© 2018 Journal of Pharmacy & Pharmacognosy Research, 6 (2), 72-80, 2018
ISSN 0719-4250
http://jppres.com/jppres

Original Article | Artículo Original

Antioxidant and neuroprotective effects of gossypitrin, a flavonoid from *Taliparititi elatum*, against chemical hypoxia-induced PC12 cell death

[Efectos antioxidantes y neuroprotectores de la gossypitrina, flavonoide de *Taliparititi elatum*, frente a la muerte inducida por hipoxia química en células PC12]

María A. Bécquer-Viart[1], José González-Yaque[2], Luis A. Fonseca-Fonseca[3], Yanier Núñez-Figueredo[3], Gilberto L. Pardo Andreu[1*]

[1]Centro de Estudio para las Investigaciones y Evaluaciones Biológicas, Instituto de Farmacia y Alimentos, Universidad de La Habana, Calle 222, # 2317 e/23 y 31, La Coronela, La Lisa, CP 13600, La Habana, Cuba.
[2]Departamento de Farmacia, Instituto de Farmacia y Alimentos, Universidad de La Habana, Calle 222, # 2317 e/23 y 31, La Coronela, La Lisa, CP 13600, La Habana, Cuba.
[3]Centro de Investigación y Desarrollo de Medicamentos, Ave 26, No. 1605 Boyeros y Puentes Grandes, CP 10600, La Habana, Cuba.
*E-mail: gpardo@ifal.uh.cu

Abstract

Context: Some flavonoids have been described as neuroprotectors. Gossypitrin (Gos) is a natural occurring flavonoid and the main bioactive substance from the flowers of *Taliparititi elatum* Sw. (Majagua azul), traditionally used in Cuba as expectorant and antiasthmatic. Only few reports have documented its antioxidant properties.

Aims: To evaluate the antioxidant and cytoprotective effects of Gos against cyanide-induced oxidative stress and cell death in PC12 cells.

Methods: Gos effects on DPPH/ABTS radical scavenging, ferric reducing power and lipid peroxidation were examined. The ischemia/reperfusion neuronal damage was produced by exposing PC12 cells to KCN in glucose-free medium. Gos was added to the incubation medium 30 min before chemical hypoxia induction. The neuroprotective potential of Gos was assessed by measuring cell viability by the MTT assay, the antioxidant enzymes SOD and CAT, and GSH and lipid peroxidation levels. Rutin (Rut), the well-known antioxidant, was used as a reference compound.

Results: Gos showed a potent intrinsic antioxidant capacity evidenced by low IC_{50} and EC_{50} values for DPPH/ABTS/malondialdehyde and ferric reducing power, respectively. Pre-treatment of PC12 cells with Gos, significantly increased their survival against KCN, restored the levels of GSH and the SOD and CAT enzymes activities, as well as reduced the level of lipid peroxidation. Its antioxidant effects were higher than those elicited by Rut.

Conclusions: The results show for the first time the neuroprotective potential of Gos against hypoxic cell damage, probably associated to its antioxidant effects.

Keywords: antioxidant; gossypitrin; hypoxia; ischemia; neuroprotection; PC12.

Resumen

Contexto: Algunos flavonoides se han descrito como neuroprotectores. La gossypitrina (Gos) es un flavonoide natural y la principal sustancia bioactiva en las flores de *Taliparititi elatum* Sw. (Majagua azul), tradicionalmente usada en Cuba como expectorante y antiasmático. Pocos reportes documentan sus propiedades antioxidantes.

Objetivos: Evaluar los efectos antioxidantes y citoprotectores de la Gos frente al estrés oxidativo y la muerte celular inducidos por cianuro en células PC12.

Métodos: Se examinaron los efectos de la Gos en el secuestro de los radicales DPPH/ABTS, su poder reductor férrico y sobre la peroxidación lipídica. El daño neuronal por isquemia/reperfusión se produjo al exponer células PC12 a KCN en un medio libre de glucosa. La Gos se añadió al medio 30 min antes de la inducción de la hipoxia química. Se determinaron la viabilidad celular, las enzimas antioxidantes SOD y CAT, y los niveles de GSH y de lipoperoxidación. La rutina (Rut), conocido antioxidante, se usó como compuesto de referencia.

Resultados: La Gos mostró una potente capacidad antioxidante evidenciada por bajos valores de CI_{50} y CE_{50} para el DPPH/ABTS/malonildialdehido y el poder reductor férrico, respectivamente. El pre-tratamiento con Gos incrementó la sobrevivencia celular frente al KCN así como las actividades enzimáticas SOD y CAT, restauró las concentraciones de GSH y redujo la lipoperoxidación. Sus efectos antioxidantes fueron superiores a los de la rutina.

Conclusiones: Los resultados muestran por primera vez el potencial neuroprotector de la Gos frente al daño celular por hipoxia, asociado probablemente a sus efectos antioxidantes.

Palabras Clave: antioxidante; gossypitrina; hipoxia; isquemia; neuroprotección; PC12.

ARTICLE INFO
Received: September 1, 2017.
Received in revised form: November 27, 2017.
Accepted: December 2, 2017.
Available Online: December 4, 2017.
Declaration of interests: The authors declare no conflict of interest.
Funding: The authors confirm that the project has no funding or grants.

INTRODUCTION

Several studies have showed that free radicals and related reactive oxygen species (ROS) mediate much of the neurological damage that occurs after an ischemic brain insult. Brain ischemia and particularly reperfusion induce generation of powerful free radicals such as superoxide and hydroxyl radicals, which overwhelm endogenous antioxidative mechanisms (Love, 1999; Allen and Bayraktutan, 2009; Manzanero et al., 2013; Zhao et al., 2016). This condition of oxidative stress in the highly vulnerable brain tissue leads to lipid peroxidation, protein oxidation, different types of DNA damage, and eventually to neuronal cell death. In this sense, antioxidants of varying chemical structures have been investigated as therapeutic agents in the treatment of this acute injury (Cuzzocrea et al., 2001; Wang et al., 2006; Alonso-Alconada et al., 2013; Ren et al., 2015). Flavonoids, one of the most potent naturally occurring antioxidants, represent the most common group of polyphenolic compounds in the human diet and are widely distributed in plants. Recently, there has been great interest in the potential of flavonoids to protect the brain from ischemic damage (Dajas et al., 2003; 2013; Lin, 2011; Panickar and Jang, 2013; Pérez-Hernández et al., 2016). Antioxidation, but also modulation of signalling cascades and gene expression as well as anti-inflammation appears as the main protective mechanisms (Dajas et al., 2013).

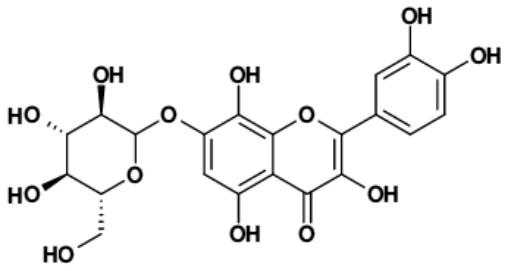

Figure 1. Gossypitrin chemical structure.

(2-(3,4-dihydroxyphenyl)-3,5,8-trihydroxy-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one).

Gossypitrin (Gos) is a flavon-3-ol glycosylated at the 7-position in ring A. It contains two free OH groups at C5 and C8 in ring A, a catechol moiety at C3′-C4′ in ring B and a free OH group at C3 in the pyrone ring C (Fig. 1). It can be found with high prevalence in the flowers from the genus *Hibiscus*, where it joins the red anthocyanins producing a co-pigmentation effect found in their extracts (Cuéllar-Cuéllar et al., 2010; 2011). It is also the main bioactive substance of the flowers of *Talipariti elatum* Sw. (Majagua azul), traditionally used in Cuban island as expectorant and antiasthmatic (Roig, 1974). Only few reports have documented the biological properties of this flavonol, despite its optimized structural arrangement for the antioxidant action, encompassing five hydroxi-aromatic groups. Therefore, it could be hypothesized that the antioxidant capacity of gossypitrin could contribute to its cytoprotective capacity *in vitro*, which would be important to define a putative neuroprotective action against ischemic brain damage. Thus, the present study was designed to address the antioxidant and cytoprotective effects of gossypitrin against cyanide-induced oxidative stress and cell death in PC12 cells.

MATERIAL AND METHODS

Plant material

Flowers were collected in January 2016 in the gardens of the Faculty of Pharmacy and Foods at Havana University, and identified at the herbarium of National Botany Garden of Havana, where the voucher specimen no. HAJB 82587 was deposited and registered as *Talipariti elatum* S.w.

Extract and samples preparation

Dark red flowering types were collected daily. The petals used were dried in an oven with controlled temperature at 40°C, during 5 days. The extracts were prepared with the ground material (60 g), using a Soxhlet apparatus and 95% ethanol (675 mL) for 20 hours. The ethanolic extracts were concentrated and roto-evaporated under vacuum to 200 mL at 120 rpm, 70°C, and 500 mBar. For to the

purification, 1 g of solid was dissolved in 25 mL of diethyl ether and the volume was completed to 100 mL with ethanol. The sample was refrigerated until the appearance of abundant yellowish-green solid, which was recovered by filtration. This process was done twice, and was monitored by thin layer chromatography (TLC) on pre-coated 60 F_{254} silica gel aluminum backed plates (Merck), using a mixture of n-butanol, acetic acid and water (4:1:5 v/v/v) as eluent (Cuéllar-Cuéllar et al., 2010). Solid obtained were characterized using 1D- and 2D NMR, and GC/MS experiments. 1D-NMR (^{1}H and ^{13}C) and 2D–NMR (^{1}H-^{1}H COSY, HSQC and HMBC) spectra were acquired on a Bruker Advance 500 spectrometer (500 MHz, DMSO-d6) (Bruker Co., Karlsruhe, Germany). Peak positions are relative to TMS for ^{1}H NMR and ^{13}C NMR spectra. GC–MS was recorded using an Agilent Gas Chromatograph (series 6890 N) (Agilent Technologies, Inc., Wilmington, DE, USA) coupled to a mass detector (series 5953 N) (Agilent Technologies, Inc., Wilmington, DE, USA). Injection of the sample was performed in the split mode with a 1:10 ratio. Temperatures in the injector, interphase and detector were 280, 280 and 150°C, respectively. Chromatographic separation was accomplished in a SPB-5 (15 m × 0.25 mm × 0.10 µm) column, using He flow (1 mL/min). The mass spectrum was recorded by electron impact as the ionization technique (70 eV). Gossypitrin was detected as [M–H] ions at m/z 481.1 (positive mode ion) and m/z 479.2 (negative mode ion). The MS spectra of the compound are dominated by the neutral loss of the O-linked hexosyl-group (-162 u), but differ in the ratio of the resulting Y_0^- and $[Y_0–H]^{\bullet-}$ ions at m/z 318.1 (Márquez et al., 1999). NMR 1D (^{1}H) and (^{13}C) revealed the presence of 21 identified carbons: (11 C sp2, 4 CH sp2, 5 CH sp3 and 1 CH2 sp3) utilizing the ^{13}C, DEPT 135 and DEPT 90 experiments. The results by 1D and 2D (proton, COSY, HSQC) allowed the confirmation of 4 aromatic protons (δ = 8.08 ppm (*d*, 1H, J_4 = 2.21 Hz, 2'-H), (δ = 7.92 ppm (*dd*, 1H, J_3 = 8.72 Hz - J_4 = 2.21 Hz, 6'-H), (δ = 6.99 ppm (*d*, 1H J_3 = 8.71 Hz, 5'-H). The observed multiplicity (ABX system) is characteristic of a catechol moiety. Observed chemical shift value of proton 6-H (δ= 6.25 ppm) and carbon 6-C (δ = 97.98 ppm) confirmed the presence of hydroxyquinol (ring A). The presence of a glucoside was confirmed by the presence of 6 carbons sp3 (5CH and 1 CH2) where the protons at (δ = 4.83 ppm (*d*, 1H, *J* = 7.32 Hz, 1''-H),), δ = 3.76 ppm (*d*, 1H, *J* = 11.96 Hz, 6''-H), δ = 3.59 ppm (*dd*, 1H, *J* = 11.96 Hz, *J* = 4.10 Hz, 6''-H), δ = 3.44-3.34 ppm (m, 4H, 2'', 3'', 4'', 5''-H) resonating in the characteristic zone of polyhydroxylated compounds. Signal at 10.39 ppm (C-7) disappeared in gossypitrin ^{1}H NMR spectra (Márquez et al., 1999).

PC12 cell culture

Pheochromocytoma (PC12) cells were kindly supplied by Beatriz Caputto, Ph.D., from Cordoba National University, and Sandra Verstraeten, Ph.D., from Buenos Aires University, Argentina. Cells at passage seven were routinely maintained in Dulbecco's Modified Essential Medium (DMEM) containing L-glutamine (Sigma, St. Louis, MO) and supplemented with 10% heat-inactivated equine serum (Gibco, Life Technologies, USA), 5% inactivated fetal bovine serum, 50 U/mL of penicillin and 10 mg/mL of streptomycin (all of which were obtained from Sigma, St. Louis, MO) in a humidified atmosphere containing 95% air/5% CO_2 at 37°C. The growth medium was replaced every three days. At day seven, the cells were trypsinized (Sigma, St. Louis, MO) and seeded at a density of 1.5×10^5 cells/mL in 96 multiwell flat bottom plates (Corning Costar, Sigma) in 200 µL of culture medium at 37°C and were flushed with 5% CO_2 in air for 24 h (Satpute et al., 2008; Figueredo et al., 2011).

Induction of chemical hypoxia in PC12 cells

For the cyanide-induced injury, the cells were exposed to 0.6 mM KCN in DMEM without glucose in the absence (control) or presence of Gos (0.001–10 mM), or Rut (0.01-0.15 mM) over the course of 1 h in a humidified atmosphere containing 95% air and 5% CO_2. Gos or Rut were added 30 min before KCN. Hypoxia was terminated by aspirating the poisoned medium and washing the cells with fresh media in all of the experimental groups, including the control and reference cultures. The reperfusion state was simulated by the addition of DMEM without KCN and with high glucose content for 24 h (Satpute et al., 2008).

In vitro antioxidant activity of Gos on cell-free system

Scavenging activity of Gos against 100 µM 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was monitored by the change in absorbance at 517 nm (U-2910 Hitachi spectrophotometer, Japan), 5 min after incubation in 40 mM sodium acetate ethanolic solution, pH 5.5 (Blois, 1958). As a positive control, the synthetic antioxidant Trolox was used. All determinations were performed in triplicate.

The antioxidant capacity was also estimated in terms of the $ABTS^{\bullet+}$ radical [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] scavenging activity following the procedure described by Long and Halliwell (2001). After the addition of 10 µL of Gos or Trolox to 4 mL of diluted $ABTS^{\bullet+}$ solution, the absorbance (U-2910 Hitachi spectrophotometer) was measured at 30 min.

Ferric ion reducing antioxidant power (FRAP) was assayed through the reduction of Fe^{3+} to Fe^{2+} by Gos or ascorbic acid as reference. The Fe^{2+}-2,4,6-tripiridils-triazine complex was detected spectrophotometrically (U-2910 Hitachi spectrophotometer) at 593 nm and Ascorbic acid was used as antioxidant control (Benzie and Strain, 1996).

Lipid peroxidation was assessed by malondialdehyde (MDA) production in rat's brain homogenate, as previously described (Genet et al., 2002). MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5$ $M^{-1} \cdot cm^{-1}$ (Buege and Aust, 1978).

PC12 cell viability assays

Cell viability was measured by the levels of blue formazan products formed from the colorless 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) by mitochondrial dehydrogenases, which are only active in viable cells. The absorbance was measured at 540 nm using a microplate reader (POLARstar Omega fluorescence spectrophotometer, Germany). The results are expressed as the percentage of absorbance relative to the undamaged control cells.

Measurement of oxidative stress parameters in PC12 cells

For the antioxidant enzymes and lipid peroxidation assays, the cultures were washed with ice cold PBS and then pooled in 0.5% Triton X-100 and 0.1 M PBS - 0.05 mM ethylenediamine tetra acetic acid (EDTA) buffered solution and homogenized. The homogenate was centrifuged for 20 min at 3000 xg at 4°C and the supernatants were used in the assays. For the absorbance readings, a U-2910 Hitachi spectrophotometer (Japan) was used.

The extent of lipid peroxidation was determined quantitatively as previously described (Buege and Aust, 1978). The amount of MDA was measured by its reaction with thiobarbituric acid, monitored at 532 nm. MDA concentration was calculated from its extinction coefficient (ε) mentioned before.

The assay of catalase was based on the consumable rate of H_2O_2 measured at λ = 240 nm (Aebi, 1984).

The superoxide dismutase activity was determined by the quantification of the superoxide anion radical ($O_2^{\bullet-}$) generated by the xanthine/xanthine oxidase system. The assay is based on the spectrophotometric determination of nitroblue tetrazolium reduction products at 560 nm (Nagai et al., 2001).

For GSH measurement aliquots (100 µL) of the supernatant were mixed with 2 mL of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5 mM EGTA. One hundred microliters of *o*-phthalaldehyde (1 mg/mL) was added, and the fluorescence was measured 15 min later using the 350/420 nm excitation/emission wavelength pair and an F-4500 fluorescence spectrophotometer (Hitachi) (Hissin and Hilf, 1976; Pardo-Andreu et al., 2007). As in the hypoxia/re-oxygenation assay, Rut (0.01-0.10 mM) was used as reference compound.

Quantitative estimation of cellular protein was made by the standard method of Lowry et al. (1951).

Statistical analysis

GraphPad Prism 6.0 software (GraphPad Software Inc., USA) was used for statistical analyses. The data are expressed as the mean ± S.E.M. Comparisons among different groups were performed using a one-way analysis of variance (ANOVA), followed by the Newman–Keuls multiple comparison *post hoc* test. Differences were considered statistically significant at $p < 0.05$. The IC_{50} and EC_{50} values were estimated using a non-linear regression algorithm.

RESULTS

Free radicals scavenging and anti-lipoperoxidative activities of Gos in a free cell system

In vitro antioxidant methods used for the evaluation of antioxidant activities of Gos included DPPH, ABTS, FRAP and lipid peroxidation assays. The results were expressed as the IC_{50} values for DPPH, ABTS and lipid peroxidation i.e., the quantity of Gos needed to scavenge 50% of the radicals or lipoperoxides produced in the reaction mixture, or EC_{50} for FRAP i.e., the quantity of Gos required to reduce 50% of total ferric iron in the assay medium. The Gos IC_{50} values for the DPPH and ABTS radicals scavenging were lower than those for Trolox, a hydrosoluble derivative of α-tocopherol, suggesting a potent intrinsic antioxidant activity (Table 1). The EC_{50} value for FRAP of this polyphenol was also lower than that of ascorbic acid, evidencing a strong reducing power that probably could explain its ability to reduce the above mentioned artificial radicals. Finally, Gos also showed a concentration dependent inhibition of lipid peroxidation, with a low IC_{50} value of 24.26 µM (Table 1).

Table 1. *In vitro* antioxidant effects of Gos on cell-free system.

Parameters	IC_{50} or EC_{50} (µM)
DPPH	19.68 ± 2.32
Trolox $_{DPPH}$	26.72 ± 1.45
ABTS	24.88 ± 2.73
Trolox $_{ABTS}$	28.52 ± 2.56
FRAP	40.65 ± 7.72*
FRAP $_{Ac. Asc.}$	52.93 ± 3.72*
MDA	24.26 ± 5.62

Each value represents as mean ± S.E.M. of triplicate experiments with at least three replicates. They were determined using a non-linear regression algorithm. * EC_{50} values.

Effects of Gos on the viability of PC12 cells submitted to KCN-mediated hypoxia

Incubation of PC12 cells with KCN (600 µM) in glucose-free medium for 1 h, followed by 24 h reperfusion (fresh growth medium without KCN and with a high glucose content), resulted in approximately 46% cell death. Fig. 2 shows a strong cytoprotective effect of Gos when it was pre-incubated with cells (the lower cytoprotective concentration was 0.001 mM), with a very low IC_{50} value (4.82 ± 0.84 µM). Rutin, the well-known glycoside derivative of quercetin showed a weaker cytoprotective effects, with an IC_{50} value around 12 times higher (57.3 ± 1.52 µM).

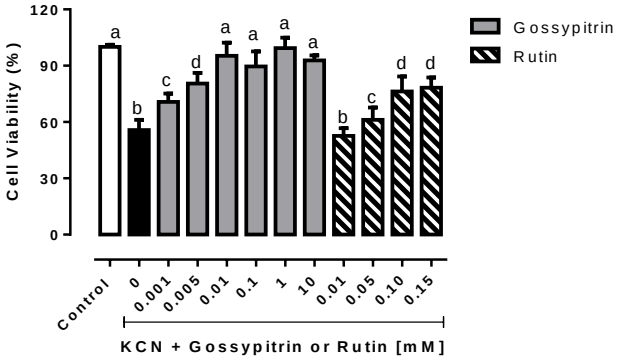

Figure 2. Protective effects of gossypitrin or rutin (reference compound) against cyanide-induced chemical hypoxia in PC12 cells.

The cells were seeded at a density of 1.5×10^5 cells/mL in 96 multiwell flat bottom plates and were exposed to 600 µM KCN for 1 h and maintained during the 24 h reperfusion period. Gos (0.001–10 mM) or Rut (0.01-0.15 mM) were added to the incubation medium 30 min before KCN. Cell viability was assessed using an MTT assay and expressed as a percentage of the control. The data are expressed as the mean ± S.E.M. (n = 3). Different letters: $p < 0.05$ according to the ANOVA and post hoc Newman–Keuls tests. The IC_{50} values were estimated using a non-linear regression algorithm.

Effects of Gos on the redox status of PC12 cells submitted to KCN-mediated hypoxia

To determine whether the protective effects of Gos on hypoxia-induced injury were mediated by their antioxidant functions, the activities of antioxidant enzymes, and lipids peroxidation in PC12 cell under hypoxia with or without Gos were examined. As shown in Table 2, the exposure of PC12 cells to low glucose and hypoxia for 1 h followed by 24 h reperfusion diminished the activities of SOD and CAT by 55 and 70%, respectively; being catalase more sensitive to cyanide than SOD as reported elsewhere (Kanthasamy et al., 1997). The levels of the endogenous antioxidant GSH was also decreased by 34% in close association to a marked increase (3.7 folds) of lipid peroxidation, evidencing the instau-

ration of an oxidative stress condition on the hypoxic-damaged cells. Gos significantly attenuated the oxidative damage on PC12 under hypoxia in a concentration-dependent manner, as reflected by the recovery of the antioxidant enzymes activities and GSH, and by a significant diminution of lipid peroxidation. The EC_{50} values for the antioxidant effects of Gos were at the very low micromolar concentrations, ranging from 1-5 µM (Table 2). Rutin, here used as a reference compound, also counteracted the oxidative damage to PC12 cells, but its antioxidant effects were attained at higher concentrations (EC_{50} values > 100 µM for CAT and SOD restoration, and 45.2 µM for MDA inhibition).

DISCUSSION

Hypoxia is a component of physiological events and multiple pathophysiological conditions, including neonatal hypoxia, and cerebral ischemia (Lendahl et al., 2009), which treatment options are still empirical due to poor mechanistic understanding. Thus, it is important to explore the mechanisms underlying the potential protective effects of compounds through hypoxia models. In this sense, the chemical hypoxia model induced by KCN exposure in PC12 cells is a useful *in vitro* model to elucidate the mechanisms associated to the hypoxic damage and to test novel neuroprotective compounds because it reproduces many hypoxic conditions, including reactive oxygen species generation, mitochondrial impairment, cytosolic calcium and pH deregulations, and apoptosis induction (Johnson et al., 1987; Maduh et al., 1990; Mills et al., 1996; Kanthasamy et al., 1997; Gao et al., 2003; Satpute et al., 2008). Using PC12 as *in vitro* model, Mills et al. (1996) demonstrated that cyanide induced apoptotic oxidative stress. Accordingly, it has been observed that treatment with the antioxidants N-acetyl cysteine (Satpute et al., 2008), ascorbate (Kanthasamy et al., 1997) and inhibitors of lipid peroxidation (Müller and Krieglstein, 1995) significantly reduced the incidence of cyanide-induced apoptosis. The antioxidant-optimized structure of Gos together with the poor documentation of its antioxidant-mediated biological effects prompted us to determine the effects of this flavonoid on PC12 cells subjected to chemical hypoxia. Our data showed that treatment of PC12 cells with 600 mM of KCN resulted in 46% decrease in the cell viability. Nevertheless, cells pre-incubation with Gos exerted a protective effect against KCN-induced cell viability decrease, reaching full protection (no difference from the Control-unpoisoned cells) between 5-10 µM. Interesting, the neuroprotective effects followed the pattern of the antioxidant actions on both the cells free and the cells lysates systems. The former data evidenced a potent intrinsic antioxidant potential (free radical scavenging and reducing power) even superior than those from the classical antioxidant Trolox and ascorbic acid used as positive controls. Gos has a privileged chemical structure that justifies is antioxidant actions.

Table 2. Antioxidant effects of gossypitrin on pheochromocytoma (PC12) cells under chemical hypoxia/reperfusion injury.

Parameters	Control	Hypoxia/reperfusion								
		Gossypitrin (µM)					Rutin (µM)			
		0	1	5	10	EC_{50}	10	50	100	EC_{50}
SOD (U/mg protein)	176.06 ± 12.34^{a}	98.34 ± 10.35^{b}	100.02 ± 9.43^{b}	134.12 ± 10.56^{c}	162.48 ± 11.47^{a}	5.35 ± 0.11	103.16 ± 12. 27^{b}	122.14 ± 8.45^{c}	131.15 ± 9.27^{c}	> 100
Catalase (U/mg protein)	22.5 ± 0.63^{a}	7.32 ± 2.56^{b}	13.57 ± 2.78^{c}	18.24 ± 2.12^{d}	21.42 ± 3.04^{a}	1.65 ± 0.33	7.65 ± 1.18^{b}	10.12 ± 0.92^{c}	12.48 ± 1.74^{d}	> 100
GSH (µg/mL)	22.4 ± 2.92^{a}	7.68 ± 1.15^{b}	10.42 ± 1.46^{c}	19. 79 ± 2.26^{d}	21.48 ± 1.78^{d}	2.20 ± 0.20	16.94 ± 2.85^{d}	13.76 ± 2.12^{c}	12.63 ± 1.42^{c}	ND
MDA (nmol/mg protein)	4.12 ± 0.68^{a}	15.2 ± 2.25^{b}	14.6 ± 3.67^{b}	8.26 ± 3.17^{c}	4.98 ± 1.12^{a}	4.1 ± 0.24	12.32 ± 1.82^{b}	9.73 ± 1.04^{c}	6.51 ± 0.94^{c}	45.2 ± 3.06

Each value represents as mean ± S.E.M. of triplicate experiments with at least three replicates. Values in the same row followed by a different superscript letters (a–d) are significantly different ($p < 0.05$) according to the ANOVA and *post hoc* Newman–Keuls tests. ND-Not determined.

On the basis of available experimental studies (Bors et al., 1990; van Acker et al., 1996; Arora and Nair, 1998), several key features of the structure–antioxidant activity relationship in flavonoids include the catechol group in ring B, the carbonyl group in the 4 position in conjugation with the double bond at C2 and C3, an OH group at C5, and a free OH group at C3. Since gossypitrin contains all these key features, it is likely to be a powerful antioxidant. In this regard it has been shown that quercetin, a well-known antioxidant flavonoid that fulfil the above mentioned structural arrangement, showed marked cytoprotective capacities in experimental conditions resembling molecular feature of ischemic brain damage (Dajas et al., 2003; 2013). It is worth to notice that the *in vitro* antioxidant effects of Gos on PC12 cells were higher than those of Rut, here used as reference compound. Rut possesses two carbohydrate structures as its side-chain, but Gos has only one carbohydrate side-chain and an extra hydroxyl group. The enhanced antioxidant effects of Gos could be correlated with higher liposolubility and stability and improved biological properties when compared to the Rut.

The protective effects of Gos in cells are probably the biological consequence of its antioxidant actions, evidenced by the Gos capacity to restore the antioxidant enzyme activities (SOD and CAT) and to recover the endogenous GSH levels that could be used for the restorative activities of the cells. In this regard, antioxidants are known to inhibit the cell injury, scavenged ROS formation, increased antioxidative enzyme activity, and attenuated the lipoperoxidative damage in oxidative-stressed neuronal cells (Hou et al., 2003; Marín-Prida et al., 2012; 2013). Excessive ROS production in the brain are believed to contribute to neurodegenerative processes and compounds inhibiting this neurotoxic process, i.e. antioxidants have been considered as promising therapeutic candidates against ischemic stroke (Broussalis et al., 2012).

Not only antioxidants, but also protein kinase C (PKC) modulators play a role in hypoxic damage. PKC down-regulation or inhibition (with chelerythrine or staurosporine) conferred protection against KCN-induced cytotoxicity (Pavlaković et al., 1995). Ca^{2+} channel blocker also protected against chemical hypoxia (Pavlaković et al., 1995). The hypoxia-inducible factor-1 (HIF-1), is also involved in the hypoxic mediated cells death since it has been shown that the protective role of ginkgolides, garlic derivative and fisetin were due to the modulation of HIF-1α protein expression (Li et al., 2008; Chen et al., 2015; Orozco-Ibarra et al., 2016). MAPK and mitochondrial mediated signaling pathways have also been involved in the neuroprotective effects of carnosic acid and resveratrol, respectively on neuronal cells under hypoxic stress (Hou et al., 2012; Liu et al., 2016). Since most of the above mentioned neuroprotective effects were elicited by antioxidants, we do not discard the contribution of some of them to the neuroprotection elicited by Gos. In this regard, additional *in vitro* and *in vivo* studies are necessary to fully understand the neuroprotective mechanism of action of Gos against neuronal hypoxic damage

CONCLUSIONS

These results showed for the first time that Gos was able to protect PC12 cells against hypoxia induced injury through direct free radical scavenging and modulation of endogenous antioxidants and antioxidant enzymes. The strong neuroprotective activity of Gos *in vitro*, as evidenced in this study, prompted us to extend our research to *in vivo* experimental models of brain damage, which are now underway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

Authors confirm that this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

Aebi H (1984) Catalase *in vitro*. Methods Enzymol 105: 121–126.

Allen CL, Bayraktutan U (2009) Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int J Stroke 4: 461–470.

Alonso-Alconada D, Alvarez A, Arteaga O, Martínez-Ibargüen A, Hilario E (2013) Neuroprotective effect of melatonin: a novel therapy against perinatal hypoxia-ischemia. Int J Mol Sci 14: 9379–9395.

Arora A, Nair MG (1998) Structure–activity relationships for antioxidants activities of a series of flavonoids in a liposomal system. Free Radic Biol Med 24: 1355–1363.

Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239: 70–76.

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181: 1199–1200.

Bors W, Michel C, Saran M (1990) Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Methods Enzymol 186: 343–355.

Broussalis E, Trinka E, Killer M, Harrer A, McCoy M, Kraus J (2012) Current therapies in ischemic stroke. Part B. Future candidates in stroke therapy and experimental studies. Drug Discovery Today 17: 671–684.

Buege JA, Aust SD (1978) Microsomal lipid peroxidation. Methods Enzymol 52: 302–310.

Chen PY, Ho YR, Wu MJ, Huang SP, Chen PK, Tai MH, Ho CT, Yen JH (2015) Cytoprotective effects of fisetin against hypoxia-induced cell death in PC12 cells. Food Funct 6: 287–296.

Cuéllar-Cuéllar A, González-Yaque J (2010) Obtención del glucósido flavonoide Gossypitrina de los pétalos de las flores de *Taliparitielatum* Sw. y evaluación de su posible efecto antioxidante. Rev Colombiana Cienc Anim 2: 338–348.

Cuéllar-Cuéllar A, Rojaz-Hernández ENM (2011) Potencialidades antimicrobianas de la gossypitrina aislada de las flores de *Taliparitielatum* Sw. y evaluación de algunos parámetros farmacognósticos de las flores. Rev Colombiana Cienc Anim 3: 125–135.

Cuzzocrea S, Riley DP, Caputi AP, Salvemini D (2001) Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev 53: 135–159.

Dajas F, Andrés AC, Florencia A, Carolina E, Felicia RM (2013) Neuroprotective actions of flavones and flavonols: mechanisms and relationship to flavonoid structural features. Cent Nerv Syst Agents Med Chem 13: 30–35.

Dajas F, Rivera-Megret F, Blasina F, Arredondo F, Abin-Carriquiry JA, Costa G, Echeverry C, Lafon L, Heizen H, Ferreira M, Morquio A (2003) Neuroprotection by flavonoids. Braz J Med Biol Res 36: 1613–1620.

Figueredo YN, Garcia Pupo L, Rubio OC, Hernandez R, Naal Z, Curti C, Pardo Andreu GL (2011) A strong protective action of guttiferone-A, a naturally occurring prenylated benzophenone, against iron-induced neuronal cell damage. J Pharmacol Sci 116: 36–46.

Gao XD, Ye WC, Yu AC, Zhang Y, Tan RX, Li M, Hsiao WL (2003) Pulsatilloside A and anemoside A3 protect PC12 cells from apoptosis induced by sodium cyanide and glucose deprivation. Planta Med 69: 171–174.

Genet S, Kale RK, Baquer NZ (2002) Alterations in antioxidant enzymes and oxidative damage in experimental diabetic rat tissues: effect of vanadate and fenugreek (*Trigonellafoenum graecum*). Mol Cell Biochem 236: 7–12.

Hissin PJ, Hilf R (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74: 214–226.

Hou CW, Lin YT, Chen YL, Wang YH, Chou JL, Ping LY, Jeng KC (2012) Neuroprotective effects of carnosic acid on neuronal cells under ischemic and hypoxic stress. Nutr Neurosci 15: 257–263.

Hou RC, Huang HM, Tzen JT, Jeng KC (2003) Protective effects of sesamin and sesamolin on hypoxic neuronal and PC12 cells. J Neurosci Res 74: 123–133.

Johnson JD, Conroy WG, Isom GE (1987) Alteration of cytosolic calcium levels in PC12 cells by potassium cyanide. Toxicol Appl Pharmacol 88: 217–224.

Kanthasamy AG, Ardelt B, Malave A, Mills EM, Powley TL, Borowitz JL, Isom GE (1997) Reactive oxygen species generated by cyanide mediate toxicity in rat pheochromocytoma cells. Toxicol Lett 93: 47–54.

Lendahl U, Lee KL, Yang H, Poellinger L (2009) Generating specificity and diversity in the transcriptional response to hypoxia. Nat Rev Genet 10: 821–832.

Li Z, Ya K, Xiao-Mei W, Lei Y, Yang L, Ming QZ (2008) Ginkgolides protect PC12 cells against hypoxia-induced injury by p42/p44 MAPK pathway-dependent upregulation of HIF-1alpha expression and HIF-1DNA-binding activity. J Cell Biochem 103: 564–575.

Lin B (2011) Polyphenols and neuroprotection against ischemia and neurodegeneration. Mini Rev Med Chem 11: 1222–1238.

Liu X, Zhu X, Chen M, Ge Q, Shen Y, Pan S (2016) Resveratrol protects PC12 cells against OGD/ R-induced apoptosis via the mitochondrial-mediated signaling pathway. Acta Biochim Biophys Sin 48: 342–353.

Long LH, Halliwell B (2001) Antioxidant and prooxidant abilities of foods and beverages. Methods Enzymol 335: 181–190.

Love S (1999) Oxidative stress in brain ischemia. Brain Pathol 9: 119–131.

Lowry OH, Rosenberg NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagent. J Biol Chem 193: 265–275.

Maduh EU, Borowitz JL, Isom GE (1990) Cyanide-induced alteration of cytosolic pH: involvement of cellular hydrogen ion handling processes. Toxicol Appl Pharmacol 106: 201–208.

Manzanero S, Santro T, Arumugam TV (2013) Neuronal oxidative stress in acute ischemic stroke: sources and contribution to cell injury. Neurochem Int 62: 712–718.

Marín-Prida J, Pavón-Fuentes N, Llópiz-Arzuaga A, Fernández-Massó JR, Delgado-Roche L, Mendoza-Marí Y, Santana SP, Cruz-Ramírez A, Valenzuela-Silva C, Nazábal-Gálvez M, Cintado-Benítez A, Pardo-Andreu GL, Polentarutti N, Riva F, Pentón-Arias E, Pentón-Rol G (2013) Phycocyanobilin promotes PC12 cell survival and modulates immune and inflammatory genes and oxidative stress markers in acute cerebral hypoperfusion in rats. Toxicol Appl Pharmacol 272: 49–60.

Marín-Prida J, Pentón-Rol G, Rodrigues FP, Alberici LC, Stringhetta K, Leopoldino AM, Naal Z, Polizello AC, Llópiz-Arzuaga A, Rosa MN, Liberato JL, Santos WF, Uyemura SA, Pentón-Arias E, Curti C, Pardo-Andreu GL (2012) C-Phycocyanin protects SH-SY5Y cells from oxidative injury, rat retina from transient ischemia and rat brain mitochondria from Ca(2+)/phosphate-induced impairment. Brain Res Bull 89: 159–167.

Márquez I, Cuéllar A, Martínez J, Alemán A, Lora J, Castro H (1999) Estudio fitoquímico de la especie *Hibiscus elatus* S.w. Rev Cubana Farm 33: 127–131.

Mills EM, Gunasekar PG, Pavlakovic G, Isom GE (1996) Cyanide-induced apoptosis and oxidative stress in differentiated PC12 cells. J Neurochem 67: 1039–1046.

Müller U, Krieglstein J (1995) Inhibitors of lipid peroxidation protect cultured neurons against cyanide-induced injury. Brain Res 678: 265–268.

Nagai T, Sakai M, Inoue R, Inoue H, Suzuki N (2001) Antioxidative activities of some commercially honeys, royal jelly, and propolis. Food Chem 75: 237–240.

Orozco-Ibarra M, Muñoz-Sánchez J, Zavala-Medina ME, Pineda B, Magaña-Maldonado R, Vázquez-Contreras E, Maldonado PD, Pedraza-Chaverri J, Chánez-Cárdenas ME (2016) Aged garlic extract and S-allylcysteine prevent apoptotic cell death in a chemical hypoxia model. Biol Res 49: 7.

Panickar KS, Jang S (2013) Dietary and plant polyphenols exert neuroprotective effects and improve cognitive function in cerebral ischemia. Recent Pat Food Nutr Agric 5: 128–143.

Pardo-Andreu GL, Cavalheiro RA, Dorta DJ, Naal Z, Delgado R, Vercesi AE, Curti C (2007) Fe(III) shifts the mitochondria permeability transition-eliciting capacity of mangiferin to protection of organelle. J Pharmacol Exp Ther 320: 646–653.

Pavlaković G, Eyer CL, Isom GE (1995) Neuroprotective effects of PKC inhibition against chemical hypoxia. Brain Res 676: 205–211.

Pérez-Hernández J, Zaldívar-Machorro VJ, Villanueva-Porras D, Vega-Ávila E, Chavarría A (2016) A potential alternative against neurodegenerative diseases: Phytodrugs. Oxid Med Cell Longev 2016: 8378613.

Ren Y, Wei B, Song X, An N, Zhou Y, Jin X, Zhang Y (2015) Edaravone's free radical scavenging mechanisms of neuroprotection against cerebral ischemia: review of the literature. Int J Neurosci 125: 555–565.

Roig JT (1974) Plantas Medicinales Aromáticas y Venenosas de Cuba, 1st edn. La Habana: Editorial Ciencia y Técnica.

Satpute RM, Hariharakrishnan J, Bhattacharya R (2008) Alpha-ketoglutarate and N-acetyl cysteine protect PC12 cells from cyanide-induced cytotoxicity and altered energy metabolism. Neurotoxicology 29: 170–178.

Van Acker SAVE, Van Der Berg DJ, Tromp MNJL, Griffioen DH, Van Bermekom WP, Van Der Vijgh WJF, Bast A (1996) Structural aspects of antioxidant activity of flavonoids. Free Radic Biol Med 20: 331–342.

Wang JY, Wen LL, Huang YN, Chen YT, Ku MC (2006) Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation. Curr Pharm Des 12: 3521–3533.

Zhao M, Zhu P, Fujino M, Zhuang J, Guo H, Sheikh I, Zhao L, Li XK (2016) Oxidative stress in hypoxic-ischemic encephalopathy: molecular mechanisms and therapeutic strategies. Int J Mol Sci 17: E2078.

Author contribution:

Contribution	Bécquer-Viart MA	González-Yaque J	Fonseca-Fonseca LA	Núñez-Figueredo Y	Pardo Andreu GL
Concepts or ideas	X	X	X	X	X
Design				X	X
Definition of intellectual content	X	X	X	X	X
Literature search	X	X	X	X	X
Experimental studies	X	X	X		
Data acquisition	X	X	X		
Data analysis	X	X	X	X	X
Statistical analysis				X	X
Manuscript preparation	X	X	X	X	X
Manuscript editing	X	X	X	X	X
Manuscript review	X	X	X	X	X

Citation Format: Bécquer-Viart MA, González-Yaque J, Fonseca-Fonseca LA, Núñez-Figueredo Y, Pardo Andreu GL (2018) Antioxidant and neuroprotective effects of gossypitrin, a flavonoid from *Taliparitiel atum*, against chemical hypoxia-induced PC12 cell death. J Pharm Pharmacogn Res 6(2): 72–80.