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ARTÍCULO ORIGINAL

Assessment of cytotoxic and genotoxic effects of Cuban endemic *Phyllanthus* (Phyllanthaceae)

Evaluación de los efectos citotóxicos y genotóxicos de especies de Phyllanthus (Phyllanthaceae) endémicas de Cuba

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RESUMEN

Las plantas pertenecientes al género *Phyllanthus* (Phyllanthaceae) son empleadas en la medicina tradicional para el tratamiento de diversas enfermedades. Varias especies presentes en la flora cubana poseen propiedades antivirales, antioxidantes y antimutagénicas. Sin embargo, los extractos vegetales constituyen mezclas complejas ricas en diversos fitocomponentes, por lo que pudieran ejercer efectos tóxicos. En el presente trabajo se evaluó la toxicidad de los extractos acuosos obtenidos de tres especies de *Phyllanthus* endémicas de Cuba: *P. williamioides* Gr., *P. chamaecristoides* Urb., and *P. microdictyus* Urb. La citotoxicidad fue determinada mediante el ensayo de Sobrevivencia Bacteriana y la genotoxicidad mediante el SOS Chromotest, en el rango de concentraciones 0,1 – 2 mg/mL y empleando células de *Caulobacter crescentus* como modelo experimental *in vitro*. Los valores de CL_{50} no se detectaron experimentalmente en el rango de concentraciones evaluadas. Los extractos acuosos de *P. chamaecristoides* y *P. microdictyus* no mostraron respuestas citotóxicas ni genotóxicas y este último mostró una sobrevivencia mayor del 90 % en todo el rango de concentraciones evaluado. Solo se encontraron respuestas cito- y genotóxicas estadísticamente significativas para el extracto acuoso de *P. williamioides* (2 mg/mL). Este efecto pudo deberse a una mayor concentración de fitocompuestos con actividad antimicrobiana como polifenoles, y/o a un alto contenido de metales. Estos resultados respaldan, desde un punto de vista pre-clínico, el empleo de estas especies con otros fines farmacológicos.

Palabras clave: Flora cubana, Ensayo de Sobrevivencia Bacteriana, SOS Chromotest, extracto acuoso de *Phyllanthus*, genotoxicidad, citotoxicidad, *Caulobacter crescentus*

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ABSTRACT

Plants belonging to the genus Phyllanthus *(Phyllanthaceae) are used in traditional medicine for the treatment of numerous diseases. Several Cuban flora species have proven strong antiviral, antioxidant, and antimutagenic properties. However, plant extracts include many different phytocomponents, and therefore they might exert undesired toxic side effects. In the present work we evaluate the aqueous extracts toxicity of three Cuban endemic Phyllanthus species:* P. williamioides *Gr.,* P. chamaecristoides *Urb., and* P. microdictyus *Urb. Cytotoxicity was measured through Bacterial Survival assay and genotoxicity was detected by means of the SOS Chromotest, both at concentrations ranging from 0.1 to 2 mg/mL and using* Caulobacter crescentus *cells as the in vitro experimental model.* LC_{50} *values were not detected at concentrations tested.* P. chamaecristoides *and* P. microdictyus *extracts were not cytotoxic, neither genotoxic. Also* P. microdictyus *extract showed a survival rate over 90% for all concentration tested. The cytotoxic and genotoxic effects were only statistically significant for* P. williamioides *aqueous extract (2 mg/mL). It might be due to the presence of high amounts of antimicrobial polyphenols, and/or high metal content. This result supports, from the preclinic point of view, the use of these species with other pharmacological purposes.*

Keywords: *Cuban flora, Bacterial Survival Assay, SOS Chromotest, Phyllanthus aqueous extract, genotoxicity, cytotoxicity,* Caulobacter crescentus

INTRODUCCIÓN

Since ancient times, human kind has trusted natural products, mainly plant-derived compounds, as sources of drugs to prevent or to ameliorate many diseases, a practice known as traditional medicine. Nowadays, a great part of the pharmaceuticals available are still derived from natural sources, and there is a growing world-wide interest in the use of phytopharmaceuticals in modern medicine.

Plants belonging to the genus *Phyllanthus* (Phyllanthaceae), comprising more than 1000 species, are widely distributed throughout most tropical and subtropical countries (Hoffmann *et al.* 2006). They are well known for their medicinal properties, mostly as part of Indian Ayurveda, Traditional Chinese Medicine and Indonesian Jamu. A great variety of *Phyllanthus* species have been phytochemically and pharmacologically studied. Many molecules have been isolated and identified, being alkaloids, lignans, triterpenes, tannins, coumarins, flavonoids, and anthocyanins the most abundant (Calixto *et al.* 1998; Bagalkotkar *et al.* 2006; Gutiérrez *et al.* 2011; Narasimhudu y Raju 2012). *Phyllanthus* plants 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 (Liu *et al.* 2001; Paithankar *et al.* 2011; Singh *et al.* 2011; Sarin *et al.* 2014; Verma *et al.* 2014).

In Cuba, there is a plentiful variety and endemism of this genus, and many studies support their antiviral, antioxidant, and antimutagenic properties. The alcoholic extracts of *P. formosus*, *P. chamaecristoides*, and *P. microdictyus* were found to *in vitro* inactivate the Hepatitis B surface antigen (del Barrio *et al.* 1995). The aqueous extract of *P. orbicularis*, has proven a broad spectrum of antiviral (del Barrio *et al.* 2014), antioxidant (Wong 2013; Sánchez-Lamar *et al.* 2015), antimutagenic (Sanchez-Lamar *et al.* 1999; Ferrer *et al.* 2001; 2002; Fuentes *et al.* 2006; Alonso *et al.* 2010), and photoprotective (Vernhes *et al.* 2013a; Vernhes *et al.* 2013b) action. The antioxidant properties of *P. williamioides*, *P. chamaecristoides*, *P. microdictyus*, *P. epiphyllanthus*, among other native species, have also been proven (Wong 2013). Although all these properties have been studied and well established in the scientific literature, to date a rather limited number of *Phyllanthus* species from Cuban flora, have been screened for possible cytotoxic and genotoxic properties. These tests are important in order to validate their safety use in medicinal practices (Posadzki *et al.* 2013).

In recent times the Gram negative alphaproteobacteria *Caulobater crescentus* has emerged as a prominent model for cellular and molecular biology studies. Bacterial cell survival studies are frequently employed as indicators of cytotoxicity of drugs and other chemicals. Cellular cytotoxicity is defined as basic cell functions impair leading to a damage that

could be detected (Arencibia *et al.* 2009). On the other hand, the SOS Chromotest assay allows identifying when bacterial DNA had been damaged. It is based on a colorimetric reaction as a result of enzimatic β-galactosidase activity, when the enzyme gene is coupled to the promoter of *imuA*, a gene involved in the SOS response in *C. crescentus* (Galhardo *et al.* 2005). The SOS response is a widely conserved bacterial stress response, which coordinates the expression of genes involved in DNA repair mechanisms, damage tolerance, cell division inhibition and mutagenesis. The SOS response of *C. crescentus* has been characterized in detail, and genetically modified strains are frequently employed to perform studies about repair mechanisms implicated in the SOS phenomenon (Martins-Pinheiro *et al.* 2007; da Rocha *et al.* 2008; Lopes-Kulishev *et al.* 2015).

In the present work the cytotoxic and genotoxic effects of *P. williamioides* Gr., *P. chamaecristoides* Urb., and *P. microdictyus* Urb. aqueous extracts were assessed by means of Bacterial Survival assay and SOS Chromotest, respectively.

MATERIALES Y MÉTODOS

Cuban endemic *Phyllanthus* plants were collected in 2011 spring, from different regions of Guantánamo province, Cuba (Wong 2013). The specimens were authenticated and stored at the Cuban Botany Garden as follow: *Phyllanthus williamioides* Griseb (TB 4523); *Phyllanthus chamaecristoides* Urb. subsp. baracoensis (Urb.) G.L. Webster (TB 4452); and *Phyllanthus microdictyus* Urb. (TB 4457).

The aqueous extracts were obtained from the leaves and stems following a previously described method (Wong 2013), in a 1:7.5 (g of dried plant: mL of distilled water) relation; further lyophilized and stored in a cool dry place until ready for use. The treatments assayed were prepared at the moment, concentrations tested were: 0.1, 0.5, 1, and 2 mg/mL for cytotoxic and genotoxic assays.

Bacterial strain and culture

Caulobacter crescentus strain NA 1 000 pP3213 LacZ was used in this study. It was obtained by the transformation of wild NA 1 000 with pP3213 plasmid, containing the *imuA* SOS response gene promoter in transcriptional fusion with the *lacZ* gene, coding for β-galactosidase enzyme (Galhardo *et al.* 2005). Cells were grown for 16 h at 30°C with constant shaking (100 rpm) in Peptone Yeast Extract (PYE) medium, supplemented with $CaCl_2$ 0.5 mM and tetracycline 2 µg/ml (Ely 1991). The culture was then diluted tenfold in fresh medium and grown under similar conditions until the optical density at 600 nm (OD_{600}) was 0.4 (6 x 10^7 cells/mL), corresponding to the phase of logarithmic growth. Then, cells were placed in tubes containing the plants extracts at different concentrations, incubated for 30 min at 4°C and then for 2 h at 30°C and constant shaking (100 rpm). As negative control, cells harvested in medium were used. Afterwards, aliquots of the same treatment were taken to perform the cytotoxicity and genotoxicity assays as described below.

Cytotoxicity assay

Cytotoxicity was evaluated by *C. crescentus* colony-forming ability, as described before (Galhardo *et al.* 2005). A 10 µL aliquot was removed after each treatment for serial dilutions and plating 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.

Genotoxicity assay

Genotoxicity was assessed through DNA primary structural damage evaluation by means of a SOS Chromotest modified protocol (Galhardo *et al.* 2005). Briefly, after the treatment explained above, the OD_{600} for each sample was determined. Then 50 µL aliquots were dispensed in tubes containing 800 µL of a permeabilization solution for cells 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. Afterwards, 200 µL of o-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_{420} was measured and β-galactosidase activity was calculated as describe previously (Zhang y Bremer 1995). Genotoxicity was estimated by β-galactosidase activity detected for each treatment respective to negative control.

Statistical analysis

Means and the corresponding standard deviation (SD) were determined for each treatment. Controls and

treatments were compared using the Kolmogorov-Smirnov test for Normality, Brown-Forsythe test for variance homogeneity, the single classification ANOVA, and the Dunnett test, all of them performed by the software Statistica v.6 (Inc 2003).

RESULTADOS

Cytotoxicity assay

The potential cytotoxicity of *P. williamioides*, *P. chamaecristoides*, and *P. microdictyus* aqueous extracts was evaluated by *C. crescentus* colony-forming capacity, in order to establish a concentration range for cellular viability. A statistically significant diminishing in survival rates were only observed in *P. williamioides* aqueous extract at 2 mg/mL (Fig. 1).

Genotoxicity assay

In order to detect whether *P. williamioides*, *P. chamaecristoides*, and/or *P. microdictyus* aqueous extracts were capable to produce primary DNA damage, SOS response induction in *C. crescentus* was measured. As shown in Fig. 2, no genotoxic effect was observed; except for the *P. williamioides* higher dose tested (2 mg/mL).

DISCUSSION

For ages, the infusion of leaves, stems, fruits and roots of several *Phyllanthus* species have been used in traditional medicine for the treatment of a broad spectrum of diseases. Most of them have shown to contain different combinations of secondary metabolites which render them medicinal properties. In recent years, the interest on many species has increased, especially regarding their therapeutic potential for the management of several pathological conditions. Numerous studies carried out on the extracts and purified compounds support most of their reported uses in folk medicine (Charoenteeraboon *et al.* 2010; Poh-Hwa *et al.* 2011; Chakraborty *et al.* 2012). In Cuba, several endemic *Phyllanthus* plants present antiviral, antioxidant, and antimutagenic properties. However, the safety use of these plants could be questioned as there are not enough studies reported in scientific literature about their possible cytotoxic and genotoxic effects.

Cytotoxicity experiments carried out in the current study through the Survival Assay, showed that *C. crescentus* cells survival rate was over 70% for all concentration tested (over 90% for *P. microdictyus*). In all cases, LC_{50} values were higher than top concentration

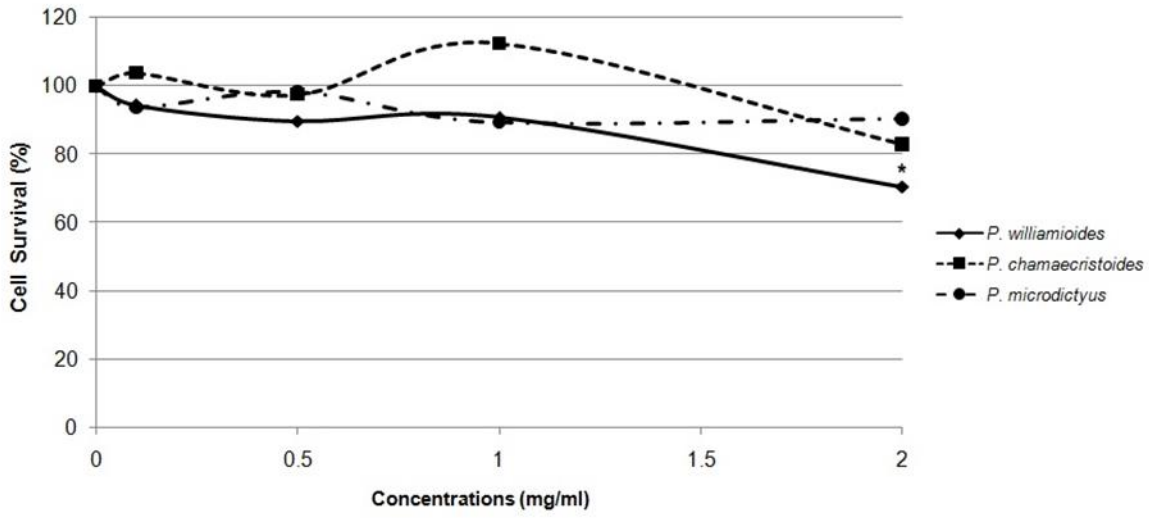

Figure 1: Influence of *Phyllanthus* plants aqueous extracts in *Caulobacter crescentus* survival curves. The results shown are the mean of at least three independent experiments, each with four replicas. (*) $p < 0.05$ Dunnett Test.

Figura 1. Influencia de los extractos acuosos de las plantas de *Phyllanthus* en las curvas de sobrevivencia de *Caulobacter crescentus*. Se muestran los valores medios de al menos tres experimentos independientes, cada uno con cuatro réplicas. (*) $p < 0,05$ en la prueba de Dunnett.

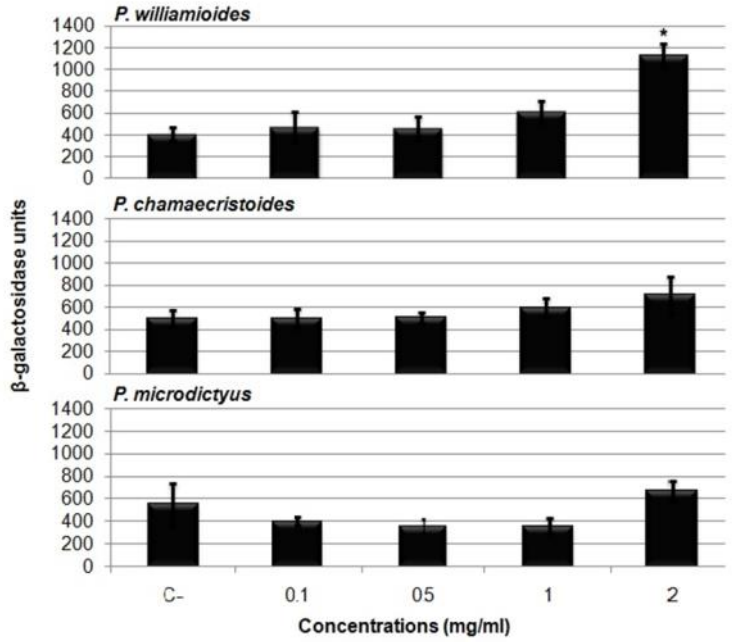

Figure 2: Effect of *Phyllanthus* plants aqueous extracts for SOS response induction in *Caulobacter crescentus*. The results shown are the mean of at least three independent experiments done in triplicate. Error bars indicate the SD. (*) $p < 0.05$ Dunnett Test.

Figura 2: Efecto de los extractos acuosos de las plantas de *Phyllanthus* en la inducción de la respuesta SOS en *Caulobacter crescentus*. Se muestran los valores medios de al menos tres experimentos independientes realizados en triplicado. Las barras de error indican la DS. (*) $p < 0,05$ en la prueba de Dunnett.

tested. This is in agreement with previous studies of their toxicity using *in vitro* and *in vivo* approaches. The cytotoxicity of the aqueous extracts of *P. williamioides* and *P. chamaecristoides*, assessed in human lung cancer cell line A549, showed no significant diminishing of cells survival tested up to 1 mg/mL, being the survival rate over 90%. In the same study, acute toxicity assays in *Artemia salina* showed that for both plants extracts LC_{50} were higher than 5 mg/mL (Wong 2013). Our results agree with these outcomes, although they have been performed in different experimental models.

In the present work, we performed the SOS Chromotest, considered one of the simplest short-term assays for genotoxicity studies. Genotoxic substances are potentially known to be mutagenic or carcinogenic. So, it results imperative to assess the aqueous extracts for possible primary DNA damage. The toxicity results were in correspondence: there is no evidence of cytotoxicity of any plant aqueous extracts (Fig. 1) at non-genotoxic doses (Fig. 2). Only *P. williamioides* 2mg/mL extract showed a significant genotoxic reaction.

The absence of DNA structural damage agrees with the absence of genotoxicity shown by other *Phyllanthus* specie endemic to Cuba, in different experimental models. The aqueous extract of *P. orbicularis* (2mg/mL) does not induce either primary DNA damage or mutation when *ex vivo* experiments with plasmidic DNA, SOS gene induction, gene reversion and conversion, and SMART assays were performed (Sanchez-Lamar *et al.* 2002; Cuétara *et al.* 2012; Vernhes *et al.* 2013b).

In general, the toxicity of *Phyllanthus* plants reported in literature tends to be low. Acute toxicity studies on aqueous leaf extract of *P. niruri* performed in rats, showed a LD_{50} > 5 000 mg/kg body weight (Asare *et al.* 2011), and no significant cytotoxicity was detected when incubated with normal human skin (CCD-1127Sk) and prostate (RWPE-1) cells (Tang *et al.* 2010). Also, no genotoxic, nor cytotoxic activities of a *P. niruri* aqueous extract was found when tested in Wistar rats bone marrow (at doses up to 250 mg/kg/day) using micronucleus assay (de Queiroz *et al.* 2013). Studies performed by Lawson-Evi *et al.*, about the acute and sub-acute toxicity of *P. amarus* aqueous extract evaluated in Swiss mice, showed that it could be considered to be safe in animals by oral route (LD_{50} > 5 g/kg) (Lawson-Evi *et al.* 2008). Non toxic effects were detected for *P. tenellus* aqueous extract tested in laboratory mice at doses up to 2500 mg/kg, although it induced agitation in animals, with spasms and increased respiratory frequency, as well as signs of depression (Silva *et al.* 2012). An standardized water extract from fruits of *P. emblica* did not produce acute (LD_{50} > 5,000 mg/kg) or chronic toxicity when tested in Sprague Dawley rats (Jaijoy *et al.* 2010). Acute and sub-chronic toxicity studies of *P. orbicularis* aqueous extract proved it did not exert any toxic

effect by oral administration at doses recommended for using as antiviral in humans (Rivero y Vidal 1998), as well as not irritant properties were detected on dermis and ophthalmic tests (Gutiérrez *et al.* 2002).

However, in the present study, toxic effects statistically significant (both, cytotoxic and genotoxic) were observed after treatment with 2 mg/mL *P. williamioides* aqueous extract (Fig. 1 and 2). Plant extracts are complex mixtures of natural substances, where active component(s) might comprise(s) a large number of constituents that could be present in highly variable amounts. There has been isolated more than 12 000 of secondary metabolites, a number estimated to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores.

Several *Phyllanthus* species extracts are known for their antibacterial activity, exerted by diverse polyphenols, among them phyllanthin (Komuraiah *et al.* 2009; Dhale y Mogle 2011; Babatunde *et al.* 2014; Mehta *et al.* 2014). As part of a preliminary qualitative study of the composition of *Phyllanthus* plants, high concentrations of phenolic compounds, such as tannins and flavonoids were found (Wong 2013). In this sense, this author detected that the amounts of polyphenols present in *P. williamioides* aqueous extracts, determined by the Folin-Ciocalteau method, where four times higher than those detected for *P. chamaecristoides* and *P. microdictyus* extracts. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins. For tannins the proposed molecular actions are to complex with proteins through hydrogen bonding, hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, among others (Haslam 1996). Flavonoids are known to be synthesized by plants in response to microbial infection; it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cowan 1999).

According to explained above, it is possible that the survival diminish observed in *Caulobacter crescentus* cells treated with *P. williamioides* extract, could be related to the presence of some polyphenols with antimicrobial action. However, the particular composition of the studied plants must be elucidated first in order to confirm the presence of any of these phytochemicals, and their biological action.

Another important feature to take into account is the possible toxic effect owed to high metal content of the extract, since others *Phyllanthus* species have shown hyperaccumulation of Ni, Co, Mg, Cd, and Ca (Reeves 2003; Berazaín *et al.* 2007; Dwivedi *et al.* 2013; Quimado *et al.* 2015). Both aspects should be subject of further analysis respective to plant extract chemical composition, for an improved comprehension of this particularly toxic response.

Because of plant extracts with medical applications consists in complex mixtures, it is imperative to determinate the possible cytotoxic and genotoxic effects they might exert. In the current work, we assessed the toxicity of three Cuban endemic *Phyllanthus* plants aqueous extracts that had previously shown promising antioxidant and antiviral properties. *P. chamaecristoides* and *P. microdictyus* aqueous extracts were not either cytotoxic nor genotoxic in concentrations up to 2 mg/mL, using *Caulobacter crescentus* cells as experimental model. *P. williamioides* extract at 2 mg/mL exerted some toxicity, but the responsible(s) phytocomponent(s) of such response remain to be detected.

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