



RESEARCH ARTICLE

REVISED Activation of the Keap1-Nrf2 pathway by specioside and the *n*-butanol extract from the inner bark of *Tabebuia rosea* (Bertol) DC

[version 3; peer review: 2 approved]

Sandra Catalina Garzón-Castaño ^{1,2}, Francisco Javier Jiménez-González ³, Luz Angela Veloza³, Juan Carlos Sepúlveda-Arias ¹

¹Grupo Infección e Inmunidad, Facultad de Ciencias de la Salud, Universidad Tecnológica de Pereira, Pereira, Colombia²Grupo de Biomedicina, Facultad de Medicina, Fundación Universitaria Autónoma de las Américas, Pereira, Colombia³Grupo Polifenoles, Facultad de Tecnologías, Universidad Tecnológica de Pereira, Pereira, Colombia

V3 First published: 21 Oct 2020, 9:1262
<https://doi.org/10.12688/f1000research.26901.1>
 Second version: 16 Nov 2020, 9:1262
<https://doi.org/10.12688/f1000research.26901.2>
 Latest published: 02 Dec 2020, 9:1262
<https://doi.org/10.12688/f1000research.26901.3>

Abstract

Background: A large number of chemical compounds exert their antioxidant effects by activation of key transcriptional regulatory mechanisms, such as the transcription factor Nrf2. The aim of this study was to evaluate the activation of the Keap1-Nrf2 pathway by both the *n*-butanol extract obtained from the inner bark of *Tabebuia rosea* (Bertol) DC and specioside isolated from this extract.

Methods: The antioxidant activity of the extract and specioside isolated from the inner bark of *T. rosea* were evaluated using the oxygen radical absorbance capacity (ORAC) and the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) techniques, whereas their effects on the viability of HepG2 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The effects of the compound and the extract on activating the Keap1-Nrf2 pathway were evaluated using a Nrf2 Transcription Factor Assay kit. Induction of the Nrf2-mediated antioxidant response genes *HMOX-1* and *NQO1* was evaluated by real-time PCR. The protective effects against H₂O₂-induced oxidative stress in HepG2 cells was determined as the percent protection using the MTT method.

Results: Both the *n*-butanol extract and specioside exhibited activity at low concentrations without affecting cellular viability, since the cell viability was greater than 80% after 24 hours of exposure at each tested concentration. In addition, Nrf2 dissociated from Keap1 after treatment with the *n*-butanol extract at a concentration of 0.25 µg/mL after 4 hours of exposure. An increase in the Nrf2 level in the cytoplasm after 4 hours of exposure to 2 µM specioside was observed.

Open Peer Review**Approval Status**

	1	2
version 3 (revision) 02 Dec 2020		 view
version 2 (revision) 16 Nov 2020		↑
version 1 21 Oct 2020	 view	 view

1. **Jaime Martin-Franco** , Universidad del Cauca, Popayán, Colombia

2. **Luca Rastrelli** , University of Salerno, Fisciano, Italy

Any reports and responses or comments on the article can be found at the end of the article.

Nrf2 levels stabilized in the nucleus 12 hours after stimulation with both specioside and the extract. After 6 hours of stimulation, both the extract and specioside induced the expression of *HMOX-1* and *NQO1*.

Conclusion: The *n*-butanol extract from the inner bark of *T. rosea* and specioside produced protective effects against H₂O₂-induced oxidative stress in HepG2 cells.

Keywords

Tabebuia rosea, specioside, Bignoniaceae, extracts, Nrf2, antioxidant agents

Corresponding authors: Sandra Catalina Garzón-Castaño (sandra.garzon@uam.edu.co), Juan Carlos Sepúlveda-Arias (jcsepulv@utp.edu.co)

Author roles: **Garzón-Castaño SC:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Jiménez-González FJ:** Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Veloza LA:** Conceptualization, Funding Acquisition, Investigation, Methodology, Supervision, Writing – Review & Editing; **Sepúlveda-Arias JC:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by Patrimonio Autónomo Fondo Nacional de Financiamiento para la Ciencia, la Tecnología y la Innovación, Francisco José de Caldas, Contrato RC-0572-2012-Bio-Red-Co-CENIVAM, Universidad Tecnológica de Pereira (Project 9-14-5) Fundación Universitaria Autónoma de las Américas and Sistema General de Regalías de Colombia (Código BPIN 2012000100050). *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

Copyright: © 2020 Garzón-Castaño SC *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Garzón-Castaño SC, Jiménez-González FJ, Veloza LA and Sepúlveda-Arias JC. **Activation of the Keap1-Nrf2 pathway by specioside and the *n*-butanol extract from the inner bark of *Tabebuia rosea* (Bertol) DC [version 3; peer review: 2 approved]** F1000Research 2020, 9:1262 <https://doi.org/10.12688/f1000research.26901.3>

First published: 21 Oct 2020, 9:1262 <https://doi.org/10.12688/f1000research.26901.1>

REVISED Amendments from Version 2

We did correct the concentration of H₂O₂ used in the evaluation of the protective effect of the extract and compounds (0.98 mM). We did correct the concentration used for specioside, catalposide and controls in [Figure 2](#) (0.5 µM) and [Figure 3](#) (2 µM).

Any further responses from the reviewers can be found at the end of the article

Abbreviations

AAPH, 2,2'-Azobis(2-amidinopropane) dihydrochloride; ALA, alpha-lipoic acid; CUR, curcumin; ORAC, oxygen radical absorbance capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CHAL, 2-trifluoromethyl-2'-methoxychalcone

Introduction

Over the years, plants have been used to treat several diseases, are considered an important source of biologically active natural products, and many have been used for the synthesis of various drugs. The natural products present in nature have great diversity in terms of their chemical structures and physicochemical properties, in addition to their biological activities. The *Tabebuia* genus belongs to the Bignoniaceae family, which includes more than 800 species and is considered as one of the most abundant family of plants in the neotropics¹. The genus *Tabebuia* is distributed from Mexico to South America and has been used in traditional medicine. Several bioactive molecules such as saponins, quinones, tannins and alkaloids^{2,3} found in *Tabebuia* species have anti-inflammatory and antioxidant activities⁴⁻⁶. There is a wide distribution of the species *Tabebuia rosea* throughout Colombia, which has motivated the study of its therapeutic properties. The antioxidant capacity of extracts obtained from *T. rosea* has not been widely studied, although this activity has been demonstrated in other species of the *Tabebuia* genus.

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant capacity, inducing oxidative damage, which plays a role in the development of premature aging, chronic diseases and cancer⁷⁻¹¹. In addition, oxidative stress contributes to pathogenesis in many neurodegenerative diseases, such as Parkinson's, Alzheimer's, Huntington's and amyotrophic lateral sclerosis, where increases in oxidative markers have been found^{12,13}. Cells respond to oxidative stress through various defense mechanisms, such as the elimination of free radicals and the generation of antioxidant molecules mediated by the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2). Nrf2 is a redox-sensitive transcription factor that plays a major role in cell defense against oxidative stress. Nrf2 belongs to a family of basic proteins with a leucine zipper domain (bZip)¹⁴. Under normal conditions, Nrf2 is localized in the cytoplasm and is inhibited by the Keap1 protein (Kelch ECH associating protein 1) and therefore degraded in the proteasome. In the presence of oxidative stress, Nrf2

translocates to the nucleus. Once there, it binds to the ARE site and induces the expression of antioxidant enzymes such as NAD(P)H quinone oxidoreductase (NQO1) and heme oxygenase 1 (HMOX-1). This response is important to modulate the homeostatic balance of the cells¹⁵⁻²⁰. A large number of molecules activate transcriptional regulatory mechanisms to induce their antioxidant activity.

Natural product research, guided by ethnopharmacological knowledge, has made significant contributions to drug innovation by providing new chemical structures and understanding of their mechanisms of action. Considering the potential health benefits and the possible pharmacological effects of extracts obtained from *T. rosea*, its abundance in Colombia and the few investigations regarding its antioxidant properties and the molecular mechanisms involved in its activity, the aim of this study was to evaluate the mechanism responsible for the *in vitro* antioxidant activity of the *n*-butanol extract obtained from the inner bark of *T. rosea* (Bertol) DC.

Methods

Plant material, extract preparation and specioside isolation

The inner bark of *T. rosea* (Bertol) DC was collected at the Universidad Tecnológica de Pereira campus in April 2011. The plant was identified at the Colombian National Herbarium (voucher no. COL 582577). The collection and processing of the material were covered by collection permission number 1133/2014 issued by the National Environmental Licensing Authority (ANLA) of Colombia.

Extracts were obtained as previously described^{21,22}. Plant material (2 Kg) was dried and macerated in analytical grade methanol for 48 hours. This was followed by homogenization, filtration, and concentration under vacuum using a vacuum rotary evaporator (Heidolph, Laborota model) at 40°C to obtain the crude extract (294.3 g, yield 14.7%). The crude extract was dissolved in 400 mL of distilled water and underwent liquid-liquid extraction with increasing polarity solvents: *n*-hexane, chloroform (CHCl₃), ethyl acetate (EtOAc), and *n*-butanol (all solvents were of analytical grade). Each extract was vacuum dried by a vacuum rotary evaporator. Endotoxin levels in the extracts were assayed using the Limulus Amebocyte Lysate Test (E-Toxate kit, Sigma Chemical Co., Saint Louis, MO, USA, Cat No. ET0200-1KT). The *n*-butanol extract was kept refrigerated at 4°C in an amber tube protected from light, heat, air and moisture. For each of the biological assays, the extract was dissolved in 0.1% DMSO (dimethyl sulfoxide, Merck, Darmstadt, Germany, Cat No. 1029521000).

The butanolic extract was concentrated with rotary evaporation under reduced pressure, obtaining a dark brown extract (12.5 g, yield 4.25%). The butanolic extract (8.0 g) was subjected to separation by column chromatography (CC) on Diaion® HP-20 (Mitsubishi Chemical Corp.), with a water-isopropanol elution gradient (90:10 to 10:90), obtaining subfractions A-J. Tr-1 (25.9 mg) was isolated from subfraction D with a semipreparative HPLC-DAD system (Hitachi-Merck) in reversed-phase

(LiChrocart 250-10, LiChrospher 100; 10 μ m, Merck) by isocratic elution with H₂O-ACN (70:30% v/v) containing 1% v/v CH₃COOH. Specioside (Tr-1, **Figure 1**) was obtained as a dark brown amorphous solid (m.p. 142-162°C). The FTIR spectrum displayed absorption bands attributable to carbonyl groups ($\nu_{\text{C=O}}$ 1698 cm⁻¹), hydroxyl groups ($\nu_{\text{O-H}}$ 3383 cm⁻¹), and aromatic rings ($\nu_{\text{C=C}}$ 1605 cm⁻¹).

Full assignments from the ¹H and ¹³C NMR spectra were made through the use of ¹H-¹H COSY, HSQC and HMBC experiments. All the experiments were performed on a 400 MHz Agilent spectrometer (125.6 MHz for ¹³C); using deuterated methanol as solvent. The ¹H NMR spectrum showed two olefinic protons at δ_{H} 6.37 (H-3, dd) and δ_{H} 4.98 (H-4, dd), characteristic of the iridoid nucleus. This structure was confirmed by correlations shown in the HMBC spectrum with carbons at δ_{C} 140.95 (C-3) and δ_{C} 101.50 (C-4). In addition, two olefinic protons at δ_{H} 7.67 (1H, d, J = 16.0 Hz, H-7'') and δ_{H} 6.38 (1H, d, J = 15.9 Hz, H-8'') suggested the presence of an *E* configuration, which is characteristic of a *p*-coumaroyl skeleton. The *p*-coumaroyl structure was confirmed by the observation of two signals at δ_{H} 7.48 (2H, d, J = 8.7 Hz, H-2'', H-6'') and δ_{H} 6.81 (2H, d, J = 8.7 Hz, H-3'', H-5''), characteristic of an AA'XX' system; these data were confirmed by the ¹³C NMR spectrum, which exhibited eight carbon signals, including carbonyl carbon δ_{C} 164.49 (C-9''), which was attributed to the *p*-coumaroyl ester. The presence of anomeric protons at δ_{H} 4.79 (1H, d, J = 7.9 Hz, H-1'), and methine signals at δ_{H} 3.42-3.23 (4H, m) are characteristic of a sugar moiety. Analysis of the 1D and 2D NMR spectra in addition to comparisons with literature data for glucoside analogs suggested that the saccharide portion was a glucose moiety. Characteristic ¹H NMR, ¹³C NMR, COSY, HSQC and HMBC spectra are supplied as *Extended data*²³.

Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity was determined using the method described by Ou *et al.* with some modifications²⁴. 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) and sodium fluorescein stock solutions were prepared in a 75 mM,

pH 7.0 phosphate buffer solution. Thirty-one microliters of each sample was diluted in 187 μ L of fluorescein (120 nM) and incubated at 37°C for 10 min. The reaction was started by the addition of 31 μ L of AAPH (143 mM) to reach a final volume of 249 μ L per well. The extract was evaluated at concentrations of 0.25, 0.5, 1 and 2 μ g/mL. Specioside and catalposide²⁵ (Sigma Chemical Co., Saint Louis, MO, USA, Cat No. SMB00094-1MG) as well as the controls (α -lipoic acid^{26,27}, curcumin^{28,29} and quercetin^{30,31}) were evaluated at concentrations of 0.5, 1, 2 and 4 μ M. A Trolox® standard curve was prepared. Changes in fluorescence were measured with a Varian Cary Eclipse 1.1 fluorescence spectrophotometer at 2 min intervals for 120 min with emission and excitation wavelengths of 515 and 493 nm, respectively. The excitation slit width was 5 nm, as was the emission slit width^{32,33}. The antioxidant capacity was calculated as the area under the curve (AUC)³⁴ and is expressed as μ mol Trolox® equivalents per liter (μ mol TE/L).

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH)

The antioxidant activity of the extract and compounds was also evaluated by the DPPH method using the methodology described by Brand-Williams *et al.* with some modifications³⁵. Thirty microliters of samples and controls were prepared at concentrations ranging from 0.25 to 2 μ g/mL and 0.5 – 4 μ M, respectively, and mixed with 2 mL of a methanol solution of DPPH (20 μ g/mL DPPH, 5×10^{-5} mol/L); each mixture was agitated and kept in the dark for 30 min at RT. The absorbance was measured at 517 nm in a Shimadzu UV-1700 spectrophotometer. Ascorbic acid (5 – 200 μ g/mL) was used for the standard curve. Each experiment was repeated three times, and the antioxidant capacity was calculated as the percent inhibition³⁶.

Cell culture

The human hepatocarcinoma cell line (HepG2; ATCC; CRL-11997) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with Glutamax (GIBCO/BRL, USA, Cat No. 10564-011)

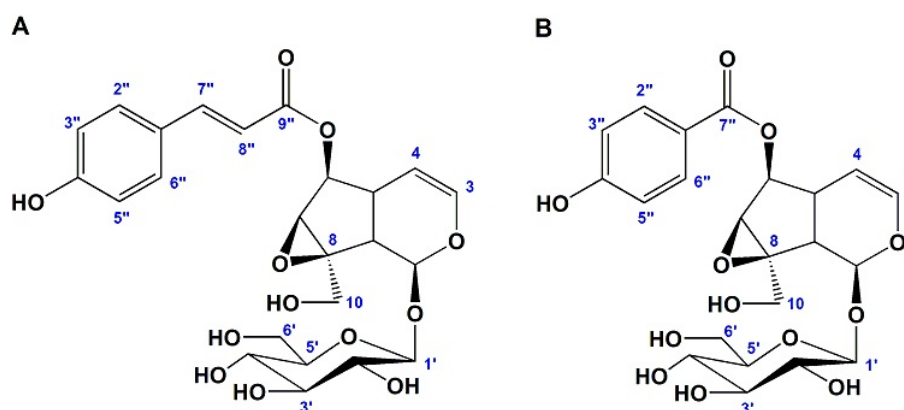


Figure 1. Chemical structure of specioside (A) and catalposide (B).

and 10% heat-inactivated FBS (GIBCO, Cat No. 16140071), 200 µg/mL penicillin, 200 µg/mL streptomycin, 400 µg/mL neomycin (GIBCO, Cat No. 15640-055), 5 µg/mL amphotericin and 1 mM sodium pyruvate (all from Sigma Chemical Co., Saint Louis, MO, USA, Cat Nos. A9528-50MG and S8636-100ML, respectively). Cells were maintained at 37°C in a 5% CO₂ atmosphere.

Cell viability test

The viability of HepG2 cells in the presence of the extracts and compounds was tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method³⁷. Cells (5×10⁴ cell/well) were treated with the extracts (0.25, 0.5 and 1 µg/mL) and compounds - 0.5, 1 and 2 µM specioside, catalposide and controls: α-lipoic acid (ALA), curcumin (CUR) and 2-trifluoromethyl-2'-methoxychalcone³⁸ (CHAL), diluted in DMSO and incubated for 24 hours. After treatment, the medium was discarded, and 200 µL of DMEM containing 0.5 mg/mL MTT (Sigma Chemical Co., Saint Louis, MO, USA, Cat No. M2128-500MG) was then added to each well. The plates were incubated for 4 hours at 37°C in a 5% CO₂ atmosphere. The medium was discarded, and 100 µL of DMSO was then added to solubilize the formazan crystals. The absorbance was measured with an ELISA microplate reader at 492 nm (ELx800; BioTek Instruments Inc., USA). The percent viability was calculated based on the nontreated control. Three independent assays were performed, each in triplicate.

Nrf2 nuclear activation

A total of 1×10⁶ HepG2 cells/well were cultured in DMEM. The medium was discarded, and the cells were exposed to the extract (0.25 or 1 µg/mL), compounds or controls (0.5 or 2 µM) for 0, 4, 12 or 24 hours. After exposure, cells were harvested and used for the simultaneous extraction of nuclear and cytosolic proteins following the specifications included in the Nuclear Extraction Kit (Abcam, Cambridge, UK, ab113474). Total protein was quantified using the BCA Protein Quantification Kit (Abcam, Cambridge, UK, ab102536). Nrf2 was detected by using the Nrf2 Transcription Factor Assay Kit (Colorimetric, Abcam, Cambridge, UK, ab207223) following the manufacturer's instructions. The absorbance of each well was measured at 450 nm in an ELx800 BioTek microplate reader.

qRT-PCR assays

HepG2 cells (3×10⁵ cells/well) were treated with extract (0.25 or 1 µg/mL), compounds or controls (0.5 or 2 µM) for 0, 4, 6 or 8 hours. After treatment, mRNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen, Maryland, USA, cat no. 74134). mRNA was quantified with a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA). The expression of the *HMOX-1* and *NQO1* genes^{21,39-41} was evaluated by RT-qPCR using predesigned TaqMan Gene Expression Assays (Hs01110250_m1 and Hs01045993_g1) and the TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems, Foster City, CA, Cat No. 4331182). The run method was holding at 48°C for 15 min, 95°C for 10 min and cycling (40 cycles) at 95°C

for 15 sec and 60°C for 1 min. β-actin (Applied Biosystems, Foster City, CA, ref. 4325788) was used as an endogenous control.

Protective effects of the extract and compounds

Hydrogen peroxide (H₂O₂) was employed as a stressor agent in order to evaluate the protective capacity of the extract (0.25 and 1 µg/mL) and compounds (0.5 and 2 µM). The controls used were ALA and CUR, since these compounds have been reported to have protective effect against oxidative stress mediated by Nrf2^{28,42}. Cells were grown to a density of 5×10⁴ cells/well in DMEM and incubated for 24 hours under a 5% CO₂ atmosphere at 37°C. The medium was discarded, and the cells were exposed for 12 hours to different concentrations of the extract (0.25 or 1 µg/mL), compounds and controls (0.5 or 2 µM). Subsequently, the medium was discarded, and one of the plates was exposed to 0.98 mM H₂O₂ (previously determined concentration) and the second plate was used as a control. After 24 hours, 200 µL of MTT (0.5 mg/mL, Sigma) was added to both plates followed by incubation at 37°C for 4 hours. The medium was discarded, and 100 µL of 99.8% DMSO (Sigma) was added to solubilize the formazan crystals. The absorbance was measured in an ELISA microplate reader at 570 nm with correction at 630 nm (ELx800; BioTek Instruments Inc., USA). The percent inhibition was calculated.

Statistical analysis

Each experiment was performed at least in duplicate. The results are expressed as the mean ± SD of at least three independent experiments. Statistical analysis was performed using the Kruskal-Wallis test, and a *p*-value less than 0.05 was considered statistically significant. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA).

Results

Antioxidant activity and cell viability after treatment with the *n*-butanol extract and pure compounds

The antioxidant activity of the *n*-butanol extract from the inner bark of *T. rosea*, the isolated compound specioside and the catalposide iridoid compound reported from the Bignoniaceae family were evaluated using the ORAC and DPPH techniques⁴³. The results showed that there was a tendency for the activity in the ORAC assay to be higher when the extract or compound concentration increased, displaying a concentration-dependent relationship, except for the control compound ALA. In addition, specioside, catalposide and the *n*-butanol extract displayed the best antioxidant activity, and this activity was significantly higher than that induced by ALA (Table 1, *p* < 0.05), whose percent DPPH inhibition was low (<25%). Specioside displayed the best antioxidant activity, followed by catalposide, quercetin, curcumin, α-lipoic acid and finally the *n*-butanol extract (Table 1). The results from the MTT assay indicated that neither the *n*-butanol extract from the inner bark of *T. rosea* nor the pure compounds affected the viability of HepG2 cells, since the viabilities were all greater than 80% after 24 hours of exposure.

Table 1. Antioxidant activity of the *n*-butanol extract, specioside and catalposide using ORAC and DPPH techniques.

Sample/Concentration ($\mu\text{g/mL}$)	ORAC ($\mu\text{mol TE/L}$)				DPPH (% Inhibition)			
	0.25	0.5	1	2	0.25	0.5	1	2
Specioside	21.42 $\pm$ 1.62	20.95 $\pm$ 2.41	27.29 $\pm$ 3.49	45.42 $\pm$ 6.93^b	15.13 $\pm$ 2.87	20.78 $\pm$ 0.53	23.04 $\pm$ 5.42	22.43 $\pm$ 6.38
Catalposide	18.08 $\pm$ 3.35	19.26 $\pm$ 3.19	16.99 $\pm$ 8.53	35.09 $\pm$ 6.79^a	16.63 $\pm$ 2.75 ^c	17.01 $\pm$ 1.04	15.08 $\pm$ 2.67	16.94 $\pm$ 1.19^c
<i>n</i>-butanol extract	13.13 $\pm$ 6.62	19.29 $\pm$ 3.75	24.92 $\pm$ 8.14	31.83 $\pm$ 4.92 ^a	5.91 $\pm$ 4.17	4.64 $\pm$ 4.34	5.96 $\pm$ 3.88	5.53 $\pm$ 4.04
α-Lipoic acid	11.90 $\pm$ 0.97	6.97 $\pm$ 2.85	12.06 $\pm$ 6.11	12.60 $\pm$ 4.27	5.48 $\pm$ 2.38	6.19 $\pm$ 3.28	5.50 $\pm$ 4.00	4.97 $\pm$ 4.33
Curcumin	18.76 $\pm$ 3.12	27.85 $\pm$ 9.39	37.44 $\pm$ 7.12	66.90 $\pm$ 5.77	7.19 $\pm$ 6.52	8.42 $\pm$ 7.37	8.96 $\pm$ 8.65	12.19 $\pm$ 9.22
Quercetin	17.23 $\pm$ 3.32	19.69 $\pm$ 1.89	27.43 $\pm$ 5.79	52.53 $\pm$ 4.89	11.38 $\pm$ 5.88	10.51 $\pm$ 7.14	9.09 $\pm$ 8.20	11.76 $\pm$ 8.51

TE, Trolox equivalents; ORAC, oxygen radical absorbance capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity. All experiments were carried out in triplicate. Data represent the mean $\pm$ SD. Kruskal Wallis, Dunn's multiple comparisons test. ^a $p < 0.01$, ^b $p < 0.0001$ compared with control (α -Lipoic acid). ^c $p < 0.05$ compared with extract and control (α -Lipoic acid).

Effects of the *n*-butanol extract and pure compounds on the activation and nuclear translocation of Nrf2

Nrf2 detection enabled comparisons of the basal Nrf2 status in both the cytosol and the nucleus. It also allowed for comparison of their associated changes after exposure to the *n*-butanol extract (0.25 and 1 µg/mL) and the pure compounds specioside and catalposide (0.5 and 2 µM) in HepG2 cells. The results showed an increase in Nrf2 levels in the cytosol and nucleus after 4 hours of exposure (Figure 2) to the extract (0.25 µg/mL) and the controls ALA, CUR and CHAL at the lowest concentration (0.5 µM), showing a significant difference ($p < 0.05$). By increasing the concentration of the *n*-butanol extract to 1 µg/mL and the pure compounds and controls to 2 µM, increases in the Nrf2 levels were observed in all samples after 4 hours of exposure (Figure 3), and this increase was maintained at 12 hours for specioside, catalposide and the *n*-butanol extract ($p < 0.05$). As shown in Figure 3b, the Nrf2 levels in the nucleus increased 4 and 12 hours after exposure to the pure compounds and the *n*-butanol extract. In addition, the level of the protein after 12 hours of exposure to specioside were significantly higher ($p < 0.05$) than that after exposure to the control CUR after 4 hours. This increase was measured in relation to the basal level (nonexposed cells).

Effects of the *n*-butanol extract and pure compounds on the expression of HMOX-1 and NQO1

The levels of expression of the *HMOX-1* and *NQO1* genes were evaluated by qRT-PCR and quantified using the $2^{-\Delta\Delta Ct}$ method. The results indicate that the molecules specioside and catalposide (0.5 µM) and the *n*-butanol extract (0.25 µg/mL) increased the expression levels of *HMOX-1* (>1.5-fold change) and *NQO1* (>1.4-fold change) after 6 hours of exposure (Figure 4). As shown in Figure 4a, the pure compounds significantly increased the expression levels of *HMOX-1* ($p < 0.05$) compared to the controls CUR and H_2O_2 . The relative expression level of the *NQO1* gene increased significantly after treatment with specioside compared with the control ALA (Figure 4b). At higher concentrations (1 µg/mL for the extract and 2 µM for the pure compounds and controls), the expression levels of *HMOX-1* and *NQO1* increased after 6 to 8 hours of exposure to specioside, catalposide and the *n*-butanol extract (Figure 5). A significantly higher expression level of *HMOX-1* was observed after 8 hours of exposure to specioside, catalposide and the *n*-butanol extract compared with the controls (CUR, CHAL and H_2O_2 , $p < 0.05$, Figure 5a). The relative expression level of *NQO1* significantly increased after exposure to specioside, catalposide and the *n*-butanol extract compared to the control CHAL ($p < 0.01$, Figure 5b).

Protective effects of the *n*-butanol extract and pure compounds against H_2O_2 -induced oxidative stress

The protective effects of the *n*-butanol extract and the pure compounds against H_2O_2 -induced oxidative stress was evaluated after 24 hours of exposure to H_2O_2 . The results showed that exposure to the *n*-butanol extract and pure compounds reduced cell viability to 60 – 70% after H_2O_2 exposure (Figure 6a). A significant protective effect ($p < 0.05$) was observed

with the *n*-butanol extract (0.25 µg/mL) and specioside (0.5 and 2 µM), greater than 10%, compared to the control CHAL (2 µM). Additionally, exposure of cells to catalposide at the same concentration (2 µM) displayed a higher protective effect than that of the control CUR and specioside (Figure 6b). These results indicate that the *n*-butanol extract, specioside, catalposide, ALA and CUR induced protective effects in HepG2 cells against H_2O_2 -induced oxidative stress at 0.98 mM.

Discussion

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant capacity and plays a critical role in the development of different neurodegenerative diseases and cancer^{12,44}. Plants are an important source of biologically active natural products, many of which are also models for the synthesis of drugs. Compounds in nature reveal great diversity in terms of chemical structure and biological properties⁴⁵. Studies carried out by Ghosh *et al.* and Fitmawati *et al.* concluded that medicinal plants are the best sources of phytochemicals and bioactive compounds that are useful for the development of drugs, antioxidants and those showing antidegenerative effects^{46,47}. Several natural compounds exert their antioxidant effects through the activation of the key transcriptional regulation mechanism of Nrf2, allowing the coordinated expression of antioxidant enzymes such as NQO1 and HMOX-1. The modulation of the Keap1-Nrf2 pathway is important to maintain the homeostatic balance of the cell⁴⁸.

The antioxidant activity results from the *n*-butanol extract by the ORAC method, which measures the oxygen radical scavenging capacity, showed a concentration-dependent relationship, obtaining the best activity ($p < 0.05$) at 2 µg/mL. There are no reports in the literature regarding the evaluation of the antioxidant capacity of *T. rosea* extracts using the ORAC technique. Studies carried out to determine the hydroxyl scavenging radical capacity from species in the *Tabebuia* genus include those from the crude extracts of the leaves from *Tabebuia chrysanth* G. Nicholson. These studies indicated that the methanolic and aqueous extracts have a significant effect on the uptake of hydroxyl radicals (between 57–86%). Moreover, it was detected that the extracts of *T. chrysanth* can also act to decrease the production of these radicals⁴⁹. The antioxidant activity reported by Kwak *et al.* for iridoid glycosides showed that specioside and catalposide had potent antioxidant activity by the ORAC method⁵⁰. When evaluating the antioxidant capacity by the DPPH method, it was observed that at 0.25–2 µg/mL (0.5–4 µM pure compound), an inhibition percentage of 50% was not obtained, and specioside and CUR showed concentration-dependent behavior. The results indicated that the extract of the inner bark of *T. rosea* and the pure compounds show the absence of the DPPH radical scavenging activity. Franco Ospina *et al.* reported the low antioxidant activity of the extracts from the inner bark of *T. rosea* using the DPPH assay. A study of the methanolic extract from the flowers, leaves, roots and inner bark of *T. pallida* showed antioxidant potential using the FRAC and DPPH methods⁵¹. Comparative analysis of the ethanolic extracts of *T. rosea* from

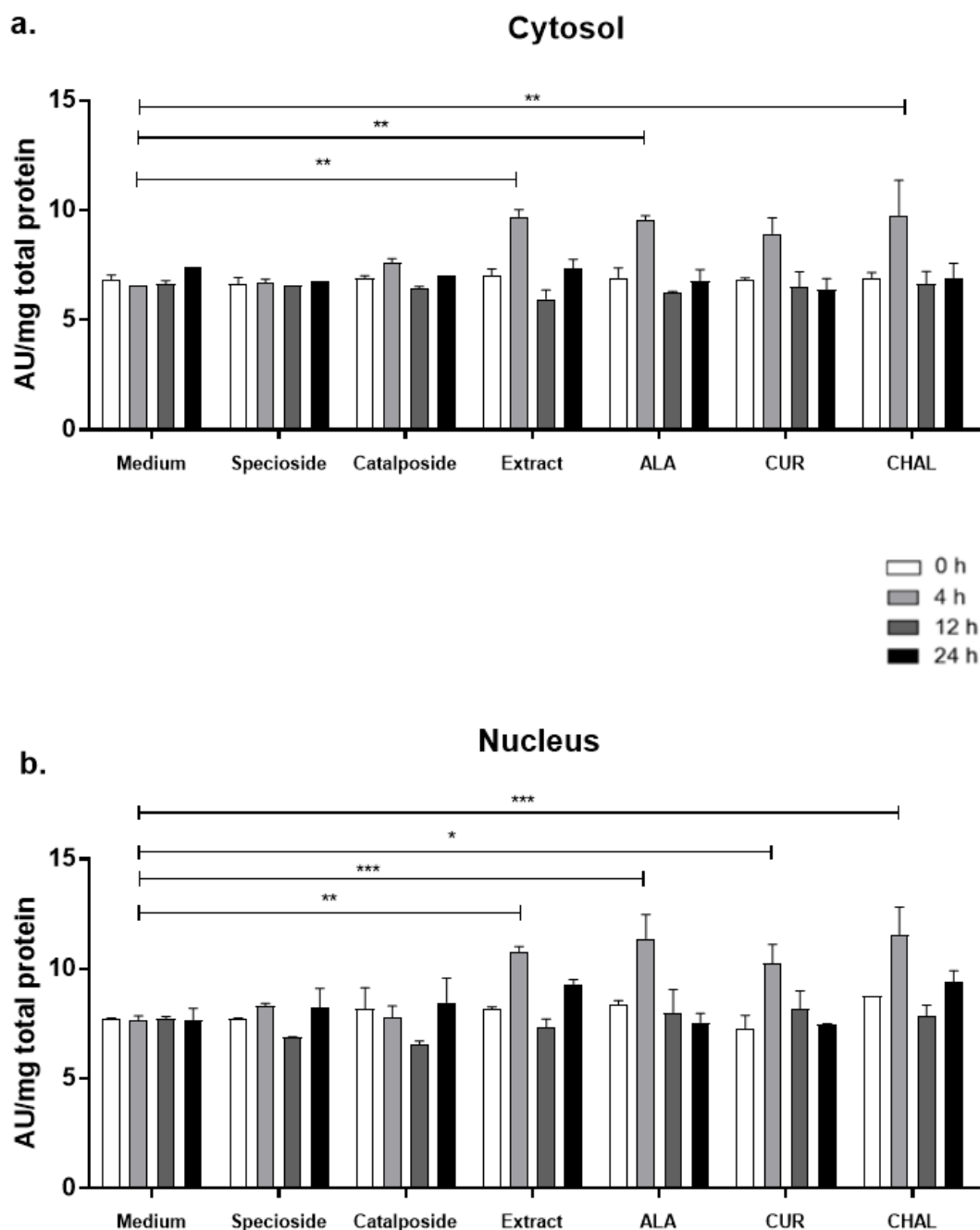


Figure 2. Nrf2 levels in cytosol (a) and nucleus (b) after 0, 4, 12 and 24 hours of exposure to 0.5 μ M specioside, catalposide, controls and 0.25 μ g/mL *n*-butanol extract. Kruskal-Wallis, Dunn's post hoc. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ALA, α -lipoic acid; CUR, curcumin; CHAL, 2-trifluoromethyl-2'-methoxychalcone.

flowers shows strong antioxidant activity against DPPH and hydroxyl radicals. On the other hand, the report by Joubouhi *et al.* on iridoid compounds found radical scavenging ability against DPPH and ABTS⁵².

Along with the previous results, the *in vitro* antioxidant activity was carried out with 0.25 and 1 μ g/mL extract and 0.5 and

2 μ M pure compounds. Activation of the Keap1-Nrf2 pathway revealed the ability of the extract and compounds (specioside and catalposide) to activate this transcription factor, with an increase in the levels of the protein in the nucleus after treatment with the extract and pure compounds. Several natural antioxidant compounds, such as curcumin, sulforaphane and resveratrol, have been reported as electrophilic regulators

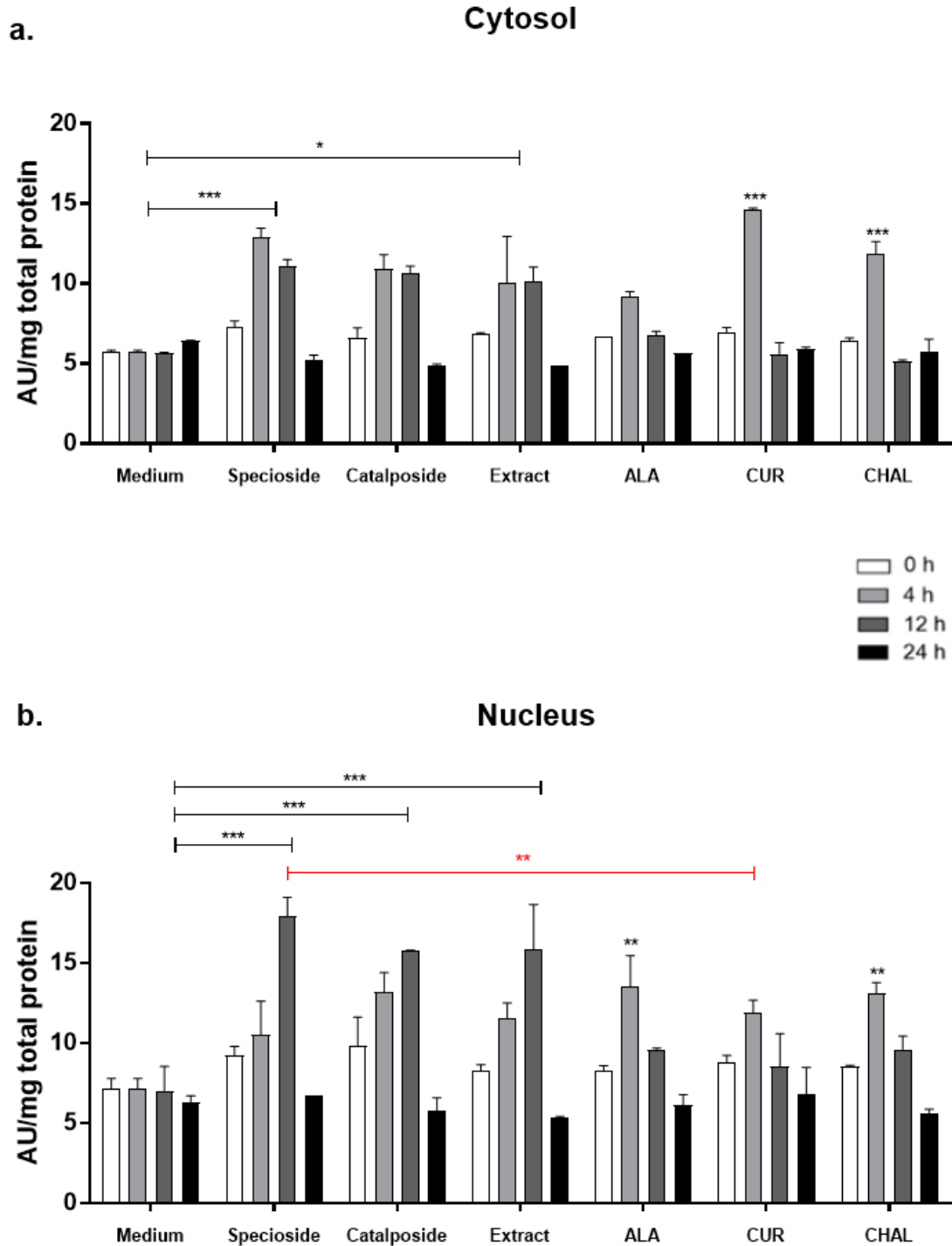


Figure 3. Nrf2 levels in cytosol (a) and nucleus (b) after 0, 4, 12 and 24 hours of exposure to 2 μ M specioside, catalposide, controls and 1 μ g/mL *n*-butanol extract. Kruskal Wallis, Dunn's post hoc. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ALA, α -lipoic acid; CUR, curcumin; CHAL, 2-trifluoromethyl-2'-methoxychalcone.

of the activation of the Keap1-Nrf2 complex. In addition, they are also used for the treatment of different pathologies, such as type 2 diabetes, asthma, and cancer⁵³. The translocation of Nrf2 to the nucleus allows the expression of antioxidant

response genes such as *HMOX-1* and *NQO1*. The results show how the compounds and extract increase the expression levels of *HMOX-1* and *NQO1*. A study carried out by our research group evaluated the antioxidant capacity of the ethyl acetate

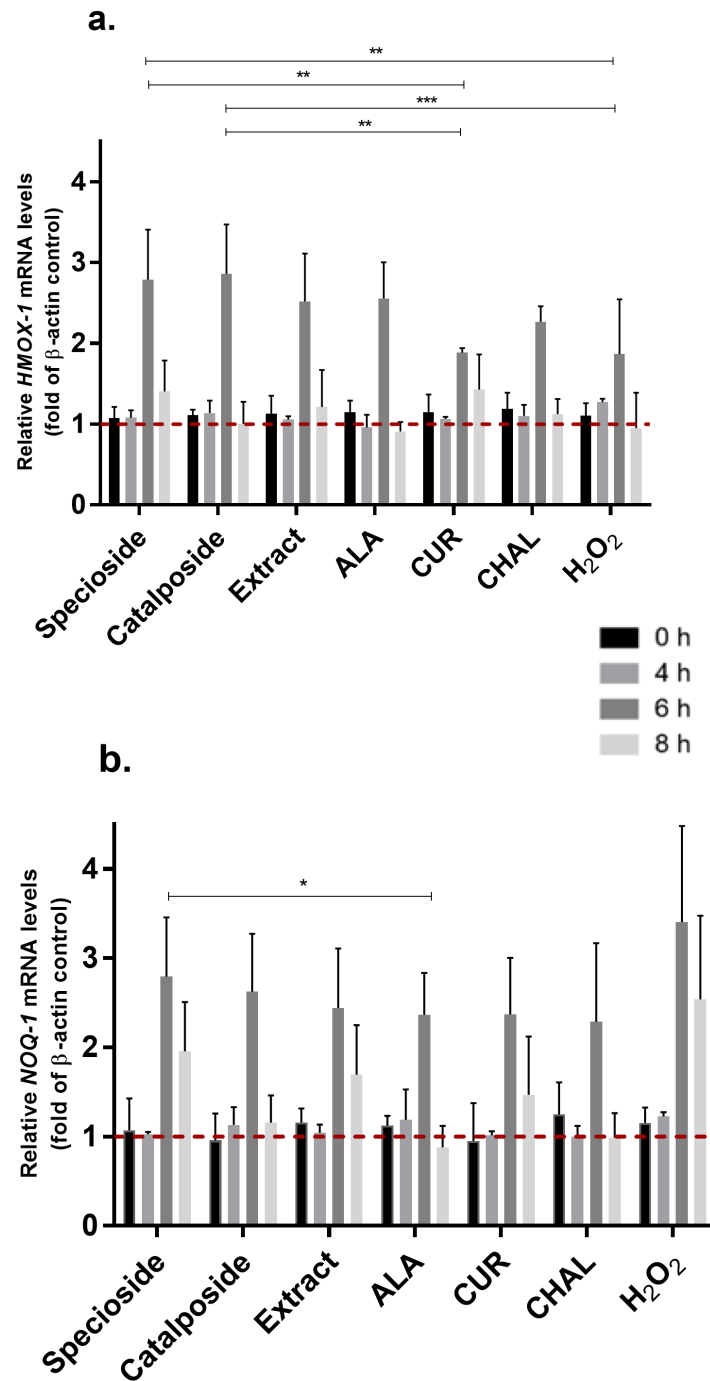


Figure 4. Relative *HMOX-1* (a) and *NQO1* (b) mRNA levels after 0, 4, 6 and 8 hours post-exposure to 0.5 μ M (pure compounds), 0.25 μ g/mL (*n*-butanol extract) and induction of oxidative stress with 0.98 mM (H₂O₂). Kruskal Wallis Dunn's post hoc. * p<0.05, ** p<0.01, *** p<0.001. ALA, α -lipoic acid; CUR, curcumin; CHAL, 2-trifluoromethyl-2'-methoxychalcone.

extract from the inner barks of *T. rosea* and *T. chrysantha*, in which the capacity of the extracts to activate Nrf2 translocation (after 4 hours of exposure) was reported to induce the expression of *NQO1*²¹. Glycosylated iridoid compounds

such as aucubin⁵⁴, catalposide and verprosides (the main compounds found in the ethyl acetate fraction of *Veronica ciliata*)⁵⁵ showed a protective effect mediated by Nrf2, increasing the expression levels of the gene and the antioxidant

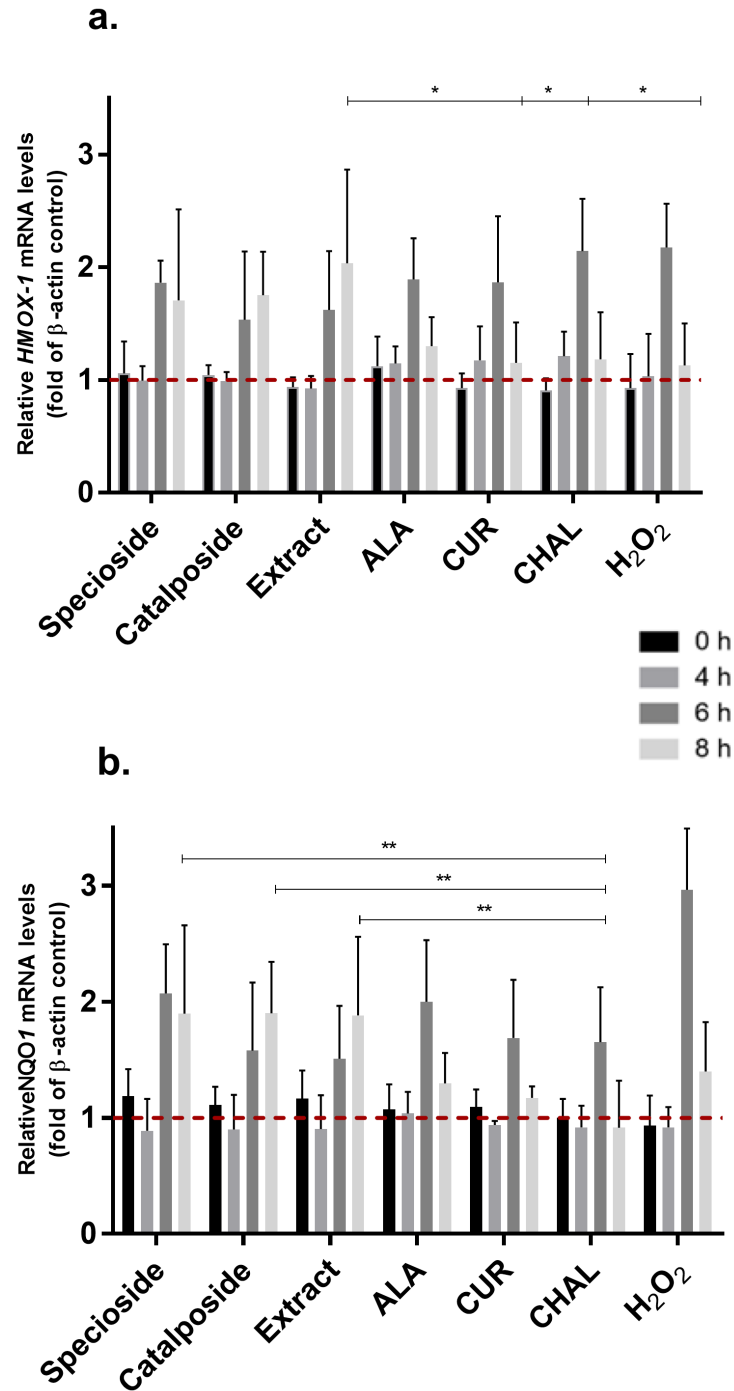


Figure 5. Relative *HMOX-1* (a) and *NQO1* (b) mRNA levels after 0, 4, 6 and 8 hours post-exposure to 2 μ M (pure compounds), 1 μ g/mL (*n*-butanol extract) and 0.98 mM (H_2O_2). Kruskal Wallis, Dunn's post hoc * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ALA, α -lipoic acid; CUR, curcumin; CHAL, 2-trifluoromethyl-2'-methoxychalcone.

protein NQO1. Ma *et al.* reported that aucubin positively regulates Nrf2 translocation and induces the response of phase II antioxidant enzymes such as HMOX-1, NQO1 and SOD, considering aucubin a promising candidate to prevent oxidative stress that induces testicular damage⁵⁶. Moon *et al.*

evaluated the ability of catalposide to induce the expression of *HMOX-1* and its protein in a concentration- and time-dependent manner and found that exposure of neuro-2A cells to catalposide generates a protective effect against H_2O_2 -induced oxidative stress, increasing the levels of the enzyme HMOX-1²⁵.

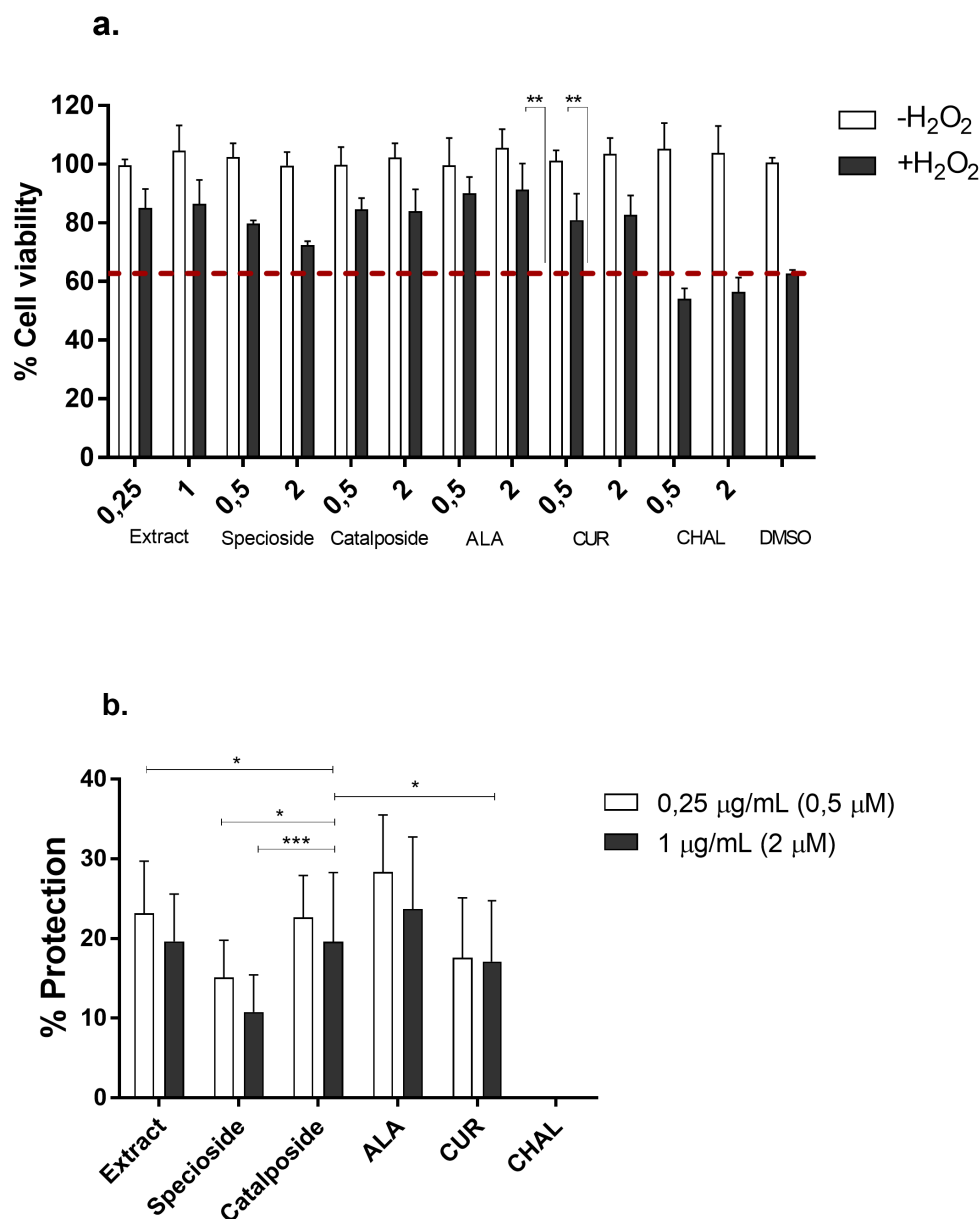


Figure 6. Effect of the *n*-butanol extract, pure compounds and controls on the HepG2 cell line against H₂O₂-induced oxidative stress. a. Percentage of cell viability. **b.** Protective effect. Kruskal-Wallis, Dunn's post hoc. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ALA, α -lipoic acid; CUR, curcumin; CHAL, 2-trifluoromethyl-2'-methoxychalcone.

In order to evaluate the protective effects of the extract and compounds, the concentration of H₂O₂ (0.98 mM) that induced death by oxidative stress in 50% of the cells was determined. The concentration found in this study for the HepG2 cell line is similar to previous reports⁵⁷. The results show that the extract and compounds exert a protective effect against oxidative stress induced by H₂O₂. The extract, specioside and catalposide had protective effects of more than 10%. The results obtained for catalposide are similar to those reported by Moon *et al.* Catalpol, another glycosylated iridoid, showed

a protective effect against oxidative stress induced by H₂O₂ in a primary astrocyte culture⁵⁸. Wang *et al.* evaluated the Nrf2-mediated neuroprotective capacity of swertiamarin, a glycosylated secoiridoid, and reported increases in the levels of the antioxidant proteins NQO1 and HMOX-1 in addition to an increase in the Nrf2 protein at the nuclear level⁵⁹. The only report of the protective effects of specioside was made by Asthana *et al.*, in which the ability of the compound to modulate antioxidant enzymes such as CAT and SOD was evaluated in a *Caenorhabditis elegans* model⁶⁰.

This study is the first report of the *in vitro* protective effects of the extract of the inner bark of *T. rosea* against oxidative stress induced by H₂O₂.

Conclusion

The present study indicates that the *n*-butanol extract from the inner bark of *T. rosea* and its isolated compound specie-side have promising antioxidant activity. Both biocompounds have the ability to activate the Keap1-Nrf2 pathway, inducing the expression of *HMOX-1* and *NQO1*, and generating a protective effect against H₂O₂-induced oxidative stress in the HepG2 cell line. These results reinforce the importance of these plants in the search for new antioxidant molecules.

Data availability

Underlying data

Open Science Framework: Activation of the Keap1-Nrf2 pathway Tabebuia. <https://doi.org/10.17605/OSF.IO/HW6X9>⁴³.

This project contains the following underlying data:

- Dataset 1 Nrf2 Levels Figure 2.csv
- Dataset 2 Nrf2 Levels Figure 3.csv
- Dataset 3 Relative HMOX-1 and NQO1 mRNA levels Figure 4.csv
- Dataset 4 Relative HMOX-1 and NQO1 mRNA levels Figure 5.csv
- Dataset 5 Relative HMOX-1 and NQO1 mRNA levels Figure 6.csv

Extended data

Open Science Framework: Garzon_et_al_Nrf22020_Supplementary .pdf. <https://doi.org/10.17605/OSF.IO/TRVB2>²³.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

References

1. Bittencourt NS Jr, Pereira EJ Jr, de Souza São-Thiago P, et al.: **The reproductive biology of *Cybistax antisiphilitica* (Bignoniaceae), a characteristic tree of the South American savannah-like “Cerrado” vegetation.** *Flora - Morphology, Distribution, Functional Ecology of Plants*. 2011; **206**(10): 872–86. [Publisher Full Text](#)
2. Sichaem J, Kaennakam S, Siripong P, et al.: **Tabebuialdehydes A-C, cyclopentene dialdehyde derivatives from the roots of *Tabebuia rosea*.** *Fitoterapia*. 2012; **83**(8): 1456–9. [PubMed Abstract](#) | [Publisher Full Text](#)
3. Blair S, Madrigal B: **Plantas antimaláricas de Tumaco: Costa Pacífica colombiana.** Universidad de Antioquia, Colombia. 2005. [Reference Source](#)
4. Park BS, Lee KG, Shibamoto T, et al.: **Antioxidant activity and characterization of volatile constituents of Taheebo (*Tabebuia impetiginosa* Martius ex DC).** *J Agric Food Chem*. 2002; **51**(1): 295–300. [PubMed Abstract](#) | [Publisher Full Text](#)
5. Sadananda TS, Chaitra RK, Govindappa M, et al.: **Antimicrobial and antioxidant activities of endophytes from *Tabebuia argentea* and identification of anticancer agent (lapachol).** *J Med Plants Res*. 2011; **5**(16): 3643–52. [Reference Source](#)
6. Suo M, Isao H, Kato H, et al.: **Anti-inflammatory constituents from *Tabebuia avellanae*.** *Fitoterapia*. 2012; **83**(8): 1484–8. [PubMed Abstract](#) | [Publisher Full Text](#)
7. Sosa V, Moliné T, Somoza R, et al.: **Oxidative stress and cancer: an overview.** *Ageing Res Rev*. 2013; **12**(1): 376–90. [PubMed Abstract](#) | [Publisher Full Text](#)
8. Reuter S, Gupta SC, Chaturvedi MM, et al.: **Oxidative stress, inflammation, and cancer: how are they linked?** *Free Radic Biol Med*. 2010; **49**(11): 1603–16. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
9. Na HK, Surh YJ: **Oncogenic potential of Nrf2 and its principal target protein heme oxygenase-1.** *Free Radic Biol Med*. 2014; **67**: 353–65. [PubMed Abstract](#) | [Publisher Full Text](#)
10. Hybertson BM, Gao B, Bose SK, et al.: **Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation.** *Mol Aspects Med*. 2011; **32**(4–6): 234–46. [PubMed Abstract](#) | [Publisher Full Text](#)
11. Cabello-Verrugio C, Ruiz-Ortega M, Mosqueira M, et al.: **Oxidative Stress in Disease and Aging: Mechanisms and Therapies.** *Oxid Med Cell Longev*. 2016; **2016**: 8786564. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
12. Kanno T, Tanaka K, Yanagisawa Y, et al.: **A novel small molecule, N-(4-(2-pyridyl)(1,3-thiazol-2-yl))-2-(2,4,6-trimethylphenoxy) acetamide, selectively protects against oxidative stress-induced cell death by activating the Nrf2-ARE pathway: Therapeutic implications for ALS.** *Free Radic Biol Med*. 2012; **53**(11): 2028–42. [PubMed Abstract](#) | [Publisher Full Text](#)
13. Gan L, Johnson JA: **Oxidative damage and the Nrf2-ARE pathway in neurodegenerative diseases.** *Biochim Biophys Acta*. 2014; **1842**(8): 1208–18. [PubMed Abstract](#) | [Publisher Full Text](#)
14. Gañán-Gómez I, Wei Y, Yang H, et al.: **Oncogenic functions of the transcription factor Nrf2.** *Free Radic Biol Med*. 2013; **65**: 750–64. [PubMed Abstract](#) | [Publisher Full Text](#)
15. Bryan HK, Olayanju A, Goldring CE, et al.: **The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation.** *Biochem Pharmacol*. 2013; **85**(6): 705–17. [PubMed Abstract](#) | [Publisher Full Text](#)
16. Dhakshinamoorthy S, Long DJ 2nd, Jaiswal AK: **Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens.** In: Earl RS, Chock PB, editors. *Curr Top Cell Regul*. Academic Press; 2001; **36**: 201–16. [PubMed Abstract](#) | [Publisher Full Text](#)
17. Jaiswal AK: **Nrf2 signaling in coordinated activation of antioxidant gene expression.** *Free Radic Biol Med*. 2004; **36**(10): 1199–207. [PubMed Abstract](#) | [Publisher Full Text](#)
18. Kaspar JW, Niture SK, Jaiswal AK: **Nrf2:INrf2 (Keap1) signaling in oxidative stress.** *Free Radic Biol Med*. 2009; **47**(9): 1304–9. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
19. Klaassen CD, Reisman SA: **Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver.** *Toxicol Appl Pharmacol*. 2010; **244**(1): 57–65. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
20. Sporn MB, Liby KT: **NRF2 and cancer: the good, the bad and the importance of context.** *Nat Rev Cancer*. 2012; **12**(8): 564–71. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
21. Garzón-Castaño SC, Lopera-Castrillon IA, Jimenez-Gonzalez FJ, et al.: **Nrf2-Mediated Antioxidant Activity of the inner bark extracts obtained from *Tabebuia rosea* (Bertol.) DC and *Tabebuia chrysantha* (JACQ) G. Nicholson [version 2; peer review: 2 approved].** *F1000Res*. 2018; **7**: 1937. [PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
22. Jimenez-Gonzalez F, Vélez-Gomez J, Melchor-Moncada J, et al.: **Antioxidant, anti-inflammatory, and antiproliferative activity of extracts obtained from *Tabebuia Rosea* (Bertol.) DC.** *Pharmacogn Mag*. 2018; **14**(55): 25–31. [Publisher Full Text](#)
23. Sepúlveda-Arias JC, Garzón-Castaño SC, Jimenez-González FJ, et al.: **Garzon_et_al_Nrf22020_Supplementary .pdf.** <https://www.doi.org/10.17605/OSF.IO/TRVB2>
24. Ou B, Hampsch-Woodill M, Prior RL: **Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe.** *J Agric Food Chem*. 2001; **49**(10): 4619–26. [PubMed Abstract](#) | [Publisher Full Text](#)

25. Moon MK, Choi BM, Oh GS, *et al.*: **Catalposide protects Neuro 2A cells from hydrogen peroxide-induced cytotoxicity via the expression of heme oxygenase-1.** *Toxicol Lett.* 2003; **145**(1): 46–54.
[PubMed Abstract](#) | [Publisher Full Text](#)
26. Shay KP, Michels AJ, Li W, *et al.*: **Cap-independent Nrf2 translation is part of a lipoic acid-stimulated detoxification stress response.** *Biochim Biophys Acta.* 2012; **1823**(6): 1102–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
27. Hwang S, Byun JW, Yoon JS, *et al.*: **Inhibitory Effects of α -Lipoic Acid on Oxidative Stress-Induced Adipogenesis in Orbital Fibroblasts From Patients With Graves Ophthalmopathy.** *Medicine (Baltimore).* 2016; **95**(2): e2497.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
28. Balogun E, Hoque M, Gong P, *et al.*: **Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element.** *Biochem J.* 2003; **371**(Pt 3): 887–95.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
29. Gonzalez-Reyes S, Guzman-Beltran S, Medina-Campos ON, *et al.*: **Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats.** *Oxid Med Cell Longev.* 2013; **2013**: 801418.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
30. Alia M, Mateos R, Ramos S, *et al.*: **Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2).** *Eur J Nutr.* 2006; **45**(1): 19–28.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Xu D, Hu MJ, Wang YQ, *et al.*: **Antioxidant Activities of Quercetin and Its Complexes for Medicinal Application.** *Molecules.* 2019; **24**(6): 1123.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
32. Alarcón E, Campos AM, Edwards AM, *et al.*: **Antioxidant capacity of herbal infusions and tea extracts: A comparison of ORAC-fluorescein and ORAC-pyrogallol red methodologies.** *Food Chem.* 2008; **107**(3): 1114–9.
[Publisher Full Text](#)
33. Atala E, Vásquez L, Speisky H, *et al.*: **Ascorbic acid contribution to ORAC values in berry extracts: An evaluation by the ORAC-pyrogallol red methodology.** *Food Chem.* 2009; **113**(1): 331–5.
[Publisher Full Text](#)
34. Cao G, Prior RL: **Measurement of oxygen radical absorbance capacity in biological samples.** *Methods Enzymol.* 1999; **299**: 50–62.
[PubMed Abstract](#) | [Publisher Full Text](#)
35. Brand-Williams W, Cuvelier ME, Berset C: **Use of a free radical method to evaluate antioxidant activity.** *LWT - Food Science and Technology.* 1995; **28**(1): 25–30.
[Publisher Full Text](#)
36. Chen Z, Bertin R, Foldi G: **EC50 estimation of antioxidant activity in DPPH assay using several statistical programs.** *Food Chem.* 2013; **138**(1): 414–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
37. Mosmann T: **Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.** *J Immunol Methods.* 1983; **65**(1–2): 55–63.
[PubMed Abstract](#) | [Publisher Full Text](#)
38. Kumar V, Kumar S, Hassan M, *et al.*: **Novel Chalcone Derivatives as Potent Nrf2 Activators in Mice and Human Lung Epithelial Cells.** *J Med Chem.* 2011; **54**(12): 4147–59.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
39. Huerta-Olvera SG, Macías-Barragán J, Ramos-Márquez ME, *et al.*: **α -Lipoic acid regulates heme oxygenase gene expression and nuclear Nrf2 activation as a mechanism of protection against arsenic exposure in HepG2 cells.** *Environ Toxicol Pharmacol.* 2010; **29**(2): 144–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
40. Koriyama Y, Kamiya M, Takadera T, *et al.*: **Protective action of nipradilol mediated through S-nitrosylation of Keap1 and HO-1 induction in retinal ganglion cells.** *Neurochem Int.* 2012; **61**(7): 1242–53.
[PubMed Abstract](#) | [Publisher Full Text](#)
41. Yap WH, Khoo KS, Ho ASH, *et al.*: **Maslinic acid induces HO-1 and NOQ1 expression via activation of Nrf2 transcription factor.** *Biomedicine & Preventive Nutrition.* 2012; **2**(1): 51–8.
[Publisher Full Text](#)
42. Koriyama Y, Nakayama Y, Matsugo S, *et al.*: **Protective effect of lipoic acid against oxidative stress is mediated by Keap1/Nrf2-dependent heme oxygenase-1 induction in the RGC-5 cell line.** *Brain Res.* 2013; **1499**: 145–57.
[PubMed Abstract](#) | [Publisher Full Text](#)
43. Sepúlveda-Arias JC, Garzón-Castaño SC, Jiménez-González FJ, *et al.*: **Activation of the Keap1-Nrf2 pathway *Tabebuia*.** 2020.
<http://www.doi.org/10.17605/OSF.IO/HW6X9>
44. Na HK, Surh YJ: **Oncogenic potential of Nrf2 and its principal target protein heme oxygenase-1.** *Free Radic Biol Med.* 2014; **67**: 353–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
45. Olga RLS: **Flora andina y amazónica: un aporte a su conocimiento químico.** *Reference Source*
46. Ghosh P, Das C, Biswas S, *et al.*: **Phytochemical composition analysis and evaluation of *in vitro* medicinal properties and cytotoxicity of five wild weeds: A comparative study [version 1; peer review: 1 approved, 2 approved with reservations].** *F1000Res.* 2020; **9**: 493.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
47. Fitmawati F, Resida E, Kholifah SN, *et al.*: **Phytochemical screening and antioxidant profiling of Sumatran wild mangoes (*Mangifera* spp.): a potential source for medicine antidegenerative effects [version 3; peer review: 2 approved].** *F1000Res.* 2020; **9**: 220.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
48. Di Domenico F, Foppoli C, Coccia R, *et al.*: **Antioxidants in cervical cancer: Chemopreventive and chemotherapeutic effects of polyphenols.** *Biochim Biophys Acta.* 2012; **1822**(5): 737–47.
[PubMed Abstract](#) | [Publisher Full Text](#)
49. Ospina LF, Aragón DM, Vergel NE, *et al.*: **Anti-inflammatory and antioxidant activities of *Phenax rugosus*. (POIR.) WEDD and *Tabebuia chrysanta* G. NICHOLSON.** *Vitae.* 2011; **18**(1): 49–55.
Reference Source
50. Kwak, Kim HJ, Lee KH, *et al.*: **Antioxidative iridoid glycosides and phenolic compounds from *Veronica peregrina*.** *Arch Pharm Res.* 2009; **32**(2): 207–13.
[PubMed Abstract](#) | [Publisher Full Text](#)
51. Rahman MM, Islam MB, Biswas M, *et al.*: ***In vitro* antioxidant and free radical scavenging activity of different parts of *Tabebuia pallida* growing in Bangladesh.** *BMC Res Notes.* 2015; **8**: 621.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
52. Sobiyana P, Anburaj G, Manikandan R: **Comparative Analysis of the *in vitro* antioxidant activity of *Tabebuia rosea* and *Tabebuia argentea*.** *J Pharmacogn Phytochem.* 2019; **8**(1): 2673–2677.
Reference Source
53. Robledinos-Antón N, Fernández-Ginés R, Manda G, *et al.*: **Activators and Inhibitors of NRF2: A Review of Their Potential for Clinical Development.** *Oxid Med Cell Longev.* 2019; **2019**: 9372182.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
54. Qiu YL, Cheng XN, Bai F, *et al.*: **Aucubin protects against lipopolysaccharide-induced acute pulmonary injury through regulating Nrf2 and AMPK pathways.** *Biomed Pharmacother.* 2018; **106**: 192–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
55. Lu Q, Tan S, Gu W, *et al.*: **Phytochemical composition, isolation and hepatoprotective activity of active fraction from *Veronica ciliata* against acetaminophen-induced acute liver injury via p62-Keap1-Nrf2 signaling pathway.** *J Ethnopharmacol.* 2019; **243**: 112089.
[PubMed Abstract](#) | [Publisher Full Text](#)
56. Ma B, Zhang J, Zhu Z, *et al.*: **Aucubin, a natural iridoid glucoside, attenuates oxidative stress-induced testis injury by inhibiting JNK and CHOP activation via Nrf2 up-regulation.** *Phytomedicine.* 2019; **64**: 153057.
[PubMed Abstract](#) | [Publisher Full Text](#)
57. Subramaniam SR, Ellis EM: **Esculetin-induced protection of human hepatoma HepG2 cells against hydrogen peroxide is associated with the Nrf2-dependent induction of the NAD(P)H: Quinone oxidoreductase 1 gene.** *Toxicol Appl Pharmacol.* 2011; **250**(2): 130–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
58. Bi J, Jiang B, Liu JH, *et al.*: **Protective effects of catalpol against H₂O₂-induced oxidative stress in astrocytes primary cultures.** *Neurosci Lett.* 2008; **442**(3): 224–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
59. Wang H, Wei W, Lan X, *et al.*: **Neuroprotective Effect of Swertiamain on Cerebral Ischemia/Reperfusion Injury by Inducing the Nrf2 Protective Pathway.** *ACS Chem Neurosci.* 2019; **10**(5): 2276–86.
[PubMed Abstract](#) | [Publisher Full Text](#)
60. Asthana J, Yadav AK, Pant A, *et al.*: **Specioside ameliorates oxidative stress and promotes longevity in *Caenorhabditis elegans*.** *Comp Biochem Physiol C Toxicol Pharmacol.* 2015; **169**: 25–34.
[PubMed Abstract](#) | [Publisher Full Text](#)

Open Peer Review

Current Peer Review Status:  

Version 3

Reviewer Report 03 December 2020

<https://doi.org/10.5256/f1000research.31026.r75623>

© 2020 Rastrelli L. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Luca Rastrelli 

Department of Pharmacy, University of Salerno, Fisciano, Italy

The authors corrected and clarified the required revisions. They welcomed the criticisms and suggestions. In my opinion this work merits being indexed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Phytochemistry, food chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 09 November 2020

<https://doi.org/10.5256/f1000research.29707.r73587>

© 2020 Rastrelli L. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Luca Rastrelli 

Department of Pharmacy, University of Salerno, Fisciano, Italy

The present study indicates that the *n*-butanol extract from the inner bark of *T. rosea* and its isolated compound specioside have promising antioxidant activity.

This work is well presented and easy to read. The experiments and the structural characterization have been conveniently described and the analyses were performed by appropriate methods. There is sufficient discussion of the results obtained. It merits being indexed after minor revision. Detailed remarks on the text are as follows:

- Extraction and isolation: The authors obtain 12.5 g of BuOH extract, please report the quantities of bark extracted to calculate the yield.
- Figure 1: Report catalposide structure.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Food chemistry, natural products, analytical chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 10 Nov 2020

Juan Carlos Sepúlveda-Arias

Dear Dr. Rastrelli. Thank you very much for your comments. In the "Plant material, extract preparation and specioside isolation" section, we did include the quantity of bark extracted and the yields.

In Figure 1 we did include the catalposide structure.

Competing Interests: No competing interests were disclosed.

Reviewer Report 27 October 2020

<https://doi.org/10.5256/f1000research.29707.r73506>

© 2020 Martin-Franco J. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Jaime Martin-Franco 

Grupo de investigación Química de Productos Naturales QPN, Departamento de Química, Universidad del Cauca, Popayán, Colombia

Plants are an important source of biologically active natural products, many of them also constitute models for the synthesis of numerous drugs. The genus *Tabebuia* includes about 100 species and is the largest genus in the Bignoniaceae family. It is important to indicate that molecules present in the inner bark of some species of this genus (used in traditional medicine) have important pharmacological potential. A large number of chemical compounds exert their antioxidant effects through the activation of key transcriptional regulatory mechanisms, such as the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2). In cells exposed to oxidative stress, Nrf2 is released and translocated to the nucleus and activates the antioxidant response. The aim of this study was to evaluate the mechanism responsible for the *in vitro* antioxidant activity of the n-butanol extract obtained from the inner bark of *Tabebuia rosea* (Bertol) DC.

Specioside was isolated and elucidated previously in the Research Group Polifenoles at Universidad Tecnológica de Pereira from the n-butanol extract obtained from the inner bark of *T. rosea*. The antioxidant activity of specioside was evaluated using the ORAC and DPPH techniques. The effect of specioside and the extract on the viability of HepG2 cells was determined using the MTT method. The effect of the compound and the extract on the translocation of Nrf2 to the nucleus was evaluated using the Nrf2 Transcription Factor Assay Kit (abcam), according to the manufacturer's instructions. Molecular docking was carried out using the AutoDock software. The antioxidant activity indicated that both the extract and the isolated compounds have antioxidant activity as measured by the ORAC (oxygen radical absorbance capacity) technique. The cell viability was determined using the MTT assay. The results indicate that specioside exhibits its activity at low concentrations without affecting the viability of the cells since viability was greater than 90% after 24 hours of exposure. They allowed performing *in vitro* tests to evaluate the effect of the isolated compounds on the activation of the Nrf2 signaling pathway in HepG2 cells. Nrf2 is dissociated from Keap1 by the n-butanol extract at a concentration of 0.5 μ M after 4 hours of exposure, showing an increase in both cytosolic and nuclear Nrf2 levels with a significant difference ($p < 0.05$) in comparison to the basal levels. Specioside at a concentration of 2 μ M did increase the Nrf2 levels in the cytoplasm after 4 hours of exposure and was stabilized in the nucleus at 12 hours after stimulation with specioside and extract (significant difference when compared to the basal levels, $p < 0.05$). The molecular docking analysis showed the interaction of the iridoid group of the ligand with the residue Tyr572 and Ser602 of Keap1 to be important for the interaction with Nrf2.

In the analysis of the ¹H NMR spectrum of the specioside shown in Fig. 1, the authors should write E instead of trans and configuration instead of conformation.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Organic Synthesis, natural products and spectral analysis by RMN

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 10 Nov 2020

Juan Carlos Sepúlveda-Arias

Dear Dr. Martin-Franco, Thank you very much for you comments. We did change "trans conformation" with "E configuration".

Competing Interests: No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research