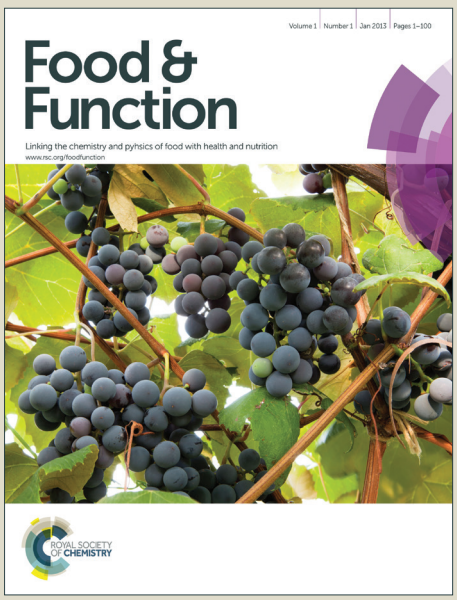
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Enhancement of flavonoid ability to cross the blood brain barrier of rat by co-administration with α-tocopherol

Paola Ferri [a], Donato Angelino [b], Lorenzo Gennari [b], Serena Benedetti [b], Patrizia Ambrogini [a], Paolo Del Grande [a], and Paolino Ninfali [b,*]

[a] Department of Earth, Life and Environmental Sciences, University of Urbino "Carlo Bo", Via Ca' Le Suore 2/4 - 61029 Urbino (PU) – Italy

[b] Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Via Saffi 2 – 61029 Urbino (PU) – Italy

Running title: Role of α-tocopherol for flavonoid blood brain barrier crossing

***Address correspondence to:**

Prof. Paolino Ninfali

Department of Biomolecular Sciences

University of Urbino "Carlo Bo"

Via Saffi 2, 61029 Urbino (PU)

Tel: +39 722 305288

Fax: +39 722 305324

web: http://www.uniurb.it/orac/

Email. paolino.ninfali@uniurb.it

ABSTRACT

Vitamin E and polyphenols could exhibit a therapeutic role in the treatment of oxidative stress-induced neurodegenerative diseases. Therefore, their ability to cross the blood brain barrier (BBB) represents an important issue to be explored by different diet combinations. In this study, we evaluated the ability of α-tocopherol to support epigallocatechin-3-gallate (EGCG), quercetin and rutin to cross the BBB, following oral administration. Eighteen rats were fed a standard diet (C), or a diet supplemented with α-tocopherol (A), or with a mixture of EGCG, quercetin and rutin (P); or with a mixture of α-tocopherol and the three flavonoids (AP). Flavonoids and their conjugated derivatives were assayed in brain and plasma by HPLC-MS, whereas α-tocopherol was detected by RP-HPLC. The oxidative damage, due to potential pro-oxidant activity of flavonoids, was evaluated by the presence of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in hippocampal Ammone's Horn, one of the most vulnerable sites in the brain.

Our results indicate that α-tocopherol is able to promote quercetin transport across the BBB. The mixture of rutin and quercetin seems to favour the accumulation of quercetin and/or its conjugated derivatives in the brain. On the contrary, α-tocopherol does not affect EGCG transport across the BBB. The densitometric analysis of 8-OHdG immunoreactivity does not reveal any difference of oxidative damage among the experimental groups. Our results suggest that α-tocopherol may promote quercetin transport across BBB, leading to a significant increase of α-tocopherol and quercetin concentration in the brain.

Key words: α-tocopherol, quercetin, rutin, epigallocatechin-3-gallate, blood brain barrier, neuroprotection.

1. INTRODUCTION

There is currently a growing interest in dietary therapeutic strategies to counteract neurodegenerative disorders associated with oxidative stress [1]. Vitamin E and flavonoids are dietary antioxidants, which might have a potential therapeutic role in prevention of oxidative stress-induced damage, such as neuro-inflammatory and neurodegenerative diseases [2,3,4,5].

Vitamin E refers to a group of ten lipid-soluble compounds that includes both tocopherols and tocotrienols [6]. Among the many different forms of vitamin E, α-tocopherol (Fig. 1) possesses the highest bioavailability in humans [7,8]. Although the transport mechanism is not entirely clear[9], it is known that α-tocopherol is able to cross the blood brain barrier (BBB) and to accumulate in brain following both intraperitoneal [10,11] and oral administration [12,13].

Flavonoids represent the largest group of phenol compounds in the human diet [2], which includes weakly hydrophilic molecules, such as catechins, and more lipophilic ones, such as quercetin [14]. Flavonoids are endowed with strong antioxidant ability, either by scavenging free radicals or, indirectly, by increasing cellular antioxidant defenses [4]. Indeed, flavonoids address neuroprotective effects throughout the modulation of signal transduction cascades or effects on gene expression [2,4].

Epigallocatechin-3-gallate (EGCG) (Fig. 1), the principal component of green tea catechins, was reported to be able to protect neurons from cell death in cellular and animal models of neurological diseases [15,16,17]. In particular, due to its iron-chelating properties, EGCG could be a promising candidate for treating or preventing neurodegenerative diseases induced by brain iron homeostasis disregulation, such as Parkinson's disease [15,18].

Quercetin (Fig. 1) is well known to exert protection of the brain from cell membrane damage [19]. Besides its antioxidant role, quercetin neuroprotective activity consists in the promotion of cell survival [2] and modulation of signalling pathways, such as PKC, MAP kinase and Sirtuin 1 activation [20,2]. Indeed, quercetin and its structurally related flavonoids, have been identified as potential leading molecules for the development of multi-targeted therapeutic tools in human brain neurodegeneration [2].

Rutin (quercetin-3β-D-rutinoside) (Fig. 1) also addresses neuroprotective abilities in animal models [21,22,23,24]. Rutin is hydrolyzed in quercetin during intestinal absorption by cecal microflora glycosidases, prior to absorption as quercetin aglycone [25,26], and a linear increase in plasma quercetin concentration, after rutin administration, has been demonstrated in healthy volunteers [25]. Recently the co-administration of quercetin and rutin showed an enhancement of plasmatic quercetin bioavailability in rats [27].

Despite of the numerous studies, supporting the neuroprotective activity of flavonoids, the main issue to be elucidated concerns the flavonoid ability to cross the BBB [28,29,30] and the interactions among the co-administered molecules [31].

This study was aimed to evaluate the plasma bioavailability of EGCG, quercetin and rutin (Fig.1) co-administered with α-tocopherol and the ability of α-tocopherol to promote the transport of the flavonoids across the BBB. Moreover, to exclude flavonoid pro-oxidant activity [32], we also examined oxidative stress occurrence in hippocampal Ammone's Horn (*Cornus Ammonis*, CA), one of the most vulnerable sites in the brain [33].

2. METHODS

2.1 Reagents

Acetonitrile, tetrahydrofuran, ethanol, methanol, water, formic acid, ammonium acetate were pure HPLC-grade from VWR (Milan, Italy). β-Glucuronidase/Arylsulfatase from *Helix Pomatia* was from Sigma Aldrich, Milan, Italy. α-Tocopherol (as tocopherol acetate), epigallocatechin-3-gallate, quercetin, rutin were provided by Polichimica s.r.l. (Bologna, Italy). The control diet was from Mucedola (Settimo Milanese, MI, Italy) and the supplemented diets were prepared by adding tocopherol acetate, epigallocatechin-3-gallate, quercetin, rutin in the due percentage, to the basic diet formulation. The chow was provided as a pelleted material by Laboratori Dottori Piccioni SRL (Gessate, MI, Italy).

2.2 Animals

Eighteen three month old male Sprague-Dawley rats (Charles River, Milano, Italy) were used in this study in accordance with European Union guidelines and Italian laws. Rats were randomly grouped into four and fed 3 months with four diet types:

- C (n = 4), controls; (4RF21 Basic diet for mice and rats [34])
- A (n = 4), Basic diet + 1.5% α-tocopherol;
- P (n = 5), Basic diet + 1% EGCG, 1% quercetin, 1% rutin;
- AP (n = 5), Basic diet + 1.5% α-tocopherol + 1% EGCG + 1% quercetin + 1% rutin.

Animals were anaesthetized with intraperitoneal injection of sodium thiopental (45 mg/kg b.w). Blood samples (2 ml) were collected and immediately centrifuged at 2,400 rpm at 4 °C for 10 min to separate the plasma. Rats were not perfused with physiological saline, as we focused our study on a comparative analysis among four groups of animals, without searching for the absolute concentrations of the flavonoids in the brain.

After blood draw, rats were sacrificed by intracardiac injection of the same anesthetic, then brains were quickly removed and dissected into two hemispheres: the left one, used for the biochemical analyses, was immediately frozen in liquid nitrogen and stored at -80 °C; the right hemisphere, was fixed in 4% paraformaldehyde in 10 mM PBS, pH 7.4 for three days, washed in PBS and embedded in Paraplast Plus (Sigma, MO, USA; melting point = 56 °C – 58°C) and used for the morphological analyses,

2.3 Biochemical analysis

2.3.1 α-Tocopherol assay

Alpha-tocopherol was assayed in brain and plasma samples by reversed-phase high-performance liquid chromatography (RP-HPLC), as described previously by Aebischer *et al.*[35]. Briefly, 100 mg of brain was homogenized in 1 mL of cold phosphate buffered saline (pH 7.4) and deproteinized with 3 mL of ethanol. Plasma samples (100 µL) were deproteinized with 300 µL of cold ethanol and incubated 10 min on ice. After centrifugation at 14,000 rpm, the supernatant was filtered using a 0.22 µm filter (Fisher, Fair Lawn, NY). α-Tocopherol was extracted from deproteinized plasma or brain with a mixture of hexane and ethanol 1:1 volume ratio, for three times. After centrifugation, the organic layer was removed and evaporated; the residue was dissolved in the eluent phase acetonitrile/tetrahydrofuran/methanol/1% ammonium acetate (680:220:70:30 v/v) and injected into the HPLC system (Jasco Corporation, Tokyo, Japan). The assay was performed using a Alltima C18 column (4.6 x 250 mm, 5 µm, Alltech, Milan, Italy) equipped with a guard column Alltima C18 (4.6 x 7.5 mm, 5 µm); the flow rate was 1.5 mL/min. The fluorescent detector was set at 298 nm excitation and 328 nm emission. Results for α-tocopherol were expressed as µg/g of wet tissue in brain samples and as µmol/L in plasma samples.

2.3.2 Flavonoid assay

Flavonoids were assayed in brain and plasma as previously described [36]. After deproteinization and extraction the flavonoid metabolites were treated with β-glucuronidase/arylsulfatase from *Helix pomatia*, (Sigma Aldrich, St.Louis, MO), according to published methods [37,38]. Briefly, 200 µL of extract were mixed with 100 µL of β-glucuronidase/arylsulfatase 1,000 units/mL in pH 5.0 acetate buffer, 50 µL ascorbic acid solution (100 mg/mL) and incubated 30 min at 37°C. After hydrolysis, samples were freeze dried and re-dissolved in 100 µL cold methanol. After 10 min centrifugation at 13,200 rpm at 4°C, the supernatant was separated, filtered and subjected to HPLC analysis.

A Series 200 Micro Pump HPLC system (Perkin Elmer, Norwalk, CT, USA) was equipped with an API 150EX (Applied Biosystems, Foster City, CA, USA) mass spectrometer with an ESI source. The sample separation was carried out using a 5 mm Alltech Alltima C18 (150 x 4.6 mm i.d.) column protected by a 5 mm Alltech C18 (7.5 x 4.6 mm i.d.) guard column (Grace Davison, Deerfield, IL, USA). The initial HPLC conditions were 20% methanol 80% water and 0.1% Formic acid, at a flow rate of 0.25 mL per minute. The column was held at the initial conditions for 2 minutes then developed with a binary gradient to 100% methanol and 0.1% formic acid over 50 additional minutes. The MS was run with the ESI probe both in positive and negative mode (ESI^+ or ESI^-) depending on the better signal/noise ratio and by comparing them with the literature databases. For flavonoids, the *m/z* signals generated by the loss of sugars were also evaluated and these data were confirmed by the analysis of aglycones obtained after acid hydrolysis of sugars. Calibration curves were performed with concentration of EGCG, rutin and quercetin ranging from 0.30 to 30 μM. The detection limit was 0.5 μM.

2.3.3. 8-OHdG immunohistochemistry and quantitative analysis

Six μm-thick serial coronal sections were cut from paraffin-embedded specimens and series of 10 coronal sections of brain, spaced 60 μm apart, were selected and used for immunohistochemical analyses.

For 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemistry, rehydrated sections were treated with 1% hydrogen peroxide in 10% methanol in PBS for 10 min at room temperature (r.t.) to block endogenous peroxidase. After rinsing with 1% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO) in PBS, sections were incubated with normal rabbit serum (D.B.A., Vector; 1:10 in PBS) for 10 min at r.t. and then incubated with a goat anti-8-OHdG (Chemicon, CA, USA; 1: 200 in PBS) overnight at 4 °C. Sections were then incubated with a biotin-conjugated rabbit anti-goat antibody (Chemicon, CA, USA; 1: 500 in PBS) for 1 hour at r.t., followed by an incubation with a kit solution (kit ABC, D.B.A., Vector; ready to use) containing peroxidase-conjugated avidin/biotinylated complex for 30 min at room temperature. Finally, color was developed with 0.5 mg/mL of 3,3'-diaminobenzidine (DAB; Sigma Aldrich, St. Louis, MO) in Tris-HCl buffer (0.05 M, pH 7.4) with 0.01% hydrogen peroxide for 20 min at r.t. Negative controls were assessed without primary antibody.

The 8-OHdG quantitative analysis was performed measuring the optical density in the radiatum layer (RL) and pyramidal cell layer (PCL) of the hippocampus CA (CA2 and CA3 areas), in at least 12 sections per animal. To avoid different labeling intensity, brain sections of all animal groups were simultaneously stained. The optical density measurements were performed on 6-8 areas

adjacent both in the RL and PCL, for each section. The optical density was measured separately in the nucleus and cytoplasm of 100 pyramidal cells per animal.

A BX51 Olympus light microscope (Olympus Italy, Milan, Italy), equipped with a digital photocamera SPOT RT (Diagnostic Instruments, Delta Systems, Rome, Italy) and the image analysis program SPOT ADVANCED was used. The resulting pictures were analyzed using the public domain Scion Image – NIH imaging software (Rasband W., National Institutes of Health, Bethesda, MD, USA) available at http://rsb.info.nih.gov/. To avoid variations in lighting, which might affect measurements, all images were acquired in one session.

2.4 Statistics

The biochemical and morphological results were expressed as mean ± standard error (SE). The One-way Anova test, followed by the post-hoc Tukey's test, and the Student's *t*-test for unpaired data were used; $p \leq 0.05$ was considered the significance threshold.

In both biochemical and morphological cases, statistical analysis were performed using SPSS® 17.0 software.

3. RESULTS AND DISCUSSION

Table 1 shows the α-tocopherol and flavonoids concentrations in plasma of treated *versus* control rats. The α-tocopherol concentrations of A, P, and AP groups were higher than the control (C), but no significant difference was detectable among the three groups. An increase was expected for A and AP groups, as the diet of the animals contained 1.5% tocopherol acetate. The increase of α-tocopherol in the P group could be explained with the ability of flavonoids to protect α-tocopherol from oxidation in plasma [39,40], thus allowing the efficient incorporation inside the cell membrane of α-tocopherol, basically present in the normal chow at 64 mg/Kg [34]. EGCG was not detectable in C and A groups, as expected from the absence of this flavonoid in the diet, whereas it was detected in P and AP groups, without any significant difference between the groups (Table 1). Quercetin was assayed as the result of the diet quercetin plus rutin contribute (Table 1). In fact, though the mechanisms of absorption of quercetin and rutin remain unclear, it has been demonstrated that enzymes able to deglycosilate rutin are present in small intestine and colon [30]. Therefore, it is very likely that rutin hydrolyzes before its absorption, which may occur as quercetin glycoside [41] and quercetin aglycone [25]. Yang *et al.* [42] proposed that rutin may undergo deglycosilation to quercetin 3-O-glucoside, by the enzyme rhamnosidase, produced by the intestinal bacteria. The method, we

applied, to detect quercetin plus rutin, was based on the hydrolysis of any quercetin or rutin glycoside, glucuronide or sulphate derivative, by means of β-glucuronidase/arylsulfatase from *Helix Pomatia*, added to the plasma or brain extracts.

Quercetin was detectable in the plasma of P and AP groups, with no significant difference between the two groups. Quercetin was not detectable in the plasma of A and C rat groups as the only source of quercetin is provided by the chow, which is roughly about 0.01% due to cereals and forage [34,43,44].

In the brain, we found non-quantifiable concentration of EGCG in all rat groups (data not shown).

On the contrary, we found α-tocopherol and quercetin in the brains of all rat groups (Figure 2). The α-tocopherol concentration was significantly higher in A group compared to C, P and AP groups, with P and AP concentrations, which were both significantly different from the control, but not between them (Fig. 2).

Quercetin was significantly higher in AP as compared to A, P and C groups, which were not significantly different among them (Fig. 2). The presence of quercetin in A and C rat groups might be explained on the basis of the chow composition, which is constituted by 66% cereals and 10% forage. This two chow components justify a 0.01% basal quercetin concentration [34,43,44], which allows to the flavonoid and its metabolites to be transported across BBB in a time-dependent manner[29].

The absolute concentration of quercetin in the brain of AP rats is striking. We may explain this result by considering that, the detected quercetin derived from the dietetic contributes of quercetin and its metabolites[45] as well as from rutin. In fact, studies demonstrated that rutin is metabolized in the gut and in the liver [46,47], to be deglycosilated into quercetin and then glucuronated or sulphated [25,42] by the phase II enzymes.

The accumulation of quercetin in the brain, when co-administered together with α-tocopherol, suggests a permissive role of α-tocopherol on flavonoid BBB crossing.

Two aspects are important to be considered here: the "chronic" administration of the antioxidant rich diet for 12 weeks and the co-administration of quercetin plus rutin, which can be able to influence the absorption of each other [27].

At present, we are not able to hypothesize a precise mechanism for this behaviour. We can only suggest two possible explanations of the significant accumulation of quercetin in the brain in the presence of α-tocopherol: i) the ability of α-tocopherol to inhibit P-glycoprotein [48,49], a membrane glycoprotein, extensively distributed in neurons and astrocytes, which allows the efflux of xenobiotics out of the cell [49]; ii) the ability of α-tocopherol to inhibit protein kinase C (PKC) by means of protein phosphatase 2A activation[50], thus impairing the

phosphorylation/dephosphorylation mechanism in quercetin transport across the BBB, which controls the in/out flux of metabolites [29,50]. The two mechanisms could also work together.

Conversely, higher quercetin concentrations in the brain seems to hamper the crossing of the BBB by α-tocopherol. In fact, when α-tocopherol was co-administered with the flavonoids, as in the AP group, the α-tocopherol concentration was significantly lower in AP as compared with the A group. Indeed, we observed that α-tocopherol promotes the transfer of quercetin and rutin metabolites in the brain, but it does not promote EGCG bioavailability either in the plasma or in the brain.

The low EGCG plasma bioavailability after oral administration and the undetectable EGCG concentration in brain, may be due to the high EGCG/protein binding ratio [51] as well as to competitive interactions with quercetin and EGCG, as proposed by Silberberg *et al.* [52]. Data on the permeability of the BBB of EGCG are contradictory in the literature. Lin and coworkers [51] proposed that EGCG may potentially penetrate through the BBB, but this might happen at a very low rate, because of the formation of protein-EGCG complexes. Other authors suggested that EGCG, as other catechins, may cross the BBB both by passive diffusion or by an unidentified membrane transporter, [29] as demonstrated by the presence of radio-labeled EGCG in brain [53]. Our data failed to detect EGCG in brain, probably because of its hydrophilic nature and ability to form protein-bound complexes.

Concerning to immunohistochemistry, the 8-OHdG immunolocalization in CA of the four experimental groups is shown in Figure 3. No difference in the CA staining pattern was observed among the four experimental groups (Fig. 2A-D). Densitometric analysis (Fig. 4) did not reveal any significant differences between the pyramidal cell and the radiatum layer in the four experimental groups (Fig. 4A). However, the 8-OHdG densitometric analysis showed a significantly higher concentration in the pyramidal cell cytoplasm than in the nucleus, without significant differences among the experimental groups (Fig. 4B). This means no particular nucleic acid oxidation due to pro-oxidant cytotoxic effect of flavonoids.

4. CONCLUSIONS

Our results indicate that α-tocopherol is able to promote the transport across the BBB of quercetin and rutin as well as of their putative metabolites. The potential mechanism of this behaviour might lie in the α-tocopherol ability to modulate the P-glycoprotein action and/or to impair the phosphorylation/dephosphorylation mechanism, which controls the in/out flux of metabolites across the BBB. α-tocopherol does not affect EGCG transport across the BBB, as prevented by the chemistry of the flavonoid, which tends to form complexes with proteins [54]. The flavonoid concentrations and their chronic administration, used in our study, do not induce any pro-oxidant or

cytotoxic side effects on hippocampus. Our results represent an attempt to identify dietary factors, that like α-tocopherol, are able to modulate flavonoid uptake to the CNS, in order to enhance the potential neuroprotective ability of these compounds.

Aknowledgements

The research was supported with a grant of the "Consiglio di Amministrazione della Fondazione Cassa di Risparmio" of Pesaro, The Marches, Italy.

Authors wish to thank Prof. Maurizio Simmaco and Dr. Luana Lionetto, Advanced Molecular Diagnostics – University of Rome "La Sapienza" and "Sant' Andrea" Hospital, Rome, for their help in the mass spectrometry analysis.

References

1. D. D. Perez, P. Strobel, R. Foncea, M. S. Diez, L. Vasquez, I. Urquiaga, O. Castillo, A. Cuevas, M. A. San and F. Leighton, *Ann. N. Y. Acad. Sci.*, 2002, **957**, 136 - 145.

2. F. Dajas, A. C. Andres, A. Florencia, E. Carolina and R. M. Felicia, *Cent. Nerv. Syst. Agents Med. Chem.*, 2013, **13**, 30 - 35.

3. M. W. Dysken, M. Sano, S. Asthana, J. E. Vertrees, M. Pallaki, M. Llorente, S. Love, G. D. Schellenberg, J. R. McCarten, J. Malphurs, S. Prieto, P. Chen, D. J. Loreck, G. Trapp, R. S. Bakshi, J. E. Mintzer, J. L. Heidebrink, A. Vidal-Cardona, L. M. Arroyo, A. R. Cruz, S. Zachariah, N. W. Kowall, M. P. Chopra, S. Craft, S. Thielke, C. L. Turvey, C. Woodman, K. A. Monnell, K. Gordon, J. Tomaska, Y. Segal, P. N. Peduzzi and P. D. Guarino, *J. A. M. A.*, 2014, **311**, 33 - 44.

4. N. A. Kelsey, H. M. Wilkins and D. A. Linseman, *Molecules*, 2010, **15**, 7792 - 7814.

5. R. Ricciarelli, F. Argellati, M. A. Pronzato and C. Domenicotti, *Mol. Aspects Med*, 2007, **28**, 591 - 606.

6. R. Brigelius-Flohe and M. G. Traber, *FASEB J.*, 1999, **13**, 1145 - 1155.

7. J. K. Lodge, *J. Plant. Physiol.*, 2005, **162**, 790 - 796.

8. J. M. Zingg and A. Azzi, *Curr. Med. Chem.*, 2004, **11**, 1113 - 1133.

9. I. Kratzer, E. Bernhart, A. Wintersperger, A. Hammer, S. Waltl, E. Malle, G. Sperk, G. Wietzorrek, H. Dieplinger and W. Sattler, *J. Neurochem.*, 2009, **108**, 707 - 718.

10. T. Cecchini, S. Ciaroni, P. Ferri, P. Ambrogini, R. Cuppini, S. Santi and G. P. Del, *J. Neurosci. Res.*, 2003, **73**, 447 - 455.

11. P. Ferri, T. Cecchini, S. Ciaroni, P. Ambrogini, R. Cuppini, S. Santi, S. Benedetti, S. Pagliarani, G. P. Del and S. Papa, *J. Neurocytol.*, 2003, **32**, 1155 - 1164.

12. C. A. Murray and M. A. Lynch, *J. Biol. Chem.*, 1998, **273**, 12161 - 12168.

13. E. O'Donnell and M. A. Lynch, *Neurobiol. Aging*, 1998, **19**, 461 - 467.

14. R. Tsao, *Nutrients*, 2010, **2**, 1231 - 1246.

15. H. S. Kim, M. J. Quon and J. A. Kim, *Redox Biol.*, 2014, **2**, 187 - 195.

16. S. Mandel, T. Amit, L. Reznichenko, O. Weinreb and M. B. Youdim, *Mol. Nutr. Food Res.*, 2006, **50**, 229 - 234.

17. S. A. Mandel, Y. Avramovich-Tirosh, L. Reznichenko, H. Zheng, O. Weinreb, T. Amit and M. B. Youdim, *Neurosignals*, 2005, **14**, 46 - 60.

18. O. Weinreb, S. Mandel, M. B. Youdim and T. Amit, *Free Radic. Biol. Med.*, 2013, **62**, 52 - 64.

19. E. O. Farombi and O. O. Onyema, *Hum. Exp. Toxicol.*, 2006, **25**, 251 - 259.

20. S. Chung, H. Yao, S. Caito, J. W. Hwang, G. Arunachalam and I. Rahman, *Arch. Biochem. Biophys.*, 2010, **501**, 79 - 90.

21. H. Javed, M. M. Khan, A. Ahmad, K. Vaibhav, M. E. Ahmad, A. Khan, M. Ashafaq, F. Islam, M. S. Siddiqui, M. M. Safhi and F. Islam, *Neuroscience*, 2012, **210**, 340 - 352.

22. M. M. Khan, S. S. Raza, H. Javed, A. Ahmad, A. Khan, F. Islam, M. M. Safhi and F. Islam, *Neurotox. Res.*, 2012, **22**, 1 - 15.

23. V. S. Motamedshariaty, F. S. Amel, M. Nassiri-Asl and H. Hosseinzadeh, *Daru*, 2014, **22**, 27 -

24. A. R. Silva, A. M. Pinheiro, C. S. Souza, S. R. Freitas, V. Vasconcellos, S. M. Freire, E. S. Velozo, M. Tardy, R. S. El-Bacha, M. F. Costa and S. L. Costa, *Cell Biol. Toxicol.*, 2008, **24**, 75 - 86.

25. I. Erlund, T. Kosonen, G. Alfthan, J. Maenpaa, K. Perttunen, J. Kenraali, J. Parantainen and A. Aro, *Eur. J. Clin. Pharmacol.*, 2000, **56**, 545 - 553.

26. S. Tranchimand, P. Brouant and G. Iacazio, *Biodegradation*, 2010, **21**, 833 - 859.

27. A. K. Kammalla, M. K. Ramasamy, J. Chintala, G. P. Dubey, A. Agrawal and I. Kaliappan, *Eur. J. Drug Metab. Pharmacokinet.*, 2014

28. F. Dajas, *J. Ethnopharmacol.*, 2012, **143**, 383 - 396.

29. A. Faria, M. Meireles, I. Fernandes, C. Santos-Buelga, S. Gonzalez-Manzano, M. Duenas, F. de, V, N. Mateus and C. Calhau, *Food Chem.*, 2014, **149**, 190 - 196.

30. S. Schaffer and B. Halliwell, *Genes Nutr.*, 2012, **7**, 99 - 109.

31. S. Passamonti, U. Vrhovsek, A. Vanzo and F. Mattivi, *J Agric. Food Chem.*, 2005, **53**, 7029 - 7034.

32. G. Galati and P. J. O'Brien, *Free Radic. Biol. Med.*, 2004, **37**, 287 - 303.

33. J. M. Billard, *Eur. J. Neurosci.*, 2013, **37**, 1931 - 1938.

34. http://www.mucedola.it/Product/ProductServices.aspx, (Accessed October 2014).

35. C. P. Aebischer, J. Schierle and W. Schuep, *Methods Enzymol.*, 1999, **299**, 348 - 362.

36. D. Angelino, M. Berhow, P. Ninfali and E. H. Jeffery, *Food Funct.*, 2013, **4**, 1339 - 1345.

37. Y. C. Hou, S. Y. Tsai, P. Y. Lai, Y. S. Chen and P. D. Chao, *Food. Chem. Toxicol.*, 2008, **46**, 2764 - 2769.

38. O. N. Pozharitskaya, M. V. Karlina, A. N. Shikov, V. M. Kosman, M. N. Makarova and V. G. Makarov, *Phytomedicine*, 2009, **16**, 244 - 251.

39. J. Frank, A. Budek, T. Lundh, R. S. Parker, J. E. Swanson, C. F. Lourenco, B. Gago, J. Laranjinha, B. Vessby and A. Kamal-Eldin, *J. Lipid Res.*, 2006, **47**, 2718 - 2725.

40. B. Zhou, L. M. Wu, L. Yang and Z. L. Liu, *Free Radic. Biol. Med.*, 2005, **38**, 78 - 84.

41. P. C. Hollman, M. N. Bijsman, G. Y. van, E. P. Cnossen, J. H. de Vries and M. B. Katan, *Free Radic. Res.*, 1999, **31**, 569 - 573.

42. J. Yang, D. Qian, S. Jiang, E. X. Shang, J. Guo and J. A. Duan, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2012, **898**, 95 - 100.

43. E. Karimi, E. Oskoueian, A. Oskoueian, V. Omidvar, R. Hendra and H. Nazeran, *J Med Plants Res*, 2013290 - 297.

44. I. Sedej, M. Sakac, A. Mandic, A. Misan, V. Tumbas and J. Canadanovic-Brunet, *J Food Sci.*, 2012, **77**, C954 - C959.

45. A. Ishisaka, R. Mukai, J. Terao, N. Shibata and Y. Kawai, *Arch. Biochem. Biophys.*, 2014, **557**, 11 - 17.

46. W. Andlauer, C. Stumpf and P. Furst, *Biochem. Pharmacol.*, 2001, **62**, 369 - 374.

47. I. B. Jaganath, W. Mullen, C. A. Edwards and A. Crozier, *Free Radic. Res.*, 2006, **40**, 1035 - 1046.

48. Y. Yang, L. Bai, X. Li, J. Xiong, P. Xu, C. Guo and M. Xue, *Toxicol. In Vitro*, 2014, **28**, 388 - 396.

49. K. A. Youdim, M. Z. Qaiser, D. J. Begley, C. A. Rice-Evans and N. J. Abbott, *Free Radic. Biol. Med.*, 2004, **36**, 592 - 604.

50. R. Ricciarelli, A. Tasinato, S. Clement, N. K. Ozer, D. Boscoboinik and A. Azzi, *Biochem. J.*, 1998, **334 (Pt 1)**, 243 - 249.

51. L. C. Lin, M. N. Wang, T. Y. Tseng, J. S. Sung and T. H. Tsai, *J. Agric. Food Chem.*, 2007, **55**, 1517 - 1524.

52. M. Silberberg, C. Morand, C. Manach, A. Scalbert and C. Remesy, *Life Sci.*, 2005, **77**, 3156 - 3167.

53. M. Suganuma, S. Okabe, M. Oniyama, Y. Tada, H. Ito and H. Fujiki, *Carcinogenesis*, 1998, **19**, 1771 - 1776.

54. N. Bordenave, B. R. Hamaker and M. G. Ferruzzi, *Food Funct.*, 2014, **5**, 18 - 34.

Table 1. Concentrations of antioxidants in plasma of the four groups of rats.

Groups of animals	α-tocopherol (µg/mL)	EGCG (µg/mL)	Quercetin (µg/mL)
C	9.73 ± 1.2 [a]	ND	ND
A	33.06 ± 4.27 [b]	ND	ND
P	23.19 ± 2.21 [b]	0.347 ± 0.012 [a]	0.151 ± 0.031 [a]
AP	26.37 ± 2.56 [b]	0.332 ± 0.005 [a]	0.180 ± 0.063 [a]

Data are the means ± standard error of 4-5 animals per group. Same letters indicate not statistical differences among the treatments. One-way Anova test: $p< 0.05$. Same letters indicate not significantly different values, among the same tissue data.
Legend: C, control ; A, α-tocopherol-supplemented; P, polyphenol-supplemented; AP, α-tocopherol + polyphenol-supplemented rats.

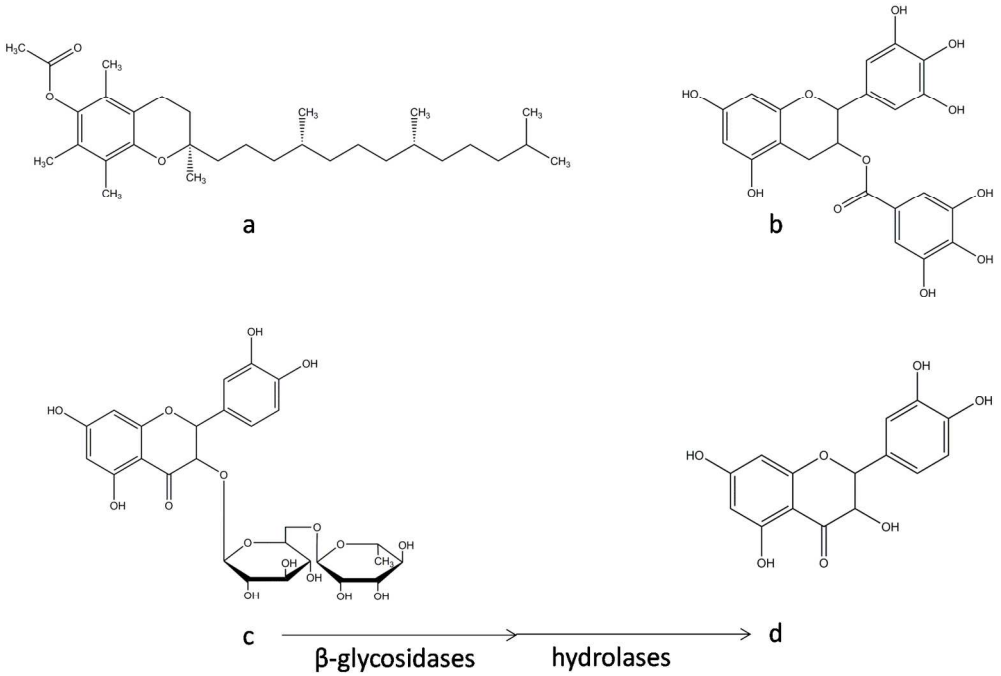
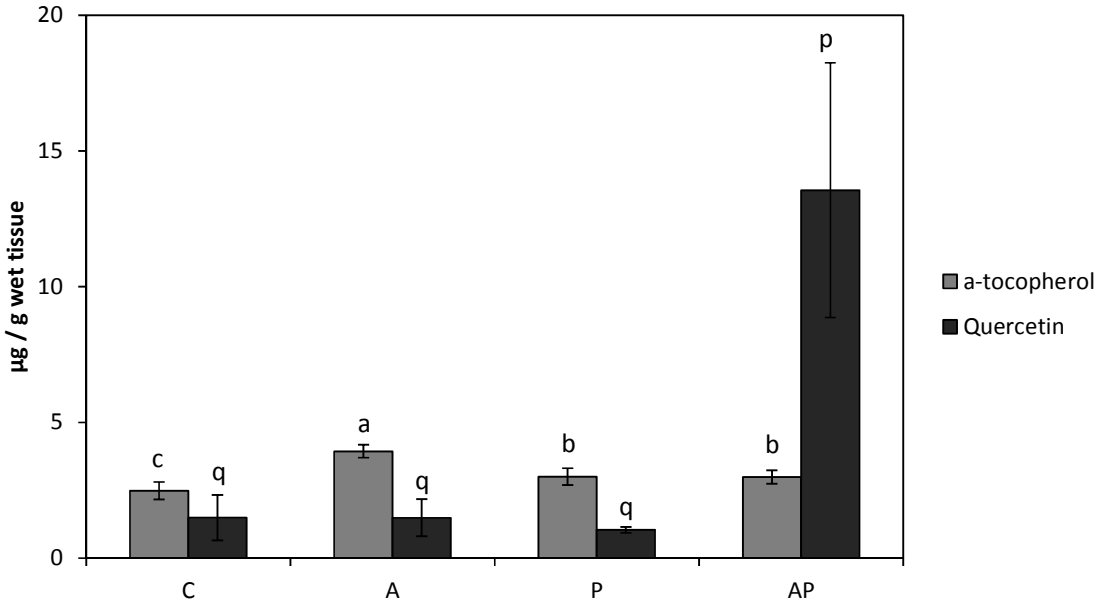


Figure 1
168x117mm (300 x 300 DPI)

Figure 2



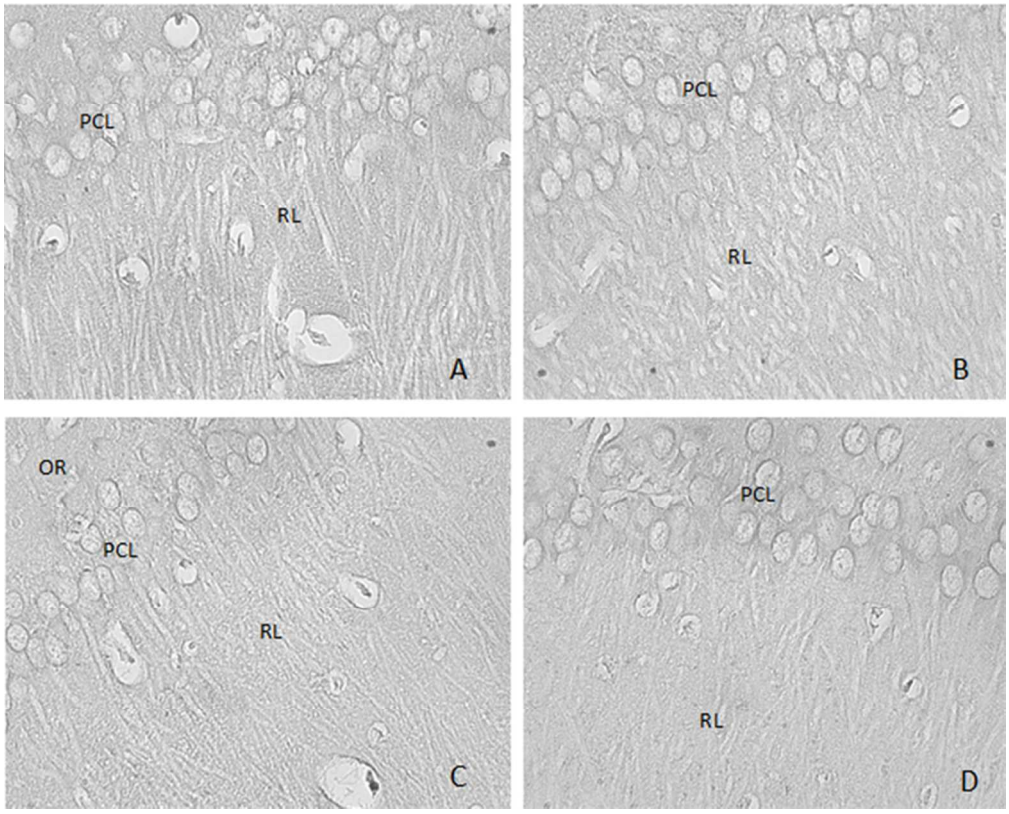


Figure 3
67x54mm (300 x 300 DPI)

Figure 4