounds. Biological activities such as antibacterial and anti-inflammatory activity in ruminants can improve dry matter digestibility, reduce methane emissions and increase propionate production (Olagaray & Bradford, 2019). Among some of the natural compounds tested *in vitro* an *in vivo*, the baccharis (*Baccharis dracunculifolia*) (Bonin et al., 2020; Campos et al., 2016; Zuccolotto et al., 2019), tamarind (*Tamarindus indica* L) (Arshad et al., 2019; Souza et al., 2018; Wang et al., 2017), cashew (*Anacardium occidentale*) (Cruz et al., 2014; Valero et al., 2014; 2016), and clove (*Syzygium aromaticum*) (Cortés-Rojas et al., 2014; Pandey & Singh, 2011; Passone et al., 2012) are easily available.

Research concerning the effects of plant extracts and essential oils (EO) on ruminal bacteria growing is still limited. Bioactive compounds extracted from plants have the capacity to affect the integrity of the cell envelope in Gram-negative bacteria.

The hypothesis of this study was that the inclusion of extracts of leaves and stems of Baccharis, tamarind seeds, cashew and cloves oils could reduce the activity of the main Gram-negative bacteria of rumen.

This study was carried out to evaluate the main class of secondary metabolites present in the seed tamarind extract by ^{1}H NMR, and the chemical composition clove oils by GC-MS. Furthermore, the antimicrobial effect *in vitro* action against principal species of Gram-negative ruminal bacteria of baccharis extract, seed tamarind extract, cashew nut shell liquid and clove oil were also determined.

Material and methods

Ethics

All experimental procedures were conducted under the surveillance of the Animal Care and Use Committee of the Universidade Estadual de Maringá, Brazil (protocol nº. 1103290719) and met the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

Origin of vegetable extracts and essential oils

The baccharis (*Baccharis dracunculifolia*) leaves and stems of the upper part was collected in Maringá, city, Paraná state, Brazil south (latitude 23º27'S and longitude 51º59' W) during the summer period. The region has a humid temperate climate with a temperate summer of 18 ºC, and an annual average rainfall of 1,114 mm. The extract of baccharis used in this study was the same used in our previous study (Bonin et al., 2020b). Analysis of the chemical constituents of baccharis using UHPLC–HRMS/MS method indicated that the extracts contained germacrene B, spathulenol, naringenin, kaempferol, artepillin C, α-pinene, hydroxycinnamic acid, apigenin, kaempferide, limonene, phenylethanol and β-caryophyllene (Bonin et al., 2020).

The tamarind (*Tamarindus indica* L.) seeds were collected in Nova Redenção city, Bahia state, Brazil northeast (latitude 12º49'S and longitude 41º03'W) during winter. This region has a tropical climate with a dry winter season, and an annual mean temperature of 23.5 ºC and an average annual rainfall of 805 mm.

The cashew nut shell liquid (CNSL) was purchased from Safeeds® (Cascavel city, Paraná state, Brazil south) and stored at -18 ºC. CNSL was chosen because it presents cardanol and cardol as the main constituents(Andrade et al., 2011; Das et al., 2004; Medeiros et al., 2020).

The essential oil (EO) from clove leaf (*Syzygium aromaticum*) was purchased from FERQUIMA® (Vargem Grande Paulista city, São Paulo state, Brazil, southeast) and stored at -18 ºC.

Preparation of natural extracts

The plant material collected from baccharis and tamarind seeds were partially dried in a forced ventilation oven (40° C) until they reached a constant weight at 72 hours. Samples were then processed in a knife mill through a 1 mm sieve (Wiley TE-650/1). To prepare the extracts of baccharis and tamarind, 10 grams of the partially dried material were weighed, mixed with 100 ml of distilled water, placed under agitation every 15 minutes for 2 hours. After the stirring period, the extract was filtered using filter papers (Whatman Nº 1, 90 mm), stored in closed flasks, overwrapped in aluminum foil, and kept stored at a temperature of 4° C until the analyses. Extracts of baccharis, tamarind, the CNSL and the EO from clove leaf were diluted in Tween® 80 solution (5%), to make the following stock concentrations: 200; 400; 1000 and 2000 mg L^{-1}. These concentrations represent typical amounts of compounds from plant extracts and EO supplied to ruminants' diets (Ornaghi et al., 2020; Rivaroli et al., 2020).

Chemical analysis

The chemical profile of the tamarind seeds was evaluated by nuclear magnetic resonance (NMR) analysis. A part of the crude extract of tamarind seeds (12.9 g) was suspended in methanol/water (1:1, 100 mL, v/v), and successively partitioned with *n*-hexane and ethyl acetate (3 x 20 mL, v/v). Ethyl acetate fraction was submitted to NMR analysis. ^{1}H NMR spectrum was recorded on a Bruker Avance III HD spectrometer (Bruker®, Billerica, MA, USA) operating at 500 MHz, using DMSO-d6 (Sigma-Aldrich) as solvent (Figure 1).

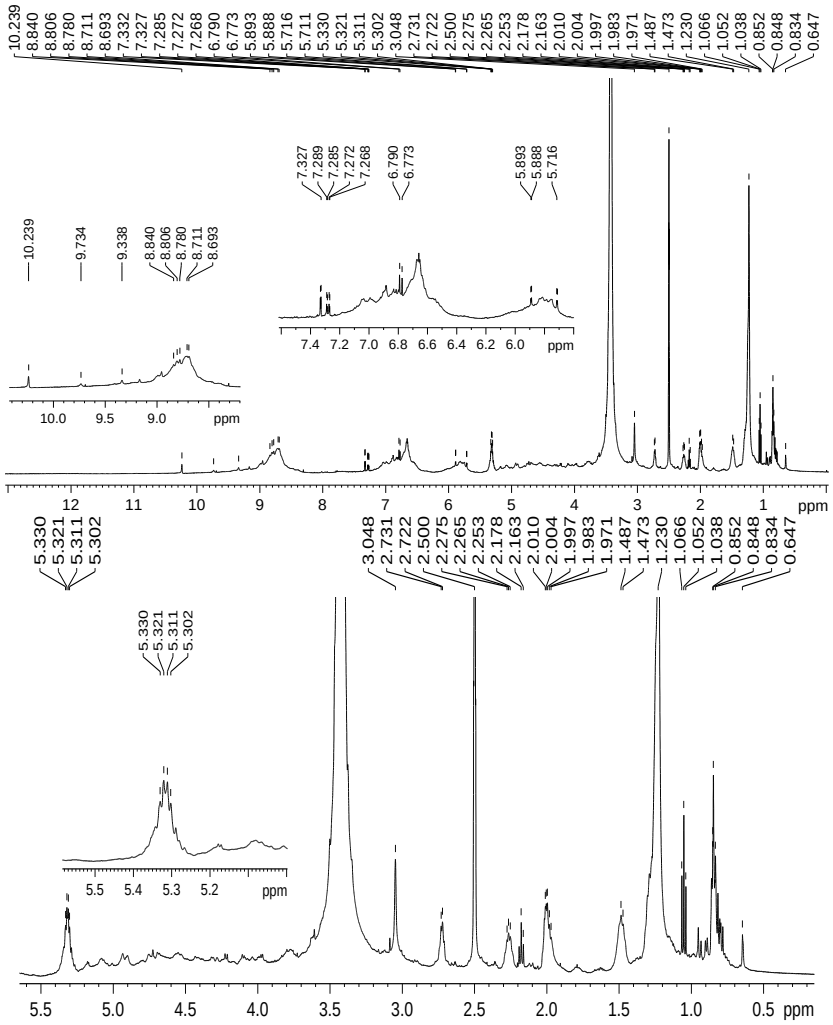

Figure 1. ^{1}H-NMR spectrum (DMSO-d_6, 500 MHz) of ethyl acetate fraction of tamarind.

The identification of bioactive compounds of clove leaf oil was performed using gas chromatography coupled to a mass spectrometer (GC-MS, USA) (Biondo et al., 2017), on a Thermo-Finnigan equipment, model Focus DSQ II, fitted with J&W Scientific DB-5 capillary column (30 m x 0.25 mm x 0.25 µm). The temperature of the ionization source was 250 °C, injector at 250° C, split injection mode 1/10. The carrier gas used was helium (99.999%) at 1.0 mL/min. A volume of 1 µL of the sample dissolved in ethyl acetate at a ratio of 1:20 was injected. The equipment operated in electron impact (70 eV) using a SCAN mode with mass spectral range of 40-650 m/z. For the separation of chemical constituents of the clove essential oil, the initial column temperature was 60° C, increased at a rate of 3°C/min to 246° C, which was held for 11 minutes, then increased at a rate of 30°C/min until the column reach a final temperature of 290° C. The comparison of the mass spectra obtained for each sample with the standard spectra (National Institute of Standards and Technology, NIST) was performed using the MS Search Program v.2.0 program spectral library (Figure 2).

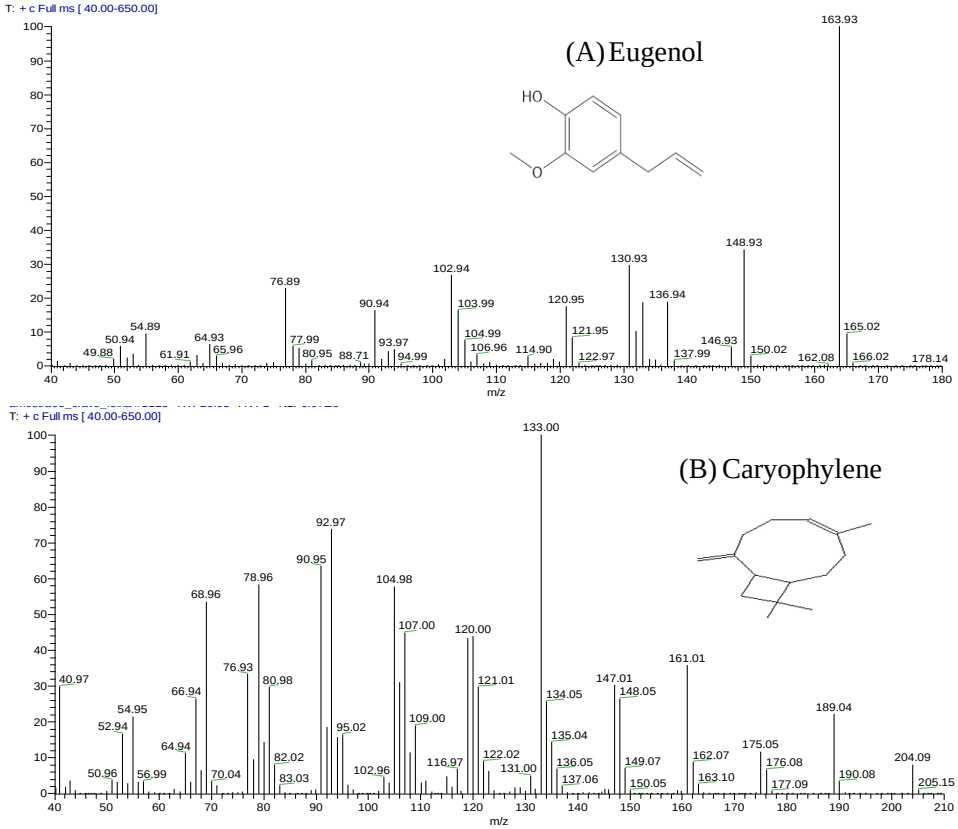

Figure 2. Chemical profile from essential oil of clove leaf, using gas chromatography coupled to mass spectrometry (GC-MS).

Microorganisms

Five strains of Gram-negative ruminal bacteria were used: *Prevotella albensis* (DSM 11370), *Prevotella bryantii* (DSM 11371), *Prevotella ruminicola* (ATCC® 19189™), *Treponema saccharophilum* (DSM 2985), *Succinivibrio dextrinosolvens* (ATCC® 19716™). All bacterial strains were cultivated in Hungate tubes under anaerobic conditions using Hobson's M2 medium (Hobson, 1969a), and at 39° C for 18 h. Stock cultures of bacteria were stored in a refrigerator at -80 °C in 15% (v/v) glycerol stock medium for further use. For every experiment, sub culturing was performed every 2 days using Hobson's M2 medium culture. The same procedure was repeated at least three times to remove any impurities coming from the glycerol.

Description and preparation of the culture medium

Ruminal contents (500 mL) were collected from three rumen-cannulated bulls (½ Zebu *vs.* ½ European) with a mean age of 24 ± 2.0 months and a mean body weight of 418 ± 4.51 kg, two hours after morning feeding. Ruminal contents were squeezed through four layers of cheesecloth and the pooled ruminal liquid sample was chilled to 5° C with ice, placed in bottle thermos and immediately transported to the laboratory. The ruminal fluid was centrifuged at 12,000 × g for (25 min, 4° C) and

the supernatant was stored at −20° C. Diet of the bulls consisted of corn silage (30% DM) and 70% concentrate (12.9% crude protein; 25.5% neutral detergent fiber and 3.3% ether extract), provided *ad libitum*.

The anaerobic medium contained (per L): glucose (2.0 g); maltose (2.0 g); sodium hydrogen carbonate (4.0 g); Bacto-casitone (10.0 g); yeast extract (2.5 g); cellobiose (2.0 g); mineral solution I (150 ml); mineral solution II (150 mL); clarified rumen Fluid (200 mL); sodium lactate solution (10 mL); resazurin Solution (1 mL); distilled water (up to 1000 mL) and cysteine-HCL (1.0 g). The mineral solution I consisted of 3 g of dipotassium phosphate, in 1 liter of distilled water and the mineral solution II consisted of 3 g of monopotassium phosphate, 6 g of ammonium sulfate, 6 g of sodium chloride, 6 g of sulfate of magnesium, 0.6 g of calcium chloride in 1 liter of distilled water. The pH of the medium (6.8) was adjusted using 2 N NaOH. The culture medium was prepared under anaerobic conditions by boiling, adding a reducing agent (cysteine), and distributing the medium in Hungate glass tubes (9.0 ml), under flux of CO_2 and then sealed with rubber septa and plastic caps (Hobson, 1969b; Hungate, 1966). Tubes were sterilized in an autoclave at 120° C for 20 minutes. After this procedure, the tubes were removed from the autoclave, waited to cool at room temperature, and then stored in the dark.

Sample preparation and analysis

The assays were performed using six replicates per bacteria (*P. albensis*, *P. bryantii*, *P. ruminicola*, *T. saccharophilum* and *S. dextrinosolvens*), which were allocated in one of the following treatments: control (0.5 mL of the test bacteria + 0.5 mL of the culture medium); 0.1 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of plant extract/oil, at a concentration of 0.1 mg mL^{-1}); 0.2 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of plant extract/oil, at a concentration of 0.2 mg mL^{-1}); 0.5 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of plant extract/oil, at a concentration of 0.5 mg mL^{-1}); 1.0 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of plant extract/oil, in the concentration of 1.0 mg mL^{-1}). To verify if Tween® 80 solution (5%) could have antibacterial effect in the tested cultures, tubes were prepared with culture medium, the tested bacteria (0.5 mL) and Tween® 80 solution (0.5 mL) and compared to the control treatment.

All tubes contained 9.0 mL of culture medium. Cultivation was performed at 39° C, and bacteria growth was evaluated by quantifying the optical density (OD) at 600 nm using a spectrophotometer (Thermo scientific, Genesys 10UV Scanning). Optical density was evaluated at 0, 8, 12, and 24 hours of incubation. Strains used in the current study reached the early stationary phase after 16 h of growth (data not shown).

Statistical analysis

After exploratory analysis of longitudinal data, all response variables showed positive asymmetric distribution. Thus, the gamma model with log connection function of the mixed generalized linear models was used. This was based on generalized estimation equations (GEE), which are the marginal distribution of mixed models, without the denotation of random effects. In this case, the sample intra-unit covariance structure was incorporated. The AR (1) was considered: first order auto-regressive. The choice of this is due to the fact that it best represented the variation of the equally spaced data and, also, the covariance decreased on average between two observations as the time interval between them increased, as suggested by (Diggle et al., 2002).

The adjusted model is as follows:
Y ~ Time + Concentration + Time: Concentration
For each Y ~ Gamma (link = log), "ar1" correlation structure wherein all models were adjusted in the R application using the geeglm command.

Results

Effect of Tween® 80 on bacterial growth

Tubes containing the culture medium, the bacteria and Tween® 80 (5%; v/v) had similar growth compared to control (data not shown), indicating that 5.0 mL Tween® 80 solution had no antimicrobial activity against the ruminal bacteria used in this study.

Baccharis dracunculifolia

The effects of baccharis extract on growth of Gram-negative ruminal bacteria are summarized in Table 1. Except for *P. ruminicola*, all cultures of ruminal bacteria reached cell high cell densities (>1.0) after 8 h of growth, and turbidity remained high until the end of the experiment. However, upon the addition of baccharis extracts, a drastic decrease ($P < 0.05$) in OD was observed for *P. albensis*, *P. bryantii* and *T. saccharophilum*. These cultures were highly susceptible to the baccharis extract and even concentrations as low as 0.1 mg mL^{-1} reduced the OD_{600nm} more than 70% after 8 h of growth. *P. ruminicola* showed little susceptibility to baccharis extracts and no differences in cell densities could be observed after 12 or 24 h of growth. The fibrolytic bacteria *Succinivibrio dextrinosolvens* initially showed a decrease of approximately 80% in the OD_{600nm} at the highest concentration of the baccharis extract tested (1.0 mg ml^{-1}). However, this effect diminished during growth, and after 12 and 24 h of incubation the reduction in OD_{600nm} was only 20% when compared to the controls.

Tamarindus indica L.

The ^{1}H NMR spectrum of ethyl acetate fraction of the tamarind seeds (Figure 1) showed characteristic signals of fatty acid, such as olefinic protons at δ_H 5.3, methylene protons in the region of δ_H 1.1 to 2.7, and terminal methyl group protons at δ_H 0.8 to 0.9 (Knothe & Kenar, 2004; Tsiafoulis et al., 2019). It was also observed signs at the region of δ_H 5.7 to 7.3 and 8.7 to 10.2, indicating the possible presence of phenolic and flavonoid compounds in the tamarind acetate fraction (Charisiadis et al., 2014; Rivero-Cruz et al., 2017).

Table 1. Effect of *Baccharis dracunculifolia* extract against Gram-negative ruminal bacteria

Concentration mg mL^{-1}	Time (hours)			^{1}SEM
	8	12	24	
	Prevotella albensis			
0.0	1.527^{A}	1.611^{A}	1.532^{A}	0.019
0.1	0.213^{bB}	0.202^{bC}	0.507^{aBC}	0.027
0.2	0.285^{cB}	0.456^{bB}	0.581^{aB}	0.027
0.5	0.266^{cB}	0.374^{bB}	0.468^{aC}	0.027
1.0	0.304^{bB}	0.367^{bB}	0.431^{aC}	0.027
SEM	0.027	0.027	0.027	
	Prevotella bryantii			
0.0	1.284^{cA}	1.374^{bA}	1.454^{aA}	0.016
0.1	0.338^{bD}	0.441^{aD}	0.506^{aBC}	0.022
0.2	0.429^{C}	0.487^{CD}	0.469^{C}	0.022
0.5	0.520^{B}	0.571^{B}	0.555^{B}	0.022
1.0	0.552^{aB}	0.525^{bBC}	0.426^{bC}	0.022
SEM	0.022	0.022	0.022	
	Prevotella ruminicola			
0.0	0.794^{AB}	0.990	0.968	0.046
0.1	0.874^{A}	0.937	1.050	0.065
0.2	0.604^{bB}	0.983^{a}	0.942^{a}	0.065
0.5	0.561^{bB}	1.133^{a}	0.953^{a}	0.065
1.0	0.911^{A}	0.966	1.061	0.065
SEM	0.065	0.065	0.065	
	Treponema saccharophilum			
0.0	1.398^{A}	1.378^{A}	1.430^{A}	0.020
0.1	0.328^{bC}	0.349^{bC}	0.461^{aC}	0.028
0.2	0.526^{B}	0.583^{B}	0.551^{BC}	0.029
0.5	0.555^{B}	0.579^{B}	0.621^{B}	0.029
1.0	0.544^{aB}	0.537^{aB}	0.459^{bC}	0.029
SEM	0.028	0.028	0.028	
	Succinivibrio dextrinosolvens			
0.0	1.045^{bB}	1.322^{aA}	1.117^{bA}	0.04
0.1	1.320^{aA}	1.086^{bB}	0.860^{cB}	0.056
0.2	1.184^{aAB}	1.038^{bB}	0.815^{cB}	0.057
0.5	0.980^{aB}	1.015^{aB}	0.836^{bB}	0.057
1.0	0.203^{bC}	0.985^{aB}	0.879^{aB}	0.057
SEM	0.057	0.057	0.057	

Different lowercase letters in the same line are significantly different and different uppercase letters in the same column are significantly different ($P < 0.05$). ^{1}SEM = standard error of the mean.

The extracts obtained from *Tamarindus indica* L. were among the least effective against the ruminal bacteria tested in this study (Table 2). Except for *P. ruminicola*, all cultures of ruminal

bacteria reached cell high cell densities (>1.0) after 8 h of growth, and turbidity remained high until the end of the experiment. However, upon the addition of *Tamarindus indica* L. extracts (12 - 24 hours), a drastic and significant decrease ($P < 0.05$) in OD was observed for *P. albensis*, and *S. dextrinosolvens*. Consistent inhibitory effects were observed at the highest concentration of the extract (1.0 mg mL^{-1}) and at later incubation times as shown for *P. ruminicola* and *S. dextrinosolvens*. *P. ruminicola* incubated with 1.0 mg mL^{-1} of tamarind extract for 24 h showed the highest reduction in OD_{600nm} (41%). For the remaining cultures, the decrease in OD_{600nm} after 24 h was much lower, and growth inhibition varied from none (*P. bryantii*) to about 25% (*T. saccharophilum*).

Table 2. Effect of *Tamarindus indica* L. extract against Gram-negative ruminal bacteria

Concentration mg mL^{-1}	Time, hours			[1]SEM
	8	12	24	
		Prevotella albensis		
0.0	1.527	1.611^{A}	1.532^{A}	0.025
0.1	1.417	1.420^{B}	1.372^{B}	0.036
0.2	1.432	1.433^{B}	1.358^{B}	0.036
0.5	1.440^{a}	1.390^{abB}	1.310^{bB}	0.036
1.0	1.395	1.415^{B}	1.395^{B}	0.036
SEM	0.036	0.036	0.036	
		Prevotella bryantii		
0.0	1.284^{bB}	1.374^{bB}	1.454^{a}	0.029
0.1	0.757^{bBC}	1.382^{aB}	1.492^{a}	0.042
0.2	0.762^{bC}	0.833^{bC}	1.456^{a}	0.042
0.5	1.581^{aA}	1.556^{abA}	1.448^{b}	0.042
1.0	1.556^{A}	1.631^{A}	1.570	0.042
SEM	0.042	0.042	0.042	
		Prevotella ruminicola		
0.0	0.794^{bB}	0.990^{a}	0.968^{aA}	0.028
0.1	0.935^{aAB}	1.067^{a}	0.465^{bC}	0.039
0.2	0.978^{aA}	1.061^{a}	0.457^{bC}	0.039
0.5	0.993^{aA}	1.060^{a}	0.406^{bC}	0.039
1.0	0.978^{aA}	1.054^{a}	0.571^{bB}	0.039
SEM	0.039	0.039	0.039	
		Treponema saccharophilum		
0.0	1.398^{A}	1.378^{A}	1.430^{A}	0.020
0.1	0.665^{cBC}	1.191^{bC}	1.413^{aA}	0.040
0.2	0.395^{bD}	1.226^{aBC}	1.311^{aA}	0.040
0.5	0.725^{bB}	1.351^{aAB}	1.326^{aA}	0.040
1.0	0.537^{bCD}	1.029^{aD}	1.075^{aB}	0.040
SEM	0.040	0.040	0.040	
		Succinivibrio dextrinosolvens		
0.0	1.045^{bBC}	1.322^{aA}	1.117^{bA}	0.04
0.1	1.235^{aAB}	0.994^{bB}	0.810^{cB}	0.055
0.2	1.277^{aA}	1.068^{bB}	0.888^{cB}	0.056
0.5	1.13^{aABC}	1.05^{aB}	0.809^{bB}	0.056
1.0	0.964^{abC}	1.000^{aB}	0.856^{bB}	0.056
SEM	0.056	0.056	0.056	

Different lowercase letters in the same line are significantly different and different uppercase letters in the same column are significantly different ($P < 0.05$). [1]SEM = standard error of the mean.

Cashew nut shell liquid (CNSL)

The CNSL extract affected the bacterial growth ($P < 0.05$) for all analyzed concentrations (Table 3). Except for *S. dextrinosolvens*, upon the addition of CNSL, all cultures of ruminal bacteria presented drastic and significant decrease ($P < 0.05$) in OD_{600nm} at intervals of 8 and 12 hours of incubation. The addition of 0.1, 0.2, 0.5 and 1.0 mg mL^{-1} resulted in a late decrease in the optical density for *S. dextrinosolvens*, the effect being evident at 12 and 24 hours of observation.

Clove leaf

The identification of bioactive compounds in clove leaf EO was performed by GC-MS, and showed that eugenol ($C_{10}H_{12}O_2$, *m/z* 164.0 [M^+]) and caryophyllene ($C_{15}H_{24}$, *m/z* 204.09 [M^+]) (Figure 2) are the main compounds present in this oil.

The effects of adding clove EO (Table 4) varied according on the concentration being used. The addition of 0.2, 0.5 and 1.0 mg mL^{-1} had no effect in reducing the OD_{600nm} for *P. albensis*, *P. bryantii* and *T. saccharophilum.* However, the 0.1 mg mL^{-1} concentration promoted decrease in OD_{600nm} after the 12-hours observation interval. For *P. ruminicola* and *S. dextrinosolvens*, the addition of 1.0 mg mL^{-1} resulted in a greater impact on growth dynamics, with a reduction in optical density in all intervals of observations. For the concentration 0.1, 0.2 and 0.5 mg mL^{-1}, when evaluated in *P. ruminicola*, a decrease in OD_{600nm} was observed only for the first 8 hours of evaluation, whereas, for *S. dextrinosolvens*, the effects of these concentrations were evident after the 8 hours of observation.

Table 3. Effect of cashew nut shell liquid against Gram-negative ruminal bacteria

Concentration mg mL^{-1}	Time, hours			[1]SEM
	8	12	24	
		Prevotella albensis		
0.0	1.527^{A}	1.611^{A}	1.532^{B}	0.025
0.1	0.100^{cB}	0.350^{bB}	1.630^{aAB}	0.038
0.2	0.160^{cB}	0.450^{bB}	1.630^{aAB}	0.038
0.5	0.120^{cB}	0.430^{abB}	1.670^{aA}	0.038
1.0	0.100^{cB}	0.420^{bB}	1.500^{aB}	0.038
SEM	0.038	0.038	0.038	
		Prevotella bryantii		
0.0	1.284^{bA}	1.374^{abA}	1.454^{aB}	0.028
0.1	0.104^{cB}	0.677^{bB}	1.554^{aAB}	0.039
0.2	0.132^{cB}	0.777^{bB}	1.488^{aAB}	0.039
0.5	0.040^{cB}	0.529^{bC}	1.477^{aB}	0.039
1.0	0.169^{cB}	0.686^{bB}	1.623^{aA}	0.039
SEM	0.039	0.039	0.039	
		Prevotella ruminicola		
0.0	0.794^{bA}	0.990^{aA}	0.968^{aA}	0.034
0.1	0.117^{bB}	0.357^{aCD}	0.476^{aC}	0.048
0.2	0.053^{cB}	0.291^{bD}	0.947^{aA}	0.048
0.5	0.165^{cB}	0.614^{bB}	1.010^{aA}	0.048
1.0	0.032^{cB}	0.484^{bBC}	0.678^{aB}	0.048
SEM	0.048	0.048	0.048	
		Treponema saccharophilum		
0.0	1.398^{A}	1.378^{A}	1.430	0.039
0.1	0.152^{cB}	0.415^{bD}	1.577^{a}	0.056
0.2	0.170^{cB}	0.456^{bCD}	1.436^{a}	0.056
0.5	0.107^{cB}	0.627^{bBC}	1.463^{a}	0.056
1.0	0.150^{cB}	0.771^{bB}	1.557^{a}	0.056
SEM	0.056	0.056	0.056	
		Succinivibrio dextrinosolvens		
0.0	1.045^{bB}	1.322^{aA}	1.117^{bA}	0.041
0.1	1.223^{aA}	0.585^{cB}	0.742^{bB}	0.057
0.2	1.250^{aA}	0.972^{bB}	0.774^{cB}	0.058
0.5	1.010^{aB}	1.022^{aB}	0.676^{bB}	0.058
1.0	1.067^{aB}	1.006^{aB}	0.775^{bB}	0.058
SEM	0.058	0.058	0.058	

Different lowercase letters in the same line are significantly different and different uppercase letters in the same column are significantly different ($P < 0.05$). [1]SEM = standard error of the mean.

Discussion

Baccharis dracunculifolia

The *in vitro* antimicrobial activity of the *Baccharis dracunculifolia* extract demonstrated in this study may be associated with synergism between the main classes of identifiedsecondary metabolites: terpenes (germacrene B, spathulenol, α-pinene, limonene and β-caryophyllene), flavonoids (naringenin, kaempferol, kaempferide and apigenin) and phenolic compounds (artepillin C and hydroxycinnmic acid) (Bonin et al., 2020; Frizzo et al., 2008; Lage et al., 2015; Paula et al., 2017; Salazar et al., 2018). Although the exact mechanism of action of these bioactive compounds has not yet been elucidated, exposure of anaerobic ruminal bacteria to these compounds as performed in the current work indicates that they can reduce cell growth and biomass production. Previous studies

indicate that plant extracts can affect the integrity of the cell membrane in bacterial cells, increasing ion permeability and causing the dissipation of the membrane potential (Mirzoeva et al., 1997; Tarahovsky et al., 2014). The electrochemical potential across the cytoplasmic membrane is essential for anaerobic bacteria to carry out ATP synthesis and substrate uptake, which is essential for biomass production (Mirzoeva et al., 1997). Further studies should be carried out to address if baccharis extracts also affect the energetics of ruminal bacteria, especially the species involved in the breakdown of proteins in ammonia in the rumen environment.

Table 4. Effect of clove essential oil against Gram-negative ruminal bacteria

Concentration mg mL^{-1}	Time, hours			^{1}SEM
	8	12	24	
	Prevotella albensis			
0.0	1.527	1.611^{AB}	1.532^{A}	0.045
0.1	1.480^{a}	1.540^{aAB}	1.070^{bB}	0.064
0.2	1.460	1.550^{AB}	1.480^{A}	0.064
0.5	1.550	1.690^{A}	1.560^{A}	0.064
1.0	1.390	1.440^{B}	1.570^{A}	0.064
SEM	0.064	0.064	0.064	
	Prevotella bryantii			
0.0	1.284^{bC}	1.374^{ab}	1.454^{aA}	0.038
0.1	1.560^{aA}	1.540^{a}	0.910^{bB}	0.053
0.2	1.510^{AB}	1.520	1.520^{A}	0.053
0.5	1.440^{ABC}	1.530	1.480^{A}	0.053
1.0	1.340^{BC}	1.470	1.410^{A}	0.053
SEM	0.053	0.053	0.053	
	Prevotella ruminicola			
0.0	0.794^{bA}	0.99^{aA}	0.968^{aA}	0.027
0.1	0.206^{bB}	0.833^{Ab}	0.839^{aAB}	0.038
0.2	0.194^{bB}	0.862^{aAB}	0.915^{aAB}	0.038
0.5	0.222^{bB}	0.847^{AB}	0.875^{aAB}	0.038
1.0	0.205^{bB}	0.832^{aB}	0.798^{aB}	0.038
SEM	0.038	0.038	0.038	
	Treponema saccharophilum			
0.0	1.398^{BC}	1.378^{B}	1.430^{A}	0.037
0.1	1.670^{aA}	1.540^{aAB}	1.040^{bB}	0.052
0.2	1.580^{AB}	1.570^{AB}	1.590^{A}	0.052
0.5	1.380^{bC}	1.650^{aA}	1.610^{aA}	0.052
1.0	1.580^{AB}	1.530^{AB}	1.480^{A}	0.052
SEM	0.052	0.052	0.052	
	Succinivibrio dextrinosolvens			
0.0	1.045^{bA}	1.322^{aA}	1.117^{bA}	0.041
0.1	1.252^{aA}	1.001^{bB}	0.798^{cB}	0.056
0.2	1.183^{aA}	0.975^{bB}	0.819^{cB}	0.057
0.5	1.124^{aA}	0.964^{bB}	0.729^{cB}	0.057
1.0	0.096^{cB}	0.229^{bC}	0.845^{aB}	0.057
SEM	0.057	0.057	0.057	

Different lowercase letters in the same line are significantly different and different uppercase letters in the same column are significantly different ($P < 0.05$). ^{1}SEM = standard error of the mean.

Tamarindus indica L.

All microorganisms tested showed susceptibility to the seed extract of tamarind. The antibacterial activity of the tamarind seed extract can be attributed to polyphenols, such as catechin, procyanidin B2, caffeic acid, chloramphenicol, quercetin, apigenin and kaempferol, that are commonly found in tamarind seeds (Abukakar et al., 2008; Razali et al., 2012, 2015; Siddhuraju, 2007; Sudjaroen et al., 2005).

The underlying mechanism of action of the antibacterial activity of the tamarind extract is not yet elucidated in the literature, but it is believed in the occurrence of synergic effects of the compounds. The flavonoids, such as the quercetin, found in the tamarind extract, are hydrophobic compounds that can translocate across lipid bilayers, exerting antimicrobial activity in the cytoplasm of the cell. Several mechanisms of action have been attributed to procyanidin and catechins, such as the destabilization/permeabilization of the cytoplasmic membrane and direct effects on the microbial

metabolism (Tarahovsky et al., 2014). Antibacterial activities have been reported for phenolic acids, like caffeic acid, both against Gram-positive and Gram-negative bacteria (Daglia, 2012).

Cashew nut shell liquid (CNSL)

The bioactive compounds the CNSL has been the subject of research, and scientific discoveries have attracted the interest of researchers, due to its antimicrobial (Boonsai et al., 2014; Stasiuk & Kozubek, 2010) and antioxidant properties (Andrade et al., 2011) and its potential to reduce methane emissions (Watanabe et al., 2010).

The results reported in the current study demonstrate the inhibitory activity of CNSL compounds against Gram-negative ruminal bacteria. Previous studies reported that CNSL is mainly effective on Gram-positive anaerobic bacteria (Kubo et al., 1993, 2003). However, the data reported in this study, demonstrate that the Gram-negative ruminal bacteria are also highly susceptible to the compounds present in CNSL extracts.

Kubo et al. (2003) indicated that the compounds present in the CNSL extract act mainly as a surfactant, causing physical damages to the bacterial cell membranes, thus triggering cell death. Corroborating with this claim, Stasiuk & Kozubek (2010) suggested that phenolic lipids (such as cardanol and cardol) interact with the cell membranes and DNA structures promoting cytotoxic effects on target cells.

Clove leaf

The results found can be associated with the presence of the bioactive compounds identified, the example of eugenol (hydrophobic nature), which allows it to penetrate the lipolysaccharide layer of the Gram-negative outer membrane causing changes the cell envelope (Devi et al., 2010). In general, it has been proposed that the synergism between eugenol and caryophyllene promotes an increase in membrane permeability (Gill & Holley, 2006; Hemaiswarya & Doble, 2009; Trombetta et al., 2005), compromising the maintenance of the electrochemical gradient and impairing the growth/survival of target organisms.

Conclusion

The present work suggests that aqueous natural extracts of baccharis, tamarind, and cashew nut shell liquid has antimicrobial activity *in vitro* against the Gram-negative ruminal bacteria analyzed in this study. In addition, the study contributes to new information about the efficacy of the extracts plant as antimicrobial agent on ruminant production.

Authorship contribution statement

Venício Macêdo Carvalho: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing, Writing - original draft. **Vicente Alfonso Díaz Ávila:** Data curation, Methodology, Writing - review & editing**. Edinéia Bonin:** Data curation, Formal analysis, Methodology. **Rodolpho Martin do Prado:** Methodology, Supervision, Writing - review & editing, Writing - original draft. **Isolde Terezinha Santos Previdelli:** Data curation, Formal analysis. **Anderson Valdiney Gomes Ramos:** Data curation, Formal analysis. **Adriano Borges Meniqueti:** Formal analysis. **Debora Cristina Baldoqui:** Conceptualization, Methodology. **Hilário Cuquetto Mantovani**: Writing - review & editing, Conceptualization, Methodology **Ivanor Nunes do Prado:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing, Writing - original draft, Project administration, Funding acquisition.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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