© 2017 Journal of Pharmacy & Pharmacognosy Research, 5 (4), 251-261, 2017
ISSN 0719-4250
http://jppres.com/jppres

Original Article | Artículo Original

Antioxidant, photoprotective and antimutagenic properties of *Phyllanthus* spp. from Cuban flora

[Propiedades antioxidantes, fotoprotectoras y antimutagénicas de *Phyllanthus* spp. de la flora cubana]

Ivette M. Menéndez-Perdomo[1,2], Maylin Wong-Guerra[3,4], Fabiana Fuentes-León[1], Elizabeth Carrazana[1], Isis Casadelvalle[3], Alexis Vidal[3], Ángel Sánchez-Lamar[1]*

[1]Departamento de Biología Vegetal, Facultad de Biología, Universidad de La Habana, calle 25, No. 455 e/ I y J, CP 10 400, La Habana, Cuba.
[2]Current address: Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, AB T2N 1N4, Canada.
[3]Departamento de Bioquímica, Facultad de Biología, Universidad de La Habana, calle 25, No. 455 e/ I y J, CP 10 400, La Habana, Cuba.
[4]Current address: Centro de Investigación y Desarrollo de Medicamentos (CIDEM), Ave 26, No. 1605 e/ Boyeros y Puentes Grandes, CP 10600, La Habana, Cuba.
*E-mail: alamar@fbio.uh.cu

Abstract

Context: Sunlight ultraviolet (UV) radiation constitutes a significant physical carcinogen in nature. It could induce direct and indirect DNA damage, which if not properly repaired may generate mutations. Plants in the genus *Phyllanthus* (Phyllanthaceae) are widely used in traditional medicine and known as natural sources of antioxidant compounds. Recent investigations support their genoprotective activity against chemical and physical mutagens, among them UV radiation.

Aims: To compare the antioxidant, photoprotective and antimutagenic activity of aqueous extracts obtained from three Cuban endemic *Phyllanthus* species: *P. chamaecristoides* Urb., *P. microdictyus* Urb., and *P. williamioides* Gr.

Methods: DPPH radical scavenging assay was use for quantify the *in vitro* antioxidant capacity. Genoprotection against UVC radiation was measured at two levels: structural DNA damage and mutations, by means of the SOS Chromotest, Survival assay, and Rif^R mutagenicity test in *Caulobacter crescentus* cells.

Results: *P. chamaecristoides* extract showed the highest antioxidant capacity (IC50 = 0.032 mg/mL), and together with *P. microdictyus* exhibited the greatest bioantimutagenic effects (RMF ≤ 5%), diminishing UVC-induced DNA damage in three and five times, respectively.

Conclusions: The reduction in DNA damage is not founded in a *Phyllanthus* aqueous extracts desmutagenic effect. It is possible that the genoprotective activity detected could be due to modulation of DNA repair mechanisms, diminishing SOS response and related mutagenicity induced by UVC radiation. Moreover, high antioxidant capacity could also decrease UV radiation oxidative-damage. Altogether, these outcomes validate future pre-clinic research regarding *Phyllanthus* photoprotective capacities.

Keywords: genoprotection; *Phyllanthus chamaecristoides*; *Phyllanthus microdictyus; Phyllanthus williamioides*; phytochemistry; plant aqueous extract; UVC.

Resumen

Contexto: La radiación ultravioleta (UV) constituye un importante carcinógeno físico natural. Puede inducir daños directos e indirectos al ADN, los que de no ser reparados correctamente pueden generar mutaciones. Las plantas pertenecientes al género *Phyllanthus* (Phyllanthaceae) son ampliamente utilizadas en la medicina tradicional y fuentes naturales de antioxidantes. Estudios recientes apoyan su actividad genoprotectora frente a mutágenos químicos y físicos, como la radiación UV.

Objetivos: Comparar las actividades antioxidante, fotoprotectora y antimutagénica de los extractos acuosos obtenidos de tres especies de *Phyllanthus* endémicas de Cuba: *P. chamaecristoides* Urb., *P. microdictyus* Urb. y *P. williamioides* Gr.

Métodos: El ensayo de atrapamiento del radical DPPH se empleó para cuantificar la capacidad antioxidante *in vitro*. La genoprotección frente a la radiación UVC fue determinada a dos niveles: estructura primaria del ADN y mutagénesis, mediante los ensayos SOS Colorimétrico, Sobrevivencia bacteriana y Rif^R en células de *Caulobacter crescentus*.

Resultados: El extracto de *P. chamaecristoides* mostró la mayor capacidad antioxidante (IC50 = 0.032 mg/mL), y junto con *P. microdictyus*, los mejores efectos bioantimutagénicos (RMF ≤ 5%), disminuyendo los daños al ADN inducidos por la radiación UVC en tres y cinco veces, respectivamente.

Conclusiones: La reducción del daño al ADN no está basada en un efecto desmutagénico de los extractos acuosos de *Phyllanthus*. La actividad genoprotectora puede deberse a la modulación de mecanismos de reparación del ADN, disminuyendo la respuesta SOS y la consecuente mutagenicidad inducida por la radiación UVC. Además, una elevada capacidad antioxidante pudiera disminuir el daño oxidativo causado por esta radiación. Estos resultados validan posteriores investigaciones pre-clínicas respecto a la capacidad fotoprotectora de *Phyllanthus*.

Palabras Clave: extracto acuoso vegetal; fitoquímica; genoprotección; *Phyllanthus chamaecristoides*; *Phyllanthus microdictyus; Phyllanthus williamioides*; UVC.

ARTICLE INFO
Received | Recibido: February 3, 2017.
Received in revised form | Recibido en forma corregida: May 16, 2017.
Accepted | Aceptado: May 21, 2017.
Available Online | Publicado en Línea: May 28, 2017.
Declaration of interests | Declaración de Intereses: The authors declare no conflict of interest.
Funding | Financiación: This work was supported by CAPES (Brazil) - MES (Cuba) international collaboration project (number 187/13).
Academic Editor | Editor Académico: Gabino Garrido.

INTRODUCTION

Sunlight ultraviolet (UV) radiation wavelength could be divided into UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm), from the most to the less energetic. It constitutes an environmental genotoxic agent that affects ecosystems and human population (Schuch et al., 2014). UV radiation is able to damage DNA directly by generating pyrimidine dimers, and indirectly by causing oxidative damage (Cadet and Wagner, 2013). These lesions, if not efficiently repaired, could lead to mutations, which can ultimately trigger skin and eye related disorders, including cancer (Pfeifer and Besaratinia, 2012).

Short wavelength UVC radiations are completely absorbed by the ozone layer, but there are important artificial sources commonly used in medicine and for cosmetic purposes (De Flora, 2013). Furthermore, stratospheric ozone layer depletion, together with climate change and global warming, predict an uncertain scenario for UV Earth's incidence in the next decades (McKenzie et al., 2011), rising concerns about its biological consequences and motivating worldwide scientific efforts to develop better photoprotection strategies (González et al., 2011).

Among current photoprotection trends, the use of naturally occurring compounds is an important emerging strategy (Afaq and Katiyar, 2011; Bandyopadhyay et al., 2014; Saewan and Jimtaisong, 2013). Plants are unique in their obligated nature to be continuously exposed to environmental UV light, hence it results logical to explore them in the quest for components, which could render photoprotection. Likewise, due to their complex secondary metabolism, phytocomponents exhibit a vast molecular diversity and biological functions.

Plants belonging to the genus *Phyllanthus* (Phyllanthaceae) are known for their ethnopharmacological properties, mostly as part of Indian Ayurveda, Indonesian Jamu, and Traditional Chinese Medicine, with infrequent side effects (Sarin et al., 2014). Aqueous extracts obtained from *P. amarus*, *P. emblica*, *P. niruri*, *P. orbicularis*, *P. urinaria*, among many others, are known as natural sources of antioxidant phytocomponents (Charoenteeraboon et al., 2010; Poh-Hwa et al., 2011; Sánchez-Lamar et al., 2015). Furthermore, *in vitro* and *in vivo* genoprotective activity of many *Phyllanthus* plants water extracts against different chemical and physical mutagens have been reported (Ahmad et al., 2015; Alonso et al., 2010; Ferrer et al., 2001; 2002).

Particularly, photoprotective capacity against UV-induced DNA damage have been demonstrated for *P. emblica* and *P. niruri* aqueous extracts (Majeed et al., 2011; Raja et al., 2011) and the effects correlated with their high antioxidant activity. Recently, it was demonstrated the Cuban endemic specie *P. orbicularis* genoprotective effect against UVB radiation in human cells (Vernhes et al., 2013a) and a significant photoprotective activity in plasmid DNA irradiated with artificial UVC light in *ex vivo* experiments (Vernhes et al., 2013b).

In the present work, for the first time to the best of our knowledge, we assess and compare the phytochemistry, antioxidant, and genoprotective effects of aqueous extracts from Cuban endemic *Phyllanthus* spp.

MATERIAL AND METHODS

Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) except for Mayer's reagent acquired from LabChem (Distributor: Caledon Laboratories Ltd., Canada), tetracycline and rifampicin antibiotics obtained from AppliChem (Darmstadtz, Germany) and the o-nitrophenyl-β-D-galactopyranoside (ONPG) acquired from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Plant materials and extracts

In 2011 spring, *Phyllanthus* plants were collected from different regions of Guantánamo province, Cuba (*P. chamaecristoides*: 20^0 28" 32.7"" N, 74^0 43" 45.4"" W; *P. microdictyus*: 20^0 30" 17.8"" N, 74^0 40" 02.5"" W; *P. williamioides*, 20^0 29" 32.3"" N, W 74^0 43" 50.2"" W). The specimens were authenticated by specialists of Cuban Botanical Garden, and stored in this scientific institution as: *Phyllanthus chamaecristoides* Urbano subsp. baracoensis (TB 4452); *Phyllanthus microdictyus* Urbano (TB 4457), and *Phyllanthus williamioides* Griseb (TB 4523).

The aqueous extracts were obtained from the leaves and stems following a previously described

methodology (Menéndez-Perdomo et al., 2016), in a 1:7.5 (g of dried plant: mL of distilled water) relation; further lyophilized (Center for Genetic Engineering and Biotechnology, CIGB, La Habana, Cuba) and stored in a cool dry place until ready for use. Qualitative phytochemical screening and total phenol content were made by diluting the lyophilized in distilled water at 2 mg/mL and 1 mg/mL, respectively. For antioxidant evaluation aqueous extracts were prepared at concentrations 0.1, 0.5, 1 and 2 mg/mL. This range of concentration was selected as it showed high antioxidant capacity for the extracts in previous assays (data not shown). Genoprotection treatments (1, 10, 100, and 1000 µg/mL) were prepared at the moment by diluting the lyophilized in Peptone Yeast Extract (PYE) medium (peptone 2 g/L; yeast extract 1 g/L; MgSO4 7H2O 0.8 g/L; supplemented with $CaCl_2$ 0.5 mM and tetracycline 2 µg/mL). The aqueous extracts concentrations used in the present work were not cytotoxic or genotoxic in *Caulobacter crescentus* cells used as the experimental model (Menéndez-Perdomo et al., 2016).

Phytochemical screening

Aqueous extracts were qualitatively analyzed using an standard procedure as described before (Chhabra et al., 1984). Briefly, 2 mg of each plant lyophilized was repeatedly extracted with ether (50 mL, 3 times), then in hot methanol (50 mL, 3 times), concentrated under reduced pressure to one third volume and divided into two portions (A and B). To portion A, chemical reactions were performed for the detection of alkaloids, reducing sugars, phenols and tannins. Portion B was hydrolyzed with hydrochloric acid (10 mL, 10%) by refluxing on a water bath for 30 min, after the contents were cooled, 15 mL of water were added and then extracted with ether (10 mL, 3 times). The ether extract was tested for the presence of flavonoids, anthocyanins and catechins.

For alkaloids detection the methanol solution was evaporated and the residue macerated with hydrochloric acid (1.5 mL, 2%), filtered, basified with ammonium hydroxide 10% and extracted with ether. Then was added Mayer's reagent and a positive response was recorded if turbidity or precipitation were produced. Reducing sugars were detected by the Fehling assay, and the appearance of red precipitate on heating the mixture was taking as a positive response. Phenols and tannins were detected by addition of ferric chloride to the sample. For flavonoids detection the solution was evaporated and dissolved in aqueous ethanol and tested by Shinoda's reaction. The appearance of red color at pH 3 and its change with pH modification (pH 9) in the solution was taken as the signal for the presence of anthocyanins. Catechins were detected by vanillin–HCl assay, carried out in 30°C water bath with a reaction time of 20 min. The vanillin reagent contained 4% concentrated HCl and 0.5% vanillin in methanol. The development of pink color was taken as a positive response.

Total phenolic content was quantified by using a modified protocol previously described (Quettier-Deleu et al., 2000). Concisely, 8 mL of distilled water were combined with 0.5 mL of Folin-Ciocalteau reagent and 0.5 mL of the plant extract (1 mg/mL). After 3 min, 1 mL of 20% Na_2CO_3 was added and samples were incubated at room temperature for 1 hour. Absorbance was recorded at 720 nm in a Rayleigh VIS-723G spectrophotometer (Beijing Beifen-Ruili Analytical Instrument, Beijing, China). Total phenolic content was estimated from a standard curve of gallic acid (Abs_{720nm}=6.76927[gallic acid] + 0.06049, R^2=0.99) plotted using concentrations between 10 and 250 µg/mL. Polyphenols content was expressed as micrograms of gallic acid equivalent per milligrams of plant lyophilized (µg GAE/mg lyophilized). All measurements were done by triplicate.

Preliminary antioxidant evaluation

Detection of scavenger capacity of the extracts was determinate using 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) assay, according to a methodology previously described (Brand-Williams et al., 1995). Briefly, 320 µL of the samples (*Phyllanthus* aqueous extracts at concentrations 0.1-2 mg/mL) and 50 µL of $DPPH^{\cdot}$ (100 µM) diluted in methanol were placed in 96 wells microplates (Falcon), incubated for 30 min, and then absorbance of the mixture was measured by spectrophotometer (BMG Labtech, Germany) at wavelength 550 nm. As a positive control (S)-(-)-6 hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was used, and the reagents mix without any sample was the negative control. Scavenger

capacity was expressed as the amount of antioxidant necessary to decrease the initial $DPPH^{\cdot}$ absorbance by 50% respecting to negative control value (effective concentration 50, EC_{50}). All trials were done by triplicate.

Transmittance quantification

The physical capacity of the extracts during UVC irradiation was quantified as the transmittance at λ=254 nm, using a spectrophotometer Genesys™ 10S (Thermo Electron Corporation, USA). Concentrations tested were 1, 10, 100, and 1000 µg/mL for each plant aqueous extract prepared at the moment by diluting the corresponding lyophilized in PYE medium. The negative control was PYE medium prepared in the same way used for diluting the lyophilized samples. Measurements were done by triplicate.

Bacterial strain and culture

Caulobacter crescentus strain NA 1000 pP3213 LacZ, kindly provided by the Department of Microbiology (Instituto de Ciências Biomédicas, Universidade de São Paulo, Brazil) was used in this study. It was previously obtained by the transformation of wild NA 1000 strain 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 overnight at 30°C with constant shaking (100 rpm) in PYE medium (Ely, 1991). The culture was then diluted ten-fold in fresh medium and grown under similar conditions until the optical density at 600 nm (OD_{600nm}) was 0.4 (6 x 10^7 cells/mL), corresponding to the phase of mid-log growth.

UVC irradiation

Caulobacter crescentus cells in the mid-log growth phase were collected by centrifugation (Cole-Parmer, Eppendorf, US) in 1.5 mL microcentrifuge tubes twice (total volume 3 mL), resuspended with the plants extracts at different concentrations (treatments: 1, 10, 100, and 1000 µg/mL), and incubated for 30 min at 4°C in a refrigerator. Then, a 1.5 mL batch of each treatment was irradiated in 3 cm diameter Petri dishes. UVC irradiation (λ=254 nm, E=45 J/m²) was carried out using a Vilber Lourmat Lamp T15M 15 W (Vilber Lourmat, Suebia, Germany) at room temperature. Afterwards, cells were collected by centrifugation, suspended in the corresponding treatments, and then incubated for 2 h at 30°C under constant shaking (100 rpm). Subsequently, aliquots of the same treatment were taken to perform the SOS Chromotest, Survival Assay, and Rif^R Test as described below. Cells harvested in medium without irradiation and irradiated were used as negative and positive controls, respectively. All steps were carried out in the dark to avoid photo-reactivation. All measurements were done by triplicate.

Assessment of genoprotective activity

DNA primary structural damage was evaluated by means of a SOS Chromotest modified protocol (Galhardo et al., 2005). Briefly, 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 cells disruption (buffer Z: Na_2HPO_4 8.5 g/L; NaH_2PO_4 7.18 g/L; KCl 0.75 g/L; $MgSO_4.7H_2O$ 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_{420nm} was measured and β-galactosidase activity was calculated as describe previously (Zhang and Bremer, 1995), by the following relationship:

$$U = (DO_{420nm} \times 1000)/(DO_{600nm} \times Vol \times t)$$

Where:

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

The statistical significant increase of the β-galactosidase enzymatic activity compared to the negative control was taken as genotoxicity criteria. All measurements were done by triplicate, and the experiments were repeated five times.

Survival experiments

The influence of the extracts in cell survival upon UVC radiation 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 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. Experiments were performed five times with four replicates each.

Mutagenesis experiments

Mutagenesis experiments were carried out as previously describe (Lopes-Kulishev et al., 2015), using the Rif^R Test. Briefly, a 50 µL aliquot of treated cells was inoculated in 200 µL of PYE liquid medium, and cultivated overnight at 30°C with constant shaking (100 rpm). Then, cultures were spread onto PYE plates containing 100 µg/mL of rifampicin for selection of Rif^R mutants, and serial dilutions were plated on PYE medium to determine the number of viable cells. Cells were incubated at 30°C and colony formation was counted after 48 h. Mutation frequencies were calculated dividing the number of Rif^R mutants by the number of viable cells in each culture, and antimutagenicity results were expressed as percentage of relative mutation frequency (RMF) with respect to the control value, where the bacterial culture was exposed to UV in the absence of any extract. The experiments were conducted three times with four replicates each.

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, and the single classification ANOVA, the Dunnett test for all the assays, except for the detection of polyphenol content and DPPH radical scavenging assay for which a Tukey HSD test was conducted. $P<0.05$ was considered as statistically significant. All the tests were performed by the software Statistica v.6.

RESULTS

Qualitative phytochemical composition and total phenolic content

The detection of main phytocomponent families in the plant extracts, based on colorimetric/precipitation principles, showed high amounts of phenolic compounds, mainly tannins (Table 1). *P. chamaecristoides* and *P. microdictyus* reached the higher concentration of reducing sugars, and the former was enriched in flavonoids. As the colorimetric reaction for detecting flavonoids turned yellow for these species, they should contain predominantly flavones, meanwhile for *P. williamioides* extract the reactants turned pink, indicating the prevalence of flavonols. All plants extracts showed moderate amounts of antocianidins and catechins. Alkaloids were not detected.

Total phenolic content of aqueous extracts was expressed as µg of GAE, the simplest form of phenolic compounds. *P. williamioides* aqueous extract showed a statistical significant higher content of polyphenols, more than six and three times the amounts found in *P. chamaecristoides* and *P. microdictyus*, respectively (Table 2).

Table 1. Qualitative phytochemical composition of aqueous extracts from *Phyllanthus* species.

Specie	Phytochemical Composition						
	Phenols	**Tannins**	**Flavonoids**	**Antocianidins**	**Catechins**	**Reducing sugars**	**Alkaloids**
P. chamaecristoides	+++	+++	+++ (yellow)	++	++	+++	-
P. microdictyus	+++	+++	++ (yellow)	++	++	+++	-
P. williamioides	+++	+++	++ (pink)	++	++	++	-

Legend: -: absence; ++: average presence; +++: high presence

Table 2. Total phenolic content detected in aqueous extracts from *Phyllanthus* species.

Specie	Total phenolic content (µg GAE/mg lyophilized)
P. chamaecristoides	19.13 ± 4.86[a]
P. microdictyus	34.75 ± 3.39[b]
P. williamioides	125.75 ± 4.64[c]

Data show the mean ± SD values obtained by triplicate. Values with different superscripts are statistically significant at p<0.05 using ANOVA and Tukey HSD test. GAE: gallic acid equivalent.

DPPH radical scavenging assay

DPPH free radical method was performed using Trolox as the positive control. *P. chamaecristoides* and *P. williamioides* (0.1 mg/mL) showed better antioxidant activity than Trolox, and the former present the best free radicals scavenger behavior at all concentrations tested. EC_{50} (mg/mL) values were *P. chamaecristoides* (0.03) < *P. williamioides* (0.15) < Trolox (0.29) < *P. microdictyus* (1.16) (Table 3). *P. chamaecristoides* (2 mg/mL) exhibited DPPH radical inhibition similar to Trolox.

Table 3. Antiradical activity of *Phyllanthus* aqueous extracts and Trolox measured by $DPPH^{\cdot}$ scavenging assay

Specie	EC_{50} (mg/mL)
P. chamaecristoides	0.03[a]
P. microdictyus	1.16[b]
P. williamioides	0.15[c]
Trolox	0.29[d]

Values with different superscripts are statistically significant at p<0.05 using ANOVA and Tukey HSD test. EC_{50}: Effective concentration 50.

Transmittance quantification

For all concentration tested (1-1000 µg/mL), transmittance values were near to 100%, allowing the passage of most of UVC radiation, which indicates low, if any, light absorption.

Photoprotection of *Phyllanthus* extracts

Primary DNA damage in *C. crescentus* was measured through SOS Chromotest assay, which is based on the induction *lacZ* gene under the transcriptional control of *imuA*, a SOS response gene, thus quantification β-galactosidase enzymatic activity is an indirect sing of DNA structural damage.

All extracts exhibited a positive response (Fig. 1B), and the best photoprotective effect was achieved by higher doses of *P. chamaecristoides* and *P. microdictyus* extracts, which diminish β-galactosidase activity in three and five times, respectively. All extracts showed a significant increase in cell survival upon irradiation, but *P. chamaecristoides* exhibited the best results, increasing more than three times the survival rate respecting to the irradiated cells (Fig. 1B).

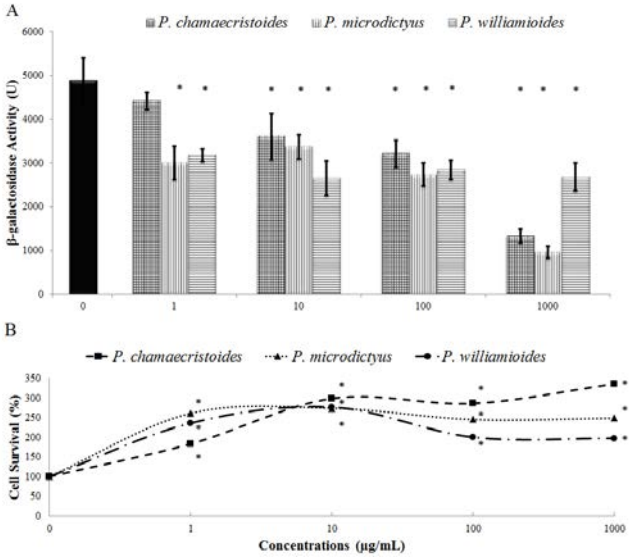

Figure 1. Induction of β-galactosidase activity **(A)** and percentage of cell survival **(B)**, after UVC irradiation in *C. crescentus* treated cells.
Data show mean and SD of 5 experiments with 3 (A) and 4 (B) replicas each. (*) Significant in Dunnett test, p < 0.05.

Antimutagenic properties of *Phyllanthus* extracts

Aqueous extracts were tested for its potential to reduce UV-induced mutation in *C. crescentus* by monitoring acquisition of resistance against rifampicin, which resulted from mutation in the beta subunit of the RNA polymerase (Campbell et al., 2001). *P. chamaecristoides* and *P. microdictyus* extracts display a positive antimutagenic response at all concentration tested, fewer than 20 and 40% of RMF values for all concentration tested. At 1 mg/mL these plants diminish mutations to less than 5%. At 1 and 10 µg/mL *P. chamaecristoides* showed the best antimutagenic results. *P. williamioides* did not exert any antimutagenic activity, by the contrary, at high concentration (1 mg/mL) this plant extract behave

as strongly mutagenic, duplicating the RMF respecting to the irradiated cells (Fig. 2).

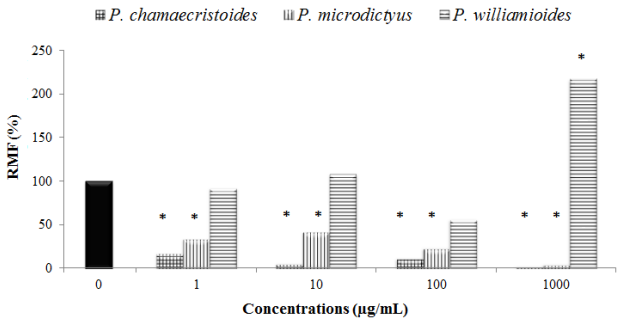

Figure 2. Relative Mutation Frequency (RMF) in *C. crescentus* cells exposed to UVC radiation in presence of the treatments.

Data represent the percentage of mean values in 3 experiments with 4 replicas each. (*) Significant in Dunnett test, $p < 0.05$.

DISCUSSION

UV radiation is able to damage DNA by direct absorption of photons or via indirect oxidative reactions, leading to mutations that can further trigger many human disorders like cataracts, immunosuppression, photoaging, and skin cancer (Pfeifer and Besaratinia, 2012). In this context, naturally occurring phytocompounds have emerged as an important modern photoprotection strategy (Afaq and Katiyar, 2011; González et al., 2011; Saewan and Jimtaisong, 2013).

Several *Phyllanthus* plants have shown antioxidant and genoprotective activity against UV radiation (Majeed et al., 2011; Raja et al., 2011), rising scientific interest in their photoprotective properties. In Cuba, there is a plentiful variety and endemism of this genus, and extensive work have been made to support their antioxidant and antimutagenic properties (Ferrer et al., 2001; 2002; Alonso et al., 2010; Vernhes et al., 2013a; 2013b; Sánchez-Lamar et al., 1999; 2015). In this work, it was presented the phytochemical composition of three Cuban endemic *Phyllanthus* species aqueous extracts, and evaluated their antioxidant capacity, as well as their photoprotective and antimutagenic properties against UVC radiation.

Herein, it was qualitatively examined the aqueous extract obtained from leaves and stems of three *Phyllanthus* plants, for the presence of phenolic compounds (phenols, tannins, flavonoids, antocianidins, and catechins), reducing sugars and alkaloids. Results showed from moderate to high amounts of compounds tested, except for alkaloids, which were not detected (Table 1).

Previous work with the aqueous extract obtained from Cuban endemic *P. orbicularis* showed the presence of flavonoids, tannins, antocianidins, and catechins, but not alkaloids (Sánchez-Lamar et al., 2015). By the contrary, in an extensive study carried out on 15 *Phyllanthus* species water extracts obtained from the leaves and stems, alkaloids were found to be the most abundant compound (Narasimhudu and Raju, 2012). In another work about the chemical composition of *P. amarus* and *P. reticulatus* aqueous extracts (which according to the above mentioned investigation had high and trace amounts of alkaloids, respectively), no alkaloids were detected (Gopinath et al., 2012). Recently, an investigation conducted with the aqueous extracts of six *Phyllanthus* species, reported alkaloids from low to high amounts in all the extracts evaluated (*P. amarus* among them), mainly in the leaf extracts (Awomukwu et al., 2014). These dissimilarities in alkaloid content might be due to different methodologies such as extraction method and solvent used by the authors for the determination of phytocomponents, as well as diverse environmental conditions that affect the chemical constitution of the plants. Phytochemical determination is also a challenging process because of the large number of compounds present in trace amounts.

It was also quantified the phenolic content (µg GAE/mg lyophilized), resulting in a *P. williamioides* > *P. microdictyus* > *P. chamaecristoides* trend (Table 2). The former exhibited around three and six times more phenolic content respecting to *P. microdictyus* and *P. chamaecristoides*, respectively, and also higher than reported for other *Phyllanthus* species aqueous extracts such as *P. amarus*, *P. emblica*, *P. niruri*, and *P. urinaria* (Charoenteeraboon et al., 2010; Poh-Hwa et al., 2011). These results reinforce the structural biodiversity found for different plant species belonging to the same genus, and also point out a possible diverse bioactivity among them.

Antioxidant capacity was evaluated herein by measuring aqueous extracts ability to *in vitro* scavenge free radical generated by $DPPH^{\cdot}$. The greater inhibition of $DPPH^{\cdot}$ was found for *P. chamaecristoides* extract (Table 3), which according to previous studies for the aqueous extracts from several *Phyllanthus* species using the same methodology, exhibited better antioxidant response than *P.*

amarus and *P. emblica* (Charoenteeraboon et al., 2010; Chandan et al., 2012).

Polyphenols are a very important family of phytocomponents involve in the antioxidant response (Afaq and Katiyar, 2011). The extract obtained from *P. williamioides*, with the highest content of polyphenols exerted a good antioxidant activity, better than the detected for Trolox at 0.1 mg/mL. However, *P. microdictyus* extract showed an intermediate amount of phenolic content, but exhibited the minor antioxidant activity for all concentrations tested and *P. chamaecristoides* aqueous extract showed modest phenolic content, but displayed the highest antioxidant response in the $DPPH^{\cdot}$ assay. This data suggest the presence of bioactive compounds others than phenols in this extract, such as tocopherols and carotenoids. Nevertheless, it could be possible that trace amounts of very active polyphenols may be present. From the results derived in the present investigation, flavonoids and reducing sugars were found in higher amounts for *P. chamaecristoides* respect to *P. williamioides* extract, and they might be implicated in the differential antioxidant response detected. In others experiments carried out with these species, for the *in vitro* reduction of ferric ions, a similar antioxidant behavior was detected: *P. chamaecristoides* > *P. williamioides* > *P. microdictyus* (data not shown).

In the present work, it was examined the genoprotective properties of these species against UVC radiation at two levels: structural DNA damage and mutations. Antimutagenic effects of the extracts might be due to different action mechanisms: desmutagens or bioantimutagens (De Flora and Ferguson, 2005). Desmutagens interfere in the radiation absorption by DNA, meanwhile bioantimutagens act after UV-induced damage, modulating DNA replication and repair mechanism or inducing apoptosis, and overall declining induced and spontaneous mutation frequency (Bhattacharya, 2011).

In order to establish if these aqueous extracts could exert photoprotection through a desmutagenic mechanism, transmittance was quantified. No absorption of UVC radiation (λ=254 nm) was detected for any of the plant extracts evaluated (1-1000 µg/mL). This result is indicative that the components present in the studied *Phyllanthus* aqueous extracts did not exert their photoprotective action through desmutagenic mechanisms.

For detection of DNA structural damage in *C. crescentus* cells upon UVC radiation in the presence of the different treatments, it was performed the SOS Chromotest, considered one of the simplest short-term assays for (anti)genotoxicity studies. All extracts significantly decrease β-galactosidase activity (Fig. 1A), principally *P. chamaecristoides* and *P. microdictyus*. Simultaneously, all treatments induced an increase in cells survival (Fig. 1B). *P. microdictyus* and *P. chamaecristoides* aqueous extracts (1 mg/mL) duplicate and triplicate, respectively, survival values for irradiated cells. Altogether, these data indicate that there are some phytocomponents that render the extracts with photoprotective properties. Although we could not establish the particular compounds responsible for such activity, we assumed that they should be chemically diverse and/or be present in different amounts, and act through varied mechanism, as the decline in SOS response showed different patterns: dose-dependent for *P. chamaecristoides*, dose-independent for *P. williamioides*, and an intermediate behavior for *P. microdictyus*.

SOS response in bacteria is highly correlated with mutagenicity, due to the induction of DNA translesionals polymerases (Janion, 2008). DNA damage caused by UVC radiation if not efficiently repaired, could trigger SOS response, and therefore induce mutations. So it is logical to assume that a significant diminish in SOS response detected may also implies a reduction in mutation frequency.

Results from the Rif^R test for *P. chamaecristoides* and *P. microdictyus* aqueous extracts showed a significant diminish in relative mutation frequency (RMF) values for all concentration tested, fewer than 20 and 35%, respectively (Fig. 2). For both species (1 mg/mL) RMF ≤ 5%, hence they could be considered as highly antimutagenic (Bandyopadhyay et al., 2014).

Overall, these results indicate that *Phyllanthus* plant extracts might act as bioantimutagens. It is probably that some phytocomponents in the extracts could be able to modulate DNA repair mechanism, diminishing SOS response and related mutagenicity induced by UVC radiation. In this regard,

the aqueous extract from *P. orbicularis* have shown bioantimutagenic activity in human fibroblast irradiated with UVB, by enhancing cyclobutane pyrimidine dimers *cis-syn* (CPDs) lesions removal in a time-dependent fashion (Vernhes et al., 2013a). These authors associate such effects to modulation of nucleotide excision repair (NER), the main DNA repair mechanism in human cells, by polyphenols present in the extract, but they could not determine which one(s). It is known that polyphenols, such as catechins and flavonoids, are involved in UV protection through modulation of NER system. Studies on the effects of green tea (*Camellia sinensis*) polyphenols, mainly catechins such as (-)-epigallocatechin-3-gallate, on the DNA repair kinetics and repair mechanisms of UV-induced CPDs, have been carried out using *in vitro* and *in vivo* models (Katiyar, 2011). Several studies indicate that these polyphenols positively influence the repair of UVB-induced DNA damage through enhancement of NER genes, in a mechanism that involves interleukin-12 (Meeran et al., 2006). Flavonoids are also very important phytocomponents in photo-protection, mostly because of their UV absorbing properties and their ability to act as antioxidants (Saewan and Jimtaisong, 2013). It has been established that silymarin, a flavononol, has the ability to decrease UVB radiation-induced DNA damage in normal human epidermal keratinocytes through NER system repair enhancement (Katiyar et al., 2011).

Other authors had evaluated the genoprotective properties of the aqueous extracts from some *Phyllanthus* species, such as *P. emblica* (Majeed et al., 2011), *P. niruri* (Raja et al., 2011), and *P. orbicularis* (Vernhes et al., 2013b) against UVB radiation in *ex vivo*, *in vitro* and *in vivo* experiments. These authors had highlighted as the main photoprotective mechanism the antioxidant activity of the extracts (Chen et al., 2012). The antioxidant properties exerted by the *Phyllanthus* species assessed in the present investigation should not be ruled out as a genoprotective mechanism for long UV wavelengths.

P. williamioides aqueous extract slightly declined SOS response (Fig. 1A), but did not decrease the UVC-induced mutagenesis (Fig. 2). By the contrary, 1 mg/mL of the extract induced a two-fold significant increase in detected mutations. This result is an indicative that there are metabolites with different bioactivity in *P. williamioides* extract. Even though there are phytocompounds that positively modulate DNA damage repair mechanisms, the extract should comprise phytocompounds able to inactivate proteins or down-regulate important genes involved in the mutations fixing process, and thus increasing the mutation frequency above the values obtained with the positive control. It has been recently reported that an aqueous mix of *P. amarus*, *P. niruri*, *P. urinaria*, and *P. watsonii*, diminish the expression of protein Msh2, involved in DNA mismatch repair mechanism (Lee et al., 2013). In the study, authors also reported high amounts of polyphenols, such as geraniin (tannin), rutin and quercetin glucoside (flavonoids). Taking into account that *P. williamioides* extract showed the higher amounts of polyphenols (Table 2), it is possible that some of them in high concentration may exert the observed mutagenic effect. Further studies are needed to a better understanding on this interesting behavior.

CONCLUSIONS

In the current work was assessed and compare the phytochemical composition, antioxidant capacity, and genoprotective properties of the aqueous extracts obtained from three Cuban endemic *Phyllanthus* plants. Based in the transmittance results, it was concluded that the detected reduction in DNA damage is not founded in a desmutagenic effect. *P. chamaecristoides* extract showed the highest antioxidant capacity for all concentrations tested, and together with *P. microdictyus* (1 mg/mL) exhibited the greatest bioantimutagenic effects in *C. crescentus* cells. It is possible that the genoprotective activity detected could be due to modulation of DNA repair mechanisms, diminishing SOS response and related mutagenicity induced by UVC radiation. Moreover, high antioxidant activity could also decrease UV radiation-induced oxidative damage. These out-comes enrich the current knowledge about the genoprotective properties of Cuban endemic *Phyllanthus* spp. that have been studied for our group during the last two decades, and shed more light into the underlying molecular mechanisms that sustain such pharmacological activities, supporting future pre-clinic assays.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

This work was supported by CAPES (Brazil) - MES (Cuba) International Collaboration (Project 187/13). The authors want to thank to Lic. Banessa Falcon for her kind help in plant material collection and authentication.

REFERENCES

Afaq F, Katiyar SK (2011) Polyphenols: skin photoprotection and inhibition of photocarcinogenesis. Mini Rev Med Chem 11: 1200-1215.

Ahmad MD, Bano S, Anwar S (2015) Cancer ameliorating potential of *Phyllanthus amarus*: *in vivo* and *in vitro* studies against aflatoxin B1 toxicity. Egypt J Med Hum Genet 16: 343-353.

Alonso A, Fuentes JL, Sanchez-Lamar A, Llagostera M (2010) Antimutagenic effect of *Phyllanthus orbicularis* against γ-radiation. Lat Am J Pharm 29: 148-152.

Awomukwu DA, Nyananyo BL, Onukwube ND, Uka CJ, Okeke CU, Ikpeama AI (2014) Comparative phytochemical constituents and pharmacognostic importance of the vegetative organs of some *Phyllanthus* species in South Eastern Nigeria. Int J Modern Bot 4: 29-39.

Bandyopadhyay N, Gautam S, Sharma A (2014) Suppression of SOS repair in *E. coli*: possible mechanism of antimutagenicity and protective effects of common vegetables. Int J Food Sci Nutr 65: 251-258.

Bhattacharya S (2011) Natural antimutagens: a review. Res J Med Plant 5: 116-126.

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol 28: 25-30.

Cadet J, Wagner JR (2013) DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harb Perspect Biol 5: a012559.

Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. Cell 104: 901-912.

Chandan S, Umesha S, Balamurugan V (2012) Antileptospiral, antioxidant and DNA damaging properties of *Eclipta alba* and *Phyllanthus amarus*. J Vet Sci Technol 1: 231.

Charoenteeraboon J, Ngamkitidechakul C, Tajoy K, Sireeratowong S (2010) Antioxidant activities of the standardized water extract from fruit of *Phyllanthus emblica* Linn. Songklanakarin J Sci Technol 32: 599-604.

Chen L, Hu JY, Wang SQ (2012) The role of antioxidants in photoprotection: a critical review. J Am Acad Dermatol 67: 1013-1024.

Chhabra SC, Uiso FC, Mshiu EN (1984) Phytochemical screening of Tanzanian medicinal plants. J Ethnopharmacol 11: 157-179.

De Flora S (2013) Genotoxicity and carcinogenicity of the light emitted by artificial illumination systems. Arch Toxicol 87: 403-405.

De Flora S, Ferguson LR (2005) Overview of mechanisms of cancer chemopreventive agents. Mutat Res 591: 8-15.

Ely B (1991) Genetics of *Caulobacter crescentus* Methods Enzymol 204: 372-384.

Ferrer M, Sánchez-Lamar A, Fuentes JL, Barbé J, Llagostera M (2001) Studies on the antimutagenesis of *Phyllanthus orbicularis*: mechanisms involved against aromatic amines. Mutat Res 498: 99-105.

Ferrer M, Sánchez-Lamar A, Fuentes JL, Barbé J, Llagostera M (2002) Antimutagenic mechanisms of *Phyllanthus orbicularis* when hydrogen peroxide is tested using *Salmonella* assay. Mutat Res 517: 251-254.

Galhardo RS, Rocha RP, Marques MV, Menck CFM (2005) An SOS-regulated operon involved in damage-inducible mutagenesis in *Caulobacter crescentus*. Nucleic Acids Res 33: 2603-2614.

González S, Gilaberte Y, Philips N, Juarranz A (2011) Current trends in photoprotection - A new generation of oral photoprotectors. Open Dermatol J 5: 6-14.

Gopinath SM, Rakesh CK, Narasimha-Murthy TP, Dayananda KS (2012) Preliminary phytochemical evaluation of leaf extracts of *Gymnema sylvestre*, *Phyllanthus amarus*, *Phyllanthus reticulatus* of Siddarabetta, Tumkur district, Karnataka. Int J Pharmacogn Phytochem Res 4: 109-111.

Janion C (2008) Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. Int J Biol Sci 4: 338-344.

Katiyar SK (2011) Green tea prevents non-melanoma skin cancer by enhancing DNA repair. Arch Biochem Biophys 508: 152-158.

Katiyar SK, Mantena SK, Meeran SM (2011) Silymarin protects epidermal keratinocytes from ultraviolet radiation-induced apoptosis and DNA damage by nucleotide excision repair mechanism. PLoS One 6: e21410.

Lee SH, Tang YQ, Rathkrishnan A, Wang SM, Ong KC, Manikam R, Payne BJ, Jaganath IB, Sekaran SD (2013) Effects of cocktail of four local Malaysian medicinal plants (*Phyllanthus* spp.) against dengue virus 2. BMC Complement Altern Med 13: 192-205.

Lopes-Kulishev CO, Alves IR, Valencia EY, Pidhirnyj MI, Fernández-Silva FS, Rodrigues TR, Guzzo CR, Galhardo RS (2015) Functional characterization of two SOS-regulated genes involved in mitomycin C resistance in *Caulobacter crescentus*. DNA Repair 33: 78-89.

Majeed M, Bhat B, Anand S, Sivakumar A, Paliwal P, Geetha KG (2011) Inhibition of UV-induced ROS and collagen damage by *Phyllanthus emblica* extract in normal human dermal fibroblasts. J Cosmet Sci 62: 49–56.

McKenzie RL, Aucamp PJ, Bais AF, Björn LO, Ilyas M, Madronich S (2011) Ozone depletion and climate change: impacts on UV radiation. Photochem Photobiol Sci 10: 182-198.

Meeran SM, Mantena SK, Elmets CA, Katiyar SK (2006) (-)-Epigallocatechin-3-gallate prevents photocarcinogenesis in

mice through interleukin-12–dependent DNA repair. Cancer Res 66: 5512-5520.
Menéndez-Perdomo IM, Fuentes-León F, Casadelvalle I, Menck CFM, Galhardo RS, Sánchez-Lamar Á (2016) Assessment of cytotoxic and genotoxic effects of Cuban endemic *Phyllanthus* (Phyllanthaceae). Rev Cubana Cienc Biol 5: 67-75.
Narasimhudu CL, Raju RRV (2012) Phytochemical constitutens of *Phyllanthus* species (Euphorbiaceae) from eastern Ghats of Andhra Pradesh, India. Int Res J Pharm 3: 184-200.
Pfeifer GP, Besaratinia A (2012) UV wavelength-dependent DNA damage and human non-melanoma and melanoma skin cancer. Photochem Photobiol Sci 11: 90-97.
Poh-Hwa T, Yoke-Kqueen C, Indu Bala J, Son R (2011) Bioprotective properties of three Malaysia *Phyllanthus* species: an investigation of the antioxidant and antimicrobial activities. Int Food Res J 18: 3887-3893.
Quettier-Deleu C, Gresier B, Vasseur J, Dine T, Brunet C, Luyckx M, Cazin M, Cazin JC, Bailleul F, Trotin F (2000) Phenolic compounds and antioxidant activities of buckwhat (*Fagopyrum esculentum* Moench) Hulls Ans Flour. J Ethnopharmacol 72: 35-42.
Raja W, Hanfi S, Pandey S, Tripathi K, Hanfi S, Agrawal RC (2011) Effect of *Phyllanthus niruri* leaf extract on antioxidant activity and UV induced chromosomal aberration in Swiss Albino mice Pharmacologyonline 3: 169-174.
Saewan N, Jimtaisong A (2013) Photoprotection of natural flavonoids. J Appl Pharm Sci 3: 129-141.
Sánchez-Lamar A, Fiore M, Cundari E, Ricordy R, Cozzi R, De Salvia R (1999) *Phyllanthus orbicularis* aqueous extract: cytotoxic, genotoxic, and antimutagenic effects in the CHO cell line. Toxicol Appl Pharm 161: 231-239.
Sánchez-Lamar A, Pérez JA, Ros J, Pons J, Ferrer M, Fuentes JL, Llagostera M (2015) Fractionation of an aqueous extract of *Phyllanthus orbicularis* Kunth and identification of antioxidant compounds. Rev Cubana Cienc Biol 4: 56-62.
Sarin B, Verma N, Martin JP, Mohanty A (2014) An overview of important ethnomedicinal herbs of *Phyllanthus* Species: present status and future prospects. Sci World J 2014: 12.
Schuch AP, Moraes MCS, Yagura T, Menck CFM (2014) Highly sensitive biological assay for determining the photoprotective efficacy of sunscreen. Environ Sci Technol 48: 11584-11590.
Vernhes M, González-Pumariega M, Andrade L, Schuch AP, de Lima-Bessa KM, Menck CFM, Sánchez-Lamar A (2013a) Protective effect of a *Phyllanthus orbicularis* aqueous extract against UVB light in human cells. Pharm Biol 51: 1-7.
Vernhes M, González-Pumariega M, Passaglia A, Martins FC, Sánchez-Lamar A (2013b) *Phyllanthus orbicularis* K aqueous extract protects plasmid DNA against the damage induced by ultraviolet radiation. Ars Pharm 54: 16-23.
Zhang X, Bremer H (1995) Control of the *Escherichia coli rrnB* P1 promoter strength by ppGpp*. J Biol Chem 270: 11181-11189.

Author contribution:

Contribution	Menéndez-Perdomo IM	Wong-Guerra M	Fuentes-León F	Carrazana E	Casadelvalle I	Vidal A	Sánchez-Lamar A
Concepts or ideas						X	X
Design	X				X	X	X
Definition of intellectual content					X	X	X
Literature search	X	X					X
Experimental studies	X	X	X	X			
Data acquisition	X	X		X			
Data analysis	X	X	X				X
Statistical analysis	X	X	X				
Manuscript preparation	X	X	X	X	X	X	X
Manuscript editing	X						X
Manuscript review	X	X	X	X	X	X	X

Citation Format: Menéndez-Perdomo IM, Wong-Guerra M, Fuentes-León F, Carrazana E, Casadelvalle I, Vidal A, Sánchez-Lamar A (2017) Antioxidant, photoprotective and antimutagenic properties of *Phyllanthus* spp. from Cuban flora. J Pharm Pharmacogn Res 5(4): 251–261.