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RESEARCH ARTICLE

Protective effect of *Uncaria tomentosa* extract against oxidative stress and genotoxicity induced by glyphosate-Roundup® using zebrafish (*Danio rerio*) as a model

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Abstract

Oxidative stress and DNA damage are involved in the glyphosate-based herbicide toxicity. *Uncaria tomentosa* (UT; Rubiaceae) is a plant species from South America containing bioactive compounds with known beneficial properties. The objective of this work was to evaluate the antioxidant and antigenotoxic potential of UT extract in a model of acute exposure to glyphosate-Roundup® (GR) in zebrafish (*Danio rerio*). We showed that UT (1.0 mg/mL) prevented the decrease of brain total thiols, the increase of lipid peroxidation in both brain and liver, and the decrease of liver GPx activity caused after 96 h of GR (5.0 mg/L) exposure. In addition, UT partially protected against the increase of micronucleus frequency induced by GR exposure in fish brain. Overall, our results indicate that UT protects against damage induced by a glyphosate-based herbicide by providing antioxidant and antigenotoxic effects, which may be related to the phenolic compounds identified in the extract.

Keywords Bioactive compounds · Antioxidant activity · Zebrafish · Acute toxicity · Pesticides

Introduction

Roundup® is glyphosate-based herbicide and is currently the major pesticide used worldwide. It consists in a post-emergence herbicide of broad spectrum that acts by inhibiting aromatic amino acid biosynthesis pathway present in plants and some bacteria (Bohn et al. 2014). Although it is generally considered safe and exerts minimal health risks to mammals and other animal species (EPA 1993; Williams et al. 2012), there are reports linking this herbicide to

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hepatic damage, antibiotic tolerance, and renal failure, among others (Bai and Ogbourne 2016). Additionally, neurological diseases such as autism, Parkinson's disease, Alzheimer's disease, depression, and anxiety are finding also related to glyphosate-based formulation exposure (Gallegos et al. 2016; Samsel and Seneff 2015).

Several data have shown the involvement of the oxidative stress in the toxicity of glyphosate-based formulations in a variety of biological systems (Cavusoglu et al. 2011; Chaufan et al. 2014; Manas et al. 2009; Richard et al. 2005; Sanchez et al. 2017). Accordingly, oxidative damage to DNA, lipids, and proteins induced by Roundup® appears to be a consequence of reactive oxygen species (ROS) generation and/or its interference with cellular antioxidant levels in fish and other organisms (Boligon et al. 2015; Chaufan et al. 2014; Ferreira et al. 2010; Guilherme et al. 2012; Sinhorin et al. 2014).

Reactive oxygen species (ROS) and free radicals are produced by the mitochondria, during normal aerobic metabolism (Adam-Vizi 2005). However, an excessive production of ROS and/or decreased cell ability in promoting an effective antioxidant response culminate in oxidative stress (Ray et al. 2012). Exposure to environmental toxicants, such as pollution, radiation, and pesticides, may also contribute to establishment of this condition in biological systems (Xu et al. 2017; Zepeda-Arce et al. 2017). Additionally, DNA damage is also an important effect resultant from herbicide exposure and may be connected to its capacity in deregulating antioxidant defenses and inducing oxidative stress (Chaufan et al. 2014; de Castilhos Ghisi and Cestari 2013; Richard et al. 2005).

There is a large body of evidence showing that oxidative stress may influence the onset of diseases such as diabetes, depression, anxiety, neurodegenerative diseases, and disturbances associated to environmental contaminant exposure (Cacabelos 2017; Cheng Li et al. 2017). For these reasons, many studies have been developed in attempt to find therapeutic and/or dietary alternatives to counteract the oxidative conditions, since antioxidants present in dietary sources (polyphenols, vitamins C and E) may help to maintain an appropriate antioxidant status in the body (Lu et al. 2010).

Uncaria tomentosa (Willd.) DC (UT) popularly known as cat's claw is a giant vine of the *Rubiaceae* family that grows in the Amazon rainforest in South America. Its use is extensive among several Peruvian tribes for the treatment of many diseases, such as arthritis and other inflammatory disorders (Reinhard 1999). Besides, there is scientific evidence regarding its antiinflammatory, immunostimulatory, and anticancer activities in human cells (Bors et al. 2012; Santos Araujo et al. 2012), some of them related to the ability of *U. tomentosa* in modulating the oxidative stress (Dreifuss et al. 2013). In fact, the active ingredients present in this plant species are the polyphenols (flavonoids, proanthocyanidins, and tannins), sterols, and alkaloids (Aquino et al. 1990; Patidar et al. 2014; Wagner et al. 1985), some of which are considered important non-enzymatic antioxidants (Rice-Evans et al. 1996; Shahidi and Wanasundara 1992). However, little is known about the protective effects of this plant species on the environmental contaminant toxicity, including pesticides, and the limited information are obtained from in vitro studies so far (Bors et al. 2012; Bukowska et al. 2012; Rebai et al. 2017).In this context, zebrafish (*Danio rerio*), a small freshwater teleost, has also been used to study the toxicological effect of environmental pollutants (Zhang et al. 2017; Bambino and Chu 2017), among them the glyphosate-based pesticides (Roy et al. 2016). This species has a rapid absorption of chemicals added directly to the tank water, which can reach a variety of tissues including the brain (Linney et al. 2004). Owing to the similarities of zebrafish genes and proteins with their mammalian counterparts, it is a suitable model not only for ecotoxicological researches but also for translational studies related to understanding of human pathologies and to environmental contaminant toxicity (Froehlicher et al. 2009; Hill et al. 2005). Additionally, this fish has become an attractive model for studies engaged in evaluating the activity of natural and synthetic compounds and their potential for therapies of different pathological conditions (Ekambaram et al. 2017; Lin et al. 2014; Tabassum et al. 2015), including those associated to environmental toxicant exposure (Hernandez and Allende 2008).

Taking into consideration that the oxidative stress is involved in the toxicity of glyphosate-based herbicides in different non-target organisms, this study investigated the protective effects of *U. tomentosa* on the oxidative stress and genotoxicity induced by a model of acute exposure to glyphosate-Roundup® in zebrafish.

Material and methods

Chemicals, apparatus, and general procedures

All chemical were of analytical grade. Acetonitrile, acetic acid, gallic acid, chlorogenic acid, caffeic acid, ellagic acid, catechin, and epicatechin were purchased from Merck (Darmstadt, Germany). Quercitrin, quercetin, luteolin, apigenin, rutin, and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector, and LC solution 1.22 SP1 software. Trizma base, bovine serum albumin, Folin & Ciocalteu's phenol reagent, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and Epinefrine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Roundup Original® (480 g/L isopropylamine salt) was obtained from commercial supplier. All other reagents used were from analytical grade.

Extract preparation

Dried vine bark *of U. tomentosa* (UT) was obtained commercially (Quimer-Ervas e especiarias, São Paulo, Brazil). For the extraction of compounds, 100 g of stalk UT was macerated and immersed in solutions containing 70:30 ethanol:water ratio (*v*/*v*). The mixtures were kept at 25 °C and atmospheric pressure for 72 h protected from light. After this period, the hydroalcoholic extract was filtered and concentrated under vacuum, frozen, and lyophilized.

HPLC-DAD

UT hydroalcoholic extract was separated at a concentration of 15 mg/mL using a reversed-phase Phenomenex C18 analytical column (4.6 × 250 mm, 5-μm particle size). The mobile phase, consisting of acetic acid (1%) in Milli-Q water deionized water (A) and acetonitrile (B), was pumped at 0.8 mL/min into the HPLC system and with injection volume of 40 μL with the following gradient elution program: 0–2 min, 1–5% B; 2–10 min, 5–20% B; 10–32 min, 20–45% B; 32–45 min, 70% B; and 45–70 min, 100% B. Subsequently, the B content was decreased to the initial conditions, and for 10 min, the column was re-equilibrated (total run time 80 min) (Boligon et al. 2015). The extract and mobile phase were filtered through 0.45-μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.050–0.300 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid; 280 nm for catechin and epicatechin; 327 for caffeic, chlorogenic, and ellagic acids; and 356 for quercetin, quercitrin, kaempferol, apigenin, and luteolin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 600 nm). Calibration curve for gallic acid was $Y = 13{,}057x + 1195.4$ ($r = 0.9998$), caffeic acid: $Y = 12{,}493x + 1269.2$ ($r = 0.9997$), catechin: $Y = 11{,}957x + 1306.8$ ($r = 0.9999$), epicatechin: $Y = 11{,}874x + 1327.5$ ($r = 0.9998$), chlorogenic acid: $Y = 12{,}743x + 1257.9$ ($r = 0.9995$), ellagic acid: $Y = 11{,}834x + 1263.2$ ($r = 0.9999$), rutin: $Y = 13{,}581x + 1176.3$ ($r = 0.9996$), quercitrin: $Y = 13{,}094x + 1308.7$ ($r = 0.9996$), luteolin: $Y = 12{,}456x + 1257.1$ ($r = 0.9998$), apigenin: $Y = 12{,}068x + 1185.6$ ($r = 0.9999$), quercetin: $Y = 11{,}387x + 1307.9$ ($r = 0.9997$), and kaempferol: $Y = 11{,}873x + 1265.4$ ($r = 0.9996$).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses (S) and the slope (σ) using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively (Sousa et al. 2015).

Animals

A total of 367 adult (4–6 months old) zebrafish (*Danio rerio*) of both sexes (50:50) were obtained from commercial supplier (Delphis Aquários) and acclimated for at least 2 weeks in 40-L tanks at a maximum density of four fish per liter. All tanks were maintained under mechanical, biological, and chemistry filtration. The temperature of the water was held at 26 ± 2 °C, whereas the pH and conductivity were set at 7.2–7.6 and 1450–1650 μS/cm, respectively. Fish were kept on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am) provided by ceiling-mounted fluorescent light tubes and fed thrice daily (Alcon BASIC®, Alcon, Brazil). The use of animals was according to the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Ethical Committee of Unochapecó (CEUA, 005/2014).

Fish exposure

Evaluation of acute toxicity of *U. tomentosa* extract

Following a 2-week adaptation period, the fish were submitted to a protocol according to OECD 203 (1992). Zebrafish were transferred to tanks in groups of seven animals at a density like that used for raising the fish in their home tanks. Acute static toxicity test was conducted with four groups as follows: control (no treatment), UT 40 mg/L, UT 50 mg/L, UT 60 mg/L, UT 70 mg/L, and UT 80 mg/L. For the treatment, a stock solution of UT hydroacoholic extract (10 mg/mL) was used. In order to maintain water quality, fish were not fed on the day before and during the experimental period. All groups were exposed for 96 h and temperature, pH, oxygenation, and conductivity of the water were checked daily. The number of dead fish was recorded every 24 h, and they were removed immediately to avoid contamination of the exposure solutions.

Exposure to glyphosate-Roundup and *U. tomentosa*

Zebrafish were transferred to tanks in groups of 20 animals at a density like that used for raising the fish in their home tanks. The groups were divided as follows: control (no treatment), GR (exposed to glyphosate-Roundup® at a concentration of 5.0 mg/L), UT (exposed to *U. tomentosa* extract at a concentration of 1.0 mg/mL), and GR + UT (simultaneously exposed to glyphosate-Roundup® and *U. tomentosa* in the concentrations previously mentioned, respectively). The concentration of glyphosate-Roundup® chosen was based in previous studies that demonstrated behavioral changes as well as in AChE activity (unpublished data), while UT concentration was based

on mortality rate and biochemical assays (data not shown). The fish were not fed on the day before or during the experimental period as well as the aquarium water was not changed during the 96 h. The exposure time of the groups was 96 h, and during each time conditions of temperature, pH, oxygenation, and conductivity were checked.

Oxidative stress analyses

Zebrafish were anesthetized in a tricaine-buffered solution (250 mg/L; pH 7.0) and euthanized by decapitation. Brain and liver were dissected out and a pool of ten zebrafish samples was used to prepare an independent homogenate fraction for each tissue. Samples were homogenized on ice into 1 mL of phosphate-buffered saline (PBS) solution, pH 7.4, containing 137 mM NaCl, 10.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4. The homogenate was further centrifuged at 800*g* for 5 min. The resultant pellets were discarded and the supernatants collected for the biochemical experiments described herein, which were performed similarly to the protocols previously reported for zebrafish (Dal Santo et al. 2014; Rosemberg et al. 2010).

Total reduced thiol content and non-protein sulfhydryl groups

To quantify the total reduced thiol content, 200 μL of the samples was incubated in the presence of 0.2 mM EDTA and 100 mM boric acid buffer, pH 8.5. DTNB (0.01 M dissolved in ethanol) was added and the intense yellow color developed was measured at 412 nm after 1 h (Ellman et al. 1961). In order to quantify the non-protein sulfhydryl groups, the protocol was similar to that previously described for assessing the total reduced thiol content, except that samples were previously centrifuged at 10.000*g* for 10 min in the presence of 15% trichloroacetic acid (TCA) and supernatants were used for the biochemical assay. The results were calculated using the molar extinction coefficient of DTNB and expressed as μmol SH. mg protein^{-1}.

Malondialdehyde

Lipid peroxidation was estimated by the measurement of malondialdehyde levels in brain and liver homogenates, which was determined by a high-performance liquid chromatographic system with visible detection (HPLC-VIS) with a detector set at 532-nm wavelength as described previously (Grotto et al. 2007). This method quantifies malondialdehyde (MDA) levels after alkaline hydrolysis and extraction with *n*-butanol.

Superoxide dismutase, catalase, and glutathione peroxidase activities

Superoxide dismutase (SOD) activity was quantified according to Misra and Fridovich (1972). Five microliters of epinephrine (60 mM) was added to a medium containing glycine buffer (50 mM, pH 10.2) and 25–75 μg of protein in a final volume of 200 μL. The inhibition of epinephrine autoxidation to adrenochrome at alkaline pH was spectrophotometrically determined at 480 nm. Catalase (CAT) activity was determined by adding hydrogen peroxide (H_2O_2, 8.8 M) in a medium containing 30 μg protein and 25 mM potassium phosphate buffer, pH 7.0 (final volume of 200 μL). The rate of decrease in H_2O_2 absorbance was monitored at 240 nm. All results were calculated and expressed as U of enzyme per mg protein (Aebi 1984).

Brain and liver glutathione peroxidase activity was determined according to Paglia and Valentine (Paglia and Valentine 1967) with minor modifications (Adeyemi et al. 2015). In short, 30 μg of protein was mixed to master mixture (final volume 212 μL) containing 0.15 mM NADPH, 1 mM GSH, 0.1 U/mL GR, and 100 mM azide, in potassium phosphate buffer, pH 7.0. Then, 20 μL of hydrogen peroxide (final concentration 0.4 mM) was added and the decrease of NADPH absorbance per minute was read at 340 nm using a Spectramax microplate reader (Molecular Devices). GPx activity was expressed as nmol NADPH/min/mg protein.

Genotoxicity assays

The genotoxic potential of glyphosate-Roundup as well as the ability of UT extract to prevent this effect was evaluated through the microscopic analysis for the presence of micronucleus, nuclear buds, and nucleoplasmic bridges in liver and brain cells. After 96 h of exposure, fish were anesthetized in a tricaine-buffered solution (250 mg/L; pH 7.0) and euthanized by decapitation and brain and liver dissected. The tissues were placed into vials containing PBS, and the cells were suspended with the aid of a pipette. Hereafter, samples (50 μL) of liver and brain cell suspension (in PBS) were added into tubes containing 50% KCl (50 μL) and fixed in methanol and acetic acid (3/1, *v*/*v*, 125 μL). Then, three to four drops of this mixture were smeared on slides and air-dried. Subsequently, slides were stained with 50% Giemsa for 5 min, rinsed in PBS, and dried at room temperature before microscopic examination. In order to determine nuclear abnormalities, for each fish ($n = 5$ for group), 1000 cells were examined under 1000-fold magnification in a light microscope. Micronuclei were considered as small inclusions of the nuclear material on cell cytoplasm with round or oval shape, coloration similar to that of the main nucleus, and size ranging from 1/3 to 1/20 of the main nucleus (Al-Sabti and Metcalfe 1995; Çavas et al. 2005). Nuclei containing

euchromatin and having a small evagination of the nuclear membrane were considered as nuclear bud while bridge-like formation between two cells was identified as nuclear bridge (Fenech et al. 2011; Anbumani and Mohankumar 2012).

Protein quantification

Protein was determined according to the method previously described by Lowry et al. (1951) using bovine serum albumin as standard (Lowry et al. 1951).

Statistics

Date were expressed as means ± standard error of mean (SEM) of three independent experiments performed in quadruplicate. Results were analyzed by one-way ANOVA. Comparison among means was performed using Tukey's test as post hoc, considering $p < 0.05$ as statistical significance.

Results

Phytochemical analysis of *U. tomentosa* extract

The HPLC profile of *U. tomentosa* extract is presented in Fig. 1. The analysis demonstrated the presence of various phenolic compounds such as gallic acid (retention time t_R = 9.95 min; peak 1), catechin (t_R = 14.87 min; peak 2), chlorogenic acid (t_R = 19.36 min; peak 3), caffeic acid (t_R = 23.71 min; peak 4), epicatechin (t_R = 28.04 min; peak 5), ellagic acid (t_R = 31.07 min; peak 6), rutin (t_R = 35.01 min; peak 7), quercitrin (t_R = 43.96 min; peak 8), quercetin (t_R = 46.57; peak 9), kaempferol (t_R = 57.11 min; peak 10), luteolin (t_R = 64.38 min; peak 11), and apigenin (t_R = 73.92 min; peak 12), especially caffeic acid present in larger quantities (Table 1).

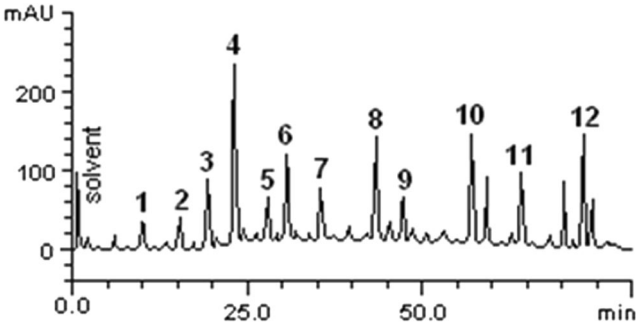

Fig. 1 Representative high-performance liquid chromatography phenol and flavonoid profile *Uncaria tomentosa* extract (327 nm). Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), epicatechin (peak 5), ellagic acid (peak 6), rutin (peak 7), quercitrin (peak 8), quercetin (peak 9), kaempferol (peak 10), luteolin (peak 11), and apigenin (peak 12)

Table 1 Phenol and flavonoid composition of the *Uncaria tomentosa* extract

Compounds	Extract (mg/g)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	3.27 a (± 0.02)	0.016	0.053
Catechin	3.41 a (± 0.04)	0.009	0.029
Chlorogenic acid	6.57 b (± 0.01)	0.023	0.072
Caffeic acid	14.95 c (± 0.01)	0.011	0.034
Epicatechin	4.08 d (± 0.03)	0.014	0.046
Ellagic acid	8.36 e (± 0.02)	0.027	0.089
Rutin	6.49 b (± 0.01)	0.024	0.076
Quercitrin	9.41 f (± 0.02)	0.008	0.026
Quercetin	4.13 d (± 0.01)	0.013	0.043
Kaempferol	9.57 f (± 0.03)	0.018	0.059
Luteolin	6.62 b (± 0.03)	0.029	0.097
Apigenin	9.59 f (± 0.01)	0.021	0.069

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters, in the lines, differ by Tukey test at $p < 0.05$

LOD limit of detection, *LOQ* limit of quantification

Acute toxicity of *U. tomentosa* in zebrafish

Table 2 shows the cumulative mortality rate for zebrafish exposed to different concentrations of UT extract. During the 96-h exposure period, no fish died in the control groups. Also, no zebrafish died when submitted to 40 mg/L of extract while the mortality rate for the groups exposed to 60, 70, and 80 mg/L was 100% and for the group exposed to 50 mg/L was 14.28%. Although the experimental period was 96 h, at UT, 70- and 80-mg/L group mortality occurred during the first 48 h and at 50- and 60-mg/L group mortality occurred during 96 h.

The water parameters related to toxicity test are described in Table 3. No significant changes were observed in physical and chemical variables of the water during treatment period that could have interfered in the obtained results.

Table 2 Mortality rate of the *Uncaria tomentosa* extract in zebrafish (*Danio rerio*) after 24-, 48-, 72-, and 96-h exposure

Concentration (mg/L)	Mortality rate					
	24 h	48 h	72 h	96 h	Total	Percent
40	0	0	0	0	0	0
50	0	0	1	0	1	14.28
60	1	0	3	3	7	100
70	6	1	0	0	7	100
80	3	4	0	0	7	100

Table 3 Water quality characteristics during the UT toxicity experiments

Parameters	Concentrations of *Uncaria tomentosa* extract (mg/L)					
	40	50	60	70	80	Range
pH	7.39	7.45	7.43	7.54	7.35	7.35–7.54
O_2 (mL/L)	8.30	7.80	7.35	7.68	8.31	7.35–8.31
Temperature (°C)	27	26	27	26	27	26–27
Conductivity (μS)	154.78	139.54	171.12	146.99	148.25	139.54–171.12

Biochemical parameters

In order to evaluate the antioxidant potential of UT extract zebrafish were exposed to glyphosate-Roundup® in the presence and in the absence of the extract and oxidative stress parameters, the water quality characteristics as well as genotoxicity in each treatment were measured.

Water chemistry

During exposure to glyphosate-Roundup® and/or UT extract, water quality characteristics in each experimental group were measured daily and maintained in accordance with Lawrence and Mason (2012). Table 4 shows that pH, dissolved oxygen, temperature, and conductivity of the water were constant throughout the experiments and the values are in accordance with recommended for the species (Lawrence and Mason 2012).

Total reduced thiol content and non-protein sulfhydryl groups

Figure 2 presents the effect of glyphosate-Roundup® and/or UT extract on the levels of total and non-protein thiols in brain and liver of zebrafish. The total thiol levels in brain were substantially decreased after exposure to GR (Fig. 2a), and this effect was effectively prevented by UT extract in the co-exposure (group GR + UT) (Fig. 2a; F (3, 12) = 3.349; p = 0.0556). However, neither glyphosate-Roundup® nor the extract altered non-protein thiol levels in the brain (F (3, 11) = 0.4565; p = 0.7181) and liver (F (3, 12) = 1.036; p = 0.4117) of zebrafish (Fig. 2c, d).

MDA levels

The MDA content in brain and liver of zebrafish was used to evaluate the level of lipid peroxidation, and the results are illustrated in Fig. 3. In the brain (Fig. 3a), there was a significant increase in MDA levels in the GR group that was prevented by the UT extract (GR + UT) group (F (3, 26) = 9.901; p = 0.0002). MDA levels in liver significantly increased in GR group but remained similar to MDA levels of control in the GR + UT group (F (3, 10) = 5.677; p = 0.0156), suggesting that the UT extract can inhibit the lipid peroxidation in the presence of glyphosate-Roundup® in a homogenate of zebrafish liver (Fig. 3b).

SOD, CAT, and GPx activities

The activities of SOD, CAT, and GPx are shown in Fig. 4. The results demonstrated that SOD activity only changed in the liver of animals exposed to GR + UT that presented an increase in activity of this enzyme (Fig. 4a, b; F (3, 10) = 3.440; p = 0.0599). glyphosate-Roundup® did not change brain CAT activity (Fig. 4c), but UT extract decreased brain CAT activity administered alone (UT group) and in co-exposure with glyphosate-Roundup® (GR + UT group) (F (3, 12) = 35.80; p = 0.0001). On the other hand, all treatments caused an increase of liver CAT activity (Fig. 4d). Concerning SOD/CAT ratio, a significant increase was found in the brain of the group GR + UT (Fig. 4e; F (3, 12) = 7.275; p = 0.0049), but no effect was observed in liver. GPx activity only decreased in the liver of fish exposed to GR, and this effect was reversed by the UT treatment (Fig. 4 g, h; F (3, 15) = 9.977; p = 0.0007).

Table 4 Water quality parameters in the experimental groups during 96-h exposure

Parameters	Experimental groups				
	Control	GR	UT	GR + UT	Values (min–max)
pH	7.83	7.83	7.69	7.93	7.69–7.93
O_2 (mL/L)	9.91	7.11	6.70	7.53	6.70–9.91
Temperature (°C)	26	27	27	26.5	26–27
Conductivity (μS)	139.94	148.32	149.49	146.22	139.94–149.9

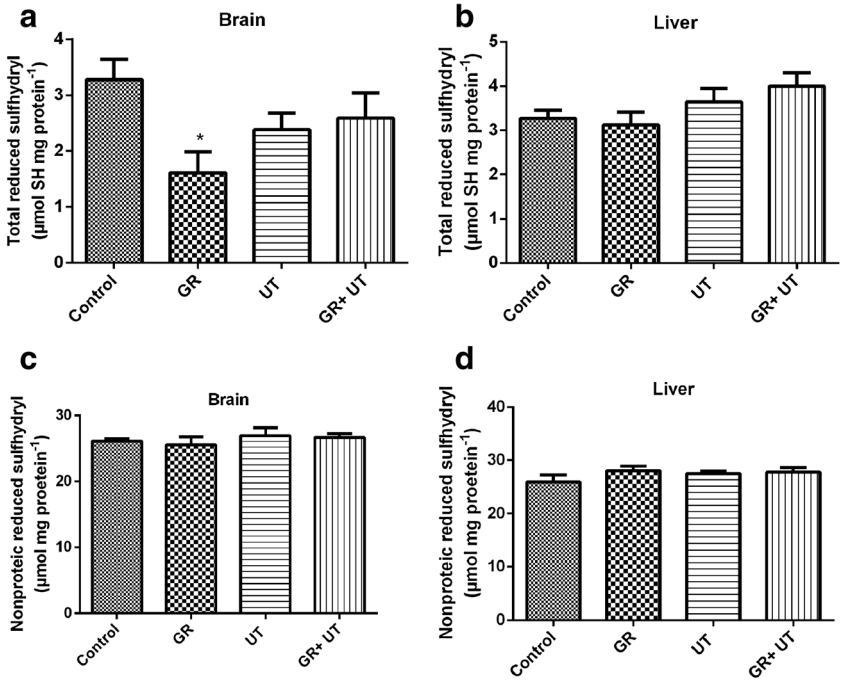

Fig. 2 Effect of glyphosate-Roundup® (GR) and/or *Uncaria tomentosa* (UT) on the levels of total thiols in the brain (**a**) and liver (**b**) and nonprotein thiols in brain (**c**) and liver (**d**) in zebrafish. *C* control group, *GR* group submitted to glyphosate-Roundup® (5.0 mg/L), *UT* group treated with *Uncaria tomentosa* extract (1.0 mg/mL), *GR* + *UT* group treated with glyphosate-Roundup® + *Uncaria tomentosa* extract. Data represent mean ± standard error of the mean. $n = 4$. One-way ANOVA followed by Tukey's post hoc test. $^{*}p < 0.05$ compared with control group

Micronucleus test

Table 5 presents the frequency of micronucleus (MN), nuclear buds, and nucleoplasmic bridges in brain and liver cells of zebrafish exposed to glyphosate-Roundup®. In brain (F (3, 14) = 12.67; $p = 0.0003$) and liver (F (3, 14) = 6.590; $p = 0.0053$), MN frequency was significantly increased in the GR group after 96 h of the treatment that was partially prevented by UT extract, as observed in the GR + UT group. In relation to the nucleoplasmic bridges, in brain (F (3, 11) = 7.051; $p = 0.0065$) but not in liver (F (3, 15) = 0.1009; $p = 0.9583$), a significant increase in the frequency that was partially prevented by the UT extract was observed. However, it was not observed significant differences in the frequency of buds in both brain (F (3, 14) = 2.333; $p = 0.1183$) and liver cells (F (3, 16) = 0.7428; $p = 0.5421$). Figure 5 representatively shows the types of nuclear abnormalities observed in fish tissues.

Discussion

Oxidative stress is an important factor involved in the toxic effects of numerous environmental contaminants. Although some toxicological studies consider glyphosate-based herbicides relatively nontoxic (WHO 1994), there are several data showing that its active ingredient is involved in the etiology of numerous diseases (Samsel and Seneff 2013; Samsel and Seneff 2015) and presents oxidant potential (Cattani et al. 2014; Manas et al. 2009).

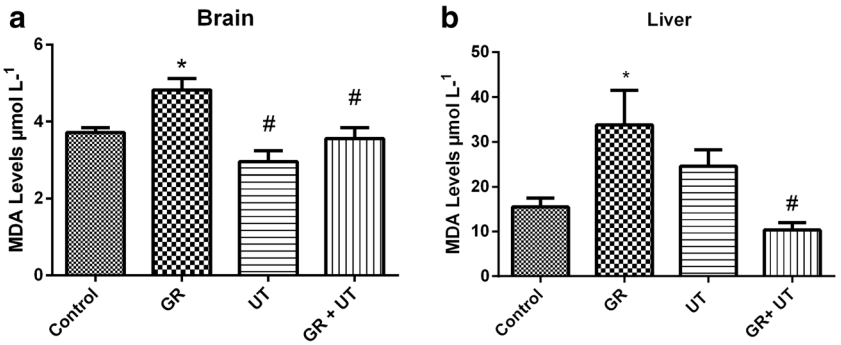

Fig. 3 Effect of glyphosate-Roundup® (G) and *Uncaria tomentosa* (UT) on lipid peroxidation measured as the formation of MDA (malondialdehyde) in brain (**a**) and liver (**b**). *C* control group, *GR* group exposed to glyphosate-Roundup® (5.0 mg/L), *UT* group treated with *Uncaria tomentosa* extract (1.0 mg/mL), *GR* + *UT* group treated with glyphosate-Roundup® + *Uncaria tomentosa* extract. Data represent mean ± standard error of mean. $n = 4$. One-way ANOVA followed by Tukey's post hoc test. $^{*}p < 0.05$ compared with control group. $^{\#}p < 0.05$ compared with GR group

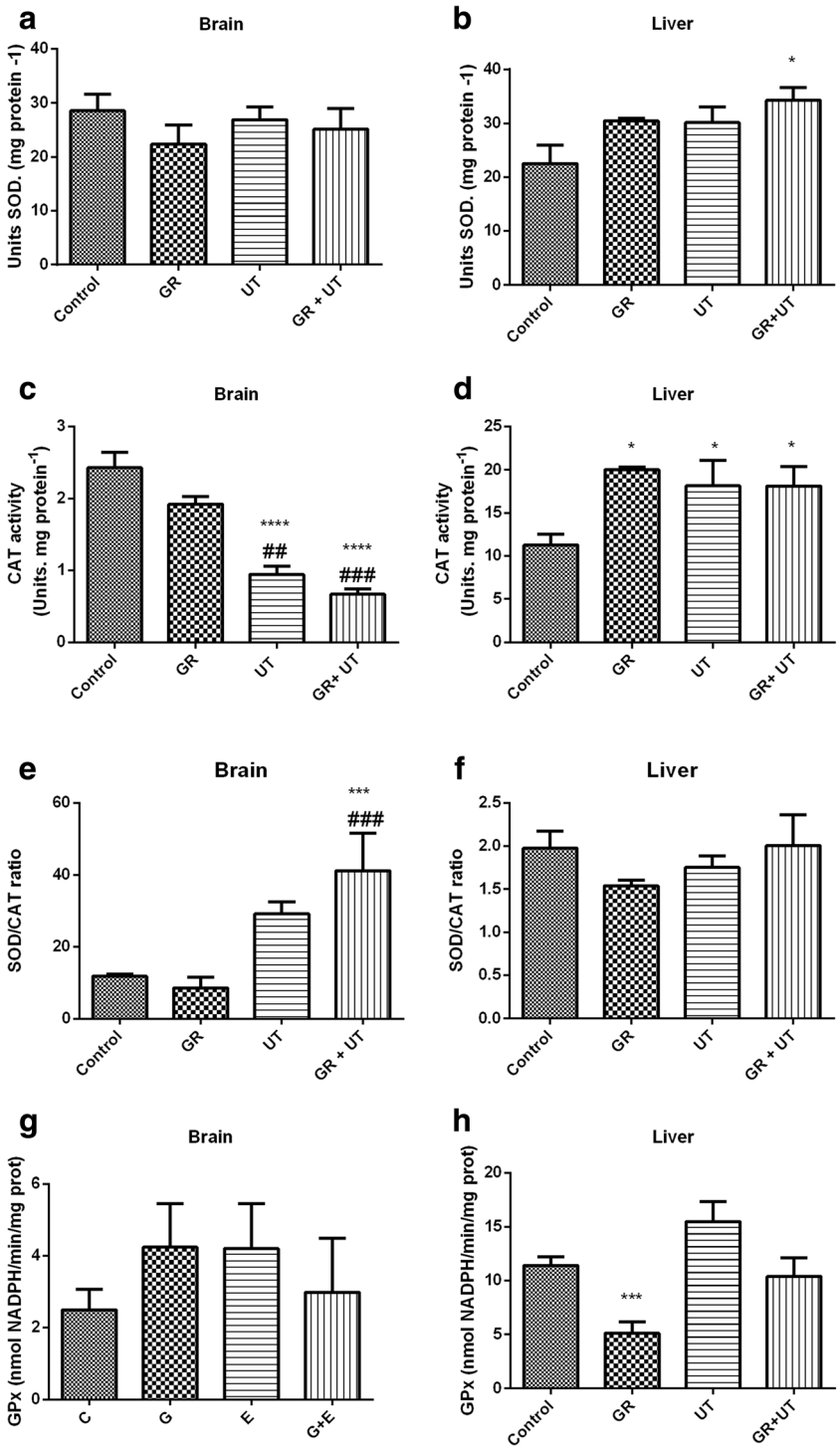

Fig. 4 Effect of glyphosate-Roundup® (GR) and *Uncaria tomentosa* (UT) on SOD (**a**, **b**) and CAT activity (**c** , **d**), SOD/CAT ratio (**e**, **f**), and GPx activity (**g**, **h**) in brain and liver, respectively. *C* control group, *GR* group submitted to glyphosate-Roundup® exposure (5.0 mg/L), *UT* group treated with *Uncaria tomentosa* extract (1.0 mg/mL), *GR + UT* group treated with glyphosate-Roundup® + *Uncaria tomentosa* extract. Data represent mean ± standard error of mean. $n = 4$. One-way ANOVA followed by Tukey's post hoc test. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with control group. $^{\#\#}p < 0.001$, $^{\#\#\#}p < 0.0001$ compared with GR group

Additionally, considering that the oxidative stress may be involved in the tissue damage induced by herbicides containing glyphosate, the use of medicinal plants with antioxidant properties could prevent or remedy the oxidative events arising from the herbicide exposure. The present study was designed to investigate the protective effect of UT extract on oxidative stress caused by acute exposure to glyphosate-Roundup® (GR) in zebrafish.

Our results showed for the first time that UT extract prevents oxidative damage induced by GR in zebrafish liver and brain. The GR concentration tested on the experiments (5.0 mg/L of isopropylamine salt equivalent to 3.7 mg/L of glyphosate base) can be considered environmentally representative considering that at application rates, a water body can have a maximum concentration of 3.7 mg glyphosate/L (Giesy et al. 2000).

Table 5 Frequency of micronucleus, nucleoplasmic bridges, and nuclear buds in liver and brain cells from zebrafish exposed to glyphosate-Roundup®

			Frequency (‰)		
Brain	Group	Number	MN	Bridges	Buds
	C	5	1.60 ± 0.93	0.0 ± 0.0	0.20 ± 0.20
	GR	5	39.67 ± 11.55***	7.33 ± 2.93**	0.0 ± 0.0
	UT	5	3.60 ± 0.60	1.00 ± 0.32	0.60 ± 0.2449
	GR + UT	7	17.20 ± 4.44$^{\#}$	1.667 ± 0.33$^{\#}$	0.6667 ± 0.33
Liver	C	5	0.0 ± 0.0	0.6 ± 0.6	0.0 ± 0.0
	GR	5	16.67 ± 4.70***	0.67 ± 0.66	0.67 ± 0.67
	UT	5	2.40 ± 1.47	1.00 ± 0.63	1.00 ± 1.00
	GR + UT	7	8.80 ± 3.65	0.83 ± 0.40	0.14 ± 0.14

Averages for the 1000 count cells per group. Control (C), glyphosate-Roundup 5.0 mg/L (GR), *Uncaria tomentosa* 1.0 mg/L (UT), glyphosate + *U. tomentosa* extract (GR + UT), respectively. Data represent mean ± standard error of mean. $n = 5$ (C, GR, UT), $n = 7$ (GR + UT). One-way ANOVA followed by Tukey's post hoc test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with control group. $^{\#}p < 0.05$ compared to GR group

Reduced total thiol content in brain and increased lipid peroxidation in both brain and hepatic tissues indicate the occurrence of alterations in the oxidative status of zebrafish exposed to GR. Oxidative effects of glyphosate-based formulations may occur due to their ability in inducing the production of ROS or directly interacting with lipid membranes. In fact, the direct interaction of organophosphorus pesticides with the cytoplasmic membrane is referred as one of the main mechanisms of their toxicity in different organisms (Hazarika et al. 2003; Kavitha and Rao 2007). The concomitant changes in two indexes of oxidative damage in the brain suggest that this tissue was more sensitive to oxidative stress induced by GR than liver. In fact, brain naturally is an organ very susceptible to oxidative stress because of its elevated oxygen utilization, its high content of polyunsatured fatty acids, and the presence of transition metals such as Fe and Cu (Valko et al. 2007). Therefore, these factors may predispose brain to suffer most prominent changes than other organs when exposed to additional oxidative insult as that involves toxicant exposure such as GR.

GR induced oxidative damage in zebrafish brain without changes in the antioxidant enzyme activity. Hence, we can hypothesize that the protection provided by UT extract against the reduction of brain total thiol levels and against the increase of brain and liver lipid peroxidation may be attributed at least in part to the ability of the bioactive compounds of the extract in directly scavenging ROS. In fact, caffeic acid, the major phenolic compound identified in UT extract, is a potent antioxidant with superoxide anion radical scavenging activity and metal-chelating properties, besides preventing the lipid peroxidation via inhibition of Fenton's reaction (Gulcin 2006). Additionally, flavonoids as kaempferol, quercitrin, and apigenin, which were found in significant amounts, may also have contributed for the antioxidant activity of the UT extract, since they exhibit important antioxidant properties and protection against lipid peroxidation as demonstrated elsewhere (Cazarolli et al. 2008; Hong et al. 2013; Singh et al. 2004).

Additionally, the ability of UT extract in preventing the lipid peroxidation in zebrafish liver may also indicate a possible indirect antioxidant effect of this extract by recovering and/or stimulating the activity of ROS-scavenging enzymes, as observed when UT extract was administered concomitantly with GR for GPx as well as for SOD and CAT activities, respectively. SOD catalyzes the dismutation of superoxide radical anion ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2), which in turn is neutralized by both CAT and GPx activities (Nordberg and Arner 2001; van der Oost et al. 2003). The combined action of these enzymes helps to avoid the hydroxyl radical ($^{\bullet}OH$) formation and therefore contributing to prevent the perpetuation of the chain reaction of polyunsaturated fatty

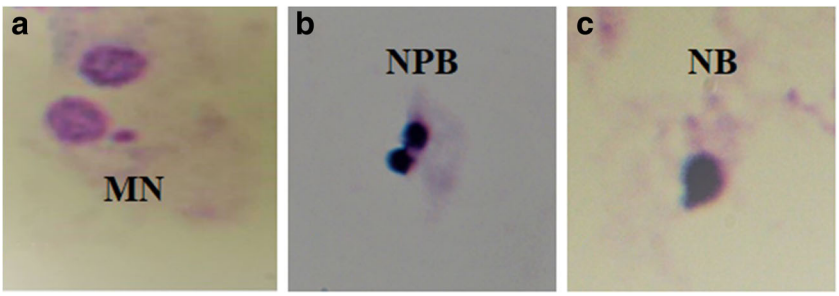

Fig. 5 Photos of a micronucleus and nuclear abnormalities found in cells of zebrafish exposed to glyphosate-Roundup. **a** Micronuclei. **b** Nucleoplasmic bridge. **c** Nuclear bud

acid oxidation and the initiation of the lipid peroxidation, since both events involve the participation of hydroxyl radical (Nordberg and Arner 2001). Therefore, the restoration of GPx activity along with increased SOD and CAT activity in UT + GR group possibly contributed to neutralize ROS and consequently to prevent the lipid peroxidation in zebrafish liver.

The decrease of brain CAT activity by the UT extract per se observed in UT and UT + GR groups may also be linked to some of its bioactive compounds such as caffeic acid, since was previously demonstrated that this compound reduced the CAT activity in glioma cells (C6) (Balkhi et al. 2013). This decrease of CAT activity appears to have contributed to the changes in SOD/CAT ratio in zebrafish brain, which tended to increase in UT group, and significantly increased in UT + GR group. Although increased SOD/CAT ratio suggests a reduction of brain antioxidant defenses against H_2O_2, the oxidative stress was unlikely to occur in these groups since no alteration in the oxidative damage parameters (thiols and MDA levels) was observed in comparison to control group. It should be stressed, however, that the reducing effect of CAT activity of glyphosate is not always likely to occur in cells treated with UT, since CAT in zebrafish liver increased in the presence of UT extract, probably as a result of action of bioactive compounds of this extract (Li et al. 2015).

Besides protection provided by UT against oxidative stress, our results also showed that the increase of the number of MN induced by GR exposure in brain could also be prevented by UT extract but not in liver. The micronucleus test and nuclear abnormalities are biomarkers of environmental genotoxicity broadly used, which detect chromosomal fragments or acentric chromosomes that are not incorporated into the main nucleus after mitosis (Torres-Bugarín et al. 2014, Udroiu 2006; Ghisi 2012). Studies have shown that micronucleus frequencies in fish from polluted regions are higher than those in fish from uncontaminated areas (Arcand-Hoy and Metcalfe 2000). Among these pollutants are the pesticides which comprise a large group of mutagenic chemicals (Ghisi 2012). Significant increase in the micronucleated cells was demonstrated in *O. niloticus* fish obtained from a lake containing organochlorines and organophosphorus residues (Nahas et al. 2017). Qin et al. (2017) showed a frequency of micronucleus significantly higher in groups of *Misgurnus anguillicaudatus* treated with glyphosate compared with the control groups. Cavalcante et al. (2008) also verified through the MN test that Roundup was able to induce genotoxic effects on *P. lineatus*, and de Moura et al. (2017) showed a significant increase in MN on peripheral blood erythrocytes of jundiara (*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*) exposed to Roundup for 96 h. DNA damage may be a consequence of the biotransformation of xenobiotics in fish, which results in the production of reactive intermediate species that target the DNA structure (Chaufan et al. 2014; Ferreira et al. 2010; Guilherme et al. 2012; Sinhorin et al. 2014). Although the mechanisms involved in the protection of UT extract against the MN formation is unknown, the antioxidant properties exhibited by UT extract may have contributed to prevent the genotoxic effects of GR (Nahas et al. 2017).

Conclusion

In summary, our results indicate that UT protects against oxidative damage induced by the glyphosate-Roundup® by providing antioxidant and antigenotoxic effects, which are probably related to the phenolic compounds identified in this extract. However, further studies are necessary to discriminate which compound of the extract could be responsible for the biological effect found, and to clarify the potential usefulness of UT extract in the protection against injuries caused by glyphosate-based formulations.

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