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Extracts of *Haematoxylon brasiletto* Inhibit Growth, Verotoxin Production, and Adhesion of Enterohemorrhagic *Escherichia coli* O157:H7 to HeLa Cells

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ABSTRACT

The extracts of 33 plants were evaluated for their effects on growth of *Escherichia coli* O157:H7 (EHEC). The extract of *Haematoxylon brasiletto* was the only one that effectively inhibited bacterial growth. The effects of ethanolic extracts of this plant on growth, verotoxin production, and adhesion of *E. coli* O157:H7 to HeLa cells were determined. The MBC for growth was 4 mg/ml. No verotoxin formation was detected at 1, 2, or 3 mg/ml. Preexposing bacteria and HeLa cells to various concentrations of extracts affected the adhesion between non-EHEC and HeLa cells. Partial purification of the active fraction suggested that polyphenols might play a role in the antimicrobial activity exhibited by *H. brasiletto* extracts.

Enteropathogens are exhibiting increasing levels of resistance to commonly used antibiotics, and resistance to new drugs is emerging at an alarming rate *(24)*. The diarrheal diseases are far more common among young children; therefore, the search for alternative treatments against enteropathogens is a public health priority. The use of higher plants and preparations from these plants to treat infections is an ancient practice. The current problems associated with the use of synthetic antibiotics have sparked renewed interest in plants with antimicrobial properties. Furthermore, natural compounds are emerging as important options for preserving stored food from contamination and in controlling various plant and human microbial diseases *(3, 7, 36)*.

Haematoxylon brasiletto (palo de brasil or palo de tinto) was well known to the Aztecs and was also widely used as a dye in Mayan weaving. Heartwood chips of palo de brasil are commonly sold in medicinal plant stores in Mexico, and many studies have cited the naturopathic remedies from extracts of this plant. A purplish red solution of the boiled chips has been reported in the traditional medicine to have many naturopathic properties, including antidiarrheal and antidysentery properties *(2)*.

Enterohemorrhagic *Escherichia coli* strains (EHECs), particularly those of serotype O157:H7, were first implicated in disease in the early 1980s on the basis of their association with hemolytic-uremic syndrome and hemorrhagic colitis *(27)*. Since then, EHECs have been associated with uncomplicated diarrhea and have been isolated from the stools of healthy individuals. EHECs are now considered to be a major cause of disease in developed countries *(20)*. EHEC infections are mainly environmental, foodborne, and waterborne, and bovine feces are an important source of food contamination by these organisms *(12)*. The principal mechanism of EHEC virulence is a combination of attaching and effacing adherence to the large bowel, followed by the production of Shiga-like toxins *(20)*. Thus, blocking bacterial adhesion or the production of toxins in the intestinal tract could prevent these bacterial infections.

In this study, we determined the antimicrobial activity of extracts derived from 33 plants commonly used to treat gastrointestinal diseases in traditional medicine in Mexico. The most active extract was analyzed to determine its ability to inhibit growth, verotoxin production, and adhesion to HeLa cells of EHEC.

MATERIALS AND METHODS

Plant extracts. *Taxodium mucronatum* Ten, *Artemisia laciniata* subsp. *parryi* (A. Gray) W.A. Weber, *Ocinum basilicum* L., *Ocinum micranthum* Willd, *Vaccinium geminiflorum* H.B. & K., *Helietta parvifolia* Benth., *Peumus boldus* Molina, *Atriplex canescens* (Pursh) Nutt., *Krameria secundiflora* ex DC., *Juliania adstringens* Schlecht., *Artemisia ludoviciana* Nutt. spp. *mexicana* (Willd. ex Spreng) D.D. Keck, *Citrus aurantium* L., *Larrea tridentata* Coville, *Euphorbia prostrata* Ait., *Psidium guajava* L., *Flourensia cernua* DC., *Lippia alba* N.E.Br., *Hyptis verticillata* Jacq., *Acacia farnesiana* (L.) Willd., *Solanum nigrum* L., *Baccharis glutinosa* Pers., *Malva parviflora* L., *Rhizophora mangle* L., *Prosopis juliflora* (Sw.) DC., *Salvia coccinea* Juss. ex Murr., *Morus alba* L., *Cnidoscolus urens* (L.) Arthur, *Lantana trifolia* L., *Rosmarinus officialis* L., *Lippia alba* (Mill.) N.E.Br., *Jatropha cordata* Müll.Arg, *Salix taxifolia* H.B. & K., and *H. brasiletto* Karst barks were purchased from retail markets in the metropolitan area of Monterrey, Nuevo León. Plants were identified by Marco A. Guzmán (Department of Botany, Universidad Autónoma de Nuevo León, San Nicolás, N.L., México). Dried plant materials were washed and 20-g samples were immersed in either 100 ml of 50 mM phosphate buffer, pH 7.2 (aqueous extracts, with distilled water for buffer preparation), or 96% ethanol (alcoholic extracts). The samples were then ground with a mortar

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and pestle to extract soluble materials. Aqueous extracts were macerated at 4°C for 8 h, and alcoholic extracts were macerated at room temperature overnight. The macerated samples were then filtered through Whatman no. 1 paper and centrifuged at 10,000 × *g* for 20 min. Supernatants were concentrated with a rotary evaporator (Buchi R 3000, Buchi Analytical, New Castle, Del.) at 60°C and 30% rotation until a small volume was obtained (20 to 30 ml). The concentrated extracts were dried at 50°C, dissolved in 10 to 15 ml of phosphate buffer, filter sterilized, and maintained at 4°C for no longer than 7 days. An aliquot was used to determine dry weight.

Cultures. The EHEC serotype O157:H7 strains ATCC 43894 and ATCC 43895 and a non-EHEC strain ATCC 25922 of *E. coli* were used in these studies. These strains were kindly provided by Dr. Lynn McLasborougth (Food Science Department, University of Massachusetts, Amherst, Mass.). The strains were maintained as stock cultures in Trypticase soy agar (TSA; Difco Laboratories, Detroit, Mich.) at 4°C. Active cultures were obtained by transferring a loop of surface growth of the stock culture into test tubes containing 10 ml of Trypticase soy broth (TSB), and tubes were then incubated overnight (16 to 18 h) at 37°C.

Purification and partial characterization of the biological active fraction from *H. brasiletto.* Plant extracts were separated by low-pressure liquid chromatography with chloroform-methanol (9.5:0.5) as an eluent in a 40-cm-tall, 3-cm-diameter column with silica gel 60 G. Each fraction was subjected to antimicrobial analysis, and those with significant levels of activity were further characterized with techniques such as nuclear magnetic resonance (NMR) assay, OH-phenolic compound analysis, Shinoda reaction, and the antrona test *(33).*

The assays of MBC determination, verotoxin production, and adhesion were conducted with the active isolated fraction, following the methodology described next.

Antibacterial assay. Antibacterial activity was measured with a diffusion technique in agar, as described previously *(11).* Petri dishes (150 mm) were filled with 25 ml of TSA. Aliquots containing 100 μl of the bacterial culture (3×10^8 CFU) were homogeneously inoculated across the agar surface. Five holes (12 mm in diameter) were made in the seeded agar plate. The holes were then filled with 200 μl of each extract. Sterile phosphate buffer was used as control. Dishes were then incubated for 24 h at 37°C. Inhibitory activity was visualized as an absence of bacterial growth in the area surrounding the holes filled with the plant extracts.

Of all the extracts tested, only the *H. brasiletto* ethanolic extract exhibited a significant inhibitory effect on bacterial growth. To further characterize the effects of *H. brasiletto* extract, we determined its MBC for bacterial growth by the method of Rotimi et al. *(28).* Activated cultures of *E. coli* O157:H7 (3×10^8 CFU) cells were grown in culture tubes containing 3 ml of TSB in the presence of various concentrations of extract (added in increments of 0.1 mg/ml). Cultures were then incubated overnight at 37°C. Bacterial growth was determined by plate count on TSA agar. The MBC was regarded as the lowest concentration of the extract that did not permit any visible bacterial colony growth on the TSA agar plate after the period of incubation.

Effect of the extracts on verotoxin production. The production of verotoxins was measured by the reverse passive latex agglutination (RPLA) technique *(5).* Samples containing 50 μl (3×10^8 CFU) of activated cultures of *E. coli* were inoculated in 5 ml of TSB in the presence of 3 mg/ml, 2 mg/ml, and 1 mg/ml of the extract, which corresponded to 75, 50, and 25% of the MBC, respectively. After 18 h of incubation at 37°C, cells were disrupted by sonication and centrifuged at 10,000 × *g* at 4°C for 15 min. The resulting supernatants were collected and freeze-dried. Samples were analyzed for verotoxin content by RPLA with a semiquantitative commercial kit (VTEC-RPLA, Seiken, Japan) *(18).* An experiment was conducted to determine the effect of plant extract on toxin that could alter quantification by RPLA. In this case, filter-sterilized supernatants from an 18-h culture were incubated with different concentrations of plant extracts (25, 50, and 75% of MBC) or phosphate buffer for 12 h at 37°C. Then RPLA was performed. No differences were observed between control and treatments (not shown).

Radiolabeling of bacterial cells. The radiolabeling of bacterial cells was performed by a modification of the assay described by Heredia et al. *(15).* An aliquot of the activated cultures containing 3×10^8 CFU was inoculated in tubes with 3 ml of fresh TSB. Methyl-1-2-[^{3}H]-thymidine (Amersham Biosciences Corp., Piscataway, N.J.) was added to the tubes at a final concentration of 10 μCi/ml. The samples were then mixed and incubated at 37°C for 3 h. Bacterial cells were centrifuged and washed twice with phosphate-buffered saline (PBS; 0.01 M, pH 7.2) and resuspended in 3 ml of fresh nonradioactive TSB.

Tissue culture. Monolayers of HeLa cells were prepared in Falcon tissue culture flasks (Becton Dickinson Labware, Oxnard, Calif.). Cells were routinely grown in minimal essential medium (MEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2–95% air. The culture medium was changed daily.

For adherence assays, postconfluence cells were used after 2 or 3 days in culture. Cells were dispersed by adding 4 ml of trypsin solution (25%, wt/vol, Gibco BRL). After centrifugation, cells were resuspended in MEM, counted, and adjusted for assays.

Adhesion assay. An experiment was conducted to determine whether extract concentrations lower than the MBC (75, 50, and 25%) affected the viability of *E. coli* or the morphology of HeLa cells. An aliquot of *E. coli* (1×10^8 CFU) or 2.5×10^4 HeLa cells were inoculated in 2.5 ml of TSB or in 500 μl of MEM, respectively, containing the extracts. After 3 h of incubation at 37°C, (i) viable bacterial cells were determined by plate count and (ii) HeLa cells were washed, new MEM medium was added, and the number and morphology of the cells were determined after incubation at 37°C for 24 h. In both cases, no effect was detected in the number of viable bacteria or in the morphology and number of HeLa cells when compared with the control (without extracts).

Adherence of EHEC to HeLa cells in the presence of *H. brasiletto* extracts was examined by a modification of the assay described by Henriksson and Conway *(14).* Two different procedures were followed, as described below. In the first procedure, bacteria were preincubated with plant extracts. An aliquot of [^{3}H]-thymidine–labeled *E. coli* (1×10^8 CFU) was inoculated in 2.5 ml of TSB in the presence of 3, 2, or 1 mg/ml *H. brasiletto* ethanolic extracts (25, 50, or 75% of the MBC). Sterile distilled water was added to the tubes to bring the final volumes to 3 ml. After 1 h of incubation at 37°C, cultures were centrifuged at 10,000 × *g* for 10 min at room temperature and washed twice with PBS. Cells were then resuspended in 3 ml of MEM. Aliquots from this culture (500 μl) were added to an Eppendorf tube containing 500 μl of a suspension of 5×10^4 HeLa cells. The mixture was incubated for 1 h at 37°C in an atmosphere of 5% CO_2–95% air. The suspension was then centrifuged at 1,000 × *g* for 10 min (this procedure allowed separation of HeLa cells with or without bound bacteria from unbound bacteria), the pellet was washed twice with PBS, and the final pellet was resuspended in 500 μl

TABLE 1. *Effect of* Haematoxylon brasiletto *extracts on production of verotoxins in various* E. coli *strains*

	% of control[a]							
	Control		1 mg/ml (25% MBC)		2 mg/ml (50% MBC)		3 mg/ml (75% MBC)	
Strain	VTI	VTII	VTI	VTII	VTI	VTII	VTI	VTII
43894 (O157:H7)	100		50	50	50	12.5	1.6	0.8
43895 (O157:H7)	100		100	100	25	50	25	6.5
25922 (non-EHEC)	0		ND	ND	ND	ND	0	0

[a] VTI, verotoxin type I; VTII, verotoxin type II; ND, not determined.

of PBS. The amount of radioactive *E. coli* adhering to HeLa cells in a 50-μl aliquot of the final suspension was determined by liquid scintillation counting (Delta 300 Liquid Scintillation system; Tracor Analytic, Elk Grove Village, Ill.). Bacterial cells preincubated with phosphate buffer were used as a control.

In the second procedure, HeLa cells were preincubated with plant extracts. In this case, 2.5×10^4 HeLa cells were washed twice in PBS and resuspended in 500 μl of MEM containing the same concentrations of extracts used in the first procedure (in which bacteria were preincubated). After 1 h of incubation at 37°C in an atmosphere of 5% CO_2–95% air, cells were centrifuged at $1,000 \times g$ at room temperature and washed twice with PBS. Cells were then resuspended in 500 μl of MEM in an Eppendorf tube containing 500 μl of radioactive *E. coli* (5×10^7 CFU). The sample was incubated for an additional 1 h at 37°C in an atmosphere of 5% CO_2–95% air. The suspension was then centrifuged at $1,000 \times g$ for 10 min, the pellet was washed twice with PBS, and the final pellet was resuspended in 500 μl of PBS. The amount of radioactive *E. coli* adhering to HeLa cells in a 50-μl aliquot of the final suspension was determined by liquid scintillation counting. HeLa cells preincubated with phosphate buffer were used as a control.

All experiments (MBC, verotoxin determination, and adhesion assays) were performed in duplicate a total of three times. The analysis of variance test ($P \leq 0.05$) was used to determine statistical significance.

RESULTS

Antimicrobial testing and MBC determination. In this study, 33 plants were analyzed for their ability to inhibit bacterial growth of *E. coli* O157:H7. Of these, the ethanolic extract of *H. brasiletto* was the only one that inhibited bacterial growth, whereas the other extracts exhibited no detectable antimicrobial activity. Thus, we focused further characterizations on this plant extract. The MBC value of ethanolic extracts of *H. brasiletto* was similar (4 mg/ml), across all strains tested.

Verotoxin production. In this study, verotoxins were quantified after bacterial cells were lysed. Verotoxin production was diminished when plant extracts were added to the cultures at concentrations lower than the MBC (Table 1). The inhibition of verotoxin production was generally higher than 90% when the extract was added at a concentration of 3 mg/ml. Because the RPLA assay is semiquantitative, the results were expressed as titers, and in all cases, the variance was 0.

Adhesion assay. We performed these experiments by two methods. Either bacterial cells were preincubated with the plant extract or HeLa cells were preincubated with plant extract. The data indicate that adhesion diminishes in response to plant extract in a concentration-dependent manner. This pattern was observed in both types of adhesion assays (Tables 2 and 3). The effect was stronger and significant in the non-EHEC strain. However, in most EHEC strains, the statistical analysis ($P \leq 0.05$) showed no differences between treatments and controls. This could suggest that the extract in some way blocks both bacterial and cellular receptors.

Activity and partial characterization of purified fraction. All the experiments were repeated with the puri-

TABLE 2. *Relative levels of adhesion of* E. coli *O157:H7 to HeLa cells; bacteria were preincubated with various concentrations of* Haematoxylon brasiletto *plant extract before adhesion assays*

	Adhesion[a]			
E. coli strain	Control (0% MBC)	1 mg/ml (25% MBC)	2 mg/ml (50% MBC)	3 mg/ml (75% MBC)
43894 (O157:H7)	11,675 ± 1,722 100%	11,244 ± 2,653 96.1% (No)	10,766 ± 2,461 92.1% (No)	10,278 ± 2,066 87.8% (No)
43895 (O157:H7)	7,436 ± 882 100%	6,720 ± 791 90% (No)	6,599 ± 239 88.7% (No)	5,340 ± 642 71.9% (Yes)
25922 (non-EHEC)	10,310 ± 451 100%	4,279 ± 176 41.5% (Yes)	3,835 ± 136 37.2% (Yes)	3,268 ± 164 31.7% (Yes)

[a] Adhesion is given in counts per minute and as a percentage of control levels. Yes or No refers to whether a significant difference was found between the treatment and control ($P \leq 0.05$).

TABLE 3. *Relative levels of adhesion of* E. coli *O157:H7 to HeLa cells, which were preincubated with various concentrations of* Haematoxylon brasiletto *plant extract before adhesion assays*

	Adhesion[a]			
E. coli strain	Control (0% MBC)	1 mg/ml (25% MBC)	2 mg/ml (50% MBC)	3 mg/ml (75% MBC)
43894 (O157:H7)	11,186 ± 1,407 100%	11,029 ± 2,286 98.6% (No)	10,940 ± 2,281 97.8% (No)	7,830 ± 1,267 70.0% (Yes)
43895 (O157:H7)	7,355 ± 440 100%	7,281 ± 312 99.0% (No)	6,987 ± 421 95.0% (No)	9,063 ± 301 90.0% (No)
25922 (non-EHEC)	12,277 ± 305 100%	11,739 ± 340 95.6% (No)	10,516 ± 226 85.6% (Yes)	10,458 ± 274 85.1% (Yes)

[a] Adhesion is given in counts per minute and as a percentage of control levels. Yes or No refers to whether a significant difference was found between the treatment and control ($P \leq 0.05$).

fied fraction obtained. The determined MBC of the purified fraction was lower (0.15 mg/ml) than that of the crude ethanolic extracts, and the value was similar in all the strains tested. Similar patterns of activity were also observed in verotoxin production and adhesion to HeLa cell assays (data not shown).

In this study, the chemical analyses performed on the bark of *H. brasiletto* extract confirmed the presence of polyphenols. Although brazilin and brazilein are polyphenols that have been reported as antimicrobials *(26, 31)*, these compounds differ from the active compounds visualized in the Cossie and NMR spectrum (not shown). At this moment, work is in progress on the complete characterization of the anti-EHEC compound.

DISCUSSION

During 1995, several studies revealed that antibiotic resistance was uncommon in *E. coli* O157:H7. However, like other foodborne pathogens, such as *Salmonella* Typhimurium *(24)*, *Campylobacter jejuni (21)*, and *Listeria monocytogenes (10)*, antibiotic-resistant strains of *E. coli* O157:H7 have emerged *(17)*. The development of such resistance by bacterial pathogens is almost certainly an inevitable consequence of the clinical use of antimicrobial drugs. Moreover, antibiotics are sometimes associated with adverse effects that include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression, and various allergic reactions *(16)*. Therefore, it is critical to identify new antimicrobial substances from natural sources that exhibit minimal side effects.

Of the 33 plants tested, *H. brasiletto* was the only one that inhibited bacterial growth. This plant was and still is widely used as a dye. It is known that high concentrations of hematoxylin and hematein (compounds present within the plant) are toxic. However, there also are traditional references that cite naturopathic antibiotic properties of this plant, as well as mild but effective astringent properties and reports that it can be taken internally for diarrhea and infections, for example *(2)*. This plant has a remarkably diverse distribution in México, from desert hillsides and small rivers in the states of Sonora and Chihuahua to the extended tropical dry forests of Oaxaca and Central America. The traditional antimicrobial properties of *H. brasiletto* and its wide distribution made it of considerable interest for further analysis.

To treat or prevent a disease, it is important not only to kill the pathogen but also to inhibit the production or activity of its virulence factors. Several studies have been conducted regarding the antimicrobial activity of plant extracts against enteropathogenic organisms *(11, 35)*. The antimicrobial activity of *H. brasiletto* has been described previously; Sanchez-Marroquín et al. *(31)* found that extracts were effective against *Brucella abortus, B. suis, B. melitensis, Shigella flexneri*, and *Staphylococcus aureus*. Later, Pratt and Yuzuriha *(26)* found that aqueous extracts of the heartwood of this plant also exhibited antibacterial activity against *Salmonella* Typhi, as well as *S. aureus*. However, no information was available regarding the effects of this plant on EHEC strains. Here, we demonstrate antimicrobial activity of this plant against various *E. coli* strains.

Several plant extracts have been shown to inhibit the production of bacterial toxins. Natural products such as oleuropein, a phenolic compound extracted from olives, inhibits the production of enterotoxin B and other exoproteins of *S. aureus (35)*. It also has been shown that garlic oil or onion oil can diminish toxin production by *C. botulinum* type A in meat slurry *(8)*. More recently, our laboratory reported that extracts of *Euphorbia postrata, H. brassileto*, and *Psidium guajava* completely inhibited enterotoxin production by *C. perfringens (11)*. Results in this work showed that *H. brasiletto* ethanolic extract was able to inhibit verotoxin production.

The precise mechanism by which verotoxins are produced has been unresolved so far. However, the *H. brasiletto* extract would be expected to act by either directly or indirectly interfering with a physiological process to reduce verotoxin production. Inhibition of protein synthesis or leakage of internal structural components such as nucleic acids, ions, ATP, or amino acids have been related to presence of polyphenols, which is the nature of the active compound of this extract *(22)*. It has been shown that several antibiotics inhibit the growth of *E. coli* O157:H7 and its

production of verotoxin. In addition, certain extracts derived from clove (*Syzygium aromaticum*), creosote (*Larrea tridentata*), *Limonium californicum*, *Cupressus lustianica*, *Salvia urica*, and *Jusiaea peruviana* effectively inhibit or reduce verotoxin production *(29, 30)*. This is the first study to report that *H. brasiletto* extract affects verotoxin production in *E. coli* O157:H7.

Adherence of bacteria to the intestinal mucosa is considered a critical initial step in the pathogenesis of bacterial infections *(4)*. Adherence of EHEC to intestinal mucosa has also been demonstrated in several studies of EHEC infection *(19, 25, 32)*. In this study, HeLa cells were used to examine the influence of *H. brasiletto* extract on bacterial adherence to host cell surfaces because the monolayer HeLa cell system has been well established as a model for human intestinal infection *(1)*. In these assays, the data indicate that adhesion diminishes in response to plant extract in a concentration-dependent manner.

The anti-adhesion activity of the plant extract was higher for the non-EHEC strain, as measured under both assay conditions. These differences could be a result of the differences between *E. coli* serotype O157:H7 and other *E. coli*, not only from certain clinical and epidemiological standpoints, but also in several bacteriological features *(23)*. The use of intestinal antibodies as another strategy to inhibit EHEC adhesion was recently reported *(34)*. However, the use of plant products offers many advantages, particularly in countries where modern medicine is not available for most people or where traditional medicine is widely used.

Several studies have attempted to characterize the metabolites responsible for the biological activity of *H. brasiletto*. Sanchez-Marroquín et al. *(31)* reported that the antimicrobial substance present in *H. brasiletto* was a pigment, the brazilin. However, Pratt and Yuzuriha *(26)* determined that the antimicrobial activity was not only from brazilin because brazilein, a derivative of brazilin, also exhibited activity. Recently, Edwards et al. *(9)* indicated that the brazilin and brazilein compounds are found at high levels in *H. brasiletto*. In this study, the chemical analyses performed on the bark of *H. brasiletto* extract confirmed the presence of polyphenols. Although brazilin and brazilein are polyphenols, these compounds differ from the activity found in this study because the spectrum of the active compound showed a different NMR spectrum.

About 20% of the investigated plants producing an antibacterial effect are rich in polyphenolic substances, tannins, catechins and polyphenolic acids. Polyphenols belong to the large family of organic natural products known as tannins and are found throughout the plant kingdom *(13)*. It is known that polyphenols can form heavy soluble complexes with proteins. Polyphenols can bind to bacterial adhesions, and by doing so, they disturb the availability of receptors of the bacterial surface *(6)*. This could explain the effect observed in the adhesion assays when bacteria were previously treated with the plant extract *(38)*. Previous investigations of natural antiplaque agents from ''Nigerian chewing sticks'' identified a group of polyphenolic compounds that significantly altered the in vitro adherence of selected oral streptococci to glass and synthetic hydroxylapatite substrates *(37, 38)*.

This study supports the selection of plants by ethnobotanical criteria to enhance the probability of finding species with activity against EHEC. Our results point to *H. brasiletto* as a potential source of compounds against EHEC. This extract could potentially be used as a preservative in foods or as a therapeutic agent for the control of the diseases caused by this bacterium. Future studies will be aimed at addressing these important issues.

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