Assessment of the genotoxic and photoprotective effects of *Phyllanthus mirificus* (*Phyllanthaceae*)

Evaluación de los efectos genotóxicos y fotoprotectores de *Phyllanthus mirificus* (*Phyllanthaceae*)

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ABSTRACT

Ultraviolet radiation constitutes a natural physical carcinogen that could induce DNA damage, which, if not repaired, might generate mutations. Plants of the genus *Phyllanthus* (*Phyllanthaceae*) are used in traditional medicine worldwide. Recent investigations support their genoprotective activity against chemical and physical mutagens, including UV radiation. The purpose of this work was to evaluate the genotoxic and photoprotective activity of the aqueous extract obtained from *Phyllanthus mirificus*. Two experimental models were used: *ex vivo* pCMUT plasmid and *Caulobacter crescentus* cells. The genotoxicity and genoprotection tests carried out were colony-forming capacity, clastogenicity and SOS Chromotest. UVC was used as an irradiation treatment. The UV absorption capacity and the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals by the extract were measured. In addition, its phytochemical composition was analyzed. The cytotoxic effect was only statistically significant at 2 mg/mL; however, it was not genotoxic at any of the concentrations tested. The *P. mirificus* extract showed antioxidant capacity and good levels of UVC absorbance from 0.1 mg/mL. However, the extract exhibited a low photoprotective capacity. It only reduced the induction of UVC-SOS in *C. crescentus* cells, at high concentrations (> 1 mg/mL), and did not prevent the generation of plasmid DNA damage. This result suggests that the antioxidant and absorptive properties are not enough to carry out photoprotection.

Keywords: Antioxidant, aqueous extract, *Caulobacter crescentus*, phytochemistry, plasmid assay

RESUMEN

La radiación ultravioleta constituye un agente carcinogénico físico, presente en la naturaleza, que puede inducir daños en el ADN, los cuales, si no son reparados correctamente, pueden generar mutaciones. Las plantas del género *Phyllanthus* (*Phyllanthaceae*), son utilizadas en la medicina tradicional alrededor de todo el mundo. Investigaciones recientes demuestran su actividad genoprotectora frente a mutágenos físicos y químicos, incluyendo la radiación UV. El objetivo de este trabajo fue evaluar la actividad genotóxica y fotoprotectora del extracto acuoso de *Phyllanthus mirificus*. Se utilizaron dos modelos experimentales: plásmido pCMUT *ex vivo* y células de *Caulobacter crescentus*. Los ensayos de genotoxicidad y fotoprotección realizados fueron: la capacidad formadora de colonias, clastogenicidad y el SOS Chromotest. La capacidad de absorción de la luz UV y el secuestro de radicales 2,2-difenil-1-picrilhidrazilo (DPPH) también fueron medidos. Adicionalmente, se analizó la composición fitoquímica del extracto. El efecto citotóxico del extracto solo fue estadísticamente significativo a 2 mg/mL; sin embargo, no resultó genotóxico a ninguna de las concentraciones evaluadas. El extracto tiene capacidad antioxidante y altos niveles de absorción de la luz UV desde los 0.1 mg/mL, sin embargo, mostró bajos niveles de actividad fotoprotectora. Solo fue capaz de reducir la inducción de SOS en las células de *C. crescentus* a concentraciones de 1 mg/mL, y no evitó la generación de daños en el ADN plasmídico. Los resultados sugieren que las propiedades antioxidantes y de absorción de la radiación UV, no son suficientes para llevar a cabo la fotoprotección.

Palabras clave: Antioxidante, extracto acuoso, *Caulobacter crescentus*, fitoquímica, ADN plasmídico

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INTRODUCTION

The ultraviolet (UV) light emitted by the sun constitutes a physical mutagenic agent that affects ecosystems and the human population as it is absorbed by DNA molecules. The direct damages generated are pyrimidine dimers, while indirect effects may produce oxidized bases. These damages, when not repaired, could lead to mutations, which can ultimately trigger skin and eye-related disorders, including cancer (Londero & Schuch 2019, Pfeifer 2020). Short-wavelength UVC radiation from sunlight is absorbed by the ozone layer; however, it is emitted by artificial sources commonly used in certain social practices of human life (De Flora 2013). This constantly motivates scientific efforts to develop better photoprotection strategies (Londero & Schuch 2019).

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The use of natural compounds is an important emerging strategy for photoprotection. Plants are exposed to ambient ultraviolet light; therefore, they have developed photoprotection mechanisms that guarantee their survival. In addition, due to their secondary metabolism, phytocomponents exhibit great molecular diversity and biological functions (Fuentes 2019).

Traditional medicine uses natural products, mainly plant-derived, as ways to prevent or ameliorate several diseases. Nowadays, a great part of the pharmaceuticals available are still derived from plants, and there is a growing worldwide interest in the use of phytopharmaceuticals in modern medicine (Anand & *al.* 2019, Calixto 2019).

Plants belonging to the genus *Phyllanthus* L. in a wide concept (*Phyllanthaceae*), comprise close to 1,200 species widely distributed throughout most tropical and subtropical countries, with high diversity in the Americas, continental Africa, Madagascar, and Australia and the Pacific Islands (Hoffmann & *al.* 2006, Ralimanana & Hoffmann 2011, Falcón-Hidalgo & *al.* 2020). They are well known for their medicinal properties, mostly as part of Indian Ayurveda, Traditional Chinese Medicine, and Indonesian Jamu, with infrequent side effects (Sarin & *al.* 2014). Extensive phytochemical and pharmacological studies have been conducted on numerous *Phyllanthus* species. These species are commonly used as antipyretics, antibacterial, antiviral, antispasmodic, and for the treatment of intestinal infections, genitourinary disorders, and diabetes. Their traditional uses and diverse pharmaceutical applications have been extensively reviewed in the last few years (Nisar & *al.* 2018, Kumar & *al.* 2020).

Several extracts obtained from *Phyllanthus acidus* (L.) Skeels, *P. amarus* Schumach. & Thonn., *P. niruri* L., *P. emblica* L., *P. indofischeri* Bennet, *P. muellerianus* (Kuntze) Exell*, P. urinaria* L., *P. debilis* J.G. Klein ex Willd. among many others, are known as natural sources of antioxidant phytocomponents (Nisar & *al.* 2018). Furthermore, *in vitro* and *in vivo* genoprotective activity of many *Phyllanthus* species aqueous extract against different chemical and physical mutagens have been reported (Ahmad & *al.* 2015, Menéndez-Perdomo & Sánchez-Lamar 2017). Particularly, photoprotective capacity against UV-induced DNA damage has been demonstrated for *P. emblica* and *P. niruri* aqueous extracts (Majeed & *al.* 2011, Raja & *al.* 2011, Menéndez-Perdomo & Sánchez-Lamar 2017) and the effects correlated with their high antioxidant activity.

In Cuba, the genus *Phyllanthus* is highly diverse, standing out for a high level of endemism (Falcón & *al.* 2020), and many studies support their antiviral, antioxidant, anti-inflammatory, and antimutagenic properties. The alcoholic extracts of *P. formosus* Urb*, P. chamaecristoides* Urb. and *P. microdictyus* Urb. were found to *in vitro* inactivate the Hepatitis B surface antigen (del Barrio & *al.* 1995). The aqueous extract of *P. orbicularis* Kunth*,* has shown a broad spectrum of antiviral (Roque & *al*. 2011, del Barrio & Roque 2014), antioxidant (Ferrer & *al.* 2002, Sánchez-Lamar & *al.* 2015), antimutagenic (Sánchez-Lamar & *al.* 1999, Ferrer & *al.* 2001, 2002, Fuentes & *al.* 2006, Alonso & *al.* 2010), and photoprotective (Vernhes & *al*. 2013a, 2013b, 2016, 2018a, 2018b) actions. The antioxidant and photoprotective properties of *P. williamioides* Griseb.*, P. chamaecristoides,* and *P. microdictyus,* among other native species, have also been proven. The latter two species also showed antimutagenic properties against UVC light (Menéndez-Perdomo & *al.* 2017).

Although all these beneficial properties have been studied and well established in the scientific literature, a limited number of *Phyllanthus* species from the Cuban flora have been examined for possible genotoxic and/or genoprotective effects. In the present work, the cytotoxic and genotoxic effects of the Cuban endemic *Phyllanthus mirificus* G.L. Webster aqueous extract were assessed as a premise. The photoprotective effects of *P. mirificus* are informed, for the first time, including antioxidant and transmittance measurements and a report of its phytochemistry.

MATERIALS AND METHODS

Plant material and extract

The Cuban endemic plants of *Phyllanthus mirificus* were collected in spring of 2011, in Guantánamo province, Cuba. The specimen voucher *T. Borsch & al. 4891* is housed at HAJB #007628 (Herbarium Johannes Bisse of the National Botanical Garden of Cuba). The aqueous extract was obtained from the leaves and stems following a previously described method by Menéndez-Perdomo & *al.* (2016). The treatments (0.001, 0.01, 0.1, 1 and 2 mg/mL) were prepared at the time of performing the assays, diluting the lyophilisate in Milli-Q water.

Phytochemical analysis

The aqueous extract was qualitatively analyzed using a standard procedure described by Menéndez-Perdomo & *al.* (2017). Total phenolic content was estimated from a standard curve of Gallic acid. Polyphenol content was expressed as micrograms of Gallic acid equivalent per milligrams of plant lyophilized (µg GAE/mg lyophilized). All measurements were done by triplicate.

LC-High Resolution-MS^n

This analysis was done as previously described in Francioso & *al.* (2019). Ultra Performance light Chromatography-Diode Array Detector-Mass Spectrometry (UPLC-DAD-MS) was performed on a Waters Acquity H-Class UPLC system (Waters, Milford, MA, USA). This equipment includes a quaternary solvent manager (QSM), a sample manager with a flow-through needle system (FTN), a photodiode array detector (PDA), and a single-quadrupole mass detector with electrospray ionization source (ACQUITY QDa). Columns Waters C18 HSST3 (100 mm × 2.1 mm i.d., 1.7 µm particle size) were used to perform chromatography. Solvent A consisted of 0.1% aqueous Formic acid (HCOOH) and Solvent B was 0.1% HCOOH in acetonitrile (CH3CN). The column temperature was set at 25 °C and the flow rate was 0.5 mL/min. Elution was performed

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isocratically for the first minute with 2% B; then, solvent B was linearly increased to 55% from minute 1 to minute 6; after that, from minute 6 to 10, solvents A and B were set at 20% and 80%, respectively. In 0.5 min solvent B was set at 100% and maintained for 2 min. Before the next injection the column was re-equilibrated with 98% A and 2% B.

Samples were dissolved in the mobile phase, and 10 µL injected through the needle. The PDA detector was set up in the range of 200 to 600 nm. The effluent from the PDA detector was connected on-line to an LTQ-Orbitrap Elite mass spectrometer equipped with high-temperature electrospray ionization (HESI) ion source, controlled by the Excalibur 2.7 software (Thermo Fisher Scientific, Bremen, Germany) and operated in the negative ion mode. The ion spray voltage was set to 4.0 kV, and the sheath and auxiliary gasses were set on 20 and 5 psi, respectively. The Orbitrap-MS spectra were acquired within the m/z range of 50–2,000 and a resolution of 30,000. The tandem mass spectra were acquired by collision-induced dissociation (CID) in a linear ion trap (LIT) at 35% normalized collision energy and isolation width of 2.0 m/z. The fragments were detected at an FT-resolution of 30,000.

Scavenger capacity evaluation

Detection of the scavenger capacity of the extract was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, according to a methodology previously described by Menéndez-Perdomo & *al.* (2017). As a positive control (S)-(-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was used, and the reagents mixed without any sample was the negative control. Scavenger capacity was expressed as the number of antioxidants necessary to decrease the initial DPPH·absorbance by 50% respecting the negative control value (effective concentration 50, EC_{50}). All measurements were done in triplicate.

Transmittance quantification

The absorption capacity of the extract against UVC, UVA, and UVB irradiation were quantified as the transmittance. The wavelength range used was 250 to 400 nm, using a spectrophotometer Genesis™ 10S UV-VIS (Thermo Scientific). The negative control was distilled water. Measurements were done in triplicate.

Experimental models systems and procedures

pCMUT: *ex vivo* experimental model

Supercoiled circular DNA pCMUT (1,762 bp) was employed. This plasmid contains a bacterial replication origin, an ampicillin resistance gene, and cut sites for the restriction enzyme *EcoRI*.

In all cases, 2 µL of plasmid DNA and 18 µL of Milli-Q water were used as negative control. As positive control, 2 µL of UVC-irradiated DNA plasmid and 16 µL of NET buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8) were used, incubated and digested with T4-endo V (2 µL) at 37 °C for 1 hour as previously described by Schuch & *al.* (2009).

Clastogenicity induced by *Phyllanthus mirificus*

For the evaluation, 2 µL of plasmid were mixed with 2 µL of aqueous extract (twice concentrated). Samples were incubated at 37 °C for 30 minutes. Then, 20 µL were completed with Milli-Q water. This same protocol was applied to evaluate the effect of increasing the exposure time at the maximum non-toxic concentration previously tested.

Photoprotection exerted by *Phyllanthus mirificus*

In the photoprotection assessment, a variation of the described protocol was used. The extract was mixed with plasmid DNA, in a 1:1 proportion (v/v), and UVC irradiated with a Vilber Loumart T15M 15 W lamp (300 J/m^2). After samples were incubated with 70 ng of T4 bacteriophage endonuclease V (T4-endo V, produced in this laboratory) to discriminate the cyclobutane pyrimidine (CPD) DNA lesions. These samples were incubated at 37 °C under the conditions previously described.

Electrophoresis and visualization

To separate the conformations of plasmid DNA: supercoiled native conformation (form S) and relaxed conformation (form R) resulting from single-strand breaks electrophoresis in 0.8% agarose gels in TBE buffer (44.5 mMTris–H_3BO_3, 50 mM EDTA, pH 8) was performed. Aliquots from each sample were mixed with 5 µL of loading buffer (75 mM EDTA; 50% glycerol; 0.2% bromophenol blue) and applied in a horizontal gel electrophoresis chamber Model MINIS-150VS, at 100 volts for 40 min. The gel was stained with ethidium bromide (0.1 mg/µL), and DNA bands were visualized using a wavelength of 312 nm in a UVITEC transilluminator, Model BTS-26.LM and photographed using an 8-megapixel Alcatel One Touch camera.

Quantification of results

The images were quantified with the program *ImageJ 1.53a*, developed by the U.S. National Institute of Health (http://imagej.nih.gov/ij). The intensity of each of the plasmid bands was measured for each of the conformations. In this way, using the formula proposed by Schuch & *al.* (2009), it is possible to obtain the number of breaks caused in the plasmid through the following formula:

$$X = -\ln (1.4 \times FI/1.4 \times FI + FII)/1.8$$

Where FI represents the intensity of fluorescence measured in the supercoiled DNA bands, FII the intensity in the relaxed DNA bands. 1.4 is a factor employed for correcting the increased fluorescence of ethidium bromide when bound to the relaxed form compared to the supercoiled form, and 1.8 is pCMUT vector size in kbp.

***Caulobacter crescentus in vitro* experimental model**

A NA1000 pP3213 strain of *Caulobacter crescentus* was kindly provided by the Department of Microbiology (Instituto de Ciências Biomédicas, Universidade de São Paulo, Brazil) and was used in this study. This strain was previously obtained by transforming the wild NA1000 strain with pP3213 plasmid, containing the *imuA* SOS response gene promoter in transcriptional fusion with the *lacZ* gene, coding

for *β*-galactosidase enzyme. The cells were grown in optimal conditions, as reported by Galhardo & *al.* (2005).

Afterward treatment with different concentrations of *P. mirificus*, aliquots were taken to perform the Survival and SOS Colorimetric assays as described below. For photoprotective analysis, cells were simultaneously treated before, during, and 2h after UVC irradiation at 45 J/m^2.

In both assays as a negative control, cells harvested in the medium were used. Only UVC irradiated cells were used as a positive control. All measurements were done in triplicate, and the experiments were repeated three times.

Survival assay

A 10 µL aliquot was removed after each treatment for serial dilutions and plated on solid PYE medium for cell viability determination after 48 h incubation at 30°C, and the number of colonies was counted. Survival was expressed as a percentage of the control values.

SOS Colorimetric assay

After the procedure explained above, the OD_{600nm} for each sample was determined. Then 50 µL aliquots were dispensed in tubes containing 800 µL of a permeabilization solution for cell disruption (buffer Z: Na_2HPO_4 8.5 g/L; NaH_2PO_4 7.18 g/L; KCl 0.75 g/L; $MgSO_4.7H_20$ 0.51 g/L); 50 µL of chloroform and 2.88 µL of *β*-mercaptoethanol; then mixed, and incubated for 5 min at room temperature. Afterward, 200 µL of nitrophenyl-*β*-D-galactopyranoside (ONPG) substrate was added at 4 mg/mL in phosphate solution (Na_2HPO_4 16.1 g/L; NaH_2PO_4 5.5 g/L), and after 5 min of incubation, the reactions were stopped using 400 µL of Na_2CO_3 1 M. Finally, the OD_{420nm} was measured and *β*-galactosidase activity was calculated as described previously (Galhardo & *al.* 2005), by the following relationship:

$$U = (DO_{420nm} \times 1\,000)/(DO_{6oo\ nm} \times Vol \times t)$$

Where: U: *β*-galactosidase enzymatic activity; OD_{420nm}: optical density value registered at 420 nm after the enzymatic reaction has occurred; OD_{600nm}: optical density value registered at 600 nm after incubation; Vol: cell culture volume = 0.05 mL; t: enzymatic reaction time = 5 min.

Statistical analysis

Means and the corresponding standard deviation (SD) were determined for each treatment. Controls and treatments were analyzed using the Kolmogorov-Smirnov test for Normality, Brown-Forsythe test for variance homogeneity, and compared using the Dunnett test, all of them performed by the software *Statistica v.10*.

RESULTS

Phytochemical analysis

The detection of the main phytocomponents families in the *Phyllanthus mirificus* extract was based on colorimetric/precipitation principles. No alkaloids were detected, and the average presence of phenols, tannins, flavonoids, anthocyanidins, catechins, and reducing sugars was obtained. The total phenolic content of aqueous extract was 200.57 ± 6.21 µg of GAE/mg, the simplest form of phenolic compound.

LC-MS Polyphenols determination

Chromatographic analysis of *Phyllanthus* aqueous extract allowed the identification of five major polyphenols and flavonoids according to the previous work of Francioso & *al.* (2019) on the Cuban endemic species *Phyllanthus orbicularis*. In Table I, the detected compounds and their chromatographic and spectroscopic features are reported.

TABLE I

Chromatographic and spectrometric features of identified compounds

*Identified by analytical standard injection.

TABLA I

Características cromatográficas y espectrométricas de los compuestos identificados

**Identificado mediante inyección de patrón analítico.*

Peak	Retention Time (min)	Compound	Molecular Formula	λ_{max}abs (nm)	MS^1 [M − H]$^-$ (m/z)	MS^2 [M − H]$^-$ (m/z)	MS^3[M − H]$^-$ (m/z)
1	3.49	Protocatechuic acid glucoside	$C_{13}H_{16}O_9$	290	315.0717	153.0196	109
2	3.52	p-Cumaroyl-glucaric acid	$C_{15}H_{16}O_{10}$	326	355.0668	191.0198	147; 85
3	4.35	Procyanidin C[1]	$C_{45}H_{38}O_{18}$	281	865.1986	847.1882; 739.1667; 695.1407; 577.1353	[865-577] 289
4*	4.51	Rutoside	$C_{27}H_{30}O_{16}$	355	609.1460		
5	4.60	Nicotiflorin	$C_{27}H_{30}O_{15}$	343	593.1514	285.0403	255; 227; 151

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Rutoside (compound 4) was identified by standard analytical comparison. Negative mode HRESIMS analyses of the other eluting peaks evidenced deprotonated ions [M−H]− (UV-Vis λ_{max}) at *m/z* 315.0717(255,sh290 nm), *m/z* 355.0668 (326 nm), *m/z* 865.1984 (280 nm), and *m/z* 593.1514 (255 and 354 nm) corresponding to compounds 1, 2, 3, 5, respectively.

The UV-Vis absorption features of compound 1 ($C_{13}H_{16}O_9$, Rt 3.49 min) fit with the presence of a protocatechuic moiety (255 nm; sh 290 nm). The MS^2 fragmentation pattern of the parent ion (*m/z* 315.0717) is consistent with protocatechuic acid glucoside, showing the presence of the major fragment at *m/z* 153.0196 derived from the loss of the sugar moiety. MS^3 of this fragment gives rise to an ion at *m/z* 109 following the structure of this molecule.

Compound 2 ($C_{15}H_{16}O_{10}$, Rt 3.52 min) shows an UV-Vis spectrum with λmax at 326 nm typical of hydroxycinnamate conjugated systems. ESI-MS^2 fragmentation of the pseudo molecular ion at *m/z* 355.0668 generates fragments at *m/z* 147.0301 and m/z 163.0405, resulting from two different cleavages of the ester bond and two complementary fragments at m/z 209.0304 ($C_6H_9O_8$) and *m/z* 191.0198, referring to glucaric acid and its dehydration product, respectively. MS^3 of the fragment at *m/z* 191 gives rise to subsequent glucaric acid decarboxylation products at *m/z* 147.1865 and m/z 85.0297. Peak 2 was consequently identified as p-cumaroyl-glucaric acid.

Compound 3 shows a molecular ion at *m*/*z* 865.1986 and a UV-vis λ_{max} at 281 nm. This molecule was previously identified in this extract as the epicatechin trimer procyanidin C1. The MS/MS fragmentation of the precursor ion generates the dimer at *m*/*z* 577.1353 with the same subsequent MS/MS fragmentation pattern as procyanidin B1/B2 type molecules, confirming the nature of this compound.

Peak 5 was identified as the flavonol glycoside nicotiflorin. MS/MS fragmentation of the pseudomolecula rion (*m*/*z* 593.1514 $[M-H]^-$) gives rise to the aglycone part at *m*/*z* 285.0403, corresponding to a kaempferol moiety. Further, MS/MS of the fragment at *m*/*z* 285 generates fragments at *m*/*z* 255, 227, and 151.

Genotoxicity evaluation: Survival and SOS Colorimetric assays

To detect whether *Phyllanthus mirificus* aqueous extract was capable of producing primary DNA damage in a cellular context, we measured SOS response induction in *Caulobacter crescentus*. As shown in Figure 1, no genotoxic effect was observed; however, the survival at the higher dose tested (2 mg/mL) was statistically decreased.

Clastogenicity evaluation: DNA plasmid *ex vivo* assay

In order to detect if *Phyllanthus mirificus* caused direct damage to the DNA molecule, the clastogenicity of different concentrations was evaluated. Figure 2 shows that the aqueous extract did not induce rupture in DNA at any of the concentrations tested.

DPPH radical scavenging assay

The DPPH radical scavenging assay was used to evaluate the antioxidant potential of *Phyllanthus mirificus* aqueous extract. The extract demonstrated better antioxidant activity compared to the positive control, Trolox (Table II).

Transmittance quantification

To evaluate photoprotective capacity of the extract, transmittance was measured (Figure 3). The values of lower concentrations tested (0.001-0.01 mg/mL) were near to 100% of transmittance, allowing the pass of all UV radiation spectra. However, it increases at concentrations higher than 0.1 mg/mL; that absorbs UV light, especially in the UVC and UVB ranges. The 1 and 2 mg/mL concentrations completely absorb these wavelength ranges.

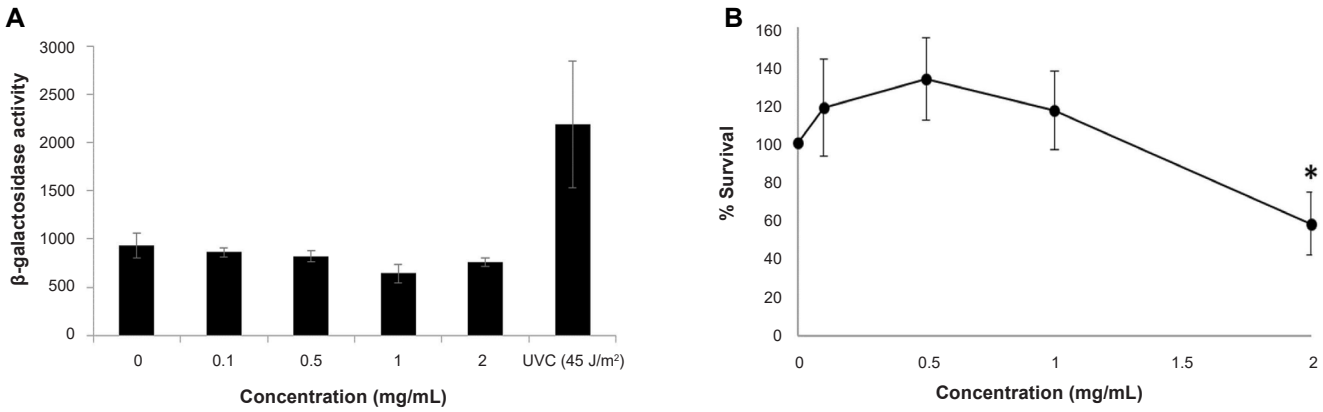

Fig. 1. Effects of *Phyllanthus mirificus* aqueous extract in *Caulobacter crescentus* cells. **A.** Induction of *β*-galactosidase activity. **B.** Percentage of cell survival. The results shown are the means of at least three independent experiments, each performed in triplicate. Error bars indicate the SD (*) $p < 0.05$ Dunnett's Test.

Fig. 1. Efectos del extracto acuoso de *Phyllanthus mirificus* en células de *Caulobacter crescentus*. **A.** Inducción de la actividad *β*-galactosidasa. **B.** Porcentaje de supervivencia celular. Los resultados mostrados son la media de al menos tres experimentos independientes, realizados por triplicado. Las barras de error indican la DE (*) $p < 0,05$ Prueba de Dunnett.

Photoprotection of *Phyllanthus mirificus* extract: *Caulobacter crescentus*

The aqueous extract concentrations used in the photoprotective assays were not cytotoxic or genotoxic in the experimental models used. The extract did not exhibit a positive response (Figure 4A). The best photoprotective effect was achieved by higher doses of *Phyllanthus mirificus* extract (1 mg/mL), which poorly diminish *β*-galactosidase activity. Also, aqueous extract concentrations used in survival assays showed a percentage of *Caulobacter crescentus* colonies survival around 50% (Figure 4B); similar survival percentage to only UV light-exposed cells was found.

Photoprotection of *Phyllanthus mirificus* extract: DNA plasmid *ex vivo* assay

The incubation with *Phyllanthus mirificus* aqueous extract, before, during and after UVC irradiation did not reduce the amount of DNA photodamage (Figure 5). Also, the extract did not inhibit the nicking T4-endo V activity on DNA damaged (treatment 7).

DISCUSSION

This study initially evaluated the potentially harmful effect of the aqueous extract of *Phyllanthus mirificus,* an endemic Cuban plant. In the bacterial model *C. crescentus* was performed the SOS Chromotest, one of the simplest genotoxic short-term assays. No primary DNA damage was observed, although cell survival was significantly reduced at the highest dose tested, 2 mg/mL (Figure 1). In lower doses, survival increased by more than 100%, suggesting that certain compounds in this species may enhance the colony-forming capacity in this model. The cell-free plasmid DNA assay is a sensitive method for the measurement of DNA damage at the primary structure level. The results did not show

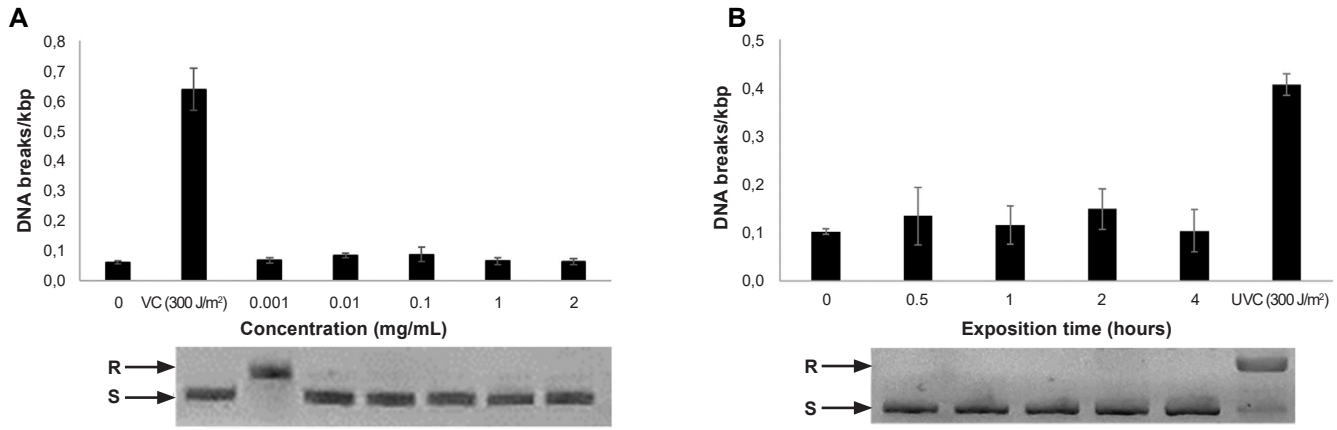

Fig. 2. Clastogenicity of *Phyllanthus mirificus* aqueous extract. **A.** Different concentrations. **B.** Increasing exposure times (1 mg/mL). The results shown are the mean of at least three independent experiments. Error bars indicate the standard error (*) $p < 0.05$ Dunnett's Test. S: supercoiled form; R: relaxed form.

Fig. 2. Clastogenicidad del extracto acuoso de *Phyllanthus mirificus*. **A.** Diferentes concentraciones. **B.** Aumento de los tiempos de exposición (1 mg/mL). Los resultados mostrados son la media de al menos tres experimentos independientes. Las barras de error indican el error estándar (*) $p < 0,05$ Prueba de Dunnett. S: forma superenrollada; R: forma relajada.

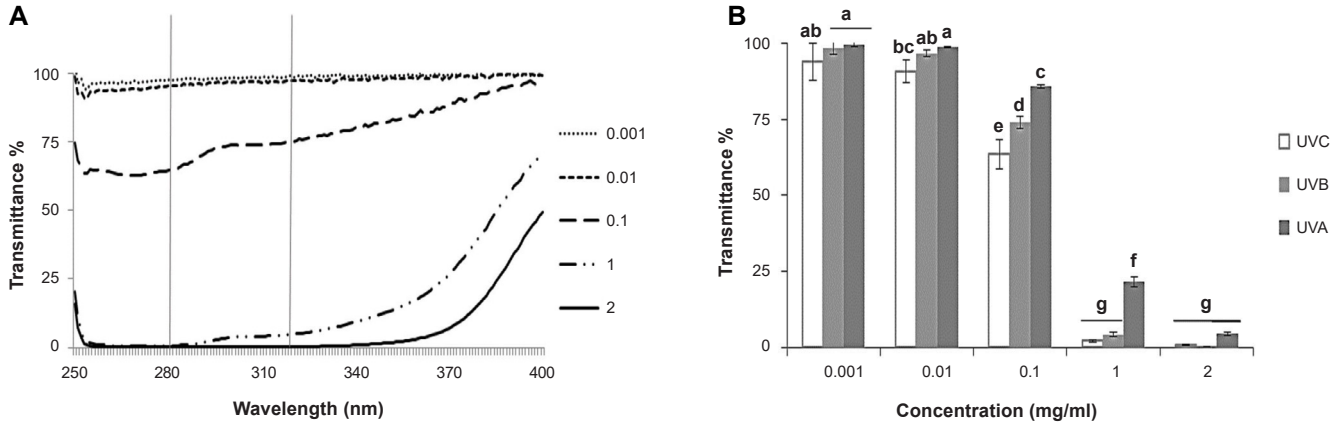

Fig. 3. The transmittance of *Phyllanthus mirificus* aqueous extract in the range of 250 to 400 nm UV light. **A.** Comparison between concentrations transmittance variation in the full range. **B.** Comparison between punctual wavelength: 254 (UVC), 312 (UVB), and 365 (UVA) nm. The results shown are the means of at least three measurements with corresponding standard deviation (SD).

Fig. 3. Transmitancia del extracto acuoso de *Phyllanthus mirificus* en el rango de luz UV de 250 a 400 nm. **A.** Comparación entre concentraciones de la variación de la transmitancia de en todo el rango. **B.** Comparación entre longitudes de onda puntuales: 254 (UVC), 312 (UVB) y 365 (UVA) nm. Los resultados mostrados son la media de al menos tres mediciones con su correspondiente desviación estándar (DE).

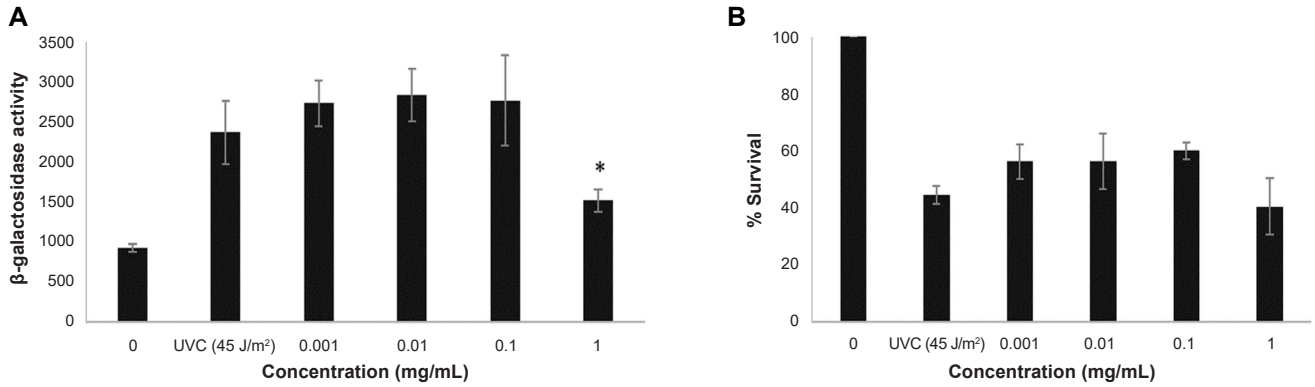

Fig. 4. Effects of *Phyllanthus mirificus* aqueous extract treatment in *Caulobacter crescentus* UVC irradiated cells. **A.** Induction of *β*-galactosidase activity. **B.** Survival assay. The results shown are the means of at least three independent experiments, each done in triplicate. Error bars indicate the SE (*) $p < 0.05$ Dunnett Test when compared to the only UVC irradiated treatment.

Fig. 4. Efectos del tratamiento con extracto acuoso de *Phyllanthus mirificus* en células de *Caulobacter crescentus* irradiadas con UVC. **A.** Inducción de la actividad *β*-galactosidasa. **B.** Ensayo de sobrevivencia. Los resultados mostrados son la media de al menos tres experimentos independientes, cada uno de ellos realizado por triplicado. Las barras de error indican el SE (*) $p < 0,05$ Prueba de Dunnett en comparación con el único tratamiento irradiado con UVC.

TABLE II

Antiradical activity of *Phyllanthus mirificus* aqueous extract and Trolox measured by DPPH scavenging assay a and b are significantly different for Student T Test, $p < 0.05$

TABLA II

Actividad antirradical del extracto acuoso de *Phyllanthus mirificus* y Trolox medida mediante ensayo de eliminación de DPPH a y b son significativamente diferentes para la prueba T de Student, $p < 0,05$

Treatment	EC_{50} (mg/mL)
Phyllanthus mirificus aqueous extract	0.12a
Trolox	0.29b

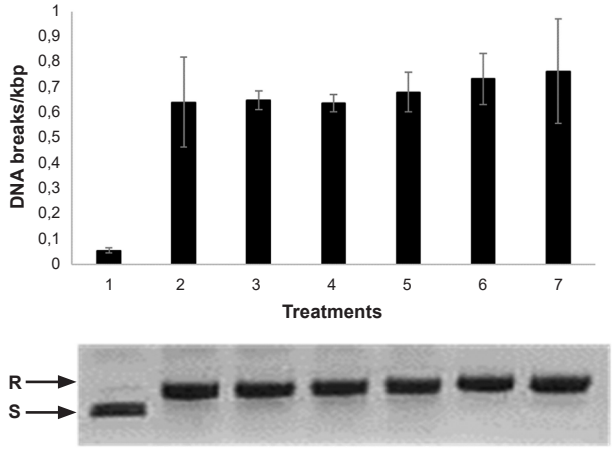

Fig. 5. Effects of *Phyllanthus mirificus* aqueous extract in UVC irradiated plasmid DNA and inhibition of T4-endo activity (1 mg/mL). (1): negative control, (2): UVC irradiated plasmid (300 J/m^2), (3-6): UVC irradiated plasmid incubated with aqueous extract (0.001, 0.01, 0.1 and 1 mg/mL), (7): inhibition of T4-endo activity. The results shown are the means of at least three independent experiments. Error bars indicate the standard error (*) $p < 0.05$ Dunnett Test. S: supercoiled form; R: relaxed form.

Fig. 5. Efectos del extracto acuoso de *Phyllanthus mirificus* en ADN plasmídico irradiado con UVC e inhibición de la actividad endo-T4 (1 mg/ml). (1): control negativo, (2): plásmido irradiado con UVC (300 J/m^2), (3-6): plásmido irradiado con UVC incubado con extracto acuoso (0,001, 0,01, 0,1 y 1 mg/mL), (7): inhibición de la actividad endo-T4. Los resultados mostrados son la media de al menos tres experimentos independientes. Las barras de error indican el error estándar (*) $p < 0,05$ Prueba de Dunnett. S: forma superenrollada; R: forma relajada.

clastogenic effects in the DNA structure at any concentration tested. Also, increasing the exposure time of 1 mg/mL dose did not induce breaks in the DNA molecule (Figure 2).

These results agree with previous studies of the toxicity of this aqueous extract using *in vitro* and *in vivo* approaches.

The evaluation tested in the human lung cancer cell line A549 showed no significant diminishing of cell survival tested up to 1 mg/mL, is a survival rate around 100% (Wong 2013). In the same study, acute toxicity assays in the *Artemia salina* showed that LC50 was higher than 5 mg/mL. Our results agree with these outcomes, although they have been performed in different experimental models.

Our findings are coherent with the reports for other Cuban species of *Phyllanthus* in different experimental models. The aqueous extract of *P. orbicularis* (2 mg/mL) did not induce either primary DNA damage or mutation when *ex vivo* experiments with plasmid DNA, SOS gene induction, gene reversion and conversion, and SMART assays were performed (Sánchez-Lamar & *al.* 2002, Cuétara & *al.* 2012, Vernhes & *al.* 2013b). The toxicity of the aqueous extract of *P. williamioides* assessed in *Caulobacter crescentus* cells results significant for the concentration of 2 mg/mL.

However, no toxic effects were observed neither for *P. microdictyus* nor *P. chamaecristoides* in the same model cells (Menéndez-Perdomo & *al.* 2016). Based on the results of toxicity in *Caulobacter crescentus* and previous reports for the genus, the concentration of 2 mg/mL was excluded for evaluating any protective effects from the extract in UVC irradiation in biological models.

Plant extracts are complex mixtures of natural substances, where active component(s) might comprise numerous constituents that could be present in highly variable amounts. As part of a preliminary qualitative study of the composition of *Phyllanthus mirificus*, this species possesses higher concentrations of phenolic compounds when compared to other endemic *Phyllanthus* species (Menéndez-Perdomo & *al.* 2017). Although fewer tannins and phenols were found, the amount of flavonoids and reducing sugars was quite the same, as were antocianidins and catechins. The chemical characterization was performed as previously for *P. orbicularis* and *P. chamaecristoides,* using the same patterns (Francioso & *al.* 2019). The results for *P. mirificus* only showed lower amounts of the same compounds already informed: Protocatechuic acid glucoside, p-Cumaroyl-glucaric acid, Procyanidin, Rutoside, and Nicotiflorin. Thus, only Table I was presented instead of the chromatogram, for comparison purposes.

There have been found that compounds that might have photoprotective action in plants are mostly polyphenols, compounds with several conjugated double bonds, and benzene rings that provide UV light absorption and antioxi-dant properties (Rojas & *al.* 2016). Phytochemical analysis confirms that *Phyllanthus mirificus* is rich in components that absorb UV light, mostly products of secondary metabolism that confers them multiple properties. DPPH and transmittance measures are previous analyses for vegetal extract photoprotective activity; it is an indicator to evaluate the antioxidant and absorbance capacities of the extract, which validates deeper studies using biological markers.

Antioxidant properties were measured by DPPH radicals scavenging assay, which evaluates the reaction capacities of the extract with DPPH radicals, and hence, inhibition of its absorbance. Higher absorbance inhibition indicates elevated extract antioxidant properties. The *Phyllanthus mirificus* extract possesses scavenging properties better than the positive control used (Trolox). However, species like *P. epiphyllanthus* L. and *P. chamaecristoides* show even better results according to their median inhibitory capacity (IC_{50}) (Wong 2013). Also, this author reports that DPPH radicals scavenging properties increase alongside extract concentration in every *Phyllanthus* species tested, including *P. mirificus*. These results show that the extract has positive antioxidant capacities so it might act as an inhibitor of UV-induced oxidative damage.

Transmittance was evaluated by scanning extract concentrations in the wavelength range of 250 to 400 nm that comprises all UV light spectra. The results showed that the extract has a lower transmittance for UVB and UVC light, which are the most dangerous (Radice & *al.* 2016, Rojas & *al.* 2016). Transmittance results for higher doses 1 and 2 mg/mL were very similar and equal for UVC, reinforcing the exclusion of 2 mg/mL for photoprotection assays. According to the chemical analysis of *Phyllanthus mirificus* aqueous extract, it is known that it has a presence of polyphenols, including phenols, tannins, and flavonoids, although the amounts are not higher than other *Phyllanthus* species such as *P. chamaecristoides* and *P. orbicularis* (Wong 2013, Menéndez-Perdomo & *al.* 2017, Francioso & *al.* 2019).

Photoprotection results show that *Phyllanthus mirificus* extract did not reduce SOS response or increment survival percentage in *Cauobacter crescentus* cells. Despite the antioxidant effects found and the low transmittance values obtained for higher concentrations, the absorbance properties cannot block UV light completely. Only the higher concentration of 1 mg/mL showed a b-galactosidase activity similar to the negative control so this one might have a certain photoprotective effect. Nevertheless, in the survival assay, this concentration did not increase survival percentage, so the decrease in SOS response appears to be only the reflection of the high level of mortality due to UVC radiation in the cellular population. The plasmid DNA assays either showed a positive response, which indicate that the extract, in direct contact with the DNA, is not capable of exerting a genoprotection effect against the UVC induced damage.

These results contrast with those obtained for other Cuban *Phyllanthus* species. *P. orbicularis* acts as a desmutagen due to its capacity to absorb all UV radiation for all concentrations tested (0.001 to 1 mg/mL) (Vernhes & *al.* 2013b). Also, *P. microdictyus*, *P. williamiodes,* and *P. chamaecristoides* presented a positive response in B- galactosidase and survival assays diminishing the damages UV generated (Menéndez-Perdomo & *al.* 2017).

CONCLUSIONS

The *Phyllanthus mirificus* extract does not have genotoxic activity, and the cytotoxic effect was only statistically significant at 2 mg/mL. Despite the antioxidant capacity and good levels of UVC absorbance, the extract exhibited a low photoprotective capacity. These results indicate that the *P. mirificus* extract cannot induce biological activity as an enhancer of DNA repair mechanisms or the compounds responsible for this property are in very low quantities, or synergism is necessary, and only the lack of one of them produces inactivity.

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AUTHOR'S CONTRIBUTION

F. Fuentes-León coordinated the research, directed the execution of the experimental work, analyzed the data and wrote the first version of the manuscript. A. Rubio performed most of the experimental work, analyzed the data and wrote the last version of the manuscript. L. Sánchez, M. Carrazco and M. Wong-Guerra participated in the execution of the experimental work. I. Spengler and A. Francioso performed, respectively, the obtaining of the extract and the phytochemical analysis of its composition. B. Falcón-Hidalgo collected the plant material and reviewed the botanical characterization of the species. J. Piloto and C.F.M. Menck participated in the discussion and interpretation of the results. A. Sánchez-Lamar conceived the original idea and design of the research, participated in the elaboration of figures and discussion of results, made all the critical revisions of the manuscript. All authors reviewed the final version of the manuscript and corrected the reviewers' comments.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that they have no conflict of interest.
Ethics approval: All authors have carried out fieldwork and data generation ethically, including obtaining appropriate permitting.
Consent for publication: All authors have consented to publishing this work.

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