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Biological activity of phototoxic compounds in *Bidens squarrosa* H.B.K. (Asteraceae)

(With 4 Tables)

Actividad biológica de compuestos fototóxicos en Bidens squarrosa H.B.K. *(Asteraceae)*

(Con 4 Tablas)

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Abstract. Phototoxic compounds (polyacetylenes and thiophenes) and their biological activity were determined on *Bidens squarrosa* plants by measuring their antibacterial and anti-inflammatory potential. Phototoxic compounds were extracted with hexane, characterized by TLC, and their presence confirmed by U.V. spectrum of their extracts and bactericidal potential. Antibacterial activity of the hexane, ethyl acetate and methanol extracts was measured against *Bacillus subtilis* and *Escherichia coli*, and their anti-inflammatory potential was determined by the mouse ear edema test.

Key Words: Asteraceae, phototoxic compounds, bactericide activity, 12-O-tetradecanoylphorbol-13-acetate (TPA).

Resumen. En *Bidens squarrosa* se caracterizaron compuestos fototóxicos (poliacetilenos y tiofenos) y se determinó su actividad biológica midiendo su potencial antibacteriano y antiinflamatorio. Los compuestos fototóxicos se extrajeron con hexano, se caracterizaron por TLC y su presencia fue confirmada por el espectro UV de su extracto y su potencial antibacteriano. La actividad antibacteriana de los extractos hexánico, acetato de etilo y metanol se midió contra *Bacillus subtilis* y *Escherichia coli*, y su potencial antiinflamatorio se determinó por el método de edema en la oreja de ratón.

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Palabras Clave: Asteraceae, compuestos fototóxicos, actividad bactericida, 12-O-tetradecanoilforbol-13-acetato (TPA).

INTRODUCTION

Most of the naturally occurring acetylenes have been isolated from the Asteraceae family. The variation in structure of these compounds is extremely wide, and their distribution is of great importance to chemotaxonomists. The biological properties of these compounds also make them of great interest to plant pathologists and pharmacologists.

Many of the acetylenes and related compounds require UV-light (300 – 400 nm) for being toxic and having other biological activities. The phototoxic acetylenes extend their toxicity to a wide range of pathogenic organisms (bacteria, yeasts, insect larvae) (Christensen & Lam, 1990).

As part of our search within the Asteraceae, we studied the species *Bidens squarrosa*, seeking for phototoxic compounds (polyacetylenes and thiophenes). This species is found in open areas and along river courses, from sea level up to 1700 m.a.s.l. In Mexico, it grows mainly in the states of Guerrero and Jalisco.

Bidens squarrosa has not been studied, but other species of this genus have several medicinal properties. For example, leaf infusions are used for influenza, swollen and sore throat and dysentery. Leaves, after being macerated in alcohol, can be used to treat rheumatic pain (Martínez et al., 2001).

The chromatographic profiles of the hexane extracts, containing the phototoxic compounds, were run for this plant. Phototoxic compound presence was confirmed by UV spectrum of their extracts, and by their bactericidal activity against *Bacillus subtilis*.

The plant bactericidal potential (against *Escherichia coli* and *Bacillus subtilis*) and anti-inflammatory activity were measured by the mouse ear edema test.

MATERIALS AND METHODS

Plant material. Plants were collected in Valle Escondido, Puebla, Mexico, and voucher specimens were deposited in the National Herbarium, Instituto de Biología, UNAM (MEXU).

Extracts preparation. Plants were divided into three parts: stems, leaves and inflorescences. Preparation of extracts was conducted at room temperature with hexane, ethyl acetate and methanol; the solvent was eliminated at a reduced pressure.

Phototoxic compounds. The polyacetylenes and thiophenes were obtained from the hexane extracts, and determined by TLC (Daniels, 1965). They were then confirmed by UV spectrums of the extracts with characteristic bands. Their bactericidal potential was evaluated after irradiating with UV-light (Table 1). Extracts (2 mg) were applied on silica gel Merck 60 F_{254} plates, using hexane-ethyl acetate (85:15) as the mobile phase; detection was carried out by using UV light (365 nm). The characteristic pale blue spots of the compounds appeared when they were developed with ceric sulphate.

Table 1. Detection of polyacetylenes and thiophenes by UV spectrum.

Tabla 1. *Detección de poliacetilenos y tiofenos utilizando el espectro de radiación ultravioleta.*

Hexane extract from *Bidens squarrosa*	Peaks (nm)	Abs (AU)
Leaves	246	4.000
	274	2.414
	354	0.791
Stems	245	7.000
	274	2.053
	346	0.752
Inflorescences	245	3.343
	275	2.080
	332	0.886

Absorption bands in the 200 and 300 nm regions are characteristic of polyacetylenes and thiophenes.

Las bandas de absorción en las regiones de 200 y 300 nm son características de poliacetilenos y tiofenos.

Phototoxic activity. The bactericidal activity of the hexane extracts the phototoxic compounds were extracted from, was measured against *Bacillus subtilis* (ATCC-6051). Two series of Petri dishes were run, one with UV-light irradiation (to activate the phototoxic compounds), and the

other under darkness (as control). The test was carried out by the Daniel's method (Daniels, 1965): Petri dishes with agar containing a bacterial concentration of 10^6 UFC, and 1, 2, 4 or 6 mg/ml extract/ml were used. Controls included the Mueller Hinton agar alone, and anhydrous ampicilline (0.02 mg) (Sigma) as a positive control. Each assay was repeated three times.

Biological activity. Anti-inflammatory and antibacterial potentials were measured on each extract. Anti-inflammatory potential was tested using the mouse ear edema test. Antibacterial activity was determined against *Bacillus subtilis* (ATCC-6051) and *Escherichia coli* (ATCC-6051). The bactericidal potential was tested by the paper disc diffusion method (Cavalieri, 2005) in Petri dishes with agar containing a bacterial concentration of 10^6 UFC. Two strains were evaluated: *Bacillus subtilis* (ATCC-6633) and *Escherichia coli* (ATCC-6051). Extracts of hexane, ethyl acetate and methanol were assayed at concentrations of 1, 2, 4 or 6 mg/ml. Petri dishes were incubated at 37 ºC for 24h. Controls included the Mueller Hinton agar without bacteria populations, and anhydrous ampicilline (0.02 mg) (Sigma) as a positive control. Each assay was repeated three times.

The anti-inflammatory activity of the 3 different extracts (hexane, ethyl acetate and methanol) was carried out by the mouse ear edema test induced with TPA (12-O-tetradecanoyl-phorbol-13-acetate) (De Young et al., 1989). For each determination, three male CDI mice (25 -30 g) were used; 10 μL of an ethanolic solution (0.25 mg/ml) of TPA (2.5 μg/ear) were applied to the surface of the right ear; the left ear was used as control. Ten minutes after the application of TPA, 20 μl of the different extracts (0.31 mg each dissolved in ethanol) were applied topically. After the fourth hour, the animals were sacrificed and a section (7 mm) of each of the ears was weighted. Greater weight of the right than the left ear indicated swelling produced by TPA. Percentage weight increase inhibition between ears was calculated. Indometacine (0.046, 0.085, 0.15 mg / ear) was used as the reference drug. Experiments were carried out to obtain (1) Edema A: edema induced by TPA alone, and (2) Edema B: edema induced by TPA plus extract sample. The percent inhibitory ratio was calculated as:

$$\text{Inhibitory ratio (\%)} = [(\text{Edema A} - \text{Edema B}) / \text{Edema A}] \times 100$$

Three mice were used on each treatment [1 Control (3 doses) + 3 extracts x 3 plant organs]. Experiments were repeated 3 times.

RESULTS AND DISCUSSION

Phototoxic activity. Tests were carried out only using the hexane extract, where the phototoxic compounds are soluble. Comparison of the three plant parts showed that stems were the most active at concentrations of 4 and 6 mg, showing an inhibition halo of 61.1% and 66.6% against *B. subtilis*, respectively. Leaves were less active at the same concentrations, showing a 5 and 5.5% of inhibition halo, respectively. Inflorescences were slightly less active than leaves, having an inhibition of 5% at the same concentrations (Table 2).

Table 2. Effect of UV - light irradiation on phototoxic bactericide activity of leaf, stem or inflorescences hexane extracts of *Bidens squarrosa* against *Bacillus subtilis*.
Tabla 2. *Efecto de la irradiación con luz ultravioleta (UV) en la actividad bactericida fototóxica de extractos conteniendo hexano obtenidos de hojas, tallos e inflorescencias de Bidens squarrosa sobre Bacilus subtilis.*

Inhibition halo (mm)						
Hexane Extract	Leaves		Stems		Inflorescences	
mg	Without UV	With UV	Without UV	With UV	Without UV	With UV
1	6.2 +/- 0.14	6.3 +/- 0.18	7.3 +/- 0.19	7.3 +/- 0.21	7.0 +/- 0.22	7.0 +/- 0.16
2	7.1 +/- 0.07	7.2 +/- 0.11	7.2 +/- 0.12	8.9 +/- 0.08**	8.0 +/- 0.06	8.1 +/- 0.06
4	7.1 +/- 0.17	9.1 +/- 0.15**	8.1 +/- 0.12	10.9 +/- 0.17**	7.8 +/- 0.08	9.1 +/- 0.1**
6	8.2 +/- 0.20	10.2 +/- 0.28**	10.3 +/- 0.10	12.2 +/- 0.09**	8.1 +/- 0.13	9.2 +/- 0.07**
Positive control (Ampicilline 0.02 mg)	18 +/- 0.06					

Microorganism concentration in plate: 10^6 UFC.
Values are the mean +/- 1 standard deviation of three replicates. Without UV= control.
Samples were analyzed between them by the Student's t - test (* $p \leq 0.05$; ** $p \leq 0.01$).

Concentración de microorganismos sobre la placa: 10^6 UFC.
Los valores son el promedio +/- 1 desviación estándar de tres repeticiones. Sin UV= Control.
Las muestras se analizaron entre sí utilizando la prueba de t de Student ($p \leq 0,05$; ** $p \leq 0,01$).*

Biological activity of the extracts. The antibacterial activity of the 3 plant parts was different in the three solvents, and against the 2 bacteria, *Escherichia coli* and *Bacillus subtilis*.

The assay with *E. coli* showed that the inflorescence was the most active plant part with the hexane extract. It showed a 71.43% inhibition of the growth halo with an hexane concentration of 4 or 6 mg; there was no activity with 1 or 2 mg hexane concentration. Diameter was 14 mm in the control against 10 mm with the inflorescence. Activity was almost the same with ethyl acetate, and it was lower with methanol (Table 3).

Bacillus subtilis showed a relatively lower activity with the hexane extract; the halo of inhibition was 5% with 2, 4 and 6 mg, and it was lower with 1 mg (Table 3). Diameters were 18 mm in the control and 0.9 mm in the inflorescences with 4 and 6 mg of the hexane extract. Activity of the extract was higher in the ethyl acetate; there were 50 and 44% of inhibition with 4 and 6 mg, respectively. There was no activity with 1 and 2 mg of the ethyl acetate extract. While diameter was 18 mm in the control, it was 9 or 8 mm with 4 or 6 mg of the ethyl acetate extract, respectively. Activity was similar with methanol (Table 3).

Stems showed a small activity with the hexane extract. Inhibition of the growth halo was 4.2% with 1, 2 and 6 mg, and 5% with 4 mg of that extract. There was no activity with the ethyl acetate extract at doses of 1, 2 or 4 mg, and at 6 mg it showed an inhibition of 44.4%. Methanol extract showed no activity with the first 3 concentrations, and inhibition was 35.7% when 6 mg were used (Table 3).

Hexane inhibition was small at the first 3 concentrations (1, 2 and 4 mg) in *B. subtilis*, but it was 71.43% when using 6 mg.. The first 2 (1, 2 mg) concentrations of the ethyl acetate extract were not active, and inhibition was 38.9 or 50% with 4 and 6 mg, respectively (Table 3). The first 3 concentrations were not active with the methanol extract, and it showed an inhibition of 47.2% when extract concentration was 6 mg (Table 3).

The leaf hexane extract with *E. coli* had a small activity with 4 and 6 mg; the inhibition halo was 5%. The first 2 concentrations (1 and 2 mg) were not active. Leaves were more active with the ethyl acetate extract. The inhibition halo was 78.5% with a concentration of 2 and 4 mg, and it was 85.7% with 6 mg. There was no activity using 1 mg extract. On the other hand, methanol extract from leaves showed low activity; there was no activity at concentrations of 1, 2 or 4 mg, and with 6 mg the inhibition halo was 53.5% (Table 3).

The hexane extract showed low activity in *B. subtilis*. The inhibition halo was 4.2 or 5% with 1 or 2 mg extract, respectively, and 6.4 or 7.1% with 4 or 6 mg concentrations, respectively. Ethyl acetate leaf extracts were

not active at concentrations of 1 and 2mg, and inhibition halo was 44.4% at 4 and 6 mg extract concentrations (Table 3). The methanol extract showed a slightly lower activity: there was no activity with 1 and 2 mg, and the inhibition halo was 38% with 4 and 6 mg extract.

Table 3. Antibacterial activity of leaves stems or inflorescences of *Bidens squarrosa* on *E. coli* and *B. subtilis* under different plant extract amounts.

Tabla 3. *Actividad antibacteriana de hojas, tallos o inflorescencias de Bidens squarrosa sobre E. coli y B subtilis utilizando diferentes cantidades de extractos vegetales.*

Extract	mg	Inhibition halo (mm)					
		Leaves		Stems		Inflorescences	
		E. coli	*B. subtilis*	*E. coli*	*B. subtilis*	*E. coli*	*B. subtilis*
Hexane	1	-	6.3 +/- 0.11	-	7.2 +/- 0.20	-	7.1 +/- 0.13
	2	-	7.2 +/- 0.05	-	7.1 +/- 0.10	-	8.2 +/- 0.21
	4	-	7.1 +/- 0.10	-	8.4 +/- 0.15	-	8.1 +/- 0.10
	6	-	8.1 +/- 0.13	-	10 +/- 0.03	-	8.0 +/- 0.15
Ethyl Acetate	1	-	-	-	-	-	-
	2	11.1 +/- 0.32	-	-	-	-	-
	4	11.5 +/- 0.5	8 +/- 0.06	-	7.3 +/- 0.11	10.4 +/- 0.36	9.3 +/- 0.26
	6	12.1 +/- 0.32	8 +/- 0.12	8 +/- 0.1	9.0 +/- 0.10	11.3 +/- 0.26	8.1 +/- 0.12
Methanol	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	4	-	7.4 +/- 0.13	-	-	7.5 +/- 0.13	8.2 +/- 0.10
	6	7.5 +/- 0.10	7.3 +/- 0.10	5 +/- 0.1	8.4 +/- 0.20	6.8 +/- 0.15	7.4 +/- 0.05
Ampicilline	0.02	14 +/- 0.05	18 +/- 0.06				

- : without activity.
Microorganism concentration in plate: 10^6 UFC.
Positive control: Anhydrous ampicilline (D[-]-α-Aminobenzylpenicillin)(Sigma).
Values are the zone of inhibition, including the diameter of the filter paper discs (5 mm). Each value is the mean +/- 1 standard deviation of three replicates. Samples were compared with the positive control (ampicilline) and analyzed by the Student's t - test. All results are significant at $p \leq 0.01$.

- : Sin actividad.
Concentración de microorganismos en placa: 10^6 UFC.
Los valores representan la zona de inhibición, incluyendo el diámetro de los discos de papel de filtro (5 mm). Cada valor es el promedio +/- 1 desviación estándar de 3 repeticiones. Las muestras se compararon con el control positivo (ampicilina) y se analizaron utilizando la prueba de t de Student. Todos los resultados son significativos a $p \leq 0,01$.

Results indicated that the bactericidal activity varied with the plant part, solvent or bacteria genus used.

Among plant parts, inflorescences or stems were the most active plant parts against *E. coli* or *B. subtilis*, respectively, using the hexane extract. With the ethyl acetate extract, leaves were more active against *E. coli* than against *B. subtilis*.

Leaves were the most active plant parts against *E. coli*, and stems were the most active against *B. subtilis* using the methanol extract.

Antiinflammatory activity. The stems were often the most active plant part on any used extract, followed by the leaves. Inflorescences showed comparatively a lower activity (Table 4).

Results obtained in this study validate the use of *Bidens squarrosa* as a medicinal plant.

Table 4. Antiinflammatory activity of *B. squarrosa* extracts [hexane (Hex), ethylacetate (AcOEt), methanol (MeOH)] coming from different plant organs (leaves, stems, inflorescences). Indometacine was used as a control.

Tabla 4. *Actidad antiinflamatoria de extractos de B. squarrosa [hexano (Hex), etilacetato (AcOEt), metanol (MeOH)] obtenidos de diferentes órganos vegetales (hojas, tallos, inflorescencias). La indometacina se utilizó como control.*

Topically administrated (TPA 2.5 mg /ear)						
	Edema (mg)			**% Inhibition of edema**		
	Hex	AcOEt	MeOH	**Hex**	**AcOEt**	**MeOH**
Leaves	9.23 +/- 0.32	9.83 +/- 0.24	12.04 +/- 0.52	**40.04****	**36.15***	**21.86***
Stems	6.87 +/- 0.55	10.1 +/- 0.78	11.7 +/- 0.22	**48.56****	**34.42***	**31.09***
Inflorescences	14.6 +/- 0.61	12.2 +/- 0.61	12.8 +/- 0.86	**-1.87**	**20.78***	**15.4**
Control (+)	Doses (mg / ear)					
Indometacine	0.046			**27 ± 4.70***		
	0.085			**50 ± 3.40***		
	0.150			**71 ± 0.62****		

Effect on TPA-induced mouse ear edema. Values are the mean of 3 replicates +/- 1 standard deviation. Results were analyzed using the Student's t - test, and values of * $p \leq 0.05$, ** $p \leq 0.01$ are considered significantly different from the control.

*Efecto sobre el edema de la oreja de ratón inducido por TPA. Los valores son el promedio de 3 repeticiones +/- 1 desviación estándar. Los resultados se analizaron usando la prueba de t de Student, y valores de * $p \leq$ 0,05, ** $p \leq$ 0,01 se consideran significativamente diferentes del control.*

REFERENCIAS

Cavalieri, S. J. (2005). Drug Resistance. Bacterial Laboratory Manuals. Departments of Laboratory Medicine and Microbiology. University of Washington. Seattle, Washington. 248 pp.

Christensen, L. P. & J. Lam (1990). Acetylenes and related compounds in Cynareae. *Phytochemistry* 29: 2753-2785.

Daniels, F.J. (1965). A simple microbiological method for demonstrating phototoxic compounds. *Journal of Investigative Dermatology* 44: 259-263.

De Young, L.M., J.B. Kheifets, S.J. Ballaron & J.M. Young (1989). Edema and cell infiltration in the phorbol ester-treated mouse ear are temporaly separate and can be differentially modulated by pharmacological agents. *Agents and Action* 26: 335-341.

Martínez, A.M., O.V. Evangelista, C.M. Mendoza, G.G. Morales, O.G. Toledo & L.A. Wong (2001). Catálogo de Plantas Útiles de la Sierra Norte de Puebla, México. Jardín Botánico, Instituto de Biología. UNAM. México D.F. 303 pp.