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Full Length Research Paper

Antimicrobial, antioxidant and toxic effects of *Senna skinneri* Bentham, Irwin and Barneby (Leguminoseae)

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Infusions of the bark of *Senna skinneri* (Leguminoseae) are used by healers in Amatlán, Morelos, Mexico for the treatment of gastrointestinal, respiratory and skin diseases. The aim of this work was to investigate antimicrobial, antioxidant and toxic effects of the bark of *S. skinneri* (Leguminoseae), validate its use and contribute to the knowledge of medicinal flora from Amatlán municipality. Hexane and methanol extracts were used. Fourteen Gram positive, nine Gram negative bacteria and six fungal strains were used in the antimicrobial assay. Antioxidant activity was determined by the scavenging of the stable Doctoral Programs in Public Health (DPPH) radical. Generally, toxicity was accessed by the *in vivo* brine shrimp lethality test. The extract showed antibacterial activity against seven bacteria species and two fungal strains. The most sensitive strains were *Staphylococcus aureus, S. epidermidis* and *S. lutea* (MIC = 125 µg/mL) and *T. mentagrophytes* (CF_{50} = 630 µg/mL). The extract revealed high antioxidant activity but was highly toxic. The present study tends to confirm the use in folk medicine of *S. skinneri* bark in infectious diseases.

Key words: Amatlán, México, antibacterial, antifungal, antioxidant, infusion, gastro intestinal.

INTRODUCTION

Senna skinneri Bentham, Irwin and Barneby (Leguminoseae) is commonly known as “Paraca”, in Mexico, infusions of the bark are used in Mexican traditional medicine by healers in the village of Amatlán, Morelos, for the treatment of gastrointestinal, respiratory and skin diseases. *S. skinneri* is a shrub, native to America and widely spread from southern Mexico to Nicaragua and Venezuela (Aguilar et al., 1994; Argueta and Cano, 1994; Zamora, 2001; Fusco et al., 2004). Phytochemical studies on *S. skinneri* have resulted in the isolation of rutin and quercetin, 5, 7-dimethoxyrutin, the aglycon 5,7-dimethoxyquercetin and D-3-O-methyl-chiro-inositol (Arrieta et al., 1999). These compounds showed mutagenic activity in *Salmonella typhimurium*. The stilbene piceatannol was isolated from roots of *S. skinneri*, and showed antimicrobial activity against *Salmonella typhimurium* and *Escherichia coli* (Arrieta et al., 1999; Agnes et al., 2004; Ku et al., 2005).

Literature appears to be scarce about the antimicrobial activities of the bark of *S. skinneri* (Since there have been few studies of the bark of this specie and has not been tested for antimicrobial activity). The aim of the study was to investigate the antimicrobial, antioxidant and general toxicity activities of the bark of *S. skinneri* (Leguminoseae), validate its use and contribute to the knowledge of medicinal flora from Amatlán municipality.

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MATERIALS AND METHODS

Plant materials

S. skinneri bark was obtained in March 2007 from the "Sonora Market", Mexico city, and was authenticated by Dr. Edith Lopez Villafranco of the IZTA Herbarium at the Factultad de Estudios Superiores Iztacala. A voucher specimen was deposited in the IZTA herbarium (Voucher no IZTA 41884).

Preparation of the extracts

S. skinneri bark (330 g) was shade-dried at room temperature, ground into powder and sequentially extracted with methanol. The extract was filtered and successively concentrated. The methanol extract (82.8 g) was redissolved in methanol and hexane was added to it in a separating funnel. After solvent-solvent extraction, the methanol phase was removed from the hexane phase. Both extracts were concentrated under low pressure and kept in the dark at 4°C until tested.

Microbial strains

The following strains of bacteria were used: *Vibrio cholerae* INDRE 206 (isolated from polluted water), *Vibrio cholerae* (clinical strain pertaining to 01 group, Inaba serotype, "El Tor" biotype, and enterotoxin producer), *Vibrio cholerae* CDC V 12, *Escherichia coli* ATCC 25922, *Enterobacter agglomerans* ATCC 27155, *Salmonella typhi* ATCC 19430, and *Staphylococcus aureus* (ATCC 12398). All the strains tested were maintained at 4°C in Mueller Hinton Agar and were subcultured every month. *E. aerogenes* (cephalosporin and ampicillin resistant), *V. cholerae* No-01 (ampicillin resistant), *Staphylococcus epidermidis* (ampicillin, cephotaxim and dicloxacillin resistant), *Sarcina lutea* (cephotaxim and dicloxacillin resistant) and *Bacillus subtilis* (cephalothin, penicillin, cephotaxim and dicloxacillin resistant) were donated by the Laboratory of Microbiology of FES-Cuautitlán, *Yersinia enterocolitica* (ampicillin resistant) was donated by the Clinical Analysis Laboratory of University Hospital Campus Iztacala. These strains were maintained at 4°C in Mueller Hinton agar, submitted to sensitivity tests (multidiscs Bigaux) and were subcultured every month.

Six fungal pathogens were used: *Candida albicans* (clinical strain) donated by the Clinical Analysis Laboratory of University Hospital Campus Iztacala, *Fusarium sporotrichum* ATCC NRLL 3299, *Aspergillus niger*, *Trichophyton mentagrophytes*, *Fusarium moniliforme* were donated by Dr. Cesar Flores (Laboratory of Plant Physiology of UBIPRO, FES-Iztacala), and *Rhyzoctonia solani* was donated by Dr. Raul Rodriguez (INIFAP-Texcoco). The stock culture was maintained on Czapek Dox Agar (Sigma).

Antibacterial activity

The antibacterial activity was measured by the disk-diffusion method (Vanden Berghe and Vlietinck, 1991). The microorganisms were grown overnight at 37°C in 10 mL of Mueller Hinton Broth (Bioxon). The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland no. 0.5 standard (1.0×10^8 CFU/mL) (Lennette et al., 1987). Petri dishes containing Mueller Hinton agar (Bioxon) were inoculated with these microbial suspensions. Concentrations of 200 mg/mL of each extract were prepared, disks of filter paper (Whatman no. 5) of 5 mm diameter were impregnated with 10 µl of each one (final doses per disk: 2000 µg of hexane and methanol extracts) and placed on the agar surface. Disks impregnated with hexane and methanol were used as negative controls. Disks with chloramphenicol (25 µg) were used as positive controls. The plates were incubated overnight at 37°C and the diameter of any resulting zones of inhibition (mm) of growth was measured. Each experiment was performed in triplicates.

The estimation of the Minimal Inhibitory Concentration (MIC) was carried out by the broth dilution method (Vanden Berghe and Vlietinck, 1991). Dilutions of methanol extracts from 2000 to 7 µg/mL were used. Test bacteria culture was used at the concentration of 10^5 CFU/mL. MIC values were taken as the lowest extract concentration that prevents visible bacterial growth after 24 h of incubation at 37°C. Chloramphenicol was used as reference and appropriate controls with no extract were used. Each experiment was made three times.

The bactericidal kinetic assay was performed by using appropriate concentrations of the methanolic extract (corresponding to ½ MIC, MIC and MBC), in accordance with the method described by Avila et al., 1999.

Antifungal activity

Yeast was assayed by the method described for bacteria, using Petri dishes containing Czapek Dox Agar (20 mL), Nystatin (30 µg) was used as reference and appropriate controls with no extract were used. Each experiment was repeated three times.

The assay of antifungal activity was carried out in Petri dishes containing Czapek Dox Agar (20 mL). After the mycelial colony had developed, sterile blank paper disks (5 mm diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot of 2000 µg of each extract were added to disks. Ketoconazole was used as reference and appropriate controls with no extracts were used. The Petri dishes were incubated at 23°C for 72 h until mycelial growth has been developed. Disks containing samples, which had formed crescents of inhibition were considered as having antifungal activity (Ye et al., 1999).

For quantitative assays, dilutions of methanol extracts from 2000 to 2007 µg/mL were added to Czapek Dox Agar (5 mL) at 45°C, these being mixed rapidly and poured into 6 cm Petri dishes. After the agar had cooled down to room temperature, a small amount (1 x 1 mm) of mycelia was inoculated. After incubation at 23°C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth and hence the IC_{50} were determined. Ketoconazole was used as reference and appropriate controls with no essential oil or extracts were used. Each experiment was repeated three times (Wang and Bun, 2002).

Antioxidant activity

Scavenging activity of the methanol extract towards DPPH radicals was assessed by using the method described by Rivero and Betancort (2006) with minor modifications. Briefly, 0.1 mL of various concentrations (2 to 20 µg/mL) of the methanol extract and quercetine (positive control) in methanol was added to 0.1 mL of DPPH (0.2 mM) in MeOH. This yielded the final test concentrations (0.5 to 8 µg/mL). After a 30 min incubation period at 25°C in the dark, the decrease in the absorbance at 517 nm was measured. The control contained methanol instead of the compound solution and blanks contained methanol in place of DPPH solution. The inhibition of DPPH radical by the samples was calculated according to the following equation: DPPH-scavenging activity (%) = [1 - (absorbance of sample - absorbance of blank)/ absorbance of control] x 100.

General toxicity assay

General toxicity was determined by the *in vivo* brine shrimp lethality

Table 1. Antimicrobial activity and general toxicity (MIC's, IC_{50} and LD_{50}) of *S. skinneri*.

Organism	Positive controls (µg/mL)			
	Chloramphenicol	Gallic acid	Ketoconazole	Methanolic extract (µg/mL)
Artemia salina	-	321.5	-	8.92
Sa	1	-	-	125
Se	2	-	-	125
Sl	1	-	-	125
Bs	2	-	-	250
Vch w	1	-	-	250
Vch cc	1	-	-	750
Ye	4	-		750
Fm			7.55	1025
Tm			1.16	630

Artemia salina (LD_{50} values); bacteria (MIC's values); Fungi (IC_{50} values); Sa, *Staphylococcus aureus*; Se*, Staphylococcus epidermidis*; Sl, *Sarcina lutea*; Bs, *Bacillus subtilis*; Vch w, *Vibrio cholerae* (isolated from polluted water); Vch cc, *Vibrio cholerae* (clinical isolate); Fm, *Fusarium moniliforme*; Tm, *Trichophyton mentagrophytes*. ds = ± 0.0 in all cases.

test. It was carried out by using brine shrimp *Artemia salina* (Leach) larvae, according to the methodology described by McLaughin (1991).

Each plant extract was tested at 1000, 100 and 10 ppm (µg/mL) and also evaluated in triplicates.

Samples were prepared by dissolving extracts in DMSO. The final DMSO concentration did not exceed 1%, which has been shown not to have any harmful effects on the larvae. Gallic acid (LC_{50} = 321.5 µg/mL) was used as positive control, while DMSO was the negative control. Survivors were counted after 24 h.

LC_{50} was determined from 24 h counts. The toxicity was considered weak when LC_{50} was between 500 and 1000 µg/mL, moderate when the LC_{50} was between 100 and 500 µg/mL, and strong when the LC_{50} ranged from 0 to 100 µg/mL (Padmaja et al., 2002).

Phytochemical screening

Preliminary phytochemical analysis was carried out using thin layer chromatography on silica gel plates developed with a mixture of toluene-ethyl acetate (93:7). Spots were revealed by the following spray-reagents: Dragendorff reagent for alkaloids, vainillin-sulphuric acid for terpenes and flavonoids, and 2% methanol solution of ceric sulfate-saturated with concentrated sulphuric acid for glucosides; the plates were dried, the presence of triterpenoids were suggested by violet spots and flavonoid by yellow or orange spots, mono and sesquiterpenes by blue-violet, red-violet, grey-blue or blue spots (Wagner et al., 2001).

The Folin-Ciocalteu (Folin-C) assay using gallic acid as standard was used for the evaluation of total phenolics (Vernon et al., 1999). A solution of methanol extract or gallic acid was mixed with distilled water, Folin-Ciocalteu reagent, and aqueous Na_2CO_3 (20%). The mixtures were allowed to stand for 120 min and the total phenols were determined by colorimetry at 760 nm. A standard curve was prepared using solutions of gallic acid in water (0.00625 to 0.2 mg/L).

Total phenolic concentrations were expressed as gallic acid equivalents (GAE) per gram of dry matter.

Statistical analysis

All experiments were performed in triplicate. The mean and standard deviation of three experiments were determined. Statistical analysis of the differences between mean values obtained for experimental groups was done by analysis of variance (ANOVA multifactorial model), *p*-values of 0.001 or less were considered statistically significant. IC_{50} values were calculated by logarithmic model. Lethal concentration 50 (LC_{50}) in the general toxicity assay and SC_{50} values, in antioxidant assay, were calculated by linear regression analysis with Microsoft Excel program.

RESULTS

The extracts yields were: hexane 1.4 % w/w, and for methanol 24.7 % w/w. The results obtained in the evaluation of the antimicrobial activity of the extracts of *S. skinneri* are shown in Table 1.

Only the methanolic extract showed antibacterial activity in four Gram positive and three Gram negative bacteria strains. *S. aureus, S. epidermidis* and *S. lutea* were the strains that are more sensitive to the methanolic extract effect (MIC = 125 µg/mL). In general, Gram positive bacteria (MIC = 125 to 250 µg/mL) were more sensitive than the Gram negative ones (MIC = 250 to 750 µg/mL).

Figures 1 and 2 show the effect of the methanolic extract (in the survival curve) on a Gram-positive (*S. aureus*) and in a Gram-negative bacteria (*V. cholerae* clinical strain). Minimum inhibitory concentrations (MIC) had a bacteriostatic effect on the microbial population, while the minimum bactericidal concentrations (MBC) had a lethal effect on both bacterial strains within the first twelve hours.

Only the methanolic extract showed antifungal activity in *T. mentagrophytes* and *F. moniliforme. T. mentagrophytes* strain was more sensitive to the methanolic extract (IC_{50} = 630 µg/mL).

The general toxicity (Table 1) was considered strong for the methanolic extract of *S. skinneri* against *A. salina*

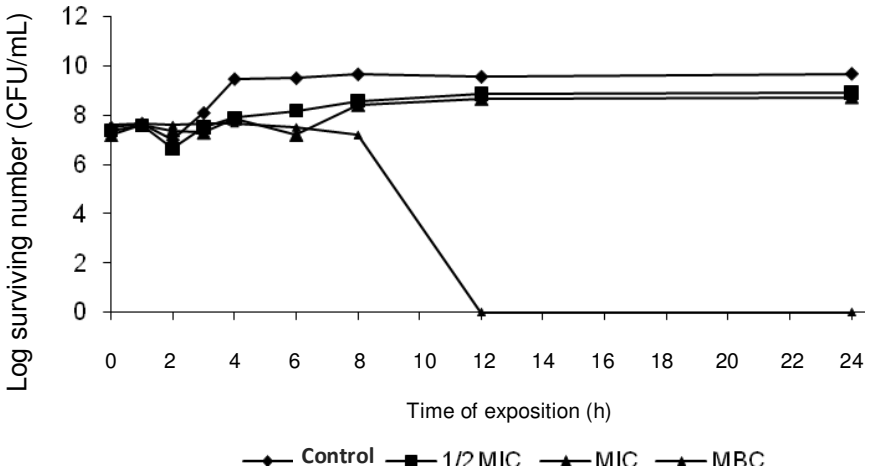

Figure 1. Survival curve of *S. aureus* exposed to methanol extract of .*S. skinneri.* The methanol extract was added to each experimental culture in zero time. The concentrations used were: 62.5 µg/mL (½ MIC), 125 µg/mL (MIC), 250 µg/mL (MBC). The control tube did not contain methanol extract.

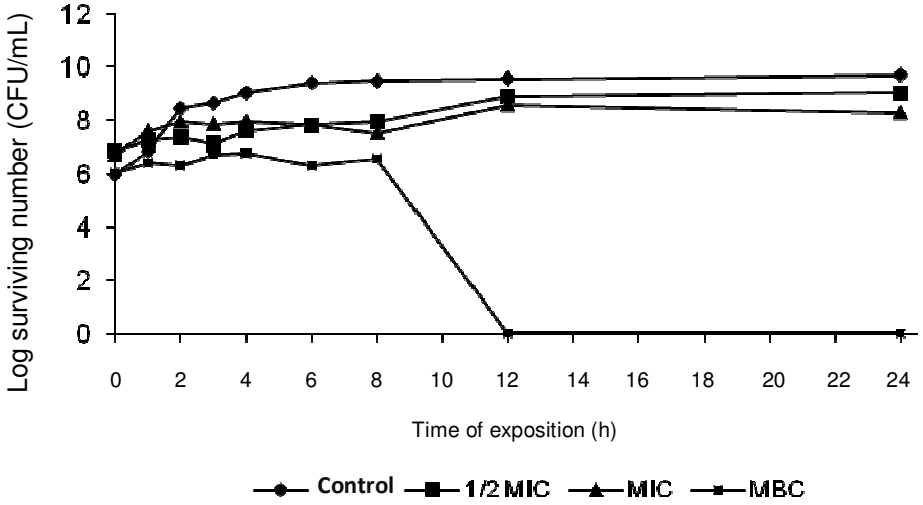

Figure 2. Survival curve of *V. cholera*e cc (clinical strain) exposed to methanol extract of *S. skinneri.* The methanol extract was added to each experimental culture in zero time. The concentrations used were: 350 µg/mL (½ MIC), 750 µg/mL (MIC), 1000 µg/mL (MBC). The control tube did not contain methanol extract.

(LC_{50} = 8.92 µg/mL).

The methanol extract presented a potent antioxidant activity, efficiently scavenging the DPPH free radical with a SC_{50} value of 8.41 µg/mL.

Phytochemical analysis revealed that the methanol extract contain triterpenes and flavonoids and was also positive to alkaloids.

In relation to the determination of total phenols, the methanolic extract of *S. skinneri* presented a 65.2% of total phenols expressed as gallic acid units (652 GAE in 1.0 g of methanol extract). This data is in accordance with SC_{50} value (8.41 µg/mL).

DISCUSSION

Only the methanolic extract of *S. skinneri* presented antibacterial activity against four Gram positive and three Gram negative bacteria. It was observed that *S. aureus, S. epidermidis* and *S. lutea* had the lowest MIC values. In general, Gram positive bacteria were more sensitive than the Gram negative ones (Jehl et al., 2004; Sepulveda-Jimenez et al., 2004). The broad spectrum antibacterial activity exhibited by the methanol extract of *S. skinneri* could be linked to its use for respiratory, gastrointestinal and dermatological infection of bacterial origin in

traditional medicine.

No significant antifungal activity was observed in the methanolic extract of *S. skinneri* since only two strains were susceptible. *T. menthagrophytes* was the most sensitive strain (Table 1). Argueta and Cano (1994) indicated that infusions of the bark of *S. skinneri* are used against skin diseases, which is related to the effect on *T. mentagrophytes*. Kim (2004) and Rogers (2005) reported the presence of emodyn in *S. obtusifolia* and *S. tora*, showing antifungal properties. Since these metabolites are common in the genus (Harborne and William, 2006), emodyn probably could be found in *S. skinneri*.

The general toxicity of the methanol extract was significant. It is important to observe that in some cases higher concentrations than LD_{50} are required to inhibit the growth of some bacterial and fungal strains, but it is known that local people use only infusions of the aerial parts of *S. skinneri* to treat infectious diseases.

Antioxidant activity determined using DPPH radical scavenging assay is expressed in terms of SC_{50}. In this study, the concentration of methanol extract needed to achieve 50% scavenging of DPPH radical under experimental condition was 8.41 µg/mL. This result can be attributed to the presence of piceatannol. Arrieta (1999) and Anges (2004) earlier reported the presence of this compound in *S. skinneri*, which possess antioxidant properties.

The methanol extract of the bark of *S. skinneri* presents 65.2% of total phenols expressed as gallic acid units (652 GAE in 1.0 g of methanol extract).

The high phenolic content may be related to the antioxidant activity observed since these compounds scavenge free radicals. Furthermore, phenolic compounds including flavonoids and alkaloids have been implicated in pharmacological activities, such as antimicrobial, antioxidant, etc. (Arrieta et al., 1999; Anges et al., 2004; Prior et al., 2005).

The present study has validated the use of *S. skinneri* in folk medicine for the treatment of gastrointestinal, respiratory and dermatological diseases.

It is hence recommended that further studies in the isolation of active components in the bark of the plant should be performed.

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