SCREENING OF ANTITOPOISOMERASE, ANTIOXIDANT, AND ANTIMICROBIAL ACTIVITIES OF SELECTED TRITERPENES AND SAPONINS

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This paper is dedicated to Professor Pedro Joseph-Nathan in recognition of his 50 years of outstanding scientific trajectory.

ABSTRACT

A baccharane-type triterpene (**1**), four oleanane-type triterpenes (**2**-**5**) and twelve oleanane-type bidesmodic saponins (**6**-**17**) were subjected to antioxidant, antimicrobial and antitopoisomerase evaluation. Moderate antimicrobial activity was observed (zone of inhibition in millimeters); triterpenes **2**-**4** were toxic to *Staphylococcus epidermidis* (13.20 ± 0.00; 11.75 ± 0.07; 9.85 ± 0.21, respectively), while baccharis oxide (**1**) inhibited the growth of *S. epidermidis* (14.75 ± 0.35), *Candida albicans* (20.55 ± 0.92), *Escherichia coli* (14.80 ± 1.13) and *Klebsiella pneumoniae* (16.35 ± 0.49). Polygalacic acid (**4**), **6**, **7, 15** and **16** showed growth inhibition of *C. albicans* (13.50 ± 0.71; 11.00 ± 00; 8.50 ± 0.71; 11.70 ± 0.14; 10.50 ± 0.71, respectively). The growth inhibition of *S. epidermidis* was achieved by **8 (**9.00 ± 1.41) and **15** (9.00 ± 0.00); while the last compound inhibited the growth of *K. pneumoniae* (9.50 ± 0.71). The tested compound concentration for antitopoisomerase activity was between 1.8-13.8 times of those used as positive controls (CPT= 0.1435 μM, ETP = 0.0849 μM), showing that **1** (1.1726 μM), is topoisomerase I inhibitor. Methylated oleanolic acid (**3a**, 0.7128 μM), together with **9** (0.3650 μM), **13** (0.3294 μM) and **14** (0.3691 μM) showed antitopoisomerase II activity. None of all tested compounds showed significant antioxidant activity using the DPPH method. *www.relaquim.com*

Keywords: Topoisomerase inhibitor, antimicrobial activity, baccharis oxide, bayogenin, polygalacic acid, saponins.

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RESUMEN

Se evaluó la actividad antioxidante, antimicrobiana y antitopoisomerasa de un triterpeno tipo bacharano (**1**), cuatro triterpenos tipo oleanano (**2**-**5**) y doce saponinas bidesmódicas tipo oleanano (**6**-**17**). Los triterpenos **2**-**4** mostraron actividad antimicrobiana (zona de inhibición en milímetros) en contra de *Staphylococcus epidermidis* (13.20 ± 0.00; 11.75 ± 0.07; 9.85 ± 0.21, respectivamente), en tanto que el óxido de baccharis (**1**) inhibió el crecimiento de *S. epidermidis* (14.75 ± 0.35), *Candida albicans* (20.55 ± 0.92), *Escherichia coli* (14.80 ± 1.13) y *Klebsiella pneumoniae* (16.35 ± 0.49). El ácido poligalácico (**4**), **7, 6**, **15** y **16** inhibieron el crecimiento de *C. albicans* (13.50 ± 0.71; 11.00 ± 00; 8.50 ± 0.71; 11.70 ± 0.14; 10.50 ± 0.71, respectivamente), el crecimiento de *S. epidermidis* fue inhibido por **8** (9.00 ± 1.41) y **15 (**9.00 ± 0.00). Este último compuesto inhibió además el crecimiento de *K. pneumoniae* (9.50 ± 0.71). En la evaluación antitopoisomerasa de **1**-**17** usando concentraciones entre 1.8 y 13.8 veces mayores que las correspondientes a los controles positivos (CPT= 0.1435 µM, ETP = 0.0849 µM) se demostró que **1** (1.1726 µM) es inhibidor tipo I de la enzima. El derivado metilado del ácido oleanólico (**3a**, 0.7128 µM) y las saponinas **9** (0.3650 µM), **13** (0.3294 µM) y **14** (0.3691 µM) mostraron actividad antitopoisomerasa II. En la evaluación antioxidante por el método del radical libre DPPH ningún compuesto mostró actividad significativa. *www.relaquim.com*

Palabras clave: Inhibidor de la topoisomerasa, actividad antimicrobiana, óxido de baccharis, bayogenina, ácido poligalácico, saponinas.

INTRODUCTION

Several assays have been developed to evaluate the compounds ability to modulate biochemical events presumed to be mechanistically linked to carcinogenesis (Shureiqi *et al.*, 2000). Examples of such assays include: a) topoisomerase inhibitors, which constitute a class of agents that inhibit carcinogenesis via their antiproliferative or cell-differentiating action and chemotherapy and chemoprevention (Cho *et al.*, 2000); b) antimicrobial activity, allows the identification of novel agents capable of interfering with a specific molecular target, that may avoid the shortcomings of conventional chemotherapy because certain antimicrobials exhibit selective cytotoxicity against a broad spectrum of human cancer cells (Schweizer, 2009); c) antioxidant activity, as 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).

Up to day, scarce studies are available on antitopoisomerase properties of oleanane-type triterpenes and its glycosides in spite of that they have shown potential in the research of anticancer drugs (Shanmugam *et al.*, 2012; Sparg *et al.*, 2004). It has been described that oleanolic acid and its derivates inhibit the activity of human DNA topoisomerase II (topo II) (Mizushina *et al.*, 2003), while Soyasaponin I and several Aesculosides were described as topo II (Suzuki *et al.*, 2003) and topo I (Wang *et al.*, 2010) inhibitors, respectively. On the other hand, the antimicrobial activities of oleanane-type triterpenes (Katerere *et al.*, 2003) and their glycosides (Bader *et al.*, 2000) have been known. For example, it was demonstrated

that oleanane type triterpenes inhibited the growth of Gram-positive and -negative bacteria (Moodley *et al.*, 2011; Djoukeng *et al.*, 2005) and polygalacic acid glycosides have shown to be potent fungicides (Bader *et al.*, 2000). Antioxidant activity of oleanane type-triterpenes has been described as moderate (Dini *et al.*, 2009). Among tetracyclic triterpenes, those of baccharane-type are less common than oleanane-type ones. For this reason biological studies of baccharane triterpenes or their glycosides are limited, for example: β-glucuronidase inhibitory activity exhibited by Baruol (Núñez *et al.*, 2004) or trypanocidal and inmunomodulatory activities described for baccharis oxide (Da Silva Filho *et al.*, 2004; Missima *et al.*, 2007).

It is known that pentacyclic triterpenes and their glycosides displayed antimicrobial and cytotoxic activities (Cipak *et al.*, 2006; Castro *et al.*, 1997). The aim of our work was to carry out the screening of antimicrobial, antioxidant and antitopoisomerase activities for one baccharane-type triterpene (**1**), four oleanane-type triterpenes (**2-5**) and saponins of bayogenin, polygalacic acid and 16-hydroxyprotobasic (**6**-**17**); herein we show the potential of baccharis oxide (**1**), polygalacic acid ester (**4a**), saponins of bayogenin (**9**) and polygalacic acid (**12E**, **13**, **14**) in the research of drugs with anticancer activity.

MATERIAL AND METHODS

General experimental procedures

Baccharis oxide (**1**): Ethyl acetate extract (4.5832 g) of *Baccharis conferta* roots (226.6 g) was dissolved in 400 mL of ethyl acetate and 571.4 mg of **1** was formed as a white solid, which was re-crystallized from methanol and identified by comparison of its spectroscopic data with those described (Nurnberg *et al.*, 1998).

Compounds **2**, **4**, **6**-**16** were obtained from *Sicyos bulbosus* and *Microsechium helleri* according to Hernández-Carlos *et al.* (2011), and **17** was obtained from *Sechium mexicanum* (Hernández-Carlos *et al.*, 2009).

HPLC analysis of **6**-**17**: Solutions of saponins (1 mg/0.1 mL) in methanol/water (65:35) were injected (20 μL) to C18 column (250 x 4.7 mm, 5 mm) with methanol/water (65:35) as mobile phase at 0.6 mL/min and IR detector. Retention times observed were 34.55 min (**6**), 13.17 min (**7**), 12.75 min (**8**), 30.66 min (**9**), 12.78 min (**10**), 11.80 min (**11**), 11.25 min (**12**), 11.47 min (**13**), 12.36 min (**14**), 27.77 min (**15**), 31.33 min (**16**) and 11.04 min (**17**).

Triterpenes **3** and **5**: The chloroform extract (1.0 g) of aerial parts of *B. conferta* was subjected to column chromatography (300 mm x 20 mm I.D.) on SiO_2 and eluted with ethyl acetate-hexane mixtures. From fractions 12 and 14, **5** (16.6 mg) and **3** (18.0 mg) were obtained, which were identified by comparison of their physical and spectroscopic data with those described (Liang *et al.*, 1988).

Saponins **10E** and **12E**: Compounds **10** (20 mg) and **12** (20 mg) were incubated with β-glucosidase (60 mg) in a buffer solution pH 5.5 at 37°C. After 16 h, each reaction mixture was extracted with *n*-butanol (10 mL x 3), the organic phase was removed under reduced pressure and subjected to separation by HPLC (Perkin Elmer series 200) using normal phase column (CNH_2, 250 x 10 mm, 5 mm, Alltech) with acetonitrile-water (5:4) as mobile phase, the flow rate of the mobile phase was 2.5 mL/min and the peaks were identified using an IR detector. Compounds **10E** (t_R = 9.2 min, 9.0 mg) and **12E** (t_R = 9.02 min, 9.8 mg) were obtained from **10** and **12**, respectively, and identified by HPLC (Perkin Elmer series 200) analysis of the mixture of each reaction (glucose t_R = 16.00 min, **10**: t_R = 10.70 min and **12**: t_R = 10.03 min).

Biological test

Chemicals: Peptone bacto, yeast extract, agar bacto, Mueller Hinton (MH) agar, MH broth, trypticase soy agar, trypticase soy

broth, sabouraud dextrose agar, sabouraud dextrose broth and dextrose were purchase from Difco (Sparks, MD). Methanol (spectrophotometric grade), hexane (HPLC grade), camptothecin (CPT), etoposide (ETP), dimethyl sulfoxide (DMSO-Hybri-Max), adenine hemisulfate salt, chloranphenicol, nystatin, ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical (St. Louis, MO).

Antioxidant activity: The antioxidant activities were measured through the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical scavenging activity by Brand-Williams *et al.*, method (1995). A methanolic solution of sample (50 µL) at four concentrations (9.5, 19, 38, 76 µM) was added to 1.95 mL DPPH radical solution (7.6 x 10^{-5} M/ methanol). The decrease in the absorbance at 515 nm was followed using a uv/ vis spectrophotometer (Beckman DU-530) until the reaction reached the steady state in the dark (Siddhuraju and Becker, 2003). The DPPH radical concentration in the reaction medium was calculated from the following calibration curve, determinated by lineal regression:

$$A_{515nm}=0.009[DPPH\cdot]_T - 0.007$$

Where $[DPPH\cdot]_T$ was expressed as µM, $r^2 = 0.998$

The percentage of remaining DPPH· (% $DPPH\cdot_{REM}$) was calculated as follows:

$$\%DPPH\bullet_{REM} = \frac{[DPPH\bullet]_T}{[DPPH\bullet]_{T=0}}$$

Where $[DPPH\cdot]_T$ was the concentration of DPPH· at the steady state time and [DPPH·]

Table 1. Inhibition zone produced by **1**-**16** and standard antimicrobial agents

Treatment	µMolar concentration	Inhibition (mm±SD)			
		C. albicans	*S. epidermidis*	*E. coli*	*K. pneumoniae*
Nystatin	30 units	35.96±0.24			
Chloramphenicol	0.0928		25.93±0.24	20.88±0.05	27.85±0.28
1	0.5863	20.55±0.92	14.75±0.35	14.80±1.13	16.35±0.49
2	0.4758	0.00	13.20±0.00	0.00	0.00
3	0.4762	0.00	11.75±0.07	0.00	0.00
4	0.4805	13.50±0.71	9.85±0.21	0.00	0.00
5	0.3047	0.00	0.00	0.00	0.00
6	0.2073	11.00±00	0.00	0.00	0.00
7	0.1575	8.50±0.71	0.00	0.00	0.00
8	0.2070	0.00	9.00±1.41	0.00	0.00
9	0.1825	0.00	0.00	0.00	0.00
10	0.1778	0.00	0.00	0.00	0.00
11	0.1661	0.00	0.00	0.00	0.00
12	0.1647	0.00	0.00	0.00	0.00
13	0.1647	0.00	0.00	0.00	0.00
14	0.1846	0.00	0.00	0.00	0.00
15	0.1667	11.70±0.14	9.00±0.00	0.00	9.50±0.71
16	0.1868	10.50±0.71	0.00	0.00	0.00

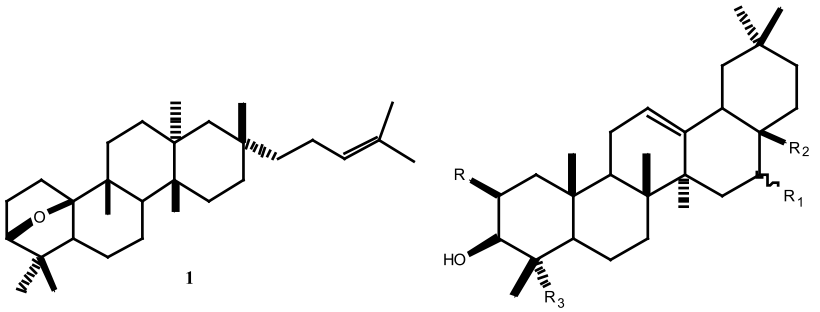
Values are means ± SD of triplicate determinations

$_{T=0}$ was the concentration of DPPH· at zero time. The % DPPH·$_{REM}$ against the standard concentration was plotted to obtain the amount of antioxidant necessary to decrease by 50% the initial DPPH·concentration (EC_{50}). The time needed to reach the steady state to EC_{50} concentration (T_{EC50}) was determined. Ascorbic acid was used as reference standard. All experiments were carried out in triplicate. The activity of each sample was expressed as percentage of that achieved for the reference standard.

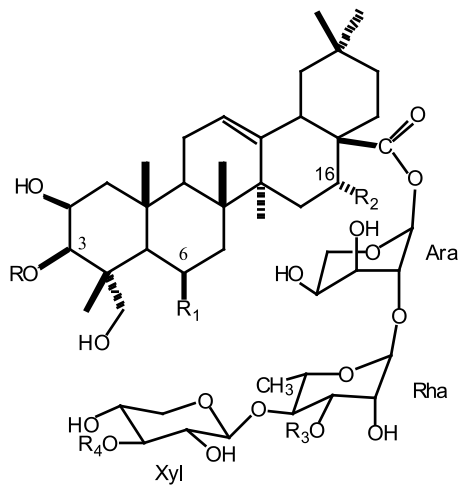
Antibacterial and antifungal activities: Culture collection (ATCC, Manassas, VA): Gram negative bacteria: *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 35218), Gram positive bacteria: *Staphylococcus epidermidis* (ATCC 12228) and the fungus *Candida albicans* (ATCC 14053). The antibacterial and antifungal activities were determined using the agar diffusion method. The bacterial strains were placed in plates of trypticase soy agar and the fungus in plates of sabouraud dextrose agar. After 24h incubation at 37°C (bacteria) and 30°C (fungus), four of five colonies were inoculated in 4 mL of Mueller-Hinton broth or sabouraud dextrose broth and incubated for 2 h at 37°C and 30°C, respectively. These inocula were adjusted to the 0.5 MacFarland standard (0.048 M $BaCl_2$ 0.5 mL + 0.18 M H_2SO_4 99.5 mL). For susceptibility testing, each 150 µL of adjusted bacterial or fungal suspension was spread on the sterile medium (trypticase soy agar or sabouraud dextrose agar) using sterile cotton swabs. The positive controls employed were chloramphenicol (30 mg) and nystatin (100 units) in the antibacterial and antifungal assays, respectively. Application of the samples (**1**-**16**) 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 prepared using the same procedures without samples or antibiotic, but with DMSO (25µL) were equally set as negative control. After incubation, the growth inhibition rings were quantified by measuring the diameter for the zone of inhibition in millimeters from the lower surface of the plates. All assays were carried out in triplicate.

Yeast antitopoisomerase assay: The yeast strains used in this study were *Saccharomyces cerevisiae* mutant cells JN362a, JN394, JN394 t_{-1} and JN394$t_{2-5,}$ containing recombinant forms of topoisomerase I and II enzymes and were kindly provided by Dr. John Nitiss of St. Jude Children's Research Hospital, Memphis, Tennessee. The requirement of topoisomerase II (Topo II) for the completion of mitosis makes this enzyme essential for cell division and cell proliferation. Differentiated cells express very low levels of Topo II, while highly proliferative and tumor cells often express 25-300 times the levels of quiescent cells (Heck and Earnshaw, 1986). A yeast *S. cerevisiae* strain clone forming assay was used as a model to measure topoisomerase I and II inhibition (Nitiss and Nitiss, 2001). Briefly, yeast cells were grown in YPD medium (yeast extract, peptone and dextrose) for 18 h in a shaking incubator. The logarithmically growing cells were then counted using a hematocytometer and adjusted to a concentration of 2 x 10^6 cells/ mL media. In all cases, cells were pre-grown at the same temperature that was used to measure drug sensitivity. Yeast cells (6 x 10^6 cells) were incubated at the optimal temperature for 24 h in the shaking incubator, in the presence of the **1**-**17**. Compounds **1**-**17** solutions in DMSO (50 µL) were prepared in concentrations given in the Table 1. The concentrations of the compounds used in this assay were based on the solubility factor in DMSO (enough DMSO to dissolve 5 - 10 mg of each sample).

The same concentrations were used with the JN362a, JN394t_{-1} and JN394t_{2-5}



	R	R_1	R_2	R_3
2	OH	H	COOH	CH_2OH
3	H	H	COOH	CH_3
3a	H	H	$COOCH_3$	CH_3
4	OH	αOH	COOH	CH_2OH
4a	OH	αOH	$COOCH_3$	CH_2OH
5	H	βOH	CH_3	CH_3



	R	R_1	R_2	R_3	R_4
6	Glc	H	H	H	Rha
7	Glc	H	OH	H	Rha
8	Glc	H	OH	Api	H
9	Glc-1→3-Glc	H	H	H	Rha
10	Glc-1→3-Glc	H	OH	H	Rha
10E	H	H	OH	H	Rha
11	Glc-1→3-Glc	H	OH	Api	H
12	Glc-1→3-Glc	H	OH	Api	Rha
12E	H	H	OH	Api	Rha
13	Glc-1→3-Glc	H	OH	Xyl	Rha
14	Glc	H	OH	Xyl	Rha
15	Glc-1→3-Glc	H	H	Xyl	Rha
16	Glc	H	H	Xyl	Rha
17	Glc-1→3-Glc	OH	OH	Api	Rha

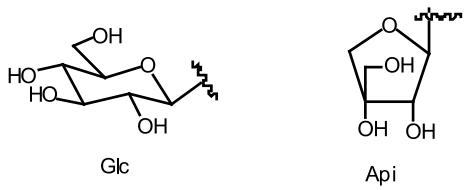


Figure 1. Compounds **1**-**17**

strains. DMSO (1.66%) was used as negative control, while CTP (50 mg/μL), a topoisomerase I inhibitor, and ETP (100 mg/μL), a topoisomerase II poison, were the positive controls. Viable counts were determined by duplicate plating to YPDA medium solidified with 1.75% agar Bacto. Plates were incubated at the optimal temperature for growth of the cells to determine viable titer (25°C for temperature sensitive top2 mutants, 30°C otherwise). The percent survival was determined by comparison of the number of colonies counted in the no-drug control culture with those in the drug-treated culture. All experiments were repeated at least three times, and the means and population standard deviations were calculated for each of the data.

Statistical Analysis: Results are expressed as the mean ± SD of values obtained in at least duplicate measurements

from three different experiments. A one-way ANOVA, with Dunnett and linear trend post test were used for statistical analysis. A probability (P) value of <0.05 indicated a significant difference.

RESULTS AND DISCUSSION

Compounds **2**-**4** and **6**-**17** (Figure 1) were obtained from *Sycios bulbosus* (**2-4**, **6**-**12**), *Microsechium helleri* (**13**-**16**) and *Sechium mexicanum* (**17**), as described in Hernández-Carlos *et al.* (2009; 2011) while saponins **10E** and **12E** were obtained by enzymatic hydrolysis of **10** and **12**, respectively, with β-glucosidase and purified by HPLC. Triterpenes **1**, **3** and **5** isolated from *B. conferta* were identified by comparing their physical and spectroscopic data with those of published values. Methyl esters (**3a**, **4a**) of **3** and **4** were obtained according to Hernández-Carlos *et al.* (2009). The pure substances and known **1**, baccharis oxide (Anthonsen *et al.*, 1970); **2**, bayogenin, (Eade *et al.*, 1963); **3**, oleanolic acid (Saad *et al.*, 1988); **4**, polygalacic acid (Seiligmann-Rodest and Polonsky, 1963); **5**, maniladiol (Quijano *et al.*, 1998); **6**, tacacoside C (Castro *et al.*, 1997); **7**, durantin III (Hiradate *et al.*, 1999); **8**, heterpappussaponin 5 (Bader *et al.*, 1994); **9**, tacacoside B3 (Castro *et al.*, 1997); **10** (Hernández-Carlos *et al.*, 2009); **11**, heterpappussaponin 7 (Bader *et al.*, 1994); **12**, (Hernández-Carlos *et al.*, 2009); **13** (Hernández-Carlos *et al.*, 2011), **14** (Hernández-Carlos *et al.*, 2011), **15**, amole F (León *et al.*, 1998); **16**, amole G (León *et al.*, 1998) and **17** (Eskander *et al.*, 2006) were subjected to antioxidant, antimicrobial and antitopoisomerase evaluation. Purity of each compound was examined by TLC (**1**-**5**) and HPLC (**6**-**17**) analysis, while the used concentration for all assays was based on the solubility factor of each compound in DMSO.

None of the studied compounds showed significant antioxidant activity. According to references (Wolska *et al.*, 2010), CH_2OH and COOH groups at C-24 and C-28 of oleanane triterpenoids are critical for bactericidal activity; in the antimicrobial assays of oleanane-type triterpenes **2**-**5,** maniladiol (**5**) showed no activity in all evaluated strains (Table 1), while bayogenin (**2**) showed activity against *S. epidermidis* (I.D. 13.20 mm) and the polygalacic acid (**4**) showed activity against *S. epidermidis* (I.D. 9.85 mm) and *C. albicans* (I.D. 13.5 mm). No difference in the extent of activity between Gram-positive (*S. epidermidis*) and -negative (*E. coli* and *K. pneumoniae*) bacteria was observed with baccharis oxide (**1**).

Antimicrobial activity of bayogenin saponins: Antimicrobial activity (Table 1) of bayogenin was improved with its glycoside amole F (**15**), which displayed the best antimicrobial activity in *S. epidermidis* (I.D. 9 mm), *C. albicans* (I.D. 11.7 mm), and *K. pneumonia* (I.D. 9.5 mm). The related saponin, amole G (**16**) with one glucose unit less than **15** (at C3 of aglycone), only showed activity against *C. albicans* (I.D. 10.5 mm) together with tacacoside C (**6**), which is equivalent to **16** without the xylose unit at C3 rhamnose inner (Figure 1). However, tacacoside B3 (**9**) did not show any antimicrobial activity in spite of its difference with **6** and **15**, which are an additional glucose unit at C3 of aglycone and a xylose unit at C3 rhamnose inner, respectively.

Antimicrobial activity of polygalacic acid saponins: Durantin III (**7**) and heterpappussaponin 5 (**8**) displayed toxic activity against strains of *C. albicans* (I.D. 8.5 mm) and *S. epidermidis* (I.D. 9.0 mm) respectively, both compounds have one glucose unit at C3 aglycone. The rest of polygalacic saponins did not show antimicrobial activity, although all saponins tested here are structurally related, for example, **12** contains an additional glucose unit at C3 of aglycone and a rhamnose unit at C3 of xylose in comparison with **8**, but did not

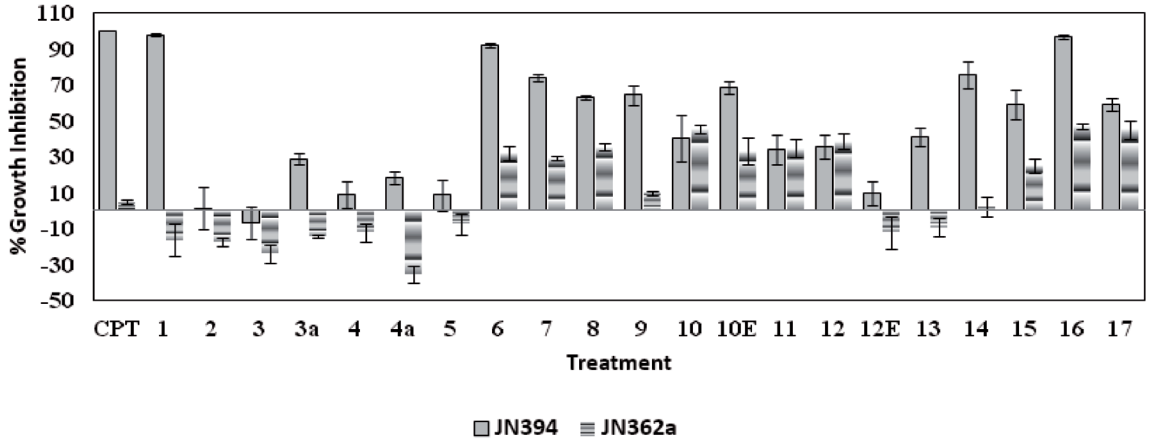


Figure 2. Growth inhibition (%) of **1**-**17** and positive control camptothecin (CPT)

show antimicrobial activity. It is noted that active saponins (**7** and **8**) contain five monosaccharide units while the rest of them contains six or seven monosaccharide units. The activity of polygalacic acid against several *C. albicans* strains is known (Bader *et al.*, 2000) and in this work we observed that the fungicide activity of polygalacic acid diminished when its glycosides were tested, this could be possible associated to the concentrations used of polygalacic acid and its glycosides (0.3202 µM and 0.1050-0.1374 µM, respectively). Then the effective concentration of polygalacic acid in the glycosides was diminished almost 50%.

Antitopoisomerase activity: As shown in Table 2 and Figure 3, the JN394 strain was hypersensitive to CPT (100.0%) which is a Topo I poison. Growth inhibition of this strain was achieved by **1** (97.57%), **3a** (28.60%), **6** (91.70%), **7** (73.90%), **8** (62.80%), **9** (64.10%), **10** (39.90%), **10E** (68.27%), **11** (33.5%), **12** (35.65%), **13** (40.80%), **14** (75.64%), **15** (59.00%), **16** (96.68%), and **17** (58.81%). The strain JN394 is DNA repair-deficient and drug permeable (carry *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-

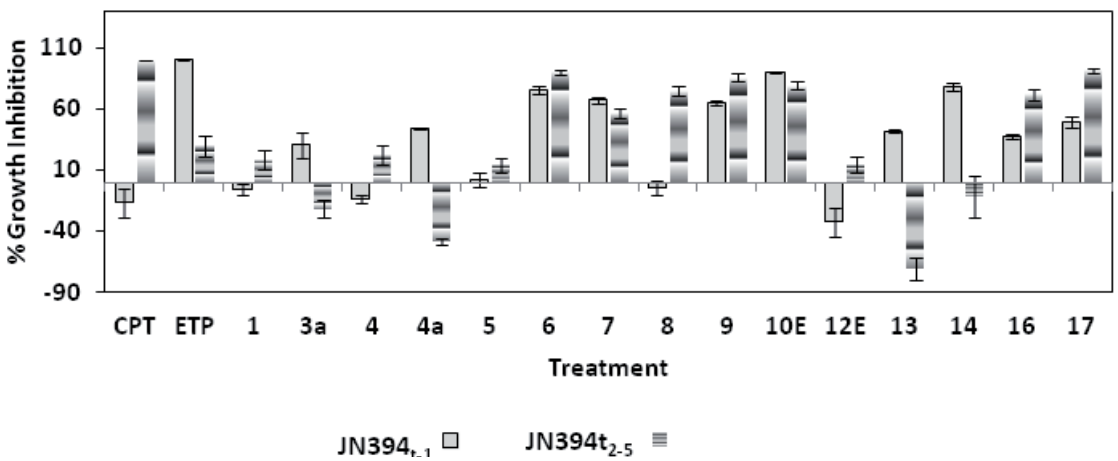


Figure 3. JN394t$_{-1}$ and JN394t$_{2-5}$ strains growth inhibition of **2**, **3a**-**10E**, **12E**-**14, 16**-**17**. Positive controls were camptothecin (CPT) and etoposide (ETP)

Table 2. Inhibition (%) of **1**-**17** on the survival of JN394, JN362a, $JN394_{t-1}$ and $JN394_{t2-5}$ strains

Treatment	Molar concentration µM	JN394	JN362a	$JN394_{t-1}$	$JN394_{t2-5}$
CPT	0.1435	*↓100.00 ± 0.0	↑4.66 ± 1.3	*↑16.60 ± 12.1	*↓99.82 ± 0.10
ETP	0.0849			*↓99.97±0.1	*↓30.03 ± 8.6
1	1.1726	*↓97.57 ± 1.0	*↑16.61 ± 8.9	↑5.95 ± 5.1	*↓18.59 ± 8.1
2	0.9515	↓1.11 ± 11.9	*↑17.29 ± 2.4		
3	0.9525	↑7.18 ± 8.9	*↑24.23 ± 5.20		
3a	0.7128	*↓28.60 ± 3.0	*↑14.25 ± 0.80	*↓30.60 ± 10.20	*↑21.97 ± 7.3
4	0.9610	↓8.79 ± 7.4	*↑12.50 ± 5.2		
4a	0.9653	↓8.79 ± 3.8	*↑12.50 ± 4.80		
5	0.6093	↓8.60 ± 8.7	↑7.73 ± 6.0		
6	0.4146	*↓91.7 ± 1.3	*↓31.64 ± 4.1		
7	0.3151	*↓73.90 ± 2.2	*↓28.83 ± 1.2		
8	0.4139	*↓62.80 ± 0.9	*↓35.01 ± 2.		
9	0.3650	*↓64.10 ± 5.3	↓9.53 ± 1.5	*↓65.15 ± 2.20	*↓86.08 ± 3.3
10	0.3556	*↓39.90 ± 12.7	*↓45.44 ± 2.3		
10E	0.4058	*↓68.27 ± 3.7	*↓32.90 ± 7.8		
11	0.3540	*↓33.50 ± 8.1	*↓34.49 ± 5.1		
12	0.3294	*↓35.65 ± 6.7	*↓38.42 ± 4.4		
12E	0.1549	↓9.59 ± 6.5	*↑12.47 ± 9.1		
13	0.3294	*↓40.80 ± 5.3	↑9.46 ± 5.3	*↓42.10 ± 1.0	*↑70.56 ± 9.1
14	0.3691	*↓75.64 ± 7.4	↓2.12 ± 5.7	*↓77.90 ± 3.5	*↑11.20 ± 17.7
15	0.3333	*↓59.00 ± 8.0	*↓24.94 ± 4.0		
16	0.3737	*↓96.68 ± 1.0	*↓46.55 ± 1.4		
17	0.3259	*↓58.81 ± 3.6	*↓44.95 ± 5.1		

DMSO (1.66%) was used as control and all the results were referred to this value. An asterisk indicates statistical differences in comparison to the control DMSO alone ($P<0.05$), using Dunnett´s test. ↓, decreaced versus DMSO. ↑, increased versus DMSO.Values are means±SD of triplicate determinations

proficient strain (Nitiss and Wang, 1988) and the compounds with antitopoisomerase activity do not inhibit the growth of this strain, but they do inhibit the JN394 strain growth. CPT as positive control, and a topoisomerase inhibitor, affects the JN394 growth but do not inhibit JN362a growth. The compounds with similar behavior to CPT were **1**, **3a**, **9**, **13** and **14** (Figure 3), which showed the following inhibition percent in the JN362a: -16.61, -14.25, 9.53, -9.46 and 2.12%, respectively. Compounds **6**, **7**, **8**, **10**, **10E**, **11**, **12**, **15**, **16** and **17**, inhibited both strains and represent cytotoxic compounds with action mechanisms different to the inhibition of topoisomerases. The compounds **2**, **3**, **4, 4a, 5** and **12E** did not inhibit any of the strains and therefore they are no considered cytotoxic. The strain $JN394t_{-1}$ is isogenic to JN394

and contains a disrupted top 1 gene (Nitiss and Wang, 1988): the absence of this gene resulted in diminution of antitopoisomerase I drugs cytotoxicity. Positive control CPT (0.1435 μM) fails to reduce the growth of these mutant cells, **1** was incapable of affecting the growth of the cells with the top1 mutation, therefore topoisomerase I is the target of **1** (Table 2 and Figure 3) at 1.1726 μM (-5.95%). Cytotoxic activity against cancer cells is known for **8** and **11** (Bader *et al.*, 1996); however **8** and **11** did not show antitopoisomerase activity. In contrast to the result for **1** no resistance was observed when JN394t_{-1} cell (contains a disrupted top1 gene) were treated with **3a, 9**, **13, 14** or the antitopoisomerase drug ETP, indicating that the observed effect is specific to antitopoisomerase II agents. The strain JN395$t_{2\text{-}5}$ carries a top2 allele that is resistant to multiple classes of topoisomerase II poisons at its permissive temperature (25°C) (Jannatipour *et al.*, 1993). Cells with the top2-5 mutation are able to grow in the presence of ETP (inhibition 30.03%), but not in the presence of CPT (99.82%). The compound **9** played as ETP. The strain has essentially the same sensitivity to CPT as JN394 (*rad52 top 2*$^{+}$ cells), indicating that the observed resistance is specific to antitopoisomerase II poison agents that trap the enzyme-mediated DNA cleavage. The compounds **3a**, **13** and **14** are inhibitors of the topoisomerase II enzyme, which have the ability to block the overall catalytic activity of the enzyme.

Baccharis oxide (**1**) resulted topoisomerase I inhibitor and it represents the first compound that is described of a baccharane-type triterpene with both antimicrobial and antitopoisomerase activities. Bayogenin (**2**) and polygalacic acid (**4**) showed not topoisomerase activity but their glycosides **9**, **13** and **14** did. Oleanolic acid (**3**) is described as topoisomerase II inhibitor when tested with an enzymatic assay (Mizushina *et al.*, 2003); in this work **3** and its methyl ester (**3a**) were subjected to evaluation using a yeast antitopoisomerase assay, being active only the derivative (antitopoisomerase II). The results suggested that **3** could be active but it is incapable of achieving the enzyme, contrary to the situation in the ester derivative (**3a**), where the polarity of **3** was diminished. This could facilitate the passing through the wall and membrane cell of the yeast. Considering these results, bayogenin (**2**), which contains one hydroxyl group less than polygalacic acid, probably could exhibit antitopoisomerase activity through its methyl ester. Biological tests of polygalacic acid and bayogenin are scarce due to several causes: a) they are not common in plants as oleanolic acid (Neto, 2011); b) they are obtained by acid hydrolysis of saponins and c) both compounds are poorly soluble in aqueous or organic solutions. These results are important examples of the biological activities of the oleanane type hydroxylated triterpenes, which are highly polar (Bader *et al.*, 2000).

Cytotoxic activity against cancer cells by maniladiol (**5**) has been described (Ling *et al.*, 1982), in this work maniladiol did not show antimicrobial or antitopoisomerase activities due to the lack of a carboxyl group at C28, which is important for both activities (Wolska *et al.*, 2010; Mizushina *et al.*, 2003), therefore maniladiol acts as cytotoxic in cancer cells through a different mechanism of topoisomerase inhibition.

Polygalacic acid glycosides that has antitopoisomerase II activity are **13** (0.3294 μM) and **14** (0.3691 μM), where the oligosaccharide chains help to enhance the availability of **4,** no matter **13** has one glucose unit more than **14** at C3 aglycone. However, **10** (0.3556 μM), **11** (0.3540 μM), and **12** (0.3294 μM) were cytotoxic to *S. cerevisiae*, even though these compounds are structurally related with the no cytotoxic saponin **12E**. As it is observed in Figure 2, compound **12** lost its cytotoxic activity when it was hydrolyzed to **12E**. All tested saponins are capable of crossing cell membranes

due to their surfactant properties and their activities seem to be related to the sugar attached at C3 and C28 of aglycone. For example, the bayogenin saponin **9** possess one additional glucose unit at C3 aglycone in comparison with **6**, therefore the antitopoisomerase II poison activity observed for **9** seems dependent on the glucose extra at C3 aglycone. Meanwhile, the antitopoisomerase activity of polygalacic acid saponins (**13** and **14**) was observed with two or one glucose unit at C3 aglycone.

Saponins tacacoside C (**6**) and tacacoside B3 (**9**) had been described as cytotoxic in cancer cells (Castro *et al.*, 1997), according to our results this activity could be due to antitopoisomerase II activity of **9** and not yet identified the cytotoxic mechanism of **6**.

CONCLUSIONS

In this work it is shown that there is antitopoisomerase I activity of baccharis oxide (**1**); the antitopoisomerase II activity was observed with methyl oleanolic acid (**3a**), polygalacic acid saponins (**13, 14**) and the bayogenin saponin (**9**). Moreover, the moderate antimicrobial activity (**1**-**4**, **6**-**8**, **15**, **16**) and the good cytotoxicity against *S. cerevisiae* through topoisomerase inhibition was shown by **1**, **3a**, **9**, **13** and **14** establishing the potential of these compounds in the development of anticancer drugs. Also, in this work it was described for the first time the antimicrobial and antitopoisomerase activity of a baccharane-type triterpene.

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