



## Multiple pharmacological and toxicological investigations on *Tanacetum parthenium* and *Salix alba* extracts: Focus on potential application as anti-migraine agents



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### ARTICLE INFO

#### Keywords:

*Salix alba*

*Tanacetum parthenium*

Cortical spreading depression

Apoptosis

Serotonin

Proteomic analysis

### ABSTRACT

Migraine is one of the most common neurological disorder, which has long been related to brain serotonin (5-HT) depletion and neuro-inflammation. Despite many treatment options are available, the frequent occurrence of unacceptable adverse effects further supports the research toward nutraceuticals and herbal preparations, among which *Tanacetum parthenium* and *Salix alba* showed promising anti-inflammatory and neuro-modulatory activities.

The impact of extract treatment on astrocyte viability, spontaneous migration and apoptosis was evaluated. Anti-inflammatory/anti-oxidant effects were investigated on isolated rat cortexes exposed to a neurotoxic stimulus. The lactate dehydrogenase (LDH) release, nitrite levels and 5-HT turnover were evaluated, as well. A proteomic analysis was focused on specific neuronal proteins and a fingerprint analysis was carried out on selected phenolic compounds.

Both extracts appeared able to exert *in vitro* anti-oxidant and anti-apoptotic effects. *S. alba* and *T. parthenium* extracts reduced LDH release, nitrite levels and 5-HT turnover induced by neurotoxic stimulus. The down-regulation of selected proteins suggest a neurotoxicity, which could be ascribed to an elevated content of gallic acid in both *S. alba* and *T. parthenium* extracts.

Concluding, both extracts exert neuroprotective effects, although the downregulation of key proteins involved in neuron physiology suggest caution in their use as food supplements.

### 1. Introduction

Migraine is one of the most common neurological disorder, whose prevalence ranges from 8 to 14.7% and serotonin (5-HT) has long been involved in its pathophysiology, with clinical evidences suggesting tight relationships between migraine attacks and neurotransmitter levels (Hering et al., 1993). Consistent with these findings, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptor activation revealed to play a key role in the control of acute migraine attacks (Lance, 1991). Conversely, the blockade of 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors resulted effective in prophylaxis therapy (Massiou and Bousser, 2005). The involvement of trigemino-vascular system in the

mechanism of pain (Noseda and Burnstein, 2013), and the role of neurogenic inflammation in pain pathogenesis (Edvinsson et al., 2018) in migraine were already demonstrated. Although the origin site and mechanisms at the basis of migraine are still largely debated, the cortical spreading depression (CSD), a supra-physiological and toxic depolarizing phenomenon, appeared to be a possible link between 5-HT depletion and trigeminal nociception (Supornsilpchai et al., 2006).

The current treatment for migraine can be divided in acute aborting attack treatment and prophylactic protocol, the latter aimed to reduce frequency, duration and severity of attacks (Terme et al., 2011). Recommended drugs for acute attack treatment include analgesics,

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<https://doi.org/10.1016/j.fct.2019.110783>

Received 26 July 2019; Received in revised form 11 August 2019; Accepted 21 August 2019

Available online 03 September 2019

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nonsteroidal anti-inflammatory drugs, and triptans (Lupi et al., 2019), whereas anti-epileptics, anti-depressants and anti-hypertensives are commonly used as preventive medications (Loder and Rizzoli, 2018). Although numerous treatment options are possible, the frequent occurrence of unacceptable adverse effects (Wider et al., 2015) supports the search for alternative medications as nutraceuticals and herbal preparations, which could display efficacy alongside with a more acceptable profile of side effects (Sangermani and Boncimino, 2017).

To this regard, *Tanacetum parthenium* represents one of the well-characterized plants for migraine prophylaxis, that showed efficacy in both adult and child migraineurs (Guilbot et al., 2017; Moscano et al., 2019). *T. parthenium* belongs to Asteraceae family and has been traditionally used as anti-pyretic, analgesic and anti-inflammatory (Pareek et al., 2011). The active phytochemical compounds include flavonoids, volatile oils and parthenolide, whose inhibition of prostaglandin and nitric oxide synthesis results in an anti-inflammatory activity (Pareek et al., 2011; Materazzi et al., 2013). The effects of *T. parthenium* were also investigated in combination with anti-inflammatory vitamins and other herbal extracts, including magnesium, coenzyme Q10 and *Salix alba* (Guilbot et al., 2017; Shrivastava et al., 2006). Interestingly, *S. alba*, when orally administered, showed a modulatory activity on 5-HT pathway, being able to reduce 5-HT turnover in the rat hippocampus (Ulrich-Merzenich et al., 2012). However, the mechanism of action at the basis of anti-migraine efficacy still needs to be clarified.

The protective effects of two *T. parthenium* and *S. alba* extracts were investigated in *in vitro* and *ex vivo* experimental paradigms, in order to improve the knowledge about the use of herbal extracts as innovative preventive strategy against migraine attacks. Particularly, the impact of extract treatment on astrocyte viability, spontaneous migration and apoptosis was investigated. In addition, anti-inflammatory/antioxidant effects were evaluated on rat cortex specimens exposed to a neurotoxic stimulus ( $K^+$  60 mM Krebs-Ringer buffer), in order to reproduce the burden of oxidative stress and inflammation occurring in CSD. To this regard, the levels of specific biomarkers of oxidative stress and inflammation, including lactate dehydrogenase (LDH) and nitrites, were assayed in rat cortexes exposed to CSD and treated with extracts. The effects of the extracts were also evaluated in the same experimental conditions on cortex 5-HT turnover, which is usually significantly increased during CSD (Supornsilpchai et al., 2006). Potential toxicological effects were explored by evaluating the impact of extract treatment on the levels of specific proteins by a validated untargeted proteomic analysis. Finally, in order to provide a better interpretation of the observed pharmacotoxicological effects, a fingerprint analysis was carried out on selected phenolic compounds, including gallic acid, catechin, epicatechin and resveratrol.

## 2. Materials and methods

### 2.1. Pharmacognostic studies

#### 2.1.1. Extract preparation

Commercial *S. alba* and *T. parthenium* water extracts were kindly provided as dried material by Cristalfarma S.r.l. (Milan, Italy). The extracts were rehydrated in a Trans-sonic T460 ultrasonic bath supplied by Elma (Singen, GER) for 10 min at room temperature and full power (35 kHz), as previously described (Menghini et al., 2018).

#### 2.1.2. High performance liquid chromatography (HPLC)-fluorimetric fingerprint analysis

*S. alba* and *T. parthenium* extracts (5 µg/mL) were analyzed for the phenol quantitative determination using a reversed phase HPLC-fluorimetric in gradient elution mode as recently described. Analyses were carried out by using a liquid chromatograph (MOD. 1525, Waters Corporation, Milford MA, USA) equipped with a fluorimetric detector (MOD. 2475, Waters Corporation), a C18 reversed-phase column (Acclaim™ 120, 3 µm, 2.1 × 100 mm, Dionex Corporation, Sunnyvale,

**Table 1**  
DPPH radical scavenging effects of the extracts.

DPPH % scavenging activity ± SD		
Concentration (µg/mL)	<i>S. alba</i>	<i>T. parthenium</i>
400	65.4 ± 1.1	Not Tested
200	46.3 ± 0.3	80.6 ± 1.1
100	29.0 ± 0.5	82.5 ± 1.1
50	20.0 ± 0.3	60.3 ± 1.3
25	13.6 ± 0.8	37.7 ± 2.7

**Table 2**  
Ferric reducing power of the extracts.

Concentration (µg/mL)	<i>S. alba</i>	<i>T. parthenium</i>
800	0.565 ± 0.014	Not Tested
400	0.304 ± 0.008	Not Tested
200	0.189 ± 0.011	0.829 ± 0.009
100	0.099 ± 0.004	0.438 ± 0.025
50	Not Tested	0.249 ± 0.010
25	Not Tested	0.150 ± 0.006
12.5	Not Tested	0.094 ± 0.004

**Table 3**  
Phenol content of the extracts.

Phenolic compound	<i>S. alba</i> µg/mg extract	<i>T. parthenium</i> µg/mg extract
Gallic acid	113.83 ± 10.24	724.71 ± 28.29
Catechin	9.72 ± 1.17	2.26 ± 0.18
Epicatechin	11.59 ± 0.70	1.17 ± 0.16
Resveratrol	Not Detected	7.92 ± 0.89

CA, USA), an *on-line* degasser (Biotech 4-CH degasi compact, LabService, Anzola Emilia, Italy). The gradient elution was achieved by a mobile phase methanol-acetic acid-water (10:2:88, v/v) as solvent A and methanol-acetic acid-water (10:2:88, v/v) as solvent B, in agreement with an already published paper (Rodríguez-Delgado et al., 2001). Accordingly to the same authors,  $\lambda_{ex} = 278$  nm and  $\lambda_{em} = 360$  nm were selected in order to analyze the following phenolic compounds: gallic acid, catechin, epicatechin and resveratrol.

#### 2.1.3. DPPH radical scavenging assay

The free radical scavenging activity of the samples was measured using the stable DPPH radical, according to previously reported methods (Vladimir-Knežević et al., 2011; Kindl et al., 2015). Briefly, a 0.1 mM solution of DPPH in ethanol was prepared and 0.5 mL of this solution was added to 1.5 mL of the sample whose concentrations ranged from 25 to 400 µg/mL. The absorbance was measured at 517 nm following an incubation of 30 min in the dark at room temperature. The capability of scavenging the DPPH radicals was calculated using the following equation: (%) =  $(1 - A_1/A_0) \times 100$ , where  $A_0$  is the absorbance of the control reaction and  $A_1$  is the absorbance in the presence of sample.

#### 2.1.4. Reducing power assay

The reducing power of the extracts were evaluated with a method previously described by Vladimir-Knežević and collaborators (2011). An aliquot of each sample (1.0 mL) at various concentrations (12.5–800 µg/mL) was mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 mL) and 1% potassium ferricyanide (2.5 mL), and incubated at 50 °C for 20 min. Following the addition of 10% trichloroacetic acid (2.5 mL), an aliquot of the reaction mixture (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron(III) chloride (0.5 mL), and the absorbance was measured at 700 nm using an appropriate blank. The IC50 was calculated as the sample concentration providing an absorbance of

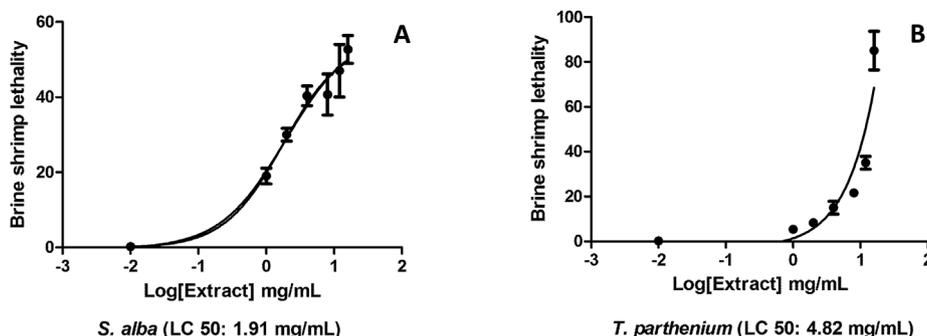


Fig. 1. Effects of water *S. alba* and *T. parthenium* extracts (0.1–16 mg/mL) on *Artemia salina* Leach viability (Brine shrimp lethality test). Data are means  $\pm$  SD of three experiments performed in triplicate.

0.5 at 700 nm.

## 2.2. Biological studies

### 2.2.1. *Artemia salina* lethality bioassay

*Artemia salina* Leach lethality bioassay was performed as previously reported (Ferrante et al., 2019). Briefly, brine shrimp larvae were bred at 25–28 °C for 24 h in presence of *S. alba* and *T. parthenium* extracts (0.1–20 mg/mL) dissolved in incubation medium (artificial sea water). After an incubation period of 24 h with extracts, the number of surviving shrimps was evaluated, and the mortality percentage was calculated with the following equation:  $((T - S)/T) \times 100$ , where T and S are the total number of incubated larvae and survival nauplii, respectively. Experiments were carried out in triplicate.

### 2.3. Cell culture and treatment

CTX-TNA2 rat astrocyte cell line was purchased from the European Collection of Cell Cultures (cat. 98102213, ECACC, Sigma-Aldrich, St Louis, MO, USA) and maintained in DMEM (cat. ECM0728L) supplemented with 10% of FBS (cat. ECS0180L) and penicillin-streptomycin ( $100 \mu\text{g mL}^{-1}$ ) (cat. ECB3001D, all from EuroClone SpA Life-Sciences-Division, Milano, Italy) according to the EACC's instructions. Cell were grown at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. When indicated the cells were treated with H<sub>2</sub>O<sub>2</sub> 300  $\mu\text{M}$  for 3 h and different concentrations of *S. alba* (100, 130, 200  $\mu\text{g/mL}$ ) and *T. parthenium* (40, 60 and 80  $\mu\text{g/mL}$ ) water extracts.

#### 2.3.1. Cell viability assay

Cell viability was measured by MTT (3 [4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) growth assay (cat. M2128, Sigma-Aldrich) as previously described (Sancilio et al., 2016).

#### 2.3.2. Cell migration assay

A wound was generated in confluent cultures of CTX-TNA2 cells using a sterile p200 tip and cells were treated with 130  $\mu\text{g/mL}$  *S. alba* and 60  $\mu\text{g/mL}$  *T. parthenium*. Photographs were taken 0, 8 and 24 h later, using a Zeiss Vert.A1 (10X magnification). The width of wounds was measured using the ZEN 2 (Zeiss, Oberkochen, Germany). Wound closure was determined by the ratio between the wound widths at 8 and 24 h and the initial wound widths (0 h) of each experimental points. Three independent experiments were performed.

#### 2.3.3. Annexin-V/PI detection of apoptotic cells in flow cytometry

To assess apoptosis, a commercial FITC Annexin V apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) was used according to Di Nisio et al. (2016).

#### 2.3.4. Ex vivo cortical spreading depression (CSD) paradigm

Sprague-Dawley rats (200–250 g) were housed in Plexiglass cages

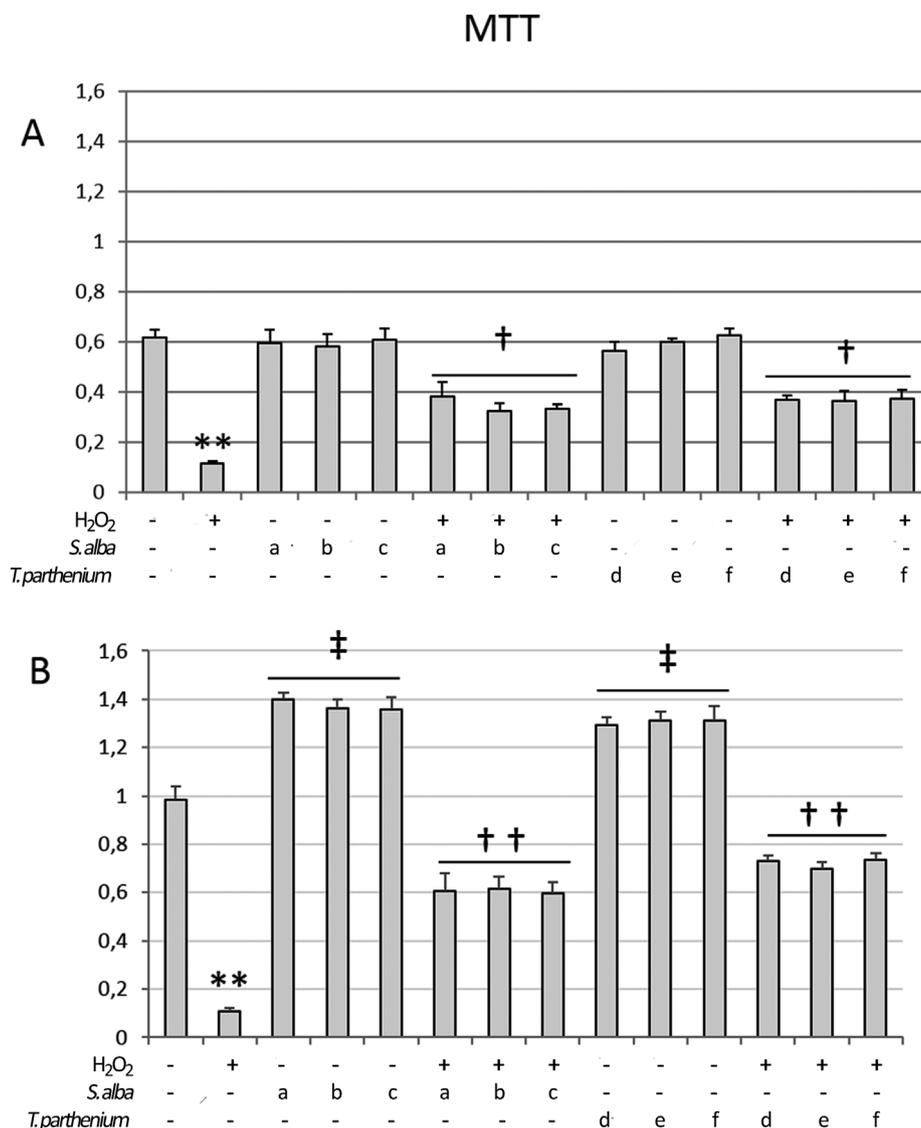
(40 cm  $\times$  25 cm  $\times$  15 cm), two rats per cage, in climatized colony rooms ( $22 \pm 1$  °C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00–19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were sacrificed by CO<sub>2</sub> inhalation (100% CO<sub>2</sub> at a flow rate of 20% of the chamber volume per min) and cortex specimens were immediately collected and maintained in thermostatic shaking bath at 37 °C for 1 h (incubation period), in Krebs-Ringer buffer at different K<sup>+</sup> concentrations (3, 15, 60 mM), as recently described (Orlando et al., 2019). During incubation, cortex specimens were exposed to *S. alba* (130  $\mu\text{g/mL}$ ) and *T. parthenium* (60  $\mu\text{g/mL}$ ). Afterwards, individual cortex slices were homogenized in perchloric acid solution (0.05 M) in order to extract and quantify 5-HT and 5-hydroxyindolacetic acid (5HIAA) (ng/mg wet tissue) via HPLC coupled to electrochemical detection, as previously reported (Brunetti et al., 2013). Additionally, a colorimetric evaluation of LDH release and nitrite level was carried out (Chichiricco et al., 2019).

#### 2.3.5. Protein extraction and filter-aided sample preparation

After protein quantification, a volume corresponding to 50  $\mu\text{g}$  of proteins was loaded onto a Nanosep 10-kDa-cutoff filter (Pall Corporation – Michigan City, Michigan USA) and digested accordingly to the protocol we routinely use in our laboratory, adapted from Distler and colleagues (2006). Briefly, each sample was washed twice with 200  $\mu\text{L}$  Urea buffer (8 M Urea, 100 mM Tris pH 8.5 in milliQ water) to remove the detergents present in the lysis buffer. The proteins on the filter were subsequently reduced and alkylated by adding 100  $\mu\text{L}$  of DTT solution (8 mM Dithiothreitol in Urea buffer) and 100  $\mu\text{L}$  of IAA solution (50 mM Iodoacetamide in Urea buffer). For protein digestion, the buffer was exchanged with 50 mM Ammonium Bicarbonate, before adding trypsin to a ratio of 1:50 (enzyme:substrate). The reaction was carried on for 16 h at 37 °C, and the mixture of peptides was collected by centrifugation, acidified with 10% trifluoroacetic acid and stored at  $-20$  °C until analysis. The detailed description of mass spectroscopy analysis is reported as “Supplementary Proteomic Analysis”.

#### 2.3.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Means  $\pm$  S.E.M. were determined for each experimental group and analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keuls comparison multiple test. Statistical significance was set at  $P < 0.05$ . As regards the animals randomized for each experimental group, the number was calculated on the basis of the “Resource Equation”  $N = (E + T)/T$  ( $10 \leq E \leq 20$ ; <https://www.nc3rs.org.uk/experimental-designstatistics>).



**Fig. 2.** MTT assay of CTX-TNA2 cell line exposed to different concentrations of *T. Parthenium* and *S. alba* extracts:

a: 100 µg/mL;

b: 130 µg/mL;

c: 200 µg/mL;

d: 40 µg/mL;

e: 60 µg/mL;

f: 80 µg/mL;

A: 24 h

\*\* H<sub>2</sub>O<sub>2</sub> vs ctrl P < 0.01

† *S. alba* H<sub>2</sub>O<sub>2</sub> + 100, 130 e 200 µg/mL and *T. parthenium* H<sub>2</sub>O<sub>2</sub> + 40, 60 and 80 µg/mL vs H<sub>2</sub>O<sub>2</sub>

P < 0.05

B: 48 h

\*\* H<sub>2</sub>O<sub>2</sub> vs ctrl P < 0.01

‡ *S. alba* 100, 130 e 200 µg/mL and *T. parthenium* 40, 60 and 80 µg/mL vs Ctrl P < 0.05

†† *S. alba* H<sub>2</sub>O<sub>2</sub> + 100, 130 e 200 µg/mL and *T. parthenium* H<sub>2</sub>O<sub>2</sub> + 40, 60 and 80 µg/mL vs H<sub>2</sub>O<sub>2</sub>

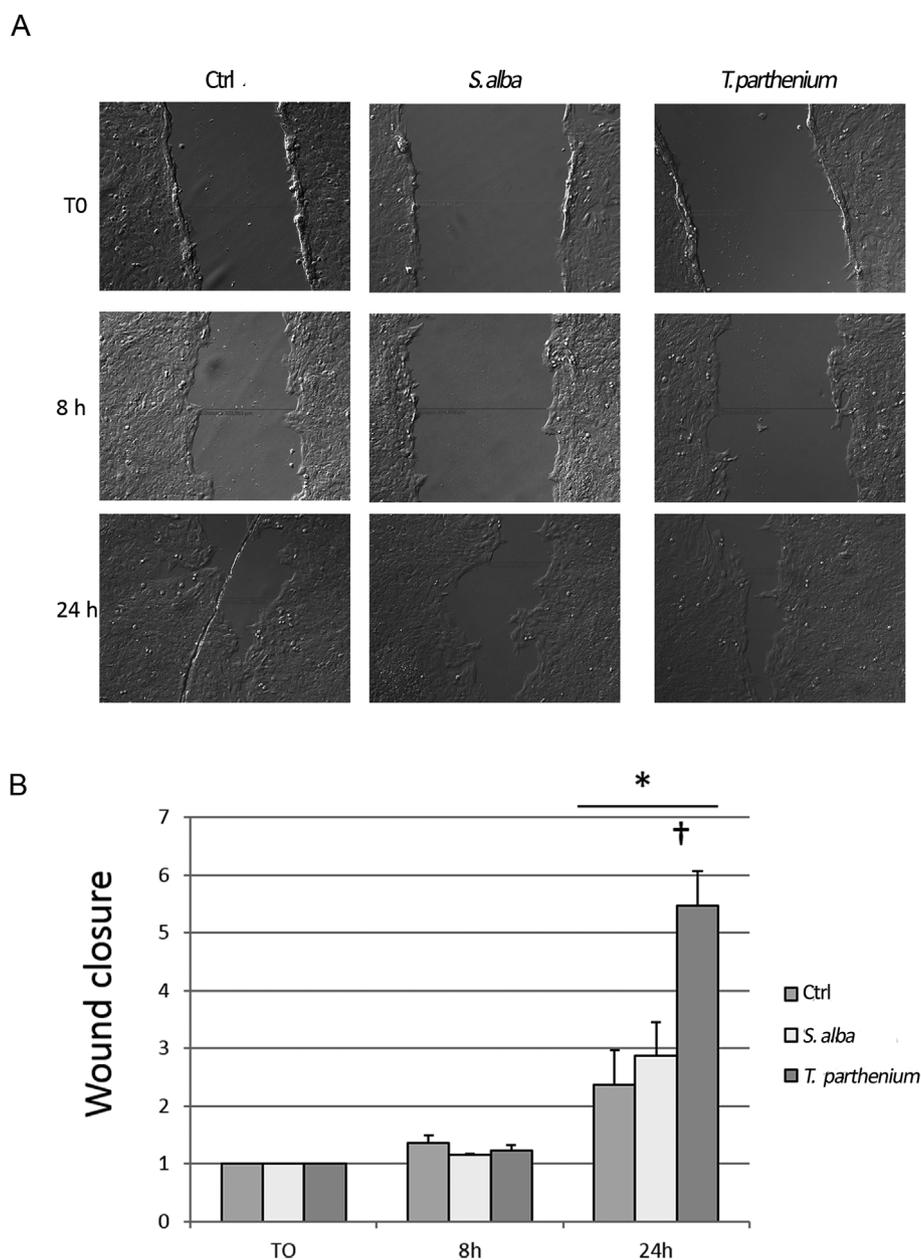
P < 0.01.

### 3. Results

#### 3.1. Pharmacognostic studies

The anti-radical/reducing properties of the extracts were evaluated testing their reducing capacity and free radical-scavenging ability. The latter was evaluated through the DPPH test and both the extracts taken into consideration in this study showed a concentration-dependent decrease of the DPPH generation (Table 1). Their DPPH-radical-

scavenging activity was quantified calculating the IC<sub>50</sub> values, which are inversely related to the anti-oxidant capacities of the plant extracts. The *T. parthenium* extract, with an IC<sub>50</sub> value of 39 ± 2 µg/mL, showed a more effective activity than *S. alba* extract, whose IC<sub>50</sub> is 239 ± 5 µg/mL. The reducing capacity of the two extracts was determined by the potassium ferricyanide reduction method (Kindl et al., 2015). Table 2 shows the reducing power for different concentrations of tested extracts. The ability to reduce iron(III) ions demonstrated by both extracts increased with concentration and consistently with the DPPH test, the



**Fig. 3.** Wound healing assay of CTX-TNA2 cell line exposed to *T. parthenium* and *S. alba* extracts.

A: Representative images (10 X)

B: The wound closure is represented as fold increase of the T0 samples.

\* Ctrl 24 h, *S. alba* 24 h and *T. parthenium* 24 h vs each relative sample at 8 h

† *T. parthenium* 24 h vs Ctrl 24 h.

*T. parthenium* extract ( $IC_{50} = 115 \pm 2 \mu\text{g/mL}$ ) was found more potent than the *S. alba* extract ( $IC_{50} = 737 \pm 13 \mu\text{g/mL}$ ).

Considering the anti-radical and reducing capacities displayed by *S. alba* and *T. parthenium* extracts, a fingerprint HPLC-fluorimetric analysis was focused on phenolic compounds, namely gallic acid, catechin, epicatechin and resveratrol, well-known for their anti-oxidant/anti-inflammatory effects. Consistently with the findings by Esatbeyoglu and colleagues (2010), *S. alba* extract contains higher levels of catechin and epicatechin, when compared to *T. parthenium* extract (Table 3). On the other hand, the levels of gallic acid and resveratrol are more elevated in the *T. parthenium* extract, being the resveratrol non-detectable in *S. alba* extract.

### 3.2. Biological studies

Basing on these results, the two extracts were tested for a potential role as neuroprotective agents, after assaying their biocompatibility (0.1–20 mg/mL) using the *Artemia salina* Leach, a lethality assay on brine shrimps (Ohikhena et al., 2016). Since the results of the test indicated an  $LC_{50}$  value of 1.91 mg/mL for *S. alba* and of 4.82 mg/mL for *T. parthenium* (Fig. 1), a concentration range at least ten-fold lower was chosen for the following *in vitro* tests.

An astrocyte cell line, CTX-TNA2, was exposed to the two plant extracts and an MTT assay was performed in order to test their effects on cell proliferation and their possible cytotoxicity in a cell type belonging to the neuroglia. In addition, cells were challenged with  $H_2O_2$  to assess the ability of *S. alba* and *T. parthenium* extracts in reverting the cytotoxicity exerted by the hydrogen peroxide. Fig. 2 shows the MTT

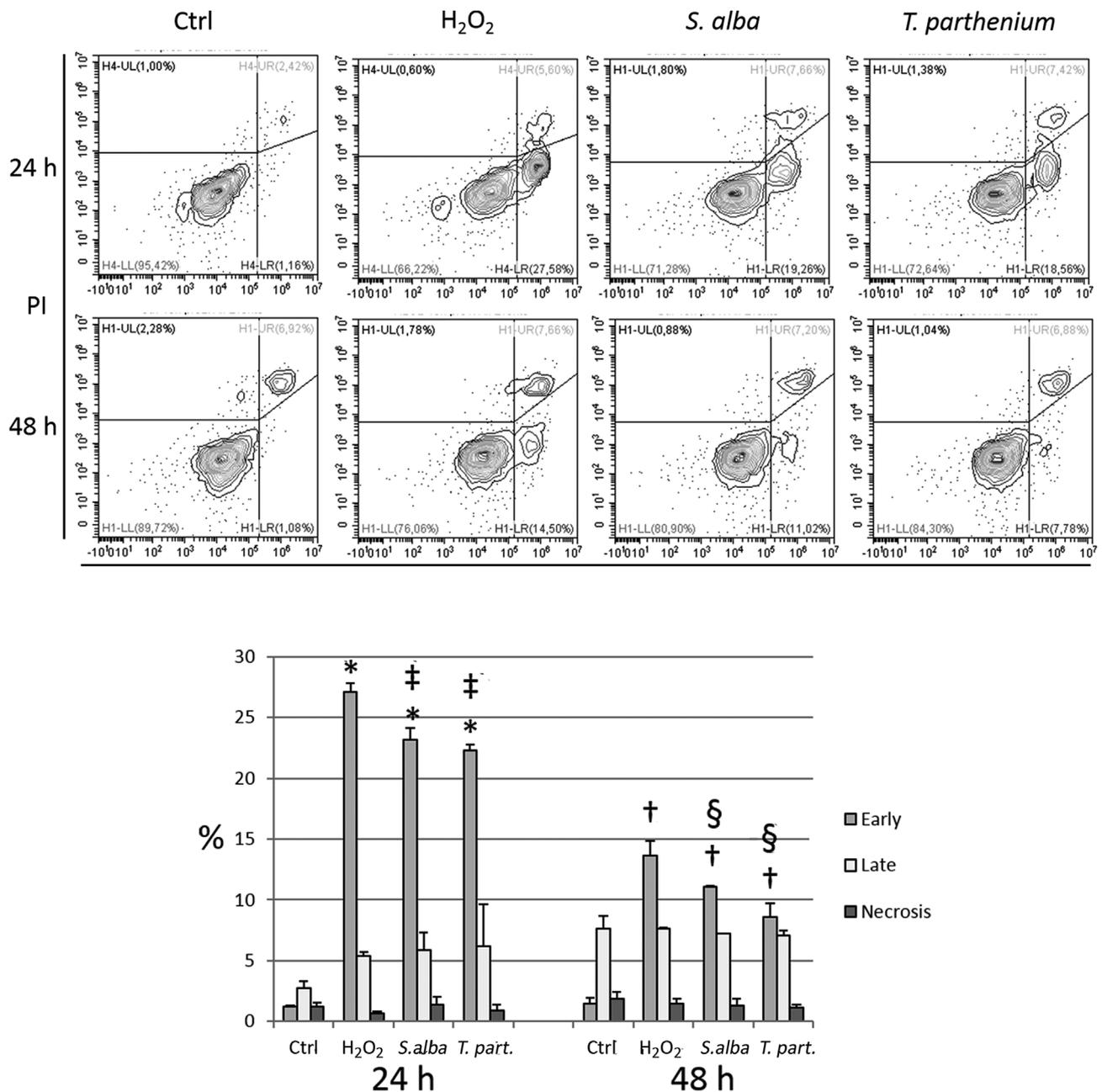


Fig. 4. Apoptosis evaluation in CTX-TNA2 cell line exposed to *T. parthenium* and *S. alba* extracts.

A: representative dot plots

B: the graph shows the mean  $\pm$  SD of three independent experiments

\* Early of H<sub>2</sub>O<sub>2</sub>, *S. alba* H<sub>2</sub>O<sub>2</sub> and *T. parthenium* H<sub>2</sub>O<sub>2</sub> 24 h vs Ctrl 24 h P < 0.05

‡ Early of *S. alba* H<sub>2</sub>O<sub>2</sub> and *T. parthenium* H<sub>2</sub>O<sub>2</sub> 24 h vs H<sub>2</sub>O<sub>2</sub> 24 h P < 0.05

† Early of H<sub>2</sub>O<sub>2</sub>, *S. alba* H<sub>2</sub>O<sub>2</sub> and *T. parthenium* H<sub>2</sub>O<sub>2</sub> 48 h vs Ctrl 48 h P < 0.01

§ Early of *S. alba* H<sub>2</sub>O<sub>2</sub> and *T. parthenium* H<sub>2</sub>O<sub>2</sub> 48 h vs H<sub>2</sub>O<sub>2</sub> 48 h P < 0.05.

assay results. As for the effects of the two plant extracts on cell proliferation, there is no variation after 24 h of treatment. On the other hand, when the astrocyte line is cultured in presence of three different concentrations of both *S. alba* and *T. parthenium* extracts for 48 h, their metabolic activity appears significantly increased when compared to the control sample. No significant difference is registered among the different concentrations, namely 100, 130 e 200  $\mu$ g/mL for *S. alba* and 40, 60 and 80  $\mu$ g/mL for *T. parthenium*. When CTX-TNA2 cells are treated with H<sub>2</sub>O<sub>2</sub>, their viability appears largely reduced but the extracts of both *S. alba* and *T. parthenium* are able to revert the cytotoxicity at both experimental times, but in a greater extent after 48 h.

Again the three different concentrations show no significant difference. Since the three concentrations of both *S. alba* and *T. parthenium* extracts affect cell viability in the same manner, one concentration for each extract (130  $\mu$ g/mL for *S. alba* and 60  $\mu$ g/mL for *T. parthenium*) was chosen for further analyses, namely wound healing assay and apoptosis detection occurrence. On the basis of the effects of the two plant extracts on cell proliferation, a wound healing assay was performed (Fig. 3). After 8 h, no significant differences were found in the three experimental conditions, whereas after 24 h the gap appeared almost completely closed in all the samples (Fig. 3a). The comparison, however, suggests an ability of the *T. parthenium* extract in improving the

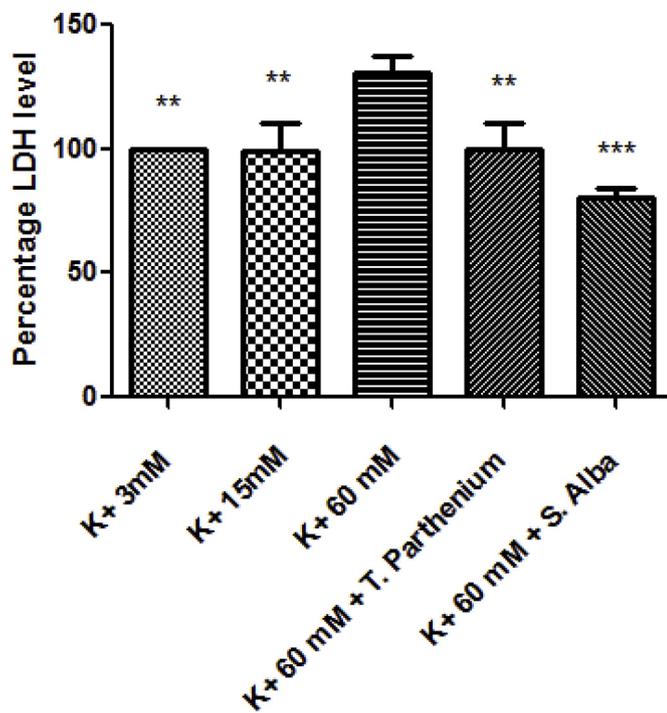


Fig. 5. Effect of *T. parthenium* (60  $\mu\text{g}/\text{mL}$ ) and *S. alba* (130  $\mu\text{g}/\text{mL}$ ) extracts on lactate dehydrogenase (LDH) release. LDH release was evaluated on isolated rat cortex challenged with basal ( $\text{K}^+$  3 mM) and depolarizing stimuli ( $\text{K}^+$  15 mM;  $\text{K}^+$  60 mM). Data are means  $\pm$  S.E.M. ANOVA,  $P < 0.001$ ; post-hoc, \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs.  $\text{K}^+$  60 mM control group.

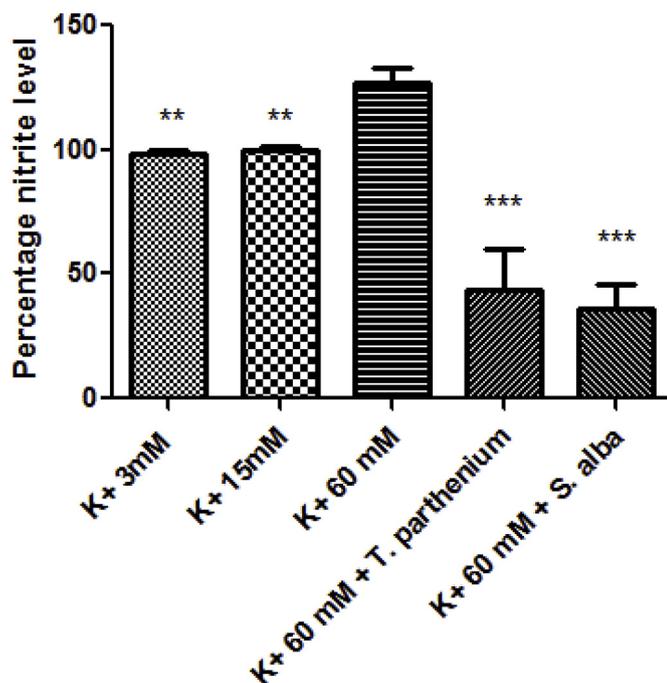


Fig. 6. Effect of *T. parthenium* (60  $\mu\text{g}/\text{mL}$ ) and *S. alba* (130  $\mu\text{g}/\text{mL}$ ) extracts on nitrite level. Nitrite level was evaluated on isolated rat cortex challenged with basal ( $\text{K}^+$  3 mM) and depolarizing stimuli ( $\text{K}^+$  15 mM;  $\text{K}^+$  60 mM). Data are means  $\pm$  S.E.M. ANOVA,  $P < 0.001$ ; post-hoc, \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs.  $\text{K}^+$  60 mM control group.

CTX-TNA2 cell proliferation with respect to the control sample (Fig. 3b). Based on the results of the MTT assay, showing a higher cell metabolic activity when cell exposed to  $\text{H}_2\text{O}_2$  are also treated with the

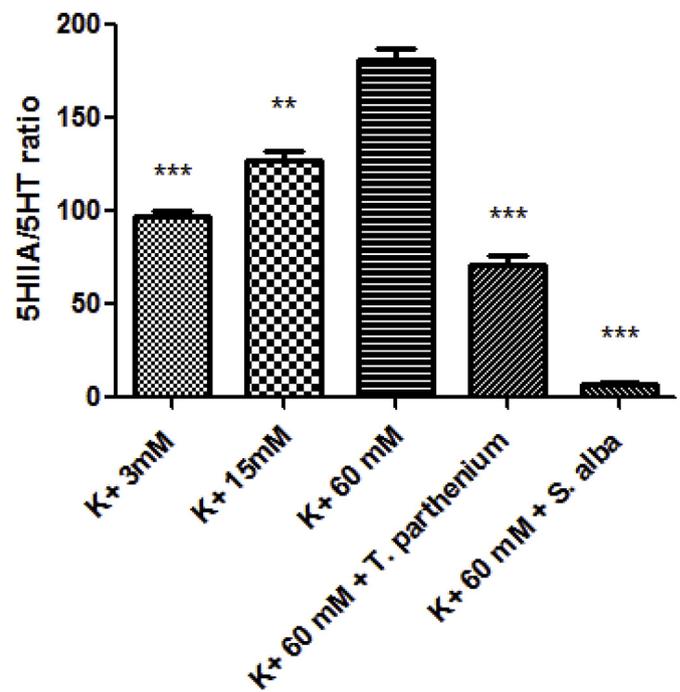
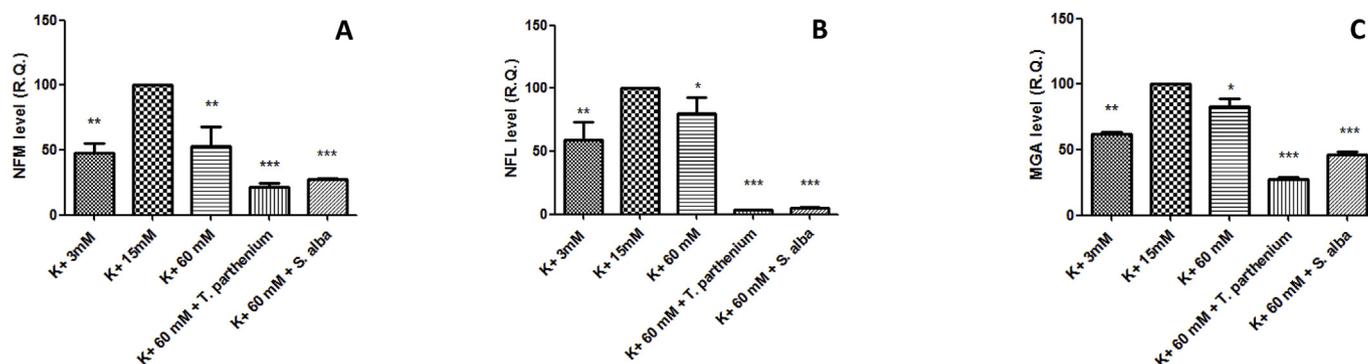


Fig. 7. Effect of *T. parthenium* (60  $\mu\text{g}/\text{mL}$ ) and *S. alba* (130  $\mu\text{g}/\text{mL}$ ) extracts on serotonin (5-HT) turnover, expressed as 5HIAA/5-HT ratio. Turnover was evaluated on isolated rat cortex challenged with basal ( $\text{K}^+$  3 mM) and depolarizing stimuli ( $\text{K}^+$  15 mM;  $\text{K}^+$  60 mM). Data are means  $\pm$  S.E.M. ANOVA,  $P < 0.001$ ; post-hoc, \*\*\* $P < 0.001$  vs.  $\text{K}^+$  60 mM control group.

plant extracts, a flow cytometry assay for the detection of apoptosis occurrence was performed. The treatment with hydrogen peroxide induces the occurrence of both early and late apoptosis (Fig. 4) already after 24 h. The percentage of early apoptotic cells is significantly reduced by both *S. alba* and *T. parthenium* extracts. After 48 h of treatment, the early apoptosis, even though reduced if compared to the earlier experimental point, is still higher than in the control sample and the benefic effect of both plant extract in reducing the apoptotic cell percentage is confirmed. The neuroprotective effects of the extracts (130  $\mu\text{g}/\text{mL}$  for *S. alba* and 60  $\mu\text{g}/\text{mL}$  for *T. parthenium*) in isolated rat cortex specimens, exposed to a neurotoxic depolarizing stimulus ( $\text{K}^+$  60 mM) in order to simulate CSD, were studied, as well. It was observed that both extracts blunted  $\text{K}^+$  60 mM-induced LDH and nitrite levels (Figs. 5 and 6) and 5-HT turnover, expressed as 5HIAA/5-HT ratio (Fig. 7). The extracts revealed also able to potentiate  $\text{K}^+$  60 mM-induced down-regulation of cortex NFM, NFL and MGA levels (Fig. 8).

#### 4. Discussion

The CSD is a pathophysiological and mass depolarization of neurons and glial cells which is related to significant alterations in ion and water distribution across neuron membrane, thus leading to subsequent cytotoxic effects, including neuron death (Richter et al., 2018) and cortical 5-HT depletion (Supornsilpchai et al., 2006). The CSD could also act as a triggering mechanism in migraine, via trigeminal nociceptive system activation (Close et al., 2019), whereas low 5-HT level could affect migraine attack, through multiple effects, including reduction of pain perception and imbalance in the control of the cerebrovascular nociception (Supornsilpchai et al., 2006). The results indicated that *S. alba* and *T. parthenium* extracts were able to completely blunt  $\text{K}^+$  (60 mM)-induced 5HIAA/5-HT, which has long been considered as a reliable marker of brain 5-HT catabolism (Lee et al., 2001; Brunetti et al., 2014). If *S. alba* results are in agreement with already published data by Ulrich-Merzenich et al. (2012), in rat hippocampus, *T. parthenium* findings appear to be discrepant with the inhibition of 5-HT



**Fig. 8.** Proteomic analysis performed on rat cortex challenged with basal ( $K^+$  3 mM) and depolarizing stimuli ( $K^+$  15 mM;  $K^+$  60 mM). Proteomic analysis showed the effects of *T. parthenium* (60  $\mu$ g/mL) and *S. alba* water extracts (130  $\mu$ g/mL) on rat cortex challenged with excitotoxic depolarizing stimulus ( $K^+$  60 mM). In subfigure A, it is showed that  $K^+$  60 mM depolarizing stimulus downregulated neurofilament (NEFs) and myelin-associated glycoprotein (MAG) levels, compared to  $K^+$  15 mM. On the other hand, as depicted in related subfigures A-C, after treating rat cortex with that both extracts, the levels of these proteins were further downregulated, compared to  $K^+$  60 mM group. ANOVA,  $P < 0.001$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs.  $K^+$  15 mM control group.

release previously demonstrated (Mitra et al., 2000). However, the reduced 5-HT turnover found after treatment with *T. parthenium*, could be, albeit partially, related to the extract capability to contrast  $K^+$  60 mM-induced 5-HT overflow (Sbrenna et al., 2000). Both extracts revealed also able to blunt  $K^+$  60 mM-induced levels of nitrites and LDH (Figs. 6 and 7) which are stable markers of nitrosative stress and tissue damage, respectively. The reduced cortex 5-HT turnover, nitrite levels and LDH release and the stimulating effect on CTX-TNA2 cell viability and spontaneous migration after treatment with these herbal extracts are consistent with the observed intrinsic antiradical activity and also with literature (Ferrante et al., 2017; Ramis et al., 2016). The neuroprotective effects showed by these extracts are also in agreement with their content in phenolic compounds, including catechin, epicatechin, gallic acid and resveratrol (Conte et al., 2003; Huang et al., 2008; Shanan et al., 2019). At the moment, there are no published studies describing gallic acid and resveratrol content in *T. parthenium* extracts, but Arituluk et al., 2016 described total phenol levels (expressed as gallic acid equivalents) in other *Tanacetum* species, finding lower concentrations than the *T. parthenium* extract investigated in the present paper. It is reasonable to hypothesize that the greater anti-radical capacity of the *T. parthenium* extract, compared to *S. alba*, could be ascribed to its higher content in gallic acid, already found able to exert a protective role in an *ex vivo* model of oxidative stress (Dutta and Paul, 2019). However, *S. alba* extract appeared to be more effective, particularly against  $K^+$  60 mM-induced 5-HT turnover, despite its poorer qualitative and quantitative phytochemical profile when compared to *T. parthenium* extract. The reason of this apparent discrepancy could be related to the different gallic acid content in the two plant extracts. Gallic acid, in fact could either act as a protective agent at pharmacological concentrations in multiple cell lines (Serrano et al., 2010), including neurons challenged with amyloid  $\beta$ -peptide (Bastianetto et al., 2006), or be potentially toxic as observed in rat bone marrow mesenchymal stem cells, stimulating the production of pro-inflammatory and oxidative stress mediators (Abnosi and Yari, 2018).

In order to deepen our knowledge about the role of the extracts in neuronal pathophysiology, a proteomic analysis was performed and the attention was focused on proteins chosen on the basis of their involvement in neuron morphology and development, namely NFM, NFL and MAG. The NFM and NFL are involved in axonal diameter regulation and their levels of expression are reduced by neurodegenerative conditions (Valdiglesias et al., 2012). The MAG myelin is instead extensively involved in axon sheath enwrapment (Kinter et al., 2013), and its expression is influenced by stressful conditions, including hypoxia (Currstin et al., 2002). Interestingly, the CSD has been related to both hypoxia and neurodegeneration (Raiteri et al., 2002; Cui et al., 2013) and the exposure of the cerebral cortex to the excitotoxic depolarizing-

stimulus ( $K^+$  60 mM) was able to downregulate both NFM, NFL and MAG levels, with respect to physiologic depolarizing-stimulus ( $K^+$  15 mM). Surprisingly, both *S. alba* and *T. parthenium* extracts resulted able to potentiate  $K^+$  60 mM-induced downregulation of the selected proteins, suggesting potential neurotoxic effects of both extracts. A possible explanation for the decreased levels of NFM, NFL and MAG following extract treatments can be found in the fact that anti-oxidants present in the extracts (i.e. *T. parthenium* and *S. alba* extracts), could exert paradoxical pro-oxidative effects, in order to activate cells to mild oxidative stress (Halliwell et al., 2000). In addition, brain is known to be highly susceptible to oxidative stress, because of many intrinsic reasons, including high membrane lipid content, modest antioxidant defense and physiological neurotransmitter autoxidation (Cobley et al., 2018).

Concluding, the present findings demonstrate multiple protective effects induced by *S. alba* and *T. parthenium* treatment, in rat astrocytes and cortex, including reduction of 5-HT turnover, thus corroborating their phytotherapeutic use in the management of clinical symptoms related to migraine (Rajapakse and Davenport, 2019; Shrivastava et al., 2006).

Nevertheless, the downregulation of the NFM, NFL and MAG levels in the cortex, triggered by *S. alba* and *T. parthenium* treatment, indicates a possible neurotoxic effect, suggesting caution in the use of food supplements enriched with *S. alba* and *T. parthenium*. Further studies are needed to clarify the mechanisms underlying the effects observed in the present study.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

This work was supported by a grant from Cristalfarma S.r.l. (Milan, Italy), within the project entitled "Applicative potential of herbal-derived phytocomplexes" (Coordinators: Luigi Menghini, Giustino Orlando and Claudio Ferrante), and by a Ministry of Education, Research and University (MIUR) grant assigned to Amelia Cataldi and Viviana di Giacomo (Fondo FAR 2018).

The animal experimental procedures were approved by Local Ethical Committee (University "G. d'Annunzio" of Chieti-Pescara) and Italian Health Ministry (Italian Health Ministry authorization N. F4738.N.XTQ, delivered on 11th November 2018).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2019.110783>.

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