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Full Length Research Paper

Evaluation of the antimicrobial activity of *Acalypha monostachya* Cav. (Euphorbiales: Euphorbiaceae)

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***Acalypha monostachya* (Euphorbiaceae) is used by the inhabitants of San Rafael and Zapotitlan Salinas, Puebla, Mexico, to alleviate illnesses of bacterial and fungal origin. We analyzed the antimicrobial activity of *A. monostachya* and some of its chemical features. The extracts were obtained by percolation. The antibacterial and antifungal activities were examined through the disc-diffusion method. The general toxicity was carried out by using A*rtemia salina.* The quenching of free radicals by extracts was evaluated by the decolouration of a methanol solution of DPPH·. Total phenols were determined by Folin Ciocalteu reagent. Gram-positive and negative bacteria were sensitive to methanol and hexane extracts. The methanol extract had a bactericidal effect on *Vibrio cholera* Tor (MIC = 1000 µg/mL). The fungi strain most sensitive to this extract was *T. mentagrophytes* (LC_{50} = 430 µg/mL). The methanol extract showed inhibition of DPPH· radical (SC_{50} = 3.45 µg/ml), 43.4% of total phenolics and was toxic against *A. salina* (LC_{50} = 4.5 µg/mL). The methanol extract showed a variety of phenolic compounds, while hexane extract showed different fatty acids methyl ester. The bacterial, antifungal, antioxidant activities and a qualitative chemical characterization of *A. monostachya*'s extracts are reported here for the first time.**

Key words: *Acalypha monostachya*, antimicrobial activity, medicinal plants, chemical characterization, plant extracts analysis.

INTRODUCTION

Euphorbiaceae is one of the largest families of plants, with more than 200 genera and 7000 species (Standley and Steyermark, 1949). *Acalypha* is the fourth largest genus in the Euphorbiaceae with about 450 species (Webster, 1994). Several species of the genus *Acalypha* has been studied and it has been demonstrated that they present antioxidant, wound healing, post-coital antifertility, neutralization of venom, antibacterial, (Okanla et al., 1990; Shirwaikar et al., 2004; Perez apoptosis, antifungal and antitrypanosomal activities Gutierrez and Vargas, 2006; Marwah et al., 2007).

Acalypha monostachya Cav. (synonyms: *Acalypha hederacea* Torr.) is a perennial herb found from the Southwestern United States (Texas and New Mexico states) to Mexico (Coahuila, Nuevo León, Zacatecas, Tamaulipas, Guerrero, Oaxaca and Puebla states) (Rzedowski and Rzedowski, 1979). *A. monostachya* is a species found in Tehuacan-Cuicatlan Valley (Mexico) and is recognized as one of the most important medicinal plants used by the inhabitants of San Rafael and Zapotitlan Salinas, Puebla, Mexico to alleviate illnesses like skin eruptions, wound healing and diarrhea (Hernandez et al.,

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2003; Canales et al., 2005, 2006). The aim of this work is to investigate whether or not the extracts of *A. monostachya* show antimicrobial activity and analyze some of its chemical features.

MATERIALS AND METHODS

Plant materials

A. monostachya was obtained from San Rafael, Coxcatlan, Puebla in August 2008, with permission from the "Secretaria de Medio Ambiente y Recursos Naturales" (SGPA/DGVS/1266). Voucher specimens (HCM61) were deposited at National Herbarium of Mexico (MEXU) at Universidad Nacional Autonoma de Mexico and at the herbarium IZTA at Facultad de Estudios Superiores Iztacala.

San Rafael is a village in the municipality of Coxcatlan, located southeast of the Tehuacan-Cuicatlan Valley, at coordinates 18°12' and 18°14' North and 97°07' and 97°09' West, and 957 m above sea level. The climate is dry or arid with summer rains and a mean temperature of 22°C (Fernández, 1999).

Extracts preparation and general procedures

Air-dried aerial plants material without flowers and seeds (100 g) was ground into powder and extracted by percolation with solvents of different polarity (hexane and methanol) at room temperature. After filtration, the extracts were then evaporated to dryness under vacuum conditions. After solvent elimination, the hexane and methanol extract that was left was 1.96 and 11.14 g, respectively.

The methanol extract was analyzed using HPLC on a HP Series 1100 separations module Hewlett-Packard (Wilmington, DE, USA), equipped with a 1100 diode array detector (DAD), and operated with Instrument ChemStation A.09.03 [1417] software. Separations were carried out on an Allsphere ODS-1 column (250 x 4.6 mm, 5µm) with a flow rate of 1.0 mL/min. At the 5th min, a linear gradient was programmed to increase the flow rate at 1.5 mL/min in 2.5 min. The sample volume injected was 20 µL and the data were analyzed at 220 to 400 nm. The standard compounds were catechin (Rt = 3.57 min), gallic acid (Rt = 3.03 min), catechol (Rt = 4.32 min) and quercetin (Rt = 8.44 min). The mobile phase for the analysis consisted of a methanol:acetic acid:water 30:5:65 system, and was developed in isocratic way.

The hexane extract was analyzed in an AGILENT 6850 (China) gas chromatograph equipped with a HP-5MS (USA) column (30 m x 0.25 mm i.d., film thickness, 0.25 µm). The temperature of the column was programmed starting at 70°C for 2 min, and then the temperature was increased with 8°C/min until it got to 270°C. At 270°C, a linear gradient is programmed to increase the temperature with 10°C/min until it gets to 290°C. The injector and detector temperatures were 250 and 290°C, respectively. The gas carrier was helium at a flowrate of 0.9 mL/min. Peak areas were measured by electronic integration. The relative amount of the individual components was based on the peak areas. GC-MS analysis was performed on an AGILENT 5975C (China) mass spectra. Mass spectra were recorded at 70 eV. The hexane extracts components were identified by comparison of their retention indices and mass spectra with the NIST/EPA/NIH Mass Spectral Library.

Microorganisms

The following strains of bacteria were used: *Vibrio cholerae* INDRE 206 (isolated from polluted water), *Vibrio cholerae* (a clinical isolate corresponding with group 01, producing enterotoxin, serotype "Inaba", biotype "El Tor"), *Vibrio cholera* CDC V 12, *Vibrio cholerae* Serotype No 01 ATCC 35971, *Escherichia coli* ATCC 25922, *Enterobacter agglomerans* ATCC 27155, *Salmonella typhi* ATCC 19430, *Staphylococcus aureus* ATCC 12398, *Enterobacter aerogenes, Staphylococcus epidermidis, Bacillus subtilis* and *Sarcina lutea* (donated by the Laboratory of Microbiology of FES-Cuatitlan UNAM), and *Yersinia enterocolitica* (donated by the Clinical Analysis Laboratory of University Hospital Campus Iztacala).

The fungi strains used include: *Fusarium sporotrichum* (ATCC NRLL3299), *Fusarium moniliforme* (CDBB-H-265), *Trichophyton mentagrophytes* (CDBB-H-1112), *Aspergillus niger* (CDBB-H-179), *Rhizoctonia lilacina* (CDBB-H-306) and *Rhizoctonia solani* (donated by INIFAP, Celaya, Mexico).

Antibacterial activity

The antibacterial activity was determined by disc-diffusion method (Vanden Berghe and Vlietinck, 1991). The microorganisms were grown overnight at 37°C in 10 mL of Müeller Hinton broth (Bioxon 260-1, Estado de Mexico, Mexico). The cultures were adjusted to turbidity comparable to that of Mc Farland no. 0.5 standard with sterile saline solution. Petri dishes containing Müeller Hinton agar (Bioxon, Edo. de Mexico, Mexico) were impregnated with these microbial suspensions. Concentrations of 200 mg/mL of each extract were prepared, and discs (Whatman no. 5) of 5 mm diameter were impregnated with 10 µL of each one (final doses per disc: 2 mg of hexane and methanol partitions). Discs impregnated with 10 µL of hexane and methanol, were used as negative controls, while discs of chloramphenicol (25 µg) were used as positive controls. The plates were incubated overnight at 37°C and the diameter of any resulting inhibition zones (mm) was measured.

Each experiment was repeated at least three times. The estimate of the minimal inhibitory concentration (MIC) was carried out by the broth dilution method (Vanden Berghe and Vlietinck, 1991). Dilutions of plant extracts from 2.0 to 0.125 mg/mL were used. The tubes were inoculated with microorganism suspension of 10^5 CFU/mL. MIC values were defined as the lowest extract concentration that prevents visible bacterial growth after 24 h of incubation at 36°C. Chloramphenicol was used as a reference, and appropriate controls with no extract and solvent were used. Each experiment was repeated at last three times.

The bactericidal kinetic assay was performed using appropriate concentrations of extract (corresponding to ½ MIC, MIC and MBC) (Lennette et al., 1987).

Antifungal activity

The assay of antifungal activity was carried out in Czapek Dox agar (30 mL) (Bioxon, Edo. de Mexico, Mexico) in Petri dishes measuring 80 mm x 10 mm. After the mycelial colony developed, discs impregnated with 2 mg of hexane extract were placed at a distance of 10 mm away from the Petri dishes inside limit. Then, they were incubated at 23°C for 72 h until mycelial growth had enveloped discs containing the control and had formed crescents of inhibition around discs containing samples with antifungal activity (Ye et al., 1999). However, Ketoconazol (7 µg/disk) was used as a positive control.

For quantitative assays, a culture plate of 24 wells was used. Seven dilutions of plant extracts from 400, 500, 600, 700, 800, 900 and 1000 µg/mL were added to Czapek Dox agar (5 mL/dilution) at 45°C. These dilutions were mixed rapidly and poured into three wells of a culture plate. After 24 h, a small amount (1 x 1 mm) of mycelia was inoculated equally in each well. DMSO (200 µL in 5 mL of agar) was used as a negative control, while Ketoconazole was used as a positive control. After incubation at 23°C for 48 h, the area of the mycelia colony was measured, and the inhibition of

Table 1. Antibacterial activity of *A. monostachya*.

Bacteria	Inhibition halos (mm)		MIC (µg/mL)	
	Hexane extract	Methanol extract	Methanol extract	Positive control chloramphenicol
S. l	na	8.3 ± 2.08	1500	1.0
B. s	na	7.5 ± 2.12	2000	2.0
S. a	7.0 ± 0	9.0 ± 2	1500	1.0
S. e	7.0 ± 0	8.6 ± 0.57	1500	2.0
Tor	11.6 ± 0.57	6.6 ± 0.57	1000	1.0
No. 01	10.6 ± 1.15	9.6 ± 1.52	2000	1.0
p. w.	7.3 ± 0.57	7.0 ± 0	1500	1.0
Clinical	11.6 ± 1.52	7.0 ± 0	2000	1.0
S. t	7.0 ± 0	na	na	2.0

S. l: *S. lutea*; B. s: *B. subtilis*; S.a: *S. aureus*; S. e: *S. epidermidis*; Tor: *V. cholerae* CDC V 12; No. 01: *V. cholerae* No 01; p. w.: *V. cholerae* INDRE 206; Clinical: *V. cholerae* which is a clinical isolate; S. t: *S. thyphi*; na: no activity. MICs values of hexane extract, in all strains of bacteria, were above 2000 µg/mL.

fungal growth and IC_{50} was determined by the following formula:

$I (\%) = d_c – d_t / d_c \times 100$

where

d_c: diameter of the colony of the control culture.
d_t: diameter of the colony of the treated culture.

The IC_{50} values were calculated by rectangular hyperbola regression of plots, where the abscissa represented the concentration of the tested plant extract and the ordinate represented the average percent of inhibition of fungal growth from three replicates.

General toxicity assay

The general toxicity *in vitro* brine shrimp lethality test was carried out by using brine shrimp *A. salina* (Leach) larvae, according to the methodology described by McLaughlin (1991). Each plant extract was tested at 1000, 100 and 10 ppm and also evaluated in triplicate. 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. As positive control, gallic acid was used (LC_{50} = 321.5 µg/mL); and as a negative control, DMSO was used. Survivors were counted after 24 h and LC_{50} was determined from the 24-h counts. The general toxicity 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 it was designated as strong when the LC_{50} ranged from 0 to 100 µg/mL (Padmaja et al., 2002).

DPPH assay

The quenching of free radicals by extracts was evaluated spectrophotometrically at 517 nm by the decolouration of a methanol solution of DPPH· (Murillo, 2006).

A freshly prepared DPPH· solution (4 mg/100 mL methanol) was used for the assays. Samples were dissolved in methanol (1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 µg/mL). However, 50 µL of each sample and 150 µL of DPPH· solution were placed in ELISA plate. The degree of decolouration indicates the free radical-scavenging efficiency of the samples. Quercetin was used as a reference free radical scavenger (SC_{50} = 4.6 µg/mL). The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH, was calculated according to the formula:

Inhibition percentage (Ip) = $[(A_B – A_A)/ A_B)] \times 100$

Where A_A is the absorbance of the sample and A_B is the absorbance of the control (Yen and Duh, 1994).

The SC_{50} was calculated when allowed according to the scavenging efficiency. The SC_{50} values were calculated by rectangular hyperbola regression of plots, where the abscissa represented the concentration of the tested plant extract, and the ordinate represented the average percent of scavenging capacity from three replicates.

Determination of total phenolics

Total phenols were determined by Folin Ciocalteu reagent (Singleton 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. The standard curve was prepared using 0.00625, 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/L solutions of gallic acid in water. However, total phenol values are expressed in terms of gallic acid equivalent.

Statistical analysis

All experiments were performed in triplicate. The mean and standard deviation of three experiments were determined. The IC_{50} and LC_{50} values were calculated by rectangular hyperbola model.

RESULTS

The results obtained in the evaluation of the antibacterial activity of the hexane and methanol extracts of *A. monostachya* are shown in Table 1. The hexane extract exhibited inhibitory effects against two gram-positive

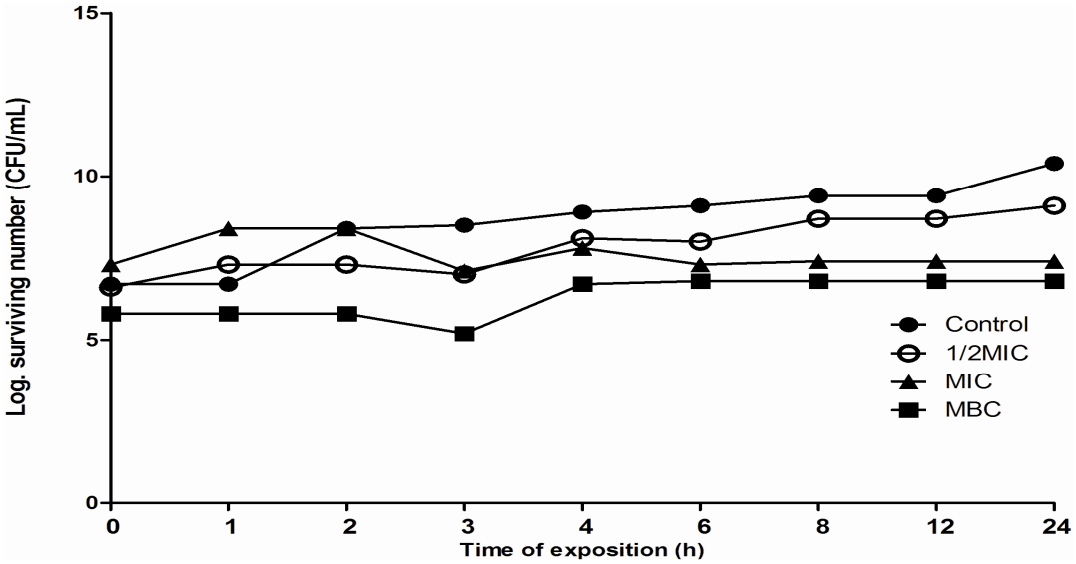

Figure 1. Survival curve of *Sarcina lutea* exposed to methanol extract of *Acalypha monostachya*. The methanol extract was added to each experimental culture in zero time. The concentrations used were: 750 µg/mL (1/2 MIC), 1500 µg/mL (MIC) and 2000 µmg/mL (MBC); moreover, the control tube did not contain methanol extract.

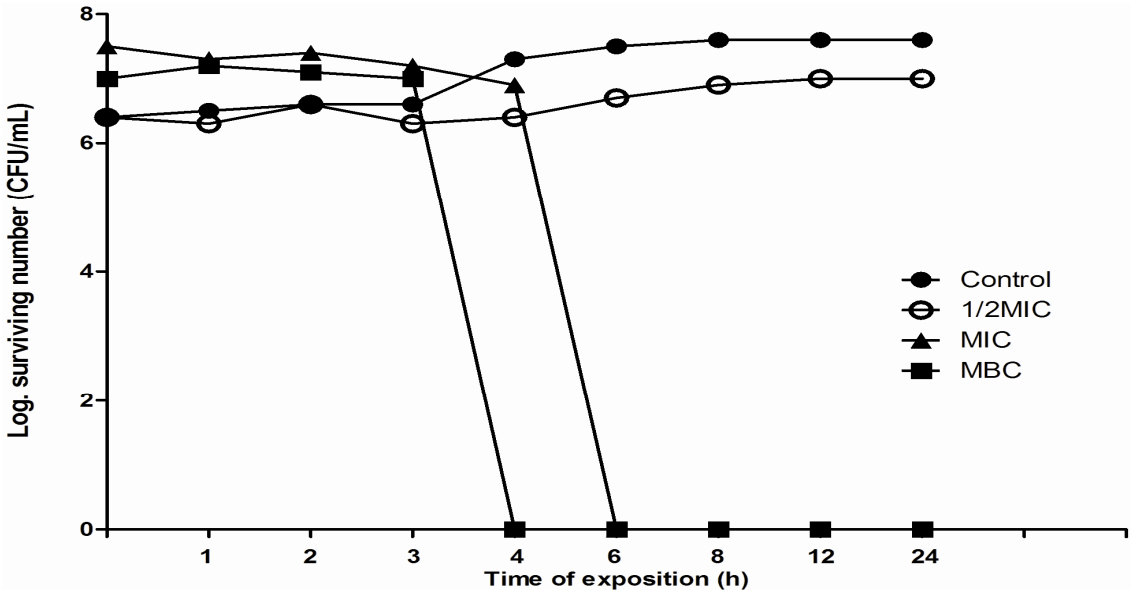

Figure 2. Survival curve of *V. cholerae* Tor exposed to methanol extract of *Acalypha monostachya*. The methanol extract was added to each experimental culture in zero time. The concentrations used were: 500 µg/mL (1/2 MIC), 1000 µg/mL (MIC) and 1500 µg/mL (MBC); moreover, the control tube did not contain methanol extract.

bacteria (*S. aureus* and *S. epidermidis*) and five gram-negative bacteria (four strains of *V. cholerae* and *S. typhi*); nevertheless, the MIC values were above 2.0 mg/mL. The methanol extract was active against all gram-positive bacteria and the four gram-negative bacteria (four strains of *V. cholera)*. As such, this extract showed the lowest MIC in *V. cholerae* Tor (MIC = 1000 µg/mL).

Figures 1 and 2 show the effect of methanol extract (in the survival curve) on gram-positive bacteria (*Sarcina lutea*) and gram-negative bacteria *(Vibrio cholerae* Torr.). Minimum inhibitory concentrations (MIC = 1500 µg/Ml)

Table 2. Antifungal IC_{50} of the methanol extract of *A. monostachya*.

Fungal strains	LC_{50} methanol extract (µg/mL)	Ketoconazole (µg/mL)
Trychophyton mentagrophytes	430	1.17
Fusarium moniliforme	1200	7.55
Fusarium sporotrichum	8000	3.90
Rhizoctonia lilacina	5800	21.56
Rhizoctonia solani	7600	19.67

Table 3. Chromatographic and spectral characteristics of phenolic compounds detected in the methanol extract of *A. monostachya* by HPLC-DAD.

Peak number	Retention time (min)	Max. (nm)	Phenolic compound assignment
1	2.6	238, 274	Benzoic acid derivative
2	4.5	236, 276, 360	Flavone derivative
3	5.0	238, 386, 330	Flavanone derivative
4	6.5	238, 274, 360	Flavone derivative
5	7.8	238, 276, 330	Flavone derivative
6	8.3	238, 284, 330	Flavanone derivative
7	13.0	238, 266, 358	Flavonol derivative
8	15.6	254, 366	Flavonol derivative

Table 4. Composition of the hexane extract of *A. monostachya*.

Compound	RT (min)	Composition (%)
Octadecanal	19.56	4.21
Palmitic acid methyl ester	20.10	6.90
Linoleic acid methyl ester	22.15	3.93
Linolenic acid methyl ester	22.23	8.21
Phytol	22.37	9.25
Eicos-9-ene-1,20-diacetate	29.11	55.56
Vitamin E	32.87	11.94

and minimum bactericidal concentrations (MBC = 2000 µg/mL) had a bacteriostatic effect on the bacterial population of *S. lutea*; while the MIC (1000 µg/mL) and MBC (1500 µmg/mL) had a lethal effect on *Vibrio cholera* Tor at 6 and 4 h, respectively.

The hexane extract did not show antifungal activity. The results of the antifungal activity of methanol extract are shown in Table 2. The methanol extract did not show antifungal activity against *A. niger*. With regards to *T. mentagrophytes*, *F. moniliforme*, *F. sporotrichum*, *R. lilacina* and *R. solani* inhibition by methanol extract was evident. With respect to IC_{50}, the methanol extract showed the lowest value in *T. mentagrophytes* (430 µg/mL). As a consequence, the methanol extract was toxic against *Artemia salina* (LC_{50} = 4.5 µg/mL).

According to the HPLC analysis, the methanol extract presented a variety of different phenolic compounds, such as: benzoic acid, flavones, flavanone and flavonol. This extract did not present any of the standards (Table 3). Actually, HPLC with multiple-wave length or diode-array UV detection is a fully satisfactory tool in studies dealing with: screening, quantification of the main aglycones and/or a provisional sub-group classification. The DAD UV spectra show the wide differing spectral characteristics of various flavonoid subclasses (Rijke et al., 2006). The hexane extract presented two unsaturated fatty acids methyl ester (Table 4 and Figure 3), while the methanol extract presented quite a potent antioxidant activity, efficiently scavenging the DPPH· free radical with a SC_{50} value of 3.45 µg/ml. The methanol extract of *A. monostachya* showed 43.4% of total phenolics as gallic acid equivalents. However, it is extremely important to point out that there was a positive correlation between antioxidant activity and phenolic content.

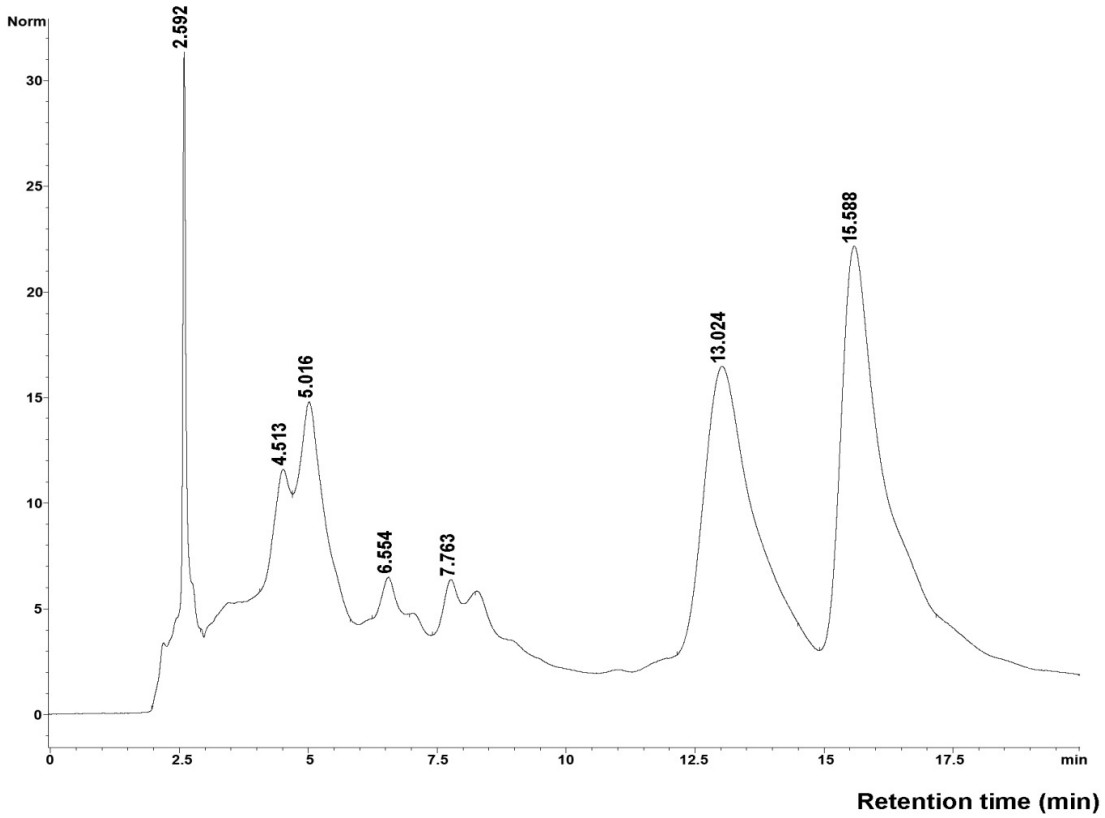

Figure 3. HPLC of *A. monostachya*'s methanol extract. Mobile phase - methanol:acetic acid:water 30:5:65.

DISCUSSION AND CONCLUSION

In this study, the antibacterial, antifungal, antioxidant and toxicity of *A. monostachya* was evaluated.

The hexanic and methanol extracts showed antibacterial activity, while the methanol extract was more active than hexane extract (Table 1). The effect of the methanol extract on the bacterial population of *S. lutea* was bacteriostatic in all concentrations tested; nevertheless, a bactericidal effect was observed on the bacterial population of *V. cholerae* Tor (MBC at 4 h; MIC at 6 h). In other species of the genus *Acalypha* (*A. guatemalensis, A. wilkesiana, A. torta, A. siamensis* and *A. indica*), it has been observed that extracts of high polarity showed antibacterial activity (Alade and Irobi, 1993; Caceres et al., 1993, 1998; Perumal et al., 1999; Wiart et al., 2004).

Interestingly, only the methanol extract showed antifungal activity and the fungi strain was more sensitive to *T. methagrophytes* (IC_{50} = 430 µg/mL) (Table 2). The antifungal activity has been observed in other species of this genus (*A. wilkesiana*, *A. hispida*) (Alade and Irobi, 1993; Ejechi and Souzey, 1999). With respect to toxicity, methanol extract of *A. monostachya* was more toxic (CL_{50} = 4.5 µg/mL) (Padmaja et al., 2002). The dichloromethane, ethanol and water extracts of *A. guatemalensis* were not toxic (CL_{50} >1000 µg/mL), nevertheless the water and ethanol extracts of *A. wilkesiana*'s seeds were cytotoxic even at 1 µg/mL (Bussing et al., 1999).

The GC-MS showed the composition of the hexane extract of these compounds as two unsaturated fatty acids methyl ester (linoleic and linolenic acid). The antibacterial activity, shown by this extract, can be attributed to the presence of the unsaturated fatty acids methyl ester. The antibacterial activity of long-chain unsaturated fatty acids have been well know, in that the MIC of linoleic acid (0.2 mM), linolenic acid (0.4 mM) and linolenic acid methyl ester (>2 mM) were reported for *S. aureus*. It was demonstrated that the antibacterial action of unsaturated fatty acids was principally mediated by inhibition of FabI, which is an essential component of bacterial fatty acids synthesis; while methyl ester derivatives were less active than the corresponding acids (Zheng et al., 2005).

The HPLC chromatogram obtained at 220 and 400 nm for methanol extract showed a variety of different phenolic compounds, such as: benzoic acid, flavone, flavanone and flavonol. Phenol compounds including: phenolic acids, flavones and flavonols have been isolated in others species of genus *Acalypha* and are the main inhibitory substances used as the antibacterial, antifungal

and antioxidant agents (Alade and Irobi, 1993; Cowan, 1999; Ejechi and Souzey, 1999; Shirwaikar et al., 2004).

The methanol extract of *A. monostachya* showed a pronounced antioxidant activity (SC_{50} = 3.45 µg/mL) which contained higher amounts of total phenolics (43.4% as gallic acid equivalents). Such a radical scavenging action contributes to prevent deleterious effects from reactive oxygen species generated by the inflammatory conditions (Halliwell and Gutteridge, 1999; Singh et al., 2006), and most probably plays a part in the wound healing properties of *A. monostachya.* However, the antioxidant capacity and wound healing activity had been studied in other species of genus *Acalypha* [*A. indica* (SC_{50} = 37.9 µg/mL and 28% as gallic acid equivalents and the ethanolic extract showed wound healing activity) and *A. langiana* (topical application of aqueous extract from leaves decreased the wounds area by 9 to 88% and 8 to 82% in excision and incision wound, respectively)] (Reddy et al., 2002; Perez Gutierrez and Vargas, 2006; Marwah et al., 2007).

This study showed that *A. monostachya* has different compounds with antibacterial, antifungal and antioxidant activities, and it is the first time that these activities are reported. This species has been traditionally implemented in the treatment of wounds, hence if a plant extract is having antioxidant potentials and antimicrobial activity, it can be a good therapeutic agent for accelerating the wound healing process.

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