Isolation and identification of bioactive compounds responsible for the anti-bacterial efficacy of *Lotus corniculatus* var. São Gabriel

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Lotus corniculatus (Fabaceae) is considered a forage plant utilized as food for ruminants in the south of Brazil. This herb is also actually used to treat intestinal infection in these animals. In our experiments, we evaluated the anti-bacterial activity of crude extract from *L. corniculatus* var. São Gabriel were assayed against Gram-positive and Gram-negative bacterium. The crude extracted did not show any anti-bacterial activity, but the hexane fraction did on *Bacillus cereus* (MIC=100 µg/mL) and on *Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermidis, Acinetobacter calcoaceticus,* and *Providencia alcalifaciens* (MIC=600, 800 or 1000 µg/mL). The Ethyl acetate fraction (AcOEt) also showed important anti-bacterial activity on *Bacillus cereus, E. faecalis,* and *Acinetobacter calcoaceticus* (MIC=800 µg/mL). The oleanolic acid isolated from hexane fraction showed the same effect on *Staphylococcus aureus* methycillin-resistant (MIC=100 µg/mL), *L. monocytogenes* (MIC=25 µg/mL), and *Bacillus cereus* (MIC=25 µg/mL). Further, Kaempferitrin isolated from ethyl acetate fraction has also shown anti-bacterial activity on *Shighella flexinerii* (MIC=100 µg/mL), *Salmonella typhimurium* (MIC=100 µg/mL), *A. calcoaceticus* (MIC=100 µg/mL), *E. faecalis* (MIC=3.9 µg/mL), and *Bacillus cereus* (MIC=8.5 µg/mL). This study suggests that *L. corniculatus* var. São Gabriel have potential pharmacological property for a new anti-bacterial drug development.

Key words: Anti-bacterial activity, Kaempferitrin, *Lotus corniculatus*, Oleanolic acid, β-sitosterol.

INTRODUCTION

Many studies have been addressed to determine the anti-bacterial activities of plant extracts. In this context, plants produce a variety of compounds named "secondary metabolites" that have many biological activities in *in vivo* experiments such as analgesic and antiinflammatory,[1] antiviral and antifungal,[2] antithrombotic and anti-platelet,[3] and anti-bacterial properties.[4]

Lotus corniculatus v. São Gabriel (Fabaceae), also known as "Cornichão" in Brazil, is a plant used as food to the ruminants since this herb promotes an important increase in essential amino acid absorption,[5] ovulation rate, and milk protein and lactose production.[6] This herb is also used as an important antihelmintic substance in these animals[7]

Nevertheless, there are few reports concerning the investigation of anti-bacterial activity of genus *Lotus*,[8] including *Lotus corniculatus* variety.[9] Therefore, this is the first report that is demonstrating the anti-bacterial effect of *L. corniculatus* variety São Gabriel and also which compounds that could be responsible for this effect. Further, this study has a significant contribution to the ethnopharmacological relevance to Brazilian veterinary medicine.

In relation to phytochemical studies of *Lotus* species, there are many reports showing many constituents including flavonoids,[10] anthocyanins,[11] sterols,[9] tannins,[12] alkaloids,[13] and cynogenic compounds.[14]

This study aimed to evaluate the anti-bacterial activity of the crude extract, fractions, and purified compounds isolated from *L. corniculatus* var. São Gabriel cultivated in Brazil. We also identify which bioactive compounds could be responsible for this biological activity.

MATERIALS AND METHODS

Plant Material

L. corniculatus var. São Gabriel was collected in November 2006, in Lages, Santa Catarina State, Brazil, at the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A. (EPAGRI). The material was identified by the botanist Prof. Dr. Daniel de Barcelos Falkenberg from the Botany Department at the Federal

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University of Santa Catarina, Florianópolis, SC, Brazil. A voucher specimen is deposited in the Herbarium at the same university (FLOR 18.770).

Preparation of Plant Extracts

The aerial parts of *L. corniculatus* var. São Gabriel were air-dried and protected from light at room temperature (25°C) for 1 week. Subsequently, the dried aerial parts (620 g) were grounded into particles (1.5 mm) using a knife mill (Mill TE-651, Tecnal, Piracicaba, SP, Brazil). The grounded material was extracted with 5 L of ethanol 96% (plant 1:8, w/v) at room temperature. After 2 days, the extract obtained was filtered (Watmann, n. 1) and the ethanol was removed by rotavapor (Fisatom- 802, São Paulo, SP, Brazil) at 55°C under reduced pressure (460 mm Hg) (Vacuum Q-355A2, Quimis, Diadema, SP, Brazil). This procedure was repeated 15 times in a period of 1 month to obtain the maximal yield of the crude extract (78 g). The CE was fractionated by liquid–liquid extraction using solvents in growing order of polarity, resulting in hexane (HEX: 7.82 g), ethyl acetate (AcOEt: 11.4 g), n-butanol (BuOH: 5.24 g), and aqueous (Aq: 30.8 g) fractions.

Preliminary Phytochemical Analysis

A preliminary phytochemical screening of the crude extract of *L. corniculatus* was carried out according to the Phytochemical Methods described by Harbone[15] to detect the presence of phenols, tannins, anthocyanins, anthocyanidins, flavonoids, xanthones, steroids, triterpenes, and saponins. In these protocols, we used the following reactions: the cianidin or Shinoda´s tests for flavonoids, antocianins, and antocianidins. Lieberman-Burchard´s reactions for sterols and triterpenes. Stanishy´s reaction characterizes the presence of condensed tannins and hydrolyzed tannins. The foam formation test to detect the saponnins presence and finally for xanthones was used the chloride acid and magnesium metallic.[15]

Chromatografic Separation and Isolation of Constituents

The hexane fraction was chromatographed using silica gel column chromatography with a HEX/EtOAc gradient resulting in the isolation of two terpenoids: a fraction eluted with HEX/EtOAc (90:10, v/v) affording 76 mg of a white crystal powder (Compound 1), and HEX/EtOAc (70:30, v/v) producing 25 mg of a white powder (Compound 2). From ethyl acetate fraction, after silica gel column chromatography eluted with EtOAc/EtOH (50:50, v/v), followed by the flavonoidic fraction purification with flash chromatography, 45 mg of a yellow powder (Compound 3) was isolated using solvent system (ethyl acetate/water/formic acid/acetic acid – 70:20:3:2, v/v/v/v).

Structure Elucidation of the Compounds

The chemical structure of each isolated compound was determined on the basis of its physical characteristics and spectral data produced by infrared analysis recorded on a Perkin Elmer FTIR 16PC infrared instrument (Beaconsfield, England). Analysis was carried out with KBr pellets and the results were registered in centimeters^{-1} (cm^{-1}). Nuclear magnetic resonance (^{1}H and ^{13}C-NMR) was recorded on a Varian AS-400 spectrometer (Palo-Alto, California, USA) operating at 400 and 100 MHz, respectively. Thyl thin layer chromatography (TLC) was carried out on silica gel 60 F_{254} plates (Macherey-Nagel, Düren, Germany). Finally, the structures of the three isolated compounds were confirmed by comparison with reference data previously reported from available literature[16,17] and co-TLC with authentic samples.

Bacterial Strains

The microorganisms used were Gram-positive bacteria: *Bacillus cereus* from the American Type Collection Culture (ATCC), *Enterococcus faecalis* from ATCC 29212, *Listeria monocytogenes* from ATCC 35152, *Staphylococcus aureus* from ATCC 25923, *Staphylococcus epidermidis* from ATCC 12228, and Methicillin-resistant *Staphylococcus aureus* (MRSA) from ATCC 43300 and Gram-negative bacteria: *Acinetobacter baumanii* from ATCC 17978, *Acinetobacter calcoaceticus* from ATCC 19606, *Escherichia coli* from ATCC 25922, *Klebsiella pneumoniae* from ATCC 31488, *Pseudomonas aeruginosa* from ATCC 27853, *Proteus mirabilis* from ATCC 25933, *Providencia alcalifaciens* from ATCC 9886, *Salmonella typhimurium* from ATCC 14028, and *Shigella flexineri* from ATCC 12022. The identification of strains was confirmed by the use of biochemical profiles according to the recommendation of the Manual of Clinical Microbiology.[18]

Anti-bacterial Assay

Minimal inhibitory concentration

Anti-bacterial activity of the crude extract, fractions, and compounds was evaluated using the micro-dilution bioassay in 96-well microplates for minimum inhibitory concentration (MIC) determination.[19] The crude extract, fractions, and compounds were dissolved in water + 10% dimethylsulfoxide (DMSO). The initial concentration of extracts and fractions was 10,000 μg/mL and for compounds it was 1,000 μg/mL. The initial test concentration was serially diluted twofold. Each well was inoculated with 5 μL of suspension containing 10^8 CFU/mL of each tested bacteria. The antibiotic gentamicin was included in the assays, as a positive control. The plates were incubated at 24 h at 37°C. Bacterial growth was tested by adding 10 μL of 5 mg/mL 2,3,5-triphenyltetrazolium chloride (TTC) to each well and the plates incubated at 37 °C for 1 h.[20] Bacterial growth in the wells was indicated by a red color, whereas clear wells indicated growth inhibition by the natural product. MIC values were recorded as the lowest concentrations of crude extract, fractions, or compounds showing clear wells. The

assay was repeated in triplicate.

To crude extract and fractions, an MIC below 100 μg/mL was considered as an excellent effect, from 100 to 500 μg/mL as moderate, from 500 to 1000 μg/mL as weak, and over 1000 μg/mL as inactive.[21] For isolated compounds, an MIC below 10 μg/mL was excellent, 10 to 100 μg/mL was good, and over 100 μg/mL was inactive.[22]

Minimal bactericidal concentration (MBC)

To determine the minimal bactericidal concentration (MBC), 10 μL aliquots of broth were taken from each well and plated in Muller-Hinton agar for 24 h at 37°C. MBC represents the concentration necessary to kill 99.9% or more of the initial inoculum.[23]

If the MBC was up to threefold the dilution of MIC, the anti-bacterial activity was considered to be bacteriostatic, and if the MBC was lower than threefold the dilution of the MIC, the anti-bacterial activity was considered to be bactericidal.[24]

Chemicals

Purchases were as follows. Muller Hinton broth and agar from Oxoid (Hampshire, UK); gentamicine from Laboratório Chile (Santiago, Chile); 2,3,5-triphenyltetrazolium chloride TTC fromVetec (São Paulo, SP, Brazil); organic solvents: acetone, chloroform, n-hexane, ethyl acetate, n-butanol, methanol, and ethanol (all analytical grade) from Synth (Diadema, SP, Brazil); sheep's blood (Newprov, Curitiba, PR, Brazil); Dimethylsulfoxide – DMSO from Sigma–Aldrich (St. Louis, MI, USA). Other reagents used were of analytical grade and obtained from different commercial sources.

RESULTS

Phytochemical Analysis

Preliminary phytochemical analysis showed that the crude extract of *L. corniculatus* var. São Gabriel had a significant presence of flavonoids, steroids, and terpenoids. From hexane fraction, we isolated the Compound 1 that was identified as β-sitosterol [Figure 1].

β-sitosterol (Compound 1): White cristal, melting point = 137 – 139°C. IR ν_{max}: 3425, 2936, 1646, 1463, 1376, 1057 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz) [Table 1] and RMN ^{13}C ($CDCl_3$, 100 MHz) [Table 2].

The second compound (Compound 2), also isolated from the same fraction, was identified as oleanolic acid [Figure 2].

Oleanolic acid (Compound 2): White amorphous solid, melting point = 279 – 282°. C. IR ν_{max}: 3453, 2942, 1692, 1644, 1464, 1382, 1273, 1183, 1087, 1034 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz) [Table 1] and RMN ^{13}C ($CDCl_3$, 100 MHz) [Table 2].

Finally, from ethyl acetate fraction we isolated a flavonoid *O*-heteroside (Compound 3) that was identified as Kaempferitrin [Figure 3].

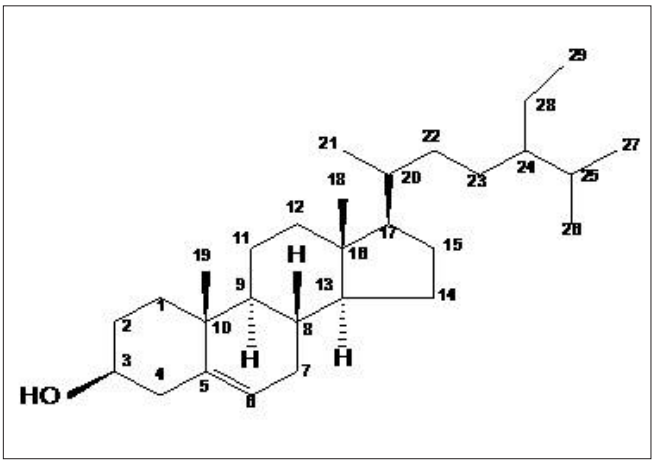

Figure 1: The chemical structure of β-sitosterol isolated from n-hexane fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae).

Table 1: ^1H NMR spectroscopic data of compounds 1, 2, and 3.

Position	Compound 1 δH[a]	Compound 2 δH[a]	Position	Compound 3 δH[a]
CH_3	0.67 (s, 3H, H18)	0.74 (s, 3H, H30)	6	6.43 (d, 2.2)
	0.80 (s, 3H, H26)	0.76 (s, 3H, H25)	7	-
	0.82 (s, 3H, H27)	0.89 (s, 3H, H26)	8	6.69 (d, 2.2)
	0.86 (s, 3H, H29)	0.90 (s, 3H, H23)	9	-
	0.92 (d, 3H, *J* = 7, 6, H21)	0.91 (s, 3H, H24)	10	-
		0.97 (s, 3H, H27)	1'	-
		1.12 (s, 3H, H29)	2', 6'	7.76 (d, 8.8)
			3', 5'	6.92 (d, 8.8)
H-sp^2	5.35 (dd, 1H, H6)	5.27 (m, 1H, H12)	4'	-
			1R1	5.55 (d, 1.8)
			2R1	4.02 (m)
			3R1	3.83 (dd)
H-3	3.50 (m, 1H, H3)	3.20 (dd, 1H, H3)	4R1	3.47 (t)
			5R1	3.60 (m)
			6R1	1.25 (d, 6.2)
			1R2	5.38 (d, 1.8)
			2R2	3.30 (m)
			3R2	4.22 (m)
			4R2	3.71 (m)
			5R2	3.33 (m)

[a]Spectra obtained in CD_3OD, 400 MHz.

Table 2: ^{13}C NMR spectroscopic data of compounds 1, 2, and 3

Position	Compound 1 δC^a	Position	Compound 2 δC^a	Position	Compound 3 δC^a
1	37.48	1	38.34	2	159.79
2	31.89	2	27.13	3	136.47
3	72.04	3	79.0	4	179.77
4	42.53	4	38.72	5	163.00
5	140.97	5	55.1	6	99.83
6	121.95	6	18.25	7	163.52
7	32.14	7	32.56	8	95.58
8	32.14	8	39.22	9	158.07
9	50.35	9	47.58	10	107.53
10	36.73	10	37.05	1'	122.37
11	21.31	11	22.87	2', 6'	132.00
12	39.99	12	122.59	3', 5'	116.57
13	42.53	13	143.5	4'	161.77
14	56.99	14	41.54	1R1	100.5
15	24.54	15	27.65	2R1	71.90
16	28.48	16	23.55	3R1	71.29
17	56.27	17	46.48	4R1	73.14
18	12.21	18	40.92	5R1	71.79
19	20.0	19	45.83	6R1	18.08
20	36.38	20	30.65	1R2	103.52
21	19.01	21	33.75	2R2	72.05
22	34.16	22	32.40	3R2	72.78
23	26.27	23	28.07	4R2	73.58
24	46.05	24	15.52	5R2	71.68
25	29.36	25	15.29	6R2	17.67
26	19.6	26	17.11	-	-
27	19.2	27	25.91	-	-
28	23.29	28	183.33	-	-
29	12.09	29	33.05	-	-
-	-	30	23.36	-	-

[a]Spectra obtained in CD_3OD, 100 MHz.

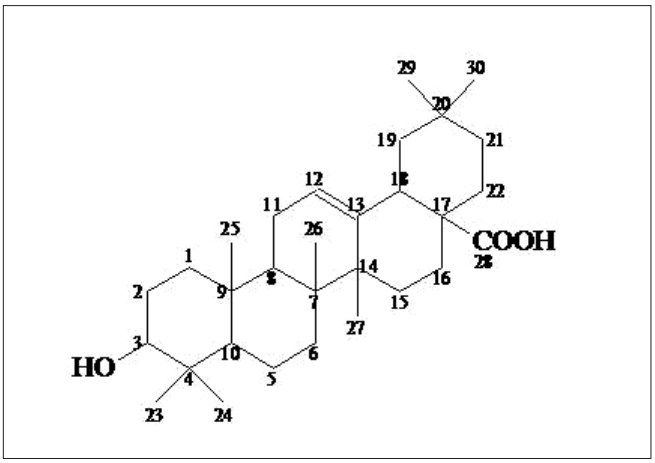

Figure 2: The chemical structure of oleanolic acid isolated from n-hexane fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae).

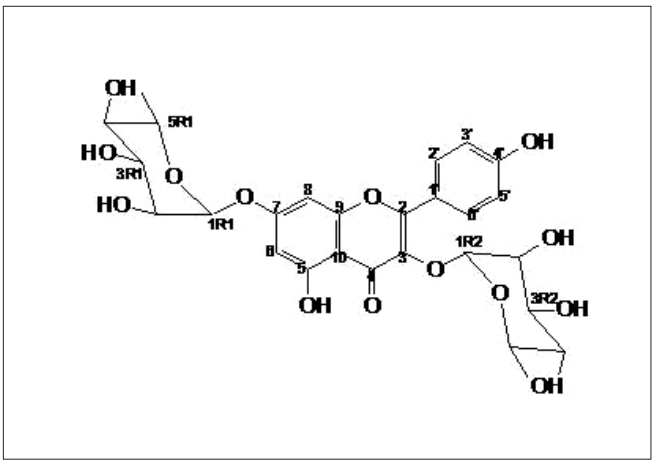

Figure 3: The chemical structure of kaempferitrin isolated from ethyl acetate fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae).

Kaempferitrin (Compound 3): yellow crystal, melting point = 198.5 – 201.3°C. IR ν_{max}: 3383, 2983, 2923, 1906, 1659, 1605, 1513, 1493, 1448 cm^{-1}; RMN ^1H ($CDCl_3$, 400 MHz) [Table 1] and RMN ^{13}C ($CDCl_3$, 100 MHz) [Table 2].

It is important to point out that these compounds have never been described for this variety of *L. corniculatus*. The β-sitosterol represented 1.42%, and the oleanolic acid 0.46% of the hexane fraction. The kaempferitrin represented 0.45% of the ethyl acetate fraction.

Anti-bacterial Analysis

The anti-bacterial activity of the crude extract, aqueous, and n-butanol fractions against all bacteria tested were considered inactive as none of them showed anti-bacterial activity below 1000 μg/mL. On the other hand, the hexane fraction demonstrated a moderate anti-bacterial activity (MIC = 100 μg/mL) on the Gram-positive bacteria *B. cereus* and a weak anti-bacterial effect (MIC = 600, 800, or 1000 μg/mL) on *E. faecalis, L. monocytogenes, Staphylococcus aureus,* and *Staphylococcus epidermidis* and the Gram-negative bacteria *A. calcoaceticus* and *P. alcalifaciens* [Table 3].

Ethyl acetate fraction (AcOEt) also had a weak anti-bacterial activity (MIC = 800 μg/mL) on the Gram-positive bacteria *B. cereus* and *E. faecalis* and the gram-negative bacterium *A. calcoaceticus* [Table 3].

All these effects were considered to be of bacteriostatic action, since the MBCs were at a dilution of more than threefold than the MICs [Tables 3 and 4]. Further, the hexane and ethyl acetate fractions demonstrated better anti-bacterial activity than the other studied fractions.

Subsequently, to elucidate which bioactive components could be responsible for the anti-bacterial activity of *L. corniculatus* var. São Gabriel. First of all, we isolated Compound 1 (β-sitosterol) [Figure 1] and Compound 2 (oleanolic acid) [Figure 2] from the hexane fraction and Compound 3 (Kaempferitrin) [Figure 3] from the ethyl acetate fraction. The second step was to investigate the

anti-bacterial effect of these three isolated compounds using the same methodology. Compound 1 [Figure 1] did not show any significant anti-bacterial activity. On the other hand, Compound 2 [Figure 2] showed a good anti-bacterial activity (MICs from 25 to 100 μg/mL) on the Gram-positive bacteria *B. cereus, L. monocytogenes,* and the methicillin-resistant *Staphylococcus aureus* [Table 3]. Compound 3 [Figure 3] also showed a good anti-bacterial activity (MICs = 100 μg/mL) on the Gram-positive bacterium *Staphylococcus epidermidis* and the Gram-negative bacteria *A. calcoaceticus, S. typhimurium*, and *Shiguella flexinerii.* These effects were considered to be bacteriostatic [Tables 3 and 4].

Surprisingly, the best activity was observed with this kaempferitrin against two Gram-positive bacteria, *B. cereus* and *E. faecalis*, with an excellent anti-bacterial activity (MIC

Table 3: Minimal inhibitory concentrations (MICs: μg/mL) of crude extract, fractions, and isolated compounds of *Lotus corniculatus* var. São Gabriel

	CE	HEX	Aq	BuOH	AcOEt	Comp. 1	Comp. 2	Comp. 3	GE
Gram-positive bacteria									
Bacillus cereus ATCC 11778	>1000	100	>1000	>1000	800	>1000	25	8.5	0.2
Enterococcus faecalis ATCC 29212	>1000	600	>1000	>1000	800	300	300	3.9	6.0
Listeria monocytogenes ATCC 35152	>1000	800	>1000	>1000	>1000	500	25	300	0.2
*MRSA** ATCC 43300	>1000	>1000	>1000	>1000	>1000	500	100	200	>100
Staphylococcus aureus ATCC 25923	>1000	1000	>1000	>1000	>1000	500	800	200	1.0
Staphylococcus epidermidis ATCC 12228	>1000	800	>1000	>1000	>1000	500	600	100	0.1
Gram-negative bacteria									
Acinetobacter baumanii ATCC 17978	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	6.0
Acinetobacter calcoaceticus ATCC 19606	>1000	600	>1000	>1000	800	800	600	100	6.0
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	6.0
Klebsiella pneumoniae ATCC 31488	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	1.0
Proteus mirabilis ATCC 25933	>1000	>1000	>1000	>1000	>1000	500	200	200	12.0
Providencia alcalifaciens ATCC 9886	>1000	800	>1000	>1000	>1000	800	500	500	2.0
Pseudomonas aeruginosa ATCC 27853	>1000	>1000	>1000	>1000	>1000	800	800	500	1.0
Salmonella typhimurium ATCC 14028	>1000	>1000	>1000	>1000	>1000	500	200	100	6.0
Shigella flexinerii ATCC 12022	>1000	>1000	>1000	>1000	>1000	400	200	100	3.1

CE = hidroalchoolic crude extract; HEX = n-hexane extract; Aq = aqueous extract; BuOH = butanol extract; AcOEt = ethyl acetate extract; Comp. **1** = β sitosterol; Comp. **2** = oleanolic acid and Comp. **3** = kaempferitrin; GE = Gentamicine; *Methicillin-resistant *Staphylococcus aureus*; ATCC – *American type collection culture* (Data from three experiments)

Table 4: Minimal bactericidal concentrations (MBCs: μg/mL) of crude extract, fractions and isolated compounds of *Lotus corniculatus* var. São Gabriel

	CE	HEX	Aq	BuOH	AcOEt	Comp. 1	Comp. 2	Comp. 3	GE
Gram-positive bacteria									
Bacillus cereus ATCC 11778	>1000	>1000	>1000	>1000	>1000	>1000	200	34	0.4
Enterococcus faecalis ATCC 29212	>1000	>1000	>1000	>1000	>1000	900	>1000	7.4	12.0
Listeria monocytogenes ATCC 35152	>1000	>1000	>1000	>1000	>1000	>1000	200	900	0.2
*MRSA** ATCC 43300	>1000	>1000	>1000	>1000	>1000	>1000	800	800	>100
Staphylococcus aureus ATCC 25923	>1000	>1000	>1000	>1000	>1000	>1000	>1000	800	4.0
Staphylococcus epidermidis ATCC 12228	>1000	>1000	>1000	>1000	>1000	1000	>1000	400	0.2
Gram-negative bacteria									
Acinetobacter baumanii ATCC 17978	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	24.0
Acinetobacter calcoaceticus ATCC 19606	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	12.0
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	12.0
Klebsiella pneumoniae ATCC 31488	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	4.0
Proteus mirabilis ATCC 25933	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	24.0
Providencia alcalifaciens ATCC 9886	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	8.0
Pseudomonas aeruginosa ATCC 27853	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	4.0
Salmonella typhimurium ATCC 14028	>1000	>1000	>1000	>1000	>1000	>1000	>1000	800	12.0
Shigella flexinerii ATCC 12022	>1000	>1000	>1000	>1000	>1000	>1000	>1000	800	3.1

CE = hidroalchoolic crude extract; HEX = n-hexane extract; Aq = aqueous extract; BuOH = butanol extract; AcOEt = ethyl acetate extract; Comp. **1** = β sitosterol; Comp. **2** = oleanolic acid and Comp. **3** = kaempferitrin; GE = Gentamicine; *Methicillin-resistant *Staphylococcus aureus*; ATCC – *American type collection culture* (Data from three experiments)

= 8.5 μg/mL and 3.9 μg/mL, respectively). In this case, the anti-bacterial activity was considered to be bacteriostatic for *B. cereus* and bactericidal for *E. faecalis* [Tables 3 and 4]. It is important to comment that for the hexane, ethyl acetate fraction, and isolated compounds the anti-bacterial activity was more enhanced to the Gram-positive bacteria than to Gram-negative bacteria.

DISCUSSION

There are few reports about the anti-bacterial activity of *L. corniculatus* concerning the *L. corniculatus* var. *ternuifolius* that showed anti-bacterial activity against Gram-positive and Gram-negative bacteria.[9] Our results demonstrated that *L. corniculatus* var. São Gabriel exhibited an important anti-bacterial activity and this effect was more enhanced with hexane and ethyl acetate fractions. The phytochemical analysis of the crude extract showed the presence of flavonoids, steroids, and tepenoids. Similar results have been presented by other authors who have also demonstrated these compounds in *L. corniculatus* var. ternuifolis.[9]

We have also isolated a terpenoid, identified as oleanolic acid (0.46%) that showed moderate anti-bacterial activity against three Gram-positive bacteria. Studies have been demonstrated that oleanolic acid presents important antiviral effect, by inhibiting HIV-1 replication,[25] as well as anti-bacterial proprieties.[26] Further, such is that published by Fontanay *et al*.[27] who also demonstrated that oleanolic acid has "good" to "moderate" anti-bacterial action against some Gram-positive bacteria.

Although, kaempferitrin represented a low concentration (0.45%) isolated from ethyl acetate fraction, this compound demonstrated important anti-bacterial activity. Other studies have also demonstrated the presence of flavonoids in aerial parts of *L. corniculatus*.[10] In addition, to their ability to cause DNA damage in bacteria, it is well known that flavonoids have important antiviral, as well as anti-bacterial activities.[28] These effects seem to be via different mechanisms of action, such as: (i) complexing with the bacterial cell wall and decreasing microbial growth,[29] (ii) inhibiting the activity of the DNA topoisomerase II (DNA gyrase),[30] and (iii) inhibiting the FtsZ protein, the bacterial analog of tubulin, which mediates bacterial cell division.[31,32] The flavonoid *O*-heteroside kaempferitrin showed excellent anti-bacterial activity against two Gram-positive bacteria comparable to the reference antibiotic gentamicine, and this result is also in accordance with Abdel-Ghani *et al*,[9] who have also demonstrated important anti-bacterial activity of kaempferitrin against both Gram-positive and Gram-negative bacteria.

Another compound isolated from hexane fraction was β-sitosterol. Although, this compound represents 1.42% of hexane fraction, this sterol did not show a significant anti-bacterial activity. These results are also in accordance with other authors who have shown that this compound has slight anti-bacterial activity against *E. coli* and activity absence against some Gram-positive bacteria.[33] It is also important to note that *in vitro* studies have also showed that oleanolic acid and β-sitosterol have a low toxicity in eukaryotic cells.[27]

High anti-bacterial activity of *L. corniculatus* var. São Gabriel was demonstrated in our study. The observed activity could be attributed from isolated bioactive compounds: kaempferitrin and oleanolic acid that showed important anti-bacterial activity. Further, kaempferitrim demonstrated good anti-bacterial effect in some Gram-negative and excellent anti-bacterial effect against two Gram-positive bacteria. These results validate the plant use in traditional veterinary medicine and show that kaempferitrin is one of the main compounds responsible for anti-bacterial effect of *L. corniculatus* and also qualify this compound to be a new source for developing a new anti-bacterial drug from natural product.

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