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

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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 neurotoxicity stimulus. The downregulation of selected proteins suggest a neurotoxic, which could be ascribed to an elevate 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.

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_{1B} and 5-HT_{1D} receptor activation revealed to play a key role in the control of acute migraine attacks (Lance et al., 1991). Conversely, the blockade of 5-HT_{2A} and 5-HT_{2C} 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 (Termine et al., 2011). Recommended drugs for acute attack treatment include analgesics, 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 phytocomplex 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 *T. parthenium* and *S. alba* water extracts were investigated in different experimental conditions, in order to better investigate their role in preventing migraine attacks. Particularly, the impact of extract treatment was evaluated *in vitro* on astrocyte viability, spontaneous migration and apoptosis occurrence. In addition, anti-inflammatory/anti-oxidant effects were investigated on isolated rat cortexes exposed to a neurotoxicity stimulus which reproduces CSD, *ex vivo*. In the same experimental model cytotoxicity was measured by evaluation of nitrite production and lactate dehydrogenase (LDH) release, and 5-HT turnover was assayed as well. The effect of the exposure to *T. parthenium* and *S. alba* water extracts on levels of specific proteins involved in neuron morphology and development, namely neurofilament (NFEMs) proteins and myelin-associated glycoprotein (MAG), was investigated by a validated untargeted proteomic analysis. Finally, in order to provide a better interpretation

of the observed pharmaco-toxicological effects, a fingerprint analysis was carried out on selected phenolic compounds, namely gallic acid, catechin, epicatechin and resveratrol.

Materials and Methods

Pharmacognostic studies

Extract preparation

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

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. 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^{TM} 120, 3µm, 2.1×100 mm, Dionex Corporation, Sunnyvale, 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, λex = 278 nm and λem = 360 nm were selected in order to analyze the following phenolic compounds: gallic acid, catechin, epicatechin and resveratrol.

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.

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 0.5 at 700 nm.

Biological studies

Artemia salina lethality bioassay

Artemia salina lethality bioassay was performed as previously reported (Ferrante et al., 2019). Brielfy, brine shrimp larvae were bred at 25-28°C for 24h 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)*100, where T and S are the total number of incubated larvae and survival napulii, respectively. Experiments were carried out in triplicate.

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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 μgmL^{-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 humified atmosphere of 5% CO_2.

When indicated the cells were treated with H_2O_2 300 µM for three hours and different concentrations of *S. alba* (100, 130, 200 µg/mL) and *T. parthenium* (40, 60 and 80 µg/mL) water extracts.

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). Cell number was quantified by the amount of tetrazolium reduction in viable mitochondria. Cultured cells were seeded into 96-well plate at $8x10^3$ cells/well and treated as described above. After 24 and 48 h, the cells were processed according to manufacturer's instructions and the absorbance of each sample was detected at 570 nm. Three independent experiments were performed under the same experimental conditions.

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 µg/mL *S alba* and 60 µg/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.

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 the manufacturer's instructions. Briefly, 10^5 cells were gently re-suspended in 100 µl of binding buffer and incubated for 15 min at room temperature in the dark with 5 µl of Annexin-V-FITC and 5 µl of Propidium Iodide (PI). After the addition of 200 µl of binding buffer, samples were analyzed with a Cytoflex flow cytometer with the FL1 and FL3 detector in a log mode using the Cytoexpert analysis software (both from Beckmann Coulter, FL, USA). For each sample, 10000 events were collected. Viable cells are Annexin-V^{neg}/PI^{neg} (unlabelled), necrotic cells are Annexin-V^{neg}/PI^{pos}, while early and late apoptotic cells are Annexin-V^{pos} and PI^{neg} and PI^{pos}, respectively.

Ex vivo cortical spreading depression (CSD) paradigm

Sprague-Dawley rats (200-250 g) were housed in Plexiglass cages (40 cm × 25 cm × 15 cm), two rats per cage, in climatized colony rooms (22 ± 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 fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, 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. The experiments 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 Novembre 2018). Rats were sacrificed by CO_2 inhalation (100% CO_2 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^+ concentrations, as described below:

K^+ 3 mM: corresponding to basal condition;

K^+ 15 mM: corresponding to physiologic depolarizing-stimulus;

K^+ 60 mM: corresponding to excitotoxic depolarizing-stimulus.

The present experimental paradigm reproduced the neural pathophysiological condition named CSD, and was designed according to previous *ex vivo* and *in vivo* studies, describing the use of elevated K^+ concentrations (up to 50-60 mM) to induce central nervous system injury (Raiteri et al., 2002). During incubation, cortex specimens were exposed to *S. alba* (130 μg/mL) and *T. parthenium* (60 μg/mL). Afterwards, individual cortex slices were homogenized in perchloric acid solution (0.05 M) in order to extract and quantify 5-HT (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 (Chichiriccò et al., 2019).

Protein extraction and Filter-aided sample preparation

After protein quantification, a volume corresponding to 50 µg 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 µL Urea buffer (8M Urea, 100 mM Tris pH 8.5 in milliQ water) to remove the detergents present in the lysis buffer. The proteins on the filter where subsequently reduced and alkylated by adding 100 µL of DTT solution (8 mM Dithiothreitol in Urea buffer) and 100 µL 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".

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Means ± 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≤E≤20; https://www.nc3rs.org.uk/experimental-designstatistics).

Results and Discussion

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_{50} values, which are inversely related to the anti-oxidant capacities of the plant extracts. The *T. parthenium* extract, with an IC_{50} value of 39 ± 2 µg/mL, showed a more effective activity than *S. alba* extract, whose IC_{50} 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 *T. parthenium* extract (IC_{50}=115 ± 2 µg/mL) was found more potent than the *S. alba* extract (IC_{50}= 737 ± 13 µg/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. At the moment,

there are no published studies describing gallic acid and resveratrol content in *T. parthenium* extracts, but Arituluk and colleagues (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 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).

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. Figure 2 shows the MTT 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 the 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 µg/mL for *S. alba* and 40, 60 and 80 µg/mL for *T. parthenium.*

When CTX-TNA2 cells are treated with H_2O_2, 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. Such findings are in line with current literature, reporting the protective effects of many phenolic compounds, including catechin, in inhibiting ROS-induced cytotoxicity in neuronal cells (Conte et al., 2003).

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 µg/mL for *S. alba* and 60 µg/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 hours, no significant differences were found in the three experimental conditions, whereas after 24 hours 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 CTX-TNA2 cell proliferation with respect to the control sample (Fig. 3b). A significant difference in the two extracts is the content in resveratrol, being this metabolite found in the *T. parthenium* extract only. Since the resveratrol was found effective in improving survival and regeneration of cerebellar neurons (Shanan et al., 2019), its presence in *T. parthenium* extract could explain the results observed in the wound healing assay.

Based on the results of the MTT assay, showing an higher cell metabolic activity when cell exposed to H_2O_2 are also treated with the 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. Such findings are not surprising, being phenolic acids well known for their antioxidant activity also in a neuronal model of apoptosis (Huang et al, 2008) and having both extracts a considerable quantity of gallic acid.

Considering the well-established phytotherapeutic use of both *S. alba* and *T. parthenium* in migraine (Rajapakse and Davenport, 2019; Shrivastava et al., 2006), the effects of the extracts in isolated rat cortex specimens, exposed to a neurotoxic depolarizing stimulus (K+ 60 mM) in order to simulate cortical spreading depression (CSD), were studied. 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., 2018), 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 K^+ (60 mM)-induced turnover, evaluated as 5HIIA/5-HT ratio (Fig. 5), 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 and colleagues (2012), in rat hyppocampus, *T. parthenium* findings appear to be discrepant with the inhibition of 5-HT release previously demonstrated (Mittra 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, 7) which are stable markers of nitrosative stress and tissue damage, respectively. The reduced 5-HT turnover, nitrite levels and LDH release after treatment with these herbal extracts are consistent with the observed intrinsic antiradical activity and with literature (Ferrante et al., 2017; Ramis et al., 2016).

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 doses in multiple cell lines (Serrano et al., 2010), including neurons challenged with amyloid β-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 neurofilament proteins (NFEMs) and myelin-associated glycoprotein (MAG). The first 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 (Curristin 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 excitotoxicity depolarizing-stimulus (K^+ 60 mM) was able to downregulate both NEFMs and MAG levels (Fig. 8), 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 NFEMs 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 use in the management of clinical symptoms related to migraine.

Nevertheless, the downregulation of the NEFMs 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.

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Conflict of interest

Authors declare no financial/commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

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Table 1 DPPH radical scavenging effects of the extracts

	DPPH % scavenging activity ± SD	
Concentration (µg/mL)	*S. alba*	*T. parthenium*
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)	*S. alba*	*T. parthenium*
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	*S. alba* µg/mg extract	*T. parthenium* 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

FIGURE CAPTIONS

Figure 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 ± SD of three experiments performed in triplicate.

Figure 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_2O_2 vs ctrl $p < 0.01$

† *S. alba* H_2O_2 + 100, 130 e 200 µg/mL and *T. parthenium* H_2O_2 + 40, 60 and 80 µg/mL vs H_2O_2 $p<0.05$

B: 48 h

** H_2O_2 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_2O_2 + 100, 130 e 200 µg/mL and *T. parthenium* H_2O_2 + 40, 60 and 80 µg/mL vs H_2O_2 $p<0.01$

Figure 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

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

A: representative dot plots

B: the grap shows the mean ± SD of three independent experiments

* Early of H_2O_2, *S. alba* H_2O_2 and *T. parthenium* H_2O_2 24 h vs Ctrl 24 h $p < 0.05$

‡ Early of *S. alba* H_2O_2 and *T. parthenium* H_2O_2 24 h vs H_2O_2 24 h $p<0.05$

† Early of H_2O_2, *S. alba* H_2O_2 and *T. parthenium* H_2O_2 48 h vs Ctrl 48 h $p<0.01$

§ Early of *S. alba* H_2O_2 and *T. parthenium* H_2O_2 48 h vs H_2O_2 48 h $p<0.05$

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

Figure 6: Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on nitrite level. Nitrite level was evaluated on isolated rat cortex challenged with basal (K^+ 3mM) and

depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Data are means ± S.E.M. ANOVA, P<0.001; post-hoc, **P<0.01, ***P<0.001 vs. K^+ 60 mM control group.

Figure 7: Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on serotonin (5-HT) turnover, expressed as 5HIIA/5-HT ratio. Turnover was evaluated on isolated rat cortex challenged with basal (K^+ 3mM) and depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Data are means ± S.E.M. ANOVA, P<0.001; post-hoc, ***P<0.001 vs. K^+ 60 mM control group.

Figure 8: Proteomic analysis performed on rat cortex challenged with basal (K^+ 3mM) and depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Proteomic analysis showed the effects of T. parthenium (60 µg/mL) and S. alba water extracts (130 µg/mL) on rat cortex challenged with excitotoxicity depolarizing stimulus (K^+ 60 mM). In subfigure A, it is showed that K^+ 60 mM depolarizing stimulus downregulated neurofilament (NEFMs) 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.

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

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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 neurotoxicity stimulus. The downregulation of selected proteins suggest a neurotoxic, which could be ascribed to an elevate 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.

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_{1B} and 5-HT_{1D} receptor activation revealed to play a key role in the control of acute migraine attacks (Lance et al., 1991). Conversely, the blockade of 5-HT_{2A} and 5-HT_{2C} 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 (Termine et al., 2011). Recommended drugs for acute attack treatment include analgesics, 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 phytocomplex 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 neurotoxicity 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 pharmaco-toxicological effects, a fingerprint analysis was carried out on selected phenolic compounds, including gallic acid, catechin, epicatechin and resveratrol.

Materials and Methods

Pharmacognostic studies

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 (35kHz), as previously described (Menghini et al., 2018).

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^{TM} 120, 3µm, 2.1×100 mm, Dionex Corporation, Sunnyvale, 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, λex = 278 nm and λem = 360 nm were selected in order to analyze the following phenolic compounds: gallic acid, catechin, epicatechin and resveratrol.

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.

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 0.5 at 700 nm.

Biological studies

Artemia salina lethality bioassay

Artemia salina lethality bioassay was performed as previously reported (Ferrante et al., 2019). Briefly, brine shrimp larvae were bred at 25-28°C for 24h 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)*100, where T and S are the total number of incubated larvae and survival nauplii, respectively. Experiments were carried out in triplicate.

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 μgmL^{-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 humified atmosphere of 5% CO_2. When indicated the cells were treated with H_2O_2 300 µM for three hours and different concentrations of *S. alba* (100, 130, 200 µg/mL) and *T. parthenium* (40, 60 and 80 µg/mL) water extracts.

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).

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 µg/mL *S alba* and 60 µg/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.

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).

Ex vivo cortical spreading depression (CSD) paradigm

Sprague-Dawley rats (200-250 g) were housed in Plexiglass cages (40 cm × 25 cm × 15 cm), two rats per cage, in climatized colony rooms (22 ± 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_2 inhalation (100% CO_2 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^+ concentrations (3, 15, 60 mM), as recently described (Orlando et al., 2019). During incubation, cortex specimens were exposed to *S. alba* (130 μg/mL) and *T. parthenium* (60 μg/mL). Afterwards, individual cortex slices were homogenized in perchloric acid solution (0.05 M) in order to extract and quantify 5-HT (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 (Chichiriccò et al., 2019).

Protein extraction and Filter-aided sample preparation

After protein quantification, a volume corresponding to 50 µg 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 µL Urea buffer (8M Urea, 100 mM Tris pH 8.5 in milliQ water) to remove the detergents present in the lysis buffer. The proteins on the filter where subsequently reduced and alkylated by adding 100 µL of DTT solution (8 mM Dithiothreitol in Urea buffer) and 100 µL 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".

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Means ± 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).

Results

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_{50} values, which are inversely related to the anti-oxidant capacities of the plant extracts. The *T. parthenium* extract, with an IC_{50} value of 39 ± 2 µg/mL, showed a more effective activity than *S. alba* extract, whose IC_{50} 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 *T. parthenium* extract (IC_{50}=115 ± 2 µg/mL) was found more potent than the *S. alba* extract (IC_{50}= 737 ± 13 µg/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.

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. Figure 2 shows the MTT 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 the 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 µg/mL for *S. alba* and 40, 60 and 80 µg/mL for *T. parthenium.* When CTX-TNA2 cells are treated with H_2O_2, 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 µg/mL for *S. alba* and 60 µg/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 hours, no significant differences were found in the three experimental conditions, whereas after 24 hours 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 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 H_2O_2 are also treated with the 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 µg/mL for *S. alba* and 60 µg/mL for *T. parthenium*) in isolated rat cortex specimens, exposed to a neurotoxic depolarizing stimulus (K+ 60 mM) in order to simulate CSD, were studied, as well. It was observed that both extracts blunted K^+ 60 mM-induced LDH and nitrite levels (Figs. 5, 6) and 5-HT turnover, expressed as 5HIIA/5-HT ratio (Fig. 7). The extracts revealed also able to potentiate K^+ 60 mM-induced down-regulation of cortex NFM, NFL and MGA levels (Fig. 8).

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 K^+ (60 mM)-induced 5HIIA/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 and colleagues (2012), in rat hyppocampus, *T. parthenium* findings appear to be discrepant with the inhibition of 5-HT release previously demonstrated (Mittra 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, 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, acid gallic 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 and colleagues (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 doses in multiple cell lines (Serrano et al., 2010), including neurons challenged with amyloid β-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 (Curristin 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 excitotoxicity 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.

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Conflict of interest

Authors declare no financial/commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

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Table 1 DPPH radical scavenging effects of the extracts

	DPPH % scavenging activity ± SD	
Concentration (µg/mL)	*S. alba*	*T. parthenium*
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)	*S. alba*	*T. parthenium*
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	*S. alba* µg/mg extract	*T. parthenium* 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

FIGURE CAPTIONS

Figure 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 ± SD of three experiments performed in triplicate.

Figure 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_2O_2 vs ctrl p <0.01

† *S. alba* H_2O_2 + 100, 130 e 200 µg/mL and *T. parthenium* H_2O_2 + 40, 60 and 80 µg/mL vs H_2O_2 p<0.05

B: 48 h

** H_2O_2 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_2O_2 + 100, 130 e 200 µg/mL and *T. parthenium* H_2O_2 + 40, 60 and 80 µg/mL vs H_2O_2 p<0.01

Figure 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

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

A: representative dot plots

B: the grap shows the mean ± SD of three independent experiments

* Early of H_2O_2, *S. alba* H_2O_2 and *T. parthenium* H_2O_2 24 h vs Ctrl 24 h $p < 0.05$

‡ Early of *S. alba* H_2O_2 and *T. parthenium* H_2O_2 24 h vs H_2O_2 24 h $p<0.05$

† Early of H_2O_2, *S. alba* H_2O_2 and *T. parthenium* H_2O_2 48 h vs Ctrl 48 h $p<0.01$

§ Early of *S. alba* H_2O_2 and *T. parthenium* H_2O_2 48 h vs H_2O_2 48 h $p<0.05$

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

Figure 6: Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on nitrite level. Nitrite level was evaluated on isolated rat cortex challenged with basal (K^+ 3mM) and

depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Data are means ± S.E.M. ANOVA, P<0.001; post-hoc, **P<0.01, ***P<0.001 vs. K^+ 60 mM control group.

Figure 7: Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on serotonin (5-HT) turnover, expressed as 5HIIA/5-HT ratio. Turnover was evaluated on isolated rat cortex challenged with basal (K^+ 3mM) and depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Data are means ± S.E.M. ANOVA, P<0.001; post-hoc, ***P<0.001 vs. K^+ 60 mM control group.

Figure 8: Proteomic analysis performed on rat cortex challenged with basal (K^+ 3mM) and depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Proteomic analysis showed the effects of T. parthenium (60 µg/mL) and S. alba water extracts (130 µg/mL) on rat cortex challenged with excitotoxicity depolarizing stimulus (K^+ 60 mM). In subfigure A, it is showed that K^+ 60 mM depolarizing stimulus downregulated neurofilament (NEFMs) 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.

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

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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 neurotoxicity stimulus. The downregulation of selected proteins suggest a neurotoxic, which could be ascribed to an elevate 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.

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_{1B} and 5-HT_{1D} receptor activation revealed to play a key role in the control of acute migraine attacks (Lance et al., 1991). Conversely, the blockade of 5-HT_{2A} and 5-HT_{2C} 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 (Termine et al., 2011). Recommended drugs for acute attack treatment include analgesics, 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 phytocomplex 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 neurotoxicity 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 pharmaco-toxicological effects, a fingerprint analysis was carried out on selected phenolic compounds, including gallic acid, catechin, epicatechin and resveratrol.

Materials and Methods

Pharmacognostic studies

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 (35kHz), as previously described (Menghini et al., 2018).

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^{TM} 120, 3µm, 2.1×100 mm, Dionex Corporation, Sunnyvale, 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, λex = 278 nm and λem = 360 nm were selected in order to analyze the following phenolic compounds: gallic acid, catechin, epicatechin and resveratrol.

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.

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 0.5 at 700 nm.

Biological studies

Artemia salina lethality bioassay

Artemia salina lethality bioassay was performed as previously reported (Ferrante et al., 2019). Briefly, brine shrimp larvae were bred at 25-28°C for 24h 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)*100, where T and S are the total number of incubated larvae and survival nauplii, respectively. Experiments were carried out in triplicate.

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 μgmL^{-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 humified atmosphere of 5% CO_2. When indicated the cells were treated with H_2O_2 300 µM for three hours and different concentrations of *S. alba* (100, 130, 200 µg/mL) and *T. parthenium* (40, 60 and 80 µg/mL) water extracts.

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).

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 µg/mL *S alba* and 60 µg/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.

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).

Ex vivo cortical spreading depression (CSD) paradigm

Sprague-Dawley rats (200-250 g) were housed in Plexiglass cages (40 cm × 25 cm × 15 cm), two rats per cage, in climatized colony rooms (22 ± 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_2 inhalation (100% CO_2 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^+ concentrations (3, 15, 60 mM), as recently described (Orlando et al., 2019). During incubation, cortex specimens were exposed to *S. alba* (130 μg/mL) and *T. parthenium* (60 μg/mL). Afterwards, individual cortex slices were homogenized in perchloric acid solution (0.05 M) in order to extract and quantify 5-HT (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 (Chichiriccò et al., 2019).

Protein extraction and Filter-aided sample preparation

After protein quantification, a volume corresponding to 50 µg 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 µL Urea buffer (8M Urea, 100 mM Tris pH 8.5 in milliQ water) to remove the detergents present in the lysis buffer. The proteins on the filter where subsequently reduced and alkylated by adding 100 µL of DTT solution (8 mM Dithiothreitol in Urea buffer) and 100 µL 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".

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Means ± 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).

Results

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_{50} values, which are inversely related to the anti-oxidant capacities of the plant extracts. The *T. parthenium* extract, with an IC_{50} value of 39 ± 2 µg/mL, showed a more effective activity than *S. alba* extract, whose IC_{50} 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 *T. parthenium* extract (IC_{50}=115 ± 2 µg/mL) was found more potent than the *S. alba* extract (IC_{50}= 737 ± 13 µg/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.

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. Figure 2 shows the MTT 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 the 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 µg/mL for *S. alba* and 40, 60 and 80 µg/mL for *T. parthenium.* When CTX-TNA2 cells are treated with H_2O_2, 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 µg/mL for *S. alba* and 60 µg/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 hours, no significant differences were found in the three experimental conditions, whereas after 24 hours 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 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 H_2O_2 are also treated with the 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 µg/mL for *S. alba* and 60 µg/mL for *T. parthenium*) in isolated rat cortex specimens, exposed to a neurotoxic depolarizing stimulus (K+ 60 mM) in order to simulate CSD, were studied, as well. It was observed that both extracts blunted K^+ 60 mM-induced LDH and nitrite levels (Figs. 5, 6) and 5-HT turnover, expressed as 5HIIA/5-HT ratio (Fig. 7). The extracts revealed also able to potentiate K^+ 60 mM-induced down-regulation of cortex NFM, NFL and MGA levels (Fig. 8).

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 K^+ (60 mM)-induced 5HIIA/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 and colleagues (2012), in rat hyppocampus, *T. parthenium* findings appear to be discrepant with the inhibition of 5-HT release previously demonstrated (Mittra 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, 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, acid gallic 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 and colleagues (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 doses in multiple cell lines (Serrano et al., 2010), including neurons challenged with amyloid β-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 (Curristin 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 excitotoxicity 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.

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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 Novembre 2018).

Conflict of interest

Authors declare no financial/commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online.

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Table 1 DPPH radical scavenging effects of the extracts

	DPPH % scavenging activity ± SD	
Concentration (µg/mL)	*S. alba*	*T. parthenium*
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)	*S. alba*	*T. parthenium*
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	*S. alba* µg/mg extract	*T. parthenium* 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

FIGURE CAPTIONS

Figure 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 ± SD of three experiments performed in triplicate.

Figure 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_2O_2 vs ctrl p <0.01

† *S. alba* H_2O_2 + 100, 130 e 200 µg/mL and *T. parthenium* H_2O_2 + 40, 60 and 80 µg/mL vs H_2O_2 p<0.05

B: 48 h

** H_2O_2 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_2O_2 + 100, 130 e 200 µg/mL and *T. parthenium* H_2O_2 + 40, 60 and 80 µg/mL vs H_2O_2 p<0.01

Figure 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

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

A: representative dot plots

B: the grap shows the mean ± SD of three independent experiments

* Early of H_2O_2, *S. alba* H_2O_2 and *T. parthenium* H_2O_2 24 h vs Ctrl 24 h $p < 0.05$

‡ Early of *S. alba* H_2O_2 and *T. parthenium* H_2O_2 24 h vs H_2O_2 24 h $p<0.05$

† Early of H_2O_2, *S. alba* H_2O_2 and *T. parthenium* H_2O_2 48 h vs Ctrl 48 h $p<0.01$

§ Early of *S. alba* H_2O_2 and *T. parthenium* H_2O_2 48 h vs H_2O_2 48 h $p<0.05$

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

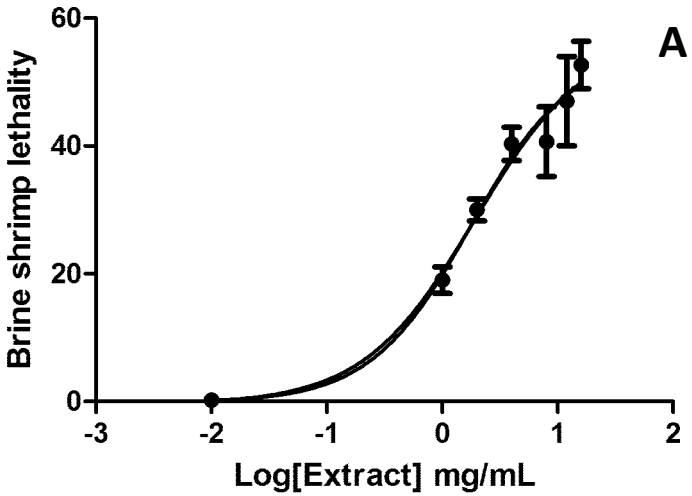
Figure 6: Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on nitrite level. Nitrite level was evaluated on isolated rat cortex challenged with basal (K^+ 3mM) and

depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Data are means ± S.E.M. ANOVA, P<0.001; post-hoc, **P<0.01, ***P<0.001 vs. K^+ 60 mM control group.

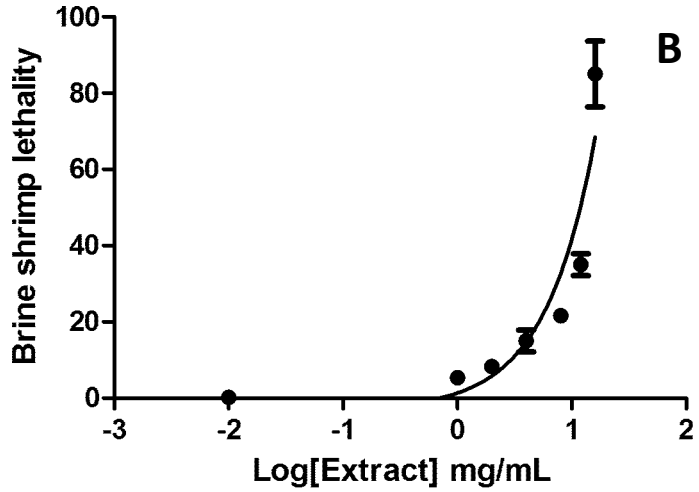
Figure 7: Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on serotonin (5-HT) turnover, expressed as 5HIIA/5-HT ratio. Turnover was evaluated on isolated rat cortex challenged with basal (K^+ 3mM) and depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Data are means ± S.E.M. ANOVA, P<0.001; post-hoc, ***P<0.001 vs. K^+ 60 mM control group.

Figure 8: Proteomic analysis performed on rat cortex challenged with basal (K^+ 3mM) and depolarizing stimuli (K^+ 15 mM; K^+ 60 mM). Proteomic analysis showed the effects of T. parthenium (60 µg/mL) and S. alba water extracts (130 µg/mL) on rat cortex challenged with excitotoxicity depolarizing stimulus (K^+ 60 mM). In subfigure A, it is showed that K^+ 60 mM depolarizing stimulus downregulated neurofilament (NEFMs) 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.

Figure 1 revised



S. alba (LC 50: 1.91 mg/mL)



T. parthenium (LC 50: 4.82 mg/mL)

Figure 2

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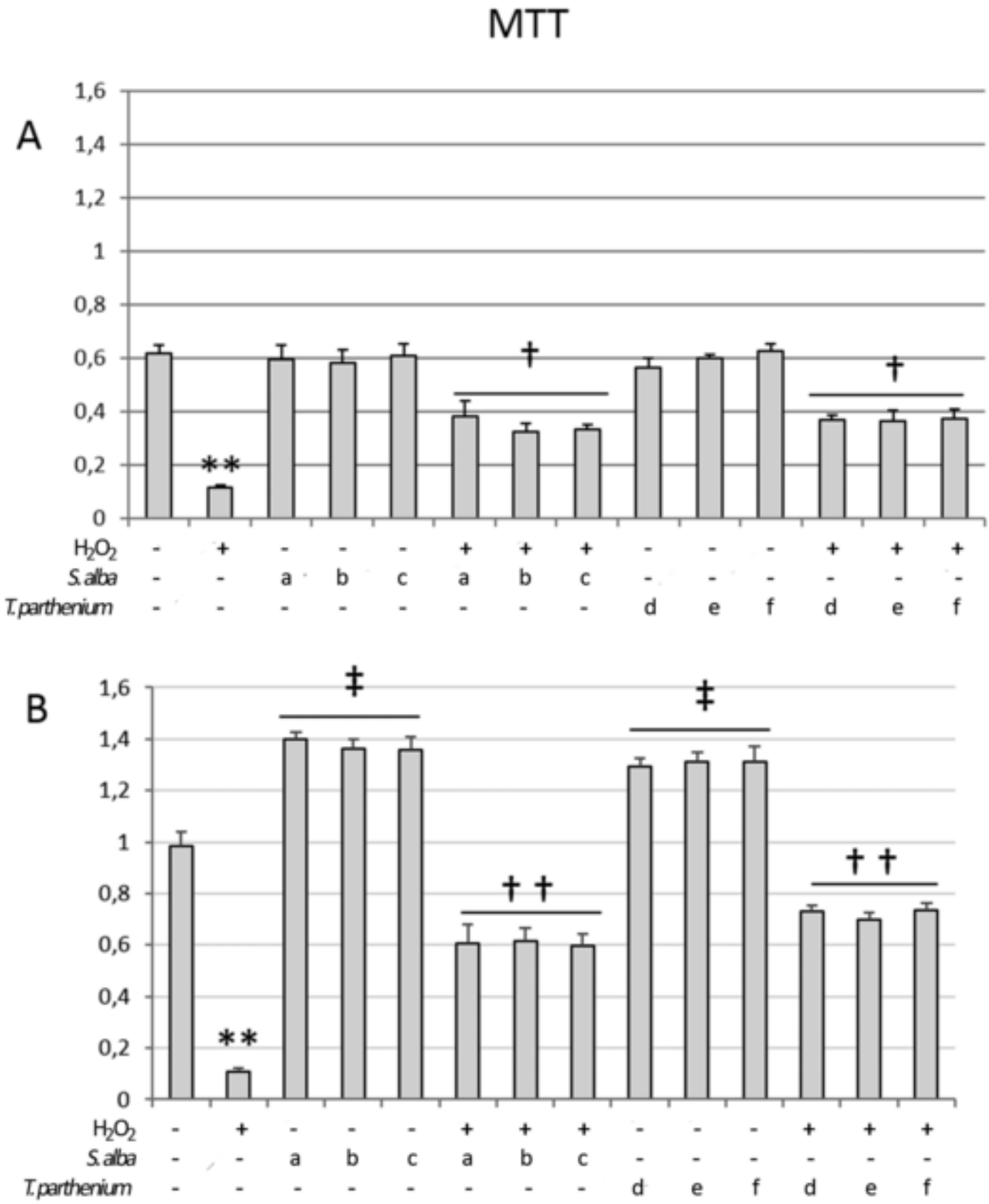

Figure 3A

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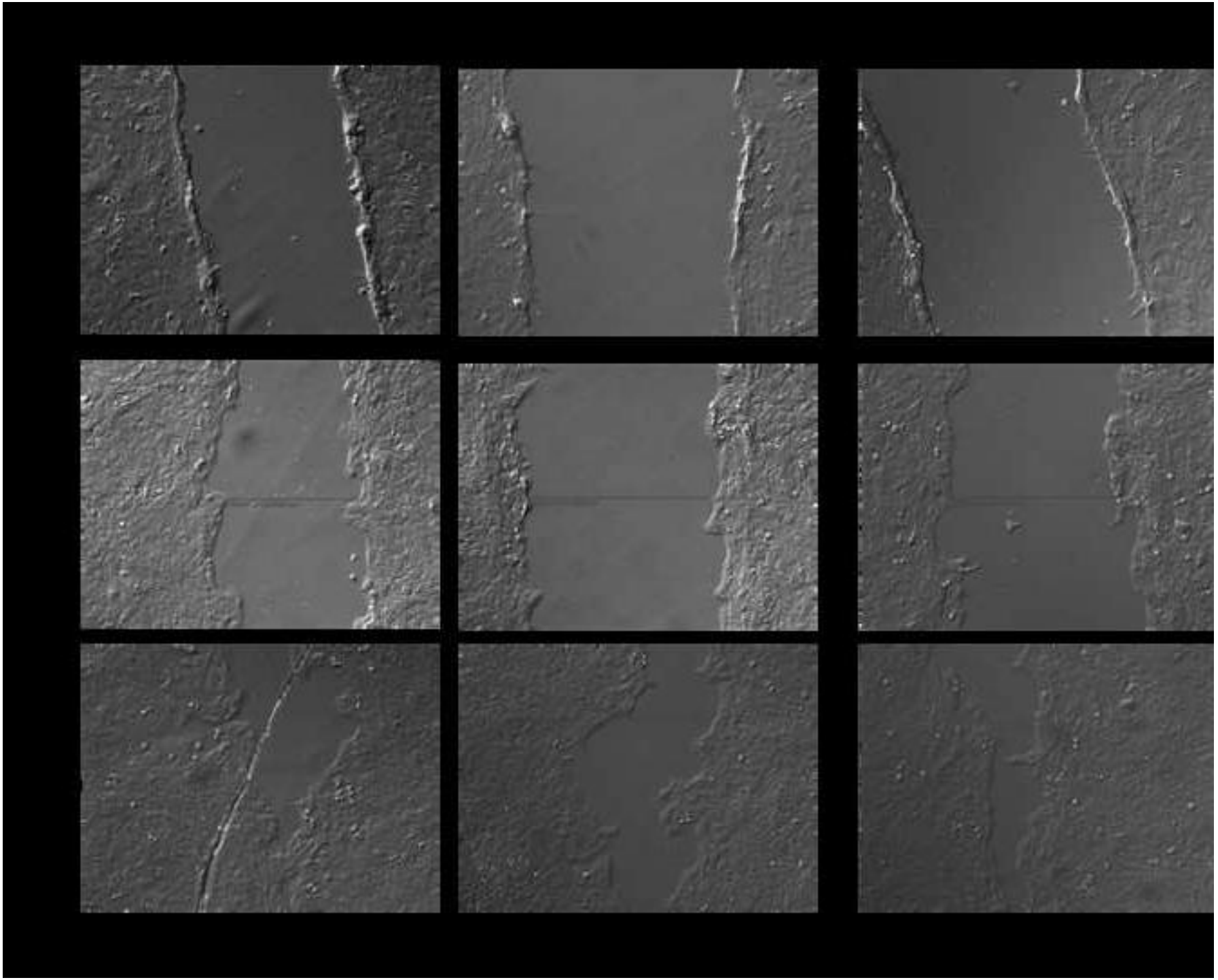

Figure 3B

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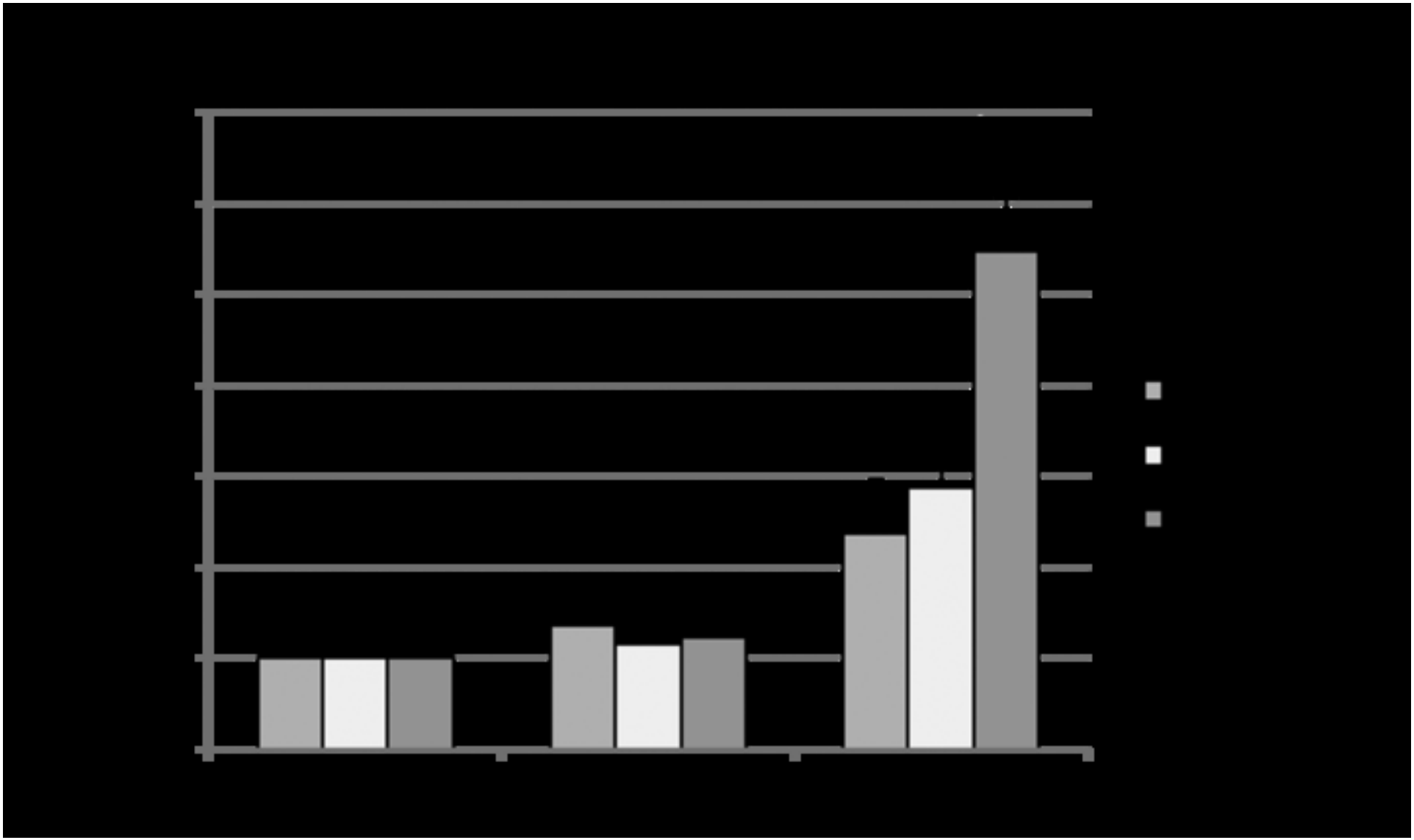

Figure 4A

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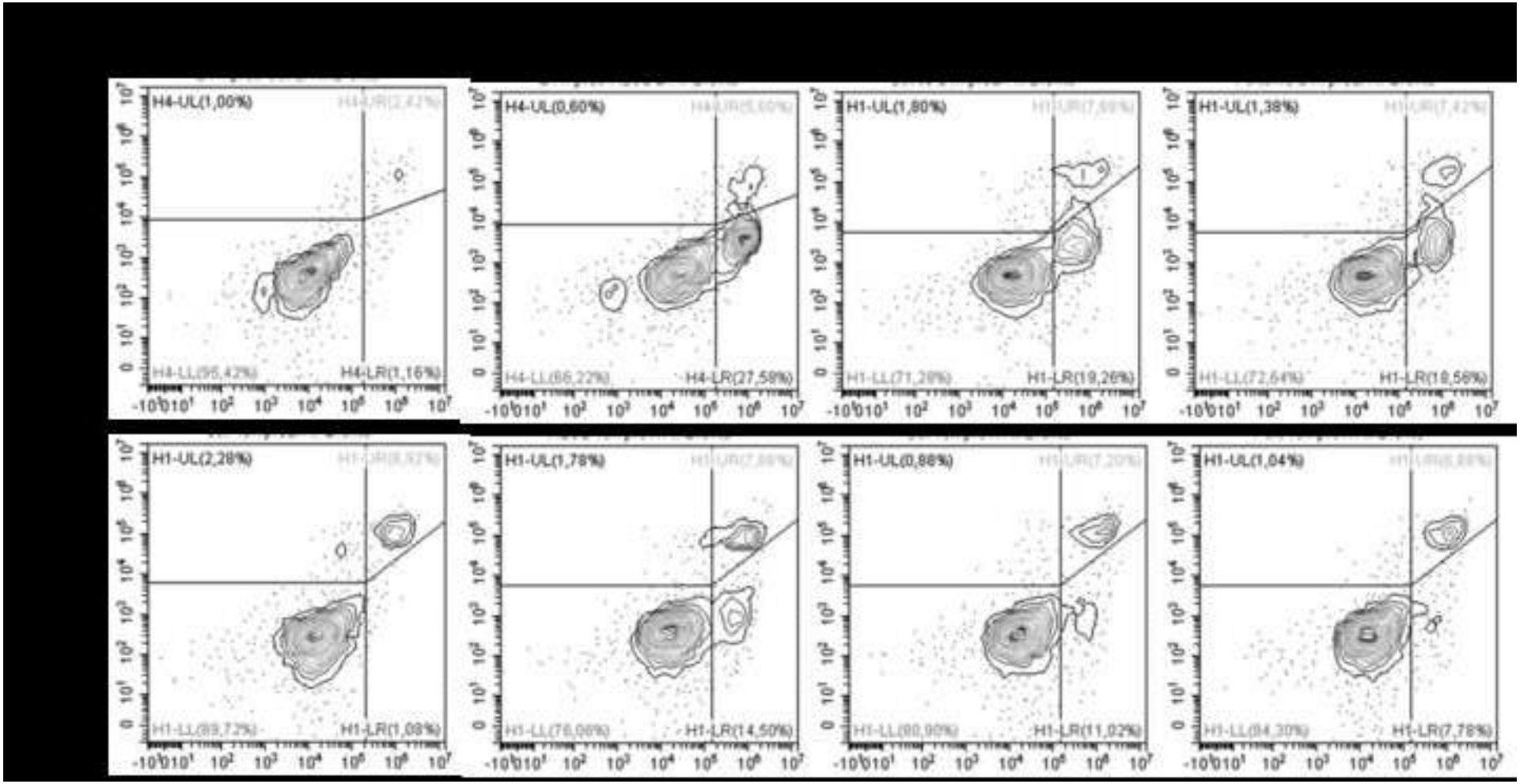

Figure 4B

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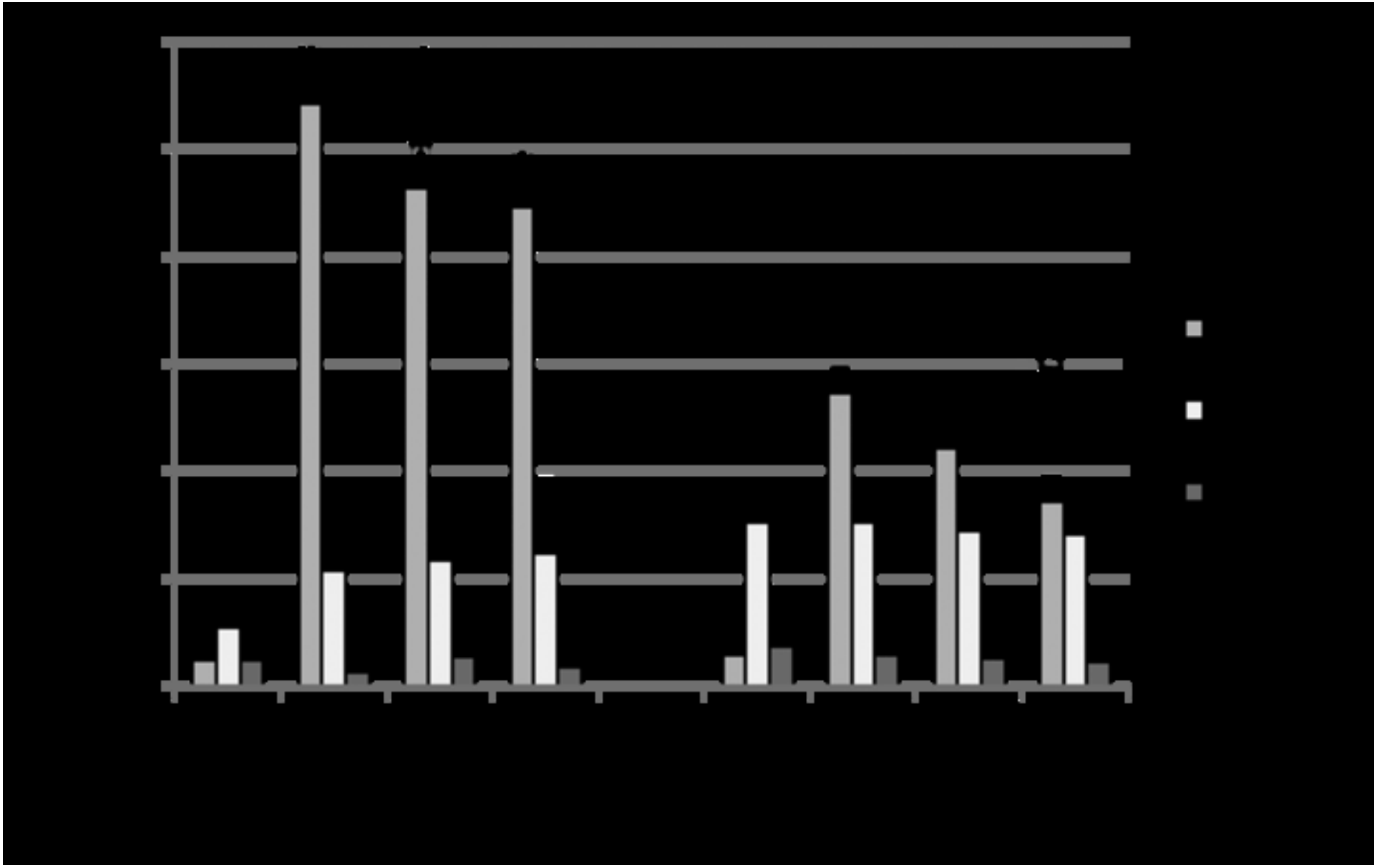


Figure 5 revised

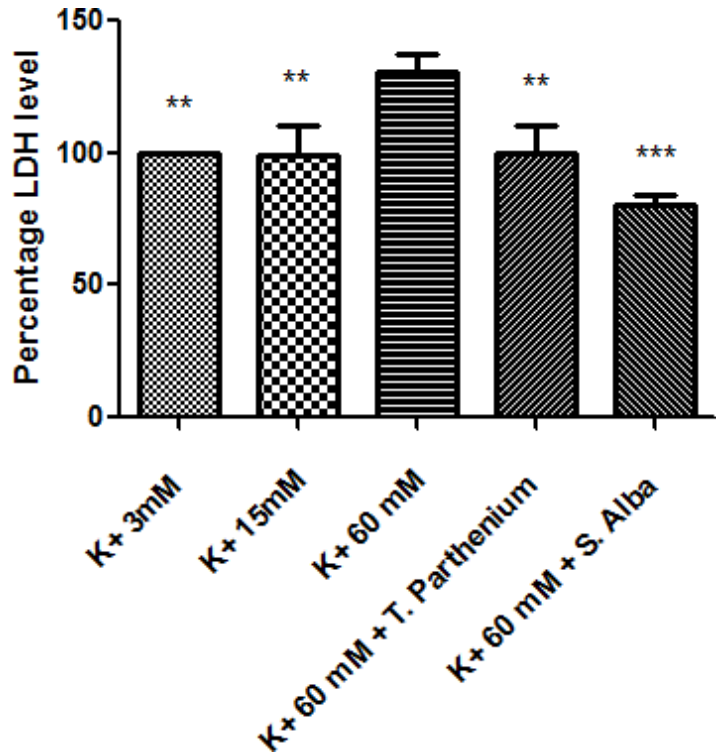


Figure 6 revised

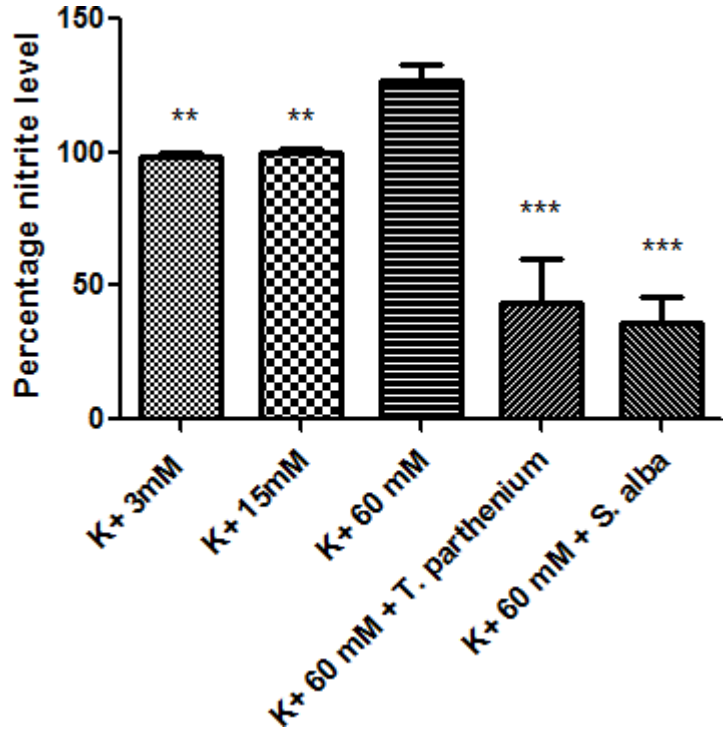


Figure 7 revised

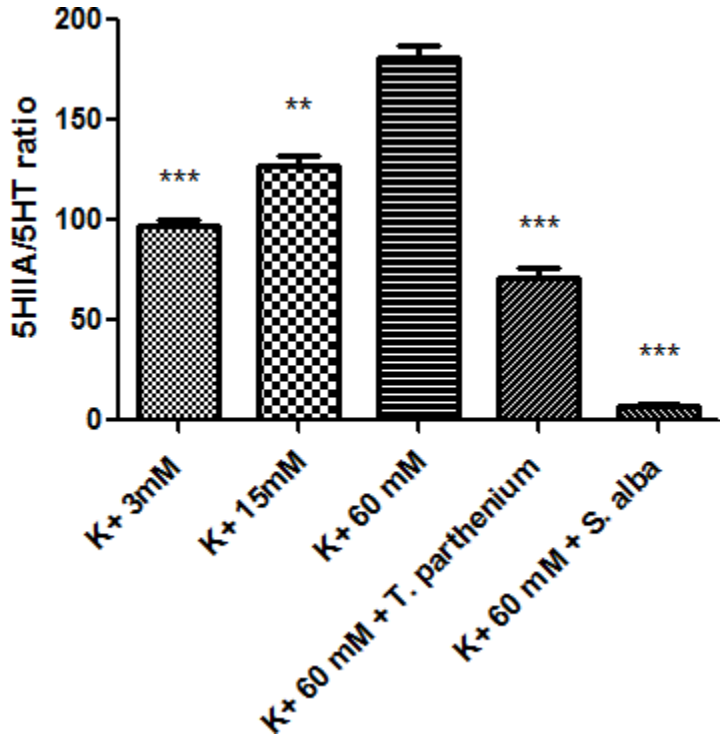
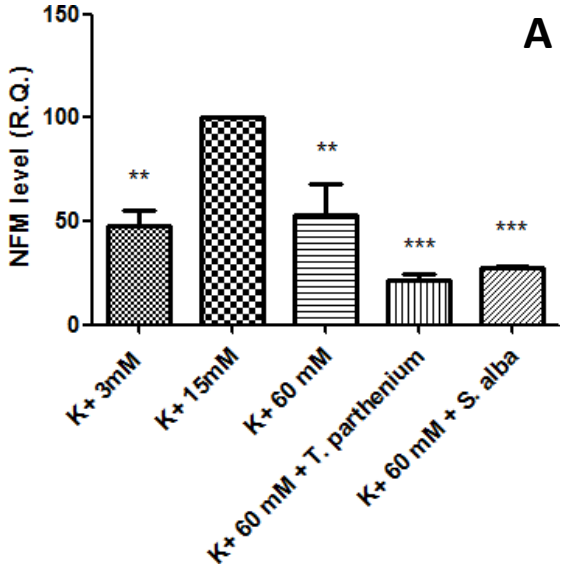
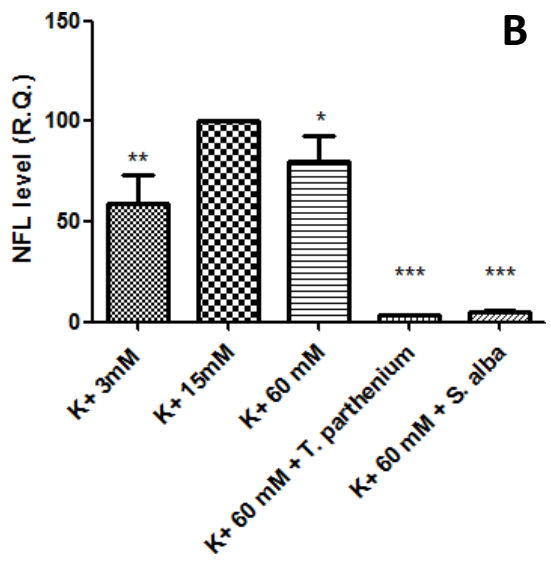
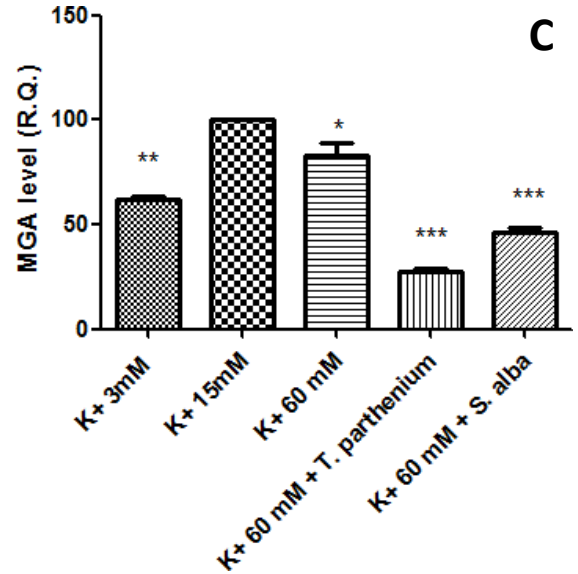


Figure 8 revised







Supplemental

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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