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Glutamate Toxicity to Differentiated Neuroblastoma N2a Cells Is Prevented by the Sesquiterpene Lactone Achillolide A and the Flavonoid 3,5,4′-Trihydroxy-6,7,3′-Trimethoxyflavone from *Achillea fragrantissima*

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Abstract Glutamate toxicity is a major contributor to the pathophysiology of numerous neurodegenerative diseases including amyotrophic lateral sclerosis and Alzheimer's disease. Therefore, protecting neuronal cells against glutamate-induced cytotoxicity might be an effective approach for the treatment of these diseases. We have previously purified from the medicinal plant *Achillea fragrantissima* two bioactive compounds which were not studied before: the sesquiterpene lactone achillolide A and the flavonoid 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone (TTF). We have shown that these compounds protect astrocytes from oxidative stress-induced cell death and inhibit microglial activation. The current study examined for the first time their effects on differentiated mouse neuroblastoma N2a cells and on glutamate toxicity. We have found that, although these compounds belong to different chemical families, they protect neuronal cells from glutamate toxicity. We further demonstrate that this protective effect might be, at least partially, due to inhibitory effects of these compounds on the levels of reactive oxygen species produced following treatment with glutamate.

Keywords Achillolide A · 3,5,4′-Trihydroxy-6,7,3′-trimethoxyflavone · Flavonoids · Sesquiterpene lactones · Mouse neuroblastoma N2a cells · Glutamate toxicity

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Introduction

Glutamate is a major excitatory neurotransmitter in the central nervous system and is involved in synaptic transmission, neuronal outgrowth, survival and plasticity, memory, learning, and behavior (Albright et al. 2000). Excessive glutamate release causes the overstimulation of glutamate receptors, which leads to a massive influx of calcium, the generation of reactive oxygen species (ROS), and subsequent death of neuronal cells. Glutamate toxicity is a major contributor to the pathophysiology of multiple neurological diseases including amyotrophic lateral sclerosis (ALS), stroke, Alzheimer's disease, Parkinson's disease, and Huntington's disease (Lewerenz and Maher 2015). Therefore, protecting neuronal cells against glutamate-induced excitotoxicity might be an effective therapeutic approach for neurodegenerative diseases.

Achillea fragrantissima (Forssk) Sch. Bip (*A. fragrantissima*) is used internally in traditional medicine of the Arabian region for the preparation of functional drinks for the treatment of various disturbances (Eissa et al. 2014; Hamdan and Afifi 2004; Mustafa et al. 1992; Segal and Dor 1987). Moreover, it was previously shown that aqueous, methanolic, and ethanolic extracts prepared from *A. fragrantissima* were well tolerated in acute and long-term (2 months) safety studies in rats when administered orally (Mandour et al. 2013). We have previously demonstrated that an extract prepared from this plant beneficially affects astrocytes (Elmann et al. 2011b) and downregulates microglial activation (Elmann et al. 2011a). Using activity-guided fractionation, we have purified and identified the bioactive compounds that are responsible for these activities in *A. fragrantissima*: a sesquiterpene lactone named achillolide A and a flavonoid named 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone (TTF) (Elmann et al. 2016; Elmann et al. 2015; Elmann et al. 2014).

Sesquiterpene lactones and flavonoids are two groups of plant secondary metabolites that exhibit a broad range of biological activities, including anti-inflammatory, antioxidant, and neuroprotective activities (Afanas'ev et al. 1989; Choi et al. 2009; Gach et al. 2015; Kim et al. 2010; Merfort 2011; Serafini et al. 2010; Song et al. 2012; Williams and Spencer 2012; Youdim et al. 2004). In the current study, we examined the effects of these compounds on glutamate toxicity to differentiated mouse neuroblastoma N2a cells.

Materials and Methods

Materials

Glutamate (≥99%, HPLC), memantine (≥98%, GC), and 2′7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM were purchased from Gibco (Paisley, UK). Glutamine, antibiotics (10,000 IU/mL penicillin and 10,000 μg/mL streptomycin), fetal bovine serum (FBS), and Trypsin-EDTA were purchased from Biological Industries (Beit Haemek, Israel); dimethyl sulfoxide (DMSO) was obtained from Applichem (Darmstadt, Germany).

Plant Material

A. fragrantissima was collected in the Arava Valley (see picture in Fig. 1). The plant was authenticated by the botanist Mrs. Mimi Ron, The Mount Scopus Botanical Garden in the Hebrew University of Jerusalem, and the voucher specimen has been kept as part of the Arava Rift Valley Plant Collection under the accession code AVPC0040.

Extraction and Isolation of Achillolide A

The dry aerial parts of *A. fragrantissima* (37 g) were homogenized and extracted with ethyl acetate (EA) (Elmann et al. 2015). Evaporation of the EA gave a brown gum that was chromatographed on Sephdex LH-20 eluted with petroleum ether/CH_2Cl_2/MeOH. Fractions containing achillolide A were combined and evaporated under vacuum, to give crude achillolide A, 290 mg. The latter was re-chromatographed by vacuum liquid chromatography (VLC) on silica gel eluted with petroleum ether EA of increasing polarity. Achillolide A (90 mg) was obtained from fraction eluted with 30% EA by evaporation of the solvent. Twice crystallization from petroleum ether/acetone mixture gave pure (98%) achillolide A (40 mg), as was determined by NMR and according to the melting point and optical activity. The structure of achillolide A is presented in Fig. 1.

Extraction and Isolation of TTF

The wild sun dried plant *A. fragrantissima* (37 g) was homogenized and extracted with ethyl acetate twice and ethyl acetate to methanol (9:1) once. The combined organic extracts were evaporated (3.8 g). The latter residue was chromatographed on a Sephadex LH-20 column, eluting with methanol to CH_2Cl_2 (1:1). These fractions were submitted to bioassay-guided fractionation by using a cellular model in which H_2O_2 was used to mimic oxidative injury and to induce astrocytic cell death (Elmann et al. 2014). A fraction of the Sephadex LH-20 column that was monitored by thin layer chromatography (TLC) and ^{1}H-nuclear magnetic resonance (NMR) and protected astrocytes from H_2O_2-induced cell death was further purified by repeated chromatographies over silica gel, using hexane with increasing proportions of ethyl acetate as eluent. TTF (38 mg, 1% dry weight) was afforded by elution with 50% ethyl acetate in hexane (strongly pH

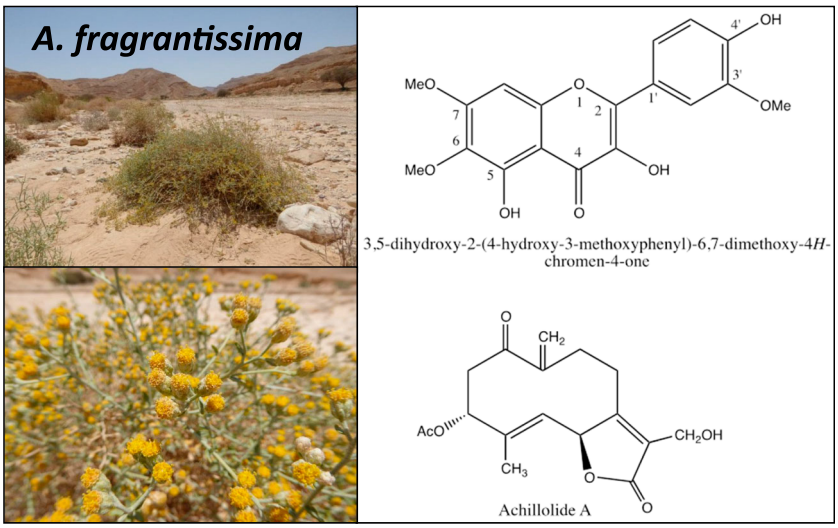

Fig. 1 The plant *A. fragrantissima* and the structures of achillolide A and 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone (TTF)

dependent). Infrared (IR) spectra were obtained with a Bruker Fourier transform infrared spectra (FTIR) Vector 22 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance-500 spectrometer. Correlation spectroscopy (COSY), heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC) experiments were recorded using standard Bruker pulse sequences. High-resolution electrospray ionization mass spectrometry (HRESIMS) measurements were performed using the instrument Waters Micromass SYNAPT HDMS mass spectrometer, time of flight (TOF). The structure of the flavonoid TTF is presented in Fig. 1.

Determination of Cytotoxicity

N2a cells were grown in a medium containing 43% DMEM (high glucose), 50% Opti-MEM, 5% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. N2a cells were re-plated at 96-well plates at a density of 1×10^4/well, in a similar medium; however, in order to induce cell differentiation by serum withdrawal (Wang et al. 2004), the concentration of FBS was reduced to 1%. Twenty-four hours later, the low serum medium of the cells was aspirated off and fresh low serum medium was added to the cells. Dilutions of glutamate, memantine, TTF, or achillolide A first in DMSO and then in the growth medium were made freshly from stock solution just prior to each experiment and were used immediately. The final concentration of DMSO in the medium was 0.2% and was not toxic to the cells. Glutamate, memantine, achillolide A, and/or TTF were added 24 h later, and cell viability was determined 20 h later using a commercial colorimetric assay (Roche Applied Science, Germany) according to the manufacturer's instructions. The absorbance was measured at 492 nm in a plate reader. The percentage of cytotoxicity was calculated according to the following equation:

$$\text{Cytotoxicity}\ (\%) = \frac{(A_{\text{treated cells}} - A_{\text{untreated cells}}) \times 100}{A_{\text{glutamate-treated cells}} - A_{\text{untreated cells}}}$$

where lactate dehydrogenase (LDH) activity in wells with untreated cells represents spontaneous cell death. In order to determine the maximum releasable LDH, untreated cells were lysed by the addition of 2% Triton X-100 (5 min, 37 °C) to the wells.

Evaluation of Intracellular ROS Levels

Intracellular ROS levels were detected using the non-fluorescent cell permeating compound, 2′7′-dichlorofluorescein diacetate (DCF-DA). N2a cells were plated onto 96-well plates (10,000 cells/0.2 μL/well) in 43% DMEM (high glucose), 50% Opti-MEM, 1% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Twenty-four hours later, cells were treated with DCF-DA (20 μM) for 30 min at 37 °C. Following incubation with DCF-DA, cultures were rinsed twice with PBS which was then replaced in fresh medium. N2a cells were then treated with glutamate in the presence or absence of TTF or achillolide A and ROS levels (fluorescence) at time zero and 20 h later were measured in a plate reader with excitation at 485 nm and emission at 520 nm.

The percentage of ROS levels was calculated according to the following equation (where F is the fluorescence):

$$\text{ROS levels}\ (\%) = \frac{\left(F_{\text{compound\&glutamate-treated cells}} - F_{\text{untreated cells}}\right) \times 100}{F_{\text{glutamate-treated cells}} - F_{\text{untreated cells}}}$$

Fluorescence in glutamate-treated cells was 31,000 ± 1000 fluorescence units (FU), and in untreated cells was ~10,000 FU.

Statistical Analysis

Statistical analyses were performed with one-way ANOVA followed by Tukey-Kramer multiple comparison tests using Graph Pad InStat 3 for windows (GraphPad Software, San Diego, CA, USA).

Results

Exposure of differentiated neuroblastoma N2a cells to 100 μM glutamate resulted in their death 20 h after exposure, as was reflected in a more than twofold increase in lactate dehydrogenate (LDH) assay (Fig. 2a) and in a 47% ± 2 damage, as was evaluated by measurement of the maximal LDH release after disruption of the cells by Triton X-100 (A_{492} = 0.95). To characterize the abilities of TTF and achillolide A (see structures in Fig. 1) to protect N2a cells against glutamate-induced cell death and to determine the optimal concentrations needed for the protective effect, cells were treated with glutamate and with different concentrations of each of these molecules. The LDH assay was used to measure cytotoxicity at 20 h after treatment. Our results showed that TTF and achillolide A exhibit a protective effect against glutamate-induced cell death, with maximal efficacy (95 and 86% protection, respectively) at concentrations of 1.4 and 1.6 μM, respectively (Fig. 2b). At their maximally effective concentrations, there was no significant difference ($P > 0.05$) between the protective effects induced by the two compounds, and their IC50 values for inhibition of cell death were very similar as well: 389 nM for TTF and 387 nM for achillolide A. At these concentrations, memantine was less effective and inhibited the glutamate toxicity only by 39%.

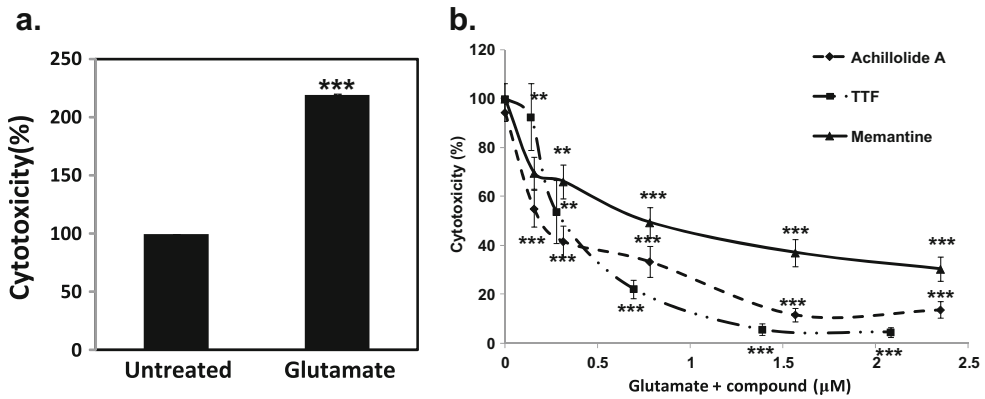

Fig. 2 TTF and achillolide A protect N2a neuroblastoma cells from glutamate-induced cell death. **a** Cells were treated with 100 μM glutamate and cell death was determined 20 h later by the LDH method. The results are means ± SEM of six experiments ($n = 48$). The absorbance value of untreated cells was 0.265 ± 0.005. ***$P < 0.001$, compared to untreated cells. The maximal LDH release after disruption of the cells by Triton X-100 was A_{492} = 0.95 ± 0.03 as was measured in five experiments ($n = 17$). **b** Cells were treated with different concentrations of TTF, achillolide A, or memantine (as a control drug). Glutamate was added and cell death was determined 20 h later by the LDH method. The results are means ± SEM of two experiments ($n = 16$). **$P < 0.01$, ***$P < 0.001$, compared to cells that were treated with glutamate only

It should be noted that at all concentrations tested, the cytotoxicities of these compounds by themselves were lower than the spontaneous cell death as was determined by the LDH assay (Table 1).

The protective activities of TTF and achillolide A were compared to that of memantine, which is used as a drug for the treatment of Alzheimer's disease. The maximally effective concentration of memantine (2.35 μM) was significantly less effective than TTF and achillolide A ($P < 0.001$ and $P < 0.01$, respectively) and provided only 70% protection (Fig. 2b).

Glutamate-induced cell death is accompanied by an increase in ROS levels, as has been described previously (Vergun et al. 2001). We therefore considered the possibility that TTF and achillolide A might protect the cells from glutamate-induced cell death by reducing the levels of ROS that are induced by glutamate. To assess the intracellular levels of ROS, cells were preloaded with the ROS indicator DCF-DA. As shown in Fig. 3a, in our experimental system, 100 μM of glutamate caused a 2.9-fold increase in ROS levels after 20 h. The elevation is ROS levels was more pronounced after 20 h than after 4 h of treatment with glutamate (Fig. 3b), and therefore the following experiments were performed under these experimental conditions (100 μM, 20 h of treatment). To examine the effect of achillolide A and TTF on glutamate-induced ROS levels, cells were treated with various concentrations of these compounds concomitant with the application of glutamate, as was done in the cytotoxicity experiments. ROS formation was assessed by examining fluorescence 20 h later. As can be seen in Fig. 3c, TTF was much more efficient (IC50 = 332 nM) in reducing glutamate-induced ROS than achillolide A, which had no inhibitory effect at this concentration.

Discussion

Substances that can protect neuronal cells from glutamate toxicity and oxidative stress are potential tools for treatment of neurodegenerative diseases like Alzheimer's diseases and ALS. In the present study, we have shown for the first time that the flavonoid TTF and the sesquiterpene lactone achillolide A, two natural compounds we have isolated from *A. fragrantissima*, can protect differentiated mouse neuroblastoma N2a cells from glutamate-induced cell death and

Table 1 Achillolide A and TTF are not cytotoxic to differentiated N2a cells

TTF										
Concentration (μM)	0	0.14	0.28	0.70	1.39	2.08	8.33	27.76	41.64	55.52
Cytotoxicity (% ± SEM)	2.07 ± 1.03	0.29 ± 0.11	0.38 ± 0.25	0.36 ± 0.32	0.11 ± 0.11	1.54 ± 1.15	0.00 ± 0.00	0.20 ± 0.04	0.22 ± 0.05	0.47 ± 0.08
Achillolide A										
Concentration (μM)	0	0.16	0.31	0.78	1.57	2.35	15.6	31.2	46.8	62.4
Cytotoxicity (% ± SEM)	2.07 ± 1.03	0.00 ± 0.00	0.07 ± 0.05	0.72 ± 0.72	0.17 ± 0.17	2.17 ± 1.79	0.02 ± 0.02	0.38 ± 0.10	0.46 ± 0.11	0.94 ± 0.16

Cytotoxicity was determined 20 h later. The values were calculated relatively to the total releasable LDH from the cells (A_{492} = 2.01)

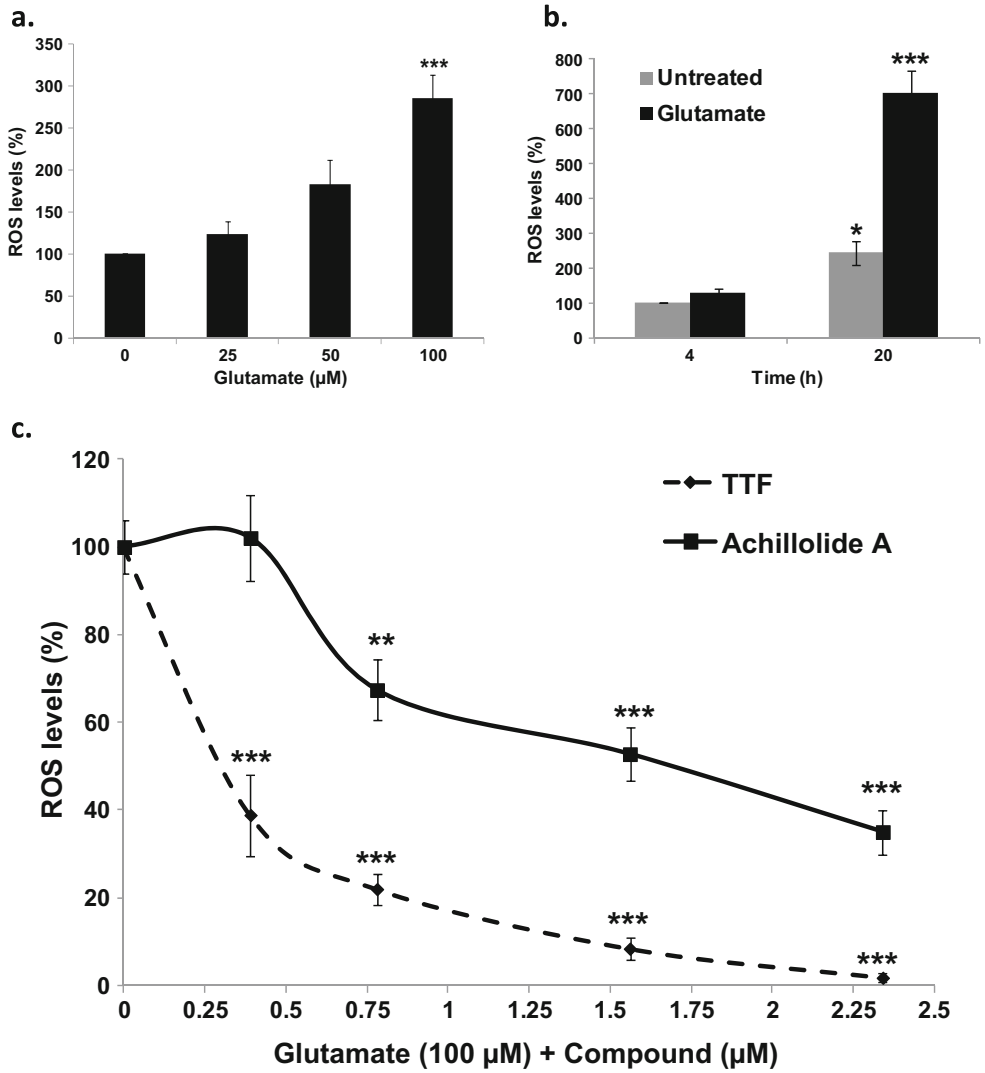

Fig. 3 TTF and achillolide A attenuate glutamate-induced ROS levels in N2a neuroblastoma cells. Cells were preloaded with DCF-DA for 30 min and washed. **a** Cells were treated with different concentrations of glutamate, and the fluorescence intensity representing ROS levels was measured 20 h later. Fluorescence intensity (FU) in untreated cells (3837 ± 460) was considered as 100%. The results represent the means ± SEM of two experiments (n = 16). ***P < 0.001, when compared to untreated cells. **b** Cells were treated with 100 μM of glutamate, and the fluorescence intensity representing ROS levels was measured 4 and 20 h later. FU in untreated cells after 4 h of incubation (6074 ± 6) was considered as 100%. The results represent the means ± SEM of five experiments (n = 40). *P < 0.05, ***P < 0.001, when compared to untreated cells after 4 h of incubation. **c** Cells were preloaded with DCF-DA for 30 min and washed and treated with various concentrations of TTF or achillolide A. Glutamate (100 μM) was added to the culture and the fluorescence intensity representing ROS levels was measured 20 h later. The results represent the means ± SEM of two experiments (n = 16). **P < 0.01, ***P < 0.001, when compared to cells that were treated with glutamate only. Fluorescence (FU; 31,000 ± 1000) in glutamate-treated cells was considered as 100%

attenuate the intracellular accumulation of ROS following treatment with glutamate. Further studies are needed to demonstrate similar effects in neuronal cells.

Interestingly, in addition to its protective activity against glutamate toxicity, we have previously shown that achillolide A inhibits the secretion of glutamate from LPS-activated microglial cells by 80% and, therefore, may further reduce the neuronal damage (Elmann et al. 2015).

No previous study has investigated the effects of TTF and achillolide A neither on neuroblastoma N2a cells nor in other models for neuronal cells nor on glutamate toxicity. Moreover, this is the first study to demonstrate a protective effect of any sesquiterpene lactone against glutamate toxicity. The protective effects of other flavonoids against glutamate toxicity have been reported previously (Chen et al. 2011; Lee et al. 2010; Shimmyo et al. 2008).

It is also interesting to note that although TTF and achillolide A belong to different chemical families, they were similarly active in protecting N2a cells against glutamate toxicity, which could be at least partially due to the inhibition of intracellular ROS generated from glutamate. We have previously demonstrated the ability of TTF (Elmann et al. 2014)

and achillolide A (Elmann et al. 2016) to attenuate oxidative stress-induced intracellular ROS levels in astrocytes. Although TTF and achillolide A were similarly active in protecting N2a cells from glutamate toxicity, TTF was much more effective in reducing the glutamate-induced intracellular levels of ROS. This observation is in accordance with the results of our previous studies, in which TTF was purified from *A. fragrantissima* by activity-guided fractionation based on its ability to protect against oxidative stress (Elmann et al. 2014) and achillolide A was purified from *A. fragrantissima* by activity-guided fractionation based on its ability to reduce microglial activation (Elmann et al. 2015).

Alzheimer's patients are usually treated with memantine alone or in combination with acetylcholinesterase inhibitors. Since memantine was shown to be less protective against glutamate toxicity than achillolide A and TTF, a combination therapy involving achillolide A and TTF might be effective, if these substances fulfill all of the necessary requirements in clinical trials.

Based on our results, and in light of the oral safety studies of extracts prepared from *A. fragrantissima* (Mandour et al. 2013), it is proposed that TTF, achillolide A, or their combination be further evaluated for the possibility of their use as drugs for the treatment of neurodegenerative diseases in which glutamate toxicity and oxidative stress play important roles.

A. fragrantissima, *Achillea fragrantissima* (Forssk) Sch. Bip; DCF-DA, 2′7′-dichlorofluorescein diacetate; LDH, lactate dehydrogenase; ROS, reactive oxygen species; TTF, 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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