USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

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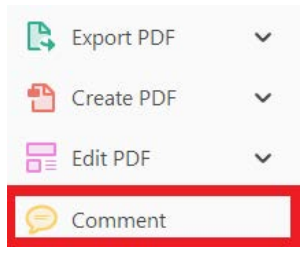
Once you have Acrobat Reader open on your computer, click on the Comment tab (right-hand panel or under the Tools menu).



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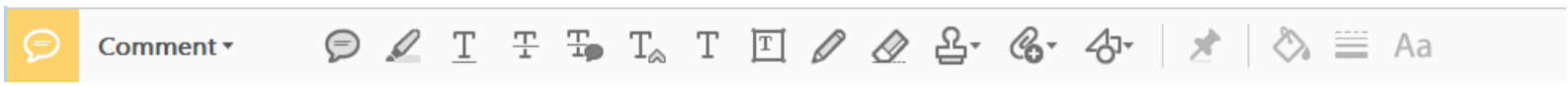
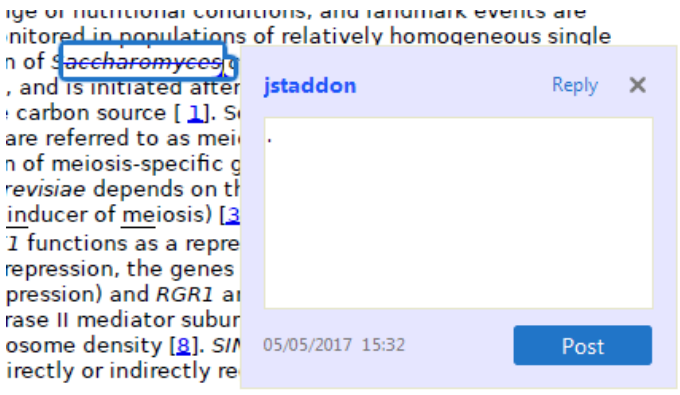


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it:

- Highlight a word or sentence.
- Click on .
- Type the replacement text into the blue box that appears.

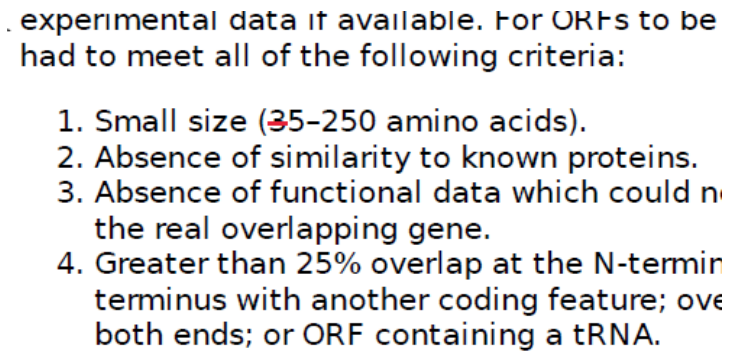


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it:

- Highlight a word or sentence.
- Click on .
- The text will be struck out in red.

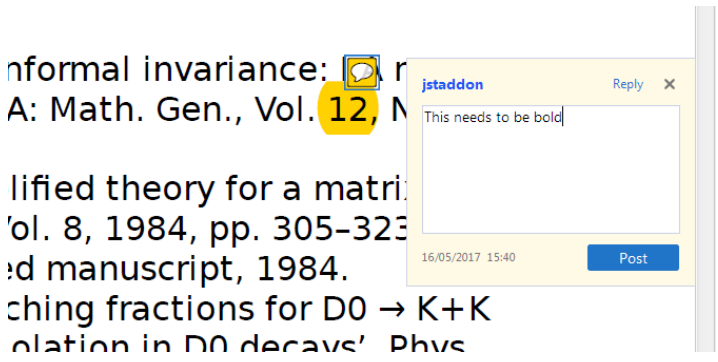


3. Commenting Tool – for highlighting a section to be changed to bold or italic or for general comments.

Use these 2 tools to highlight the text where a comment is then made.

How to use it:

- Click on .
- Click and drag over the text you need to highlight for the comment you will add.
- Click on .
- Click close to the text you just highlighted.
- Type any instructions regarding the text to be altered into the box that appears.

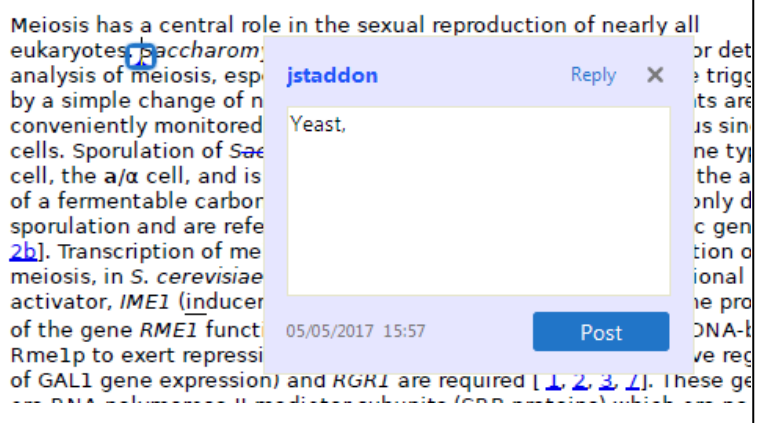


4. Insert Tool – for inserting missing text at specific points in the text.

Marks an insertion point in the text and opens up a text box where comments can be entered.

How to use it:

- Click on .
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the box that appears.



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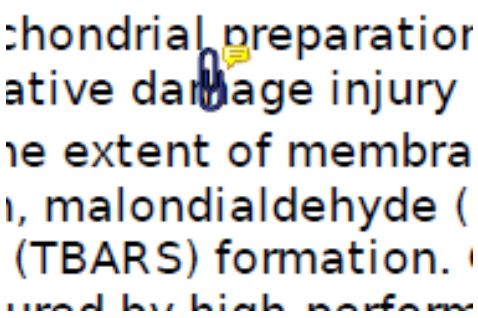
5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it:

- Click on  .
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

  The attachment appears in the right-hand panel.

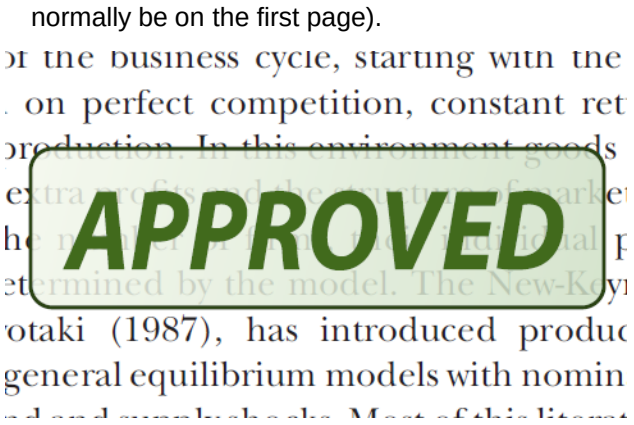


6. Add stamp Tool – for approving a proof if no corrections are required.

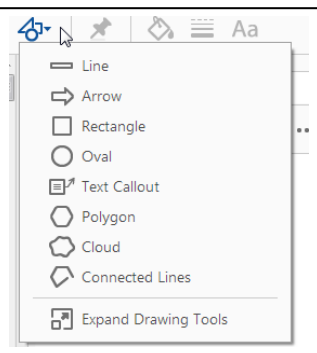
Inserts a selected stamp onto an appropriate place in the proof.

How to use it:

- Click on  .
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears. Others are shown under *Dynamic*, *Sign Here*, *Standard Business*).
- Fill in any details and then click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



Drawing tools available on comment ribbon

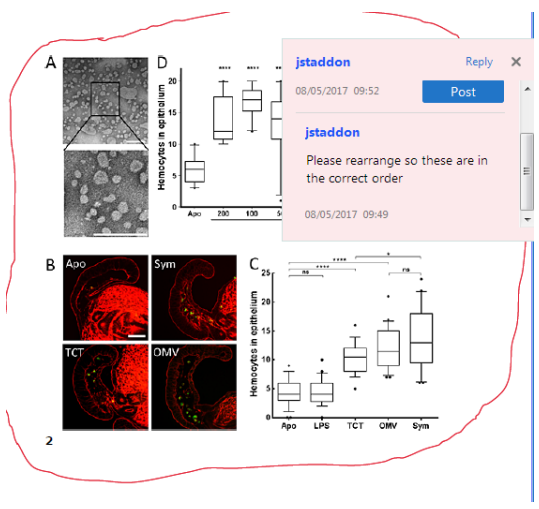


7. Drawing Markups Tools – for drawing shapes, lines, and freeform annotations on proofs and commenting on these marks.

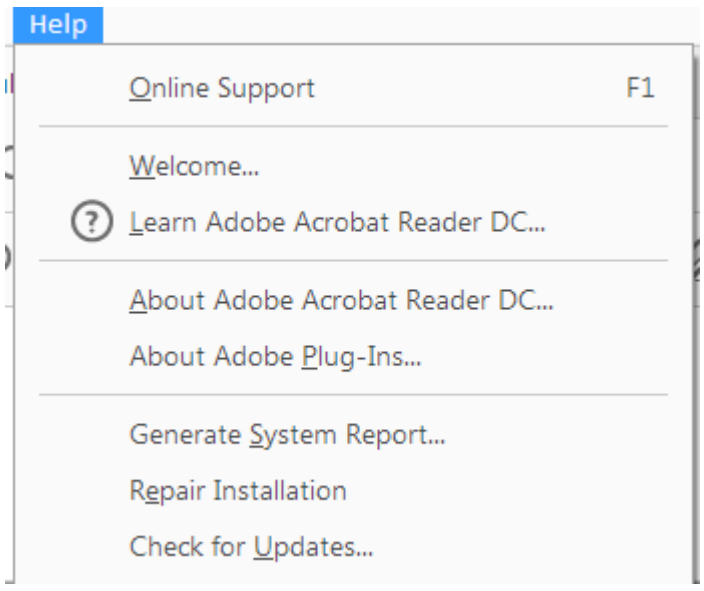
Allows shapes, lines, and freeform annotations to be drawn on proofs and for comments to be made on these marks.

How to use it:

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, right-click on shape and select *Open Pop-up Note.*
- Type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



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Protective Effects Induced by Microwave-Assisted Aqueous Harpagophytum Extract on Rat Cortex Synaptosomes Challenged with Amyloid β-Peptide

Q2 Q1 **Claudio Ferrante,[1]* Lucia Recinella,[1] Marcello Locatelli,[1] Paolo Guglielmi,[2] Daniela Secci,[2] Lidia Leporini,[1] Annalisa Chiavaroli,[1] Sheila Leone,[1] Sara Martinotti,[1] Luigi Brunetti,[1] Michele Vacca,[1] Luigi Menghini[1] and Giustino Orlando[1]**

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Harpagophytum procumbens **is a plant species that displays anti-inflammatory properties in multiple tissues. The iridoid glycosides arpagoside, harpagide, and procumbide appear to be the most therapeutically important constituents. In addition, harpagoside treatment exerted neuroprotective effects both *in vitro* and *in vivo*. Considering these findings, the aim of the present work is to explore the possible protective role of the previously described microwave-assisted aqueous extract of *H. procumbens* on rat hypothalamic (Hypo-E22) cells, and in rat cortex challenged with amyloid β-peptide (1–40). In this context, we assayed the protective effects induced by *H. procumbens* by measuring the levels of malondialdehyde, 3-hydroxykynurenine (3-HK), brain-derived neurotrophic factor, and tumor necrosis factor-α, 3-HK. Finally, we evaluated the effects of *H. procumbens* treatment on cortex levels of dopamine, norepinephrine, and serotonin. *H. procumbens* extract was well tolerated by Hypo-E22 cells and upregulated brain-derived neurotrophic factor gene expression but down-regulated tumor necrosis factor-α gene expression. In addition, the extract reduced amyloid β-peptide stimulation of malondialdehyde and 3-HK and blunted the decrease of dopamine, norepinephrine, and serotonin, in the cortex. In this context, our work supports further studies for the evaluation and confirmation of Harpagophytum in the management of the clinical symptoms related to Alzheimer's disease. Copyright © 2017 John Wiley & Sons, Ltd.**

Keywords: Harpagophytum procumbens; cell viability; oxidative stress; neurotransmitter; neuropeptide.

INTRODUCTION

Oxidative/nitrosative stress plays a key role in age-related cellular degeneration, particularly in the brain, where the increase of highly peroxide-modified unsaturated fatty acids is coupled to elevated oxygen consumption and a significant deficiency of antioxidant systems (Uttara *et al.*, 2009; Jomova *et al.*, 2010).

The high content of unsaturated fatty acids, in the brain, could be easily peroxidable by the elevated levels of reactive oxygen/nitrogen species produced by brain Q4 oxygen metabolism (Floyd and Hensley, 2002), and lipid peroxidation has been long linked to the onset of Q5 neurodegenerative processes (Marnett, 1999; Plàtenik *et al.*, 2001; Praticò, 2002). On the other hand, preclinical studies indicate clinical protective effects of antioxidants naturally present in food or supplemented in the diet of rats subjected to oxidative brain damage (Joseph *et al.*, 1999). Previously, we observed the inhibitory effects on lipid peroxidation induced by ginkgo and allium extracts (Brunetti *et al.*, 2006, 2009), and by resveratrol (Chiavaroli *et al.*, 2010), a natural compound found in grapes, on rat cortex neuronal endings (synaptosomes) challenged with β-amyloid peptide, a validated *ex-vivo* model of Alzheimer's disease (AD) (Brunetti *et al.*, 2004).

Harpagophytum procumbens, traditionally known as devil's claw or Harpagophytum, is a plant species that displays anti-inflammatory properties in multiple tissues (Chantre *et al.*, 2000; Kaszkin *et al.*, 2004). The iridoid glycosides arpagoside, harpagide, and procumbide, found in the tubers of the plant, appear to be the most therapeutically important constituents (Bradley, 1992). Harpagoside treatment exerted neuroprotective effects in rats injected with β-amyloid (1–40) peptide (Li *et al.*, 2015). Sun *et al.* (2012) also observed a protective effect exerted by harpagoside treatment on isolated mesencephalic neuron degeneration induced by MPTP/MPP$^+$. In addition, the ethyl acetate soluble fraction of the roots of Harpagophytum displayed inhibitory effects on cholinesterase, with possible therapeutic applications for the treatment of AD (Bae *et al.*, 2014). Recently, we found that a microwave-assisted aqueous extract of Harpagophytum roots (containing about 1.5% harpagoside) was able to inhibit lipid peroxidation and inflammatory markers, in colon specimens challenged with lipopolysaccharide (Locatelli *et al.*, 2017a). Considering these findings, the aim of the

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present work is to explore the possible protective effect of the previously described microwave-assisted aqueous extract of Harpagophytum on rat hypothalamic (Hypo-E22) cells. In addition, we evaluated the effects of the extract on an *ex vivo* experimental model of AD, constituted by cortex synaptosomes of young (3-month age) and old (24-month age) rats, challenged with amyloid β-peptide (1–40). In this context, we assayed the protective effects induced by Harpagophytum treatment by measuring the levels of malondialdehyde (MDA), marker of brain oxidative stress and lipid peroxidation (Mancuso *et al.*, 2012), and the activity of neuromodulators such as brain-derived neurotrophic factor (BDNF), and tumor necrosis factor (TNF)-α, 3-hydroxykynurenine (3-HK), markers of inflammation, neuroprotection and neurotoxicity, respectively (Almeida *et al.*, 2005; Maddison and Giorgini, 2015). Finally, we evaluated the effects of Harpagophytum treatment on cortex levels of dopamine (DA), norepinephrine (NE), and serotonin (5-HT), whose concentrations are decreased in the cortex of AD subjects (Romano *et al.*, 2015).

MATERIAL AND METHODS

Chemicals and standards

Harpagoside, as chemical standard used for the HPLC-PDA analyses (purity ≥95%), and 0.2-μm syringe filter were obtained from Sigma-Aldrich (Milan, Italy). Methanol and acetonitrile (HPLC-grade) were purchased from Sigma-Aldrich (Milan, Italy), while HPLC-grade acetic acid was bought from Carlo Erba Reagents (Milan, Italy). Double distilled water (Milli-Q system, Millipore, Bedford, USA) was used.

Plant material and extraction

Dry roots of *H. procumbens* DC. ex Meisn. were purchased in a local market in Namibia. It is characterized by margins partially rolled or wrinkled, a cortex with evident longitudinal wrinkles. *H. procumbens* root material was ground to a fine powder, passing through a 40 mesh to obtain a uniform granulometry and was stored in a vacuum box in the dark at 4 °C until use.

Microwave-assisted extraction was performed using an automatic Biotage Initiator™ 2.0 (Uppsala, Sweden), 2.45-GHz high-frequency microwaves, power range 0–300 W, as previously reported (Mollica *et al.*, 2016). An IR sensor probe controlled strictly the internal vial temperature. Plant powder (500 mg) was protected from the light and placed in a 20-mL sealed vessel suitable for an automatic single-mode microwave reactor. Then, 20 mL of the water was added to the sample to form a brown suspension. MAE was carried out, after a pre-stirring of 5 s, heating the sample by microwave irradiation for 8 min at 80 °C (±1 °C), followed by cooling with pressurized air. The suspension was then filtered through a 0.2-μm syringe filter, and the extraction solvent was directly injected into the HPLC system. Each extraction was performed in triplicate. Microwave-assisted extracts from the same batch powder were freshly prepared the day of the experiment, sterilized through the 0.2-μm syringe filter and immediately used for biological tests.

HPLC-PDA analyses

Extracts of *H. procumbens* were analyzed for the harpagoside quantitative determination using a reversed phase HPLC-PDA in gradient elution mode. Analyses were carried out by using a Waters liquid chromatograph equipped with a photodiode array detector, a C18 reversed-phase column (Prodigy ODS(3), 4.6 × 150 mm, 5 μm; Phemomenex, Torrance, CA, USA), an *online* degasser (Biotech 4-CH degasi compact, LabService, Anzola Emilia, Italy), a column oven set at 30 °C (±1 °C). The gradient elution was achieved by a solution of water-acetonitrile (93:7 ratio, with 3% of acetic acid) as initial conditions. The complete separation was achieved in 60 min by means of a validated method (Locatelli *et al.*, 2017b) and herein applied after evaluation of analytical performances and the absence of matrix interferences specifically for the harpagoside quantification.

In vitro studies

Hypo-E22 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (Euroclone) supplemented with 10% (*v*:*v*) heat-inactivated fetal bovine serum and 1.2% (*v*:*v*) penicillin G/streptomycin in 75-cm^2 tissue culture flask (n = 5 individual culture flasks for each condition). The cultured cells were maintained in humidified incubator with 5% CO_2 at 37 °C. For cell differentiation, cell suspension at a density of 1 × 10^6 cells/mL was treated with various concentrations (10, 50, and 100 ng/mL) of phorbol myristate acetate (PMA, Fluka) for 24 or 48 h (induction phase). Thereafter, the PMA-treated cells were washed twice with ice-cold pH 7.4 phosphate buffer solution to remove PMA and non-adherent cells, whereas the adherent cells were further maintained for 48 h (recovery phase). Morphology of cells was examined under an inverted phase-contrast microscope (Sintiprungrat *et al.*, 2010). To assess the basal cytotoxicity of *H. procumbens*, a viability test was performed on 96-microwell plates, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Cells were incubated with extracts (ranging concentration 10–200 μg/mL) for 24 h. About 10 μL of MTT (5 mg/mL) were added to each well and incubated for 3 h. The formazan dye formed was extracted with dimethyl sulfoxide and absorbance recorded as previously described (Menghini *et al.*, 2011). Effects on cell viability were evaluated in comparison with untreated control group. Following viability tests, Hypo-E22 cells were treated with Harpagophytum extract (10–50 μg/mL) for 24 h (stimulation). At the end of the stimulation period, we evaluated the gene expression of BDNF and TNF-α, as previously reported (Brunetti *et al.*, 2012). Total RNA was extracted from the cells using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and 1 μg was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reactions were

incubated in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) initially at 25 °C for 10 min, then at 37 °C for 120 min, and finally at 85 °C for 5 s. Gene expression was determined by quantitative real-time polymerase chain reaction (PCR) using TaqMan probe-based chemistry (Applied Biosystems, Foster City, CA, USA). PCR primers and TaqMan probes were obtained from Applied Biosystems (Assays-on-Demand Gene Expression Products, Rn02531967_s1 for BDNF; Rn01525859_g1 for TNF-α). β-actin (Applied Biosystems, Foster City, CA, USA, Part No. 4352340E) was used as the housekeeping gene. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. Data were elaborated with the Sequence Detection System software version 2.3 (Applied Biosystems, Foster City, CA, USA).

Ex vivo studies

Male adult Sprague–Dawley rats (200–250 g) were housed in Plexiglas cages (40 × 25 × 15 cm), two rats per cage, in climatized colony rooms (22 ± 1 °C; 60% humidity), on a 12/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, and 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Union ethical regulations on the care of animals for scientific research. According to the recognized ethical principles of 'Replacement, Refinement and Reduction of Animals in Research', colon specimens were obtained as residual material from vehicle-treated rats randomized in our previous experiments approved by Local Ethical Committee (University "G. d'Annunzio" of Chieti-Pescara) and Italian Health Ministry (Project N. 880 definitely approved by Italian Health Ministry on 24 August 2015).

Synaptosomes were prepared from a pool of frontal and parietal cortex, which are more sensitive to oxidative stress, compared with other areas such as occipital and dorsal cortex (Brunetti *et al.*, 2002). Briefly, the frontal and parietal cortex quickly dissected, homogenized in 0.32-M saccharose solution and centrifuged, first at 4000× g for 10 min, and then at 12000× g for 20 min, to isolate neuronal endings from cell nuclei and glia. The purified synaptosomes were suspended at 37 °C, under O_2/CO_2 95%/5%, pH 7.35–7.45, in Krebs–Ringer buffer (mM: NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, Tris–HCl 10, glucose 10). Then, the synaptosome suspension was divided in fractions (each containing 100 mg of tissue in 3-mL medium) that were incubated at 37 °C, under agitation for 30 min (incubation period), and treated with a pharmacological stimulus as follows: (i) Krebs–Ringer buffer (vehicle); (ii) vehicle plus oxidant stimulus [amyloid β-peptide (1–40) (1 μM)]; and (iii) vehicle plus oxidant stimulus and Harpagophytum extract (10–50 μg/mL). After the incubation period, synaptosome suspension was treated for the extraction and quantification of thiobarbituric acid reactive substances, DA, NE, 5-HT, and 3-HK. Malondialdehyde level was determined by thiobarbituric acid reactive substances method (Mihara *et al.*, 1980). Briefly, synaptosomes were added with 1% H_3PO_4 and 0.6 thiobarbituric acid, and then incubated at 96 °C for 20 min. Finally, absorbance was recorded at 532 nm, and MDA level was expressed as μg/mL. As regards DA, NE, 5-HT, and 3-HK analysis, 1 mL synaptosome suspension was added with 1 mL of 0.05-N perchloric acid containing 0.004% sodium EDTA and 0.010% sodium bisulfite, and then vortexed for 1 min and centrifuged at 4000 g for 10 min. Thereafter, supernatant was analyzed by isocratic HPLC (Jasco PU-2080 chromatographic pump) coupled to electrochemical detection consisting of ESA Coulochem III detector equipped with ESA 5014B analytical cell, as previously reported (Brunetti *et al.*, 2014; Pocivavsek *et al.*, 2012). In a first set of experiments, we analyzed DA, NE, and 5-HT levels in the samples by using the following analytical conditions. The chromatographic separation was performed by isocratic elution on Phenomenex Kinetex reverse phase column (C18, 150 × 4.6 mm i.d., 2.6 μm). The mobile phase was (10:90, *v*/v) acetonitrile and 75 mM pH 3.00 phosphate buffer containing octanesulfonic acid 1.8 mM, EDTA 30 μM, and triethylamine 0.015% *v*/v. Flow rate was 0.8 mL/min, and the samples were manually injected through a 20-μL loop. The analytical cell was set at −0.150 V, for detector 1 and at +0.300 V, for detector 2, with a range of 100 nA. Regarding 3-HK analysis, the mobile phase was consisted of 1.5% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM EDTA, and 8.9 mM sodium heptane sulfonic acid, and a flow rate of 0.8 mL/min. The HPLC column and electrode potentials were the same of DA, NE, and 5-HT analysis. Dopamine, NE, 5-HT, and 3-HK levels were expressed as ng/mL. The analyses were performed in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Means ± SEM were determined for each experimental group and analyzed by one-way analysis of variance, followed by Newman–Keuls comparison multiple test. Statistical significance was set at $p < 0.05$. As regards to gene expression analysis, the comparative $2^{-\Delta\Delta Ct}$ method was used to quantify the relative abundance of mRNA and then to determine the relative changes in individual gene expression (relative quantification) (Livak and Schmittgen, 2001). Finally, as regards the animals randomized for each experimental group, the number was calculated on the basis of the 'Resource Equation' $N = (E + T)/T$ ($10 \leq E \leq 20$) (Charan and Kantharia, 2013), according to the guidelines suggested by the 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3RS) and reported on the following web site: https://www.nc3rs.org.uk/experimental-designstatistics. In particular, N is the number of animals per treated group. E represents the degrees of freedom of the analysis of variance. T is the number of treatments. Considering that E values should be between 10 and 20, the animal number N for *ex vivo* analysis was chosen in accordance to an E value of 20. Being $E = 20$ and $T = 5$, for the evaluation, N was 5.

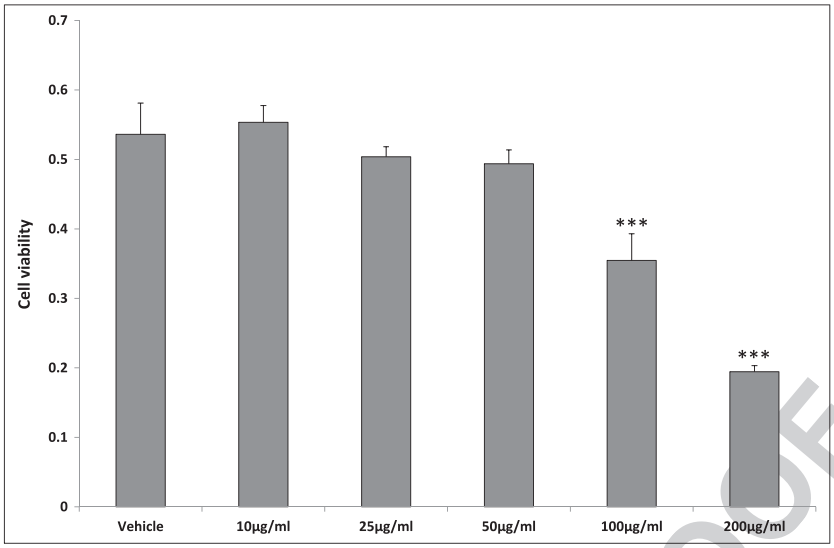

Figure 1. Effect of microwave-assisted aqueous Harpagophytum extract (10–200 µg/mL) on hypothalamic (Hypo-E22) cell line viability. Analysis of variance, $p < 0.0001$, *post hoc* $***p < 0.001$ versus vehicle-treated group.

RESULTS AND DISCUSSION

Our *in vitro* study showed that the microwave-assisted aqueous Harpagophytum extract was well tolerated by the Hypo-E22 cells in the range 10–50 µg/mL. On the other hand, a significant decrease in cell viability was observed in the range 100–200 µg/mL (Fig. 1). According to this, we evaluated the gene expression of BDNF and TNF-α, in the same cell line, following treatment with Harpagophytum extract (10–50 µg/mL), finding a marked increase of BDNF and significant decrease of TNF-α gene expression only at the concentration of 10 µg/mL of extract, corresponding to an harpagoside concentration of approximately 30 nM (Fig. 2). Our observations on Hypo-E22 cells are consistent with the study of Sheu *et al.* (2015) that observed the highest microglial cell viability at a concentration of 10-nM harpagoside.

Actually, the observed stimulatory effect on BDNF gene expression, following Harpagophytum extract treatment, could be related to the stimulation of neurotrophic factors induced by harpagoside (Sun *et al.*, 2012; Li *et al.*, 2015). Brain-derived neurotrophic factor is a neuropeptide deeply involved in neuronal survival as well as in learning and memory (Almeida *et al.*, 2005). In addition, BDNF gene expression is significantly decreased in AD patients (Peng *et al.*, 2005) and possibly related to the cognitive impairments (Siuda *et al.*, 2017). Our finding of increased BDNF gene expression is consistent with a possible protective effect related to Harpagophytum extract treatment. In order to test this hypothesis, we performed further *ex vivo* pharmacological tests on cortex synaptosomes challenged with β-amyloid peptide, in order to reproduce the neurodegenerative damages in the brain of AD patients. In the *ex vivo* experiments, Harpagophytum treatment (10–50 µg/mL) was able to

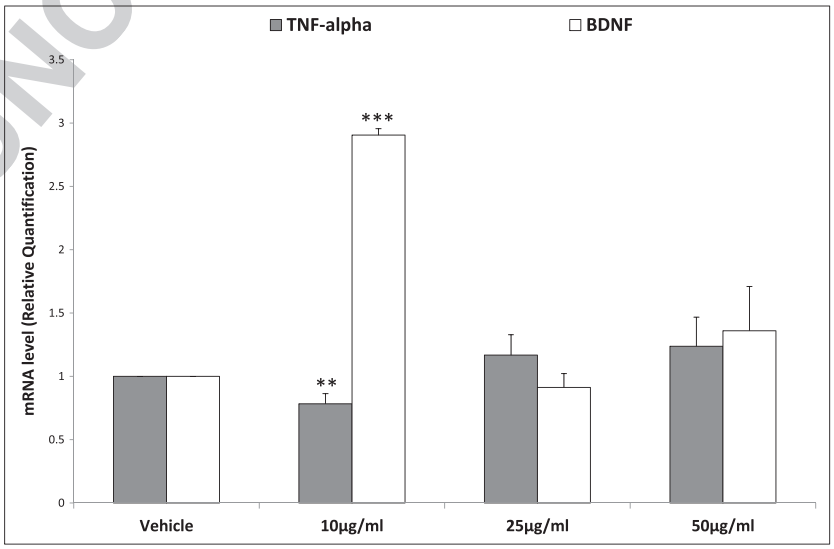

Figure 2. Effect of microwave-assisted aqueous Harpagophytum extract (10–50 µg/mL) on brain-derived neurotrophic factor (BDNF) and tumor necrosis factor (TNF-α) gene expression in hypothalamic (Hypo-E22) cell line. Analysis of variance, $p < 0.001$, *post hoc* $**p < 0.01$, $***p < 0.001$ versus respective vehicle-treated group.

significantly inhibit amyloid β-peptide-induced production of MDA and 3-HK (Figs 3 and 4), in both young and aged rats. In addition, we observed higher basal and amyloid β-peptide-induced levels of MDA and 3-HK, in aged rats, consistently with our previous observations of increased lipid peroxidation in aged rats, compared with young animals (Brunetti *et al.*, 2004). Malondialdehyde is a well-established marker of lipid peroxidation, in rat brain (Mancuso *et al.*, 2012), and previous works have also indicated the stimulatory effects of amyloid β-peptide on lipid peroxidation (Butterfield *et al.*, 1994; Cecchi *et al.*, 2007). Amyloid β-peptide could quickly accumulate near the plasma membrane and shuttle redox active metals, such as iron or copper, with consequent production of hydrogen peroxide (Rottkamp *et al.*, 2001). This results in an early and sharp increase in membrane oxidative injury. Actually, the observed inhibitory effects on MDA production could be related to the radical scavenging activities of Harpagophytum (Grant *et al.*, 2009; Schaffer *et al.*, 2016). In addition, we observed an inhibitory effect induced by Harpagophytum extract on 3-HK level, a marker of neurotoxicity synthesized by kynurenine monooxygenase (KMO) which generates free radicals in the brain (Maddison and Giorgini, 2015). Amyloid β-peptide could induce the activity of KMO, through the stimulation of proinflammatory cytokines in the brain (Maddison and Giorgini, 2015). On the other hand, AD is also characterized by reduced brain kynurenic acid level, a kynurenine metabolite that displays antioxidant and neuroprotective effects (Maddison and Giorgini, 2015). The reduced 3-HK levels, following Harpagophytum treatment, are consistent with the reduction of TNF-α gene expression, in Hypo-E22 cells, and confirm the reported inhibitory effects of Harpagophytum on TNF-α in human

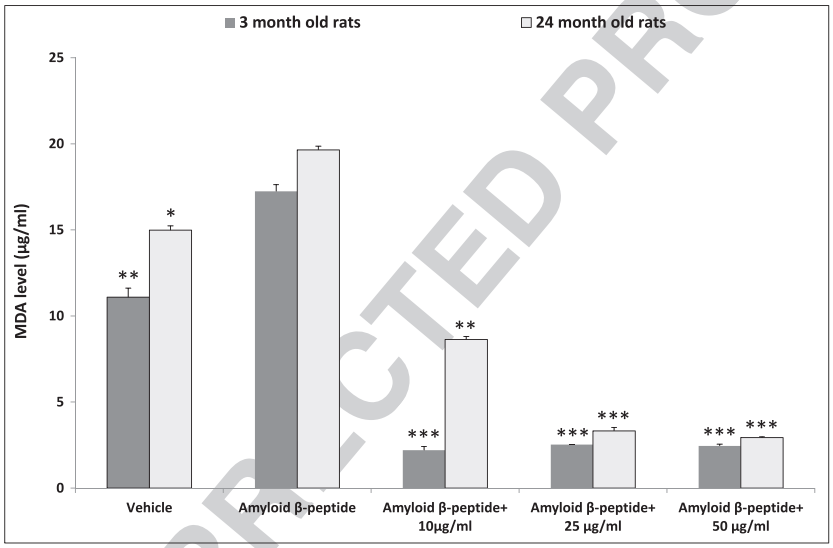

Figure 3. Effect of microwave-assisted aqueous Harpagophytum extract (10–50 µg/mL) on malondialdehyde (MDA) level (µg/mL), in cortex synaptosome of young (3-month-old) and aged (24-month-old) rats. Analysis of variance, $p < 0.05$, *post hoc* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus respective amyloid β-peptide-treated group. [Colour figure can be viewed at wileyonlinelibrary.com]

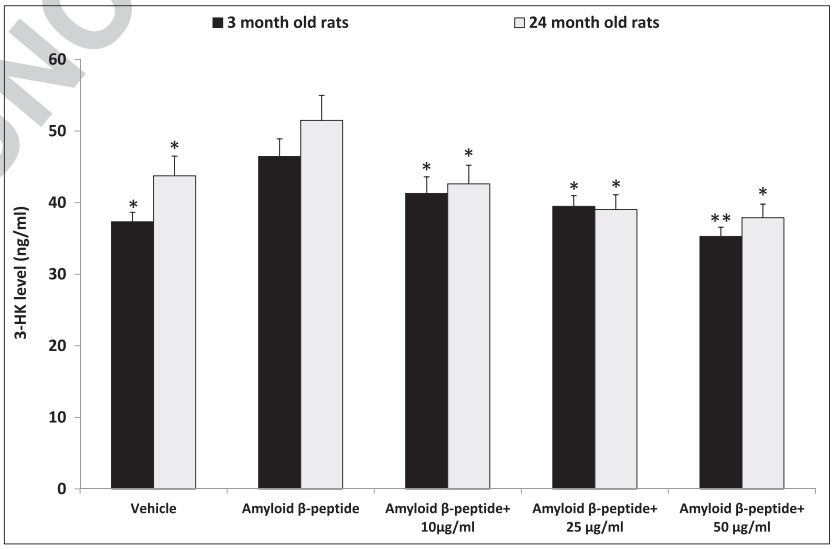

Figure 4. Effect of microwave-assisted aqueous Harpagophytum extract (10–50 µg/mL) on 3-hydroxykynurenine (3-HK) level (ng/mL), in cortex synaptosome of young (3-month-old) and aged (24-month-old) rats. Analysis of variance, $p < 0.001$, *post hoc* $^{*}p < 0.05$, $^{**}p < 0.01$ versus amyloid β-peptide-treated group. [Colour figure can be viewed at wileyonlinelibrary.com]

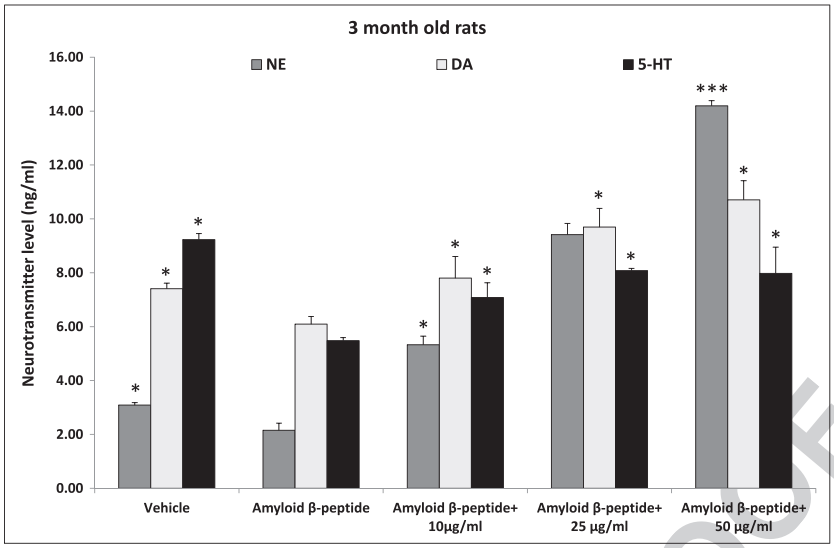

Figure 5. Effect of microwave-assisted aqueous Harpagophytum extract (10–50 μg/mL) on dopamine (DA), norepinephrine (NE), and serotonin (5-HT) level (μg/mL), in cortex synaptosome of young (3-month-old) rats. Analysis of variance, $p < 0.01$, *post hoc* $^{*}p < 0.05$, $^{***}p < 0.001$ versus amyloid β-peptide-treated group. [Colour figure can be viewed at wileyonlinelibrary.com]

monocytes (Fiebich *et al.*, 2012). Our findings of reduced MDA and 3-HK levels in cortex synaptosomes are consistent with a protective role of Harpagophytum extract on amyloid β-peptide-induced toxicity. Finally, we evaluated the possible modulatory effect of Harpagophytum extract on cortex levels of DA, NE, and 5-HT. We observed that Harpagophytum was able to contrast the reduction of DA, NE, and 5-HT induced by amyloid β-peptide, in both young and aged rats (Figs 5 and 6). In addition, aged rat synaptosomes are more sensitive to the effect of amyloid β-peptide. This is consistent with the physiological decline in DA, NE, and 5-HT levels in aged rats (Lee *et al.*, 2001). Their brain levels are also decreased in a murine model of AD and could explain, albeit partially, the onset of depression in AD patients (Romano *et al.*, 2015). On the other hand, the increase of brain monoamines induced by chronic natural antioxidant supplementation was related to improvements of age-related cognitive and motor decline (Ramis *et al.*, 2016).

Finally, as regards the dose–response relationship, we observed that Harpagophytum extract was effective in the whole dose range (10–50 μg/mL), in reducing oxidative markers and stimulating monoamine levels. This contrasting results, compared with the protective effects observed on Hypo-E22 cells, could be related, at least in part, to the higher complexity of the *ex vivo* experimental model. Brain synaptosomes are extremely sensitive to the burden of oxidative stress induced by amyloid β-peptide (Brunetti *et al.*, 2004). In addition, despite their limited viability (until 2 h following brain dissection), synaptosomes result extremely suitable to study aminergic neurotransmission, because they display the same neurotransmitters biosynthesis and release pathways as intact neuronal endings, *in vivo* (Raiteri *et al.*, 1976).

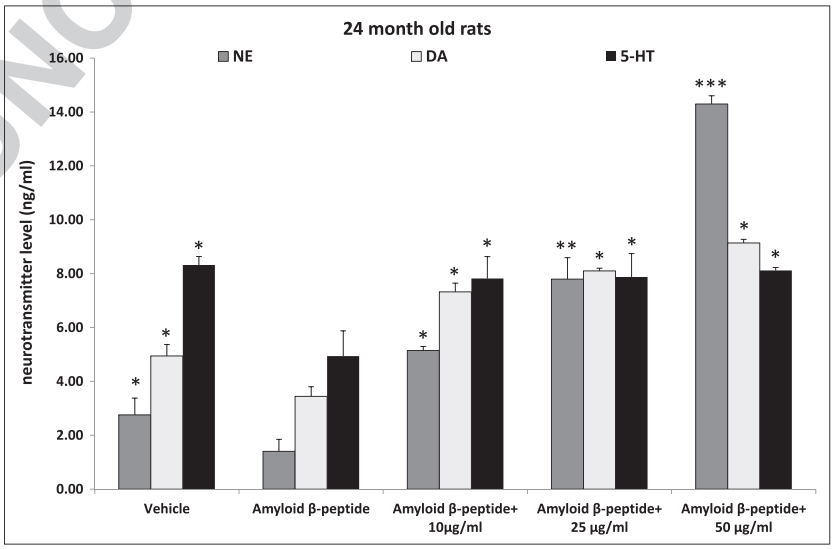

Figure 6. Effect of microwave-assisted aqueous Harpagophytum extract (10–50 μg/mL) on dopamine (DA), norepinephrine (NE), and serotonin (5-HT) level (μg/mL), in cortex synaptosome of aged (24-month-old) rats. Analysis of variance, $p < 0.01$, *post hoc* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus amyloid β-peptide-treated group. [Colour figure can be viewed at wileyonlinelibrary.com]

In conclusion, with the present study, we demonstrated that microwave-assisted aqueous Harpagophytum extract was effective in reducing the burden of oxidative stress and the consequent decrease on monoaminergic signaling, in cortex presynaptic endings challenged with amyloid β-peptide. These findings add to our previous observation of efficacy of aqueous home-made Harpagophytum extracts in blunting the burden of oxidative stress in inflamed tissues (Locatelli *et al.*, 2017a). Despite the preliminary data, our findings corroborate previous observations concerning the efficacy of harpagoside to contrast amyloid β-peptide neurotoxicity and improve cognitive and motor functions. In this context, our work supports further studies for the evaluation and confirmation of Harpagophytum in the management of the clinical symptoms related to AD.

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Conflict of Interest

Authors declare no financial/commercial conflicts of interest.

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