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EVALUATION OF ANTICANCER POTENTIAL OF EIGHT VEGETAL SPECIES FROM THE STATE OF OAXACA

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Abstract

Background: Eight plant species from Oaxaca, some of them used in traditional medicine, were subjected to screening of several biological activities to provide data regarding their anticancer potential, although no scientific information is available about their pharmacological effects.
Materials and methods: Methanol extracts from stems or roots of the eight plants were tested for antioxidant activity by the DPPH· method. Antimicrobial activity was determined using the agar diffusion method and the minimal inhibitory concentration (MIC) was obtained by broth dilution method. Antitopoisomerase activity was assessed using mutant strains of *Saccharomyces cerevisiae* JN362a, JN394, JN394t_{-1}, JN394t_{2-4} and JN394t_{2-5}. The mutagenic activity was evaluated using the Ames test (*Salmonella typhimurium* TA1535).
Results: No extract showed significant antioxidant activity. The best antimicrobial activity was observed for *Salpianthus arenarius* (MIC 56.25 µg/mL) and *Lantana achyranthifolia* (MIC 78.12 µg/mL) against *Staphylococcus aureus*. Extracts of *Acalypha cuspidata, Alloispermum integrifolium* and *L. achyranthifolia* stems showed antitopoisomerase II activity with JN394$_{t-1}$ growth of -30.88±0.0%, -38.11±4.95%, and -70.97±12.02% respectively. *Galium mexicanum* stem extract showed antitopoisomerase I activity with growth of 35.31±6.36% on the same mutant strain. All plant extracts were non-mutagenic. Fractionation of *A. cuspidata* extract led to identification of two subfractions with antitopoisomerase I and II activity at 154µg/mL (Positive controls 50 and 100µg/mL).
Conclusion: Methanol extracts of *A. cuspidata, A. integrifolium, G. mexicanum,* and *L. achyranthifolia* stems showed antitopoisomerase and non-mutagenic activities, and consequently could be promising as a source of anticancer drugs.

Key words: Antitopoisomerase activity, mutagenic activity, vegetal extracts, *Acalypha cuspidata.*

Introduction

In the search for bioactive compounds from vegetal species, the value of ethno-medicine and traditional pharmacology is recognized to increase the probability of finding new molecules with useful biological activities in the treatment of diseases or disorders, such as cancer, with important economic and social impact. Established anticancer drugs from plants have had significant influence on the study of anticancer molecular mechanisms and have provided new structural models for future drugs to treat or prevent cancer (Khazir et al., 2014). The research to discover these bioactive compounds is based on several biological tests. *In vitro* bioassays were used to evaluate the anticancer activity of a compound. These assays examined antimicrobial activity, which allows the identification of novel agents capable of interfering with a specific molecular target. These may avoid the shortcomings of conventional chemotherapy because certain antimicrobials exhibit selective cytotoxicity against a broad spectrum of human cancer cells (Schweizer, 2009). Assays to determine the topoisomerases inhibition mechanism were done. This mechanism is considered an attractive targeting strategy in both chemotherapy and chemoprevention because antitopoisomerase agents showed potential to inhibit carcinogenesis via antiproliferative or cell-differentiating action (Cho et al, 2000). Antioxidant activity was investigated because potent scavengers of Reactive Oxygen Species (ROS) may serve as a possible preventive intervention for free radical-mediated diseases such as cancer (Ralph et al., 2010).

In Mexico, ethnomedicine has led to knowledge of some useful plants for the treatment of cancer (Alonso-Castro et al., 2011), however there is a lack of scientific evidence about biological activity of most of them. Therefore, the aim of the present study is to evaluate the anticancer potential of eight vegetal species from Oaxaca, belonging to six botanic families (Asteraceae, Euphorbiaceae, Rubiaceae, Verbenaceae, Nictaginaceae and Boraginaceae), which are used in traditional medicine (Table 1), by screening the antioxidant, antimicrobial, and antitopoisomerase activities of methanol extracts of stems or roots of each specie. Additionally, two antitopoisomerase fractions were obtained from an active extract. Mutagenic evaluation using *Salmonella thyphimurium* TA1535 strain was done to investigate the possible toxic effects of vegetal extracts in addition to their medicinal properties (Déciga-Campos et al., 2007).

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Materials and Methods

Biological material

Dr. John Nitiss of St. Jude Children's Research Hospital, Memphis, Tennessee, kindly provided *Saccharomyces cerevisiae* mutant cells JN362a, JN394, JN394t-$_1$ JN394t$_{2-4}$ and JN394t$_{2-5}$. Standard microorganisms were purchased from American Type Culture Collection (ATCC, Manassas, VA) and from National Collection of microbial and cell cultures of CINVESTAV-IPN (CDBB, México, D.F.). These included *Salmonella thyphimurium* TA1535, a histidine-mutant bacterial strain obtained from ATCC and used in antimutagenicity experiments, and Gram negative bacteria *Klebsiella pneumoniae* (CDBB-B-1024), *Escherichia coli* (CDBB-B-1000), *Proteus mirabilis* (ATCC12453) and *Proteus vulgaris* (ATCC49132). Gram positive bacteria; *Staphylococcus aureus* (CDBB-B-1005) and *Staphylococcus epidermidis* (CDBB-B-1012) and the fungus *Candida albicans* (CDBB-L-1003) were also purchased for use in this work.

Chemicals

All extracting and column chromatography (CC) reagents were reagent grade. Merck silica gel (100-200 mesh ASTM) was used for CC chromatography. TLC was performed on precoated silica gel aluminum sheets (silica gel 60 F254, 0.20 mm, Merck). Fractions were monitored by UV (254 nm), and by a ceric sulfate reagent followed by heating. Peptone bacto, yeast extract, agar bacto, Mueller Hinton (MH) agar, MH broth, trypticase soy agar, Luria broth (LB), trypticase soy broth, sabouraud dextrose agar, sabouraud dextrose broth, minimal glucose agar (MGA) and dextrose were purchased from Difco (Sparks, MD). Methanol (HPLC grade), methanol (spectrophotometric grade), camptothecin (CPT), etoposide (ETP), dimethyl sulfoxide (DMSO-Hybri-Max), adenine hemisulfate salt, chloranphenicol, nystatin, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), benzo [a] pyrene (B[a]P), and sodium azide (NaN_3) were obtained from Sigma Chemical (St. Louis, MO).

Collection of plant material

All vegetal species were collected in the State of Oaxaca, México. *Acalypha subviscida* S. Watson var. *lovelanddii* McVaugh, *Alloispermum integrifolium* (DC.) H. Rob., and *Galium mexicanum* H.B.K. were collected in August 2012 in San Miguel Suchixtepec, Miahuatlán. *Acalypha cuspidata* Jacq. (Euphorbiaceae) was collected in July 2011 in Candelaria Loxicha, San Pedro Pochutla. *Adenophyllum aurantium* (L.) Strother, *Salpianthus arenarius* Bonpl. (Nyctaginaceae) and *Tournefortia densiflora* M. Martens & Galeotti (Boraginaceae) were collected from the Chepilme Botanic Garden of the Universidad del Mar (UMAR), San Pedro Pochutla in July 2012. *Lantana achyranthifolia* Desf. (Verbenaceae) was collected in October 2010 in Huajuapan de León (Table 1). The vegetal material was identified at the genus and specie level by Gerardo Salazar Chávez Ph.D. from the Biology Institute of the Universidad Autónoma de México (UNAM), M. C. Ernestilla Cedillo Portugal and M. C. Lilian López Chávez from Universidad Autónoma de Chapingo.

Plant samples were air-dried at room temperature without exposure to sunlight. The dry material was chopped into pieces and stored in a cool dry place until it was processed.

Preparation of extracts

Organic extracts were prepared with 200 g of the stem (S), bulb (B) or root (R) of selected plants. Vegetal material was macerated with 1000 ml of methanol for two weeks at room temperature (25^{o}C). The extracts were filtered using Whatman No 1 filter paper and the vegetal residue was further macerated twice with the same amount of solvent for one week more and filtered. The filtrates obtained from each extraction were mixed and concentrated until dry, under vacuum. All extracts were kept at 4^{o}C for further use (Table 1). Prior to biological evaluation, each extract was dissolved in a minimum amount of dimetylsulfoxide (DMSO) to produce solutions free of solids. Concentration of each treatment for all tests, except mutagenicity, was based on the solubility factor of each solid extract in DMSO. These concentrations were 100 mg/ml for *A. aurantium, A. aurantium (*R), *A. subviscida, A. integrifolium, L. achyranthifolia* and *T. densiflora*; 50 mg/mL for *A. cuspidata, G. mexicanum*; 67 mg/mL for *S. arenarius* and 70 mg/ml for *T. densiflora* (R).

Fractionation of *A. cuspidata* extract

Methanol extract (13.0g) was subjected to silica gel column using hexane-ethyl acetate (Hx/AcOEt) mixtures of increasing polarity. All 287 fractions obtained were analyzed over TLC (SiO_2, Hx: AcOEt) and grouped according to their chromatographic profile. This process resulted in 21 groups: AaA1 (7.37%). AaA2 (1.44%), AaA3 (1.08%), AaA4 (5.52%), AaA5 (1.41%), AaA6 (1.15%), AaA7 (0.58%), AaA8 (1.69%), AaA9 (0.54%), AaA10 (1.27%), AaA11 (1.13%), AaA12 (1.54%), AaA13 (0.68%), AaA14 (0.70%), AaA15 (2.13%), AaA16 (2.59%), AaA17 (5.09%), AaA18 (1.17%), AaA19 (3.41%), AaA20 (11.17%), and AaA21 (7.98%). AaA8 (200mg) was the only active fraction, so it was subjected to CC on reverse phase (C18) using methanol/water 1:1. Thirty-four eluates of 10 mL were collected and grouped in six sub-fractions: AaA8a (5.4%), AaA8b (28.5%), AaA8c (16.5%), AaA8d (17.2%), AaA8e (5.6%), AaA8f (1.9%). All fractions and sub-fractions were evaluated for antitopoisomerase activity.

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Antioxidant activity of the extracts

The antioxidant activity of extracts was assessed based on the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical. A methanolic (spectrophotometric grade) solution (5µl) of the herbal extract at five different concentrations was added to 195 µl of DPPH• solution (7.6 x 10^{-5} M in methanol). When the reaction reached the steady state (in the absence of light), absorbance was measured at 515 nm in a microplate reader (Benchmark plus BIO-RAD) (Siddhuraju and Becker, 2003). The DPPH• concentration in the medium was calculated from the following calibration curve:
A_{515nm} = 0.0072 $[DPPH•]_T$ – 0.0097, where $[DPPH•]_T$ was expressed as µM and correlation coefficient (r^2) = 0.999.

The percentage of remaining DPPH• (% $DPPH•_{REM}$) at the steady state was calculated as follows:

$$\% DPPH•_{REM} = \{[DPPH•]_T / [DPPH•]_{T=0}\} \times 100$$

Where $[DPPH•]_T$ was the concentration of DPPH• at the time of steady state and $[DPPH•]_{T=0}$ was the concentration of DPPH• at zero time. The $\% DPPH•_{REM}$ against the standard concentration was plotted to obtain the amount of sample necessary to decrease the initial DPPH• concentration ($EC_{50,}$ g/kg $_{DPPH}$) by 50%. The time needed to reach the steady state to EC_{50} concentration (T_{EC50}) was calculated graphically and the Antiradical Efficiency (AE), which represents Kg DPPH/g sample, was calculated by the equation: $AE=1/(EC_{50}T_{EC50})$ (Sánchez-Moreno et al. 1998). Ascorbic acid was used as a reference standard. All experiments were carried out in triplicate. The activity of each extract was expressed as a percentage of that achieved for the ascorbic acid.

Antimicrobial screening

The test microorganisms used in this study included six bacteria species, four Gram negative (*K. pneumoniae, E. coli*, *P. mirabilis*, and *P. vulgaris*), two Gram positive (*S. aureus, S. epidermidis*) and the fungus *C. albicans*. Antimicrobial activity was tested using the agar diffusion method using a cell suspension of approximately 1.5×10^6 CFU/ml (colony forming units per milliliter) obtained using a 0.5 McFarland turbidity standard. The bacterial strains were inoculated in plates of trypticase soy agar and the fungus in plates of sabouraud dextrose agar.
For susceptibility testing, each 150 µl of adjusted bacterial or fungal suspension was spread on a sterile medium (trypticase soy agar or sabouraud dextrose agar) using sterile swabs. The positive controls employed were chloranphenicol (30 µg) and nystatin (100 units) for antibacterial and antifungal assays respectively. Application of the samples and controls (25 µl) was done directly in the solid medium. The application point was marked on the lower surface of the Petri dish. The preparations were left to diffuse. Subsequently the plates were incubated at 37°C for 24 h in the case of the bacteria; while the fungus was cultured at 30°C for 48 h. Plates were prepared using the same procedures without extract or positive control, but with DMSO (25 µl) and were equally set as negative controls. After incubation, the growth inhibition rings were quantified by measuring the diameter for the zone of inhibition (I.D.) in millimeters from the lower surface of the plates. All assays were carried out in triplicate.

Determination of minimal inhibitory concentration (MIC)

The Greenwood method (Greenwood, 1989) was used to determine the MIC of all eight extracts against each test organism. Each extract (50 µl) was mixed with 950 µl of sterile water to get an X concentration. Eight sterile test tubes were arranged in a test tube rack and 1 ml of sterile water was pipetted into each test tube. Thereafter, there was a twofold serial dilution of the extract to obtain 1 ml of each one of the following concentrations: X/2, X/4, X/8, X/16, X/32, X/64, X/128 and X/256. Each test organism (1 ml = 5 x 10^5 CFU/ml) was pipetted into each of the test tubes containing the extract. The final concentrations were: X/4, X/8, X/16, X/32, X/64, X/128, X/256 and X/512. Finally, the tubes were incubated at 37°C for 24 h in the case of the bacteria, and the fungus was cultured at 30°C for 48 h. The MIC was recorded as the least concentration of extract that completely inhibited the growth of the test organism.

Yeast antitopoisomerase assay

The antitopoisomerase activity was assessed using mutant strains *Saccharomyces cerevisiae* JN362a, JN394, JN394 t_{-1}, JN394t_{2-4} and JN394t_{2-5} (Nitiss and Nitiss, 2001). Yeast cells were grown in YPDA media at 30°C for 18 hours in a shaking incubator. The logarithmically growing cells were then counted using a hematocytometer and adjusted to a concentration of $2x10^6$ cells/ml. Yeast cells ($6x10^6$ cells) were incubated at 30°C (JN362a, JN394, JN394t_{2-4}, JN394 t_{-1}) or 25°C (JN394t_{2-4} and JN394t_{2-5}) for 24 hours in the shaking incubator, with each extract dissolved in 50 µL DMSO. DMSO (1.66%) was used as the negative control. CPT (50 µg/ml), a topoisomerase I inhibitor; ETP (100 µg/ml), a topoisomerase II poison and SBZ (150 µg/ml), a topoisomerase II inhibitor, were the positive controls. Treated cells from each mixture were then duplicate plated to Petri dishes containing 1.75% agar Bacto solidified YPDA as media. Cells were incubated at growth temperature of 30°C (JN362a, JN394, JN394t_{2-4}, JN394t_{-1}) or 25°C (JN394t_{2-4} and JN394t_{2-5}) for 48 hours. Antitopoisomerase activity was then measured by comparing the number of counted colonies in each plate to that of the negative control plate (DMSO).

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Mutagenicity test

A preliminary cytotoxic experiment was performed using the bacterial growth assay on nutrient agar plates to identify the concentration at which the extracts have no significant effect on the viability of *Salmonella typhimurium* TA1535. Each plant extract (5 or 10 mg/mL) was incubated with 100 µL of 15 h growing bacteria (1 x 10^9 UFC/mL) on LB medium and mixed with 2.5 mL of warm 0.6% top agar (NaCl/Agar). The mix was then added to nutrient agar plates, the plates were incubated at 37°C for 24 h and finally the colonies on triplicate plates were counted and compared to control plates containing no extracts. The concentration investigated hereafter for all extracts was 5 mg/mL. To screen the mutagenic potential of the plant extracts, the reverse bacterial mutation assay was performed as described by Maron and Ames (1983). Each plant extract (5 mg/mL) was preincubated with 100 µL of 15 h growing bacteria (1 x 10^9 UFC/mL) on LB medium at 37°C for 30 minutes, with and without the addition of S9 mix (microsomal fraction). The incubated strain (TA1535) was then added to top agar containing 50 µM of histidine/biotin and poured on to MGA plates. Revertant colonies were counted after incubation at 37°C for 36-48 h. Spontaneous revertant colonies arising on plates containing neither mutagens nor extracts were also counted; DMSO was used as the negative control. Revertant colonies produced with NaN_3 (1µg/plate) or 20 µM B[a]P with S9 mix were used as positive controls. The number of visible revertant colonies (His^+) was counted in each treatment and blanks. For a treatment to be considered mutagenic in the Ames test, the number of His^+ revertant colonies on the plates containing the treatments must be more than twice the number of His^+ revertant colonies on the negative control plate (Fawole et al., 2009). All assays were done in triplicate.

Table 1: Values of extraction yield (expressed as percentage of dry plant weight) obtained for eight different medicinal plants used in the treatment of infectious and chronic diseases in Mexico

Plant species Family	Traditional uses	References	Plant part collected	Common name/Collection site/voucher number	(%)Yield extracts /MeOH
Acalypha cuspidata Euphorbiaceae	Treatment of skin infections.	Note [a]	Stem and leaves	Hierba de la araña Candelaria Loxicha UACh 25068	3.1
Acalypha subviscida Euphorbiaceae	Treatment of skin infections.	(UNAM, 2009)	Stem and leaves	Hierba del pastor San Miguel Suchixtepec UACh 24007	9.8
Adenophyllum aurantium (L.) Strother Asteraceae	Treatment of gastro-intestinal disorders and Cancer.	(Alonso-Castro et al., 2011)	Stem and leaves	Ruda montes Chepilme Botanic Garden UACh 25173	9.8
			Roots (R)		1.1 [b] 2.5
Alloispermum integrifolium Astearaceae	Plant (stem) infusion is used as hypoglycemic.	(Andrade-Cetto and Heinrich, 2005)	Stem and leaves	Prodijiosa San Miguel Suchixtepec UACh 24024	7.2
Galium mexicanum var mexicanum/ Rubiaceae	Oral infusion of leaves is used as abortifacient Oral infusion of leaves is used in the treatment of stomachache, diarrhea.	(Andrade-Cetto, 2009) (UNAM, 2009)	Stem and leaves	Pegarropa or pega pega San Miguel Suchixtepec UACh 23994	7.6
Lantana achyranthifolia Verbenaceae	Treatment of gastrointestinal disorders, hepatic and respiratory complaints, fever	(Frei et al., 1998)	Stem and leaves	Cancerina Huajuapan de León UACh 25185	3.9
Salpianthus arenarius Nictaginaceae	Hypoglycemic	(Andrade-Cetto and Heinrich, 2005)	Stem and leaves Roots (R)	Chichuca Chepilme Botanic Garden UACh 25084	27.4
Tournefortia densiflora Boraginaceae	Stem is used for the treatment of gastrointestinal disorders.	(Alonso-Castro et al., 2011)	Stem and leaves	Hierba del negro, Hoja de cáncer Chepilme Botanic Garden UACh 25221	2.2
			Roots (R)		3.6

[a] *A. cuspidata* is recommended by the community of Candelaria, Loxicha.
[b] Solvent AcOEt UACh Chapingo Autonomous University; MeOH methanol: AcOEt: ethylacetate

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Statistical Analysis

All statistical analyses were performed using Graph Pad Prism 6 Program for Windows. Dunnett posttest was used for comparison of two groups. Data are expressed as means ± standard deviation (SD). Analysis of variance was determined by one-way (ANOVA). P value < 0.05 was considered as significant.

Results

In our search for natural products with anticancer activity, we prepared methanol crude extracts of eight native plants used in the treatment of degenerative or infectious diseases. No scientific information is available about the pharmacological, biological and toxicological effects of the plant extracts studied in this work. Moreover, there are no references about the medicinal use of *A. cuspidata*. However, in the rural community where the plant was collected, the population said that *Acalypha cuspidata* stems and leaves are useful to treat skin infections. Table 1 summarizes the plant species, the parts of plants used for extract preparation, the percentage of yield extracts as well as the voucher numbers of the eight plants tested. Organic extracts were subjected to antioxidant, antimicrobial, antitopoisomerase, and mutagenicity evaluations to justify further studies searching for new anticancer molecules. In addition, two fractions with antitopoisomerase activity were identified from *A. cuspidata* extract.

Antioxidant activity

An antioxidant assay was carried out for all plant extracts (Table 2) and only *A. subviscida* extract showed weak antioxidant activity (EC_{50} = 480.97±2.34 g/kg DPPH•, T_{EC50} = 1 ± 0.17 min, % AE = 31.04).

Antimicrobial activity and MIC

The results of antibacterial activity on six bacteria and one fungus (Table 3) indicated that all extracts (except *A. aurantium* R) showed inhibition in at least one strain (≥7.02 mm) and were tested for their MIC values. *S. epidermidis* was sensitive to *S. arenarius* (MIC = 130 µg/mL). The extracts from *A. cuspidata* (97.65 µg/mL), *L. achyranthifolia* (78.12 µg/mL) and *S. arenarius* (56.25 µg/mL) showed better antimicrobial activities with MIC < 100 µg/mL values, all of them tested against *S. aureus* (Table 4).

Table 2: Estimation of free radical-scavenging activity of crude plant extracts of some Mexican flora from Oaxaca.

Treatments	EC_{50} g/kg $_{DPPH}$	T_{EC50} min	AE x 10^{-3}	% of efficiency
Ascorbic acid	102.67 ± 2.34	1.45 ± 0.36	6.70 ± 0.00051	100
A. aurantium	4599.61 ± 1.88	2.45± 0.45	0.08 ± 0.000008	1.19
A. aurantium (R)	6576.54 ± 2.05	2.50± 1.00	0.06 ±0.000008	0.90
A. integrifolium	9007.23 ± 2.62	0.78± 0.24	0.28 ± 0.00021	4.18
A. subviscida	480.97 ± 3.15	1.00± 0.17	2.08 ± 0.0001	31.04
L. achyranthifolia	838.85 ± 2.05	2.91± 0.54	0.41 ± 0.00005	6.12
S. arenarius	851.54 ± 1.74	6.95± 0.63	0.16 ± 0.00001	2.39
T. densiflora	1147.90 ± 3.73	10.5± 2.03	0.08 ± 0.000003	1.24
T. densiflora (R)	2315.52 ± 3.11	7.56± 0.71	0.05 ± 0.000004	0.75

Results are means ± S.D. (n=3).
(R) = roots

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Table 3: Zone of inhibition (mm) produced by crude plant extracts of some Mexican flora from Oaxaca and standard antimicrobial agents.

	Microbial strains						
Treatment	*S. aureus*	*S. epidermidis*	*K. pneumoniae*	*E. coli*	*P. mirabilis*	*P. vulgaris*	*C. albicans*
*Control (+)	22.75 ± 0.92	29.78 ± 1.62	29.56 ± 1.28	31.16 ± 1.93	23.03 ± 0.96	24.24 ± 1.52	21.45 ± 1.42
A. aurantium	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	11.64 ± 1.18	9.98 ± 0.63	9.17 ± 2.21
A. aurantium R	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
A. cuspidata	16.56 ± 0.99	0 ± 0.00	0 ± 0.00	0 ± 0.00	15.35 ± 0.75	19.13 ± 1.91	0 ± 0.00
A. subviscida	14.90 ± 0.74	15.44 ± 0.00	0 ± 0.00	10.03 ± 1.84	27.09 ± 1.83	27.37 ± 0.84	0 ± 0.00
A. integrifolium	0 ± 0.00	0 ± 0.00	0 ± 0.00	7.02 ± 0.97	19.60 ± 1.44	16.35 ± 2.90	0 ± 0.00
G. mexicanum	10.97 ± 0.99	0 ± 0.00	0 ± 0.00	0 ± 0.00	15.17 ± 1.58	16.38 ± 1.21	0 ± 0.00
L. achyranthifolia	21.84 ± 0.93	0 ± 0.00	0 ± 0.00	7.44 ± 0.44	18.21 ± 1.52	17.19 ± 1.91	11.46 ± 0.92
S. arenarius	15.66 ± 0.73	14.17 ± 0.00	0 ± 0.00	0 ± 0.00	14.64 ± 1.31	15.05 ± 0.79	13.26 ± 1.15
T. densiflora	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	9.6 ± 1.25	0 ± 0.00
T. densiflora R	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	13.54 ± 0.51	15.38 ± 0.88	12.74 ± 1.64

* Chloramphenicol (bacteria) and nystatin (fungus)
Root indicated by "R". Results are means ± S.D. (n=3).

Table 4: Minimal inhibitory concentration of crude plant extracts of some Mexican flora from Oaxaca and standard antimicrobial agents.

	MIC x 10^{-3} (mg/ml)						
Treatment	*S. aureus*	*S. epidermidis*	*K. pneumoniae*	*E. coli*	*P. mirabilis*	*P. vulgaris*	*C. albicans*
*Control (+)	5.62	2.81	1.40	5.62	11.25	11.25	250000[#]
A. aurantium	ND	ND	ND	ND	3125	6250	25000
A. aurantium R	ND	ND	ND	ND	ND	ND	ND
A. cuspidata	**97.65**	ND	ND	ND	6250	12500	ND
A. subviscida	781.2	390.6	ND	12500	781.1	390.6	ND
A. integrifolium	ND	ND	ND	25000	25000	6250	ND
G. mexicanum	781.25	ND	ND	ND	6250	6250	ND
L. achyranthifolia	**78.12**	ND	ND	3125	3125	390.62	25000
S. arenarius	**56.25**	130.85	ND	ND	1046.87	1046.87	8375
T. densiflora	ND	ND	ND	ND	ND	ND	25000
T. densiflora R	ND	ND	ND	ND	17500	8750	8750

*Chloramphenicol (bacteria) and nystatin (fungus)
Root is indicated by "R", ND: Not Determined, [#] (U/ml)

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Antitopoisomerase activity

The strain JN394 is DNA repair-deficient and drug-permeable (*ise2* and *rad52* mutations) (Nitiss and Wang, 1988). These mutations increase the sensitivity of these cells to drugs. The yeast JN362a is a DNA repair-proficient strain (Nitiss and Wang, 1988). As shown in Figure 1, the strain JN394 was hypersensitive to CPT (-99.69±0.0 % growth) and ETP (-97.96±0.0 % growth), which are Topo I and Topo II poisons, respectively. A topoisomerase inhibitor affects the JN394 growth but does not inhibit JN362a growth (CPT 84.49%). Treatments with *A. cuspidata* (JN394, -71.23±2.12% and JN362a, 84.69± 3.54%), *A. integrifolium* (JN394, -81.19±2.12% and JN362a, 126.06±12.02%), *G. mexicanum* (JN394, -34.28±7.07% and JN362a, 66.06±15.56%), and *L. achyranthifolia* (JN394, -49.74±3.54% and JN362a, 1.63±12.73%) showed similar behavior to CPT. *A. aurantium, A. aurantium* (R), *T. densiflora* and *T. densiflora* (R) were toxic extracts with different mechanisms of action to topoisomerase inhibition because of inhibited growth of JN394 and JN362a strains. *A subviscida* did not show growth inhibition on either strain.

To find the topoisomerase inhibitor type, the extracts were tested with strain JN394t_{-1}, which is isogenic to JN394 and contains a disrupted topI gene (Nitiss and Wang, 1988). The absence of the gene resulted in diminished cytotoxicity of antitopoisomerase I drugs. As shown in Figure 2, CPT topo I poison failed to reduce the growth of these mutant cells (76.50±2.12%); similar behavior is observed with *G. mexicanum* (35.31±6.36%). Therefore, the Topo I enzyme is the target of this extract. In contrast, when topI cells were treated with antitopoisomerase II ETP (-99.08% growth) no resistance was observed. The extracts that showed growth inhibition of JN394t_{-1} strain were *A. cuspidata* (-30.88±0.0%), *A. integrifolium* (-38.11±4.95%), and *L. achyranthifolia* (-70.97±12.02%). Sensitivity in the topI cells was higher than in the parent cells (JN394). This finding provided evidence that Topo I was not the cellular target of these plant extracts.

The strain JN394$t_{2\text{-}5}$ expresses at 25 °C the mutation top2-5, which consists of changing three different amino acid II TOP domains which are important in the interaction of protein with topoisomerase II poison. Thus, the strain has a resistance at 25 °C to active compounds with topoisomerase II poison (Jannatipour et al., 1993). Figure 2 shows that cells with the top2-5 mutation can grow in ETP (55.77±3.54%). The sensitivity of the top2-5 strain to CPT (-100.00±0.00 %) and SBZ (-38.46±7.07%) is shown in Figure 2. The strain has essentially the same sensitivity to CPT as JN394 (*rad52 top2*$^{+}$ cells), indicating that the observed resistance is specific to antitopoisomerase II agents. Among the plant extracts tested, none showed similar behavior to ETP. This means that none of the extracts exhibited antitopoisomerase II poison activity. Plant extracts of *A. cuspidata* (-15.38 ± 2.83%), *A. integrifolium* (-17.31 ± 2.12%) and *L. achyranthifolia* (-61.54 ± 4.24%) were shown to have topoisomerase II catalytic inhibitors compounds.

To complete the analysis, we also used the yeast strain JN394$t_{2\text{-}4}$, that expresses the temperature-sensitive top2-4 mutant in place of the wild type top2 gene (Jensen et al., 2000, Nitiss and Nitiss, 2001). JN394$t_{2\text{-}4}$ cells have problems growing at 30 °C, because topoisomerase II activity at this temperature is reduced (DiNardo et al., 1984; Dong et al., 2000). Figure 2 shows that cells with the top2-4 mutation at 25°C can grow with ETP (18.29 %) and SBZ (25.14 %), but they are sensitive to CPT (-40.57 %). Cells were hypersensitive at 30°C, displaying cytotoxicity with CPT (-85.45%), ETP (-69.35 %) and SBZ (-81.89 %). Similar behavior was observed for extracts of *A. cuspidata, A. integrifolium. L. achyranthifolia* showed no growth inhibition at 25°C (8.57 to 44.57%), but at 30°C, growth was inhibited (-63.47 to -72.29 %).

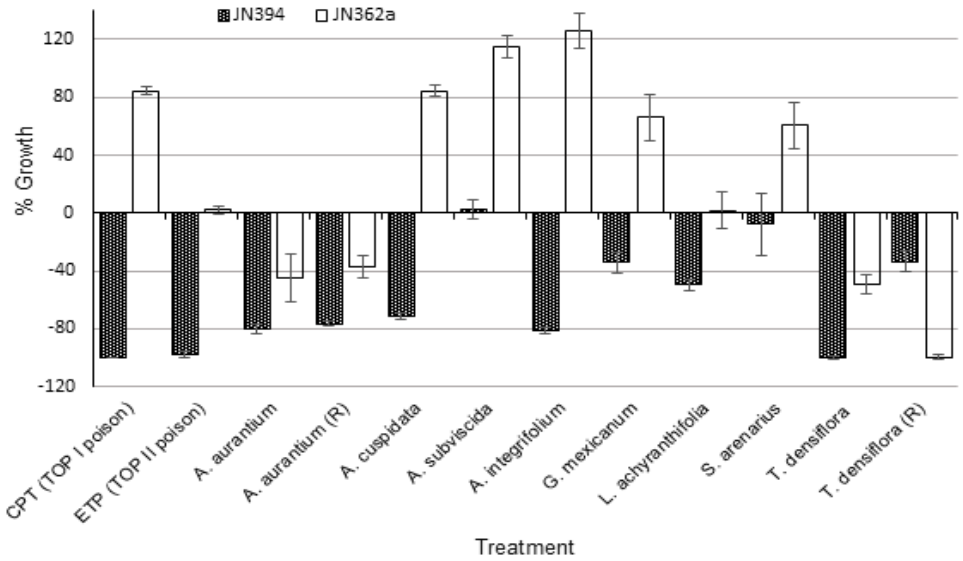

Figure 1: Effects of controls CPT (camptothecin topo I inhibitor), and ETP (etoposide topo II poison), and extracts on JN394 (30°C) and JN362a (30°C) growth. Treatments with similar behaviors to CPT or ETP are *A. cuspidata, A*

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integrifolium, G. mexicanum, and *L. achyranthifolia,* which inhibited growth of JN394 and promoted or did not affect that of JN362a. *A. aurantium, A. aurantium* R, *T. densiflora* and *T. densiflora* R were toxic to both strains.

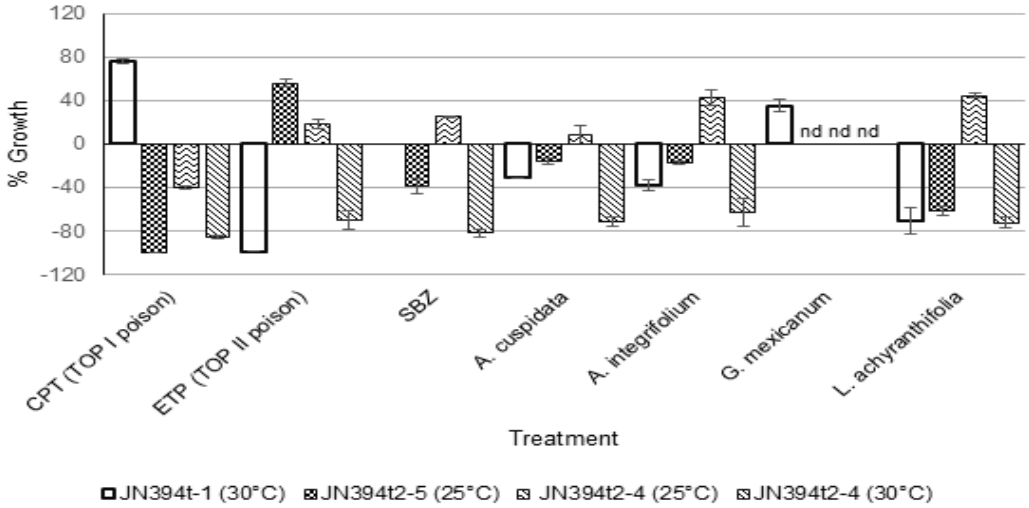

Figure 2: Effects of controls CTP (camptothecin topo I inhibitor), ETP (etoposide topo II poison), and SBZ (Sobuzoxane topo II inhibitor), and extracts from *A. cuspidata, A. integrifolium, G. mexicanum*, and *L. achyranthifolia* on JN394t_{-1} (30°C), JN395t_{2-5} (25°C), JN394t_{2-4} (25°C), and JN394t_{2-4} (30°C) growth. *G. mexicanum* was identified as topoisomerase inhibitor I, meanwhile these extracts showed similar behavior to that of SBZ, a topo II inhibitor (catalytic). JN395t_{2-5} (25°C), JN394t_{2-4} (25°C), and JN394t_{2-4} (30°C) assays with *G. mexicanum* were not determinate (ND).

Antitopoisomerase activity of fractions and sub-fractions of *A. cuspidata*

Growth inhibition of JN394 and JN362a cells was observed with AaA1 (both strains -100 % growth) and AaA17 sub-fractions (-24.57 and -27.2%, respectively) (Table 5). However, both fractions exhibited active cytotoxic mechanisms different from topoisomerase inhibition. Fractions (Table 4) with similar behavior to CPT or ETP were AaA7 (at 336 µg/mL) and AaA8 (at 583 µg/mL) due to the growth inhibition on JN394 (-20.15±11.58% and -43.10±2.58%, respectively) and no growth inhibition on JN362 (27.09±12.14% and 9.56±5.06%, respectively). These are the fractions with antitopoisomerase activity, which showed no toxicity to JN394t_{-1} (-8.11±3.12 and 32.73±2.55 %) and JN394t_{2-5} (0.75±5.45 and 29.38±1.96%), where the first strain has diminished cytotoxicity of topo I inhibitors and the second one has a resistance at 25°C to antitopoisomerase II compounds. Thus, AaA7 and AaA8 contain topo I and II inhibitors. Chromatographic separation showed behavior similar to CPT (15.84±1.11%, -89.37±3.57%) with JN394t_{-1} and JN394t_{2-5} 25°C strains (Figure 3); thus, sub-fraction (-23.42±4.30%, -61.28±6.86%) was toxic to JN394t_{-1} and JN394t_{2-5}; therefore, it contains topo II inhibitors (Figure 3).

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Table 5: Percentage of growth of JN394, JN362a, JN394 t_{-1}, and JN394t_{2-5} strains from *A. cuspidata* fractions.

Fraction	Concentration µg/mL	JN394 30°C	JN362a 30°C	JN394$_{t-1}$ 30°C	JN394t_{2-5} 25°C
CPT	50	-100.00±0.00	39.60±6.40	15.84±1.11	-89.37±3.57
ETP	100	-99.76±0.42	5.00±3.67	-85.53±8.35	27.88±4.51
AaA1	0.16µL	**-100.00±0.00**	**-100.00±0.00**	ND	ND
AaA2	501	-10.58±2.36	-4.81±3.94	ND	ND
AaA3	685	-14.07±4.94	-8.50±4.00	ND	ND
AaA4	111	-9.33±1.90	-11.42±2.08	ND	ND
AaA5	99	19.67±14.05	ND	ND	ND
AaA6	319	-27.19±13.23	-93.60±2.45	ND	ND
AaA7	336	-20.15±11.58	27.09±12.14	-8.11±3.12	0.75±5.45
AaA8**	**583**	**-43.10±2.58**	**9.56±5.06**	**32.73±2.55**	**29.38±1.96**
AaA9	514	-16.51±1.72	-6.76±5.33	ND	ND
AaA10	253	54.00±40.58	ND	ND	ND
AaA11	293	12.74±4.22	ND	ND	ND
AaA12	293	21.55±31.67	ND	ND	ND
AaA13	223	21.91±17.75	ND	ND	ND
AaA14	302	19.65±13.13	ND	ND	ND
AaA15	296	59.43±17.60	ND	ND	ND
AaA16	166	9.95±13.29	ND	ND	ND
AaA17	**164**	**-24.57±7.09**	**-27.20±7.47**	ND	ND
AaA18	306	27.90±11.95	ND	ND	ND
AaA19	287	11.25±13.64	ND	ND	ND
AaA20	144	59.00±66.91	ND	ND	ND
AaA21	267	7.14±5.13	ND	ND	ND

[a]Values are means ± SD of triplicate determinations.
ND: Not Determined.
**Topo I and II inhibitors

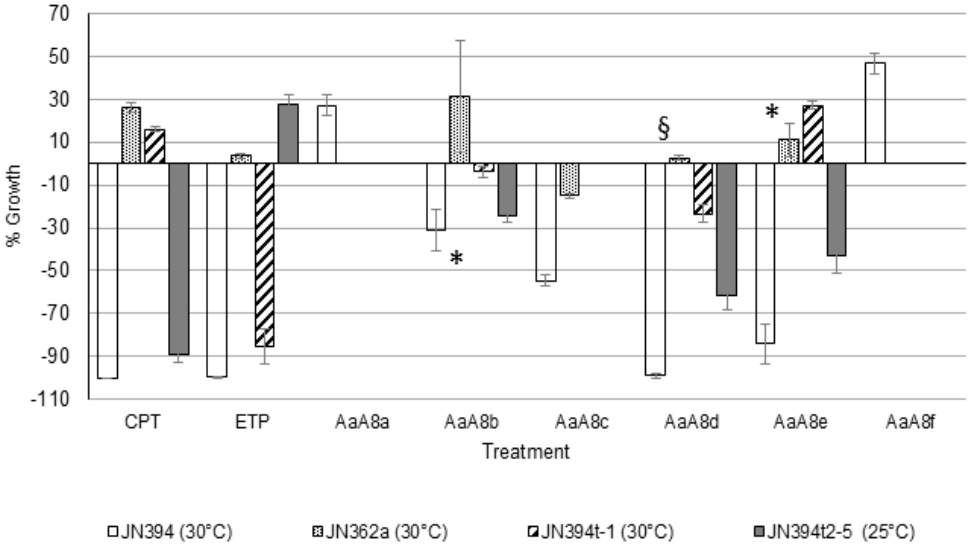

Figure 3: Effects of controls CTP (topo I inhibitor, 50µg/mL), and ETP (topo II poison 100µg/mL), and the subfractions AaA8a (154µg/mL), AaA8b (154µg/mL), Aa8c(213µg/mL), AaA8d (154µg/mL), AaA8e (154µg/mL) and AaA8f (63.9µg/mL) on the JN394 (30°C), JN362a (30°C), JN394t_{-1} (30°C), and JN394t_{2-5} (25°C) growth. AaA8b and AaA8e showed behavior as topo I inhibitors (*) while AaA8d showed behavior as a topo II inhibitor (§)

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Mutagenicity test

Three extracts at 10 mg/mL resulted in significant reductions in the viability of *Salmonella typhimurium* and all extracts at 5 mg/mL had no significant effect on the bacterial viability. Therefore, we decided to proceed with 5 mg/mL, which is well below any concentration that could compromise the bacterial viability. Table 6 shows the number of His^+ revertant colonies with and without S9 activation against *S. typhimurium* tester strain TA1535 for all extracts. His^+ revertant colonies/plate of all extracts without S9 mix ($S9^-$) were < 27.7±1.8, a value less than twice that of the negative control, therefore extracts are not direct mutagens. When the extracts are tested in the presence of S9 ($S9^+$), which mimics *in vivo* an activation metabolic process (Ames et al., 1973), none of the extracts showed a His^+ revertant colonies/plate number large enough to characterize extracts as indirect mutagens (His+ revertant colonies/plate <31.3).

Table 6: Mutagenic effect of different extracts (5 mg/mL) using *Salmonella typhimurium* TA1535 with and without S9 metabolic activation

Treatment	Concentration	His^+ revertant colonies/plate
Control	-----	27.6 ± 1.4
NaN_3	1µ g/plate	297.7 ± 16.8*
B[a]P	20µM/plate	350.3 ± 10.1*
	His^+ revertant colonies/plate	His^+ revertant colonies/plate
	$S9^-$	$S9^+$
A. aurantium	18.0 ± 1.2	21.0 ± 2.1
A. aurantium (R)	21.7 ± 2.4	25.3 ± 4.2
A. cuspidata	15.7 ± 1.8	20.3 ± 1.5
A. subviscida	26.0 ± 3.2	31.0 ± 2.1
A. integrifolium	30.3 ± 0.9	30.3 ± 5.0
G. mexicanum	21.7 ± 2.7	18.3 ± 2.6
L. achyranthifolia	23.3 ± 1.5	25.3 ± 2.2
S. arenarius	27.7 ± 1.8	31.3 ± 2.2
T. densiflora	24.7 ± 3.5	26.7 ± 4.5
T. densiflora (R)	18.7 ± 2.6	20.0 ± 1.5

Number of His+ revertant colonies/plate: mean values of three replicates
B[a]P = Benzopyrene
(R) = Root
$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation
S. typhimurium TA1535 without treatment differs significantly from control (P<0.05) (spontaneous mutation).

Discussion

Among the species that were studied, the *S. arenarius* and *L. achyranthifolia* extracts showed significant antimicrobial activity (< 100 µg/mL) (Cos et al., 2006) against *S. aureus* with MIC values of 56.25 and 78.12 µg/mL respectively. Cyclitol-2-methoxy-5-hydroxymethyl cyclopentane-1,3,4-triol with parasympathomimetic activity was described from flowers of *S. arenarius* (Pérez-Gutiérrez and Pérez-Gutiérrez, 1992) but there are no reports about antimicrobial activity for this species. On the other hand, studies of *L. achyranthifolia* described the isolation of two furanonaphthoquinones: diodantunezone and isodiodantunezone (Abeygunawardena et al., 1991) from roots, and eighteen monoterpenes from essential oils (Hernández et al., 2005). Essential oils, and ethanol, chloroform and *n*-hexane extracts were weakly toxic to eleven pathogenic bacteria strains, including *S. aureus*, whose MIC value was 1.0 mg/mL (Hernández et al., 2003). We found significant activity of methanol extract against *S. aureus* with a MIC of 78.12 µg/mL. The differences in MIC values observed could be due to the solvents used for the extract preparation (chloroform, hexane and ethanol) and the disc-diffusion and well-diffusion methods used for treatments that do not easily diffuse into agar. Topoisomerase (type II catalytic) inhibition has not been described for extracts or compounds from the *Lantana* genus.

Some species of *Acalypha* genus (Euphorbiaceae) have been described as useful in the treatment of conditions related to infections and inflammation (Hassan-Abdallah et al., 2013; Hernández et al., 2003). Among the biological studies reported for *Acalypha* genus, there are antimicrobial compounds (Emeka et al., 2012; Alade et al., 1993) and cytotoxic compounds (Amarnath et al, 2014; Lim et al., 2011). Weak to moderate antimicrobial activity was reported from *A. cuspidata* leaf and stem methanolic extracts against five methicillin-resistant *Staphylococcus* strains (Rocha-Gracia et al., 2011). However, *A. cuspidata* showed a MIC value of 97.65 µg/mL against *S. aureus* and catalytic antitopoisomerase II activity (at 50 µg/mL). The antitopoisomerase activity of *Acalypha* genus has not been described, although there is a report about the probable antitopoisomerase II activity of *A. wilkesiana* extracts, which induce

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apoptosis by single and double strand DNA breaks (Lim et al., 2011). Chemical studies of the *Acalypha* genus demonstrated the presence of sesquiterpenes and diterpenes (Siems et al., 1996). Thus, the observed antimicrobial and antitopoisomerase activities could be due to these compounds, which are known for biological activities such as those observed for the diterpene ingenol. Ingenol, isolated from the Euphorbiaceae family, exerts antimicrobial and antitopoisomerase type II activities (Miyata et al., 2006).

Fractionation of *A. cuspidata* extract led to the identification of the following:

i) The toxic fractions to *S. cerevisae* cells: AaA1 (at 0.16µL/mL) and Aa17 (at 164 µg/mL) (Table 4) which is activity unrelated to topoisomerase inhibition;

ii) Two antitopoisomerase fractions AaA7 (at 336 µg/mL) and AaA8 (at 583 µg/mL); (AaA8 which had a better yield (1.69%) was subjected to chromatographic separation);

iii) Two subfractions AaA8d and AaA8e (at 154 µg/mL) with similar antitopoisomerase activity (-99.07±0.89 and -84.10±9.22%) to 50 µg/mL CPT (-100±0.0) and 100 µg/mL ETP (-99.76±0.42%) on JN394 strain (Figure 3);

iv) Two subfractions AaA8b and AaA8e with topo I inhibitors and a subfraction AaA8d containing topo II inhibitors;

v) The overall effect of the *A. cuspidata* extract as a topo II inhibitor but with fractions and sub-fractions showing topo I, topo II or both mechanisms.

A. subviscida showed no significant antimicrobial or antitopoisomerase activities but exhibited the best antiradical efficiency (AE = 31.04%).

Anti-inflammatory activity and antibacterial effects have been described against *S. aureus* (MIC 67µg/mL), and *Bacillus subtilis* (MIC 67µg/mL) of hexane and chloroform *G. mexicanum* extracts (Bolívar et al., 2011). The current study is the first to report that methanolic extract of *G. mexicanum* showed antitopoisomerase type I activity.

Methanol extract of *A. integrifolium*, which is used in the treatment of diabetes in Mexico (Andrade-Cetto and Heinrich, 2005), showed no significant antioxidant (AE = 4.18 %) or antimicrobial activities (MIC ≥ 6250 µg/mL), but it displayed antitopoisomerase type II activity. The isolation of an acetylene compound containing seventeen carbon atoms from stems of *A. integrifolium* (Bohlmann and Zdero, 1976) could explain the biological activity observed, because these metabolites display important biological activities such as anticancer, antibacterial, antifungal, among others (Kuklev et al., 2013). Another Asteraceae species studied was *A. aurantium*, whose extracts, from roots and stems, showed no significant antioxidant (EA = 1.19 %), antimicrobial (MIC ≥ 3125 µg/mL) or antitopoisomerase activities. In this work, neither *A. aurantium* nor the *T. densiflora* methanolic extracts showed growth inhibition of *S. cerevisae* JN394 and JN362a strains, meaning that they are cytotoxic through a mechanism different from topoisomerases inhibition. The toxicity of *A. aurantium* extract agrees with the toxic effects observed on *Alternaria alternata* and *Fusarium solani* (Lira-De León et al., 2014). The biological activity of *A. aurantium* is probably because it contains α-terthienyl and 5-(4´´-hydroxy-1´´-butynyl)-2-2´-bithiophene (Herrera-Martínez et al., 2016), which are known for their antiviral, cytotoxic (Hudson et al., 1989), antiamoebic (Herrera-Martínez et al., 2016), and fungicidal activities (Kourany and Arnason, 1988).

In Mexico, many vegetal species are used as complementary and alternative medicines with the constant or frequent consumption by sick people of extracts which have generally unknown mutagenic potential. None of the plant extracts tested in this work showed mutagenic effects at 5 mg/mL using *S. typhimurium* TA1535 strain. This evaluation was particularly significant for those vegetal species such as *S. arenarius* and *A. integrifolium*, which are known as hypoglycemic (Andrade-Cetto and Heinrich, 2005) and are used for the treatment of chronic diseases. However, more toxicity assays are needed to assure that the plant extracts tested in this work can be used safely.

Conclusions

In this work, we identified four plant species with antitopoisomerase and non-mutagenic activities, and consequently with anticancer potential: *A. cuspidata*, *A. integrifolium*, *G. mexicanum*, and *L. achyranthifolia* (Figure 4). None of these plants showed significant toxic effects (MIC>390 µg/mL) against growth of the Gram-negative bacteria tested or the fungus *C. albicans*. They also showed no antioxidant activity. Only *L. achyranthifolia* and *S. arenarius* showed significant antimicrobial activity (< 100 µg/mL) against *S. aureus*. We obtained three antitopoisomerase sub-fractions from *A. cuspidata*, demonstrating increasing activity at concentrations similar to reference substances (CPT and ETP). Therefore, further work is required to isolate the phytochemicals responsible for the observed activities. In addition, more extensive experimentation is needed to evaluate the toxicity to JN394 and JN362a strains caused by *T. densiflora*, *A. aurantium*, and two fractions of *A. cuspidata* (AaA1 and AaA17). The current work represents the first report on the topoisomerase enzyme inhibition of extracts from *Alloispermum*, *Acalypha* and *Galium* genus.

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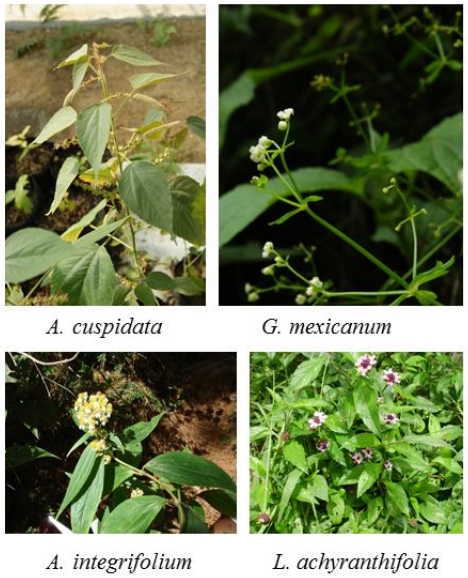

Figure 4: *A. cuspidata, G. mexicanum, A. integrifolium* and *L. achyranthifolia* images

Declaration on interest section

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