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Antibacterial activity of flower extracts from *Helenium mexicanum* H.B.K.

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Abstract: *Helenium mexicanum* (Asteraceae) is used in traditional Mexican medicine. Several studies have shown its insecticidal activity but its antibacterial properties have not been studied. The aim of this work was to assess the antibacterial activity of flowers extracts from *H. mexicanum* on ten human pathogen bacteria by disc-diffusion, broth dilution and bioautographic methods. Eight different extracts were obtained from *H. mexicanum* flowers and the antibacterial activity was evaluated. The acetone macerates (MA) and the soxhlet-chloroform (SC) extracts showed the highest activity against seven bacterial isolates. The MA extract was fractionated and the highest activity against *S. aureus* was shown by chloroform (CF) and methanol (MF) fractions, with MICs values of 0.125 mg/mL. Bioautography analysis of CF revealed at least six antibacterial components with different inhibition zones, whereas MF revealed only one inhibitory spot. Given the solubility properties of the active fractions, sesquiterpene lactones may comprise most of the antibacterial active compounds of *H. mexicanum*. This work systematically demonstrated the antibacterial activity of flower extracts from *H. mexicanum*.

Key words: Medicinal plant, *Helenium mexicanum*, antibacterial activity, bacterial pathogens

نشاط مضادات البكتيريا المستخلصة من زهرة نبات *Helenium mexicanum* H.B.K.

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الملخص: استخدم نبات *Helenium mexicanum* (Asteraceae) في الطب التقليدي المكسيكي . وقد أظهرت عدة دراسات احتواء تلك النباتات خاصية الطارد الحشري ولم تدرس خصائصها المضادة للبكتريا . وتهدف هذه الدراسة لتقييم النشاط المضاد للبكتيريا لمستخلص من زهرة نبات *H. mexicanum* على عشرة أمراض بكتيرية يصاب بها الإنسان بواسطة طرق disc-diffusion, broth dilution . تم الحصول على ثماني مستخلصات مختلفة من أزهار نبات *H. mexicanum* وتم تقييم النشاط المضاد للبكتريا . وجد إن المستخلص acetone macerates (MA) وكان المستخلص soxhlet-chloroform (SC) أعلى نشاطاً ضد *S. aureus* . والمستخلص acetone macerates (MA) وجد أن متبقياته كانت اعلي نشاط ضد MICs التي استكشفت من خلال الكلورفورم والميثانول مع قيم 0.125 mg/mL و Bioautography. و أظهرت تحليل الكلورفورم إن ما لا يقل عن ستة مركبات مضادة للبكتريا مع مناطق تثبيط مختلفة ، حيث كشفت طريقة الميثانول بقعة واحدة مثبطة ونظرا لخصائص المركبات المتبقية قد تضم أعلى مواد نشطة مضادة للبكتريا لنبات *H. mexicanum* واثبت منها عمليا ومنهجيا نشاط مضاد البكتريا من مستخلص زهرة نبات H.B.K. *Helenium mexicanum*.

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Introduction

Antimicrobial agents are widely used for control of bacterial infections but their use is subject to considerable debate due to the emergence of antibiotic resistance in bacteria (Payne and Tomasz, 2004). Increasing bacterial resistance has important implications for society in terms of morbidity, mortality, and health-care costs in medical facilities. Hence, extensive attention has been focused on understanding the antimicrobial resistance mechanisms and on searching new antimicrobial agents for the treatment of infections caused by resistant microorganisms (Mahmoud and Louis, 1999).

Secondary metabolism of plants is a rich source of novel and complex molecules that are part of plant defense mechanisms against predation by microorganisms, insects and herbivores (Cowan, 1999). Many of these metabolites also show activity against human pathogens (Rios and Recio, 2005). For example, extracts of *Quercus ilex*, one of the most common plants used in Morocco's traditional medicine, displayed activity against several bacterial strains (Berahou et al., 2007). A similar example is *Cordia curassavica,* a plant used in Mexican traditional medicine which exhibited *in vitro* activity against *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Vibrio cholerae, Yersinia enterocolitica* and *Escherichia coli* (Hernández et al., 2007).

Helenium mexicanum H.B.K. (Asteraceae), known as "chapuz", "cabezona" and "exoxóchitl", is a plant widely spread in countries like Mexico, El Salvador and Costa Rica. Its flowers are used in Mexican folk medicine as antiseptic, acaricide and sternutative (Díaz, 1977; Sánchez, 1994; Waizel and Waizel, 2005). The insecticidal properties of *H. mexicanum* have been reported (Martinez, 1959; Giral and Ladabaum, 1961).

Despite the widespread use of *H. mexicanum* as antiseptic in traditional medicine, it is important to notice the lack of systematic studies directed to characterize these properties. In order to achieve this, the aim of this work was to evaluate the antibacterial activity of flower extracts from *H. mexicanum* on human pathogenic bacteria.

Materials and methods

Bacterial strains

Ten clinical bacterial isolates and two reference strains were used in this work. Clinical isolates were obtained from the Laboratory of Microbiology and Parasitology of the Central Children Hospital "Eva Sámano de López Mateos" at Morelia, Michoacán, Mexico. Clinical isolates were *Shigella flexneri, Escherichia coli, Staphylococcus aureus, Proteus mirabilis, Enterobacter* spp., and *Salmonella* spp., *Klebsiella pneumoniae* RS, *K. pneumoniae* TN, *Pseudomonas aeruginosa* G53 and *P. aeruginosa* G71. The later four strains were characterized for being resistant to multiple antibiotics according to clinical trials carried out at the source hospital. *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853 were used as the reference strains. The identity of the strains was confirmed by standard biochemical procedures (Winn et al., 2006). All strains were maintained at 4 °C and subcultured every month in Luria agar.

Plant material

Helenium mexicanum was collected in Morelia, Michoacán, México and taxonomic identification of the plant material was confirmed by the Botanic Laboratory of Faculty of Biology (Universidad Michoacana de San Nicolás de Hidalgo). A voucher specimen was deposited in the herbarium of the same Faculty with the code "Barrera 23/04/01".

Preparation of crude extracts

H. mexicanum flowers were dried in the shade and grounded to a fine powder. From this material, eight crude extracts were obtained using the following solvents and procedure: acetone macerate (MA, 25°C for 96h), methanol macerate (MM, 25°C for 96h), chloroform macerate (MC, 25°C for 96h), methanol-chloroform macerate (MMC, 1:2 v/v, 4°C for 24h), aqueous extract (AQ, 60°C for 30min), soxhlet-ethanol (SE, 65°C for 4 h), soxhlet-ethyl acetate (SA, 65°C for 4h) and soxhlet-chloroform (SC, 65°C for 4h).

Disc-diffusion assay

The antibacterial activity assay was carried out following the NCCLS disc-diffusion method (NCCLS, 1997). Briefly, the bacterial strains were grown overnight at 37°C in 10ml of Luria broth. The cultures were adjusted to reach turbidity comparable to standard, McFarland´s scale 0.5, corresponding to 1.0 X 10^8 CFU/mL. Petri dishes containing Muller Hinton agar (Bioxon) were inoculated with these microbial suspensions. Discs of sterile filter paper (6mm of diameter) were impregnated with 20µl of each crude extract concentrated at 125 mg/mL (2.5 mg/disc) and placed on extraction hood for 1 h to accelerate the evaporation of solvents. The discs were placed on the plate's surface already inoculated with the bacterial suspensions. After incubation at 37°C for 18h the diameters of inhibition halos were registered in millimeters. Discs impregnated with the solvents were used as negative controls, while discs containing 10µg of gentamicin (Sigma) were used as positive controls of inhibition. Each assay was repeated three times.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericide Concentration (MBC)

The determination of MIC and MBC of the active extracts was carried out following the NCCLS method for broth dilution (Tamashiro, 1992; NCCLS, 1999). The extracts were dissolved in 1% Tween 20/Luria broth to a final concentration of 50 mg/mL and sterilized by filtration through a 0.45 µm membrane filter (Invitrogen). Then, serial dilutions were prepared from the stock solution to give concentrations ranging from 1 to 10 mg/mL in Luria broth. These test dilutions were inoculated with bacterial cultures at 1-5 x 10^5 CFU/mL final concentrations and incubated at 37°C for 24h. MIC values were taken as the lowest extract concentration that prevented visible bacterial growth after 24h of incubation. Test tubes with 1% aqueous Tween 20/Luria broth or different concentrations of gentamicin (Sigma) were used as negative or positive controls of inhibition, respectively. To discriminate a bactericidal effect from a bacteriostatic effect, MCB values were obtained (Frost, 1994). MCB are those concentrations that did not showed bacterial growth, after reinoculated in extract-free Luria broth and incubated to 37°C for 12h. Each experiment was replicated three times.

Fractionation of crude extracts and bioautography

To begin the preliminary isolation of the antibacterial (s) compound (s) from the most active extract, solvent/solvent fractionation (Baker) was carried out following the method described by Martini and Eloff (1998). In this way, six fractions were obtained: aqueous fraction (AF), butane fraction (BF), chloroform fraction (CF), hexane fraction (HF), methanol fraction (MF) and tetrachloride fraction (TF). These fractions were assayed for MIC and MBC as described above. The fractions with the highest antibacterial activity were analyzed by Thin Layer Chromatography (TLC) in silica Gel plates (G60 F_{254} Merck). After assaying different solvent systems, chloroform/methanol 9:1 rendered the best separation of compounds in the active fractions. Bioautography was performed on these TLC plates after the solvents were evaporated. Briefly, the plates were covered with Luria agar containing *S. aureus* at 1.0 X 10^7 UFC/mL and incubated at 37°C for 24 h (Hamburguer and Cordell, 1987). Bacterial growth was developed by spraying a 2mg/mL tetrazolium salt solution (TNBT, Sigma). Dark blue color indicated growth zones in the chromatogram, while colorless halos indicated growth inhibition zones (Beghe and Kline, 1972).

Results and discussion

In this work, eight crude extracts from *H. mexicanum* flowers were tested against human pathogen bacteria. The results showed that among the tested extracts, the acetone macerate (MA) and the soxhlet-chloroform (SC) extracts displayed the highest activity against seven bacterial isolates, including the multiresistant strain *Pseudomonas aeruginosa* G71 (Table 1). The less active extracts were aqueous (AQ) and methanol macerate (MM), showing limited activity on few isolates (Table 1). These results

indicate that compounds in flowers crude extracts from *H. mexicanum* have antibacterial effect against Gram positive and Gram negative bacteria, being *S. aureus* the most sensitive organism (Table 1). This data suggests a potential correlation between the use of *H. mexicanum* as an antiseptic to treat dermatological infections and the involvement of *S. aureus* as a common causal agent in such diseases. In addition, *S. aureus* is an important opportunistic pathogen that causes a variety of diseases in humans and animals which has a notorious raise in the frequency of resistant strains in the last years (Werckenthin et al., 2001).

Table 1. Antibacterial activity of flower extracts from *Helenium mexicanum*.

Bacterial isolate	***H. mexicanum* flowers extracts (2.5 mg/disc) Diameter of inhibition (mm)**								
	MA	**SC**	**SE**	**SA**	**MC**	**MMC**	**MM**	**AQ**	**Gm**
Staphylococcus aureus	21	23	18	20	17	16	9.6	-	25
Shigella flexneri	18	15	16	16	16	16	11	15	23
Escherichia coli	9	9	8	8	8	10	-	-	21
Salmonella spp.	11	10	8	10	-	-	-	-	23
Proteus mirabilis	10	9	-	-	-	-	-	-	19
Enterobacter spp.	8	-	-	-	-	-	-	-	19
Klebsiella pneumoniae TN	-	-	-	-	NT	NT	NT	NT	18
Klebsiella pneumoniae RS	-	-	-	-	NT	NT	NT	NT	18
Pseudomonas aeruginosa G53	-	-	-	-	NT	NT	NT	NT	19
Pseudomonas aeruginosa G71	-	8	-	-	NT	NT	NT	NT	16
Escherichia coli ATCC25922	9	8	-	-	NT	NT	NT	NT	22
Pseudomonas aeruginosa ATCC27853	-	-	-	-	NT	NT	NT	NT	18

MA acetone macerate ; SC: soxhlet-chloroform; SE: soxhlet-ethanol; SA: soxhlet-ethyl acetate; MC: chloroform macerate ; MMC: methanol-chloroform macerate; MM: methanol macerate; AQ: aqueous extract. (-): no inhibition. NT: not tested. Gm: Gentamicin discs (10 μg).

The extracts MA and SC were then tested in a broth dilution assay to determinate the MIC and MBC values against of sensitive bacteria. The results showed that MIC and MBC values of both extracts ranged from 1 to 10 mg/mL between the tested isolates (Table 2). These results correlate with the effects observed in disc diffusion assays. According to MIC and MBC values, the strains with the highest sensitivity for both extracts were *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella* spp. whereas multiresistant *K. pneumoniae* strains were the less sensitive (Table 2). On the other hand, multiresistant *P. aeruginosa* strains displayed slightly higher sensitivity to the extracts when compared to the reference strain *P. aeruginosa* ATCC27853 (Table 2). The obtained results confirmed the antibacterial activity of MA and SC extracts from *H. mexicanum* flowers and showed that the MA extract has the highest activity against pathogen bacteria, for this reason we carried out the fractionation of this extract.

Six fractions were obtained from MA extract which were tested against *S. aureus* by the broth dilution assay. The highest antibacterial activity against *S. aureus* was shown by chloroform (CF) and methanol (MF) fractions, with MICs in the lowest concentrations tested (0.125 mg/mL) for both fractions (Table 3). These fractions were subject to TLC and used for detection of antibacterial activity by bioautography.

Bioautography analysis of CF fraction revealed at least six antibacterial components against *S. aureus* (R_f 0.19, 0.23, 0.35, 0.41, 0.64 and 0.68). Three of these components (R_f 0.35, 0.41 and 0.64) showed zones of strong inhibition (Figure 1). With regard to MF fraction, the chromatogram just revealed one inhibitory spot with R_f 0.34 (Figure 1). These results indicate that the activity of MA crude extract can be attributed mainly to compounds

soluble in chloroform, but there also could be compounds of intermediate polarity contributing to its antibacterial activity which are extracted in the methanol fraction.

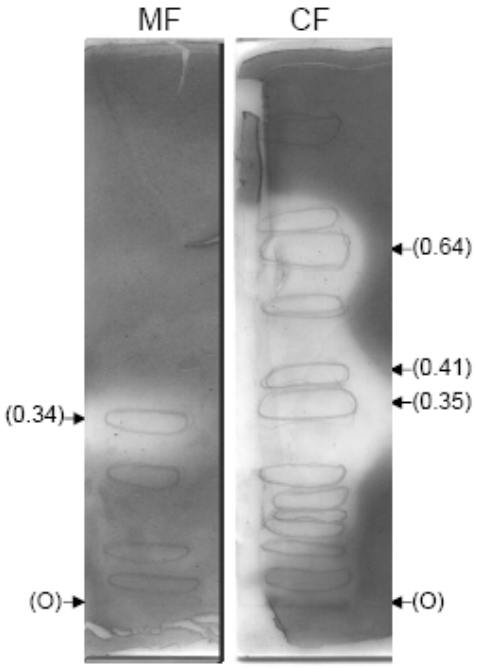

Figure 1. Bioautography of methanol (MF) and chloroform (CF) fractions with *Staphylococcus aureus*. Growth was visualized with Tetranitro blue tetrazolium.
Arrows indicate the Rf values of major active spots (0.34, 0.35, 0.41 and 0.64) and loading origin (O).

Table 2. Minimal Inhibitory Concentration (MIC) and Minimal Bactericide Concentration (MBC) values of extracts from *Helenium mexicanum* flowers.

Bacterial isolate	Concentration (mg/mL)				
	MA		SC		Gm
	MIC	MBC	MIC	MBC	MIC
Staphylococcus aureus	1.0	1.0	2.0	2.0	0.001
Shigella flexneri	1.0	1.0	3.0	3.0	0.001
Escherichia coli	4.0	6.0	5.0	7.0	0.001
Salmonella spp.	1.0	1.0	1.0	2.0	0.001
Proteus mirabilis	5.0	6.0	6.0	10.0	0.002
Enterobacter spp.	4.0	10.0	6.0	10.0	0.002
Klebsiella pneumoniae TN	8.0	>10.0	9.0	>10.0	0.002
Klebsiella pneumoniae RS	10.0	>10.0	>10.0	>10.0	0.002
Pseudomonas aeruginosa G53	3.0	5.0	3.0	8.0	0.004
Pseudomonas aeruginosa G71	4.0	6.0	2.0	4.0	0.004
Escherichia coli ATCC25922	2.0	4.0	3.0	5.0	0.001
Pseudomonas aeruginosa ATCC27853	5.0	10.0	5.0	10.0	0.002

MA: acetone macerate; SC: soxhlet-chloroform; MIC: minimal inhibitory concentration; MBC: minimal bactericide concentration. Gm: Gentamicin

Previous phytochemical characterization of *H. mexicanum* showed the presence of sesquiterpen lactones, such as helenalin and 8 mexicanins (Romo de Vivar and Romo, 1959;

Herz et al., 1963; Romo de Vivar, 1977). In many Asteraceae family members, sesquiterpene lactones has been reported as active compounds with insecticidal (Guillet et al., 1999), parasiticidal (Jimenez-Ortiz et al., 2005) and antimicrobial properties (Lee et al., 1977; Boulanger et al., 2007). According to known solubility properties of sesquiterpene lactones, is likely that they were enriched in the extracts from *H. mexicanum* that showed the highest antimicrobial activity in this work. Therefore, they may be the main compounds responsible for the properties that have made *H. mexicanum* a plant favored by traditional medicine. Studies aimed to identify the specific molecules acting as bioactive compounds from CF and MF fractions are been carried out in our laboratory.

Table 3. Minimal Inhibitory Concentration of acetone macerate (MA) fractions on *Staphylococcus aureus.*

Fractions	MIC (mg/mL)
AF	>1.00
BF	1.00
CF	0.125
HF	>1.00
MF	0.125
TF	>1.00

AF: aqueous fraction; BF: butane fraction; CF: chloroform fraction; HF: hexane fraction; MF: methanol fraction and TF: tetrachloride fraction.

Conclusions

It is worth noting that, to our knowledge, this work is the first study that demonstrates systematically the antimicrobial activity of diverse extracts from *H. mexicanum* flowers against human pathogen bacteria (Gram negative and Gram positive). Thus, this work is part of an effort to validate the use of *H. mexicanum* in traditional medicine and to explore the use of this plant as a source for future discovery of antimicrobial drugs.

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