

Antioxidant and antitopoisomerase activities in plant extracts of some Colombian flora from La Marcada Natural Regional Park

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Abstract: Many plants have been used to treat some diseases and infections since time immemorial, and this potential has been exploited by the pharmaceutical industry in the search of new analgesic, anticarcinogenic and antimicrobial agents, among other active agents. In order to contribute with bioprospection studies on the Colombian flora, 35 extracts from 13 plant species belonging to seven families (Apocynaceae, Cactaceae, Costaceae, Eremolepidaceae, Passifloraceae, Solanaceae and Urticaceae) were collected from La Marcada Natural Regional Park (LMNRP), Colombia. Dichloromethane, *n*-hexane and aqueous-methanol crude extracts were prepared and evaluated for their activity against *Saccharomyces cerevisiae* RS322N, R52Y and RS321 strains in the yeast mutant assay and their antioxidant capacity through the DPPH test. The dichloromethane extract from *Myriocarpa stipitata* (Urticaceae) showed moderate inhibitory activity against the three *S. cerevisiae* strains tested. The capacity of the dichloromethane extract from *M. stipitata* to inhibit the enzyme topoisomerase I and to cause DNA damage was inferred from these results. In the DPPH assay, the *n*-hexane crude extract from *Costus* sp. (Costaceae) showed good antioxidant activity (48%); in addition, the crude dichloromethane and aqueous-methanol extracts from *Rhipsalis micrantha* (Cactaceae) showed moderate antioxidant activity with percentage of 29 and 21%, respectively. Rev. Biol. Trop. 59 (3): 1089-1097. Epub 2011 September 01.

Key words: DNA damage agent, DPPH, DNA topoisomerase inhibitors, *Saccharomyces cerevisiae*.

Many bioactive compounds and their derivatives have been shown to inhibit carcinogenesis in a number of experimental systems involving initiation, promotion and progression steps of this disease. Therefore, the discovery of novel naturally occurring anticarcinogens which would prevent, slow down and/or reverse cancer induction and its subsequent development are of paramount importance to produce new effective medicines (Rajeshkumar *et al.* 2002).

DNA topoisomerases play important functions in transmission and expression of genetic information, because these enzymes carry out critical roles in DNA replication, transcription and recombination, as well as chromosome

condensation and segregation (Colley *et al.* 2004). Two major classes of topoisomerase enzymes have been identified, type I and type II. The former one introduces one break, while the last type produces transient double strand breaks in DNA (Dong *et al.* 2000). Topoisomerase inhibitors constitute a class of chemopreventive agents that hamper carcinogenesis via their antiproliferative or cell-differentiating action (Ramirez-Mares *et al.* 2004). Antitopoisomerase drugs bind and stabilize the complex (DNA Topoisomerase) and the enzyme cannot relax (Zhang & Siede 2003). In this manner, several studies have shown the usefulness of the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen as a model organism

for characterization and screening of anticancer drugs with inhibitory properties against topoisomerases I and II (Simon *et al.* 2000, Niño *et al.* 2007).

On the other hand, in living organisms the reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to cause damage to lipids, proteins, enzymes and nucleic acids leading to cell or tissue injury, implicated in the processes of aging as well as in a wide range of degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer, Parkinson and coronary heart pathologies, among others (Moon & Shibamoto 2009). However, cells have defence systems to protect and keep their cellular constituents in the right redox state; this includes two different pathways: enzymatic and non enzymatic ones. The former one includes enzymatic systems such as the superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase. *In vivo*, these antioxidant enzymes should have the proper equilibrium to reduce and to prevent ROS and RNS species generation within the cells and to protect cellular constituents from oxidative damage (Gasparri 2005). The non enzymatic one includes small antioxidant molecules like ascorbate, chlorogenic acids, polyunsaturated fatty acids, sugars and vitamin E, among others (Reddy *et al.* 2008).

Continuing with the bioprospection study on the flora from La Marcada Natural Regional Park (LMNRP, Risaralda, Colombia) as a source of new secondary metabolites with diverse grade of biological activities and taking in consideration the fact that there is still great interest in finding novel and better inhibitory agents against topoisomerases or with antioxidant activities, prompted us to screen *n*-hexane, dichloromethane and aqueous-methanol crude extracts belonging to the selected families collected to study these activities.

MATERIAL AND METHODS

Plant material: The adult aerial plant samples (leaves and branches) used for this study were collected in June-July 2005 in La Marcada Natural Regional Park (LMNRP, Colombia) with an altitudinal range between 1 600-2 000m.a.s.l., a temperature variation between 16-22°C and an average rainfall of 2 464mm/year. The plants were identified taxonomically by Dr. F.J. Roldán and a voucher specimen for each plant material was deposited at the University of Antioquia Herbarium, Medellín, Colombia (Table 1).

Plant samples (Table 1) were oven dried at 50°C for 48h, the dry materials were ground to a fine powder and aliquots of 300g were extracted by maceration with 900mL of methanol (three times each). Then, the solvent was pulled out and concentrated to dryness in a rotary evaporator at 45°C. A total of 25g of each concentrated methanol extract was dissolved in 100mL of a mixture of methanol-water (2:8) and were successively partitioned with 300mL of *n*-hexane and dichloromethane to obtain the *n*-hexane, dichloromethane and aqueous-methanol extracts, for each plant. The different extracts were concentrated to dryness at reduce pressure and stored at -10°C until assayed.

In this study, the solvents *n*-hexane, dichloromethane, dimethylsulfoxid (DMSO), methanol and ethanol (analytical grade) were purchased from Mallinckrodt (Phillipsburg, NJ, USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH), Nystatine and D-glucose were obtained from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract, peptone and agar were acquired from Oxoid (Basingtoke, United Kingdom). Silica gel plates (F₂₅₄ 0.2mm layer thickness) were purchased from Merck (Darmstadt, Germany).

Yeast mutant bioassay: The bioactivity of the crude plant extracts against *Saccharomyces*

cerevisiae was determined by the well diffusion method according to Ríos *et al.* (1988). *S. cerevisiae* mutant strains RS322N, R52Y and RS321 were supplied by Dr. David G.I. Kingston and were used for the topoisomerase assay. *S. cerevisiae* strains were cultured in erlenmeyers containing YPD broth. Each microorganism was diluted with a sodium chloride isotonic solution to obtain 25% of transmittance (optical density at 600nm). One milliliter of this microorganism suspension was thoroughly mixed with 20mL of sterile YPD agar in each Petri dish.

After solidification, seven wells (6.0mm diameter each) were made in each plate under sterile conditions. Each well was filled with 20 μ L of each one of the five different concentrations tested (4 000, 2 000, 1 000, 500 and 250mg/L) for each plant extract. A volume of 20 μ L of nystatin at 30, 150, 40mg/L for RS322N, R52Y and RS321 strains, were used as positive control, respectively; 20 μ L of DMSO-EtOH-H₂O (1:1:2) (for no-polar extracts) and EtOH-H₂O (1:4) (for polar extracts) were used as negative controls. All plates were incubated at 30°C for 24h. Inhibition zone diameters around each well were measured and recorded at the end of the incubation time. All determinations were performed in triplicate with two replicates, and the mean standard deviations (\pm SD) were obtained with these values. Activity was determined from a dose to response curve and is reported as IC₁₂(μ g/mL) values, which is the concentration required for an extract to produce an inhibition zone of 12mm in diameter, around each 6.0mm diameter well in this bioassay (Gunatilaka & Kingston 1998).

In this bioassay, an extract is considered active if it displays selective activity against one or more repair deficient yeast. For instance, an agent that displays greater activity against RS321 rather than to RS322N, with an IC₁₂ less than one third between both yeast strains and in general if they exhibit an IC₁₂ less than 2 000, most probably mediates its inhibitory activity through topoisomerase II. Conversely, greater activity against RS322N implies an inhibitory

DNA topoisomerase I mechanism (Gunatilaka & Kingston 1998, Zhou *et al.* 2000).

Antioxidant activity: The antioxidant activities were measured through the DPPH radical scavenging activity by the method of Brand-Williams *et al.* (1995), with minor modifications; for example, the *n*-hexane, dichloromethane and aqueous-methanol extracts were dissolved in the same mixtures used in the yeast mutant assay and they were also used as blank in the DPPH assay (A_{517 Blank}). All plant extracts were evaluated at 250mg/L by mixing 0.75mL of each one with 1.5mL of a freshly prepared DPPH solution (20mg/L) and stood for 20 minutes under light protection at room temperature (A_{517 Sample}). Then, the absorbances were measured at 517nm and the percentage of DPPH radical scavenging activity for each plant extract was calculated by applying the equation: (I%)=(A_{517 Blank}-A_{517 Sample})/A_{517 Blank}) \times 100. Each assay was performed in triplicate with two replicates in different times. With these values the mean standard deviations (\pm SD) were calculated.

Phytochemical screening: For each plant extract a phytochemical screening was performed testing the presence of secondary metabolites by using TLC analysis. The solvent systems *n*-hexane-ethyl acetate (6:4) was used for development of *n*-hexane extracts; while, ethyl acetate-methanol-water (100:13.5:10) was used for elution of dichloromethane and the aqueous-methanol extracts, respectively. The following spray reagents Dragendorff, anisaldehyde-sulphuric acid, 1% vanillin in sulphuric acid-ethanol, 2% aluminium chloride in ethanol, 1% ferric chloride, and hydroxylamine-ferric chloride were used as chromogenic agents for alkaloids, sterols, saponins, flavonoids, tannins and lactones, respectively (Wagner & Bladt 1996). All determinations were done in duplicates.

RESULTS

Yeast mutant bioassay: A total of 35 different crude plant extracts (*n*-hexane,

TABLE 1
Inhibition concentration (IC₁₂ µg/mL) of 35 crude plant extracts collected at La Marcada Natural Regional Park (LMNRP).

Family	Species (Voucher number)	Extract ¹	<i>Saccharomyces cerevisiae</i> strains IC ₁₂ (µg/ml)±SD			
			RS322N	R52Y	RS321	
Apocynaceae	<i>Mandevilla veraguensis</i> Hemsl. (FJR 3955)	H ¹	- ⁴	-	-	
		DC ²	-	-	-	
		A-Mt ³	-	-	-	
	<i>Blepharodon mucronatum</i> (Schltdl.) Decne. (FJR 3952)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
	<i>Mandevilla fendleri</i> (Mull.Arg.) Woodson (FJR 3951)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
Cactaceae	<i>Rhipsalis micrantha</i> DC (FJR 3948)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
Costaceae	<i>Costus</i> sp. (FJR 3958)	H	-	-	-	
		DC	NE ⁵	NE	NE	
		A-Mt	-	-	-	
Eremolepidaceae	<i>Antidaphne viscoidea</i> Poepp. & Endl. (FJR 3956)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
Passifloraceae	<i>Passiflora suberosa</i> L. (FJR 3954)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
	<i>Passiflora rubra</i> L. (FJR 3953)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
Solanaceae	<i>Solanum aturense</i> Humb. & Bonpl. Ex Dunal (FJR 3947)	H	-	-	-	
		DC	-	-	-	
		A-Mt	-	-	-	
Urticaceae	<i>Myriocarpa stipitata</i> Benth (FJR 3959)	H	6 607±1	2 910±2	5 208±1	
		DC	835±4	434±5	1 306±3	
		A-Mt	-	-	-	
	<i>Urera baccifera</i> Gaudich (FJR 3957)	H	-	-	-	
		DC	NE	NE	NE	
		A-Mt	-	-	-	
	indet. sp. 1 (FJR 3946)	H	-	-	-	
		DC	-	-	-	
		A-Mt	NE	NE	NE	
	<i>Phenax rugosus</i> (Poir.) Wedd. (FJR 3960)	H	-	-	-	
		DC	-	-	-	
		A-Mt	NE	NE	NE	
	Positive control		Nystatin	30	150	40

¹ H=*n*-Hexane; ² DC=Dichloromethane; ³ A-Mt=Aqueous-methanol; ⁴ (-)=Not active;

⁵ NE=Not evaluated.

dichloromethane and aqueous-methanol) were screened for their activity against *S. cerevisiae* strains in the yeast mutant assay. The results are shown in Table 1. The dichloromethane extracts from *Myriocarpa stipitata* Benth. (Urticaceae) was the only one that showed moderate topoisomerase I activity.

Phytochemical screening: The phytochemical screening of the *n*-hexane, dichloromethane and aqueous-methanol extracts showed the presence of different types of secondary metabolites, namely saponins, tannins, flavonoids and terpenes (Table 2). These phytochemicals were present in almost all the

extracts tested. However, *M. stipitata* (Urticaceae) extracts did not show evidence of flavonoids and *Costus* sp. (Costaceae) did not display the presence of tannins neither lactones (Table 2).

Antioxidant activity: Table 3 shows the percentage of antioxidant activity from the plant extracts studied. The aqueous-methanol extracts were the most effective in the DPPH assay. But, the *n*-hexane extract of *Costus* sp. exhibited the highest percentage (48%) of antioxidant activity while hydroquinone (the positive control) showed a percentage of 26%.

TABLE 2
TLC phytochemical screening of crude plant extracts collected at La Marcada Natural Regional Park (LMNRP)

Family	Species	Extract	Phytochemicals					
			Alkaloids	Terpenes	Flavonoids	Tannins	Saponins	Lactones
Apocynaceae	<i>Mandevilla veraguensis</i>	H	-	+++	-	+++	+++	-
		DC	-	++	-	+++	+++	-
		A-Mt	-	-	+++	+++	+	-
	<i>Blepharodon mucronatum</i>	H	-	-	-	-	+++	-
		DC	-	+	+++	-	+++	-
		A-Mt	-	-	+++	++	+	-
	<i>Mandevilla fendleri</i>	H	-	+++	-	-	+++	-
		DC	-	++	-	+	+++	-
		A-Mt	-	-	-	-	-	-
Cactaceae	<i>Rhipsalis micrantha</i>	H	-	-	-	-	++	-
		DC	-	-	-	-	++	-
		A-Mt	-	+	-	-	++	-
Costaceae	<i>Costus</i> sp.	H	-	-	-	-	++	-
		DC	+	+++	+++	-	++	-
		A-Mt	-	-	+++	-	-	-
Eremolepidaceae	<i>Antidaphne viscoidea</i>	H	-	-	-	+++	++	-
		DC	NE	NE	NE	NE	NE	NE
		A-Mt	-	-	-	-	++	-
Passifloraceae	<i>Passiflora suberosa</i>	H	-	+++	-	+++	+++	-
		DC	+	+	-	++	++	+++
		A-Mt	-	-	++	-	-	-
	<i>Passiflora rubra</i>	H	-	-	-	++	++	-
		DC	-	+	-	++	++	+
		A-Mt	-	+++	-	-	+++	-
Solanaceae	<i>Solanum aturense</i>	H	-	-	-	+	+++	++
		DC	-	-	-	-	++	-
		A-Mt	-	-	++	-	-	-

TABLE 2 (Continued)
TLC phytochemical screening of crude plant extracts collected at La Marcada Natural Regional Park (LMNRP)

Family	Species	Extract	Phytochemicals					
			Alkaloids	Terpenes	Flavonoids	Tannins	Saponins	Lactones
Urticaceae	<i>Myriocarpa stipitata</i>	H	-	++	-	+	++	-
		DC	+++	-	-	-	++	-
		A-Mt	-	-	-	-	+	-
	<i>Urera baccifera</i>	H	-	-	-	-	++	++
		DC	NE	NE	NE	NE	NE	NE
		A-Mt	-	-	-	-	-	-
	indet. sp. 1	H	-	++	-	-	++	++
		DC	+	+++	-	++	++	-
		A-Mt	NE	NE	NE	NE	NE	NE
	<i>Phenax rugosus</i>	H	-	-	-	-	++	++
		DC	-	-	-	-	++	-
		A-Mt	NE	NE	NE	NE	NE	NE
Standards		Papaverine	Taxol	Kaempferol	Gallic Acid	Digitonin	Digitoxin	
		+++	+++	+++	+++	+++	+++	

¹H=*n*-Hexane; ²DC=Dichloromethane; ³A-Mt=Aqueous-methanol.

(-)=Absent; (+)=Weak content; (++)=Moderate content; (+++)=Strong content; NE=Not evaluated.

TABLE 3.
Percentage of antioxidant activity from the plant extracts studied

Family	Species	Percentage of antioxidant activity \pm SD*		
		<i>n</i> -Hexane	Dichloromethane	Aqueous-methanol
Apocynaceae	<i>Mandevilla fendleri</i>	-	18 \pm 1.8	14 \pm 1.4
	<i>Blepharodon mucronatum</i>	-	-	17 \pm 2.8
	<i>Mandevilla veraguensis</i>	-	-	18 \pm 2.2
Cactaceae	<i>Rhipsalis micrantha</i>	-	29 \pm 1.6	21 \pm 1.5
Costaceae	<i>Costus</i> sp.	48 \pm 2.4	NE	-
Eremolepidaceae	<i>Antidaphne viscoidea</i>	-	-	13 \pm 1.7
Passifloraceae	<i>Passiflora suberosa</i>	-	-	-
	<i>Passiflora rubra</i>	-	-	-
Solanaceae	<i>Solanum aturense</i>	-	22 \pm 1.5	22 \pm 1.3
Urticaceae	<i>Myriocarpa stipitata</i>	-	19 \pm 1.8	20 \pm 1.2
	<i>Urera baccifera</i>	-	NE	-
	indet. sp. 1	-	-	NE
	<i>Phenax rugosus</i>	-	-	NE
Control	Hydroquinone		26	

* Values are the mean of triplicate readings; (-) = Not active; NE = Not evaluated

DISCUSSION

Since the crude dichloromethane extract from *M. stipitata* (Urticaceae) showed an IC₁₂ against the RS322N strain (835±4µg/mL) rather than to RS321 strain (1 306±3µg/mL), this implies that this plant extracts showed moderate DNA topoisomerase I inhibitory activity; the other plant extracts did not show any DNA topoisomerase I or II inhibitory activity at all. The last results correlate for instance, with the extract of some Cactaceae species (*Rhipsalis baccifera* (Mill.) Stearn and *Rhipsalis mesembryanthemoides* Steud.) that were not active against any of *S. cerevisiae* strains assayed (Valente *et al.* 2007).

The result with *M. stipitata* is in concordance with those found for the antineoplastic activity from *Boehmeria siamensis* Craib (Urticaceae), because both plants contain alkaloid. From *B. siamensis* has been isolated the phenanthroquinolizidine type alkaloids boehmeriasin A and B, and the latter one showed antitumor activity as evidenced by an *in vitro* bioassay (Luo *et al.* 2003, Yan *et al.* 2006). According to the phytochemical screening (Table 2), it can be deduced that the biological activity showed by the crude dichloromethane extract from *M. stipitata* could be attributed to phytochemicals such as alkaloid or saponin present on this extract. The relevance of DNA topoisomerase I inhibitory activity displayed by *M. stipitata* extract is based on the fact that there are not reports on the phytochemicals neither on the biological activity for this species or genus in the yeast mutant assay (Dyer *et al.* 2003).

Furthermore, these results on the DPPH assay suggest that the *n*-hexane extract from *Costus* sp. is a powerful natural antioxidant and according to the phytochemical screening this activity could be explained based on its saponin contents. From *Costus speciosus* SM. and *Costus spicatus* Sw. (Costaceae) there has been isolated a series of furostanol saponins (Sigh & Thakur 1982, Da Silva *et al.* 1999) which could explain their *in vitro* antioxidant activity.

Recently, interest has increased considerably in finding saponins with antioxidant properties. They have been described as antioxidant agents against free radical mediated cellular damage (Somova *et al.* 2003, Garcez *et al.* 2006, Dini *et al.* 2009), inhibitors of membrane lipid peroxidation (Yoshiki *et al.* 1996, Keum *et al.* 2000) and protection against low density lipoproteins oxidation (Li *et al.* 2008).

In addition, the antioxidant activity of *Rhipsalis micrantha* DC (Cactaceae) correlates well with the antioxidant activities of *Opuntia ficus-indica* (L.) Mill.; however, in the last species this activity could be explained based on its flavonoid contents such as quercetin and myricetin present in their extracts (Lee *et al.* 2002); while in the former one this activity could not be explained based on its flavonoid contents, since in our phytochemical screening this type of phytochemicals were not detected.

On the other hand, the aqueous-methanol extract from *Mandevilla veraguensis* Hemsl. (Apocynaceae) showed a moderate percentage of antioxidant activity. This result is in concordance with the rate and the antioxidant activity displayed by *Holarrhena pubescens* Wall. (Apocynaceae) in the total antioxidant *in vitro* assay (Palasuwan *et al.* 2005), and with a moderate antioxidant activity of aqueous extract from *Mandevilla pentlandiana* (A.DC) Woodson (Borneo *et al.* 2009); furthermore, *Rauvolfia sellowii* Müll. Arg. (Apocynaceae) also displayed good scavenging activity in the DPPH test (Menezes *et al.* 2004). Moreover, the aqueous-methanol extract from *Solanum aturense* Humb. & Bonpl. Ex Dunal (Solanaceae) also showed a moderate percentage of antioxidant activity and this result correlates with the high antioxidant activity exhibited by the methanol extracts of *Solanum nigrum* L. fruits in the DPPH assay (Al-Fatimi *et al.* 2007).

Our results suggest that the crude dichloromethane extracts from *Myriocarpa stipitata* (Urticaceae) and *Rhipsalis micrantha* (Cactaceae), as well as the *n*-hexane extract from *Costus* sp. (Costaceae) showed good bioactivity that provide evidence related to the potentiality from the LMNRP flora in the

search for novel antioxidant compounds. In addition, the fact that the dichloromethane extract from *M. stipitata* (Urticaceae) displayed topoisomerase I inhibition is a good argument in the search of anticarcinogenic agents from plant sources. Further research is needed to isolate the compounds responsible for these biological activities.

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RESUMEN

Desde tiempos inmemoriales, muchas plantas han sido usadas para el tratamiento de varias enfermedades e infecciones, este potencial ha sido explotado por la industria farmacéutica en la búsqueda de nuevos agentes analgésicos, anticancerígenos y antimicrobianos, entre otros. Consientes con esto, se evaluó la actividad de 35 extractos de 13 especies de plantas recolectadas en el Parque Regional Natural La Marcada (PRNLM, Colombia) contra las cepas mutadas de *Saccharomyces cerevisiae* RS322N, R52Y y RS321 en el ensayo de la levadura mutada y la capacidad antioxidante de los extractos a través del método del DPPH. El extracto crudo de diclorometano de *Myriocarpa stipitata* (Urticaceae) presentó actividad moderada contra las tres cepas de *S. cerevisiae* evaluadas. Lo cual permitió inferir la capacidad del extracto de diclorometano de esta especie para inhibir la enzima topoisomerasa I y causar daño al ADN. Además, en el ensayo del DPPH, el extracto de *n*-hexano crudo de *Costus* sp (Costaceae) mostró actividad antioxidante buena (48%), mientras que los extractos de diclorometano y acuoso metanólico crudos de *Rhipsalis micrantha* (Cactaceae) tuvieron actividad antioxidante moderada, con valores del 29 y 21%, respectivamente.

Palabras clave: agentes que dañan el ADN, DPPH, inhibidores de las ADN topoisomerasas, *Saccharomyces cerevisiae*.

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