IJPSR (2021), Volume 12, Issue 2 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 02 January 2020; received in revised form, 03 June 2020; accepted, 17 June 2020; published 01 February 2021

ANTIBACTERIAL ACTIVITY OF THREE URTICA SPECIES

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Keywords:

Infectious diseases, Antibacterial activity, Genus Urtica, *Urtica dioica*

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ABSTRACT: The antimicrobial activity of *Urtica dioica* L. and *Urtica urens* L. has been reported; however, there are no reports regarding *Urtica mexicana* Liebm., which is widely distributed in central Mexico. *U. urens* and *U. mexicana* were collected in Amecameca and *U. dioica* was collected in Toluca Valley, both located in the state of Mexico. Crushed leaves of the plants were soaked consecutively in hexane, dichloromethane, methanol and water. The minimum inhibitory concentration (MIC) was determined with 0.039 mg/mL to 5 mg/mL solutions of the extracts, which were added to cultures of *Salmonella typhimurium*, *Shigella flexneri*, *Salmonella typhi*, *Escherichia coli*, *Proteus mirabilis*, *Bacillus subtilis*, and *Staphylococcus aureus* (5×10^6 CFU/mL) with Resazurin sodium salt and Mueller–Hinton agar. The lowest MIC (0.039 mg/mL) was observed for the hexane, dichloromethane, and the methanol extracts of *U. urens* against *Bacillus subtilis* and the hexane and dichloromethane extracts of *U. urens* against S. aureus (0.156 mg/mL). *U. mexicana* showed a greater spectrum of activity at higher concentrations; *U. dioica* showed exhibited the least activity. *U. mexicana*, *U. urens* and *U. dioica* produced 14, 15 and 7 inhibitory extracts, respectively. The plants collected in Amecameca demonstrated higher antibacterial activity.

INTRODUCTION: According to the World Health Organization (WHO), in 2015, respiratory tract infections and infections that occur with diarrhea were responsible for 3.2 and 1.4 million deaths, respectively, ranking as the third and eighth leading causes of death worldwide (OMS/WHO) [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(2).1293-99

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(2).1293-99

In Mexico, diarrheal infections and gastroenteritis of presumed infectious origin were responsible for 5.1% of deaths in children between 1 and 4 years of age (INEGI) [2].

Treatment of these infections is based on the administration of antibiotics; however, many of these drugs have lost effectiveness due to the development of bacterial defense mechanisms, leading to the expression of genes that confer resistance to the antimicrobial agents used in clinical practice. Additionally, some antibiotics have adverse effects, such as suppression of the antimicrobial immune response and some patients

are hypersensitive or allergic to certain antibiotics as a result of the excessive and indiscriminate use of antibiotics [3]. It is estimated that bacterial infections will be the leading cause of death by the year 2050. This prediction has motivated the search for antimicrobials from natural sources, such as medicinal plants. Herbal remedies are widely employed to combat various types of infection, but only some plants have been studied chemically and biologically to determine their pharmacological action and constituents [4].

One of the most important genera known for their pharmacological properties is Urtica, which belongs to the family Urticacea, the largest group of angiosperms. At least 46 species of the genus Urtica have flowers, and *Urtica dioica* L., *Urtica urens* L., *Urtica pilulifera* L., *Urtica cannabina* L., *Urtica membranacea* Poiret., *Urtica haussknechtii* Boiss., *Urtica atrovirens* Req., *Urtica rupestris* Guss., *Urtica chamaedryoides* Pursh., *Urtica ferox* Forst. *Urtica dioica* L., and *Urtica urens* L. are the most prominent species and are found in Europe, Asia, Africa, and in the Americas [5]. Approximately 10 species of the Urtica genus can be found in Mexico. The plants of the genus Urtica are annual or perennial herbs that have urticating trichomes. Within the genus, the dioica specie has the greatest distribution worldwide and is the most widely studied [6].

***Urtica dioica* L:** *U. dioica* L. is of economic importance due to its potential in medicinal, nutritional, and textile production, and it has an urban distribution. In Mexico, it is known as ortiga (nettle), chichicastle, and mala mujer, and it can be found in the states of Hidalgo, Jalisco, Morelos, Puebla, and Oaxaca.

The plant grows to 1 m in height, and the stem and leaves are covered by urticating hairs. The leaves are round or elongated with serrated edges. The flowers are green, small, and grouped in spikes that emerge from the axils of the leaves. The plant has traditionally been utilized to treat genitourinary disorders (cystitis, urethritis, urolithiasis, nephritis), benign prostatic adenoma, gout, hypertension, edema, skin conditions (acne, burns, skin ulcers, alopecia) and diabetes, anemia (due to vitamin or mineral deficiency), internal bleeding, gastro-intestinal tract conditions (diarrhea, dysentery and gastric hyperacidity), allergies, musculoskeletal pain and osteoarthritis [7].

Chemical Composition: The presence of formic acid, acetylcholine, serotonin, and histamine has been reported in urticating trichomes, and some glycosidic flavonoids, such as rutin, isoquercetin, quercetin, and kaempferol have been found in the flowers and leaves. Also, the presence of fatty acids, caffeic acid, acetic acid, butyric acid, citric acid, formic acid, fumaric acid, ascorbic acid, proteins, essential oils, tannins, mucilages, vitamins (A, B_1, B_2, C, folic acid and pantothenic acid iron, sulfur, magnesium, manganese, copper, zinc, cobalt, potassium and calcium salts, and nitrates has been reported. The aqueous extract of the aerial structures of the plant has antioxidant, anti-microbial, antiulcer, and analgesic activity [8].

***U. urens* L:** *U. urens L.* is an annual, monoecious herb that is 10-60 cm in height, light green in color, with ovate, 1- to 4-cm by 1- to 6-cm, with deeply serrated leaves. The male and female flowers are numerous, and the fruits are achenes. The plant has smaller leaves and shorter flowers than *U. dioica*, is frequently used in place of *U. dioica* and is known as small nettle, burning nettle, and annual nettle. *U. urens* has a distribution pattern similar to that of *U. dioica* and is employed medicinally as an expectorant, purgative, diuretic, hemostatic agent, hypoglycemic agent, and anthelmintic (vermifuge), and to combat rheumatism, hemorrhoids, bronchitis, hyperthyroidism, gastrointestinal infections, and cancer. *U. urens L.* has been reported to have analgesic and anti-inflammatory activity; its seeds are used to reduce serum creatinine levels and in tests with mice, it has prevented carbon tetrachloride-induced hepa-totoxicity [9].

***Urtica Mexicana* Liebm:** *U. mexicana* Liebm is an herbaceous plant approximately 40 cm high that has a branching stem and oval, crenelated long petiolate leaves (2.5-5 cm), with androgynous inflorescences, usually shorter than the petioles. *U. mexicana Liebm*, has been used by the Nahuatlxihuitl (Organization of Midwives and Traditional Indigenous Physicians) for conditions related to joints and bones (rheumatism) and for gastrointestinal and skin infections.

This plant is abundantly distributed in the central region of Mexico [7]. As mentioned previously, *U. dioica* is the most widely studied species of the genus. The plant is cultivated for commercial purposes and sold in tablets or capsules as a nutritional supplement and used alone or together with Serenoa repens in the treatment of benign prostatic hyperplasia[10]. These three previously noted species grow in the same regions, have similar morphologies, are all sold as medicinal plants under the name of nettle, and are employed in traditional Mexican medicine for the same purposes. The objective of our study was to determine the antibacterial activity of *U. urens* and *U. mexicana* in comparison to that of *U. dioica* L, because the genus, and particularly *U. dioica*, is recommended for combatting various types of infections.

MATERIALS AND METHODS:

Plant Material and Preparation of Extracts: *U. urens L*. and *U. mexicana* Liebm were collected from Parque Esmeralda in Amecameca in October 2016, while *U. dioica* L. was collected in the Toluca Valley in November 2016, both located in the State of Mexico. The specimens were identified at the Herbario Metropolitano by Profs. Jorge Santana and Reyna Cerón of the Universidad Autónoma Metropolitana (UAM) where voucher specimens were deposited with the registration numbers *U. dioica* (70445), *U. urens* (79750), and *U. mexicana* (79753).

The leaves of the plants were allowed to dry at room temperature; then, 500 g of the crushed material was ground separately and soaked consecutively for 48 h in 3 L of hexane, dichloromethane, methanol (Xalostoc, Mexico) and water. The extracts were filtered, and the solvents were removed under reduced pressure in a Rotavapor (Buchi RII, Switzerland); water was removed by evaporation in a water bath. Solutions of 0.03 mg/mL-5 mg/mL in 10% dimethyl sulfoxide (DMSO)/H_2O were prepared from the solid extracts (J.T. Baker, New Jersey, USA).The percentage of recovery of the extracts was evaluated, while the aqueous extracts underwent a preliminary phytochemical study using colorimetric and precipitation reactions as reported previously. Total protein content was determined using the Lowry method [11].

Bacterial Strains: The *Salmonella typhimurium* ATCC 13311, *Shigella flexneri* ATCC 29003, *Salmonella typhi* ATCC 6539, *Escherichia coli* SOS, *Proteus mirabilis*, *Bacillus subtilis*, and *Staphylococcus aureus* ATCC 6538 bacterial strains were used. To determine the minimum inhibitory concentration (MIC), the Drummond and Waigh protocol (modified by Satyajit), in which 96-well plates and resazurin were used as indicators of viability, was followed.

This method is based on the reduction capacity of resazurin into resorufin by means of the oxidase–reductase enzymes in the surviving bacteria. When the extract inhibits bacterial growth, there is no bacterial oxide–reductase activity. Thus, the resazurin remains blue, whereas when the bacteria survive, reduction to resorufin yields a pink color into the culture medium due to the indicator [12].

Antibacterial Activity of the Extracts: The bacteria were cross-streaked in plates with Mueller-Hinton agar (Bioxón, México) and incubated for 24 h at 37 °C. A single colony was collected and seeded in duplicate in 50 ml of Mueller-Hinton broth (Bioxón, México). The culture was incubated for 24 h at 37 °C. Then, a 2.5-ml aliquot was taken and centrifuged (SOL-BAT, México) at 2,500 g for 5 min the supernatant was removed, and the cell pack was suspended in the same volume of sterile physiological saline solution (PSS).

The bacterial concentration was adjusted to 4×10^6 CFU/mL with the turbidity standard of 0.5 of the McFarland Nephelometer and incubated for 24 h at 37 °C. With the solid extracts of *U. urens* and *U. dioica*, an initial solution of 5 mg/mL in 0.8% DMSO (J. T. Baker, USA) was prepared, and double dilutions were performed. Fifty microliters/ well were deposited in 96-well plates (Nunclon) as follows: 10 µL of the bacterial suspension (4×10^6 CFU/mL), and 10 µL of resazurin sodium [(Sigma-Aldrich, St. Louis, MO, USA) (0.675% w/v in sterile distilled water)], and 30 µL Mueller–Hinton culture medium 3X (Bioxón, México), DMSO 0.8% and sterile distilled water were used as negative controls. A Penicillin–Streptomycin 1×10^4 IU/mL-1×10^4 mg/mL (Sigma Chemical Co., St. Louis, MO, USA) solution served as positive control. The culture plates were incubated at 37 °C

for 22 h in an atmosphere of 5% CO_2 and 90% relative humidity (Lab-Line, USA). Each extract was tested in triplicate at least three times.

RESULTS: The percentage of recovery and total protein content in extracts of the genus Urtica are presented in **Table 1**.

TABLE 1: PERCENTAGE OF TOTAL RECOVERY OF EXTRACTS OF *U. DIOICA* L., *U. URENS* L. AND *U. MEXICANA* LIEBM AND THEIR TOTAL PROTEIN CONTENT

	Extract	Recovery (g)	Performance (%)	Protein µg/mL
U. dioica L.	Hexane	6.52	1.30	0.00
	Dichloromethane	5.35	1.07	7.12
	Methane	14.22	2.94	24.13
	Aqueous	47.60	9.52	39.62
U. urens L.	Hexane	5.70	1.14	0.00
	Dichloromethane	7.32	1.46	6.35
	Methane	16.27	3.52	28.16
	Aqueous	47.60	9.06	45.02
U. mexicana Liebm	Hexane	7.22	1.44	0.10
	Dichloromethane	4.37	0.87	8.32
	Methane	15.16	3.03	33.16
	Aqueous	50.03	10.05	48.15

Phytochemical analysis of the aqueous extract revealed the presence of flavonoids, phenolic compounds and tannins.

Antibacterial Activity:

***Urtica urens* L:** The minimum inhibitory concentration was 0.039 mg/mL; the hexane, dichloromethane and methane extracts of *U. urens* revealed activity against *B. subtilis*, and these extracts also inhibited *S. aureus* at concentrations of 0.156-5 mg/mL, the latter indicating the maximal dose used. At the same concentration, the previously mentioned extracts also inhibited *Shigella flexneri* and *Salmonella typhi*. The aqueous extract inhibited *E. coli* and *B. subtilis* **Table 2**.

TABLE 2: ANTIBACTERIAL ACTIVITY OF THREE SPECIES OF THE GENUS URTICA

	Bacterial strains						
	A	**B**	**C**	**D**	**E**	**F**	**G**
***Urtica urens* L.**							
Hexane	-	5	5	-	-	0.039	0.156
Dichloromethane	-	5	5	-	-	0.039	0.156
Methanol	-	5	5	-	-	0.039	5
Aqueous/DMSO 1%	-	-	-	5	-	5	-
***Urtica mexicana* Liebm.**							
Hexane	-	5	5	5	-	1.25	2.5
Dichloromethane	-	5	5	5	-	0.625	-
Methanol	-	5	5	5	-	0.3125	-
Aqueous/DMSO 1%	-	5	-	-	-	5	-
***Urtica dioica* L.**							
Hexane	-	-	-	-	-	0.25	0.25
Dichloromethane	-	-	-	-	-	0.125	0.5
Methanol	-	1	1	-	-	-	-
Aqueous/DMSO 1%	-	-	1	-	-	-	-

A = *Salmonella typhimurium*; B= *Shigella flexneri*; C = *Salmonella typhi*; D = *Escherichia coli*; E = *Proteus mirabilis*; F = *Bacillus subtilis*; G = *Staphylococcus aureus*:– Indicates no observed growth inhibition.

***Urtica mexicana* Liebm:** At a concentration of 5 mg/mL, the hexane, dichloromethane, and methane extracts inhibited the growth of *S. flexneri*, *S. typhi*, and *E. coli* and inhibited *B. subtilis* at concentrations of 1.25, 0.625, and 0.312. The aqueous extract of this plant inhibited to *S. flexneri* and *B. subtilis*. This plant had the greatest number of inhibitory extracts **Table 2**.

***Urtica dioica* L:** This plant, which is the reference plant, had the lowest antibacterial activity. Seven extracts exhibited an inhibitory effect compared

with the 14 and 15 extracts of *U. urens* and *U. mexicana* respectively **Table 2**.

DISCUSSION: As mentioned previously, *U. dioica* is the most widely studied plant of the genus and it possesses notable activity against Gram-positive and Gram-negative bacteria, comparable to that exhibited by compounds such as Amoxicillin-clavulanic acid and Methylmycin. This activity has been demonstrated both with crude extracts and the fractions obtained from the plants. The aqueous extract of the leaves, roots, or seeds inhibits *B. subtilis*, *E. coli*, *Pseudomonas aeruginosa* and *Lactobacillus plantarum* in a range of concentrations from 36.21 mg/mL, 76.43 mg/mL and an inhibitory effect on *Proteus mirabilis*, *Citrobacter koseri*, *Micrococcus luteus* and *Candida albicans*. In contrast, in our results, the aqueous extract of *U. dioica* leaves only inhibited *S. typhi* at a concentration of 1 mg/mL, which is very low compared with the results of the previously mentioned studies. In our study, this species demonstrated the lowest antibacterial activity [13, 14].

Other authors reported the antibacterial activity of aqueous extracts of *U. dioica* obtained by the following: a) consecutive treatment with solvents of low-to-high chemical polarity, including hexane, ethyl acetate, chloroform, methanol, and water, and b) partition with methanol, methanol–chloroform, ethyl acetate, and water. The authors reported antibacterial activity ranging from 0.130-66.66 mg/mL [15]. In general, the crude extracts obtained by means of the first method showed better activity against Gram-positive than Gram-negative bacteria. The resistance of the latter has been associated with the more complex structure of the cell wall, with lipopolysaccharide in the outer membrane and other defense mechanisms that allow these bacteria to neutralize the action of antimicrobials, dyes and various agents that affect their viability [16].

Unlike the findings reported by these previous authors, in our study, the aqueous extract of *U. dioica* showed low antibacterial activity, while the corresponding extracts of *U. urens* and *U. mexicana* inhibited *B. subtilis* at the highest concentration utilized (5 mg/mL). This bacterium was the most sensitive because its growth was inhibited by 10 of the 12 extracts evaluated at concentrations ranging from 0.039 mg/mL-5.0 mg/mL. The other Gram-positive bacterium examined, *S. aureus*, was inhibited only by six extracts at concentrations ranging from 0.156 mg/mL-5.0 mg/mL. Such results differed from ours, and the differences are likely related to the method employed to obtain the extracts (theirs was obtained at 30-32 °C for 72 h, while ours was obtained at an average temperature of 25 °C for 24 h), and to the method used to evaluate and obtain the MIC (by diffusion in their case and by the resazurin oxidation-reduction method in our case [12].

An aqueous extract of the *U. dioica* leaf inhibited the growth of *S. aureus* and *Listeria monocytogenes*. The ethanolic extract of the leaf showed a lower effect than its aqueous extract [17]. These authors reported that the alcoholic extract of the stem possesses better activity than the corresponding extract of the leaf on Gram-positive bacteria. This extract also inhibited Gram-negative bacteria and the yeast Candida albicans, and its activity was higher than that of the root extract. According to findings reported [17, 18, 19, 20], the respective aqueous extracts of U. dioicademonstrated activity against *S. aureus*, *E. coli* and *C. albicans*.

With respect to other species of the genus Urtica, antibacterial activity has also been reported for the ethanolic extract of *U. urens* [21]. The aqueous extract of *Urtica pilifera* seeds and methanolic extract of the leaves, roots, and seeds have been reported to have antibacterial activity against several species of Gram-positive and Gram-negative bacteria [22, 23]. In the present study, the methanol extract of the leaves of *U. urens* and *U. mexicana* inhibited *Shigella flexneri*, *Salmonella typhi*, *Bacillus subtilis*, *U. mexicana* and *E. coli*.

Our results with the aqueous extract differ from those reported by other authors, likely primarily due to the extraction method; in the majority of studies, extraction is achieved through heating, while in our case, the extract were obtained at room temperature. In addition, in our study, the plant was previously treated with hexane, dichloromethane, and methanol and the aqueous extract comprised the residual material. It is possible that, in the soaking of the plant with methanol, certain

compounds were extracted that are also soluble in water, leaving the aqueous extract poor in components. As mentioned previously, among the outstanding components of *U. dioica* and other species of the genus Urtica are alkaloids, flavonoids, phenols, tannins, and saponins, which are compounds thought to have antibacterial activity [24].

Alkaloids are considered to have antibacterial activity due to their ability to react with the amino, carboxyl, and with sulfhydryl groups of bacterial proteins, as well as with nucleic acids [24]. Tannins precipitate proteins, including structural or enzymatic bacterial proteins, preventing the microorganism from nourishing itself.

Although the majority of phenolic compounds are hydrophobic, the hydroxyl group (-OH) allows them to introduce aryl and alkyl groups into proteins, modifying the three-dimensional structure of proteins. The OH- group can also affect DNA stability when the group interacts with amino and carbonyl groups of the purine and pyrimidine bases, forming new hydrogen bonds, which inhibits the functionality of microorganisms. The antibacterial action of flavonoids is attributed to their ability to form complexes with extracellular and soluble proteins and with the bacterial cell wall; moreover, flavonoids can disorganize the lipids of the bacterial membrane [25, 26].

It is noteworthy that *U. urens* and *U. mexicana* were collected in Amecameca at an altitude of 2,420 meters above sea level (masl) and at a latitude of 98 º45 '46 West, while *U. dioica* was collected in the Toluca Valley at an altitude of 2,667 masl and at a latitude of 99º 39’ 38” West, both cold-weather locations. The results obtained are attributable to the specific characteristics of each species in these habitats.

The results show that the Amecameca environment is more favorable for the antibacterial activity of plants than that of the Toluca Valley, which is consistent with the preliminary results of a comparative study of the antibacterial activity of *U. mexicana* collected in Amecameca and in Toluca. The results revealed greater activity of the samples collected from Amecameca, with 15 active extracts *vs.* seven active extracts from the Toluca Valley samples (results not included). This study shows that *U. mexicana* has antibacterial activity, which has not, to our knowledge, been previously reported.

This finding is important because conducting a comparative study on the attributed and demonstrated properties of *U. dioica* with those of other species opens a path to further comparative studies of species with a wide distribution in central Mexico, which would permit additional comparative studies with *U. dioica* and confirm the activities described for *U. mexicana* in traditional medicine in the country. This study exhibits a relationship between the ethnomedicinal use of Urtica plants for the treatment of gastrointestinal infections and experimental findings.

CONCLUSION: *U. mexicana* showed the highest antibacterial activity but at higher concentrations than *U. urens* and *Urtica dioica*, which had the lowest MIC. The plants collected in Amecameca had greater antibacterial activity than plants collected in the Toluca Valley.

ACKNOWLEDGEMENT: We thank Dr. Everardo Curiel Quesada of the National Polytechic Institute, Mexico, for the donation of the *B. subtilis* and *E. coli* strains.

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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How to cite this article:
Velasco LR, Tapia AR, Ruíz CJ, Sánchez Siles TE, Herrerasolis SB, Cerón R and Santana CJ: Antibacterial activity of three urtica species. Int J Pharm Sci & Res 2021; 12(2): 1293-99. doi: 10.13040/IJPSR.0975-8232.12(2).1293-99.