Acta Pharm. **62** (2012) 371–382
DOI: 10.2478/v10007-012-0026-x

Original research paper

In vitro antioxidant activity of stem bark of *Trichilia catigua* Adr. Juss

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Antioxidant activity of the ethanolic extract and fractions from the stem bark of *T. catigua* was investigated. IC_{50} (for DPPH scavenging) by *T. catigua* varied from 9.17 ± 0.63 to 76.42 ± 5.87 μg mL^{-1} and total phenolic content varied from 345.63 ± 41.08 to 601.27 ± 42.59 μg GAE g^{-1} of dry extract. Fe^{2+}-induced lipid peroxidation was significantly reduced by the ethanolic extract and fractions. Mitochondrial Ca^{2+}-induced dichlorofluorescein oxidation was significantly reduced by the ethanolic extract in a concentration-dependent manner. Ethanolic extract reduced mitochondrial $\Delta\psi_m$ only at high concentrations (40–100 μg mL^{-1}), which indicates that its toxicity does not overlap with its antioxidant effects. Results suggest involvement of antioxidant activities of *T. catigua* in its pharmacological properties.

Keywords: Trichilia catigua (*Meliaceae*), antioxidant, flavonoids, phenolics, reactive oxygen species, oxidative stress

Accepted June 11, 2012

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Many plants contain substantial amounts of antioxidants such as vitamins C and E, carotenoids, flavonoids and tannins that can scavenge free radicals from the human body (1). Since ancient times, a high percentage of the populations of many developed countries have been using medicinal plants in the treatment of different pathologies, including neurodegenerative diseases in which free radical assaults are implicated in their etiology.

Trichilia catigua, is a plant used in Brazil as an aphrodisiac and neurostimulant. It exhibits a variety of pharmacological properties, including antidepressive and anti-inflammatory ones, and its use has been reported to be safe with no known side effects or toxicity in healthy human volunteers (2). Phytochemical reports on *T. catigua* indicated that the plant contains omega-phenyl alkanes, omega-phenyl alkanoic acids, omega-phenyl--gamma-lactones, alkyl-gamma-lactones, alkenyl-gamma-lactones, fatty acids, β-sitosterol, stigmasterol, campesterol, epicatechin, cinchonains (Ia, Ib, IIa, IIb), catiguanins A and B, procyanidins B2 and C1, tannins and a mixture of flavalignans (3, 4).

It is of particular pharmacological significance that many pathological processes in which *T. catigua* exerts its beneficial action can be associated with overproduction of reactive oxygen species (ROS) which can impair energy metabolism *via* oxidative changes in key mitochondrial components (5).

Considering the fact that *T. catigua* has been widely employed empirically in folkloric medicine in the management of free radical related diseases, and that there is little information in the literature about the potential antioxidant properties of *T. catigua*, we investigated whether the ethanolic extract and fractions of different polarities extracted from the stem bark of *T. catigua* exhibited *in vitro* antioxidant activity using chemical and biological models.

EXPERIMENTAL

Chemicals

All chemicals used, including solvents, were of analytical grade. 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Folin Ciocalteu's phenol reagent, malonaldehyde bis-(dimethyl acetal) (MDA), thiobarbituric acid, sodium dodecyl sulfate, ascorbic acid, 2',7'-dichlorofluorescein diacetate (DCFH-DA), Tris-HCl, ethylene glycol tetraacetic acid (EGTA), quercetin, rutin, chlorogenic acid and gallic acid were purchased from Sigma Chemical Co. (USA). 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), ferrous sulfate, mannitol and sucrose were obtained from Vetec (Brazil).

Plant collection and separation of the different fractions

Extract of *Trichilia catigua* bark was obtained from Ely Martins (Ribeirão Preto, São Paulo, Brazil), in 2007, registered under the number CAT-i0922 (Farm. Resp.: Ely Ap. Ramos Martins). The stem bark powder of *T. catigua* (100 g) was macerated at room temperature with 70 % ethanol and extracted for a week. On the seventh day, the combined ethanolic extract was filtered and the solvent was fully evaporated under reduced pres-

sure to give a brown solid (11.61 g). This was divided into two parts and one part was suspended in water and partitioned successively with dichloromethane, ethyl acetate and *n*-butanol (3 × 50 mL for each solvent). Dichloromethane was added to one part of the extract (1 : 1, *V*/*V*) and the mixture was allowed to remain at room temperature for 15 min. The solution was decanted and the solvent was evaporated to obtain the dichloromethane fraction (CH_2Cl_2, 1.98 g). The other fractions (ethyl acetate, AcOEt) and butanolic fraction (*n*-BuOH) were processed as described for the dichloromethane fraction and the quantities obtained after evaporation were 1.05 g and 1.52 g, respectively.

In this procedure, the extract was suspended 3 times with each solvent (3 × 50 mL). The fractions and EtOH extract were then diluted in ethanol in order to prepare different concentrations (10, 40, 100 and 400 μg mL^{-1}). *T. catigua* is normally used as a tea; consequently, hot and cold water extracts from *T. catigua* were tested to compare their antioxidant capacities with the ethanolic extract and fractions.

Animals

Male Wistar rats, weighing 270–320 g and aged from 2.5 to 3.5 months, from our own breeding colony (Animal House-holding, UFSM, Brazil) were kept in cages with free access to food and water in a room with controlled temperature (22 ± 3 °C) and in 12 h light/dark cycle. The protocol of this study has been approved by the Brazilian Association for Laboratory animal Science (COBEA).

Antioxidant assays

The free radical scavenging activity of the *T. catigua* extract was measured with the stable radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) in terms of hydrogen-donating or radicals scavenging activity. A solution of DPPH (0.3 mmol L^{-1}) in ethanol was prepared, and 100 μL of this solution was added to 20 μL of each fraction and ethanolic extract at different concentrations (10, 40, 100 and 400 μg mL^{-1}). Ethanol and ascorbic acid, at the same concentrations used for fractions and ethanolic extract, were used as negative and positive controls, respectively. After 30 minutes, absorbances were measured at 518 nm in an ELISA plate reader (TP-Reader, Brazil).

Analysis of phenolics

For the determination of total phenolic content, samples of the extract/fraction (10–400 μg mL^{-1}) were added to a test tube and the volume was adjusted to 1.4 mL with distilled water. Then, 0.2 mL of 10 % Folin-Ciocalteu reagent (diluted 1 : 1 with water) and 0.4 mL of sodium carbonate solution (7.5 %) were added sequentially to the test tube. The tubes were then incubated for 40 min at 45 °C and the absorbance was measured at 725 nm in a spectrophotometer (SP-2000UV, Biospectro, Brazil). The standard curve was prepared using 0, 1, 2.5, 5, 10 and 15 μg mL^{-1} solutions of gallic acid (0.1 mg mL^{-1}). Total phenol value was calculated and expressed as the microgram gallic acid equivalent (μg GAE g^{-1}) of dry extract.

In vitro *Fe^{2+}-induced lipid peroxidation in the brain*

Rats were decapitated; whole brain was dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 10 mmol L^{-1} Tris–HCl, pH 7.4 (1/10, mass/volume). The homogenate was centrifuged for 10 min at 3600 × *g* to yield a pellet, which was discarded, and a low-speed supernatant (S1) was used for the thiobarbituric acid reactive substances (TBARS) assay.

Aliquots of the brain homogenate and the pro-oxidant agent (10 μmol L^{-1} $FeSO_4$) were incubated for 1 h at 37 °C in the presence or absence of the *T. catigua* extract (10–20 μg mL^{-1}). To the reaction mixture, 8.5 % sodium dodecyl sulfate (SDS), acetic acid/HCl (pH 3.4) and 0.6 % thiobarbituric acid (TBA) were subsequently added and the mixture was incubated at 100 °C for 1 h. Lipid peroxidation (LPO) was measured by TBARS formation as described by Puntel *et al*. (6). Color was read at 532 nm using an ELISA plate reader. Standard curve of malondialdehyde (MDA) was used to quantify TBARS production in brain homogenates.

Quantification of phenolics and flavonoids by HPLC-DAD

The phenolics and flavonoids in the extract were quantified by reverse phase chromatographic analysis by the method described by Laghari *et al*. (7), with slight modifications. Reverse phase chromatographic analysis was carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5-μm diameter particles. The mobile phase was water containing 2 % acetic acid (A) and methanol (B), and the composition gradient was: 5 % (B) for 2 min; 25 % (B) until 10 min; 40, 50, 60, 70 and 80 % (B) every 10 min. All samples and the mobile phase were filtered through a 0.45-μm membrane filter (Millipore, USA) 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 0.031–0.250 mg mL^{-1} quercetin and rutin, and 0.006–0.250 mg mL^{-1} for gallic and chlorogenic acids. Quantification was carried out by integration of the peaks using the external standard method, at 257 nm for gallic acid, 325 nm for chlorogenic acid and 365 for quercetin and rutin. The flow rate was 0.8 mL min^{-1} and the injection volume was 40 μL. Chromatographic peaks were confirmed by comparing their retention time and diode-array-UV spectra with those of the reference standards. All chromatography operations were carried out at ambient temperature and in triplicate.

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated as previously described by Puntel *et al*. (8), with some modifications. The livers were rapidly removed (within 1 min) and immersed in ice-cold "isolation buffer I" containing in mmol L^{-1}: 225 manitol, 75 sucrose, 1 K^+-EGTA and 10 K^+-HEPES, pH 7.2. The tissue was minced using surgical scissors and then extensively washed. The tissue was then homogenized in a power-driven, tight-fitting Potter-Elvehjem (Reviglass, Brazil) homogenizer with a teflon pestle. The resulting suspension was centrifuged for 7 min at 2,000 × *g* in a Hitachi CR 21E centrifuge (Japan). The supernatant was centrifuged again for 10 min at 12,000 × *g*. The pellet was resuspended in "isolation buffer II" containing in mmol L^{-1}: 225 manitol, 75 sucrose, 1 K^+-EGTA (ethyleneglycol tetraacetic acid) and 10 K^+-HEPES [4-(2-hydroxyethyl)-1-piperazine ethane-

sulfonic acid], pH 7.2, and recentrifuged at 12,000 × g for 10 min. The supernatant was decanted, and the final pellet was gently washed and resuspended in respiration buffer containing in mmol L^{-1}: 100 sucrose, 65 KCl, 10 K^+-HEPES and 0.05 EGTA, pH 7.2, to a protein concentration of 0.6 mg mL^{-1}.

Determination of reactive oxygen species (ROS)

ROS production in isolated mitochondria was measured using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence probe. Mitochondrial suspensions (0.25 mg mL^{-1}) in respiration buffer containing 100 mmol L^{-1} sucrose, 65 mmol L^{-1} KCl, 10 mmol L^{-1} K^+-HEPES and 50 μmol L^{-1} EGTA, pH 7.2, were incubated with 10, 40, 100 μg mL^{-1} of the ethanolic extract *T. catigua* in the presence or absence of $CaCl_2$ (80 μmol L^{-1}) (13). Then, 3.33 μmol L^{-1} of DCFH-DA was added to the solution. The formation of the oxidized fluorescent derivative 2',7'-dichlorofluorescain (DCF) was monitored using a spectrofluorimeter (Shimadzu RF-5301, Japan) with excitation and emission wavelengths of 488 and 525 nm, respectively, and with slit widths of 1.5 nm.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was estimated by fluorescence changes of safranine (5 μmol L^{-1}) recorded by a RF-5301 Shimadzu spectrofluorimeter operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 3 nm. Values of mitochondrial membrane potential ($\Delta\psi_m$) were expressed as the percent of control.

Protein estimation

Protein concentration was measured by the method of Lowry *et al*. (9), using bovine serum albumin (BSA) as a standard.

Statistical analysis

Results were expressed as mean ± SEM (standard error of mean). One-way or two-way ANOVA followed by Duncan's multiple range tests were utilized to evaluate the differences between the groups when appropriate. The data of cold and hot water extracts were compared using *t*-test. Pearson's correlation coefficient was calculated to determine the relationship between two variables.

RESULTS AND DISCUSSION

Various extracts from the stem barks of *T. catigua* scavenged DPPH radical in a concentration-dependent manner (Fig. 1a,b), which can be mediated by the different polyphenolic components found in these extracts. The total phenolic content of different crude extracts from *T. catigua* is given in Table I. The concentration varied from 345.63 μg GAE g^{-1} (in butanolic fraction) to 601.27 μg GAE g^{-1} (in ethyl acetate fraction) of plant

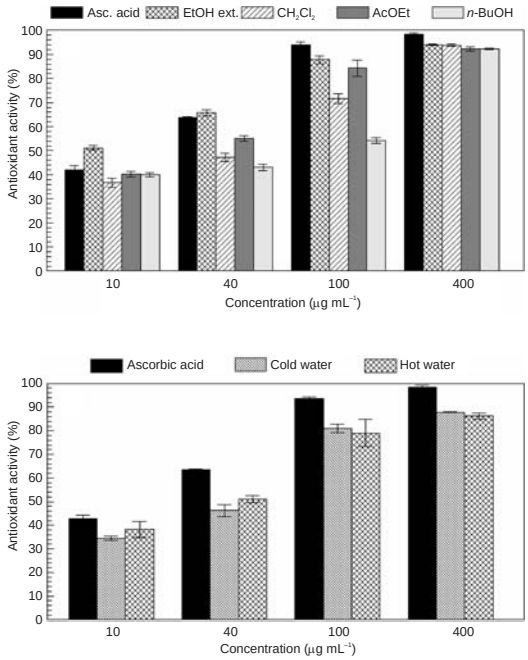

Fig. 1. Quenching of DPPH color by extracts from the stem barks of *T. catigua vs.* asorbic acid: a) ethanolic, ethyl acetate, dichloromethane and butanolic, b) aqueous (cold and hot water extracts). Mean ± SEM, n = 3–4 independent experiments.

extract. Surprisingly, we observed that the highest content of total phenol in ethyl acetate fraction did not correlate with the highest antioxidant activity evaluated by the DPPH assay. The effectiveness order of IC_{50} (the extract concentration required to inhibit 50 % of DPPH radical) for decolorizing DPPH was: EtOH extract (9.17 μg mL^{-1}) > AcOEt (30.28 μg mL^{-1}) > CH_2Cl_2 (42.42 μg mL^{-1}) > *n*-BuOH (76.35 μg mL^{-1}) (Table I).

Table I. Phenolics and flavonoids from different fractions of T. catigua *stem barks and their* IC_{50} *values (DPPH)*

	Reference (ascorbic acid)	EtOH extract	CH_2Cl_2	AcOEt	*n*-BuOH
Total phenolics (μg GAE g^{-1})	–	443.87 ± 22.23	594.03 ± 31.32	601.27 ± 42.59	345.63 ± 41.08
Gallic acid (mg g^{-1})	–	16.04 ± 1.68	1.90 ± 0.19	25.40 ± 0.30	0.90 ± 0.10
Chlorogenic acid (mg g^{-1})	–	27.30 ± 0.20	5.10 ± 0.30	14.90 ± 0.40	1.70 ± 0.30
Rutin (mg g^{-1})	–	7.90 ± 0.20	–	10.50 ± 0.20	2.80 ± 0.10
Quercetin (mg g^{-1})	–	14.2 ± 0.10	1.10 ± 0.20	23.70 ± 0.50	0.70 ± 0.40
IC_{50} (μg mL^{-1})	20.72 ± 1.30[a]	9.17 ± 0.63[a]	42.42 ± 4.92[a]	30.29 ± 1.37[a]	76.35 ± 5.92[a]

Mean ± SEM, n = 3–4.
[a] Significantly different from ascorbic acid (reference) (*t*-test; $p < 0.05$).

Several authors (10, 11) have described a positive correlation between phenolic content and antioxidant activity using similar assay systems. However, we have not observed such type of correlation. This could be explained by the fact that factors other than total phenolics can play a major role in the antioxidant activity of these extracts. Boligon *et al.* (12) and Kiliçgün and Altiner (13) found no correlation between phenolic content and antioxidant activity measured by various methods, either.

All the extracts exhibited a significant inhibitory effect on Fe^{2+}-induced TBARS production in brain homogenates ($p < 0.05$) and at 10 μg mL^{-1} a maximal inhibitory effect was attained for all the fractions (Fig. 2a). Similarly to what was observed with extracts obtained with organic solvents, cold and hot water extracts of *T. catigua* significantly inhibited Fe^{2+}-induced TBARS production in brain homogenates in a concentration-dependent manner (Fig. 2b) ($p < 0.05$).

Free Fe^{2+} can induce neurotoxicity *via* stimulation of the Fenton reaction and its levels are increased in some degenerative diseases. *T. catigua* extracts inhibited Fe^{2+}-induced lipid peroxidation in brain homogenates and this antioxidant effect can, at least partly, be associated with iron chelation. In fact, the chelating effects of some plant extracts could be attributed to the presence of flavonoids, which are well known to be chelator compounds. *T. catigua* extracts possess flavonoids, among which are quercetin and rutin (Fig. 3, Table I) that may form redox inactive complexes with Fe^{2+}, rendering this pro-oxi-

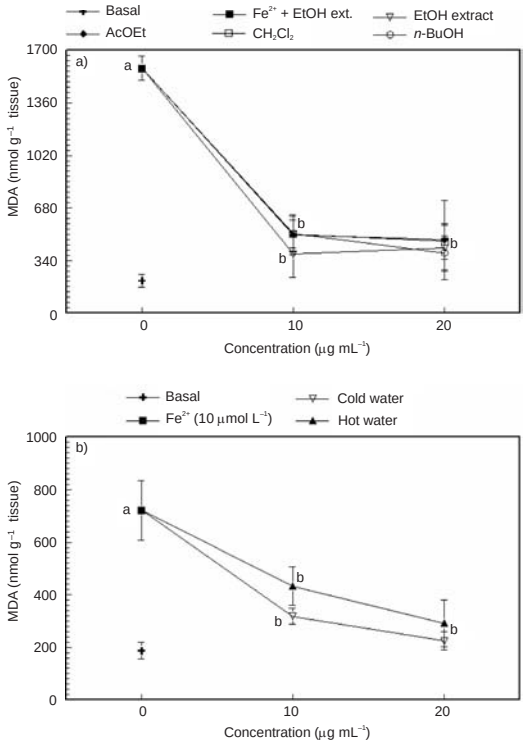

Fig. 2. Effects of (a) crude extracts and (b) aqueous extracts from the stem bark of *T. catigua* on Fe^{2+} (10 μmol L^{-1})-induced TBARS production in brain homogenates. The samples were incubated for 1 h with Fe^{2+} in the presence or absence of plant extracts (basal). Mean ± SEM, n = 3–4 independent experiments. Significant difference: a) $p < 0.05$ *vs*. basal, b) $p < 0.05$ *vs*. Fe^{2+} + ethanol (used as solvent), $p < 0.05$ *vs*. Fe^{2+} alone.

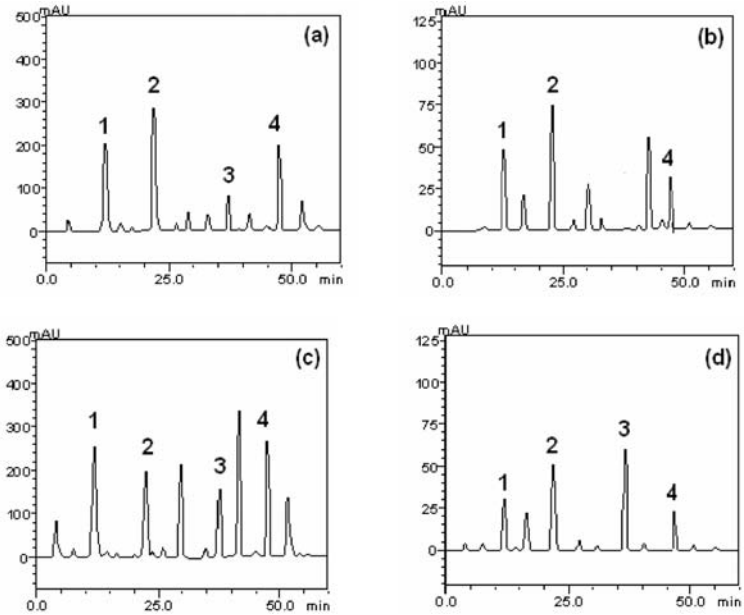

Fig. 3. High performance liquid chromatographic profile of phenolics and flavonoids in: a) ethanolic, b) dichloromethane, c) ethyl acetate, d) butanolic extract of *T. catigua*. Gallic acid (peak 1), chlorogenic acid (peak 2), rutin (peak 3) and quercetin (peak 4).

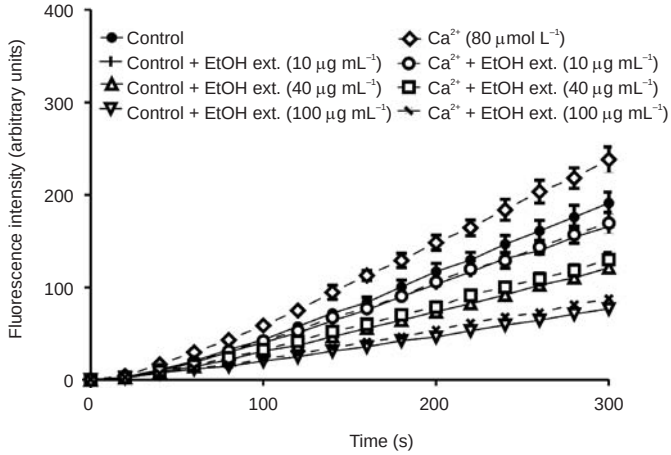

Fig. 4. Effect of calcium and ethanolic extract of *T. catigua* on rat liver mitochondrial DCFH oxidation. Mitochondria (0.25 mg protein mL^{-1}) were suspended in respiration buffer and mitochondrial ROS generation was determined by monitoring the fluorescence of DFCH oxidation (emission at 525 nm with excitation at 488 nm. Mean ± SEM, n = 4 independent measurements.

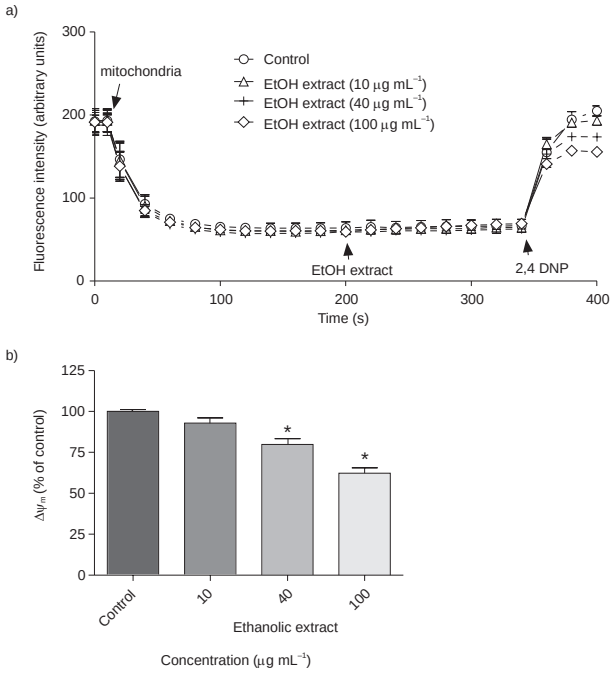

Fig. 5. Effect of ethanolic extract of *T. catigua* on mitochondrial membrane potential. Isolated rat liver mitochondria (0.6 mg mL^{-1}) were incubated in standard medium and the $\Delta\psi_m$ was monitored as described in experimental session. a) Effect of ethanolic extract (10–100 μg mL^{-1}) on mitochondrial membrane potential; b) values of $\Delta\psi_m$ after adding the mitochondrial uncoupler 2,4-dinitrophenol (2,4-DNP). The mitochondria (0.6 mg mL^{-1}), ethanolic extract or 2,4-DNP were added where indicated by arrows. Experiments were performed three times using independent mitochondrial preparation. Mean ± SEM, $n = 3$. * Significant difference *vs*. control: $p < 0.05$.

dant unavailable for Fenton reaction. Accordingly, quercetin and its glycoside form, rutin, effectively block Fe^{2+}-induced TBARS production in brain homogenates (14).

Mitochondrial oxidation of DCFH was stimulated by Ca^{2+} (80 μmol L^{-1}) and the ethanolic extract of *T. catigua* prevented ROS production stimulated by Ca^{2+} in a concentration-dependent fashion (Fig. 4). Substantial evidence in the literature has indicated that Ca^{2+} can increase mitochondrial oxidative stress (15). In line with this, Ca^{2+} increased the rate of DCFH oxidation compared to the control. Interestingly, the production of ROS induced by Ca^{2+} in the presence of ethanolic extract was not significantly different from those produced during basal conditions (control). This suggests that ROS production induced by Ca^{2+} was fully suppressed by the ethanolic extract, which is in accord with previous data from our laboratory indicating that quercetin, quercitrin and rutin protected brain mitochondria from Ca^{2+}-induced oxidative stress (16).

Ethanolic extract of *T. catigua* at high concentrations (40–100 μg mL^{-1}), produced a decrease in $\Delta\psi_m$ (~20 % and ~ 38 % depolarization, respectively) compared to the control

($p < 0.05$) whereas no effect was observed at 10 μg mL^{-1} (Figs. 5a,b). However, a partial decrease in $\Delta\psi_m$ can be associated with cardioprotection, which may be related to a reduction in mitochondrial ROS production (17). Consequently, the *in vitro* decrease in mitochondrial ROS production by *T. catigua* can be related to the partial depolarization of mitochondria.

CONCLUSIONS

Crude extracts from the stem bark of *T. catigua* have *in vitro* antioxidant activity in different chemical and biological models, which can be, in part, attributed to flavonoids and phenolic compounds present in the plant extracts. Taken together, our results indicate that *T. catigua* has promising compounds to be tested not only as potential antioxidant drugs for the treatment of diseases resulting from oxidative stress, but also for the use in different fields such as pharmaceuticals and cosmetics.

Acknowledgements. – JPK would like to thank especially TWAS-CNPq for financial support. JPK is a beneficiary of the TWAS-CNPq postgraduate (doctoral) fellowship. Work was supported by CNPq, CAPES, FAPERGS, FAPERGS-PRONEX-CNPq, VITAE Fundation, Rede Brasileira de Neurociências (IBNET-FINEP), FINEP-CTINFRA and INCT for excitotoxicity and neuroprotection-CNPQ.

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S A Ž E T A K

In vitro antioksidativni učinak kore stabljike *Trichilia catigua* Adr. Juss

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U radu je opisano ispitivanje antioksidativnog učinka etanolnog ekstrakta i pojedinih frakcija kore stabljike *T. catigua*. IC_{50} (za DPPH test) varirao je između 9,17 ± 0,63 i 76,42 ± 5,87 μg mL^{-1}, a ukupni sadržaj fenola od 345,63 ± 41,08 i 601,27 ± 42,59 μg GAE po gramu suhog ekstrakta. Etanolni ekstrakt i frakcije značajno su reducirale Fe^{2+}-induciranu lipidnu peroksidaciju. Nadalje, reducirana je oksidacija diklorfluoresceina inducirana ionima kalcija u mitohondrijima, a redukcija je ovisila o dozi etanolnog ekstrakta.

Etanolni ekstrakt smanjio je mitohondrijsku $\Delta\psi_m$ samo pri visokim koncentracijama ($40 \pm 100\ \mu g\ mL^{-1}$), što ukazuje da se toksičnost ne preklapa s antioksidativnim učinkom. Rezultati pokazuju da u farmakološko djelovanje *T. catigua* treba uključiti i antioksidativni učinak.

Ključne riječi: *Trichilia catigua* (*Meliaceae*), antioksidans, flavonoidi, fenoli, reaktivne kisikove specije, oksidativni stres

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