

# Anti-Pathogenic Activity of Herbs Used in Argentinean Traditional Infusion

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**Abstract:** The anti-pathogenic activity of *Acantholippia deserticola*, *Haplopappus baylahuen*, *Lippia integrifolia* and *Satureja parvifolia* herbs used as traditional infusion was investigated. The extracts obtained were compared with respect to chromatographic profiles and antimicrobial activities. The dichloromethane and methanol extracts of the four herbs showed significant inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth and biofilm formation. The dichloromethane extract of *Lippia integrifolia* that showed the highest inhibitory effect on *P. aeruginosa* biofilm formation, was fractionated by column chromatography using a gradient of polarity, and the activities of the fractions were evaluated. In general, the lower polar fractions inhibited biofilm in correlation with bacterial growth. However, in more polar fractions the biofilm diminution is well correlated with the inhibition of autoinducers production more than the bacterial development. The results provide scientific support for the usage of these herbs to the protection against foodborne diseases. This effect is noteworthy in *L. integrifolia* because the fractions showed higher growth, biofilm, and autoinducer inhibitory activity than the crude extract.

**Keywords:** Traditional infusions, South American herbs, Biofilm inhibition, Anti-pathogenic activity, *Lippia integrifolia*.

## 1. INTRODUCTION

People from many South American countries use to consume a traditional beverage called “mate” (drink prepared as an infusion of *Ilex paraguariensis* leaves and twigs, commonly named “yerba mate”). It is prepared in a very sui generis way by large populations in South America, having evolved from a tea drunk by the Guarani ethnic group to a beverage that has a social and almost ritualistic role in some South American modern societies [1, 2]. In fact, it was informed as the most commercialized plant of South America [3].

The mate is an infusion that is consumed by sucking a straw or bulb, which is shared by a group of people. Once the infusion is ready, a special drinking straw, “bombilla” (literally: small pump) is usually made of metals such as stainless steel that “bombilla” which finish in a closed perforated bulblike filter is inserted into the mate [4]. This bulb has perforations, which are the size of a pinhole; avoid the aspiration of fine solids powdered mate leaves when the infusion is sucked up through it. This peculiar method of brewing allows for a continuous extraction of the compounds in the dried leaves, indeed, a portion of the compacted tea is left dry on top.

Each extract of about 30 ml is drunk by a person. After that, the mate is filled and served to another person. Many people consume between 1–2 liters per day. Therefore, the risk of microbial infection is high [5,6]. For this reason, the use of herbs that may have antimicrobial properties is important from a hygienic-sanitary point of view. Populations of Northern Argentina add different herbs to the “mate” because they traditionally attribute medicinal properties to these herbs. *Acantholippia deserticola*, *Haplopappus baylahuen*, *Lippia integrifolia* and *Satureja parvifolia* are the four very popular herbs added to the mate infusion [7]. *Acantholippia deserticola* (Phil.) Moldenke is a small bush, commonly known as rica-rica, this herb is used in herbal medicine for diarrhea, gastrointestinal bloating, dyspepsia and by treating liver disorders, and digestive complaints [8]. *Haplopappus baylahuen* Remy (Asteraceae) known as Baila bien and its aerial parts are widely used for its liver stimulating properties [9]. *Lippia integrifolia* (Gris.) Hieronymus (Verbenaceae) is a woody aromatic shrub, known as Incayuyo, and it is used for gastrointestinal disorder and has been included in the Argentina Food Code [10]. *Satureja parvifolia* (Phil.) Epling, the synonym of *Clinopodium gilliesii* (Benth Kuntze) is a species growing in the Andean countries, popularly known as “Muña-Muña”, used in food as an aromatic plant, but also in traditional medicine [11].

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Due to the risk of foodborne disease and the low people infected due to mate consumption, we investigated the properties of these herbs to control the growth and biofilm formation in a Gram-negative and a Gram-positive bacterium.

Several foodborne disease outbreaks have been associated with microbial biofilm [12]. The biofilm formation is regulated by the bacteria cells-cells communication called *Quorum sensing* (QS), mediated by small diffusible signal molecules called autoinducers (AI). Gram negative bacteria produce N-acyl-homoserine lactones (AHLs) as QS molecules [13] and several AHLs had been found in food [14]. Moreover, the bacterial signal molecules per se may influence the outcome of an infection by modulating the host immune response [15]. *Pseudomonas* sp. is one of the main biofilm producing bacteria found in food.

On the other hand, Staphylococcal foodborne intoxication is reported to be one of the most common bacterial foodborne diseases in several countries and the food processing promotes biofilm formation by *Staphylococcus aureus* [16].

The inhibitory potential of Northern Argentina herbs extracts on the growth and biofilm formation by *S. aureus* and *P. aeruginosa* strains, as well as, on the production of N-acyl-homoserine lactones (AHLs) by *P. aeruginosa* was evaluated.

## 2. MATERIALS AND METHODS

### 2.1. Plant Material

Four dried herbs including *Acantholippia deserticola*, *Haplopappus baylahuen*, *Lippia integrifolia* and *Satureja parvifolia* were purchased from local herbalism's of San Salvador de Jujuy (Jujuy, Argentina) and San Miguel de Tucuman (Tucuman, Argentina).

### 2.2. Preparation of Plant Extracts

Dried plant material (200 g) for each species was extracted by soaking, first in dichloromethane (DCM) (200 ml) and after that in methanol (MeOH) (200 ml) at room temperature for 7 days with each solvent, with discontinuous agitation and then vacuum filtered. Both extracts were dried by a rotary evaporator until 45°C. The weight of dried extracts represented the extracted material for each extract. Extracts were stored at -20°C until use.

### 2.3. Thin Layer Chromatography (TLC) of Extracts

All extracts were loaded onto analytical TLC plates (Silica gel 60 F<sub>254</sub>, 3.5 x 7 cm, Merck), and developed using ethyl acetate/chloroform (EA:C, 4:1, v/v) as the mobile phase. After drying, bands were located by viewing under short (254 nm) and long (366 nm) UV radiation. The following sprays were used to locate the bands on the TLC: Godin reagent (1 vol 1% vanillin in ethanol +1 vol 3% perchloric acid in water) and heating to 85°C for 5 min was used for reveal polyols, phenols, and ketones [17] (Godin, 1954) and Lieberman-Bouchard reagent (LB) was used for reveal steroidal nuclei (triterpenes and/or steroids).

### 2.4. Lippia Integrifolia DCM Extract Purification by Column Chromatography (CC)

A total of 3 g of DCM extract material was loaded on a column (54 x 3.5 cm) packed with Silica technical grade (pore size 60 Å, particle size: 63–200 µ, 230-70 Mesh; Sigma-Aldrich) and eluted first with 100% chloroform, then with increasing amount of EA (solvent of increasing polarity) and finally with MeOH (washing). One hundred and twenty fractions of 40 ml were collected and analyzed by TLC (Silica gel 60 F<sub>254</sub>). According to the chromatographic profile, thirty-five pools were made. The solvent was evaporated under rotary evaporator until 45°C.

### 2.5. Infrared Spectrometry

DCM extracts of four selected herbs were analyzed by infrared spectrometry by Fourier transform (FTIR-Perkin Elmer Spectrum I Serie 1600) in order to determine the presence of functional groups of diagnostic value.

### 2.6. Bioassays

Two strains were used, *Staphylococcus aureus* F7 methicillin-resistant isolated from infected patients of a San Miguel de Tucumán hospital (Collection of Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina), and a *Pseudomonas aeruginosa* ATCC 6738.

### 2.7. Bacterial Growth

Overnight cultures of *P. aeruginosa* and *S. aureus* were diluted to reach an OD of 0.125 ± 0.01 at 560 nm in Luria-Bertani (LB) and Mueller Hinton (MH) media, respectively. A 180 µl aliquot of the diluted culture was placed in each one of the 96 wells of a plastic microtitre plate.

Solutions containing 10, 1 and 0.1 mg/ml extracts or fractions in a dimethyl sulfoxide and water mixture (DMSO:H<sub>2</sub>O, 1:1) were prepared separately, and 20 µl of each solution was pipetted into the microtitre plate wells individually, with eight replicates of each solution. The final concentration of the samples assayed was 1000, 100 and 10 µg/ml respectively. Positive control wells (eight replicates) contained 180 µl of the diluted culture with 20 µl of DMSO: H<sub>2</sub>O (1:1) and negative control wells were performed using sterile culture media.

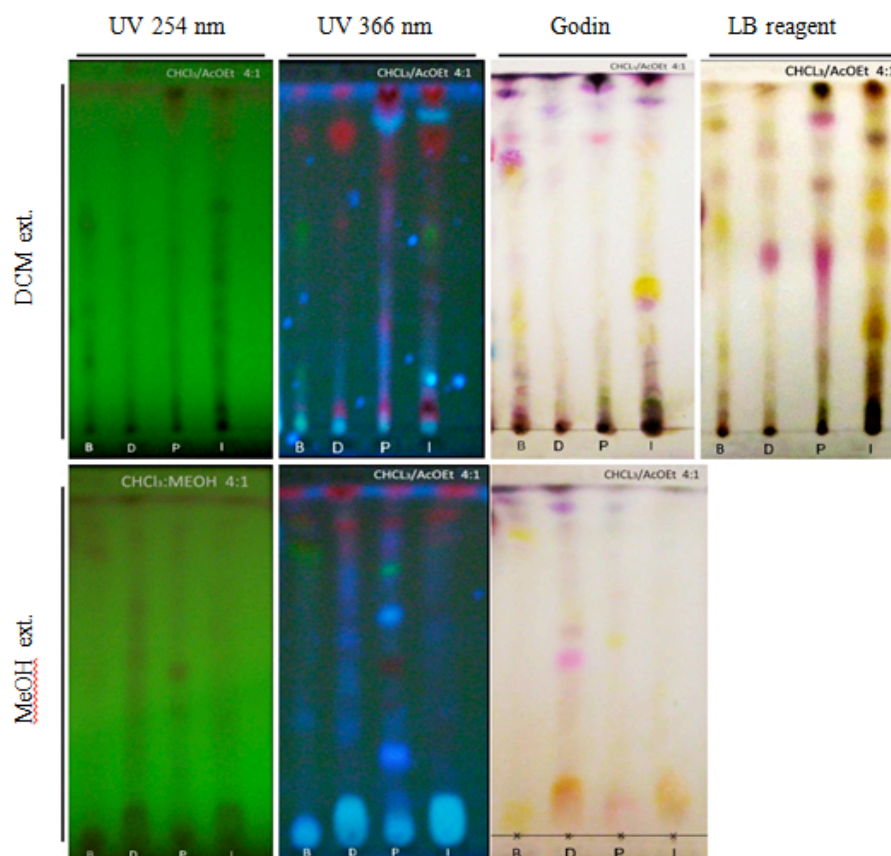
Bacterial cultures were carried out in LB and MH media at 37°C. The bacterial growth was detected as the turbidity at 560 nm using a microtitre plate reader (Power Wave XS2; BioTek, Vermont, USA).

### 2.8. Biofilm Formation Assay

For biofilm quantification, a micro-method based on a protocol previously reported was employed [18] with some modifications [19].

### 2.9. Effect of *Lippia Integrifolia* Fractions on *P. Aeruginosa* AI Production

*P. aeruginosa* qsc 119 (reporter strain) is a mutant donated by P. Greenberg [20] unable to produce its own AHL (QS signal molecules). However, produce β-galactosidase in presence of exogenous AHL. In consequence, the β-galactosidase activity is proportional to the AHL concentration present in the supernatant obtained from *P. aeruginosa* ATCC 27853 cultured in LB media containing the fractions obtained from DCM extract of *L. integrifolia*. To determine the overall AI production in each condition, the control wells contained cell-free culture supernatant (100 µl) obtained from *P. aeruginosa* ATCC 27853 cultured in LB media (190 µl) plus 10 µl of DMSO:H<sub>2</sub>O (1:1). β-galactosidase activity was measured spectrophotometrically by Miller test [22]. The experiments were repeated independently three times with six replicates.



**Figure 1:** Chromatography profile of plant extracts. Plants were extracted with dichloromethane and methanol, and then chromatographed as described in 2.3. The chromatograms were developed with UV (at 254 nm and 366 nm), Godin and Lieberman-Bouchard (LB) reagents. For each lane, the amount applied to the TLC plate corresponds to the minimum detectable amount. Abbreviations: (DCM ext.) dichloromethane extract, (MeOH ext.) methanol extract, (B) *H. baylahuen*, (D) *A. deserticola*, (P) *S. parvifolia* and (I) *L. integrifolia*. CHCl<sub>3</sub>: chloroform, AcOEt: ethyl acetate, MeOH: methanol.

### 3. RESULTS AND DISCUSSION

#### 3.1. Extraction Yields

The choice of extraction conditions is a critical step for research of natural bioactive compounds [23]. Two extractive forms were assayed to select the best solvent and experimental conditions. Samples (200 g of each herb) extracted with DCM showed the best recovery rate of *A. deserticola* and *H. baylahuen*: 23,27 g (12%) and 36,72 g (18%); compare with the MeOH extraction: 18,95 g (9%) and 21,46 g (11%), respectively.

On the other hand, for *L. integrifolia* and *S. parvifolia* the MeOH extraction yields [19,83 g (10%) and 20,19 g (10%)] were higher than DCM extraction yields [7,52 g (3%) and 4,19 g (2%), respectively]. These results indicate the higher amount of polar components present in the *L. integrifolia* and *S. parvifolia* with respect to *A. deserticola* and *H. baylahuen*.

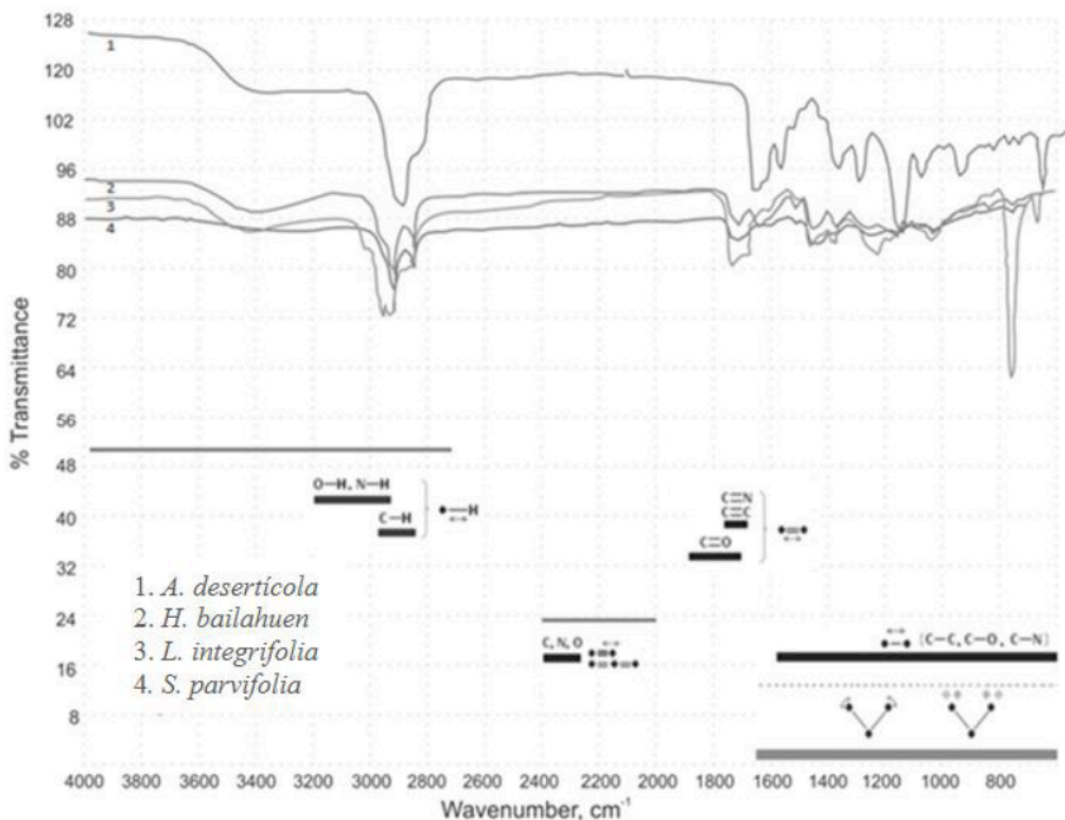
#### 3.2. Characterization of Chemical Compounds

A TLC was developed for the MeOH and DCM extracts of the four herbs studied: *A. deserticola*, *H.*

*baylahuen*, *L. integrifolia* and *S. parvifolia*. The chromatographic profiles show that all extracts are complex samples (Figure 1). The different eluates resulting mixture, have a wide range of polarities about cholesterol, taken as a reference compound. To improve the characterization in all herbs infrared spectrometry properties were screened in DCM extracts. In the spectra, diagnostic value signals at 3300, 2925, 1735, 1380  $\text{cm}^{-1}$  were observed corresponding to the stretching of OH, CH sp<sup>3</sup> bonds, C=O tension and methyl groups bending, respectively. The signals between 900 and 800  $\text{cm}^{-1}$  correspond to C-O stretching links (Figure 2).

The TLC results suggest that *A. deserticola* possesses a complex composition and differ with respect to its essential oil that had 95% of thujone [24]. In hydroalcoholic extracts of *A. deserticola* the presence of phenolic compounds (non flavonoids, flavonoids and tannins), saponins and triterpenes/steroids, with anti-inflammatory was previously reported [25].

Previous works in aerial parts of *H. baylahuen* revealed the presence of anthraquinone glycosides as emodin or chrysophanic acid, flavonoids, coumarins as



**Figure 2:** Infrared spectra of *L. integrifolia*, *S. parvifolia*, *A. deserticola* and *H. baylahuen*, in selected spectral range 4000-600  $\text{cm}^{-1}$ .

esculetin, eupatilina [26], prenetina, haplopine and haplopinol [27]. The characteristic compounds of this genus are flavones, flavonols, flavonoids glycosides, some diterpenes and coumarins [28]. From *Haplopappus multifolius* some compounds (prenetina and haplopine) with antibacterial activity against *S. aureus* were previously informed [27].

Through a literature review, we can see that gender *Lippia* has been very little studied, except for the essential oils [29]. Iridoids are secondary metabolites found in this gender and have several properties as sedative, antipyretic, skin diseases, antimicrobial activity and hepatoprotective [30, 31].

Reports on the chemical composition of *S. parvifolia* indicate its essential oil composition [32, 33]. Serialized extracts in petroleum ether, DCM and MeOH, obtained from the aerial parts of Muña- Muña showed antimicrobial activity against *Escherichia coli* and *Trichophyton mentagrophytes* [34]. Antimicrobial activity of flavonoids contained in Muña-Muña among other medicinal plants of Tafi del Valle, Tucuman, Argentina was also reported [35]. Most of the identified

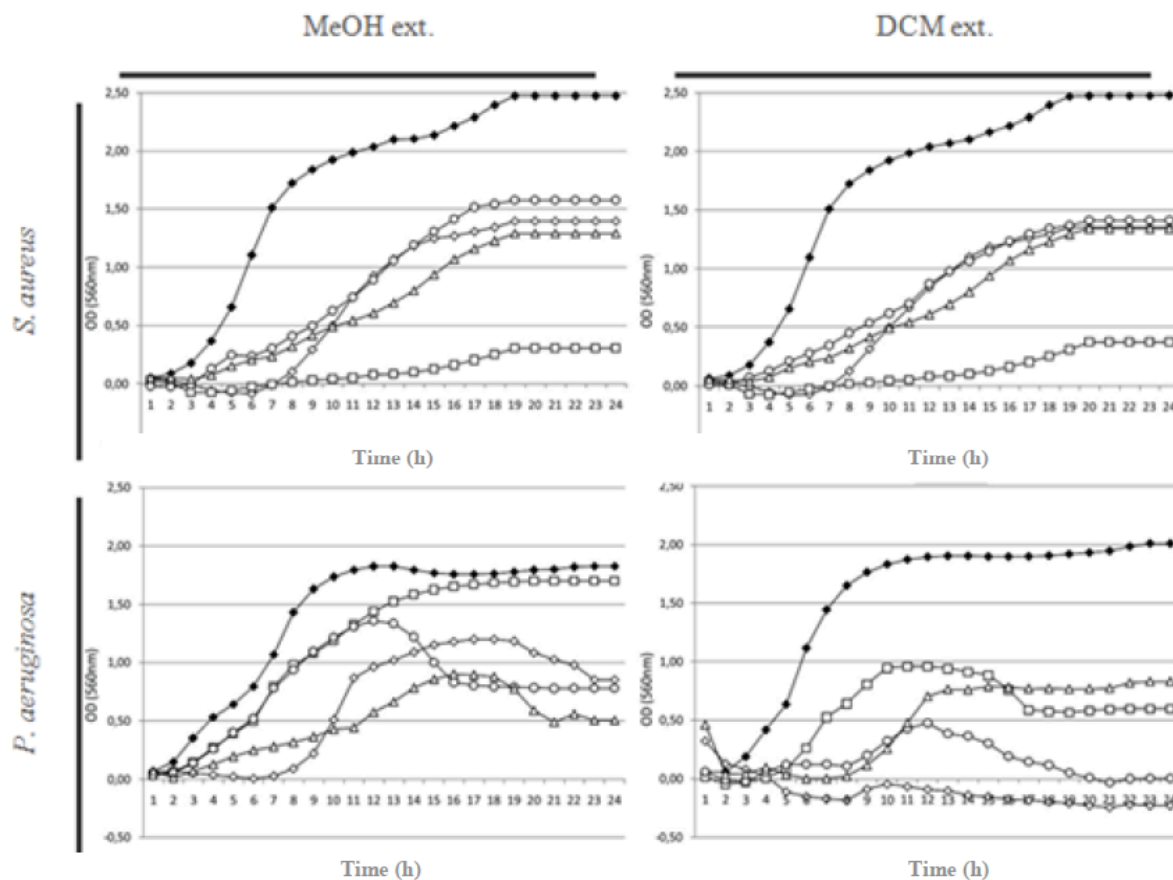
compounds were flavonoids mono- and di-hydroxylated.

In all the extracts the presence of pink, purple and yellow spots observed using the Liebermann-Buchard revelator suggest the presence of triterpene and steroids with low polarity.

### 3.3. Antibacterial Activity of Herbal Extracts

None of the extracts exhibits significant inhibition of bacterial growth at low concentrations (data not shown). At the highest concentration assayed (1000  $\mu\text{g/ml}$ ) both extracts of *A. deserticola* diminished the bacterial growth of *S. aureus* more than 85%. In the same challenge all extracts of *H. baylahuen* and *L. integrifolia* inhibited near to 45% the bacterial growth. The MeOH extract of *S. parvifolia* inhibited 36% the bacterial growth and the DCM extract decreased it by 45% (Figure 3).

The *P. aeruginosa* growth inhibition observed with 1000  $\mu\text{g/ml}$  of MeOH and DCM extracts was 5 and 52% for *A. deserticola*, respectively; 40 and 100% for



**Figure 3:** *S. aureus* and *P. aeruginosa* growth inhibition by *A. deserticola* ( $\square$ ), *H. baylahuen* ( $\diamond$ ), *L. integrifolia* ( $\Delta$ ) and *S. parvifolia* ( $\circ$ ) extracts (1000  $\mu\text{g/ml}$ ). Control without extract ( $\blacklozenge$ ). (DCM ext.) dichloromethane extract, (MeOH ext.) methanol extract.

*H. baylahuen*; 67 and 60% for *L. integrifolia*, and 56 and 81% for *S. parvifolia*, respectively (Figure 3).

The MeOH and DCM extracts of *A. deserticola* showed the highest percentage of inhibition of *S. aureus* bacterial growth. It is noteworthy that there were no previous reports on the antimicrobial effect *A. deserticola* on bacteria. On the other hand, the DCM extract of *H. baylahuen* suppressed completely the *P. aeruginosa* growth.

The inhibitory effect of genus *Lippia* against many Gram-positive bacteria was previously reported [29, 36]. In addition, *Lippia* essential oils have been widely shown to exhibit antimicrobial activity against other organisms [37]. Several extracts of *S. parvifolia* obtained from the aerial parts showed antimicrobial activity against *E. coli* [34].

### 3.4. Influence of Herbal Extracts in Biofilm Formation

The growth inhibitory effect was achieved at an extract concentration so higher to be considered as antimicrobials. However, at a concentration 100 times lower, the extracts showed anti pathogenic properties. The results of the influence of MeOH and DCM extracts from *A. deserticola*, *H. baylahuen*, *L. integrifolia* and *S.*

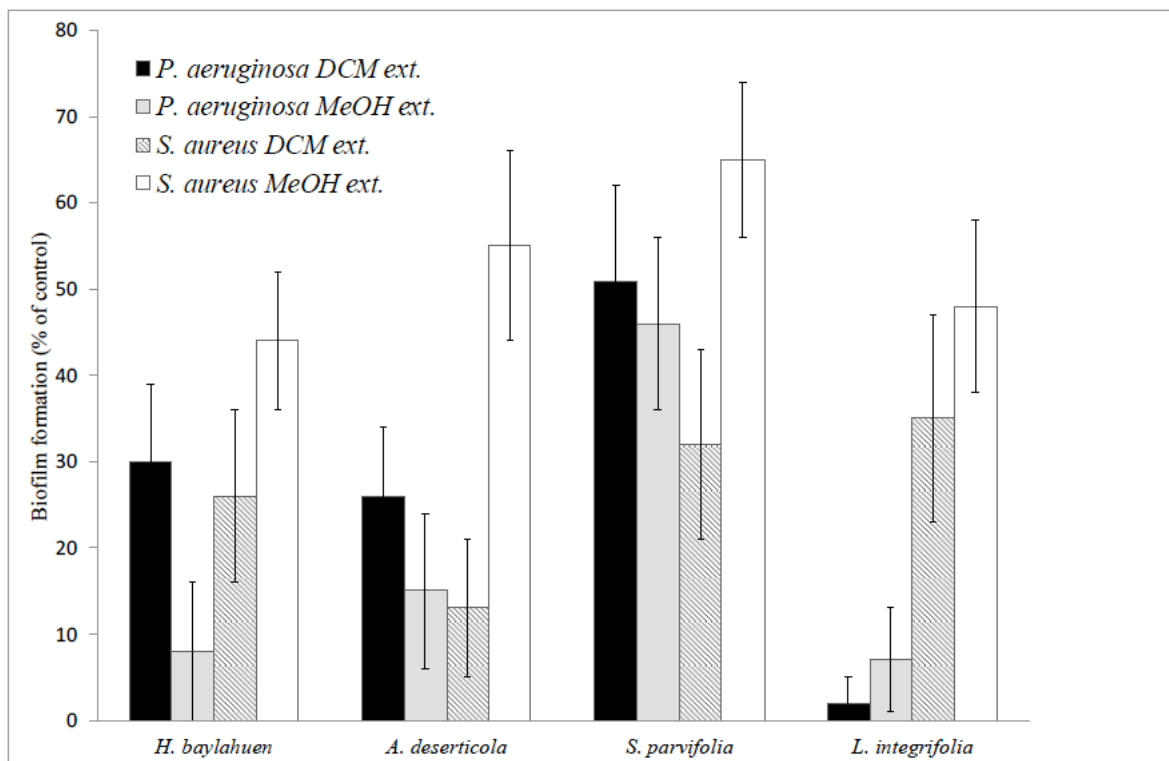
*parvifolia* on biofilm formation by *P. aeruginosa* and *S. aureus* are shown in Figure 4.

*A. deserticola* inhibit the biofilm formation by *S. aureus* 87 and 45% at a concentration of 10 µg/ml of DCM and MeOH extracts, respectively. While for the Gram negative bacterium, *P. aeruginosa*, the inhibition was 74 and 85%, respectively.

The DCM and MeOH extracts of *H. baylahuen* (10 µg/ml) inhibited *S. aureus* biofilm formation, 74 and 56%, respectively and *P. aeruginosa* biofilm formation 70 and 92%, respectively.

In presence of *L. integrifolia* DCM and MeOH extracts, the biofilm formation by *S. aureus* decreased 65 and 52%, respectively. Of all samples assayed, the highest *P. aeruginosa* biofilm inhibition was observed for *L. integrifolia* with 98 and 93% decrease for DCM and MeOH extracts.

The incubation in presence of DCM and MeOH extract of *S. parvifolia* affect the biofilm formation of *S. aureus* by 68 and 35%, respectively. On *P. aeruginosa* the inhibition these extracts was 49 and 54%, respectively.



**Figure 4:** Inhibitory effect of *A. deserticola*, *H. baylahuen*, *L. integrifolia* and *S. parvifolia* extracts (10 µg/ml) on biofilm formation by *P. aeruginosa* and *S. aureus*. The inhibitory effect was expressed as percentage referred to the control without extract (100 %). Abbreviations: (DCM ext.) dichloromethane extract, (MeOH ext.) methanol extract.

To our knowledge, there are no data about *P. aeruginosa* and *S. aureus* biofilm inhibition caused by these plant species, so this work is the first report of it. Due to the high ability of *L. integrifolia* DCM extract to inhibit the biofilm formation of both strains, with emphasis on *P. aeruginosa* biofilm inhibition, this extract was chosen and processed by column chromatographic to found the active fractions.

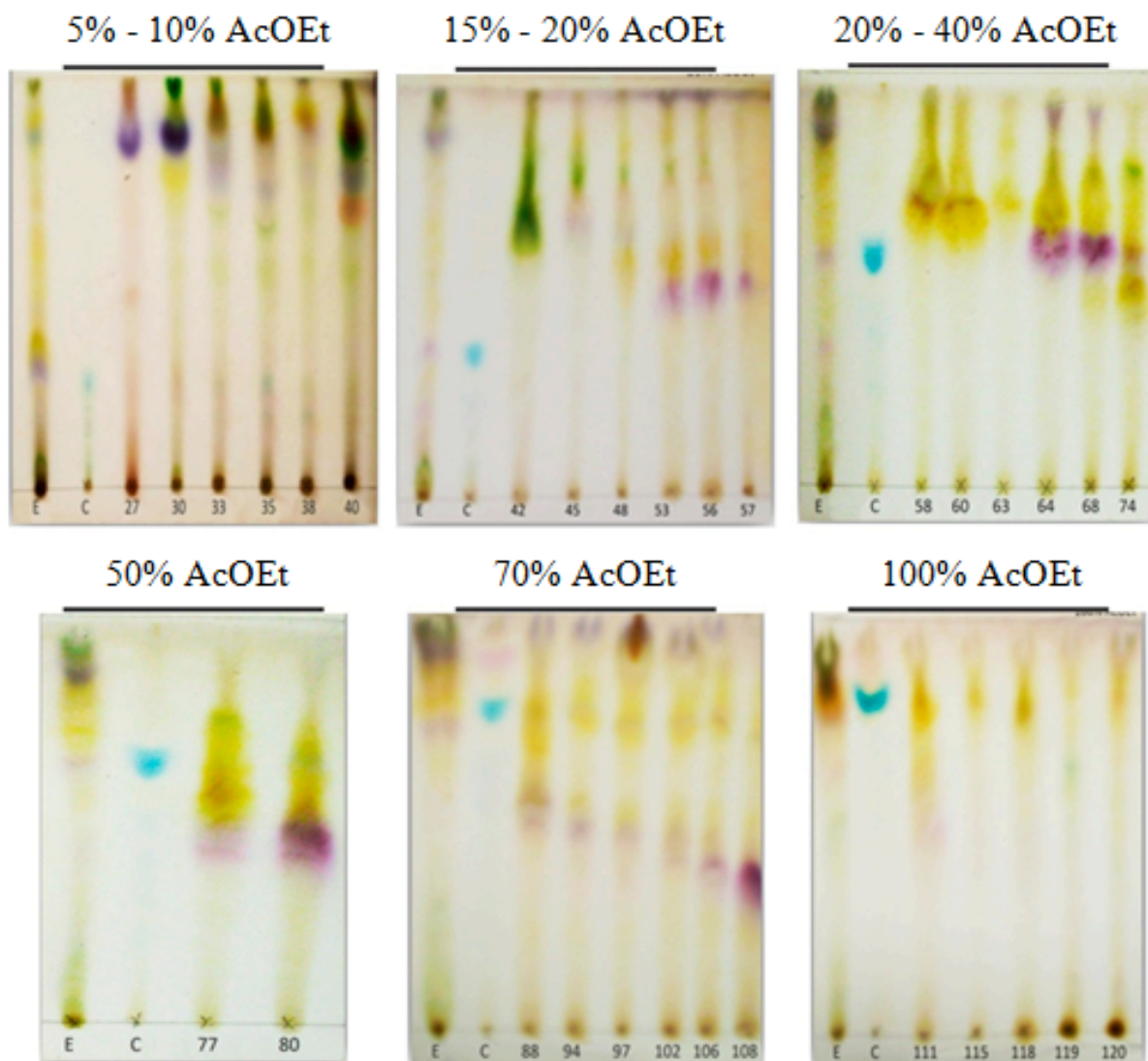
### 3.5. Column Chromatographic of *L. Integrifolia*

The DCM extract of *L. integrifolia* was chemically processed as mentioned in material and methods. One hundred twenty fractions were collected and analyzed by TLC. The eluates that showed the same TLC profiles were pooled in 35 fractions (Table 1; Figure 5).

**Table 1: Bioactivity Detected in *Lippia Integrifolia* DCM Extract and Chromatographic Fractions (F). The Inhibitory Effect was Express as Percent of Control (Raw Sample)**

Sample 10 µg/ml	Inhibition of <i>Pseudomonas Aeruginosa</i> (%)		
	Growth	Biofilm	Auto Inducer
Crude Extract	14	98	78
F1 - EA: C (5:95)	22	77	68
F12 - EA: C (5:95)	20	69	44
F25 - EA: C (5:95)	10	78	17
F26 - EA: C (5:95)	87	91	1
F27 - EA: C (5:95)	33	58	55
F30 - EA: C (5:95)	30	39	28
F33 - EA: C (5:95)	20	86	77
F35 - EA: C (15:85)	24	100	84
F38 - EA: C (15:85)	37	93	3
F40 - EA: C (15:85)	20	77	5
F42 - EA: C (15:85)	35	41	45
F45 - EA: C (15:85)	36	68	27
F48 - EA: C (15:85)	92	79	20
F53 - EA: C (15:85)	89	75	51
F56 - EA: C (20:80)	89	71	-3
F57 - EA: C (20:80)	91	86	-11
F58 - EA: C (20:80)	95	82	70
F60 - EA: C (20:80)	72	73	17
F63 - EA: C (20:80)	96	38	-113
F64 - EA: C (20:80)	60	81	92
F68 - EA: C (20:80)	88	98	85
F74 - EA: C (50:50)	69	90	96
F77 - EA: C (50:50)	83	86	83
F81 - EA: C (50:50)	23	93	89
F88 - EA: C (50:50)	16	56	92
F94 - EA: C (50:50)	35	59	92
F97 - EA: C (50:50)	44	67	88
F102 - EA: C (50:50)	28	99	76
F106 - EA: C (70:30)	36	79	81
F108 - EA: C (70:30)	29	99	82
F111 - EA: C (70:30)	36	97	86
F115 - EA: C (70:30)	93	86	81
F118 - EA: C (70:30)	69	67	89
F119 - EA: C (70:30)	51	88	80
F120 - EA: C (70:30)	53	94	61

Abbreviation: (EA) ethyl acetate, (C) chloroform.



**Figure 5:** TLC bioassay of *L. integrifolia* fractions. Fractions were obtained as described in 2.4. The chromatograms were developed with Liebermann-Bouchard (LB) reagent. For each lane, the amount applied to the TLC plate corresponds to the minimum detectable amount. Abbreviation: (E) *L. integrifolia* crude extract, (C) cholesterol. AcOEt: ethyl acetate.

The fractions were tested for growth, biofilm and autoinducers inhibition of *P. aeruginosa* (Table 1).

According to the polarity of the fractions, different inhibitory effects were observed on *P. aeruginosa* (Table 1). The total extracts of *L. integrifolia*, not showed a notable antibacterial effect against *S. aureus* and *P. aeruginosa* at the tested concentrations thereof. However, several column fractions of DCM extract showed an inhibition greater than 80% for *P. aeruginosa* growth. The eluate 26 (F26) obtained with 5% EA and 95% C, inhibited 87% the bacterial growth. And almost all the fractions that elute with 20% EA and 80% C inhibited more than 80% the bacterial development, showing several of them a purple band with a retention factor (Rf) between 0.5 and 0.75 (Figure 5). A good correlation was observed between

bacterial growth and biofilm inhibition for the fractions above mentioned, with the exception of the fraction 63 that inhibit less the biofilm.

On the other hand, it is well known that several compounds are able to modify the biofilm formation by inhibiting the autoinducers production and without altering the bacterial development. Antipathogenic compounds do not kill bacteria or stop their growth. They rather control bacterial virulence factors and prevent the development of resistant strains [38]. The available data indicate that plants might produce a wide range of AI-inhibitory compounds [39]. Anti-QS activity has been shown in a few terrestrial plants [40, 41,42]. In addition, the influence of few secondary metabolites from South American plants on biofilm formation has been reported by our laboratory [41, 43, 44, 45, 46].



Moreover, several food components such as honey [47], *Zuccagnia punctata* [48] or citrus essential oil [19] are able to inhibit *P. aeruginosa* biofilm.

Since our results demonstrated that many fractions of *L. integrifolia* inhibit the biofilm formation, we wanted to know how the influence in the overall production of QS signals is. To investigate if the activation of the QS systems takes place during the biofilm formation a quantification of the overall AI production was carried out in each chromatographic condition during the biofilm formation experiments on microtitre plates using *P. aeruginosa*.

In this way, we found several fractions that inhibit the biofilm formation without modify the bacterial growth, such as the less polar fraction 33 and 35, as well as, almost all the fractions that elute with EA:C (50:50) and (70:30). A similar behavior was observed for the crude extract of *L. integrifolia* (Table 1).

The biofilm inhibition could be due to the lower bacterial growth or because the lower AI production. In general, the lower polar fractions inhibited biofilm in correlation with bacterial growth. However, in more polar fractions the biofilm diminution is well correlated with the inhibition of AI production more than the bacterial development. These results suggest different mode of actions of the fractions studied. An important point to emphasize is that at not-inhibitory-growth-concentrations the biofilm is inhibited, indicating an antipathogenic effect of some extracts and fractions, rather than an antibacterial effect.

## CONCLUSION

The extracts of *A. deserticola*, *H. baylahuen*, *L. integrifolia* and *S. parvifolia*, exhibited anti-pathogenic properties by inhibiting the growth and biofilm formation by *S. aureus* and *P. aeruginosa*. The DCM extract of *L. integrifolia* presented many fractions with stronger inhibitory activity of *P. aeruginosa* growth, biofilm and autoinducer production. This study demonstrated that these plant species traditionally consumed as infusions in mate may be the responsible for the protection against foodborne diseases when this drink is socially ingested.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest related to this work.

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