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REGULAR ARTICLE

Antibacterial activity of methanolic and aqueous extracts of *Ligaria cuneifolia* and *Tripodhantus flagellaris*

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ABSTRACT

Ligaria cuneifolia and *Tripodanthus flagellaris* are two species belonging to the *Loranthaceae* family which is widely distributed in the central and northern areas of the Argentina Republic. The present study was conduced to test the antibacterial activity of aqueous and methanol extracts of both plants against *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* CLIP 74910, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 strains. Antibacterial activity was determined using the agar diffusion method (variant holes) and the minimum inhibitory concentration (MIC) by broth microdilution test at concentration ranges from 5000 to 156.25 μg/mL. The greatest effect was observed for the aqueous and methanol extracts of *T. flagellaris* against *S. aureus* and *L. monocytogenes* for which the average diameters of the zones of inhibition were 15 to 16 mm and the MIC values were 625 to 312.5 μg/mL. Considering the MBC/MIC ratio, MIC values of *T. flagelaris* aqueous extracts and MIC values of both extracts of *L. cuneifolia* showed bactericidal effect against *L. monocytogenes*. Also, both extracts of *L. cuneifolia* showed bactericidal effect against this bacterial species (2500 μg/mL) and bacteriostatic effect against *S. aureus*. These preliminary findings demonstrate the antibacterial activity of both plants and contribute to improve the knowledge of these species, reinforcing the importance of the ethnobotanical approach as a potential source of new bioactive substances.

Key words: Antibacterial activity; *Ligaria cuneifolia; Tripodanthus flagellaris*

INTRODUCTION

Plant extracts were regarded by ancient civilizations as significant agents for the treatment of various ailments. However, for some decades there was an increasing interest in plant uses and in the detection of their constituents with antibacterial activity (Alcaráz et al., 2012; Savoia et al., 2012; Abreu et al., 2012). In many places of Argentina, there is a rich tradition of using herbal medicine for the treatment of various infectious diseases, inflammations, injuries, and other diseases. (Del Vitto et al., 1997).

The mistletoe is one of the oldest known herbs with vast folkloric usage as a medicinal plant in many countries and regions of the world. Mistletoe was described as "an all purpose herb" due to its rich traditional uses and it has been widely used in ethnomedicine for various purposes (Kafaru, 1993). Mistletoes are the semiparasitic plants because they normally grow on various host trees and shrubs and they are dependent on their respective host for mineral nutrition and water, although they produce their own carbohydrates through photosynthesis (Griggs, 1991).

Since ancient times, the "mistletoe, *Viscum album L.*" (*Viscaceae*) has been recognized in Europe and Asia as a therapeutic herb, with effects on the cardiovascular system and blood pressure (Griggs, 1991). In Argentina, mainly in the interior provinces, as in the rest of the world, the word "mistletoe" is applied to plants similar to *V. album*, for example *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh (*Loranthaceae*) which belongs to a different botanical family but keeps some degree taxonomic relationship with that species (Wagner et al., 1996). In Argentina, there are 7 genera and 11 species of *Loranthaceae* family (Del Vitto et al., 1997).

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Ligaria cuneifolia (Ruiz & Pav.) Tiegh, popularly known as "liga", "liguilla","liga roja", or "muérdago criollo" and *Tripodanthus flagellaris* (Cham& Schlchdtl). Tiegh, known as "liga blanca", "liguilla", "corpo", "liga", "pupusa", are two species belong to the *Loranthaceae* family widely distributed in the central and northern areas of the Argentina Republic between 1800 and 2700 m on the sea level. These species are shrubby plants hemiparasite, evergreen, living in epiphyte on trees and shrubs. *L. cuneifolia* present woody stems and leaves are alternate, simple, lanceolate. Its flowers are very showy, red (or yellow) color (Figure 1).

In folk medicine, the infusion of these plants are used as hypotensive and to reduce excess cholesterol. Also, their anti-carcinogenic, anti-diabetic and anti-HIV activities have been reported (Dominighini et al., 2010; Fernandez et al., 1998). *Tripodanthus flagellaris* is used mainly as cardioactive drug in Cuyo region (Argentina) (Del Vitto et al., 1997; Fusco et al., 2010).

There are some studies on the biological activities of *L. cuneifolia* and *T. flagellaris* (Fusco et al., 2001; Fusco et al., 2004; Fusco et al., 2010; Ferrero et al., 2006; Dominighini et al., 2010). Few researchers have reported antibacterial activity of *Loranthaceae* species (Varela et al., 2001; Ukwueze et al., 2013). However, some researches suggested that several factors play important roles in the phytochemical composition and pharmacological activities of the mistletoe plants. Such factors include: the host, specie of mistletoe used, season of harvest, etc. (Osadebe and Ukwueze, 2004; Wagner et al., 1996; Osadebe et al., 2008; Yusuf et al., 2013).

Prevoius phytochemical studies on *L. cuneifolia* indicated the presence of tannins, flavonoids, saponins, anthraquinones, alkaloids and cardiac heterosides (Fusco et al., 2001; Fusco et al., 2004). Antibacterial activity of some of these phytochemicals has been shown (Alcaráz et al., 2000; Takhi et al., 2011. In Argentina, has been reported only a study on the antibacterial activity of *L. cuneifolia* in Tucuman (Soberón et al., 2014), while no information regarding the antibacterial activity of *T. flagellaris* was found.

Fig 1. Flowers and leaves of *L. cuneifolia*

In the present study, our purpose was evaluated the antibacterial activity of aqueous and methanolic extracts from these two native plants against Gram positive and Gram negative pathogenic bacteria species.

MATERIALS AND METHODS

Plant material

The *L. cuneifolia* and *T. flagelaris* samples (branches with leaves and flowers) were collected in the Province of San Luis, Argentina in El Milagro (Dpto. Pueyrredón) and La Florida (Dpto. Coronel Pringles), respectively, and were authenticated by Dr. Del Vitto, Botany Department, San Luis National University (UNSL). The voucher specimen of *L. cuneifolia* was deposited in the herbarium of UNSL under the number 9245 (UNSL) and *T. flagelaris* under the number 8553 (UNSL).

Preparation of extracts

The plants materials were dried in open air at room temperature, and finely ground with a hammer mill.

Aqueous extracts

Dried and powdered plant material (5g) was boiled with 70 ml of water for 20 min. After cooling to 40-45 ºC, the liquid was filtered and the volume adjusted to 100 ml with distilled water and then lyophilized (Kuklinski, 2000).

Methanolic extracts

A known amount of powdered plant material (100 g) was extracted overnight with methanol (Merck), under gentle shaking. The extracts were filtered through Whatman Nº 4 filter paper, dried under reduced pressure at 40ºC, and weighed (Kuklinski, 2000).

The methanolic extract was dissolved in dimethylsulfoxide (DMSO) and the aqueous extract was dissolved in water. Later, they were diluted to the highest concentration to be tested (5,000 µg/mL) and then, serial two-fold dilutions were made in concentration ranges from 5,000 to 156,25 µg/mL.

Microorganisms

Antibacterial activity of the extracts was tested individually on clinically important reference bacterial strains. The Gram-positive bacterial strains used were *Staphylococus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* CLIP 74910. The Gram-negative

bacterial strains used were *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853. ATCC strains were provided by the Institute Malbrán (Argentina) and *L. monocytogenes* CLIP 74910 was obtained from the *Listeria* Collection of the Pasteur Institute, Paris. Bacterial strains were maintained on tryptycase soy broth supplemented with 20% glycerol at -80 ° C until use. Before testing, the suspensions were transferred to nutrient broth (Difco) and cultured overnight at 37°C. Inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standard (10^8 bacterial cells). Then, they were diluted 10 times.

Antibacterial testing

Agar diffusion assay

Antibacterial activity of the crude methanol extracts and aqueous extract were determined by the modified agar well diffusion method (Perez et al., 1990). Mueller-Hinton agar (MHA, Britania, Argentina) 25 mL was poured into each petri plate. Once the agar solidified, the microorganisms were inoculated on the surface of the plates (1×10^8 CFU/mL). Subsequently, the surface of the agar was punched with a 6-mm-diameter wells. Each well was filled with 50 µL of each plant extract. Simultaneously, wells containing the same volume de DMSO and distilled water served as negative controls. Gentamicin sulfate (1µg per well) was used as positive control. After a 24-hours incubation at 35ºC, all plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimetres.

All tests were performed in duplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

Determination of Minimum Inhibitory Concentration (MIC)

Methanol and aqueous extracts were further tested to determine the minimum inhibitory concentration (MIC) for each bacterial strain. The MIC values were determined by broth microdilution test using 96-well microplate (CLSI, 2011). In each well, 95 µL triptone soy broth supplemented with 0.01% 2,3,5,-trifenyltetrazolium as visual indicator of bacterial growth, 5 µL of a suspension of 10^7 CFU/mL strains and 100 µL serial dilution in base two (8000 to 125 µg/mL) of the extracts were added. The final volume in each well was 200 µL. The plates were covered with sterile plate sealer and then incubated aerobically at 37 °C for 24 h and read visually. MIC was defined as the lowest concentration of the extract in the medium in which there was no visible growth after incubation. Media, extract and strains controls were included. The test was performed in duplicate and then replicated at least three times. The plates were incubated at 37 °C 24 h.

Determination of Minimal Bactericidal Concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bacterial growth. MBC was determined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37 ºC.

Statistical analysis

The experiments were replicated three times and were expressed as mean value ± standard error of the mean of growth inhibition zones diameters obtained with those natural products which amount was sufficient to perform repetitions.

RESULTS AND DISCUSSION

The antibacterial activity of aqueous and organic extracts from *L. cuneifolia* and *T. flagelaris* was assayed *in vitro* conditions by agar well diffusion and broth microdilution methods against *S. aureus, L. monocytogenes, E. coli and P. aeruginosa*. Inhibition of bacterial growth by the action of the aqueous and methanolic extracts according to the agar well diffusion method is summarized in Table 1. Although the diameters of the inhibition halos are minor compared to commercial antibiotic, the results are interesting, because the total extract was assayed, and no pure compounds.

All extracts were active against gram-positive bacteria and showed no activity against Gram-negative bacteria at the concentrations tested. A slightly greater inhibitory effect was observed in both extracts of *Tripodhantus flagelaris* against *S. aureus* and *L. monocytogenes* for which the average diameters of the zones of inhibition were ranged between 15 and 16 mm. Table 2 show the results of the MIC of aqueous and methanolic extracts of the two plants against strains studied using the broth microdilution method.

There was correlation between the two methods tested. The MIC values of both extracts of *T. flagellaris* against both strains of *S. aureus* were the lowest (MIC values were of 625 µg/mL) being the methanolic extract of *T. flagellaris* (MIC values were of 321.5 µg/mL) slightly more active against *S. aureus* ATCC 43300 (methicillin resistant). There was no difference in the activity of the extracts of both plants among methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. These results indicate that antibiotic resistance does not interfere with the antimicrobial activity of plant extracts and extracts could have different modes of action on microorganisms studied.

Table 1: Antibacterial activity of aqueous and methanolic extracts of *L. cuneifolia* and *T. flagelaris* by agar well diffusion assay

Microorganisms	Inhibition (mm)				
	L. cuneifolia		*T. flagelaris*		Gentamicin
	AE	ME	AE	ME	
S. aureus ATCC 43300	17.4±0.3	17.2±0.2	15.1±0.1	15.2±0.2	21.2±0.2
S. aureus ATCC 25923	17.4±0.3	17.3±0.2	15.3±0.2	15.4±0.3	20.1±0.2
E. coli ATCC 35218	n/i	n/i	n/i	n/i	20.4±00
L. monocytogenes CLIP 74910	18.2±0.2	18.3±0.1	15.2±0.1	16.2±0.2	30.2±0.4
P. aeruginosa ATCC 27853	n/i	n/i	n/i	n/i	18.0±0.1

*AE: Aqueous extracts; *ME: Methanolic extracts; n/i: No inhibition. The dates represent the mean±standard error of the mean values of three different experiments

The methanolic and aqueous extracts of *T. flagellaris* were the most active*s* (MIC values 312.5 and 625 μg/mL respectively) against *Listeria monocytogenes*. Although no differences in antibacterial activity of aqueous extract compared to methanol *L. cuneifolia* extract were observed, the methanol extract from *T. flagellaris* was slightly more active against both Gram-positive bacteria, partially coinciding with other studies reporting that alcohol is a better solvent for extraction of antimicrobial substances from medicinal plants than water (Eloff, 1998).

According to MIC, tested extracts were inactive against gram negative bacteria coinciding with results obtained previously by agar well diffusion method. These results are consistent with some studies where the Gram-positive bacteria are more sensitive to plant extracts than Gram-negative bacteria. Probably, this fact may be due to the relatively impermeable outer membrane that surround Gram-negative bacteria.

There is little scientific information concerning to the antimicrobial activity of *L. cuneifolia* and *T. flagelaris* in Argentina. A study on *L. cuneifolia* performed in Tucumán, Argentina, reported MIC values inferior to our results (Soberón et al., 2014). However, it is difficult to compare the data because several variables influence the results, such as the environmental and climate conditions of the plant and the choice of the extraction method and antimicrobial tests. Furthermore, some authors report the presence of different active metabolites in *Loranthaceae* species according to the host tree (Osadebe and Ukwueze, 2004; Osadebe and Akabogu, 2006).

Table 3 shows MBCs values of methanolic and aqueous extracts against gram-positive bacteria. In order to elucidate if the observed antibacterial effects were bactericide or bacteriostatic, MBC/MIC ratios were calculated. Extracts with ratios greater than 1 were considered as bacteriostatic, while extracts with ratio less than or equal to 1 were bactericide. These data allow concluding that MIC values of both extracts of *L. cuneifolia* were bactericidal against

Table 2: Minimal inhibitory concentration (MIC) of aqueous and methanolic extracts of *L. cuneifolia* and *T. flagelaris* by micro-well dilution method

Microorganisms	MIC (μg/mL)			
	L. cuneifolia		*T. flagelaris*	
	AE	ME	AE	ME
S. aureus ATCC 43300	1250	1250	625	312.5
S. aureus ATCC 25923	1250	1250	625	625
E. coli ATCC 35218	n/i	n/i	n/i	n/i
L. monocytogenes CLIP 74910	2500	2500	625	312.5
P. aeruginosa ATCC 27853	n/i	n/i	n/i	n/i

AE: Aqueous extracts; ME: Methanolic extracts ; n/i: No inhibition

Table 3: Minimal bactericidal concentration (MBC) of methanolic and aqueous extracts of *L. cuneifolia* and *T. flagelaris* against gram-positive bacteria

Microorganisms	MBC (μg/mL)			
	L. cuneifolia		*T. flagelaris*	
	AE	ME	AE	ME
S. aureus ATCC 43300	2500	2500	1250	1250
S. aureus ATCC 25923	2500	2500	1250	1250
L. monocytogenes CLIP 74910	2500	2500	625	625

AE: Aqueous extracts; ME: Methanolic extracts; n/i: No inhibition

L. monocytogenes (2500 μg/mL). Similarly, the MIC values of aqueous extracts of *T. flagelaris* were bactericidal against these bacterial species.

Further, according to the MBC/MIC relationship, the antibacterial effect of the other active extracts against these Gram-positive species is considered bacteriostatic.

Previous phytochemical screening of these plants were positive for tannins, flavonoids, saponins, anthraquinones, alkaloids and cardiac glycosides (Fusco et al., 2001). Also, these authors, in another study, demonstrate, by spectrophotometric methods the presence of betulin and betulinic acid in the dichloromethane extract *L. cuneifolia* (Fusco et al., 2004). It is known that some metabolites such as flavonoids and terpenoids are synthesized by plants in response to microbial infection, and in fact, has been found in various studies that they are effective antimicrobial agents against a broad range of microorganisms (Himejima et al., 1992). While quercetin

glycosides had previously been found in *Ligaria cuneifolia* as the only one flavonol, other authors first described the presence of a kaempferol glycoside in this plant (Soberon et al., 2014) While these plants are used in folk medicine as hypotensive and to reduce excess cholesterol, the data found demonstrate the antimicrobial activity of some of its components.

These preliminary findings represent a contribution to a better knowledge of these plants, used in folk medicine and suggest extending the popular use to other conditions and further, reinforce the importance of the ethnobotanical approach as a potential source non expensive as well as available of new bioactive substance.

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Author contributions

L.E.A: Designed the study, did the analysis and wrote the article. M.R.F: performed harvesting of plants and obtaining extracts. C.M.M and S.E.S: participated in designing and carrying out experiments. A.L.L: supervised this research work and corrected the article.

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