**ENZYMATIC SYNTHESIS OF A NOVEL NEUROPROTECTIVE
HYDROXYTYROSYL GLYCOSIDE**

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ABSTRACT

The eco-friendly synthesis of non-natural glycosides from different phenolic antioxidants was carried out using a fungal β -xylosidase in order to evaluate changes in their bioactivities. Xylosides from hydroquinone and catechol were successfully formed although the best results were obtained for hydroxytyrosol, the main antioxidant from olive oil. The formation of the new products was followed by thin layer chromatography, liquid chromatography and mass spectrometry. The hydroxytyrosyl xyloside was analyzed in more detail, in order to maximize its production and evaluate the effect of glycosylation on some hydroxytyrosol properties. The synthesis was optimized up to the highest production reported for a hydroxytyrosyl glycoside. The structure of this compound was solved by 2D-NMR and identified as 3,4-dihydroxyphenyl-ethyl-*O*- β -D-xylopyranoside. Evaluation of its biological effect showed an enhancement of both its neuroprotective capacity and its ability to ameliorate intracellular levels of reactive oxygen species.

KEYWORDS

Glycosylation; xyloside, regioselectivity, plant phenols, olive oil

INTRODUCTION

Olive oil is one of the main commodities of the Mediterranean agro-industry and, as a consequence, olive production in Southern Europe amounted to 9 million tons in 2014 (FAOSTAT). Both olive oil and its by-products contain high amounts of phenols, which implies an invaluable source of bioactive compounds¹. Among them, the most potent antioxidant is 3,4-dihydroxyphenylethanol, a phenylethanoid commonly named hydroxytyrosol (HT), known for its strong anti-inflammatory and neuroprotective

properties^{2;3}. In recent years several studies have demonstrated the effect of HT on the prevention of cardiovascular problems, cancer and other chronic diseases⁴⁻⁶.

HT is well absorbed in a dose-dependent manner, but it displays a poor bioavailability for being quickly converted into its sulphate and glucuronide metabolites by a classic phase I/II biotransformation⁷. In this sense, glycosylation has demonstrated to be a useful approach not only for enhancing bioavailability, but also other specific properties of phenolic antioxidants, such as solubility, stability or biosafety⁸⁻¹⁰. Glycosylation can be accomplished by synthetic chemical approaches, but they imply many steps of protection and de-protection^{11;12}, low specificity and toxic side-products. On the contrary, the use of glycosidases or glycosyltransferases for the same purpose is an eco-friendly and highly specific alternative^{9;13}. Several reports deal with *in vitro* synthesis or isolation of physiological metabolites of HT^{14;15}, but there is little information about the production of non-natural glycosides, which may possess enhanced properties or simplify *in vivo* studies¹⁶ to determine its role.

The aim of this work was double. On the one hand, to develop a one-step method to enzymatically synthesize non-natural glycosides of polyphenolic antioxidants with commercial interest and, on the other hand, to assess the effect of xylosylation on some of the bioactivities reported for these antioxidants. The GH3 β -xylosidase BxTW1 from *Talaromyces amestolkiae*, heterologously expressed in *Pichia pastoris* (rBxTW1), was used as catalyst. This enzyme, previously characterized in our group, catalyzed the regioselective transxylosylation of substrates and showed to have remarkable acceptor promiscuity^{17;18}. The current work reports the screening of several antioxidants, the regioselective and efficient xylosylation of HT, and positive results for catechol and hydroquinone (HQ). In addition, as an approach for improving the properties of bioactive compounds, the ability to reduce the level of intracellular reactive oxygen

species (ROS) and the neuroprotective effect of this novel HT xyloside have been evaluated.

MATERIALS AND METHODS

Materials

Hydroxytyrosol was provided by Seprox Biotech (Madrid, Spain). Quercetin, ferulic acid, gallic acid, ($\pm$)- α -tocopherol, L-ascorbic acid, hesperidin, hesperetin, lipopolysaccharides from *Escherichia coli* 055:B5 (LPS), MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay kit, 2',7'-dichlorofluorescein diacetate (H₂DCFDA) and 4-nitrophenyl- β -D-xylopyranoside (pNPX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xylobiose was from TCI Europe (Zwijndrecht, Belgium). Hydroquinone was from Acros Organics. Catechol was purchased from Fisher Scientific (Loughborough, UK). Resveratrol (RES) was from Seebio Biotechnology, Inc. (Shanghai, China). Epigallocatechin gallate (EGCG) was provided from Zhejiang Yixin Pharmaceutical Co., Ltd. (Jinhua Shi, China). Silica gel G/UV254 polyester sheets (0.2 mm thickness and 40 x 80 mm plate size) for thin layer chromatography (TLC) were provided by Macherey-Nagel (Düren, Germany). BxTW1 from *T. amestolkiae*, heterologously expressed in *P. pastoris*, was produced and purified as previously reported¹⁸.

Enzyme activity and acceptor screening

Enzyme activity was defined and determined as previously reported¹⁷. Transxylosylation reactions were carried out using 0.5 and 5 U/mL of rBxTW1 in the presence of 0.1% BSA and 50 mM sodium formate buffer (pH 3.0). 40 mM xylobiose (11.3 g/L) was used as donor and a series of plant phenolic antioxidants were tested as

acceptors. Negative control reactions were carried out with no enzyme added. Concentration of acceptors and selected co-solvents are listed in Table 1. Acceptor stocks of gallic, ferulic and ascorbic acids were neutralized to pH 3 with sodium hydroxide to prevent pH changes of the reactions. Reactions were carried out at 50 °C and 1200 rpm with a final volume of 200 µL.

Samples (2 µL) were collected at different time intervals from 10 min to 72 h and reactions were followed by TLC developed with ethyl acetate/methanol/H₂O 10:2:1 (v/v). Detection was performed under 254 nm UV light, and the pattern of spots in each reaction and its no-enzyme control were compared.

Mass Spectrometry

Conventional mass spectrometry analyses were performed on a hybrid QTOF analyzer, model QSTAR, Pulsar I, from AB Sciex (Framingham, MA, USA). Reaction samples were analyzed by direct infusion and ionized by electrospray (ESI-MS) with methanol as mobile phase in positive reflector mode.

HR-MS analysis was carried out by FIA-ESI-MS on a QTOF Agilent G6530A Accurate Mass Q-TOF LCMS system (Agilent Technologies, Santa Clara CA, USA). The sample was directly infused and ionized by ESI in negative reflector mode. Ionization was enhanced by JetStream technology and the mobile phase was H₂O/Formic acid 99.9:0.1 (v/v). Data were processed with Masshunter Data Acquisition B.05.01 and Masshunter Qualitative Analysis B.07.00 software (Agilent Technologies).

Optimization of transxylosylation

The reaction with HT or HQ as acceptors and xylobiose as donor was optimized using a range of donor and acceptor concentrations as well as different reaction times. The

reactions were carried out in 50 mM sodium formate buffer (pH 3.0), at 50 °C and 1200 rpm (see Supporting Information for the assayed conditions).

At the selected time intervals, reactions were stopped by enzyme denaturation at 100 °C for 5 min and stored at 4 °C. The concentration of the new xylosides was determined by HPLC as described below. Concentration data, in terms of molarity, were used to calculate reaction yields according to the following equation:

$$\text{Yield (\%)} = [\text{Product}]/[\text{Acceptor}]_0 \times 100$$

Reactions were performed in triplicate. Standard deviations were in all cases lower than 5%.

In order to identify the HT-xyloside (HTX) produced and to assay its biological activity, the reaction was scaled-up to 1 mL in the conditions of highest production: 350 mM xylobiose, 300 mM HT, 50 mM sodium formate buffer (pH 3.0) at 50 °C and 1200 rpm for 2 h. The reaction was stopped by incubation at 100 °C for 5 min without loss of product by physicochemical hydrolysis (see Supporting Information). The reaction mixture was analyzed by HPLC as described below. Fractions corresponding to HTX were collected and the solvent was evaporated. The product was stored at 4 °C.

HPLC analysis

HPLC analysis was performed in an instrument equipped with a semipreparative HPLC quaternary pump 600E (from Waters Corporation, Milford, MA, USA), an autosampler ProStar (Agilent Technologies) and a photodiode-array detector controlled by software Varian LC Workstation 6.41 (Agilent Technologies). The reaction products were detected monitoring their absorbance at 241 and 268 nm, using the chromatogram recorded at 268 nm for quantitation. The sample was loaded in an ACE 3 C18-PFP column (15 x 4.6 mm, 5 µm, Advanced Chromatography Technologies Ltd., Aberdeen,

United Kingdom) that was kept constant at 40 °C. The mobile phase was methanol/H₂O 20:80 v/v (both containing 0.1% acetic acid) at 0.7 mL/min for 12 min in the case of samples with HT as acceptor. Methanol/H₂O 5:95 v/v (both containing 0.1% acetic acid) at 0.7 mL/min for 15 min was selected for HQ assays. The quantitation was carried out using the Varian Star LC Workstation 6.41. Calibration curves of HT and HQ standards were used for the quantification of HT- and HQ-xylosides, respectively.

HTX was purified by semipreparative HPLC. The 1 mL reaction mixture was loaded onto the semipreparative column ACE 5 C18-PFP 15 x 10 mm, 5 µm (Advanced Chromatography Technologies Ltd.). The mobile phase was methanol/H₂O 20:80 v/v (both containing 0.1% acetic acid) at 5.5 mL/min for 12 min. A three-way flow splitter (model Accurate, Dionex, Sunnyvale, CA, USA) was used. The fractions containing the product were pooled and the solvent evaporated.

Nuclear Magnetic Resonance (NMR)

The structure of the xyloside was determined using a combination of 1D (¹H, 1D-selective NOESY experiments) and 2D (COSY, DEPT-HSQC, NOESY) NMR techniques. The sample was dissolved in deuterated water (*ca.* 10 mM), recording spectra on a Bruker AV-III 800 spectrometer (Bruker, Billerica, MA, USA) equipped with a TCI cryoprobe with gradients in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in parts per million with respect to the 0 ppm point of DSS (4-dimethyl-4-silapentane-1-sulfonic acid), used as internal standard. All pulse sequences used were provided by Bruker. The values set for the DEPT-HSQC experiment were of 7 ppm and 2K points, for the 1H dimension, and 160 ppm and 256 points for the ¹³C dimension. For the homonuclear experiments COSY and NOESY, 7 ppm windows were

used with a 2K x 256 points matrix. For the NOESY and 1D-selective NOESY experiments, mixing times of 500-600 ms were applied.

Evaluating the biological properties of HTX

The potential of the HTX as neuroprotective compound and its capacity to ameliorate intracellular ROS levels were assayed and compared with those of HT and RES, a well-characterized antioxidant. The tests were carried out *in vitro*, in cell cultures. SH-S5Y5 neurons were cultured in collagen-pretreated Petri-dishes with Dulbecco's Modified Eagle's Medium (DMEM): Nutrient Mixture F-12 medium supplemented with Penicillin/Streptomycin and 10% inactivated fetal bovine serum (iFBS). RAW 264.7 macrophages were cultured in DMEM high glucose medium supplemented as above.

To carry out all the following assays, the tested compounds were dissolved in dimethyl sulfoxide (DMSO) and added at different concentrations (1, 10 and 100 μ M) to the cell cultures. The final DMSO percentage in each cell culture was adjusted to 1%. Averages and standard deviations of at least eight different readings from various experiments were calculated.

The cytotoxicity of the tested compounds to macrophages and neurons was evaluated before conducting the ROS and neuroprotection assays. Neuron assays were done in collagen-pretreated 96-well plates by seeding 2×10^4 neurons per well in a 100- μ L volume, incubating for 24 h before compound addition. Macrophage assays were done in 96-well plates by seeding 2.5×10^4 macrophages per well in a 100- μ L volume and incubating for 4 h before compound addition. Cell viability was evaluated 24 h after compound addition by the mitochondrial MTT assay, according to the manufacturer.

The intracellular ROS level was determined on macrophages that were cultured and plated as described for the cell viability assay. After 2-h incubation of the cells with

solutions of the tested compounds in DMSO, 100 ng/mL of LPS were added in order to induce ROS production. ROS levels were evaluated 24 h after compound addition by using the ROS-sensitive H₂DCFDA staining method. Following treatments, the medium was removed and cells were incubated with 25 µM H₂DCFDA for 2 h at 37 °C in the dark. H₂DCFDA, a cell-permeable non-fluorescent compound, is intracellularly de-esterified and turns into the highly fluorescent permeant molecule 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS upon oxidation. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a multimode microplate reader (TECAN, Männedorf, Switzerland). The direct effect of the assayed compounds on the fluorescence intensity was evaluated in cell-free controls, in order to discard potential artifacts (see Supporting Information).

To determine the neuroprotective effect, neurons were also cultured and plated as described in the cell viability assay. The compounds were added at three different concentrations and incubated for 10 min before adding 100 µM hydrogen peroxide. Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay. Neuron viability was normalized to the H₂O₂ negative control.

RESULTS AND DISCUSSION

Screening of phenolic plant antioxidants as transxylosylation acceptors

A previous report on rBxTW1 described the transxylosylation of xylobiose with 2,6-hydroxynaphthalene, obtaining the anti-tumorigenic 2-(6-hydroxynaphthyl) β-D-xylopyranoside with a modest yield of 0.47 g/L. In the same work the wide acceptor versatility of rBxTW1 was demonstrated by a high-throughput analysis¹⁸.

Because of its acceptor promiscuity, the enzyme was considered a potential candidate for glycosylating polyphenolic antioxidants, although the presence of bulky aromatic rings and their high insolubility were supposed to decrease transxylosylation. In this sense, the bioactive compound hydroxytyrosol was a good candidate for this assay for containing an aliphatic chain with a primary alcohol group, which was expected to be a suitable target for efficient glycosylation by rBxTW1. Besides this compound, a panel of phenols from biomass were further explored because of their commercial interest: resveratrol, quercetin, ($\pm$)- α -tocopherol, hesperetin, hesperidin, gallic acid, L-ascorbic acid, epigallocatechin gallate (EGCG), ferulic acid, HQ and catechol.

The screening was carried out at pH 3, using 40 mM xylobiose as the donor and 10 g/L of each phenolic antioxidant. This low pH value is optimum for rBxTW1 action, enhances the stability of the acceptors, preventing self-oxidation, and allowed long reaction times and long-term storage of the potential xylosides. The reactions were followed by TLC, and spots likely corresponding to newly formed xylosides, were detected under UV-light in reactions involving HT, HQ and catechol as the acceptors (Fig. 1 and Supporting Information). No sign of glycoconjugates was observed for other screened acceptor candidates.

The reaction mixtures obtained with HT, HQ and catechol were analyzed by ESI-MS. The existence of mono-xylosylated derivatives was confirmed for each of the three acceptors (Fig. 2 and Supporting Information). In addition, xylotriose was also detected in the reactions with HQ and catechol, indicating that xylobiose itself could also serve as acceptor¹⁷.

Hydroquinone and catechol are well-known phenolic antioxidants. HQ is the most used skin-whitening agent for its role as tyrosinase inhibitor, but there is a rising

concern about its safety on human health and industry is looking for safer and active hydroquinone derivatives¹⁹. The xyloside from hydroquinone has been reported as an alternative for inhibiting tyrosinase²⁰, so it constitutes an interesting compound to be synthesized by rBxTW1.

Regarding catechol, it is the core of many compounds used as drugs in the treatment of bronchial asthma, hypertension, Parkinson's disease, myocardial infarction or even HIV^{21;22}. Catechyl glycosides, as the catechyl xyloside reported in this work, may lead to novel drugs with improved functionalities²³. The enzymatic synthesis of catechyl xyloside was previously reported by Chiku *et al.*²⁴ using a bacterial xylanase, but the process produced a mixture of glycoconjugates with one to four units of xylose.

Reaction monitoring by HPLC and evaluation of catalysis specificity

Upon preliminary analysis of the reactions by TLC, the positive hits were analyzed by HPLC for a deeper insight into the regio- and stereo-specificity in transxylosylation of these compounds catalyzed by rBxTW1. Different mixtures of methanol and water were employed as mobile phases in order to get the best separation. Surprisingly, peaks corresponding to the catechyl-xylosides were not detected, which could be probably attributed to the low concentration of the xyloside formed.

The number of HT- and HQ-xylosides was determined by HPLC. Compounds containing an aromatic ring (derived from HQ or HT) were detected by measuring the absorbance at 241 and 268 nm, and hence the remaining xylobiose, xylose or other side products did not interfere with the analysis. The chromatograms displayed a single new peak for both acceptors, thus confirming monoglycosylation. These results suggest that rBxTW1-catalyzed transxylosylation was highly selective, although the possibility of producing other secondary products as xylobiosides or, in the case of HT, also of

different mono-xylosides in amounts below the detection limits of the techniques used, cannot be ruled out.

Determination of the acceptor/donor balance for maximizing the synthesis yield

Trying to find optimal conditions, we tested the influence of donor and acceptor concentration in the reaction mixture, as well as the degree of hydrolytic activity of the enzyme on the synthesized products. HPLC was used to determine the concentration of the xyloside under the different experimental conditions (see Supporting Information).

All assays using HT as acceptor contained 18 U/mL of rBxTW1. The effect of HT concentration was analyzed in the range of 75-600 mM, setting xylobiose at 40 mM and following HTX production along the time (Fig. 3A). Then, the HT concentration that yielded the highest amount of xyloside was selected for assessing a range of concentrations of xylobiose (Fig. 3B).

Figure 3A shows that the maximal production of the xyloside was quickly achieved at all the HT concentrations tested. However, with 75 and 150 mM HT a dramatic decrease of the product occurred beyond the first 10 min. This suggests that the HT-xyloside is also a substrate for rBxTW1, causing the kinetic equilibrium transglycosylation-hydrolysis to shift towards hydrolysis when the transglycosylation product is available at similar concentrations to the donor. However, when the reaction medium contained 300 and 600 mM HT, the amount of xyloside remained stable at least for 4 hours after reaching the maximum. Then, at high HT concentrations, the hydrolysis-transxylosylation balance shifts towards the synthesis. HTX can act as the donor of a new transxylosylation reaction, attaching the xylosyl moiety to another HT molecule, maintaining the overall concentration of the product. The lower production

reached with 600 mM HT in comparison to 300 mM could be due to substrate inhibition.

Figure 3B illustrates how the production of xyloside increases in parallel to the donor concentration, with 300 mM HT as acceptor according to the previous experiment. The amount of xyloside produced in reactions with 40 mM xylobiose was virtually twice of that obtained with 20 mM of the donor. Nevertheless, this trend was less significant at xylobiose concentrations over 80 mM, with HTX yields around 30% higher for each donor increment (2-fold). Taking into account that the disaccharide is also an efficient acceptor for rBxTW1¹⁸, as demonstrated by the synthesis of xylotriose as co-product (Fig. 2), this effect may be due to the competition between xylobiose, when present in high excess, and HT.

As defined before, the reaction yield describes the molar ratio between product and acceptor. Among the conditions assayed, the best result (26.6% yield) was attained with 300 mM HT and 350 mM xylobiose, yielding 77.7 mM of HT-xyloside (22.2 g/L) in 2 h. This production is the highest reported for a non-natural hydroxytyrosyl glycoside synthesized by transglycosylation. Trincone *et al.*¹⁶ reported 45% conversion for the glucosylation of HT catalyzed by the α -glucosidase from *Aplysia fasciata*, but they used a low concentration of HT (5 mM), which led to a scarce net production, and a high excess (30:1) of maltose. In addition, the process was not chemo- or regioselective, giving rise to the three possible glucosides: one from the aliphatic alcohol and one from each of the phenolic hydroxyls. On the contrary, rBxTW1 catalyzes the synthesis of high concentrations of a single xyloside (or at least of a very predominant one), which simplifies product purification and reaction control.

The same experimental design was followed to evaluate the effect of HQ and xylobiose concentration on the production of HQ-xyloside (HQX). The preliminary

assay, with xylobiose at 40 mM, revealed that synthesis and hydrolysis of the product occurred very quickly (data not shown). Then, the enzyme concentration was lowered to 0.9 U/mL, and the formation of HQX was analyzed along a range of concentrations of HQ (Fig. 4A) and xylobiose (Fig. 4B) over time. The highest yield (4.7%) was obtained using 110 mM HQ and 160 mM xylobiose in 2 h, producing 5.2 mM (1.2 g/L) of HQ-xyloside. This value is similar to those reported for other transglycosylation products of HQ^{8;25} but far away from the high levels reported by Seo *et al.*²⁶ and remarkably low in comparison to the results displayed for HT. The HPLC profiles of the reaction mixtures for HTX and HQX production, in optimum conditions, are presented in the Supporting Information.

Product characterization by NMR and MS

The synthesis of HTX, the xyloside produced at higher yields, was scaled-up to 1-mL in order to confirm its structure by NMR and to assay its biological properties. The HTX was purified by semipreparative HPLC as described above, obtaining 21.4 mg of a mixture of xyloside and acetic acid. The molar ratio of the mixture was determined to be 1:0.5 by NMR, corresponding to a purity of 90.5% of the xyloside. The reaction yield was 23.4%, close to the theoretical value determined by HPLC (26.6%), and the isolated product had the appearance of highly viscous oil with a pale yellow color.

The NMR signals were assigned using standard ¹H, COSY and HSQC-edited experiments. The observed chemical shifts (C₁ and C_A, see Fig. 5 and Table 2) as well as the existence of a strong NOE between the anomeric Xyl H₁ and the methylene H_A protons (see Supporting Information for the NMR spectra) permitted to unequivocally identify the glycosylation product as 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside. To the best of our knowledge, the glycoside synthesized by rBxTW1 is a novel

compound. The purified product was also analyzed by HR-MS obtaining the signals corresponding to the $(M-H)^-$ anion of the xyloside (see Supporting Information for the HR-MS spectrum). The theoretical molar mass and the exact mass of 3,4-dihydroxyphenyl-ethyl-*O*- β -D-xylopyranoside were calculated to be 286.28 g/mol and 286.1053 Da (85.0090% abundance) respectively. The theoretical exact mass of the $(M-H)^-$ anion was determined to be 285.0980 Da and the experimental value obtained by HR-MS was 285.0985, validating the identity of the new xyloside.

These results confirmed the high chemo- and regioselectivity of rBxTW1 for this reaction and the preference of the enzyme for primary-alcohols as acceptors, proposed in a previous paper¹⁷, as only this functional group of HT was confirmed to be modified by transglycosylation. This preference may also serve to explain the higher yields obtained for HT in comparison with HQ, with no primary alcohols.

Biological activity of 3,4-dihydroxyphenyl-ethyl-*O*- β -D-xylopyranoside (HTX)

Neuroprotection and oxidative stress amelioration are well-known effects of HT itself^{3,27}, therefore the assays focused on comparing its effects with those produced by HTX and resveratrol, a well-known antioxidant with proven neuroprotective and ROS inhibition capacities²⁸.

First, cell viability in the presence of the tested substances was evaluated by adding different concentrations of HTX, HT and RES to RAW 264.7 macrophage and SH-SY5Y neuroblastoma cultures. None of these compounds but RES at 100 μ M was toxic to macrophages (Fig. 6A) or neurons (Fig. 6C). Treatment with 100 μ M RES induced a remarkable viability decrease, reducing it up to 44% in RAW macrophages, which indicated certain cytotoxicity of this compound. This effect has been previously reported in the literature²⁹. Interestingly, the cell viability of neurons in the presence of

100 μ M HTX increases markedly, to values above those of the controls, suggesting its beneficial effect even in the absence of an external stress.

Once established the safety of HTX, its potential to alleviate intracellular ROS levels and its neuroprotective activity were assayed and compared to those of the other compounds tested. The former assays were carried out in the presence of LPS as intracellular ROS trigger. ROS levels (Fig. 6B) were measured from the fluorescence intensity of DCF as explained in the Experimental section. As a general rule, all compounds produced a decrease in ROS regardless of the dose added to the cell culture, but this effect was greater for HTX and RES, comparing to HT. It should be noted that treatment with 100 μ M HTX reduced ROS levels below 40% of the value detected in the negative control. A similar trend was found upon treatment with 100 μ M RES, but considering the results from the cell viability tests, this effect can be attributed to RES toxicity.

Regarding neuroprotection, the activity of the compounds evaluated was very different (Fig. 6D). While for RES and HT non-significant neuroprotection from oxidative damage was observed in the concentration range assayed, HTX is neuroprotective at 10 and 100 μ M. In fact 100 μ M of HTX allow restoration of cell viability to values equal or above those of non-challenged cells.

The absence of a protective effect of HT may seem surprising, since it has been reported as a strong neuroprotective agent. However, the methodological differences among different studies make it difficult a precise comparison of the results, and may explain the lack of neuroprotection found in the current study^{3;30-32}.

The results presented here confirm that, in the tested conditions, HTX is non-toxic and lowers intracellular ROS levels to a greater degree than HT itself, displaying also considerably higher neuroprotective effect. Thus, this xyloside might be a stronger

activator of the Nrf2 signaling pathway³³. These preliminary analyses on the biological activity of HTX suggest that uptake of the xyloside by the cell and its potential to cross the blood-brain barrier may merit attention in further research.

Beyond the specific bioactivities evaluated, this work demonstrates that xylosylation is a valid approach for the valorization of biomass' compounds with pharmacological interest. Given the abundance of xylan, a natural source of xylobiose, the synthesis of novel bioactive xylosides by β -xylosidases may be an expanding field in the near future.

ABBREVIATIONS

DCF: 2,7-dichlorofluorescein; DMEM: Dulbecco's Modified Eagle's Medium; EGCG: Epigallocatechin gallate; H₂DCFDA: 2',7'-dichlorofluorescein diacetate HQ: Hydroquinone; HT: hydroxytyrosol; HQX: Xyloside of HQ; HTX: 3,4-dihydroxyphenyl-ethyl-*O*- β -D-xylopyranoside; *p*NPX: 4-nitrophenyl- β -D-xylopyranoside; RES: Resveratrol.

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SUPPORTING INFORMATION

List of tested conditions for the transxylosylation of hydroxytyrosol and hydroquinone; stability assay of HTX; cell free controls for the amelioration of ROS levels; TLCs from screening reactions with HT, HQ and catechol; HPLC profiles from transxylosylation reactions with HT and HQ; complete NMR data for HTX identification; complete data of mass spectrometry from transxylosylation reactions and pure HTX.

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FIGURE CAPTIONS

Figure 1. Positive acceptors for transxylosylation with rBxTW1. Hydroxytyrosol (A), hydroquinone (B) and catechol (C).

Figure 2. Mass spectra of a transxylosylation reaction mixture with hydroxytyrosol (A), hydroquinone (B) and catechol (C). The identified adducts are indicated, a: Xylobiose+Na⁺; b: HT-Xyloside+Na⁺; c: 2HT+Na⁺; d1: HQ-xyloside+Na⁺; d2: Catechyl-xyloside+Na⁺; e: Xylotriose+Na⁺.

Figure 3. Optimization of acceptor (A) and donor (B) concentration for the synthesis of HTX. Reactions were performed at pH 3.0 and 50 °C. (A) Time course of the HT-xyloside production varying the concentration of HT. Initial concentration of xylobiose was fixed as 40 mM. (B) Relation between xyloside production and initial xylobiose. Reactions contained 300 mM HT as acceptor.

Figure 4. Optimization of acceptor (A) and donor (B) concentration for the synthesis of HQX. Reactions were performed at pH 3.0 and 50 °C. (A) Time course of the HQ-xyloside production using 40 mM xylobiose and varying concentration of HQ. (B) Relation between xyloside production and initial xylobiose by using the optimum concentration of HQ (110 mM).

Figure 5. Structure of 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside. Atoms are labeled in agreement with Table 2.

Figure 6. Biological activity of 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside: Cells viability (A, C), intracellular ROS levels (B) and neuroprotection (D) were evaluated in the presence of HTX and three commercial antioxidants. Assays were performed on macrophage (A, B) and neuron (C, D) cell cultures. Controls with (+) and without (-) the stress agent were included in every case.

TABLES

Table 1. Phenolic antioxidants and co-solvents selected for the acceptor screening.

Acceptor	Concentration (g/L)	Co-solvent
α -tocopherol	10	20% Acetonitrile
	40	50% Ethyl acetate
Catechol	10	-
EGCG	10	-
	40	-
Ferulic acid	10	-
Gallic acid	10	-
Hesperetin	10	20% Acetonitrile
	40	50% Ethyl acetate
Hesperidin	10	20% Acetonitrile
	40	50% Ethyl acetate
Hydroquinone	10	-
Hydroxytyrosol	10	-
L-ascorbic acid	10	-
Quercetin	10	20% Acetonitrile
	40	50% Ethyl acetate
Resveratrol	10	20% Acetonitrile
	40	50% Ethyl acetate

Table 2. Chemical shifts data (ppm) from 3,4-dihydroxyphenyl-ethyl-*O*- β -D-xylopyranoside.

	δ_H (Multiplicity/J/nH)	δ_C
Aryl-H6'	6.57 (dd/8.1,2.1 Hz/1H)	123.6
Aryl-H2'	6.67 (d/2.0 Hz/1H)	119.0
Aryl-H5'	6.70 (d/8.1 Hz/1H)	119.0
H1-Xylose	4.21 (d/7.9 Hz/1H)	105.7
H3-Xylose	3.25 (t/9.2 Hz/1H)	78.3
H2-Xylose	3.07 (dd/9.3,7.9 Hz/1H)	75.6
CH ₂ (A) α	3.65 (dt/10.2,7.1 Hz/1H)	73.6
CH ₂ (A) β	3.84 (dt/10.1,7.0 Hz/1H)	
H4-Xylose	3.43 (ddd/10.5,9.1,5.5 Hz/1H)	71.8
H5-Xylose α	3.11 (dd/11.6,10.5 Hz/1H)	67.8
H5-Xylose β	3.76 (dd/11.6,5.5 Hz/1H)	67.8
CH ₂ (B)	2.64 (t/7.0 Hz/2H)	37.1

δ : chemical shift; d: doublet; dd: doublet of doublets; ddd: doublet of doublet of doublets; dt: doublet of triplets; t: triplet; J: coupling constant; nH: number of protons.

FIGURES

A)

B)

C)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

TOC graphic