

ISSN- 0975-7066

Vol 12, Issue 2, 2020

**Original Article** 

## ANTIMICROBIAL AND ANTIOXIDANT EFFECT OF NATURAL EXTRACTS FROM LEAVES, ROOT, STEM AND FLOWERS OF *BACCHARIS LATIFOLIA* FROM ECUADOR

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#### Received: 22 Nov 2019, Revised and Accepted: 21 Jan 2020

#### ABSTRACT

**Objective:** The increase in chronic and degenerative diseases and the use of synthetic antioxidants such as (butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT)) are being restricted because they can be considered carcinogenic. Therefore, there is a growing interest in the search for natural antioxidants, especially from plants, due to their content in different bioactive compounds, such as antioxidants and antimicrobials.

To evaluate the antibacterial and antioxidant activity of *Baccharislatifolia* extracts.

**Methods:** For the determination of the antimicrobial activity of extracts of leaves, root, stem and flowers of *Baccharislatifolia* (*BI*), the disk plate diffusion method was used, the strains of *Listeria, Salmonella* and *E. coli* were studied; antibiotics Penicillin G and Ciprofloxacin were the controls. For the antioxidant activity, a solution of  $H_2O_2$  (Abs at 230 nm) was prepared in Potassium Phosphate Monobasic-Sodium Hydroxide buffer.

**Results:** The antimicrobial activity against *Listeria* and *Salmonella*, showed that the extracts of leaves and flowers were more effective with inhibition zones>15 mm and>20 mm respectively. In front of E. coli, the extracts of flowers and stem were the best with zones>7.0 mm. Antibiotics studied inhibited the development of *Listeria* and *Salmonella*. However, *E. coli* isolates were resistant. In the antioxidant activity, the flower extract of Bl in 60 mg/ml presents a higher effect with 47.25%.

Conclusion: Bl extracts from leaves and flowers were more efficient both in their antimicrobial and antioxidant capacity.

Keywords: Baccharislatifolia, Natural extracts, Antimicrobial, Antioxidant

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#### INTRODUCTION

The continuous increase of microorganisms resistant to different antimicrobial agents has been a major problem for health and food safety, it is also known that with the appearance of antibiotics the lives of millions of human beings have been saved, however, is approaching a reality of dimensions not yet considered.

Among the most used antibiotics, are the antibiotics from the group of Fluoroquinolones [1]. On the other hand, Penicillin G, according to Flores *et al.* [2], is an antibiotic belonging to the group of Beta-lactams, they have a mechanism of action inhibiting the cell wall of the bacteria, especially Gram+such as *Listeria, Salmonella, E. coli* [3-5].

The world could be in a serious phase caused by multiple lethal bacteria resistant to antibiotics, this due as the lack of innovation and development of new antibiotics, especially of a natural origin [6]. The antibiotics derived especially from vegetables (medicinal plants), have been proven to be less toxic than synthetic agents [7].

The use of extraction techniques to obtain substances with bioactive principles is of great importance at the time of obtaining the component, even, when orthodox medicines are available, a large percentage of the population still uses herbal remedies together with conventional medicines [8, 9].

These compounds are obtained by contact with solvents through extraction techniques like maceration. The effectiveness of extraction generally depends on the polarity and nature of the solvent used [10].

#### Antioxidant activity of plants

Oxidative damage caused by free radicals is related to the development of various diseases such as atherosclerosis, cancer, arthritis and other inflammatory diseases [11]. The existence of

synthetic substances (Butylated hydroxyanisole (BHA) or Butylated hydroxytoluene (BHT)) that are efficient scavengers of free radicals; but they are being restricted because they can be considered carcinogenic [12]. So, there is a growing interest in the search for antioxidants of natural origin, especially from plants. In most cases, the antioxidant activity of these plants is mainly due to the presence of phenolic compounds, which are powerful oxygen scavengers and are also capable of inhibiting enzymes that produce free radicals [13].

The chilca (*Baccharis latifolia*) (fig. 1), is one of the 46 species of *Baccharis* genus that is widely distributed in Ecuador, in provinces such as Pichincha, Imbabura, Cañar, Cotopaxi, Chimborazo, Bolívar, Azuay, Loja, Napo, Sucumbíos and Zamora Chinchipe [14].



Fig. 1: Baccharis Latifolia plant

Baccharis latifoliait is commonly used in poultices to relieve external inflammations, fractures, dislocations and rheumatic

pains; in infusions, it is used as an antidiarrheal, for asthma, menstrual pains, antidiabetic and insomnia [15]. Also, *Baccharislatifolia*, has been used in Latin America for medicinal purposes, such as antidiarrheal, anti-inflammatory, antidiabetic, antidepressant, analgesic and disinfectant of wounds, ulcers and antimicrobial [16].

#### Objective

The objective of the present study was; to determine the antimicrobial and antioxidant activity of the extracts of *Baccharislatifolia*.

#### MATERIALS AND METHODS

The leaves, stems and roots of *Baccharislatifolia* (Bl) were collected from young plants during the months of July 2017 to July 2018 on the grounds of the Faculty of Agricultural Sciences of the Universidad Estatal de Bolívar (Ecuador). The selected plants were clean and free of damage.

#### **Preparation of extracts**

The selected leaves, stems and flowers were exposed to maceration during 6 d in 96% ethyl alcohol in a ratio 50 gr of the vegetal matter: 100 ml of alcohol, after this time, the extracts were obtained by centrifugation (Sigma, 3-16C, United Kingdom) at 10,000 rpm and filtration using of cellulose filters pore size 2.5  $\mu$ m (Whatman, 1001-110, USA). The preparation of the extracts was done in duplicate and the extracts with better performance were used to Antimicrobial analysis

#### **Collection of bacterial inocula**

*Baccharislatifolia* Extracts (*Bl*-E) were tested against three bacterial genera, *Escherichia coli*, *Salmonellas*pp and *Listerias*pp, these bacterial strains were provided by the Molecular Biology laboratory of the Research Department of the Universidad Estatal de Bolívar. A number of 10 isolated for each bacterial genus isolated from meats were used in the study, as control were used the type strains of the *Listerias*pp, *Salmonellas*pp and *Escherichia*, the bacterial strains used are described in table 1.

#### Table 1: Microorganisms used in the study

Type of meat (origin)	Sample number and selected colony	Code	Identified microorganism
Beef	Sample 1-Colony 2	B1C2	Listeria spp
Beef	Sample3-Colony2	B3C2	Listeria spp
Chicken	Sample3-Colony1	C3C1	Listeria spp
Chicken	Sample4-Colony2	C4C2	Listeria spp
Chicken	Sample5-Colony1	C5C1	Listeria spp
Chicken	Sample6-Colony1	C6C1	Listeria spp
Chicken	Sample8-Colony2	C8C2	Listeria spp
Chicken	Sample14-Colony3	C14C3	Listeria spp
Chicken	Sample18-Colony1	C18C1	Listeria spp
Pork	Sample19-Colony1	P19C1	Listeria spp
ATCC 33090			Listeria innocua
Beef	Sample3-Colony2	B3C3	Salmonella spp.
Beef	Sample5-Colony1	B5C1	Salmonella spp.
Beef	Sample15-Colony3	B15C3	Salmonella spp.
Beef	Sample27-Colony1	B27C1	Salmonella spp.
Chicken	Sample2-Colony1	C2C1	Salmonella spp.
Chicken	Sample13-Colony2	C13C2	Salmonella spp.
Pork	Sample1-Colony3	P1C3	Salmonella spp.
Pork	Sample1-Colony5	P1C5	Salmonella spp.
Pork	Sample5-Colony3	P5C3	Salmonella spp.
Pork	Sample14-Colony3	P14C3	Salmonella spp.
ATCC 13314			Salmonella arizonae
Beef	Sample2-Colony1	B2C1	Escherichia coli
Beef	Sample3-Colony1	B3C1	Escherichia coli
Beef	Sample3-Colony3	B3C3	Escherichia coli
Beef	Sample5-Colony1	B5C1	Escherichia coli
Chicken	Sample2-Colony3	C2C3	Escherichia coli
Chicken	Sample3-Colony2	C3C2	Escherichia coli
Pork	Sample2-Colony1	P2C1	Escherichia coli
Pork	Sample4-Colony1	P4C1	Escherichia coli
Pork	Sample5-Colony1	P5C1	Escherichia coli
Pork	Sample14-Colony3	P14C3	Escherichia coli
ATCC 10536			Eschericha coli

The microorganisms used in this study were previously identified by biochemical and molecular methods.

#### Antimicrobial analysis

The antibacterial activity of the four *Baccharislatifolia* extracts (*Bl*-E) of root, stem, leaves and flowers against *Listerias*pp; *Salmonellas*pp and *Escherichia coli* strains were tested by the paper disc diffusion method applied by Shokeen *et al.* [17].

Colonies of fresh pure culture from each isolate and of the reference strains were suspended in the physiological saline solution until turbidity of 0.5 McFarland standard (equivalent to  $1.5 \times 10^8$  UFC/m). Bacteria from each suspension were inoculated onto Muller Hilton Agar (MHA) (Neogen, 7101A, USA) using a sterile cotton-tipped swab and the plates were left standing for 10 min.

The sterile filter paper discs (Oxoid, CT0998B, United Kingdom) of 6 mm diameter were immersed in 10 ml of each extract for 7 min, then applied to the surface of the agar [18]. Sterile water was used as a

negative control. The commercially available standard antibiotics, Penicilin G (Oxoid, CT0043B, UK) and Ciprofloxacin (Bioanalyse, 181129B, Turkey) were used as reference antibiotic controls. All assays were performed in duplicate. The sensitivity of microorganisms to natural extracts is related to the size of the microbial grown inhibition zone. According to the diameter of inhibition zone, microorganisms are classified in: resistant (d<8 mm), sensitive (9 mm.<d<14 mm), very sensitive (14 mm<d<19 mm) and extremely sensitive (d>20 mm) [19].

#### Antioxidant capacity of the Baccharislatifolia extracts

The ability of plant extracts to remove  $H_2O_2$  can be estimated according to the method of Ruch *et al.* [20]. In this work a solution of  $H_2O_2$  (40 mmol) was prepared in Potassium Phosphate Monobasic-Sodium Hydroxide buffer (50 mmol, pH 7.4) (SB108-1, Fisher Chemical, Belgium), the concentration of  $H_2O_2$  was determined by absorption at 230 nm in a spectrophotometer Genesys 10 UV (Thermo Scientific 335902, USA). Subsequently, the extracts were added individually in concentrations of 20, 40 and 60 µg/ml on  $H_2O_2$ , the ABS was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without  $H_2O_2$ . It should be noted that there are several methods to evaluate antioxidant capacity *in vitro*, although there is still no consensus on the most appropriate method, as well as, although there are markers of oxidative damage to biomolecules, the results *in vivo* remain contradictory [21].

The percentage of the sweep of hydrogen peroxide is calculated with the following formula:

Antioxidant capacity (% of  $H_2O_2$  sequestered) =  $[(A_i-A_t)/A_i]x100$ .

Where:  $A_i$  = absorbance of the reference standard;  $A_t$  = Absorbance of the sample.

## **RESULTS AND DISCUSSION**

#### Antimicrobial activity of BI-E against Listeria isolates

Through the inhibition analysis, it was determined that the extract of leaves and flowers presented greater effectiveness against *Listeria* isolates, in fact, the size of the inhibition zone presented a mean greater than 15 mm. The third extract that showed effectiveness in the analysis was root, but with smaller zone size, as shown in fig. 2. The isolates: B1C2, B3C2 and CH5C1 showed resistance to the root extract of *Bl*; isolates: B3C2, CH5C1 and CH14C3 resisted the stem

extract; the extracts of leaves and flowers did not show effectiveness against the isolated CH5C1 and CH14C3.



Fig. 2: Antimicrobial effect of Bl-E agaists Listeria, R-E: root extract; S-E: stem extract; L-E: leave extract; F-E: flower extract

On the other hand, in the control strain (*Listeria innocua*, ATCC 33090) presented 23 and 22 mm in diameter of the zone in the extracts of leaves and flowers respectively, however, these two extracts did not inhibit the development of the isolate, as shown in table 2. Resistant isolates were considered to those that presented a zone size  $\leq 8$  mm in diameter [19].

#### Table 2: Antibacterial activity of BI-E against strains of Listeria spp

Antibiogram in <i>Listeria</i> Inhibition diameter in mm (24 h/incubation)							
N °	Code	R	S	L	F	Р	Ср
1	B1C2	8	12	12	18	21	24
2	B3C2	4	4	12	18	-	12
3	C3C1	10	11	14	11	22	-
4	C4C2	12	14	20	21	18	-
5	C5C1	8	2	-	-	9	14
6	C6C1	15	12	22	24	-	24
7	C8C2	24	18	20	15	22	20
8	C14C3	10	8	2	2	21	14
9	C18C1	20	24	16	9	4	27
10	P19C1	12	11	20	18	18	18
Mean		12,3	11,6	15,3	15,1	16,9	19,1
Listeria inno	zua, ATCC 33090	20	18	23	22	21	14

R = root; S = stem; L = leaves; F = flowers; Cp = ciprofloxacin; P = penicillin

#### Anti-listerial effect of BI-E and antibiotics for clinical use

For penicillin G (gr), an average of 16.9 mm in diameter was obtained, not so far from the average of our best *Bl* extracts. It should be noted that this antimicrobial is specific to fight infections caused by *Listeria*, taking as reference the parameters established by CLSI2012, [22], it can be said that of the 10 isolates studied, 4 were resistant to this agent (B3C2, C5C1, C6C1 and C18C1) with zone sizes  $\leq 14$  mm.

The control strain was susceptible to the antibacterial with 21 mm diameter. On the other hand, after applying ciprofloxacin discs, 5 isolates (B3C2, C3C1, C4C2, C5C1, C14C3), showed resistance with a zone size  $\leq$ 15 mm, according to the CLSI 2012 [22], the control strain proved susceptible to the quinolone (table 2).

#### Antimicrobial activity of BI-E against Salmonella isolates

After having measured the inhibition diameters, it was determined that all the *Salmonella* isolates were susceptible to the *Bl* extracts studied, where the extracts of leaves and flowers presented greater effectiveness, in fact, the size of the zone had a mean greater than 20 mm fig. 3.

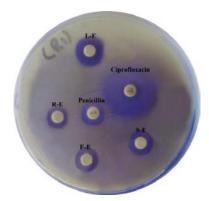


Fig. 3: Antimicrobial effect of *BI*-E against *Salmonella*, R-E: root extract; S-E: stem extract; L-E: leave extract; F-E: flower extract

On the other hand, in the control strain, the root of Bl extract with a zone of 22 mm acted better, followed by the leaves extract with 21 mm of inhibition zone, as shown in table 3.

Antibiogram	1 in Salmonella	Inhibition diameter in mm (24 h/incubation)					
N°	Code	R	S	L	F	Р	Ср
1	B5C1	20	20	18	14	-	28
2	B3C3	26	28	22	18	-	35
3	B15C3	16	24	25	25	22	28
4	B27C1	14	24	28	20	21	35
5	C2C1	20	16	14	16	-	31
6	C13C2	23	16	22	16	-	31
7	P1C3	20	20	24	31	-	35
8	P1C5	20	20	18	26	-	33
9	P5C3	24	26	30	28	-	31
10	P14C3	22	16	20	14	-	30
Mean		20,5	21	22,1	20,8	21,5	31,7
Salmonella arizonaeATCC 13314		22	18	21	18	14	32

Table 3: Antibacterial activity of BI-E against strains of Salmonella spp

R = root; S = stem; L = leaves; F = flowers; Cp = ciprofloxacina; P = penicilina

# Anti-Salmonella effect of Bl extracts and antibiotics for clinical use

In the clinical antimicrobials, for penicillin G, a mean of 21 mm in diameter was obtained (in strains with inhibitory effect) value not so far from the average of the extracts of Bl, in fact, the extracts of Bl leaves acted better than this antibiotic. There were 8 strains of *Salmonella* that showed total resistance to Penicillin (B5C1, B3C3, C2C1, C13C2, P1C3, P1C5, P5C3, P14C3) zone size<14 mm as established by the CLSI 2012 [22], (table 2). However, it is important to consider that in the group of penicillins they are specific for Gram+microorganisms [23], so that it justifies the ineffectiveness of the antibiotic against *Salmonella*.

With ciprofloxacin, all isolates were shown to be susceptible to this antimicrobial agent with a zone diameter size>14 mm, according to the CLSI, 2012 [22]. The control strain was also susceptible to this quinolone.

#### Antimicrobial activity of BI-E against Escherichia coli isolates

After finishing each of the zones of inhibition, it was determined that the extract of flowers and stem showed greater effectiveness against isolates of *Escherichia coli* with a zone size of 7.2 and 7.1 mm respectively. However, according to Ponce *et al.* [19], it should be considered that the microorganism is susceptible to the natural extract or oil, if the zone size is greater than 9 mm, so that the root extract alone inhibited the P2C1 strain; stem extract, the strains:

B3C3 and P4C1; leaves extract, the strains: B2C1 and C3C2 and flowers extract only strain B3C3, as shown in fig. 4.

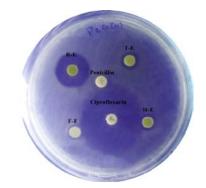


Fig. 4: Antimicrobial effect of *BI*-E against *Escherichia coli*, R-E: root extract; S-E: stem extract; L-E: leave extract; F-E: flower extract

On the other hand, in the control strain, the root of *Bl*extract with a zone size of 12 mm acted better, definitively all the extracts acted better than penicillin against *E. coli* isolates, see table 4.

Antibiog	ram in Escherichia coli	Inhibition diameter in mm (24 h/incubation)					
N °	Code	R	S	L	F	Р	Ср
1	B2C1	8	4	10	8	6	30
2	B3C1	4	6	8	8	4	15
3	B3C3	2	14	4	20	8	26
4	B5C1	4	6	8	4	4	24
5	C2C3	4	8	4	6	6	22
6	C3C2	4	4	10	6	4	30
7	P2C1	20	8	6	4	6	38
8	P4C1	8	9	4	8	4	16
9	P5C1	4	8	4	4	4	24
10	P14C3	8	4	8	4	2	22
Mean		6,6	7,1	6,6	7,2	4,8	24,7
Escherich	a coli ATCC 10536	12	5	7	4	4	21

Table 4: Antibacterial activity of BI-E against strains of Escherichia coli

R = root; S = stem; L = leaves; F = flowers; Cp = ciprofloxacina; P = penicilina

#### Anti-E. coli effect of Bl extracts and antibiotics for clinical use

For penicillin G, an average of 4,0 mm in diameter was obtained. Considering the parameters established by the CLSI 2012 [22], all the isolates showed resistance to this antibiotic, the size zone<12 mm. However, it is important to consider that in a group of penicillins they are specific for Gram+microorganisms [23], so that they justify the ineffectiveness against these isolates. After applying

#### Antioxidant capacity of Bl extracts

After this analysis it was possible to determine that the flower Bl extract in 60 mg/ml presents a higher percentage of sequestration of  $H_2O_2$  with 47.25%; followed by the concentration of 20 mg/ml with

46.36%. In relation to the extract obtained from the stem of *Bl*, the concentration of 20 mg/ml was enough to be able to sequester  $H_2O_2$  by 42.08%; followed by the 40 mg/ml concentration with 19.23%.

The leaves of *Bl* extract with the greatest effect on the retention of  $H_2O_2$  was that of 20 mg/ml concentration. While the root extract did not show any positive effect for this analysis, as shown in table 5.

Extract	Concentration (mg/ml)	% of kidnapped peroxide	
	20	46.36	
Flower	40	41.04	
	60	47.25	
	20	42.08	
Stem	40	19.23	
	60	18.72	
	20	5.62	
Leaves	40	3.92	
	60	0.84	
	20	0.00	
Root	40	0.00	
	60	0.00	

#### DISCUSSION

The extracts of medicinal herbs showed inhibitory activity, as determined by a work developed by Yoon and Choi [24], where the extracts of Bogolji and Gosam showed antibacterial capacity with zone diameter>10 mm; also, Eruteya and Badón [25], obtained antilisteria activity of ethanol extracts of Moringa oleifera, with zone of inhibition>11 mm from extract concentrations of 200 mg/ml. Similar results were obtained by Ruilova et al. [18], obtained antilisteria effect of ethanolic extracts of *Physalis peruviana* fruits, but with zone sizes<7 mm. Odedina et al. [26], used Rhodomyrtustomentosa ethanolic leaf extract as biocontrol against Listeria monocytogenes. Also, in the work carried out by Carrizo et al. [27], reported that the essential oil of B. salicifolia inhibits the growth of Listeria monocytogenes CLIP 74904, but was inactive against the Gram-negative organisms analyzed. It should also be noted that there are no studies on the antilisterial activity of Baccharislatifolia, which shows that our research group is the first to work with extracts of this plant against Listeriaspp isolates.

In a study developed by Shan *et al.* [28], reported that the extracts of 26 medicinal herbs positively inhibited the development of *Salmonella anatum* (mean = 7.2 mm, 4.7-19.2 mm). On the other hand, the acid environment improved the antibacterial activity of the extract of *Filipendulaulmaria* when tested against *S. enteritidis* PT4, whose aqueous methanol extract contains a variety of phenolic compounds [29].

In a study conducted by N'guessan *et al.* [30], the aqueous extracts of *Thonningiasanguinea* showed an antimicrobial effect for all *Salmonella* strains of multiple drug resistance (*S. typhi, S. typhimurium* and *S. hadar*). In another study conducted in South Korea, by Lee *et al.* [31], the aqueous and methanolic extracts of *Schizandraefructus* showed antibacterial activity against the three *Salmonella* serotypes (*S. typhi* ATCC 19943, *S. paratyphi* A and *S. gallinarum* ATCC 9184). In addition, the root of *Euphorbia balsamifera* had shown high activity against *S. typhimurium* in comparison with the extracts of leaves and stems [32]. As well, in the study developed by our research group, the inhibitory effect of extracts of *Physalis peruviana* L against isolates of *Salmonella* spp. with inhibition zones between 8 and 10 mm [33].

In the research developed by BachirRaho and Benali [34] shows that the essential oil of *Eucalyptus globulus* is effective to inhibit the development of *Escherichia coli* with zone sizes ranging from 8 to 26 mm in diameter. According to Argote-Vera *et al.* [35], mentions that the essential oils of *eucalyptus* and mandarin inhibit in a 13.2 µl/ml and lemon 14.6 µl/ml, demonstrating that the essential oils of *eucalyptus*, lemon peel and mandarin have the inhibitory capacity to the bacteria *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Also, in the research developed by Bastos [36], oregano oil was more effective against *Escherichia coli* with 0.35% CBM (minimum bactericidal concentration), with an inhibition zone of 29.5±3.4 mm. Similarly, Sequeda *et al.* [16], studied extracts of *Baccharislatifolia* obtained by percolation, maceration and soxhlet, but did not observe an inhibitory effect on E. coli, although it did act positively against other pathogens.

In general, the chemical compound of the plant extract has revealed the presence of several components, most of which have important antimicrobial properties [37]. The properties present in *Baccharis* species are constituted mainly in flavonoids, monoterpenes, diterpenes, triterpenes, tannins, quinones, saponins, as well as some phenolic compounds, where flavonoids are distinguished by confer protection/resistance against attack of microorganisms [38, 37, 16]. Coumarins and essential oils have also been obtained of *Baccharis* species [39].

In addition, there are studies that show that monoterpenes are the components that also act in the inhibition of microorganisms, [40]. Other researchers state that the phenolic compound in the plant contributes significantly to its antimicrobial and antioxidant properties [41]. This study is the first to analyze the antibacterial effect of extracts of *Baccharislatifolia* (root, stem, leaves and flowers) on isolated *Listeria, Salmonella* and *E. coli*.

In a study developed by Guerra [42], they analyzed the antioxidant activity of the essential oil of the *Baccharislatifolia*- $\beta$  carotene test, with concentrations of 26 and 64 mg/ml of *Bl* oil obtained values of 40.56% and 46.20% respectively. In our study, the extracts of flowers were the only ones that approximate these results. *Cucurbita pepo* extracts inhibited the peroxidation of linoleic acid at 5.1-30.4% after incubation for 96 h [43].

In another study conducted by Hossain *et al.* [44], obtained a value of 48.6% with lipophilic extracts of mixed *Cucurbita*, in Peru a study by Doroteo *et al.* [45], determined the antioxidant effect of cat's claw (*Uncariatomentosa*) with an effect of 47%.

So also, Rodriguez *et al.* [46], determined the effect of an extract of *Boccaniafrutencens* L with a capture value of 40% with an extract concentration of 25 mg/l.

#### CONCLUSION

Extracts of leaves and flowers of *Baccharislatifolia* acted better against *Listeria* and *Salmonella* isolates, whereas in *E. coli* isolates; Flower and stem extracts were the best. In short, these extracts proved to be equal to or better than the antibiotics for clinical use, thus considering the extracts of *Bl* as an alternative natural product to inhibit the development of pathogens.

#### ACKNOWLEDGMENT

The authors express their gratitude to the Departamento de Investigación of the Universidad Estatal de Bolívar for allowing the experiments in their facilities, as well as to the debt Exchange program Ecuador–Spain, for the support received for carrying out the present work.

#### FUNDING

Nil

## AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

#### **CONFLICT OF INTERESTS**

Declare none

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