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Fractionation of an aqueous extract of *Phyllanthus orbicularis* Kunth and identification of antioxidant compounds

Identificación de fitocompuestos antioxidantes en el extracto acuoso de Phyllanthus orbicularis *Kunth*

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ABSTRACT

The aqueous extract of Phyllanthus orbicularis *contains bioproducts able to significantly reduce the mutagenesis mediated by oxidative compounds. A bioactive-directed fractionation of the aqueous extract of P. orbicularis has been applied with the aim of identifying antimutagenic fractions, which allowed isolating and identifying some biocompound responsible for this activity. Antimutagenesis of all fractions was determined by the Salmonella retromutation assay, using hydrogen peroxide as model mutagen. Applying this methodology, two potent antimutagenic fractions have been obtained able to totally abolish the mutagenicity produced by hydrogen peroxide. In these fractions 2,4-di-tertbutylphenol, OO'-diphenol-4,4',6,6'-tetra-tert-butyl and 2,6-di-sec-butylphenol were identified by Gas Chromatography/Mass Spectrometry.*

Keywords: P. orbicularis; *2,4-di-tert-butylphenol; OO'-diphenol-4,4',6,6'-tetra-tert-butyl; 2,6-di-secbutylphenol; antimutagenesis; oxidative damage; chemical fractionation*

RESUMEN

El extracto acuoso de *Phyllanthus orbicularis* es capaz de reducir significativamente la mutagénesis inducida por agentes oxidantes como el peróxido de hidrógeno y aminas aromáticas. Con el objetivo de identificar algún fitocomponente responsable de esta propiedad, se aplicó un fraccionamiento químico del extracto, guiado por el monitoreo de la referida bioactividad. La antimutagenicidad de cada fracción frente al peróxido de hidrógeno se determinó mediante el ensayo de retromutación en la cepa TA102 de *Salmonella typhimurium* (Test Ames). La identificación de los compuestos específicos se realizó por cromatografía gaseosa y espectrometría de masa. Se obtu-

vieron dos fracciones con alto grado de pureza cuya actividad antimutagénica fue capaz de abolir totalmente la mutagenicidad producida por el peróxido de hidrógeno. En una de estas fracciones se identificaron los compuestos 2,4-di-ter-butilfenol y su forma dimérica, OO'-diphenol-4,4',6,6'-tetra-ter-butil; en la otra, se identificó un solo compuesto, el 2,6-di-sec-butilfenol. Estos tres metabolitos secundarios pertenecen a la familia fitoquímica de los fenoles, en la cual se incluyen muchos de los agentes antioxidantes informados por la literatura especializada.

Palabras clave: *P. orbicularis*; 2,4-di-*ter*-butilfenol; OO'-difenol-4,4',6,6'-tetra-*ter*-butil; 2,6-di-*sec*-butilfenol; antimutagénesis; daño oxidativo; fraccionamiento químico.

INTRODUCTION

The efficient detection of compounds responsible for biological effects is one of the major challenges for the isolation and purification of bioactive products within a complex natural mixture. In this sense, the establishment of strategies and methodologies for the isolation of active compounds from plants has always been a constant field of interest due to the frequent presence of substances with potential applications as therapeutics. The most efficient methods for compound isolation are bioactivity-guided fractionations, although it is certain that some natural products, which could be very useful for the treatment of human illnesses, will probably, remain undiscovered.

Chemoprevention of mutation-related diseases is an area of increasing research. The search for chemopreventive agents, the assessment of their efficacy and safety, and the knowledge of the involved mechanisms are certainly of essential importance in order to develop fine chemoprevention strategies (De Flora and Izzotti, 2007; Nagy *et al.*, 2009; De Flora and Bonanni, 2011). In the last several years an extensive number of studies have reported different medicinal plants and dietary components as an excellent source of chemopreventive agents; likewise, interest is addressed to the isolation of plant compounds, and the elucidation of their mechanisms of action has received particular attention (El-Mahdy *et al.*, 2008; Katiyar *et al.*, 2010; Katiyar, 2011). The mutations induced by hydroperoxides are a direct consequence of radicals generated upon their decomposition. The multiplicity of alterations produced in DNA after exposure to reactive oxygen species is associated with both the early steps in carcinogenesis and many types of chronic degenerative diseases (Cervelli *et al.*, 2012; Zech *et al.*, 2013; Deng *et al.*, 2014). In this direction, the isolation of new products with major antioxidant activities and the elucidation of their mechanisms of action are a crucial step for the establishment of fine chemoprevention strategies for degenerative diseases.

Among the different species included in the genus *Phyllanthus*, known for its extended use in traditional medicine and confirmed therapeutic properties (Forzza *et al.*, 2012; González-Ramírez, 2010; Idárraga -Piedrahita *et al.*, 2011), *Phyllanthus orbicularis* Kunth is an endemic plant from Cuba, the aqueous extract of which has different antiviral activities (del Barrio and Parra, 2000; Alvarez *et al.*, 2009; Roques, 2011). Besides this activity, we have reported the antimutagenic activity of this plant extract against some promutagenic aromatic amines by two major mechanisms: the direct interaction of plant compounds with promutagens and the modulation by components of the extract of the activity of hepatic enzymes, which are responsible for promutagen activation (Ferrer *et al.*, 2001). Furthermore, we have also reported that the aqueous extract of *Phyllanthus orbicularis* contains antimutagenic compounds able to protect DNA of oxidative-induced damage by hydrogen peroxide and g-radiations, through reducing the number of chromosome aberrations in the Chinese hamster ovary cell-line (Sánchez-Lamar *et al.*, 1999), the number of mutants of *Salmonella* cells (Ferrer *et al.*, 2002), and the induction of SOS-repair in *E. coli* cells (Alonso *et al.*, 2005; Fuentes *et al.*, 2006).

The present work is focused on applied methodologies based on a bioactive-directed fractionation of the aqueous extract of *Phyllanthus orbicularis* that allowed isolating, purifying and characterizing compounds responsible for *P. orbicularis* antimutagenesis against oxidative damage.

MATERIALS AND METHODS

Phyllanthus orbicularis aqueous extract

Phyllanthus orbicularis plants were collected from Cajálbana, Pinar del Río, Cuba. The specimens were authenticated and stored at the Cuban Botany Garden (No.7/220 HAJB). *P. orbicularis* aqueous extract employed in this work was obtained from leaves and stems and further lyophilized, following the method previously described (del Barrio and Parra, 2000).

Phytochemichal studies of the *P. orbicularis* extract have demonstrated the presence of the compounds above on Table 1.

Table 1. Phytochemichal composition of *P. orbicularis* extract.

Tabla 1. Composición fitoquímica del extracto de P. orbicularis.

PHYTOCHEMICAL COMPONENTS	REFERENCES
Polyphenol compounds	
Flavonoids	
Tannins	
Quinonas	
Antocianidins	
Coumarins	
Quercetin glycosides	
Flavanols	Gutiérrez *et al.*, 2000
Condensed tannins (pro- cyanidin dimers)	Alvarez *et al.*, 2009
Gallic acid-derivatives	Gutiérrez *et al.*, 2010
Quercetin- 3-O-rutinoside (rutin)	
Kaempherol-3-O-rutinoside	
Procyanidin B1 and procyanidin B2	
Catechin	
Epicatechin	
Protocatechuic acid	
Hydrocarbon	
Cylooctacosane	Gutiérrez *et al.*, 2011
Terpene	
Esterol b-sitosterol	

Fractionation Process

The fractionated process was started by dissolving the plant extract in methanol for 48 hours with gentle shaking. The solvent was removed under reduced pressure (Fraction A) and the insoluble fraction was discharged. The complete Fraction A was dissolved in water and extracted 3 times with chloroform (3:1), obtaining an organic fraction which contains fewer polar compounds (Fraction B) and a white aqueous fraction (Fraction C). Both fractions were dried and Fraction C was extracted 3 times with a water/dichloromethane mix (3:1), rendering an aqueous (E) and an organic (D) fraction, the latter being fractionated by silica 60 Å column chromatography, eluted with ethyl acetate/ hexane (2:1), giving 5 sub-fractions (Rf1 to Rf5). Fraction Rf2 was chosen to continue the fractionation process, following the chromatographic method described before, six different sub-fractions (Rf2p, Rf1', Rf2', Rf3', Rf4' and Rf5') being obtained.

Chemical analysis

^{1}H NMR and $^{13}C\{^{1}H\}$ NMR spectra of Rf1 and Rf2p compounds were recorded on a NMR-FT Bruker 250 MHz spectrometer in $CDCl_3$ solution at room temperature. Chemical shifts are reported as d (ppm) with tetramethylsilane (TMS) as the internal standard. The chemical components in Rf1 and Rf2p were identified by Gas Chromatography/Mass Spectrometry (GC-MS) (Hewlett-Packard 6890 GC and MS Engine 5973 mass spectrometer, Beckman Coulter Inc., USA) (Günther, 1980; Hesse *et al.*, 1987; Pretsch *et al.*, 1989; Williams and Fleming, 1995).

Antimutagenesis assay

The antimutagenesis of the different fractions against hydrogen peroxide (H_2O_2) from Aldrich was determined by the plate-incorporation mutagenicity assay as described (Ferrer *et al.*, 2002) and using *Salmonella enterica Typhimurium* tester strain TA102 (Levin *et al.*, 1982), kindly supplied by Dr. B. N. Ames. Seventy five µg of each fraction per plate were used to test its antimutagenic activity in front 100 µg of H2O2. Two independent assays were performed with each fraction and the antimutagenic activity of each fraction was considered optimal when the remaining mutagenesis (% RM) (Ferrer *et al.*, 2002) obtained was below 20%, which means an antimutagenic activity higher than 80%. This criterion was used to select the most active fractions along the fractionation process. Nevertheless, in particular cases, the antimutagenicity against m-PDA, PhIP, 4-ABP and B(a)P, was used as a complementary criterion.

RESULTS

The fractionation strategy here developed was designed according to the purpose to first extract the most polar compounds and then in a decreasing order of polarity five separation steps were performed (Fig. 1), searching for to obtain active products responsible for the antimutagenesis activity against H_2O_2, detected in the crude extract of *P. orbicularis*

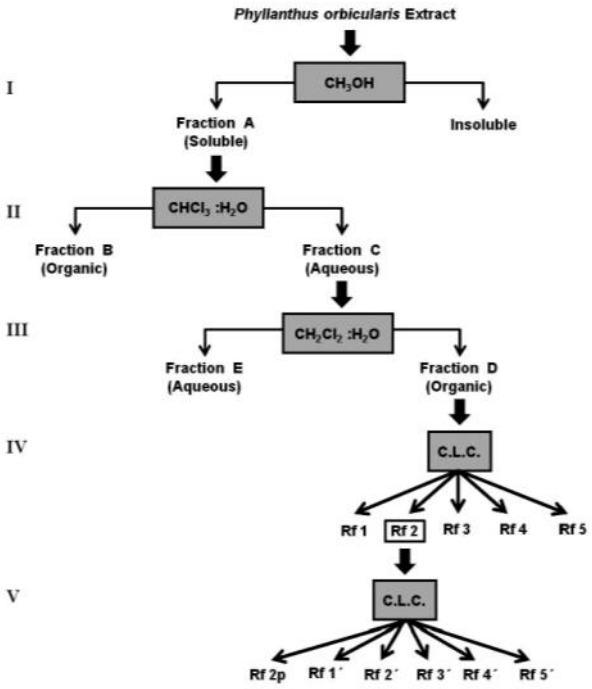

Figure 1. Flow chart of the procedure followed for the chemical fractionation of *Phyllanthus orbicularis* extract. C.L.C = column liquid chromatography.

Figura 1. Diagrama de flujo del procedimiento seguido para el fraccionamiento químico del extracto de Phyllanthus orbicularis*. C.L.C = cromatografía de columna líquida (siglas en inglés).*

(Sánchez-Lamar *et al.*, 1999; Ferrer *et al.*, 2002). Five separation steps were performed. The standardized method of the fractionation of the plant extract allowed us to obtain active fractions in a reproducible way, which showed different levels of activity in front of H_2O_2 (Table 2).

The methanol soluble fraction (Fraction A) showed an antimutagenic activity and a level of chemical complexity almost identical to the crude plant extract (Ferrer *et al.*, 2002). Likewise, the insoluble fraction that was also tested for antimutagenic activity was discharged due to its considerable lower activity. Fraction A was used for the following fractionation steps. The second step rendered a very active organic fraction (Fraction B) and another (Fraction C) with certain activity level. Fraction C was again fractionated, giving an aqueous fraction (E), practically inactive, and a very active organic fraction (D). When different fractions had near values of antimutagenic activity front H_2O_2, other mutagens were used to decide the most effective antimutagenic fraction (Table 2). Using silica column chromatography, Fraction D was further subfractionated, yielding 5 sub-fractions (Rf1 to Rf5). The best activity was detected for Rf1, Rf2 and Rf4 with 100%, 93% and 93% of antimutagenic activity, respectively. Then Rf2 was again sub-fractionated by liquid chromatography in column with ethyl acetate/hexane (2:1), obtaining 6 sub-fractions (Rf2, Rf1' to Rf5'). Among them, Rf2p was extremely active against H_2O_2, because the H2O2-mediated mutagenesis was totally abolished (Table 2).

Results obtained indicated that Rf1 and Rf2p contain some of the bioproducts of *P. orbicularis* able to avoid

Table 2. Remainder Mutagenesis (%RM), in *Salmonella typhimurium* mutagenicity assay, when chemical fractions from *Phyllanthus orbicularis* aqueous extract were used vs. model mutagens.

Tabla 2. Mutagénesis remanente (%RM) en el ensayo de mutagenicidad con Salmonella typhimurium al emplear el extracto acuoso de Phyllanthus orbicularis *frente a diferentes modelos de mutágenos.*

75 µg/plate of: Organic Fractions	%RM for:				
	H_2O_2 (100 µg/plate)	m-PDA (200 µg/plate)	PhIP (5 µg/plate)	4-ABP (3 µg/plate)	B(a)P (2,5 µg/plate)
B	9	15	93	75	54
D	12	9	8	10	10
Sub-fractions* derived from: D					
Rf2	**7**	**0**	47	49	67
Rf4	7	40	46	29	41

*: Obtained by column liquid chromatography ND: Not determined.
m-PDA: meta-fenilendiamina; PhIP: 2-amino-1-metil-6-fenilimidazo[4,5-b]piridina; 4-ABP: 4-aminobifenil; B(a)P: Benzo(a)pireno.

the oxidative damage in *Salmonella* cells. The chemical analysis indicated the presence of phenolic substance in Rf1 and Rf2p sub-fractions. As result of Gas Chromatography/Mass Spectrometry analysis, two compounds [2,4-di-*tert*-butylphenol and OO'-diphenol -4,4',6,6'-tetra-*tert*-butyl] were identified in Rf1 (Fig. 2A), while only one compound, 2,6-di-*sec*-butylphenol (DSBP), was present in Rf2p (Fig. 2B). The physical and spectral data for each one of them were:

2,4-di-*tert*-butylphenol:

MS (ESI) [*m/z (%)*]: 206 (15%) [M+], 191 (100%) [M+ - Me], 91 (7%) [M+ - 2 *t*Bu], 57 (23%) [*t*Bu]; ^{1}H NMR ($CDCl_3$ solution, 250 MHz). δ: 7.28 (1H, d, J = 2 Hz, H_{bz}), 7.01 (1H, dd, J_{orto} = 8 Hz, J_{meta} = 2Hz, H_{bz}), 6.82 (1H, d, J_{orto} = 8 Hz, H_{bz}), 1.43 (18H, s, C-(C*H*3)3), 1.35 (18H, s, C-(C*H*3)3).

^{13}C{^{1}H} NMR ($CDCl_3$ solution, 250 MHz). δ: 151.2 (*C*-OH), 143-122 (C_{bz}), 35.2 (*C*-$(CH_3)_3$), 32.8 (C-(*C*$H_3)_3$), 31.4 ($(CH_3)_3$), 31.2 ($(CH_3)_3$).

OO'-diphenol-4,4',6,6'-tetra-*tert*-butyl:

MS (ESI) [*m/z (%)*]: 410 (50%) [M+], 395 (39%) [M+ - Me], 339 (63%) [MH+ -Me -*t*Bu], 190 (27%) [M+/2 – Me], 57 (100%) [*t*Bu].

^{1}H NMR ($CDCl_3$ solution, 250 MHz). δ: 7.31 (2H, d, J_{meta} = 2 Hz, H_{bz}), 7.11 (2H, d, J_{meta} = 2Hz, H_{bz}), 1.41 (18H, s, C-(C$H_3)_3$), 1.30 (18H, s, C-(C$H_3)_3$).

^{13}C{^{1}H} NMR ($CDCl_3$ solution, 250 MHz). δ: 149.7 (*C*-OH), 142-117 (C_{bz}), 36.8 (*C*-$(CH_3)_3$), 34.2 (*C*-$(CH_3)_3$), 31.3 (C-(*C*$H_3)_3$), 30.7 (C-(*C*$H_3)_3$), 29.8 ($(CH_3)_3$), 29.6 ($(CH_3)_3$).

2,6-di-*sec*-butylphenol:

MS (ESI) [*m/z (%)*]: 206 (17%) [M+], 177 (100%) [MH+ - 2Me], 91 (11%) [M+ - 2 *s*Bu].

^{1}H NMR ($CDCl_3$ solution, 250 MHz). δ: 6.98 (2H, d, H_{bz}), 6.81 (1H, t, H_{bz}), 3.35 (2H, m, C*H*-CH_3), 2.85 (4H, m, CH_2-CH_3), 2.65 (12H, m, C*H*3).

^{13}C{^{1}H} NMR ($CDCl_3$ solution, 250 MHz). δ: 149.7 (*C*-OH), 142-117 (C_{bz}), 36.8 (*C*-$(CH_3)_3$), 34.2 (*C*-$(CH_3)_3$), 31.3 (C-(*C*$H_3)_3$), 30.7 (C-(*C*$H_3)_3$), 29.8 ($(CH_3)_3$), 29.6 ($(CH_3)_3$).

It is noteworthy that Rf1 contains the monomer 2,4-di-*tert*butylphenol (DTBP) and its dimer OO'-diphenol-4,4',6,6'-tetra-*tert*-butyl (bis-DTBP). We believe that DTBP is probably rendered by the breakdown of the dimer (bis-DTBP), perhaps through the fractionation process. However other hypothesis is possible: may be the bis-DTBP is formed by dimerization of some DTBP radicals form.

DISCUSSION

Plant phenolic compounds are the most frequently reported substances, responsible for antimutagenic properties shown to be fine antioxidants and potent modulators of xenobiotic metabolism enzymes, among other activities (Krizková *et al.*, 2008; Svobodova *et al.*, 2009; Chakraborty and Verma, 2010). In many reports about isolation of plant compounds, searching for anticarcinogens or antimutagens, a first step is frequently used that allows for the total recovery of these phenolic compounds (Schwikkard *et al.*, 2000; Miyazawa and Hisama, 2003), and the utilization

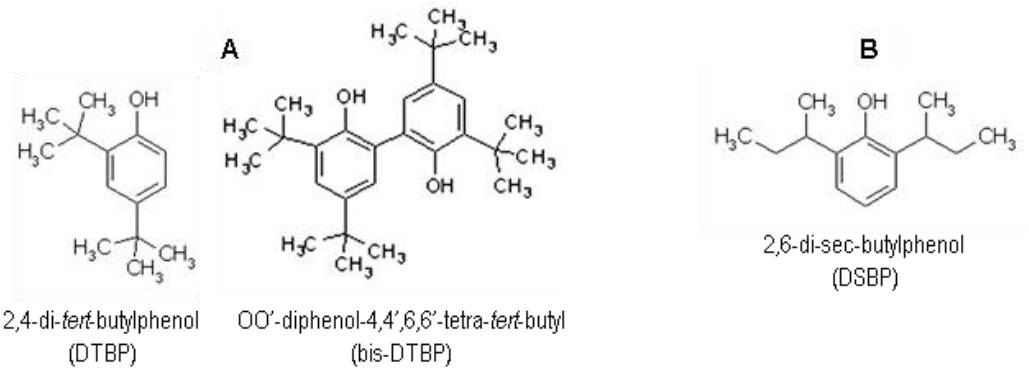

Figure 2. Molecular structure of phenol compounds identified in Rf1 (A) and Rf2p (B) sub-fractions obtained from *Phyllanthus orbicularis* extract.

Figura 2. Estructura molecular de los compuestos fenólicos identificados en las subfracciones Rf1 (A) and Rf2p (B) obtenidos del extracto de Phyllanthus orbicularis.

of methanol is strongly recommended, which usually yields fine extraction of this kind of chemicals.

Little information exists about the biological activities of these three compounds identified by us. Recently, it has been show that DTBP and bis-DTBP are radical scavengers and present a high cytotoxic activity against a human submandibular gland carcinoma cell line and human gingival fibroblasts (Fujisawa *et al.* 2004). Structurally similar to DTBP compounds: 4-metil-2,6-di-tertbutilfenol (ionol), 2-amino-4-tertbutylphenol, and 2-amino-4,6-di-tertbutylphenol, has been proposed as powerful antioxidant agents (Serbinova *et al.*, 2004; Fujisawa *et al.*, 2004; Grigorenko *et al.*, 2007). The DSBP, an analogue of the propofol which is a clinically useful general anaesthetic (Langley and Heel, 1988), is known as an effective intravenous anaesthetic in mice (James and Glen, 1980) and it has been recently demonstrated its high potency at g-amino butyric acid type A (GABAA) receptors and to promote the loss of righting reflex in tadpoles (Krasowski *et al.*, 2001).

The identification of these three phenolic compounds is a novel contribution to the knowledge about the phytochemical constitution of *P. orbicularis*. This report shows a natural origin of these three substances that, until now, are usually obtained by chemical synthesis. Our results evidence that these bioproducts must be some of the compounds responsible for the antimutagenic effect of *P. orbicularis* crude extract against oxidative damage.

Endogenous and exogenous sources of hydroperoxides are believed to play an important role in the generation of free-radical DNA damage. The mutations induced by hydroperoxides are a direct consequence of the reactive oxygen species (ROSs) generated upon their decomposition. However, metabolic intermediates of most xenobiotics can also form adducts with bases of DNA as well as yield oxygen radicals which are also DNA damaging agents, producing DNA strand scissions in addition to base modifications (Sugimura, 1998; Termini, 2000; Jackson and Loeb, 2001). In this sense, obtained results reinforce our proposal in previous papers about the potential usefulness of this plant extract in different fields of application such as chemoprevention or as a source of antimutagenic and/or anticarcinogenic bioproducts.

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