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Study of Antimicrobial Potential Of Verbena litoralis Organic Extracts

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— Abstract—

Verbena litoralis is a plant used in traditional medicine in Mexico by the multicultural population and is effective in relieving cold symptoms, stomach aches, fever, and diarrhea, among others. Scientific reports of its pharmacological potential are limited, so in this study, the antimicrobial potential of extracts from *V. litoralis* on pathogenic strains responsible for gastrointestinal infections was determined. The crude organic extracts were prepared by sonication and the bacterial growth inhibition effect was performed using the disk diffusion method and the minimum inhibitory concentrations of the extracts were obtained through plate microdilution. Phytochemical analysis was also done by Thin Layer Chromatography (TLC) and visible light spectrophotometry. The methanolic leaf extract and the ethanolic and ketonic extracts of the stem from *V. litolaris* showed a positive effect of inhibition of growth in all the strains evaluated, *E. coli* was the strain with the highest sensitivity to the components of the ethanolic stem extract with a minimum inhibitory concentration (MIC) of 5 mg/mL. The methanolic extract leaf from *V. litolaris* presented the highest concentration of phenylpropanoids with 11.75 + 0.03 µg Eq rutin/mL. This is the first report of the presence of coumarins, anthrones, and anthraquinones in *V. litoralis*, and with our results, we contribute to validating their use as an alternative to inhibit the growth of the evaluated strains.

Keywords:

Antibacterial, Verbena litolaris, phytochemicals, gastrointestinal infections.

Gastrointestinal ailments are among the first reasons for medical consultation, and their low level of care has resulted in it currently being considered one of the leading causes of death not only in Mexico but also worldwide. Gastrointestinal symptoms due to acute infections are the most frequent and can occur at any time of the year, but the risk of suffering from these diseases increases in the hot season (Hernández Cortez et al., 2011).

Regarding the monitoring of infections caused by bacteria, the global health sector faces an increase in the number of cases that require treatment and medical surveillance in order to avoid complications and reduce the use of antibiotics that eventually lose effectiveness (World Health Organization [WHO], 2020). Of the enterobacteria monitored by the WHO, those that have shown the highest degree of resistance to various generations of antibiotics in recent years are *Escherichia coli, Klebsiella pneumoniae*, and *Salmonella* spp. Within the list of critical priority are *Pseudomonas aeruginosa* and Enterobacterial resistant to carbapenems; in the list of elevated and medium are *Salmonella* spp and *Shigella* spp, resistant to fluoroquinolones, respectively (WHO, 2021).

Traditional knowledge and healing practices developed by rural communities around the world represent an important alternative in health care, on par with their importance, in some cases, to Western medicine. The use of medicinal and aromatic plants is of vital importance for the preservation of the health of people around the world, especially in developing countries. Traditional Mexican medicine dates from pre-Hispanic times in primary health care (Campos et al., 2018). The process of making herbal medicines produces various responses in the metabolism of those who consume them because they contain multiple molecules that act as active ingredients; therefore, they must comply with pharmacopoeial and quality control specifications (Gallegos-Zurita, 2016).

The *Verbena litoralis* plant (Schoch CL et al., 2020) is native to Mexico and is distributed in several states such as Sonora, Sinaloa, Chihuahua, Tamaulipas, Durango, San Luís Potosí, Nuevo León, Guanajuato, Querétaro, Hidalgo, Nayarit, Jalisco, Colima, Michoacán, Mexico City, Michoacán, Morelos, Puebla, Veracruz, Guerrero, Oaxaca, Tabasco and Chiapas (Rzedowski & Rzedowski, 2002, as cited in CONABIO, 2010), where it is also known as verbena fina or yakan k 'ulub wamal (tzotzil), yaxal nich jomol (tzeltal) (Digital Library of Traditional Mexican Medicine, 2009).

The *V. litoralis* is used to cure stomach pain, vomiting, cough (CONABIO, 2010), fever (Willmann et al., 2000, as cited in CONABIO, 2010) and for biliary colic; the latter is recommended to be prepared in an infusion with the leaves (Digital Library of Traditional Mexican Medicine, 2009). Chemical or pharmacological studies that validate its therapeutic appli-

cation are scarce (Digital Library of Traditional Mexican Medicine, 2009), it has been reported to have antioxidant, contraceptive (Braga et al., 2012), hepatoprotective (Vestena et al., 2019), and anti-inflammatory activity, and it has been suggested that these are due to the presence of phenolic compounds (Lima et al., 2020), or to terpenes that have been identified in other Verbena species. Therefore, it is important to expand the information on the phytochemical composition of *V. litoralis* and check its ability to treat bacterial infections. Therefore, the objective of this study was to determine the antibacterial effect of organic extracts of *Verbena litoralis* on *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Salmonella typhimurium* strains, in addition to phytochemical screening.

2. MATERIALS AND METHODS

2.1. Vegetal material

20 complete plants were collected from several randomly distributed individuals of *Verbena litoralis*, in the city of Comitán de Domínguez, Chiapas, in August 2022, the geographical coordinates being 16°15' N and 92° 08' W, and with an altitude of 1,600 masl.

2.2. Microorganisms

The strains evaluated were *Salmonella typhimurium* ATCC 14028 (donated by the Center for Research and Assistance in Technology and Design of the State of Jalisco, Southeast Subsite), *Escherichia coli*, ATCC 25922 (donated by the State Public Health Laboratory of the state of Chiapas), *Pseudomonas aeruginosa* and *Staphylococcus aureus* (donated by the laboratory of the Faculty of Medicine of the Universidad Autónoma de México).

2.3. Experimental design

A completely random design was made, the plant sample was divided into stem and leaves, and three solvents of different polarity were used: ethanol, methanol, and acetone, from which six extracts were obtained that when including the positive control (antibiotic) and the negative controls (solvents), 10 treatments were evaluated in triplicate with a total of 30 experimental units for each microorganism, the response variables being: the inhibition halos and the inhibitory effect.

2.4. Preparation of organic extracts

The dried and pulverized plant samples were weighed and the corresponding solvent according to the experimental design was added to each in a ratio of 1:10 (m/v). The six mixtures were sonicated for 2.5 hours at 20°C in a 40 Hertz VEBOR sonicator model: JPS-20ª. At the end of that time, each extract was vacuum filtered and centrifuged at 3500 rpm for 15 min; the supernatants were concentrated under reduced pressure (16 inHg) at 40°C on a HEIDOLPH rotary evaporator and the liquid recovered from each crude extract was deposited in amber bottles, which were kept under refrigeration for later use (Kuete et al., 2006).

2.5. Antibacterial activity by impregnated discs

For the antimicrobial evaluation, the diffusion method on agar with impregnated discs was used, based on the Kirby-Bauer method with an inoculum concentration of 1 x 10^8 UCF mL^{-1} distributed using an elbow rod on the surface of the Mueller Hinton agar Petri dishes. Subsequently, Whatman No. 5 sterile filter paper discs were placed, and impregnated with 15 µL of the crude extracts (Pandey, 2019), as a negative control the solvent of the extract was used, and as a positive control 30 µg mL^{-1} of chloramphenicol per disc (Vaghasiya & Chanda, 2007; Pandey, 2019). Each experiment was performed in triplicate. The boxes were incubated for 48 h at 37°C, the inhibition halo was measured and the relative inhibitory effect was determined, based on the positive control, by the formula:

$$\%\ Inhibitory\ effect\ (\%\ EI) = \left(\frac{mean\ inhibition\ halo\ diameter}{positive\ control\ inhibition\ halo\ diameter}\right) x\ 100$$

2.6. Determination of the Minimum Inhibitory Concentration

For those microorganisms that showed greater susceptibility to crude extracts, their minimum inhibitory concentration (MIC) was determined by the broth microdilution method, using sterile 96-well flat-bottomed microplates (Canche, 2019). The incubation of the microplates was at 37°C for 24 h. All experiments were done in triplicate.

2.7. Phytochemical analysis

It was performed qualitatively by Thin Layer Chromatography (TLC), using chloroform-acetone-acetic acid (9:1:0.2) as the mobile phase (Wagner et al., 1996). Quantitative analysis of phenolic compounds was performed by visible light spectrophotometry using the colorimetric methods: aluminum chloride

for flavones and flavonols (Chang et al., 2002), 2-Aminoethoxydiphenyl borate for total flavonoids (Robertson & Hall, 1989), and Folin Ciocalteau for total phenols (Singleton et al., 1999), using a Hach Dr 5000 spectrophotometer.

2.8. Statistical Analysis

The data obtained were analyzed using a one-way analysis of variance (ANOVA), and the comparison of means was performed by Tukey's test ($P<0.05$). A statistical analysis was carried out using Statgraphics software Centurion XIX® (Statgraphics Technologies, Inc., Madrid, Spain).

3. RESULTS AND DISCUSSION

Six crude *V litoralis extracts were obtained* with which the antibacterial effect on the pathogenic strains *S. aureus, P. aeruginosa, S. typhimurium*, and *E. coli* (which cause gastrointestinal diseases) was analyzed, the values obtained from inhibition halos as well as inhibitory effect, showed that only three extracts had a positive effect of inhibiting bacterial growth, and the statistical analysis indicated that there was a significant difference concerning the sensitivity of the strains with the extracts with halos between 6.17 to 9.9 mm (Figure 1), being the methanolic leaf extract with which we obtained 40.5 and 42.4% of inhibitory effect on *E. coli* and *S. aureus* as shown in Table 1. Bacterial growth was disrupted within 24 hours, where the lowest MIC (5 mg/mL) was determined to be with the ethanolic stem extract for all microorganisms tested.

The reported evidence of the use of *V. litoralis* in traditional herbal medicine, as well as the differences in the antibacterial effect of the extracts in this study led to phytochemical studies to relate this effect to the interaction of the compounds present. The qualitative analysis of leaf and stem extracts (Table 2) revealed the presence of the three main groups of secondary metabolites: alkaloids, saponins, flavonoids, coumarins, anthrones and anthraquinones, being abundant, mainly, in the methanolic and ethanolic extracts, which suggests that the positive inhibition effect may be due to the interaction of high polarity molecules, mainly by the hydroxyl groups presented by phenolic compounds, of which the presence of phenolic acids such as chlorogenic acid, caffeic acid, p-coumaric acid, vanillic acid and ferulic acid as well as flavonoids such as luteolin and apigenin has been reported (Lima et al., 2020), other metabolites with biological activity that have been identified for *V. officinalis*, a species belonging to the same genus, are: limonene, 1,8-cineole, ar-curcumene, epoxycaryophyllene, spatulenol, citral, geraniol, and verbenene of lipophilic nature, which correspond to monoterpenes and sesquiterpenes, which together with artemitin, sorbifolin, pedalitin, nepetin, and 7-O-β-D-glucopyranosyl-apigenin are considered

responsible for their biological properties (Deepak et al., 2000; Zhang et al., 2000). The high content of phenolic compounds of the *V. litoralis* also stands out in the crude extracts, mainly flavonoids with 11.75 + 0.03 µg Eq Routine/mL for methanolic leaf extract (Table 3).

The richness and content of secondary metabolites in these extracts may explain their effect on the growth of microorganisms. In this regard, Rodríguez-Pava et al. (2017) mention that metabolites such as alkaloids, flavonoids, tannins, and other compounds of a phenolic nature are responsible for antimicrobial activities in higher plants. For their part, Díaz-Solares et al. (2017) indicated that the pharmacological properties of plant extracts are attributed to the high content of phenolic compounds, which, in turn, are related to antioxidant and antimicrobial activities.

The antimicrobial activity of phenolic compounds, as mentioned by Aguilar-Mendez et al. (2020), involves the reaction of phenols with cell membrane proteins or sulfhydryl groups of proteins, which causes bacterial death by precipitation of membrane proteins and inhibition of some enzymes. The effect of flavan-3-ols has been shown to suggest that these classes of flavonols inhibit nucleic acid synthesis through the inhibition of topoisomerase or dihydrofolate reductase (Gradišar et al., 2007; Navarro-Martínez et al., 2005). Ikigai et al., (1993) used liposomes as models of bacterial membranes to test the activity of (-)-epigallocatechin-3-gallate (EGCG) on them and found that this catechin caused the leakage of small molecules from the intraliposomal space. The EGCG exhibited activity against *E. coli*, this was described by Nakayama, et al. (2013). EGCG was found to interact with the porin protein of the outer membrane of *E. coli*, thus inhibiting the main function of porin, namely the transport of small hydrophilic molecules such as glucose, which eventually leads to the inhibition of *E. coli growth*. Phosphatidic acid is the universal intermediate in the synthesis of membrane glycerophospholipids (Machinandiarena et al., 2020) in bacteria this synthesis is carried out by a complex of multiple individual enzymes known as fatty acid synthase II (FAS II) (Machinandiarena et al., 2020; Zhang & Rock, 2004). Being an attractive target for antibiotic development, several studies showed that various flavonoids exhibit inhibitory action on some of these enzymes in fatty acid synthetase.

CONCLUSION

Polar crude extracts of *V. litoralis leaves and stems* are a source of phenolic, terpenic, and alkaloid compounds, with the ability to inhibit the growth of bacteria such as *E. coli, P. aeruginosa, S. aureus*, and *S. typhimurium*, which lends validity to the empirical use of infusions to treat the symptoms of a gastrointestinal infection. However, other studies are required to define whether the antibacterial activity is due to a particular molecule or is a synergistic action of the metabolites present, as well as to test their cytotoxic effect.

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ANNEX 1

Table 1

Antimicrobial activity of the crude extracts of Justicia spicigera on the strains of "Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium, and Escherichia coli"

Crude extracts	S. aureus		P. aeruginosa		S. typhimurium		E. coli		CMI mg/mL
	HI mm	EI %	HI mm	EI %	HI mm	EI %	HI mm	EI %	
MH	8.87 a	42.4 a	6.67 b	25.0 b	6.67 a	32.6 a	9.67 a	40.5 a	50
ET	7.67 b	36.6 b	9.90 a	37.1 a	8.00 a	39.1 a	9.02 a	37.7 a	5
AT	6.33 bc	30.2 bc	6.17 b	23.2 b	7.51 a	36.7 a	6.42 b	26.9 b	18.33
CL	20.94		26.65		20.42		23.9		

Note. Average values followed by at least one letter are not significantly different between extracts for each strain studied at P ≤ 0.05 (Tukey's Test). M: Methanol, A: Acetone, H: Leaf, T: Stem. CL: chloramphenicol. HI: Halo of inhibition; EI: Inhibitory effect; CMI: Minimum inhibitory concentration.

Table 2

Phytochemical characterization of crude extracts of "Verbena litoralis" by thin-layer chromatography

Crude extracts	Alkaloid	Saponins	Flavonoids	Cumarin	Anthrone	Anthraquinone
MH	+	+	+++	++	+	++
MT	++	++	++	++	+	++
AH	-	-	++	-	-	+
AT	-	++	+	+	+	++
EH	+++	++	+	+	-	-
ET	+++	+++	+++	+	+	++

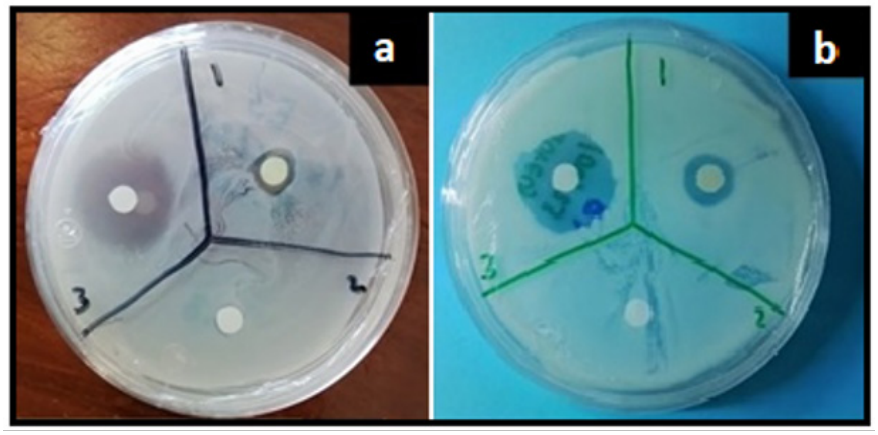
Note. M: Methanol, A: Acetone, E: Ethanol, H: Leaf, T: Stem. Abundant (+++), moderate (++), slight (+), and null (-) presence according to Kamatenesi-Mugisha et al., (2013).

Table 3

Quantification of phenolic compounds in crude extracts of "Verbena litoralis"

Crude extracts	Flavones and flavonols µg Eq quercetin/mL	Total flavonoids µg Eq rutina/mL	Total phenols µg Eq ác. gálico/mL
MH	2.63 + 0.01 b	11.75 + 0.03 a	9.20 + 0.02 a
ET	0.53 + 0.02 c	0.53 + 0.01 c	1.80 + 0.02 b
AT	4.88 + 0.07 a	2.05 + 0.01 b	1.46 + 0.01 c

Note. Mean values followed by at least one same letter are not significantly different between extracts at P ≤ 0.05 (Tukey's test). M: Methanol, A: Acetone, E: Ethanol, H: Leaf, T: Stem.



Note. a) ethanolic extract of the stem, b) methanolic extract of the leaf. 1) extracts, 2) negative control (solvent), 3) positive control (antibiotic)

Figure 1. Growth inhibition halos of Saureus in the presence of organic extracts of V. litoralis