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**Pharma-toxicological and phytochemical investigations on
Tanacetum parthenium and Salix alba extracts: Focus on
potential application as anti-migraine agents**

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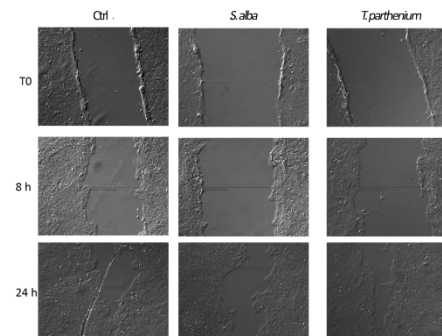


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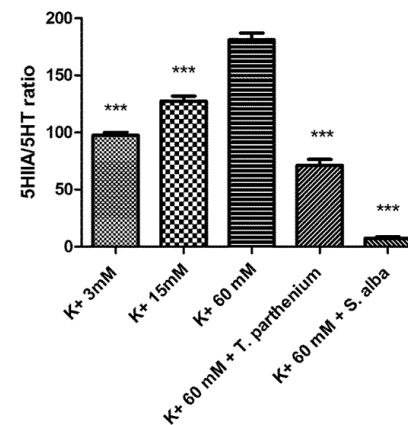
Pharma-toxicological and phytochemical investigations on *Tanacetum parthenium* and *Salix alba* extracts: Focus on potential application as anti-migraine agents

Graphical Abstract

Increased wound healing



Reduced 5-HT turnover



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.

Keywords: *Salix alba*; *Tanacetum parthenium*; cortical spreading depression; serotonin; proteomic analysis



Introduction

Migraine is one of the most common neurological disorder, whose prevalence ranges between 8 to 14.7%, according to European data. Serotonin (5-HT) has long been involved in migraine, with clinical evidences suggesting tight relationships between migraine attacks and neurotransmitter levels. 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 attack. Conversely, the blockade of 5-HT_{2A} and 5-HT_{2C} receptors resulted effective in prophylaxis therapy. Multiple studies also confirmed the involvement of trigeminovascular system in the mechanism of pain, and the role of neurogenic inflammation in pain pathogenesis. Despite there still being a matter of debate about migraine origin site and mechanism, cortical spreading depression (CSD), a supraphysiological and toxic depolarizing phenomenon, appeared to be a possible link between 5-HT depletion and trigeminal nociception. Actually, migraine treatment can be classified in acute aborting attack treatment and prophylactic protocol, the latter aimed to reduce frequency, duration and severity of attacks. Recommended drugs for acute attack treatment include analgesics, nonsteroidal anti-inflammatory drugs, and triptans. Despite there being multiple treatment options, the frequent occurrence of unacceptable adverse effects further supports the research toward nutraceuticals and herbal preparations which could display efficacy alongside with a more acceptable profile of side effects. To this regard, *Tanacetum parthenium* (L.) Sch.Bip. represents one of the well-characterized plants for migraine prophylaxis, that showed efficacy in both adult and children migraineurs, The efficacy of *T. parthenium* was also investigated in combination with anti-inflammatory vitamins and herbal extracts, including *Salix alba* L. Intriguingly, *S. alba* showed modulatory activity on 5-HT pathway, being able to reduce 5-HT turnover, in the hippocampus of rats orally administered, and stimulate 5-HT_{1D} receptor activity. With the aim to improve our knowledge about the use of herbal extracts as innovative preventive strategy against migraine attacks, in the present work we investigated the protective effects of two commercial *T. parthenium* and *S. alba* extracts, in multiple *in vitro* experimental paradigms. Particularly, we evaluated the impact of extract treatment on astrocyte viability, spontaneous migration and apoptosis. Moreover, anti-inflammatory/antioxidant effects were investigated on rat cortex specimens exposed to a neurotoxicity stimulus (K⁺ 60 mM Krebs-Ringer buffer), in order to reproduce CSD, *ex vivo*. Contextually, we assayed specific biomarkers of oxidative stress and inflammation, including lactate dehydrogenase (LDH) and nitrites. Additionally, we evaluated extract effect on cortex 5-HT turnover. Through a validated untargeted proteomic analysis, we also explored potential toxicological effects by evaluating the impact of extract treatment on the levels of specific proteins involved in neuron morphology and development, namely neurofilament (NFEMs) proteins and myelin-associated glycoprotein (MAG). Finally, in order to provide a better interpretation of the observed pharmaco-toxicological effects, a fingerprint analysis has been carried out on selected phenolic compounds, including gallic acid, catechin, epicatechin and resveratrol.



Results and discussion

We observed that both extracts revealed able to exert antioxidant effect, in a selected astrocyte model. Additionally, extract treatment revealed effective in reverting apoptosis, as well. *S. alba* and *T. parthenium* extracts showed efficacy in reducing K⁺ 60 mM-induced LDH and nitrite levels, and 5-HT turnover. On the other hand, untargeted proteomic analysis indicated potential neurotoxicity induced by the extracts, that resulted able to potentiate K⁺ 60 mM-induced downregulation of NEFMs and MAG. Actually, we cannot exclude that the observed neurotoxic effect is the result of elevated content of gallic acid (**113.83 ± 10.24 and 724.71 ± 28.29 µg/mg**), in *S. alba* and *T. parthenium*, respectively.



Phytochemical analysis

Phenol content of the extracts

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



Phytochemical analysis

DPPH radical scavenging effects of the extracts		
Concentration ($\mu\text{g/mL}$)	DPPH % scavenging activity \pm SD	
	<i>S. alba</i>	<i>T. parthenium</i>
400	65.4 \pm 1.1	Not Tested
200	46.3 \pm 0.3	80.6 \pm 1.1
100	29.0 \pm 0.5	82.5 \pm 1.1
50	20.0 \pm 0.3	60.3 \pm 1.3
25	13.6 \pm 0.8	37.7 \pm 2.7



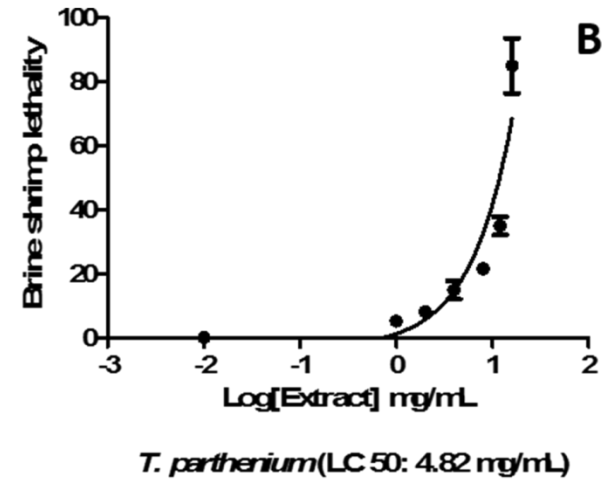
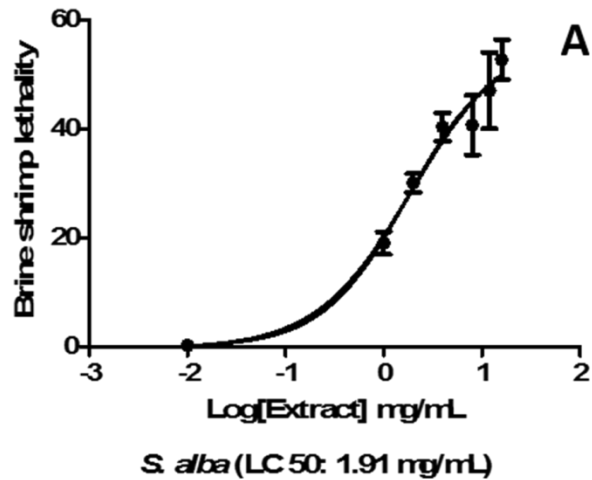
Phytochemical analysis

Table 2 Ferric reducing power of the extracts

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



In vitro studies



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



In vitro studies

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

** H₂O₂ vs ctrl p <0.01

† *S. alba* H₂O₂ + 100, 130 e 200 µg/mL and *T. parthenium* H₂O₂ + 40, 60 and 80 µg/mL

vs H₂O₂ p<0.05

B: 48 h

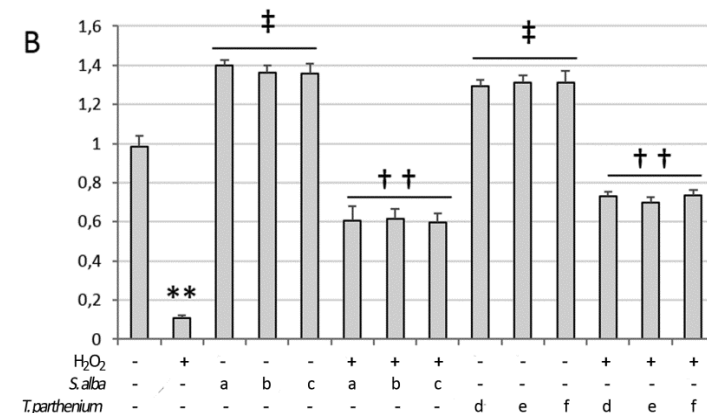
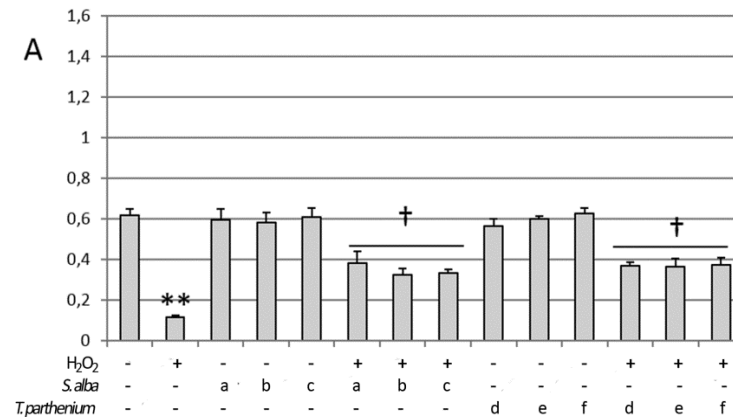
** H₂O₂ vs ctrl p <0.01

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

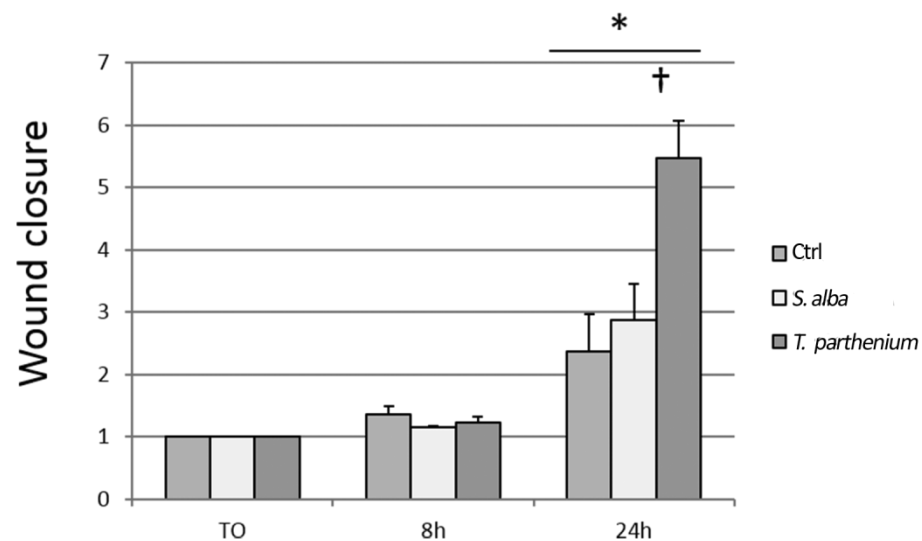
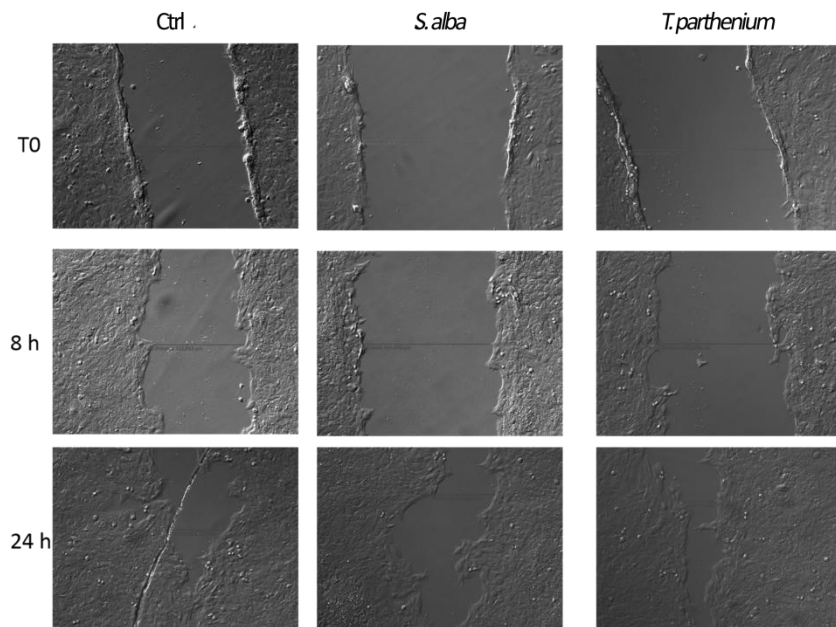
†† *S. alba* H₂O₂ + 100, 130 e 200 µg/mL and *T. parthenium* H₂O₂ + 40, 60 and 80 µg/mL

vs H₂O₂ p<0.01

MTT



In vitro studies



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

A: Representative images (10 X)

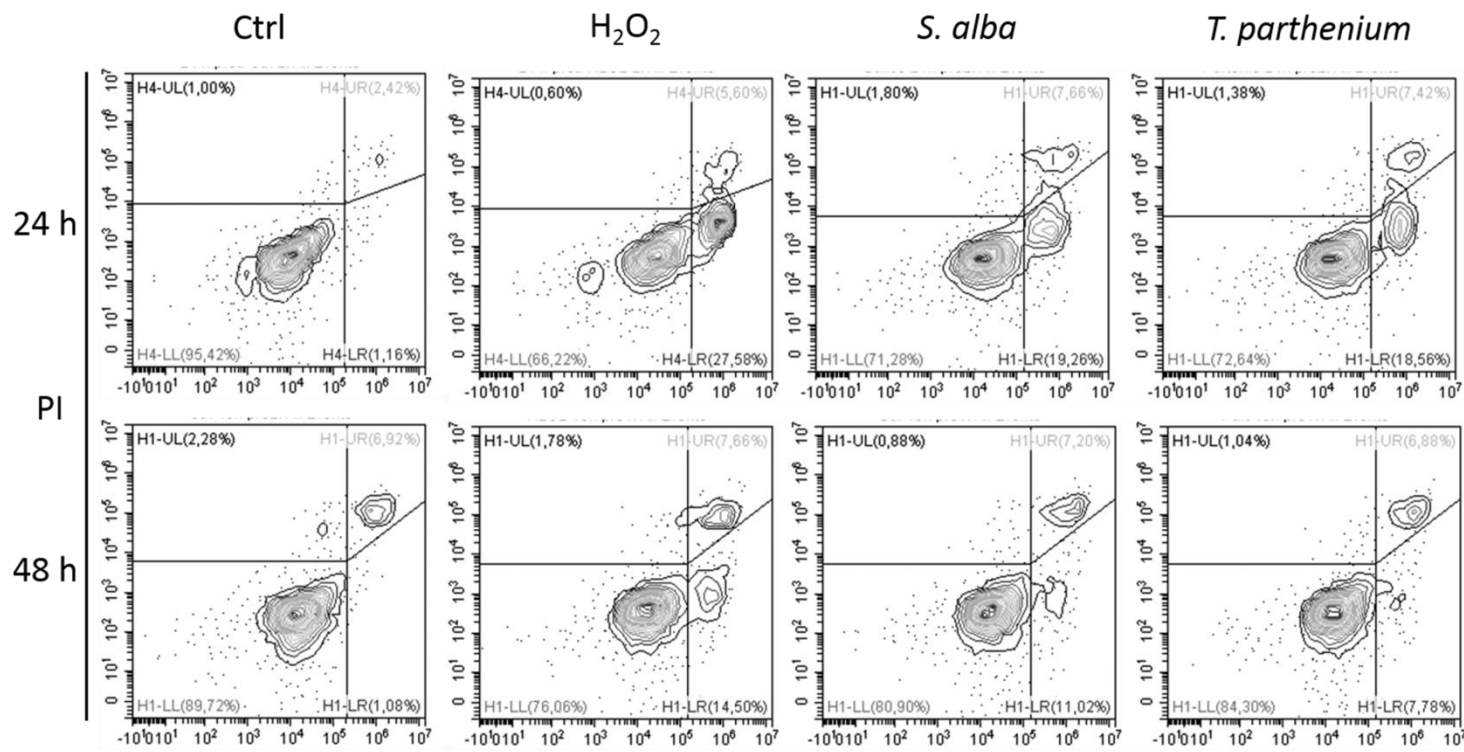
B: The wound closure is represented as fold increase of the T0 samples.

* 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



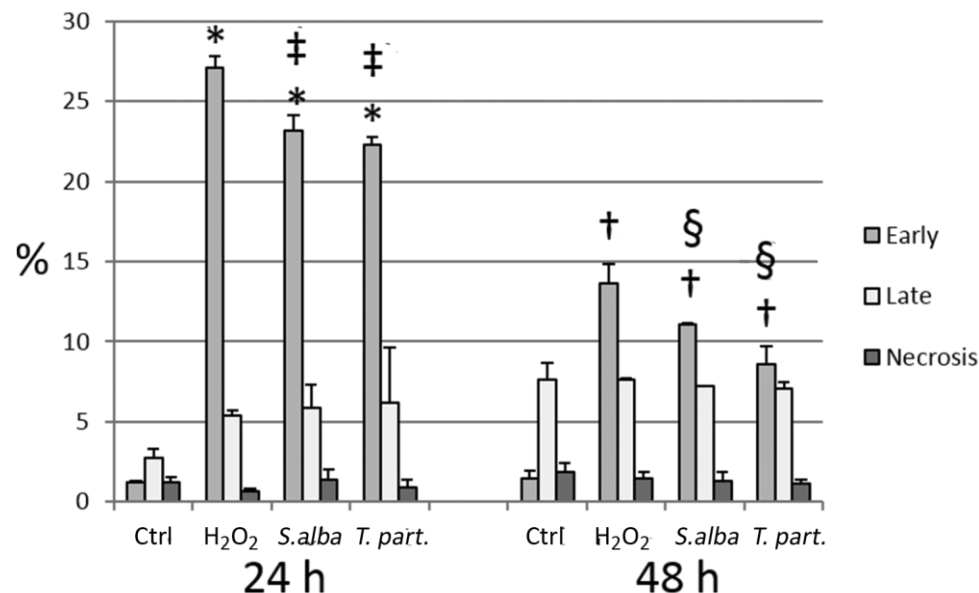
In vitro studies



Apoptosis evaluation in CTX-TNA2 cell line exposed to *T. parthenium* and *S. alba* extracts. Representative dot plots



In vitro studies



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

The graph shows the mean \pm SD of three independent experiments

* Early of H₂O₂, *S. alba* H₂O₂ and *T. parthenium* H₂O₂ 24 h vs Ctrl 24 h p < 0.05

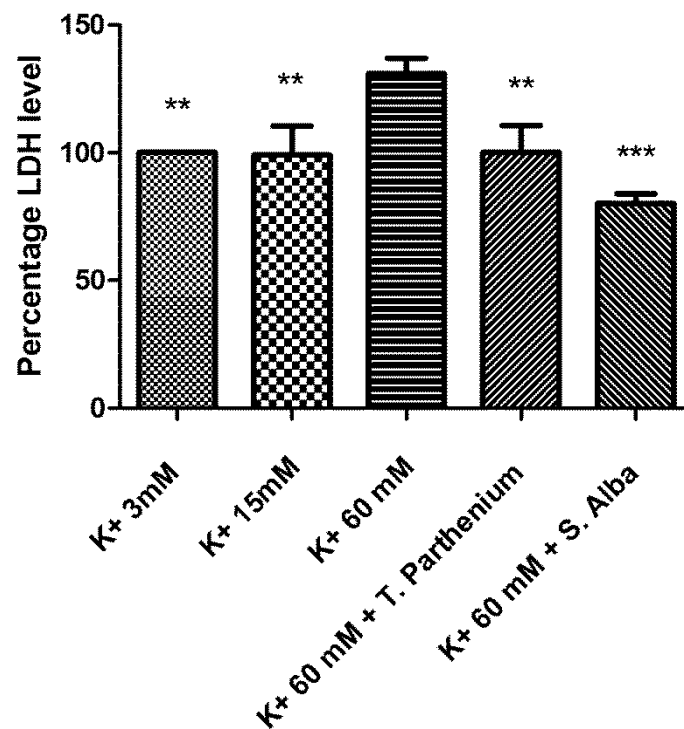
‡ Early of *S. alba* H₂O₂ and *T. parthenium* H₂O₂ 24 h vs H₂O₂ 24 h p < 0.05

† Early of H₂O₂, *S. alba* H₂O₂ and *T. parthenium* H₂O₂ 48 h vs Ctrl 48 h p < 0.01

§ Early of *S. alba* H₂O₂ and *T. parthenium* H₂O₂ 48 h vs H₂O₂ 48 h p < 0.05



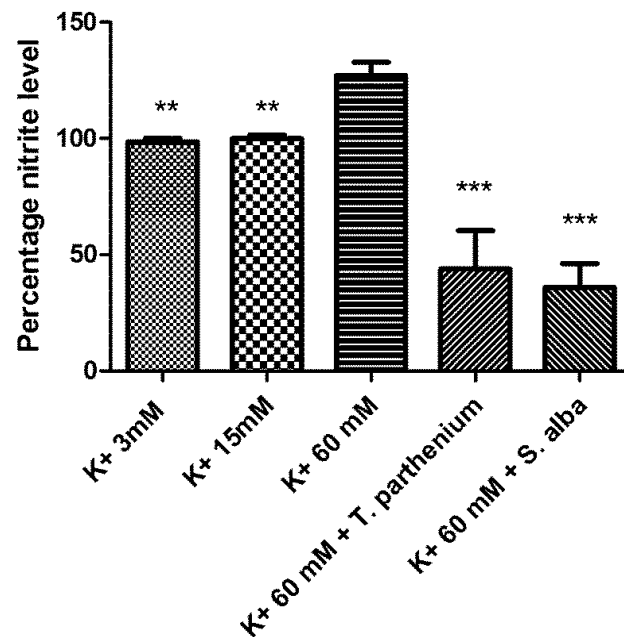
Ex vivo studies: CSD model



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



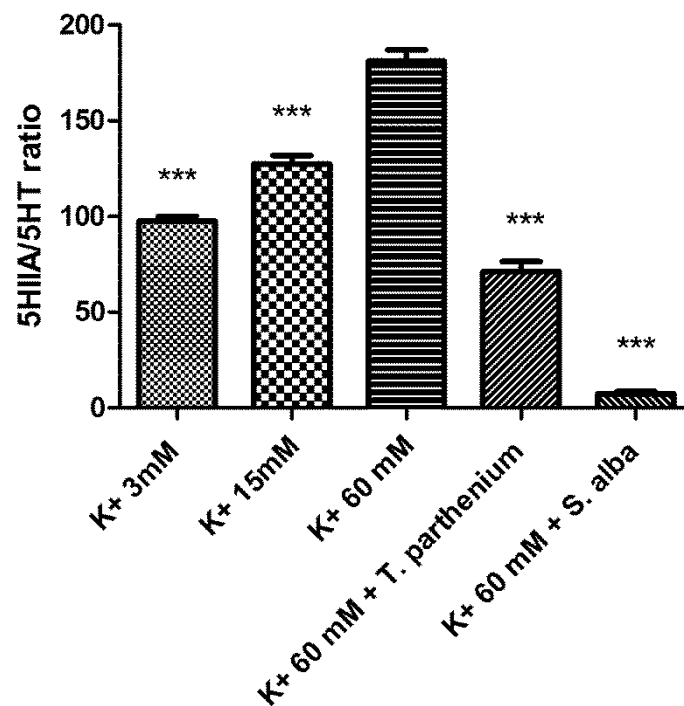
Ex vivo studies: CSD model



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.



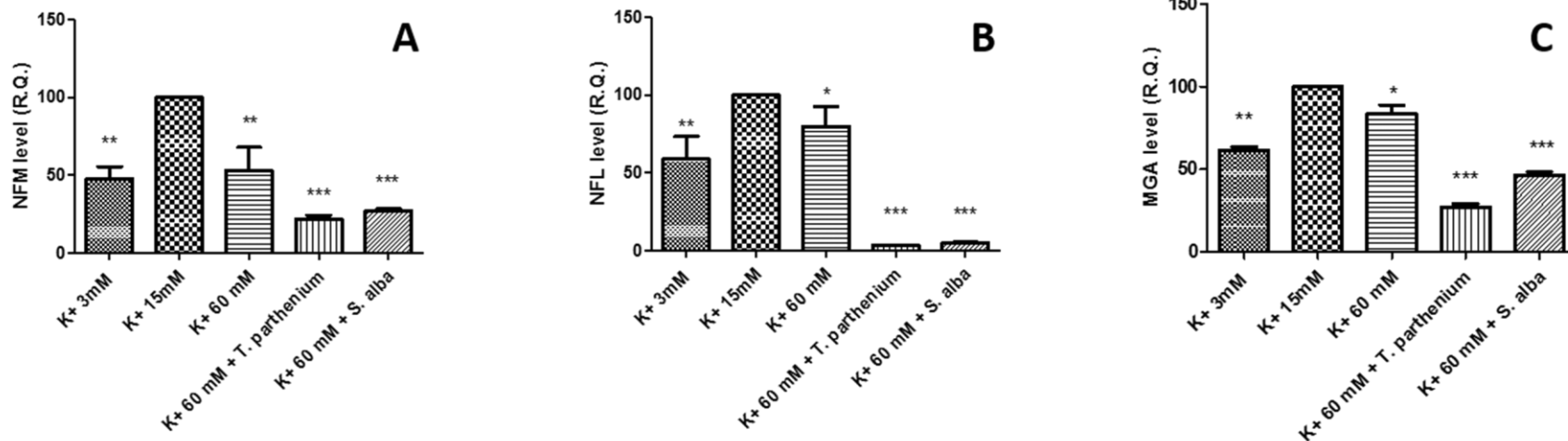
Ex vivo studies: CSD model



Effect of *T. parthenium* (60 µg/mL) and *S. alba* (130 µg/mL) extracts on serotonin (5-HT) turnover, expressed as 5HIA/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.



Ex vivo studies: CSD model



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.



Conclusions

Concluding, the present findings demonstrate multiple protective effects induced by *S. alba* and *T. parthenium* treatment, in rat cortex, including reduction of 5-HT turnover, thus corroborating their use in the management of clinical symptoms related to migraine.

On the other hand, the potentiating effect on K⁺ 60 mM-induced downregulation of cortex NEFMs and MAG levels, suggest caution in the use of *S. alba* and *T. parthenium*-enriched food supplements for potential neurotoxicity effects that should confirmed and characterized with further studies, possibly employing independent methods.



Acknowledgments

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