BLACPMA ISSN 0717 7917

Artículo Original | Original Article

Antimicrobial activity of *Argemone ochroleuca* Sweet (Chicalote).

[Actividad antimicrobiana de *Argemone ochroleuca* Sweet (Chicalote)]

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Abstract
Argemone ochroleuca Sweet (Papaveraceae) is used to treat eye infection, respiratory and dermatological disorders in Tepotzotlán, State of México (México). The aim of this work was to investigate antimicrobial activity of hexane, ethyl acetate and methanol extracts from aerial parts of *A. ochroleuca*. The antimicrobial activity was evaluated against thirteen bacteria and nine fungal strains. Only methanol extract showed antimicrobial activity. *S. aureus* (MIC= 125 µg/mL) and *C. neoformans* (MIC= 500 µg/mL) were the most sensitive strains. The bioassay-guide purification of the methanolic extract resulted in the isolation and identification of the isoquinoline alkaloid berberine. The present study confirms the antimicrobial activity of methanolic extract of *A. ochroleuca*.

Keywords: Antibacterial activity, Antifungal activity, *Argemone ochroleuca*, Berberine, Papaveraceae, Tepotzotlán, México.

Resumen
Infusiones de la parte aérea de *Argemone ochroleuca* Sweet (Papaveraceae) son utilizadas por los pobladores de Tepotzotlán, Estado de México, para el tratamiento de infecciones en los ojos, enfermedades respiratorias y dermatológicas. El objetivo de este trabajo fue investigar la actividad antimicrobiana de los extractos hexánicos, acetato de etilo y metanólico de la parte aérea de *A. ochroleuca*. La actividad antimicrobiana fue evaluada frente a trece cepas bacterianas y nueve cepas fúngicas. Solo el extracto metanólico presentó actividad antimicrobiana. *S. aureus* (MIC= 125 µg/mL) y *C. neoformans* (MIC= 500 µg/mL) fueron las cepas que presentaron mayor sensibilidad. La purificación biodirigida del extracto metanólico dio como resultado el aislamiento e identificación del alcaloide isoquinolínico berberina. El presente estudio confirma la actividad antimicrobiana del extracto metanólico de *A. ochroleuca*.

Palabras Clave: *Actividad antibacteriana, Actividad antifúngica,* Argemone ochroleuca, *Berberina, Papaveraceae, Tepotzotlán, México.*

Recibido | Received: August 30, 2010.
Aceptado en versión corregida | Accepted in revised form: February 21, 2011.
Publicado en línea | Published online: March 30, 2011.
Declaración de intereses | Declaration of interests: This research could not have been done without the cooperation of many people in Tepotzotlán, Estado de México. The authors are grateful to Dr. Edith López Villafranco and Simon Hernandez for their technical assistance. This research has been supported by UNAM PAPCA 2009-2010 (Project N°: 21).
Este artículo puede ser citado como / This article must be cited as: Francisco Daniel REYES, Celia Jimena PEÑA, Margarita CANALES, Manuel JIMÉNEZ, Samuel MERÁZ, Tzasna HERNANDEZ. 2011. Antimicrobial activity of *Argemone ochroleuca* Sweet (Chicalote). Bol Latinoam Caribe Plant Med Aromat 10(2): 139 – 146.

INTRODUCTION

A. ochroleuca Sweet (Papaveraceae) is known as "chicalote", and is a weed native of North America, widely spread from southern USA to central México (Martinez, 1996). It is used in traditional medicine by the inhabitants of Tepotzotlán, State of Mexico. Based on information obtained from healers in the region is known that infusions of the aerial part are used to relieve eye infections, (Neri, 1995), spots, and warts, combat insomnia, cough and dermatological disorders (Argueta & Cano, 1994). *A. ochroleuca* is an annual or perennial herb of short life (Rzedowski & Rzedowski, 2001).

There are some publications about the chemical composition of *A. ochroleuca* and it have been identified fatty acids (Fletcher *et al*., 1993), flavonoids (Stermitz *et al*., 1969, Bhardwaj *et al*., 1982, Saleh *et al*., 1987, Fletcher *et al*., 1993; Chang *et al*., 2003) and several alkaloids such as atropine, berberine (Espinosa & Sarukhan, 1997), chelerythrine, protopine, sanguinarine, dihidrosanguinarine, dihidroquelerithrina, α-allacriptopine, heleritrine, queilantifoline, scouletrine, reticuline and copsitine (Haisova & Slavik, 1973; Israilov *et al*., 1986; Chelombit'ko & Nazarova 1988; Takken *et al*., 1993).

The purpose of this study was to evaluate the antimicrobial activity of hexane, ethyl acetate and methanol extracts from aerial parts of *A. ochroleuca*.

MATERIALS AND METHODS

Plant Material

Flowering specimens of *A. ochroleuca* were collected in September 2007 in Tepotzotlán, State of Mexico. Voucher specimens were deposited in the National Herbarium of Mexico (MEXU) at the Universidad Nacional Autónoma de Mexico, and the herbarium IZTA at the Facultad de Estudios Superiores Iztacala (Voucher n° 41638).

Preparation of the Extracts

Air-dried aerial part (7 days at room temperature, 1 kg) of the plant was extracted with solvents of different polarity: hexane, ethyl acetate and methanol. The extracts were filtered, successively concentrated and kept in dark at 4 °C until tested. The extracts yields were: hexane 1.77 % w/w, ethyl acetate 3.22 % w/w and for methanol 10.03 % w/w.

Microbial Strains

The following strains of bacteria were used: *Vibrio cholerae* INDRE 206 (isolated from polluted water), *Vibrio cholerae* (clinical strain pertaining to 01 grup, 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. *Enterobacter aerogenes* (cephalosporin and ampicillin resistant), *Vibrio 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.

Nine fungal pathogens were used: *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). *Candida albicans* ATCC 14065, ATCC 10231, *Candida albicans* (clinical strain), were donated by the Clinical Analysis Laboratory of University Hospital Campus Iztacala. *Cryptococcus neoformans*, donated by the Mycology and Parasitology Laboratory of Medicine, UNAM. The stock culture was maintained on Czapek Dox Agar (Sigma).

Antibacterial Activity

The antibacterial activity was measured by the disc-diffusion method (Vanden Berghe & 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 (Lennett et al., 1987). Petri dishes containing Mueller Hinton agar (Bioxon) were inoculated with these microbial suspensions. Discs of filter paper (Whatman Nº 5) of 5 mm diameter were impregnated with 2000 μg of each extract and placed on the agar surface. Disks impregnated with 10 μL of

hexane, ethyl acetate and methanol were used as negative controls. Discs with 25 μg (5 μl) of chloramphenicol (5 mg/mL Sophia Labs, Mexico) 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 repeated three times.

The estimation of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were carried out by the broth dilution method (Vanden Berghe & Vlietinck, 1991). Dilutions of the extracts from 2000-7 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, and MBC as the lowest concentration that completely inhibited bacterial growth.

Chloramphenicol was used as reference and appropriate controls with no extract were used. Each experiment was repeated 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

For yeast we used 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 had enveloped. Disks containing samples and had formed crescents of inhibition were considerate with antifungal activity (Ye *et al*., 1999).

General toxicity assay

The general toxicity *in vivo* brine shrimp lethality test was carried out by using brine shrimp *Artemia salina* (Leach) larvae, according to the methodology described by McLaughin (1991). The methanolic extract was tested at 1000, 100 and 10 ppm (μg/mL) and also evaluated by triplicate.

Samples were prepared by dissolving the extract in DMSO. The final DMSO concentration did not exceed 1% which has been shown not to have any harmful effects on the larvae. As positive control, gallic acid was used (LC_{50} =321.5 μg/mL); and as negative control DMSO was used. Survivors were counted after 24 h. LC_{50} was determined from 24 h counts. The general toxicity activity was considered weak when the 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).

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. Lethal concentration 50 (LC_{50}) values, in the general toxicity assay, were calculated by linear regression analysis with Microsoft Excel program.

Active compound isolation

For the separation of the active compound, column chromatography (silica gel mesh 70-230 Sigma 5-2509) was used. The methanolic extract (70 g) was chromatographed on silica gel column and was eluted with the following gradient of solvents chloroform-methanol (10:0, 9:1, 8,2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10); obtaining thirty-two collected fractions. The purification of the active compound was making by assay-guided isolation. The antibacterial activity of all collected fractions was measured by disc-diffusion method (Vanden Berghe & Vlietinck, 1991). Fractions 16-20 were active. The purification of the active compound was made by recrystalization in chloroform and methanol. The yield obtained of the active compound at the end of the process was of 18.9 mg.

The active compound was identified by: melting point 144-146 °C; IR ART (Perkin Elmer to FT-IR Spectrum 2000), 3421cm^{-1} assignable to a tertiary amine belonging to a heterocyclic; 2993-2849 cm^{-1} assignable to –CH, -CH_2 and CH_3,1506 cm^{-1} assignable to double bonds of an aromatic compound, 1274 cm^{-1} assignable to C-O (methoxyl group); and at 1103 cm^{-1} a sign that suggests the presence of a cyclic

esther; and confirmed with X-ray crystallography (Bruker AXS-Detector de Area con Radiaciones MoKalfa; (Kariuki and Jones, 1995).

RESULTS

The results obtained in the evaluation of the antimicrobial activity of the methanolic extract of *A. ochroleuca* are shown inTable 1.

Table 1. Antimicrobial and general toxicity activities (MIC's, IC_{50} and LD_{50}) of *A. ochroleuca*.

	Positive controls (μg/mL)			
Organism	Chloramphenicol	Gallic acid	Nystatine	Methanolic extract (μg/mL)
Artemia salina	-	321.5	-	10
Sa	1	-	-	125
Se	2	-	-	500
Sl	1	-	-	>2000
Bs	2	-	-	250
Vch No01	1	-	-	1500
Vch Tor	1	-	-	1500
Cn	-	-	4	500
Ca	-	-	4	1750

Artemia salina (LD_{50} values); bacteria (MIC's values); Fungi (IC_{50} values); Sa, *Staphylococcus aureus*; Se, *Staphylococcus epidermidis*; Bs, *Bacillus subtilis*; Sl, *Sarcina lutea*; Vch No-01, *Vibrio cholerae*; Vch Tor, *Vibrio cholerae* CDC V12; Cn, *Cryptococcus neoformans;* Ca, *Candida albicans* (clinical strain). The hexane and ethyl acetate extracts showed no activity. sd ± 0.0 in all cases.

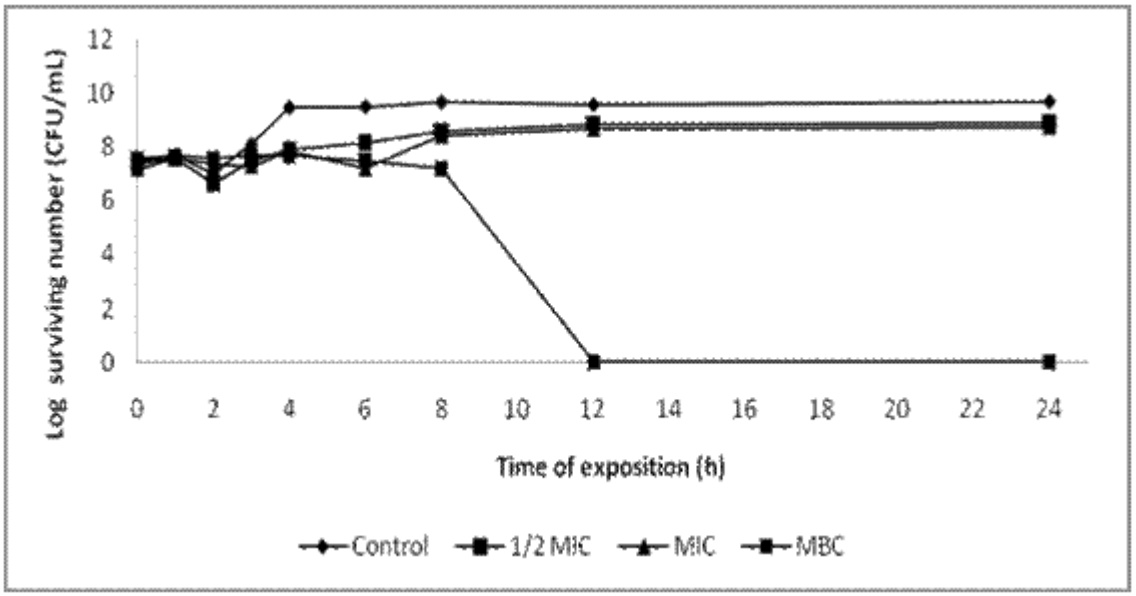
Only the methanolic extract showed antibacterial activity in four Gram positive and two Gram negative bacteria strains.

S. aureus was the strains more sensitive to the methanolic extract effect (MIC=125 μg/mL). In general Gram positive bacteria (MIC=125 - 500 μg/mL) were more sensitive than the Gram negative ones (MIC=1500 μg/mL).

Only the methanolic extract showed antifungal activity in *C. neoformans* and *C. albicans* (clinical case). *C. neoformans* was the strain more sensitive to the methanolic extract effect (IC_{50}=500 μg/mL).

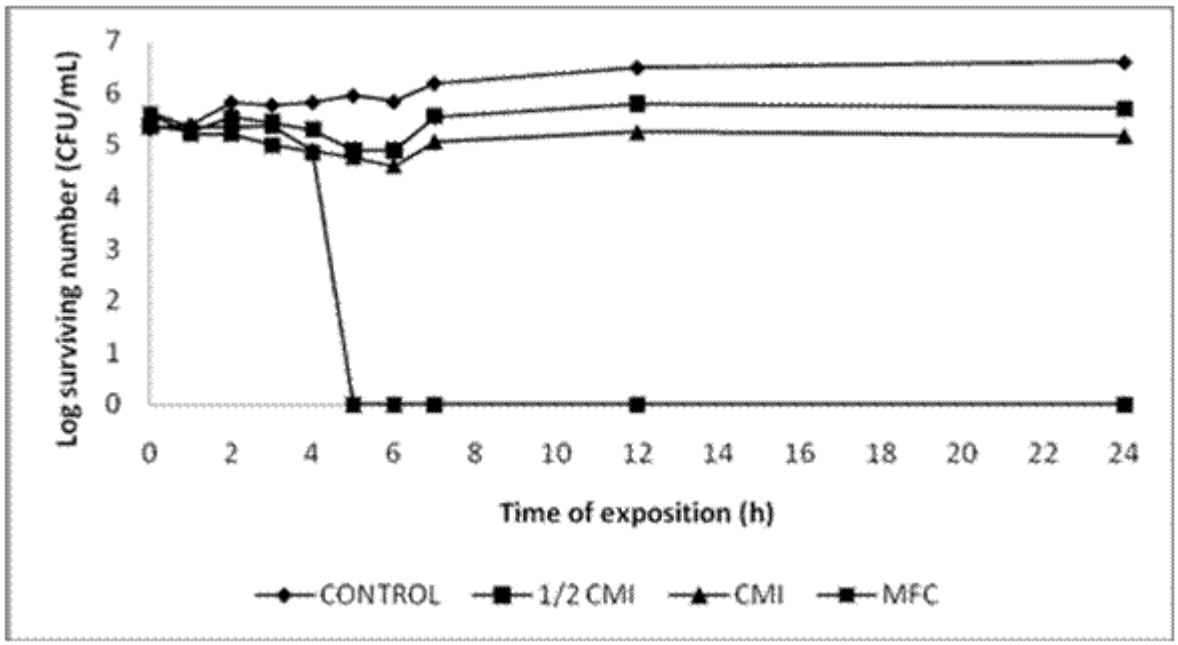
Figure 1 and 2 show the effect of the methanolic extract (in the survival curve) on a Gram-positive bacterium (*S. aureus*) and in a fungal strain (*C. albicans* isolated of a clinical case). Minimum inhibitory concentrations (MIC) had a bacteriostatic effect on the microbial population, while the minimum bactericidal concentrations (MBC) had a lethal effect on bacteria and in the fungal strain within the first twelve and five hours respectively.

Figure 1. Survival curve of *Staphylococcus aureus* exposed to methanol extract of *A. ochroleuca.*



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 *Candida albicans* (clinical strain) exposed to methanol extract of *A. ochroleuca.*

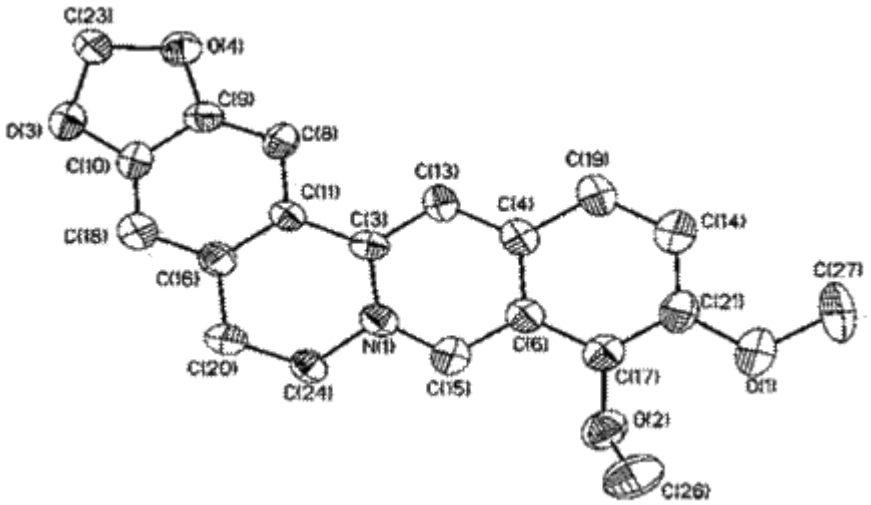


The methanol extract was added to each experimental culture in zero time. The concentrations used were: 875 µg/mL (½ MIC), 1750 µg/mL (MIC), 3500 µg/mL (MBC). The control tube did not contain methanol extract.

The general toxicity activity (Table 1) was considered strong for the methanolic extract of *A. ochroleuca* against *A. salina* (LC_{50} = 10 µg/mL).

IR spectra and the X-Ray Cristallography showed that the structure of the active compound is the isoquinoline alkaloid berberine (Figure 3).

Figure 3. Perspective view of the X-ray structure of the Berberine



DISCUSSION

Only de methanolic extract of *A. ochroleua* presented antibacterial activity against four Gram positive and two Gram negative bacteria. It was observed that *S. aureus* presented the lowest values of MIC. In general Gram positive bacteria were more sensitive than the Gram negative ones (Inbaraj *et al*., 2001; Freile *et al*., 2003; Sepulveda-Jimenez et al., 2004; Enriz & Freile, 2006; Abdel-Sattar *et al*., 2010; Alamri & Moustafa, 2010).

The methanolic extract of *A. ochroleuca* presented antifungal activity against two strains. It was observed that *C. neoformans* (MIC=500 μg/mL) was the most sensitive strain (Table 1).

These results showed that the methanolic extract has potential antimicrobial effects against representative human pathogenic bacteria and fungi, such as *S. aureus, S. epidermidis*, *B. subtilis*, *Vibrio cholerae* CDC V12, *Cryptococcus neoformans*, and *Candida albicans.*

The antimicrobial activity showed by the methanolic extract of *A. ochroleuca* can be attributed to the presence of the isoquinoline alkaloid berberine, which is already known to exhibit antimicrobial activity (Harborne & Williams, 1995; Birdsall & Kelly, 1997; Inbaraj *et al*., 2001; Freile *et al*., 2003; Sepulveda-Jimenez *et al*., 2004; Enriz & Freile, 2006; Abdel-Sattar *et al*., 2010; Alamri & Moustafa, 2010). Is important to considerate that berberine is not the only active component because there are reports that the total methanol extract also has flavonoids and other biologically active compounds (Stermitz *et al*., 1969, Bhardwaj *et al*., 1982, Saleh *et al*., 1987, Fletcher *et al*., 1993; Chang *et al*., 2003).

The general toxicity activity of the methanolic extract was considerate strong. It is important to observe that in some cases bigger concentrations to LD_{50} are required to inhibit the growth of some bacterial and fungal strains, but as we know local people use infusions of the aerial part of *A. ochroleuca* to treat infectious diseases.

CONCLUSION

The present study confirms the antimicrobial activity of methanolic extract of *A. ochroleuca.*

ACKNOWLEDGEMENTS

This research could not have been done without the cooperation of many people in Tepotzotlán, Estado de México. The authors are grateful to Dr. Edith López Villafranco and Simon Hernandez for their technical assistance. This research has been supported by UNAM PAPCA 2009-2010 (project number: 21).

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