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Antimicrobial activity of five plants from Northern Mexico on medically important bacteria

María del Carmen Vega Menchaca[1], Catalina Rivas Morales[1], Julia Verde Star[1], Azucena Oranday Cárdenas[1], María Eufemia Rubio Morales[1], Maria Adriana Núñez González[1] and Luis Benjamín Serrano Gallardo[2]

[1]Department of Analytical Chemistry, Biological Sciences Faculty, Autonomous University of Nuevo Leon, México.
[2]Department of Pharmacology, Biomedical Research Center, Medicine Faculty, Autonomous University of Coahuila, Mexico.

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The aim of this study was to evaluate the potential antimicrobial activity of five medicinal plants from Northern Mexico against ATCC bacteria *Klebsiella pneumoniae* (9183), *Staphylococcus aureus* (BAA44), *Escherichia coli* (O157), *Enterobacter aerogenes* (9180) and *Enterobacter cloacae* (9235) and eight clinical isolated strains (CI) *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Sarcina lutea* and *Streptococcus pyogenes*. Methanolic extracts of the leaves were tested against these bacterial strains using diffusion on agar method. The extracts showed antimicrobial activity against at least one of the microorganisms tested. *Leucophyllum frutescens* showed antimicrobial activity against *Staphylococcus aureus* (CI) and Escherichia *coli* O157 the minimal inhibitory concentration (MIC) was 28.0 and 30.0 μg/ml, respectively; *Tecoma stans* inhibited the growth of *Staphylococcus aureus* (CI) MIC 36.1 μg/ml; *Fouquieria splendens* showed antimicrobial activity against *Staphylococcus aureus* and *E. coli* O157 MIC 25.0 and 27.1 μg/ml, respectively, *Euphorbia antisyphylitica* resulted active against *E. aerogenes* 9183 and *S. aureus* (CI) MIC 30.1 and 26.8 μg/ml respectively. *Acacia farnesiana* did not show any antimicrobial activity. With the bioassay of *Artemia salina*, only the extract of *L. frutescens* showed toxicity (DL_{50} de 196.7 μg/ml). The dichloromethane soluble fraction of methanolic extract of *L. frutescens* with bioautography assay revealed three bands in the TLC, showed a broad spectrum activity against *S. aureus* (IC). This findings could increase scientific knowledge of medicinal plants from North of Mexico with antibacterial properties.

Key words: Toxicity, antimicrobial activity, methanolic extracts, medicinal plants. bioautography.

INTRODUCTION

Infectious diseases represent an important health problem and represent one of the main causes of morbidity and mortality worldwide, due to the inadequate use of antibiotics and to bacterial resistance (Ortiz et al., 2009). In past years, the problem of bacterial resistance has increased due to the appearance of pathogenic bacteria resistant to antibiotics (Kahkashan et al., 2012). In traditional medicine diverse infectious diseases have been treated with herbal products. Approximately the 80% of the world population have used herbal products to

*Corresponding author. E-mail: carmelitavega2006@yahoo.com.mx. Tel: 871-2033020; mobile 871-7276638. Fax: 871-7121717.

Table 1. Plants species from Northern Mexico screened for antimicrobial activity.

Plant (family)	Common Name	Part	Popular use
Tecoma stans (L.) Juss. Ex Kunth (Bignonaceae)	Tronadora	Leaves	Dysentery, diabetes, liver injury.
Acacia farnesiana (L) Willd (Mimosaceae)	Huizache	Flowers and leaves	Dysentery, antispasmodic, antituberculosis.
Euphorbia antisiphylitica (Zucc) (Euphorbiaceae)	Candelilla	Leaves	Purgative, tooth pain, syphilis. infections urinary.
Fouquieria splendens (Engelm) (Fouquieriaceae)	Ocotillo	Leaves	Diuretic, stomach pain, cough.
Leucophyllum frutescens (Berl.) I.M. Johnst (Scrophulariaceae)	Cenizo	Leaves	Dysentery, fever, cough, asthma, liver injury, cataracts.

Sher, 2009; Adame, 2000; Monroy, 2000.

satisfy their primary health care (Shubhi et al., 2010; OMS, 2004). It has been scientifically demonstrated that plants contain secondary metabolites to which biological properties are attributed, and from these properties, drugs have been developed to cure some diseases. Due to this, there is a constant need to find and develop new compounds with antimicrobial potential, and to continue the search of medicinal plants with new mechanisms of action to treat infectious diseases (Egwaikhide et al., 2009).

Mexico has a rich vegetal biodiversity with a long tradition in folk medicine among indigenous communities. These days the interest in traditional medicine is major on treatment of infectious diseases that affect the poorest sector of the population because this practice plays an important role in primary health care (Adame 2000; Wadud et al., 2007). There are various researches focused on the new compounds with biological activity from natural sources. In them, a great number of articles have been directed to antimicrobial activity evaluation on extracts and essential oils from medicinal and aromatic plants. For this, *in vitro* techniques have been used because of its simplicity and reproducibility (Rodriguez et al., 2010).

With this background, the aim of this study was to evaluate the antimicrobial activity *in vitro* of the methanol extracts of five plants *Leucophyllum frutescens* (Berl.) I.M. Johnst, *Acacia farnesiana (L) Willd*, *Tecoma stans* (L.) Juss. ex Kunth, *Euphorbia antisyphilitica* (Zucc), *Fouquieria splendens* Engelm against negative and positive bacteria that cause infectious diseases and reference ATCC strains, determine the toxicity of the extracts with the *Artemia salina* bioassay as well as partially identify the active compounds by assay bioautography. The vegetal species evaluated were selected for their use in traditional medicine to treat tuberculosis, fever, skin diseases gastroenteritis, urinary infectious, gastroenteritis and respiratory infections or conditions related to them (Table 1).

This study will contribute to increase the knowledge of the antimicrobial properties of these five medicinal plants from Northern Mexico on the development of new compounds with potential antimicrobial activitiy.

MATERIAL AND METHODS

Vegetal material

For this investigation, older people and herbalists were interviewed for the selection of the plants with anecdotic evidence for the treatment of infectious diseases on respiratory and gastrointestinal diseases. Fresh plants were recollected during the months of May and July of 2010 from the towns Nazas, Tlahualilo and San Pedro located on Chihuahuan semidesert in Northern Mexico with an altitude of 1200 m above sea level. The plants were authenticated by Eduardo Blanco Contreras of the Universidad Autonoma Agraria Antonio Narro and one sample was kept on the Herbarium in this university.

Extract preparation

The vegetal material was washed with deionized water to eliminate the excess of dust and damaged materials. The leaves were selected as the vegetal species except for *Acacia farnesiana* where the flower was used. The plants were dried in an oven at fixed temperature of 40°C with internal thermometer monitored until dried and ground in a mill.

To obtain the extracts, a mixture of powdered plant material and methanol (CTR Scientific®) in 1:10 proportion was prepared for every plant in an Erlenmeyer flask. The mixture was macerated for five days, and was left in a constant shake. The macerated material was filtered using a vacuum pump. The solvent was eliminated under reduced pressure with a rotating evaporator (Buchii,® R-205, Switzerland) set to 30 rpm and 50° C. The extracts were then completely dried (Rodríguez et al., 2010).

Microorganisms

Five referenced bacteria strains were provided by the Analytical Chemistry Laboratory of the Universidad Autonoma de Nuevo Leon from Monterrey México, and isolate clinical strains were obtained

Table 2. Evaluated microorganism.

ATTC bacterial strain	Clinical isolated bacteria strains (CI)
Klebsiella pneumoniae 9183	*Klebsiella pneumoniae*
Staphylococcus aureus BAA44	*Staphylococcus aureus*
Escherichia coli O157	*Escherichia coli*
Enterobacter aerogenes 9180	*Proteus mirabilis*
Enterobacter cloacae 9235	*Proteus vulgaris*
	Pseudomonas aeruginosa
	Sarcina lutea
	Streptococcus pyogenes

from the Hospital Infantil Universitario from Torreon, Mexico (Table 2).

Identification and conservation of the bacterial strains

The identification and typing of the bacterial strains was realized on biochemical profiles and recommendation of the Clinical Manual of Microbiology (Koneman et al., 2008; Hernández, 2003). All isolates were kept on a liquid medium (Rivas et al., 2007).

Assay microbiological

For the microbiological assay, the crude extracts were screened against eight strains bacterial pathogens (IC) and five reference strains by agar disc diffusion. In this method, 5 ml of culture medium (C. Rivas) was poured in test tubes of 13x100 mm sterilized to 15 lb/15min at 121°C. The tubes were inoculated with each bacterial strain and incubated for 18-24 h at 37°C. With sterile cotton swabs were dipped in the bacterial suspension 100 µl of $1x10^6$ colony forming units (CFU) adjusted with the Mc Farland Nephelometer (Clinical Laboratory Institute, 2006) and evenly streaked over the entire surface of the agar plate to obtain uniform inoculums (Khan et al., 2010).

Crude extracts 50 µL was poured on a Whatman No. 1 (Whatman® International LTD England) paper disc to 1,000, 500 and 250 µg/mL concentration and sterilized by filtration with 0.25 µm Millipore® membranes, over the solid agar. C Rivas was used as positive control 50 µL Cefotaxime (Sigma Aldrich®, St Louis MO, USA) and 50 µL Dimetil sulfóxido was used as the negative control. Each assay was analyzed in triplicate. All the plates were incubated at 37°C for 24 h after this time, the diameter inhibition halo was measured (NCCLS, 2001).

Determination of minimum inhibitory concentration (MIC)

The MIC assay was performed by the microdilution method; the medium used was C. Rivas broth. In 96-well microplate, 100 µL of C. Rivas broth was deposited in each well, and was added to the extract at 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 µg/mL concentration, followed by 100 µL of bacterial suspension containing the inoculum $1x10^6$ CFU adjusted with the Mc Farland Nephelometer. Next the microplate was incubated for 24 h to 37°C; for each trial, 500 µg/mL cephotaxime was used as positive control. All assays were performed in triplicate. To determine the MIC extract, 10 µL of indicator solution p-iodine tetrazolium at a concentration of 2.5 mg/mL was added to each well. The microplate was incubated for 8 h at 37°C; absorbance was read in a microplate reader (Dynatech® MR500) at 570 nm. (Yasunaka et al., 2005; NCCLS, 2002; Umeh et al., 2005: Vega et al*.,* 2013).

Bioautography

Evaluation of active chemical compounds was performed by bioautography using *Leucophyllum frutescens* against *Staphylococcus aureus* (IC). The assay was performed by agar overlay bioautography technique. Plant extract sample (5 µl) was applied 2.5 cm from the base of the silica plate (60W Merck)®. After drying, the plates were developed using solvent Benceno-Acetona (8:2), after which chromatography thin layer (TLC) plates were carefully dried for complete removal of solvents. Bioautography was performed with a culture of *S. aureus* (IC) which showed a better antibacterial sensitivity to the dichloromethane fraction extract of *Leucophyllum frutescens.* Aliquot of 20 mL of C. Rivas agar was overlaid on dried TLC plate under aseptic condition in laminar airflow by adding 200 µL of bacterial inoculum (1×10^6 CFU). The TLC plate were incubated at 37°C and examined for the zone of inhibition (Ncube et al., 2008, Schmourlo et al., 2004). Figure 1 shows the three active fractions of *L. frutescens* and inhibition on *S. aureus* isolate (IC).

Toxicity test with *Artemia salina* nauplii.

For the toxicity test, 0.1 g eggs of *Artemia salina* (Brine Shrimp Eggs® San Francisco Bay Brand, INC) were incubated on artificial sea water on a dark container divided by a middle wall with a space of 2 mm in the bottom. To prepare the sea water, 40 g of sea salt (Instant Ocean®, Acuarium System) were weighed with 0.006 g of yeast (Mead Johnson®) after which one liter of bidistilled water was added.

The pH was adjusted to 7.8, the containers were kept in condition of artificial white light and oxygenation, 48 h later, the hatched larvae called nauplii were taken with a Pasteur pipette and transferred to another container and kept in conditions of light, oxygen and temperature 22-29°C for 24 h. On a microplate of 96 wells was placed 100 µl of seawater containing 10 nauplii per well plus 100 µL of vegetal extract having concentrations of 10. 50, 250, 500 and 1,000 µg/mL a four replicates. Was used as positive control potassium dichromate a 400 ppm concentration and sea water was used as negative control. After 24 h with the help of a stereoscopic microscope, the total count of live and dead nauplii per dose was done. Probit method was used to determine the LD_{50} (Bastos et al., 2009, Déciga et al*.,* 2007).

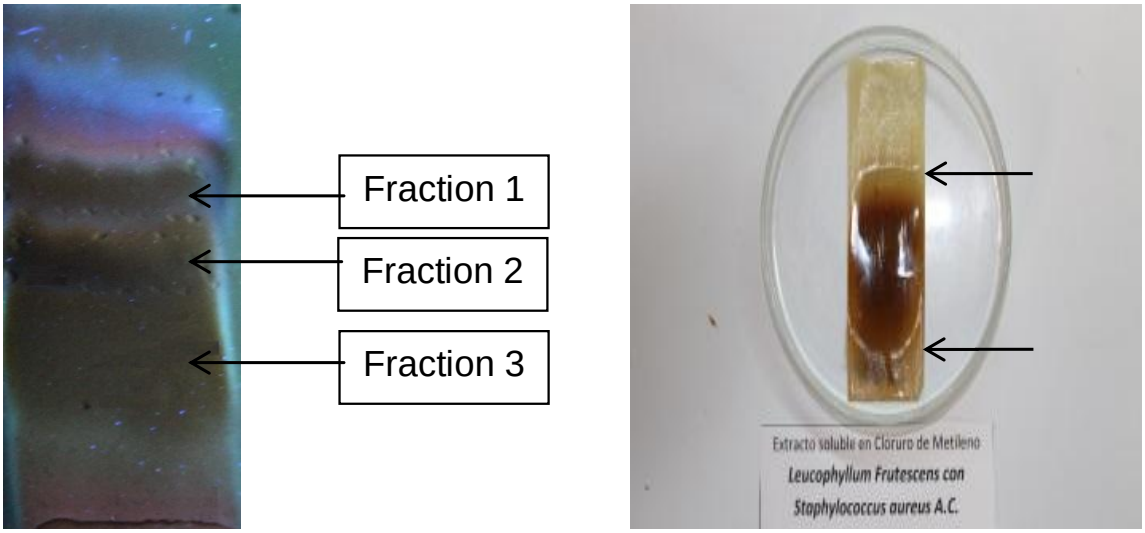

Figure 1. Dichloromethane fraction of *L. frutescens* and inhibition on *S. aureus* (IC).

Table 3. Biological activity of methanolic extracts of vegetal species on study from Northern Mexico against reference bacterial strains ATCC with method agar disc diffusion.

Plant	Microorganism				
	***K. pneumoniae* No 9183**	***S. aureus* No BAA44**	***E. coli* O157**	***E. aerogenes* 9180**	***E. cloacae* 9235**
L. frutescens	+	+ + +	+ +	-	+
F. splendens	-	-	+ +	-	-
E. antisiphylitica	-	-	+ +	+ +	-
A. farnesiana	-	-	-	-	-
T. stans	-	+ +	-	-	-

Scale: 5-10 mm weakly active (+), moderately active 11-15 mm (++), highly active > 15 mm (+++) at the discretion of García et al., 2006.

RESULTS AND DISCUSSION

Plants evaluated were selected by their use on folk medicine and by the few scientific reports on the antimicrobial activity of the flora from North of Mexico. All plant species showed antibacterial activity against tested microorganisms (Tables 3 and 4). Extracts of *L. frutescens, T. stans*, *F. splendens* and *E. antisiphylitica* showed antibacterial activity at concentrations of 250 - 1000 μg/ml against *S. aureus* (IC). In studies performed by Molina-Salinas et al. (2007), methanol leaves extracts of *L. frutescens* were reported to possess antibacterial activity which inhibited the growth of multirresistant strains of *M. tuberculosis*, *S. aureus* and *H. influenzae* b type. Extracts of *L. frutescens* and *T. stans* inhibited the growth of *E. coli* O157 at the concentrations tested. *E. antisiphylitica* showed antibacterial activity against *E. aerogenes* (9183), *F. splendens* inhibited the growth of *K. pneumoniae* (IC). The antibacterial activity showed a linear range with the extracts concentrations. The methanolic flowers extract of *A. farnesiana* showed no significant activity with the bacteria tested. Zaidan et al. (2005) evaluated the methanol leaves extracts of *Morinda citrifolia*, *Piper sarmentosum, Vitex negundo, Andrographis paniculata* and *Centella asiatica* found a high antibacterial activity against *S. aureus* and *S. aureus* methiciline resistant, but none of the five plants studied showed antibacterial activity against Gram negative bacteria; these findings are similar to our study, but different plants.

Other studies with native plants from Northern Mexico desert performed by Cespedes et al. (2006) found that *T. lucida* extracts ($MeOH/CH_2Cl_2$) inhibited the growth of *E. coli, P. mirabilis, K. pneumoniae, Salmonella* spp. and

Table 4. Biological activity of methanolic extracts of vegetal species on study from Northern Mexico against bacterial strains (IC) with method agar disc diffusion.

Plant	Microorganism				
	K. pneumoniae	*S. aureus*	*E. coli*	*E. aerogenes*	*E. cloacae*
L. frutescens	+	+ + +	+ +	-	+
F. splendens	-	++	+ +	-	-
E. antisiphylitica	-	++	+ +	+ +	-
A. farnesiana	-	-	-	-	-
T. stans	-	+ +	-	-	-

Scale: 5-10 mm weakly active (+), moderately active 11-15 mm(++), highly active > 15 mm (+++) at the discretion of García et al., 2006

Table 5. Minimum inhibitory concentration of methanolic extracts of vegetal species on study.

Plant species	Minimum concentration inhibition μg/Ml			
	***E. aerogenes* ATCC 9183**	***E. cloacae* ATCC 9235**	***S. aureus* (IC)**	***E. coli* ATCC O157**
L. frutescens			25.4	30.0
T. stans			36.1	
F. splendens			25.0	27.1
E. antishypilitica	30.1		26.8	

ATCC, American type culture collection; IC, isolate clinical.

Shigella spp. unlike the results of our study; where, Gram negative bacteria showed no activity. Adelou et al. (2009) reported the antibacterial activity of methanol extracts of leaves and stems of *Buddleja saligna* against Gram positive bacteria *S. aureus* and *S. epidermidis*, where they found inhibition of these bacteria, unlike the Gram negative strains of our study. This suggest that in our study the Gram negative bacteria were most resistant than the Gram positive bacteria. *A. farnesiana* did not show activity with bacteria tested. Ruiz et al. (2009) evaluated the antimicrobial and antifungal activity of methanol extract of six medicinal Mexican plants; *Amphypteringium adstrigens, Castella tortuosa, Coutarea latiflora, Ibervillea sonorae, Jatropha cuneata, Selaginella lepidophylla*, plant species different used to in our study, reported that *S. aureus* bacteria was more susceptible to all that plants tested.

The minimum inhibitory concentrations (MICs) for the methanolic extract of *L. frutescens, T. stans, E. antisiphylitica and F. splendens* are shown in Table 5. Our results show that all tested strains bacteria was less or equal to 30 μg/ml, but *S. aureus* show better inhibitory effect. do Nascimento et al. (2013) reported results similar with plant Pimenta malagueta (*Capsicum frutescens*). Bioautography assay of the dichloromethane soluble fraction obtained of methanol leaves extract of *L. frutescens* revealed three bands with chromatography thin layer at final concentration of 1000 μg/mL (Figure 1) which were active against *S. aureus* with halo inhibition of 24 mm. In the results obtained, the fraction were compared with the standard antibiotic used in this study with halo inhibition of 30 mm. Taiwo et al. (2013) reported activity against *S. aureus* concentration of 20 mg/mL with an inhibition even 27 mm with the fraction dichloromethane de *Cassia occidentalis* linn.

The chemical compounds identified in all three bands were two flavonoids and a quinone (Table 6). Mehrotra et al. (2010) studied five plant extracts for evaluating the bioactive components by bioautography, they reported that the extract *Zyzygium aromaticum* (clove) showed at least two active components against *S. aureus*. Bastos et al. (2009) conducted a study with the chloroform extract of *Zeyheria tuberculosa* (Vell.) Bur, found four flavones which were evaluated by bioautography against *S. aureus* and reported that two compounds were active against *S. aureus*.

Based on the fact that the objective was to evaluate the biological activity of extracts of plants, including toxicity, the LD_{50} was determined with the assay of *Artemia salina* lethality, screening test considered biological systems (Lagarto-Parra et al., 2001; Bastos et al., 2009) thereby ensuring their effectiveness and no toxicity. In this paper, we found a LD_{50}> 1,000 mg / mL for the methanol extract of the plants under study, except that *L. frutesens* showed an LD_{50} of 196.7 μg/mL. This result indicates that the extract tends to be toxic in agreement to the study of

Table 6. Rf of three chromatography bands of dichloromethane soluble fraction developed with UV light y $CoCl_2$ with eluent Benceno-Acetona (8:2) and chemical compounds.

Fraction	Rf	Light UV	Cobalt chloride	Chemical compounds
1	0.4	Brown	Brown	Flavonoides
2	0.5	Brown	Brown	Quinonas
3	0.6	Brown	Brown	Flavonoides

Déciga et al. (2010). In a study by Morales (2006) LD_{50} of 64.57 µg/mL of the methanol extract of *Lophocereus schottii* was obtained a on the lethality of crustacean *A. saline*.

Conclusions

The methanolic extracts of five plants native from Northern Mexico: *L. frutescens, T. stans, F. splendens, E. antisiphylitica* and *A. farnesiana* were evaluated against pathogen bacteria of clinical isolates and reference strains. *L. frutescens, T. stans, F. splendens, E. antisiphylitica* extracts showed significant activity over *Staphylococcus aureus* (IC) with MIC 25.4, 36.1, 25.0, 26.8 µg/ml, respectively. The extract that showed higher activity was *L. frutescens* (CMI) 25.4 µg / mL against *S. aureus*. The dichloromethane fraction of *L. frutescens* with Rf 0.4, 0.5 and 0.6 showed activity on *S. aureus* by bioautography. The tested extracts of the five study plants in *A. salina* nauplii showed no toxicity except for *L. frutecens* with a LD_{50} of 196.7µg/mL.

In this study, we obtained results of the antimicrobial activity of the five native species from Northern Mexico, contributing to increasing the knowledge of the plant used to in traditional medicine and could be the basis for further studies to isolate the active compounds of the studied plants and evaluate their effectiveness against other microorganisms.

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