



Article

Unravelling the Neuroprotective Effects of a Novel Formulation Based on Plant Extracts, Mg, and Vitamin B6

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Abstract: The aim of the present study was to investigate the phenolic composition and the efficacy of an innovative formulation based on Mg, Vitamin B6, and water extracts from *Vitex agnus-castus*, *Crocus sativus*, *Melissa officinalis*, *Betula pendula*, and *Betula pubescens* developed as an effective tool to face neuroinflammation and depression symptoms occurring in premenstrual syndrome (PMS). The formulation was analyzed through colorimetric and liquid chromatography methods for determining the content in phenols and flavonoids. Additionally, scavenging/reducing properties were investigated via 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and horseradish peroxidase assays. The biocompatible limits were determined via allelopathy, the brine shrimp lethality test, and *Daphnia magna* cardiotoxicity assay. The formulation was then assayed in an experimental model constituted by isolated mouse cortex specimens exposed to K⁺ 60 mM Krebs–Ringer buffer, a toxic depolarizing stimulus able to reproduce the burden of inflammation/oxidative stress and the increased serotonin (5-hydroxytryptamine, 5-HT) impoverishment occurring in different neurological and psychiatric conditions, including depression. The results of the phytochemical analysis showed that the formulation is rich in benzoic acids, namely gentisic acid (155.31 µg/mL) and phenylethanoid compounds, namely hydroxytyrosol (39.79 µg/mL) that support the antioxidant effects measured via DPPH (IC₅₀: 1.48 mg/mL), ABTS (IC₅₀: 0.42 mg/mL), and horseradish peroxidase (IC₅₀: 2.02 mg/mL) assays. The ecotoxicological models indicated the formulation as non-toxic, permitting the identification of a biocompatible concentration (1000 µg/mL) to be used in isolated mouse cortex exposed to K⁺ 60 mM Krebs–Ringer buffer. In this model, the gene expression of cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), estrogen receptor-1 (ESR1), prolactin receptor (PRLR), brain-derived neurotrophic factor (BDNF), and serotonin transporter (SERT) was determined by real-time PCR. In the isolated mouse cortex, the formula reduced COX-2, IL-6, SERT, ESR1, and PRLR gene expression and increased BDNF and IL-10 gene expression. Overall, the study corroborated the use of the formulation as an innovative tool to contrast inflammation, oxidative stress, and neurotransmitter impairment associated with PMS.



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1. Introduction

Premenstrual syndrome (PMS) is defined as a cluster of psychological and physical syndromes including anxiety, depression, fatigue, headache, pain, inflammation, and weight gain that is experienced by almost 80–90% of women, with a higher incidence in obese persons [1–3]. The symptoms could be related, albeit partially, to different risk factors among which hormonal changes, distress, altered diet, and pattern of neurotransmission, with serotonin reduction possibly playing a key role in the onset of PMS [4]. Furthermore, new research in the field of neuroendocrinology has highlighted close relationships between inflammation and premenstrual syndrome, as demonstrated by the concomitant increase and decrease in circulating levels of pro-inflammatory markers and neurotrophic factors, respectively [5]. Different pharmacological treatments were shown to be effective in managing PMS, namely the progesterone metabolite allopregnanolone, for its allosteric modulation of GABA neurotransmission, and selective serotonin reuptake inhibitors (SSRIs). These drugs could be effective with different mechanisms including (i) an increase in serotonin (5-HT) concentration in the synaptic cleft; (ii) stimulation of allopregnanolone synthesis; and (iii) modulation of neurosteroid activity [4]. In contrast, in light of the incidence of considerable side effects, alternative therapeutic options are also considered by women including the use of medicinal plants and nutritional supplements, especially vitamins [6]. It is worth mentioning that, besides pharmacological and phytotherapeutic options, alternative approaches including regular aerobic exercise, cognitive-behavioral therapy, and yoga are strongly recommended [4].

Medicinal plants and their derivatives have been historically used worldwide both as drugs and as health foods, and still represent an important source of innovative remedies. Research about natural products, including extracts from medicinal plants, has increased significantly in recent decades, as a direct consequence of new pharmaco-toxicological studies that highlight unexpected side effects of synthetic drugs [7]. In this context, especially in the case of mild chronic pathologies, herbal preparations are often preferred due to the lower degree of side effects. This further stimulated not only the demand for herbal products but also scientific research to lay rational foundations for the use of remedies of plant origin, both from medicinal plants with well-established ethnopharmacological use and from those whose value has yet to be assessed [8].

Vitex agnus-castus L. is a medicinal plant that has been long considered effective in treating PMS. Its fruits are rich in different specialized metabolites, including flavonoids (casticin, isovitexin, luteolin), terpene compounds (agnuside, negundoside, and vitegnoside), and glycosides. The presence of these phytochemicals could explain, albeit partially, the efficacy in modulating neurosteroid, neurotransmitter, opioid, pain, and inflammatory signalings [9].

Crocus sativus L. (Iridaceae Family) stigmas are recognized as the most expensive spice in the world. They are rich in numerous specialized metabolites, including flavonoids (kaempferol, rutin, apigenin, vitexin, isoorientin, naringenin, orientin, populin, myricetin, quercetin, and rhamnetin), kaempferol glycosides, carotenoids (crocins), and monoterpenes (safranal). Flavonoids, crocins, and safranal are the main compounds responsible for the numerous pharmacological effects attributed to this medicinal plant, including its effectiveness against PMS [10–12]. Regarding PMS, the efficacy could be related, albeit partially, to the capability of crocins and safranal in modulating the serotonergic system [13,14], but also in blunting the burden of neuroinflammation associated with depressive behaviors [15].

Melissa officinalis L., belonging to the Lamiaceae Family, is an aromatic and medicinal plant containing more than 100 compounds in its phytocomplex, among which monoterpenes (citral, citronellal), cariophyllane sesquiterpenes, flavonoids (kaempferol, luteolin, quercetin, apigenin), and hydroxycinnamic acids, which includes rosmarinic acid. The plant has been demonstrated to improve cognitive functions, like other aromatic plants such as rosemary, and to reduce clinical symptoms related to PMS, among which anxiety, depression, and sleep disorder [16]. These effects could be related, at least in part, to the modulation of the 5-HT pathway [17].

Betula pendula Roth. and *Betula pubescens* Ehrh., belonging to the Betulaceae Family, are widespread trees in the northern hemisphere. Their leaves, that form the elective plant material, contain numerous quercetin glycosides, among which hyperoside, kaempferol and myricetin glycosides, hydroxycinnamic acids (caffeic acid, chlorogenic acid), lignans, triterpenic alcohols [18,19]. It is traditionally used for treating genitourinary and inflammatory disorders, and also to regulate menstrual flux [19]. Recent findings suggest the efficacy of *Betula* extracts in contrasting lipid peroxidation and neuroinflammation [20,21], closely related to PMS [15].

Other than medicinal plants, the administration of mineral salts and vitamins such as magnesium (Mg) and Vitamin B6 has been positively related to the efficacy in blunting the clinical symptoms of PMS [22,23], with their pharmacological association being more effective than the single ingredient [24]. This also adds to the capability of Mg and coenzyme Q10, in improving the efficacy of *Tanacetum parthenium* L. and *Salix alba* L. Indeed, all these ingredients are co-administered for migraine treatment [25,26], which is one of the main symptoms characterizing PMS [2].

Therefore, the aim of the present study was to investigate the efficacy of the pharmacological association of Mg, Vitamin B6, and water extracts from *V. agnus-castus*, *C. sativus*, *M. officinalis*, *B. pendula*, and *B. pubescens* in an experimental model constituted by isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. The present experimental paradigm reproduces a supraphysiological and toxic depolarizing stimulus capable of increasing the burden of inflammation/oxidative stress. This stimulus causes 5-HT impoverishment that is related to different neurological and psychiatric conditions, including migraine and depression. This is of relevance considering the use of serotonergic drugs in PMS [4]. In isolated mouse cortex challenged with K^+ 60 mM Krebs–Ringer buffer, the gene expression of cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), estrogen receptor-1 (ESR1), prolactin receptor (PRLR), brain-derived neurotrophic factor (BDNF), and serotonin transporter (SERT) was determined by real-time PCR. These biomarkers have been assayed in order to measure the capability of the formulation in contrasting inflammation and oxidative stress that is related to PMS [4]. Regarding the formulation, it is important to highlight that the use of water extracts was chosen for the high degree in biocompatibility [18], especially in view of the long-term use of the extracts. The pharmacological study also included an accurate evaluation of the biocompatibility limit of the formulation, that was assessed through three different and independent ecotoxicological models, namely allelopathy, *Artemia salina* lethality, and *Daphnia magna* cardiotoxicity assays. These are reliable and alternative in vivo models for predicting toxicity in eukaryotic organisms; thus, permitting to define safe doses in pharmacological tests [27–29]. The formulation was also subjected to a rigorous phytochemical analysis to determine the main compounds present in the formulation. Finally, a bioinformatics study, including protein–protein interactions and components–targets analysis, was conducted to unraveling the putative mechanisms underlying the observed effects.

2. Materials and Methods

2.1. Formulation

Dry water extracts from fruits of *V. agnus-castus* L. (0.5% agnuside), stigmas of *C. sativus* L. (2% safranal and 3% crocins), leaves of *M. officinalis* L. (12% rosmarinic acid), and leaves of *B. pendula* Roth and *B. pubescens* Ehrh. (2.5% flavonoids), Vitamin B6 (pyridoxine hydrochloride) powder and tribasic anhydrous magnesium citrate powder (magnesium 14.50%, citrate 85.50%) were provided by Cristalfarma s.r.l. (Milan, Italy). The samples were deposited at the Herbarium of the Botanical Garden, "G. d'Annunzio" University with following voucher specimen numbers: GDS_VAGNUSCASTUS_2024, GDS_CSATIVUS_2024, GDS_MOFFICINALIS_2024, GDS_BPENDULA_2024, and GDS_BPUBESCENS_2024. The dry extracts and the powders were rehydrated in water via ultrasound-assisted extraction (Trans-sonic T460 ultrasonic bath; Elma, Singen, Germany). The formulation concentration after rehydration was 10 mg/mL. The components were used in combination with the proportions reflecting the composition of the commercial food supplement Elsa[®] property of the Cristalfarma S.r.l. (Milan, Italy), at the following percentages: magnesium 50.86%, *B. pendula* and *B. pubescens* 22.6%, *M. officinalis* 16.95%, *V. agnus-castus* 4.52%, *C. sativus* 4.52%, and Vitamin B6 0.54%.

2.2. HPLC

The formulation was subjected to reversed-phase HPLC-UV analysis, in gradient elution mode, to quantitatively determine the phenolic composition. The HPLC apparatus consisted of a two PU-2080 PLUS chromatographic pump, a DG-2080-54-line degasser, a mix-2080-32 mixer, UV, an AS-2057 PLUS autosampler, and a CO-2060 PLUS column thermostat (all from Jasco, Tokyo, Japan). ChromNAV2 Chromatography software was used for integration. The separation was conducted within 48 min of the chromatographic run, starting from the following conditions: 97% water with 0.1% formic acid, and 3% methanol with 0.1% formic acid. The flow was set at 0.6 mL/min throughout the analysis. Details about the gradient are listed in Table S1. The analytes were separated on an Infinity lab Poroshell 120-SB reverse phase column (C18, 150 × 4.6 mm i.d., 2.7 µm; Agilent, Santa Clara, CA, USA). Column temperature was set at 30 °C. Quantitative determination of phenolic compounds was performed via UV detector at 254 nm. The injection volume was 5 µL. Quantification was performed through 7-point calibration curves, with linearity coefficients (R²) > 0.999, in the concentration range 2–140 µg/mL. The final concentration of the extract was 40 mg/mL before injecting in HPLC system.

2.3. Colorimetric Assays

Total phenolic and flavonoid contents were determined according to the Folin–Ciocalteu assay, and the results were expressed as gallic acid (mg GAE/g dry extract) and rutin (mg RE/g dry extract) equivalents. The antioxidant activity of hemp extract was determined by neutralization methods like DPPH, ABTS, and horseradish peroxidase assays. The comprehensive procedures are reported in the literature [30] and Supplementary Materials.

2.4. Ecotoxicological Investigation

2.4.1. Preparation of Test Sample Concentrations

A stock solution of the formulation was prepared at a concentration of 10 mg/mL and further diluted to obtain the desired concentration range for testing. For the allelopathy assay, distilled water was used for the dilution, while artificial sea water was used for the brine shrimp lethality assay, with a concentration range from 0.625 to 10 mg/mL for both assays.

2.4.2. Allelopathy Assay

A Petri dish-based experiment was carried out to evaluate the potential phytotoxic activity of the tested formulation in a concentration range from 1.25 to 10 mg/mL. Commercial dicotyledon seeds of *Cichorium intybus* (CI), *Dichondra repens* (DR), *Raphanus sativus* (RS), and monocotyledon seeds of *Avena sativa* (AS) and *Secale cereale* (SC) were selected for the test due to their fast germination rate and high sensitivity to external compound, which makes them ideal bioindicators for detecting even subtle inhibitory effects caused by the tested samples. By incorporating these diverse species, the assay ensures a robust and meaningful exploration of the phytotoxic potential of the formulation, offering valuable data on how it could impact various plants in real-world scenarios. The inclusion of both dicotyledons and monocotyledons also ensures a more comprehensive analysis, as the two plant groups possess distinct physiological characteristics. This selection helps to assess whether the formulation exhibits a broad-spectrum allelopathic effect or if it shows selective inhibition based on plant type. The assay was conducted in 90 mm Petri dishes, each containing a double-layered filter paper disk, previously soaked with 3 mL of the plant extract at different concentrations. Distilled water was used as a negative control. The seeds were surface-sterilized using a diluted (NaClO:dH₂O, 1:9) commercial bleaching liquid for 10 min. After thoroughly rinsing the seeds with sterile distilled water to remove any traces of bleach, 10 seeds of the corresponding seed variety were arranged on the filter paper disks, previously divided into 3 distinct areas for dicotyledon and 2 distinct areas for monocotyledon seeds. Petri dishes were then sealed with parafilm, to ensure a closed-system model and incubated in darkness at room temperature (25 ± 2 °C) for 96 h. The criterion for determining germination was the emergence of a radicle at least 2 mm in length, exhibiting a typical geotropic curvature. Seeds that only swelled but did not fully germinate were excluded. The seedling length was measured and categorized by elongation rate: low (<0.4 cm), medium (0.5–0.9 cm), or high (>1 cm). The number of germinated seeds and their seedling lengths were recorded after 96 h, and results were reported as Relative Germination Percentage (RGP) and Seedling Length (SL), that is the mean of the seedling's length compared to control samples.

2.4.3. Brine Shrimp Toxicity Bioassay

Artemia salina L. cysts were hatched in oxygenated artificial sea water (1 g cysts/L). After 24 h, the toxicity assessment via BSLA (brine shrimp lethality assay) was carried out as previously described on freshly hatched brine shrimps (nauplii) [31,32]. Each sample concentration was tested in triplicate over a range of concentrations between 0.625 and 10 mg/mL in glass tubes containing 5 mL of artificial sea water and 10 nauplii each. The surviving brine shrimp nauplii were counted after 24 h and the results were expressed as the percentage of mortality, and calculated as follows: $[(T - S)/T] \times 100$, where T is the total number of incubated larvae, and S is the number of surviving nauplii. The median lethal concentration (LC₅₀) was calculated using GraphPad software. For the acceptability of the test, up to 10% of mortality in the control was admitted.

2.4.4. *Daphnia magna* Cardiotoxicity Assay

Three non-pregnant *Daphnia magna* specimens were collected per experimental group and transferred into corresponding wells of a 6-well plate, with three specimens per well. One well contained spring water as a control group, and another well contained the formulation at a concentration of 5.837 mg/mL (corresponding to the LC₅₀ value obtained from the previously described BSLA). After transfer, the *D. magna* specimens were exposed to the treatment for 15 min at room temperature. Subsequently, each specimen from the respective groups was individually transferred to a microscope slide in a 50 µL droplet of

the tested extract, and the heartbeat rate was recorded using microscopy for 15 s. Following the basal condition observations, each specimen was exposed to a cardiotoxic stimulus (10% ethanol solution) for 2 min. All measurements were conducted in triplicate, and the experiment was repeated three times. The decrease in heart rate was compared to untreated and 10% ethanol-treated groups, serving as negative and positive controls, respectively.

2.5. *Ex Vivo Study*

In accordance with the recognized principles of “replacement, refinement and reduction in animals in research”, the cortex specimens were obtained as residual materials from vehicle-treated mice randomized in our previous experiments, approved by the local ethical committee (‘G. d’Annunzio’ University, Chieti, Italy) and Italian Health Ministry (Project no. F4738.N.5QP). The specimens were maintained in a thermostatic shaking bath at 37 °C for 1 h (incubation period), in a Krebs–Ringer buffer at different K⁺ concentrations (3, 60 mM), as previously described [33,34] in a humidified incubator and challenged with the formulation (1000 µg/mL). The samples were then subjected to gene expression analyses of BDNF, SERT, IL-6, IL-10, ESR1, and PRL, as previously reported [35]. Details about gene expression analysis are included in Supplementary.

2.6. *Bioinformatics Prediction*

Direct (physical) and indirect (functional) protein–protein interactions were predicted using the bioinformatics platform STRING [36], and results have been reported as protein–protein network. The prediction was limited to the species *Homo sapiens* and the interaction score was set to a confidence 0.4 as the threshold. Putative targets were identified through the bioinformatic method recently described by Gu and colleagues [37]. Briefly, chemical structures were prepared and converted in canonical SMILES using ChemSketch software version 12.01. The SMILES were then processed by the Swiss Target Prediction (<http://www.swisstargetprediction.ch/>, accessed on 10 January 2025) for predicting putative targets. The name of the identified targets was normalized according to the UniProt database (<https://www.uniprot.org/>, accessed on 30 September 2024). Finally, Cytoscape software (3.9.0 version, National Institute of General Medical Sciences (NIGMS), Bethesda, MD, USA) was used to create a components–targets illustration network.

2.7. *Statistical Analysis*

Statistical analysis was performed by GraphPad Prism™ (Version 5.01) software (GraphPad Software, Inc., San Diego, CA, USA). The statistical significance ($p < 0.05$) was evaluated through analysis of variance (ANOVA) followed by Newman–Keuls comparison multiple test.

3. Results and Discussion

3.1. *Formulation*

In the present study, an innovative formulation based on commercial water extracts from *V. agnus-castus* L., *C. sativus* L., *M. officinalis* L., *B. pendula* Roth, and *B. pubescens* Ehrh., standardized in agnuside, crocins and safranal, rosmarinic acid, and total flavonoids, respectively, was investigated. The herbal extracts were selected for their potential use in managing PMS symptoms [9–11,16]. The formulation was analyzed in order to determine the qualitative and quantitative composition in phenolic compounds and flavonoids, that are known to play a primary role in neuroprotection [38,39].

3.2. Phytochemical Analysis

As depicted in Table 1, the colorimetric analysis of the total phenols and flavonoids showed that phenolic compounds, expressed as gallic acid equivalents, are almost four times more concentrated compared with total flavonoids, expressed as rutin equivalents. This is also confirmed, albeit partially, by the chromatographic analysis (Table 2, Figure 1) that demonstrated the gentisic acid (peak #5; Rt 15.383 min.), a phenolic acid, as the phytochemical present at a higher concentration in the formulation, followed immediately by 3-hydroxytyrosol (peak # 2; Rt 11.700 min.), a phenylethanoid, and hydroxycinnamic acids such as caftaric acid and chlorogenic acid (peak #3, Rt 12.733 min.; peak #8, Rt 17.042 min.) and benzoic acid (peak # 17, Rt 26.033). Phenylethanoids have been reported in *Betula* species [40]. This suggested that *B. pendula* and *B. pubescens* extracts are the main sources of hydroxytyrosol. Although hydroxycinnamic and benzoic acids are ubiquitous in angiosperms [41], the extract of *M. officinalis* could be the main source of caftaric acid [42]. In contrast, benzoic acids were identified in all medicinal plants included in the formulation [43–46].

Table 1. Total phenolic content and flavonoid content of the tested formulation. Values are reported as mean \pm S.D. of three parallel measurements. TPC, total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalents; RE, rutin equivalents; dm, dry material.

Parameters	Results
Total phenolic content (mg GAE/g _{dm})	52.13 \pm 3.48
Total flavonoid content (mg RE/g _{dm})	14.01 \pm 0.51

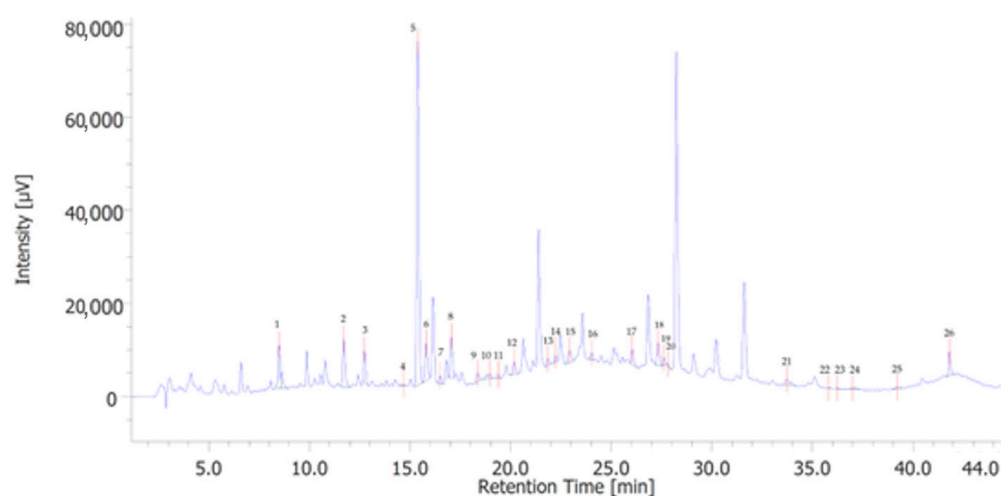


Figure 1. Chromatographic analysis of the phenolic composition of the formulation. Among the identified phenolic compounds, the most prominent were 3-hydroxytyrosol (peak #2, Rt 11.700 min.), caftaric acid (peak #3), gentisic acid (peak #5, Rt 15.383 min.), and benzoic acid (peak #17, Rt 26.033). The separation of the terpenophenols was conducted in gradient elution mode on an Infinity lab Poroshell 120-EC reverse phase column (C18, 150 mm \times 4.6 mm i.d., 2.7 μ m; Agilent Santa Clara, CA, USA). Details about chromatographic conditions are reported in Table 1.

Table 2. Quantitative determination chromatographic analysis of the phenolic composition of the formulation.

Peak Number	Phytochemical	Retention Time (min)	Concentration ($\mu\text{g/mL}$)
1	Gallic acid	8.483	3.62
2	3-Hydroxytyrosol	11.700	39.79
3	Caftaric acid	12.733	10.47
4	Catechin	14.700	2.87
5	Gentisic acid	15.383	155.31
6	4-Hydroxybenzoic acid	15.792	1.33
7	Loganic acid	16.492	2.06
8	Chlorogenic acid	17.042	6.81
9	Vanillic acid	18.358	0.37
10	Caffeic acid	18.958	0.40
11	Epicatechin	19.383	3.44
12	Syringic acid	20.175	1.62
13	Syringaldehyde	21.850	5.84
14	Chicoric acid	22.217	3.15
15	p-Coumaric acid	22.925	3.43
16	t-Ferulic acid	24.033	0.39
17	Benzoic acid	26.033	10.27
18	Hyperoside	27.317	1.60
19	Rutin	27.608	0.58
20	Resveratrol	27.800	0.78
21	t-Cinnamic acid	33.717	0.28
22	Quercetin	35.792	0.03
23	Naringenin	36.225	0.36
24	2,3-Dimethylbenzoic acid	36.983	0.78
25	Hesperetin	39.200	1.99
26	Kaempferol	41.792	1.48

3.3. Antioxidant Assays

The content of phenolic compounds is consistent with the antioxidant effects reported in Tables 3 and 4. Indeed, the formulation was effective as a radical scavenger in DPPH and ABTS assays and at the same time it was effective in inhibiting the peroxidase activity. This further demonstrated the antioxidant effects that could be crucial for a neuroprotective role, which will be described. Among the cell-free antioxidant assays, the ABTS test showed the highest potency, with a IC_{50} value of 0.42 mg/mL that also permitted the definition of the formulation as a moderate antioxidant [47,48]. This could depend, albeit partially, on different antioxidant mechanisms involved in the radical-antioxidant reactions [49]. For instance, the single electron transfers (SETs) in ABTS reactions are faster than those occurring in DPPH assay, due to the difficult accessibility of phenolic compounds to the radical site of the DPPH molecule [50]. This suggested that ABTS could be superior to DPPH in predicting the antioxidant activity of a formulation rich in phenolic compounds.

Actually, the antioxidant effects displayed by the formulation could be related, albeit partially, to the total content of phenols and flavonoids [51]. However, it is reasonable to hypothesize that most of the antiradical efficacy could be ascribed to the content in genticic acid [52] and hydroxytyrosol [53].

Table 3. Antioxidant properties of the tested formulation. Values are reported as mean \pm S.D. of three parallel measurements.

Sample	DPPH	ABTS
	IC ₅₀ (mg/mL)	IC ₅₀ (mg/mL)
Formulation	1.48 \pm 0.02	0.42 \pm 0.06
Trolox	0.08 \pm 0.01	0.03 \pm 0.01

Table 4. Enzyme inhibitory properties of the tested formulation. Values are reported as mean \pm S.D. of three parallel measurements.

Sample	Peroxidase Inhibition (IC ₅₀ : mg/mL)
Formulation	2.02 \pm 0.20
Gallic acid	0.004 \pm 0.001
Quercetin	0.039 \pm 0.005

3.4. Eco-Toxicological Assays

3.4.1. Allelopathy Assay

Allelopathy is a biological process where plants secrete biochemicals, known as allelochemicals, into their environment, affecting the growth, survival, and reproduction of other plants. These allelochemicals can be present in various plant components, such as leaves, stems, roots, flowers, and seeds, and are released through volatilization, root exudation, leaching, and the decomposition of plant residues [54]. Understanding allelopathy is essential as it reveals the complex interactions within plant communities and ecosystems thereby enriching our knowledge of plant competition and biodiversity. In this study, the phytotoxic activity of the tested formulation was evaluated using five plant species: *C. intybus* (CI), *D. repens* (DR), *R. sativus* (RS), *A. sativa* (AS), and *S. cereale* (SC), representing both dicotyledons and monocotyledons. The results are presented in Figures S1–S5 (Supplementary Materials) and Table 5, depicting the effects of different concentrations of the formulation (0.62 to 10 mg/mL) on the relative seed germination percentage (RGP) and seedling growth, in terms of length (SL). The results demonstrated a concentration-dependent inhibition of seed germination for all tested species, with the exception of *R. sativus*. This suggested a higher tolerance to the allelopathic effects of the formulation at all concentrations. The dose-dependent phytotoxic effect induced by the extracts is consistent, albeit partially, with their content of phenolic compounds, which are well-documented as allelochemicals once released into the soil [55]. These compounds can disrupt seed germination and early plant growth by affecting key physiological processes. The observed variations in sensitivity among the tested species suggested that the allelochemical action was influenced by both the concentration of phenolic compounds in the extracts and the intrinsic tolerance mechanisms of each species. The data in Table 5 reflect the effects of varying concentrations of the tested formulation on the seedling length, suggesting a different response between the dicotyledonous and monocotyledonous species, with varying levels of sensitivity among the species. Dicotyledonous *C. intybus* and *R. sativus* species exhibit some level of resilience, especially at lower concentrations. Whilst *D. repens* and monocotyledonous species, particularly *S. cereale*, appear more susceptible displaying an immediate and substantial growth

inhibition, even at lower doses of the extract. This suggests that the allelochemicals in the formulation may differentially affect the physiological processes of these two groups, with monocotyledons being generally more susceptible. These variations in sensitivity emphasize the importance of considering species-specific responses when evaluating the biocompatibility limits of such extracts.

Table 5. Tested formulation effect (concentration range: 0.62–10 mg/mL) on the seedling length (SL) of the selected plant species, expressed in millimeters.

Concentration mg/mL	Seedling Length (mm)				
	Dicotyledon Seeds			Monocotyledon Seeds	
	<i>C. intybus</i>	<i>R. sativus</i>	<i>D. repens</i>	<i>A. sativa</i>	<i>S. cereale</i>
CTRL	12.7 ± 0.9	24.6 ± 1.5	5.4 ± 0.6	38.6 ± 5.0	6.4 ± 1.1
0.62	12.4 ± 1.3	25.9 ± 1.2	3.6 ± 0.3 ***	13.8 ± 1.2 ***	4.4 ± 0.3 ***
1.25	14.6 ± 2.1	22.1 ± 1.8	2.9 ± 0.8 ***	15.2 ± 1.5 ***	3.7 ± 0.2 ***
2.5	10.0 ± 1.2	16.3 ± 1.1 ***	3.2 ± 0.2 ***	16.0 ± 1.0 ***	3.7 ± 0.5 ***
5	17.3 ± 1.8 *	19.6 ± 1.5 *	3.3 ± 0.6 ***	17.0 ± 1.1 ***	3.5 ± 0.4 ***
10	11.2 ± 2.1	19.9 ± 2.7 *	2.2 ± 0.1 ***	11.3 ± 1.0 ***	0.0 ± 0.0 ***

Data are presented as means ± SD. ANOVA, $p < 0.0001$; post hoc (Newman–Keuls test), * $p < 0.05$, *** $p < 0.001$ vs. CTRL (Control) group.

3.4.2. Brine Shrimp Toxicity Bioassay

Artemia salina, also known as brine shrimp, is a zooplanktonic crustacean ubiquitous in saline aquatic environments ranging from lakes to oceans and it is extensively utilized as a model system for the evaluation of acute toxicological responses. In particular, the brine shrimp lethality assay (BSLA) is widely used in preliminary screenings for bioactive compounds due to its simplicity, rapidity, reliability, and cost-efficiency [33]. This assay also demonstrates a high correlation of results with cytotoxic activity in higher organisms in particular with the toxicity data of rodents and humans and shows a good correlation with cytotoxicity tests making these measurements suitable as preliminary results [56,57]. *Artemia* species have been used in testing acute toxicity of toxic materials, such as heavy metals and pesticides [58], nanoparticles [59], bioactive molecules, natural extracts, and metal complexes [56]. The present study was conducted to test the toxicity limits of the formulation under study and evaluate them in terms of LC₅₀ (lethality concentration) value, using the standard toxicity indices established by Meyer and Clarkson. This classification helps in assessing the potential toxicity of various substances, including plant extracts, chemicals, and pharmaceuticals. According to Meyer's classification, extracts are considered toxic if the LC₅₀ is less than 1000 µg/mL, and non-toxic if the LC₅₀ is greater than 1000 µg/mL [32]. Clarkson's classification categorizes substances as non-toxic for LC₅₀ values above 1000 µg/mL, low toxic for LC₅₀ values between 500 and 1000 µg/mL, medium toxic for LC₅₀ values between 100 and 500 µg/mL, and highly toxic for LC₅₀ values between 0 and 100 µg/mL [60]. Table 6 and Figure 2 show that the tested formulation can be classified as non-toxic against *Artemia salina* nauplii, with LC₅₀ values of 5.837 mg/mL.

Table 6. Brine shrimp lethality assay results in terms of LC₅₀ value and toxicity levels according to Meyer’s and Clarkson’s classifications. Tested samples: formulation in a concentration range between 0.625 and 10 mg/mL.

Sample	Concentration Range [mg/mL]	LC ₅₀ (mg/mL)	95% Confidence Interval	R ²	Toxicity Class	
					Meyer’s Classification	Clarkson’s Classification
Formulation	[0.625–10]	5.837	5.465–6.235	0.971	non-toxic	non-toxic

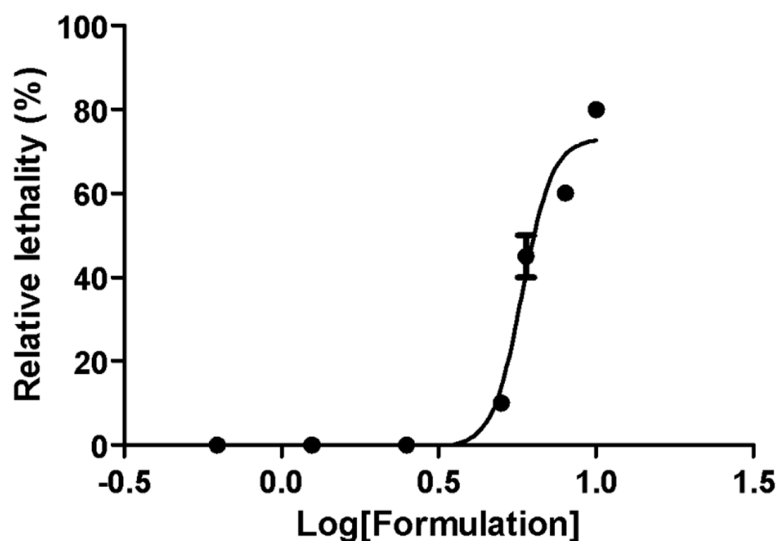


Figure 2. Dose–response curve displaying the lethality effects induced by the tested formulation (0.625–10 mg/mL) on brine shrimps (*Artemia salina*). LC₅₀ value was 5.837 mg/mL.

3.4.3. *Daphnia magna* Cardiotoxicity Assay

The formulation at the tested concentration of 5.837 mg/mL, which corresponds to the LC₅₀ obtained from the previous brine shrimp lethality assay, was found to be biocompatible and non-cardiotoxic under basal conditions. Furthermore, it did not demonstrate any protective effect following the cardiotoxic stimulus induced by 10% ethanol (Figure 3).

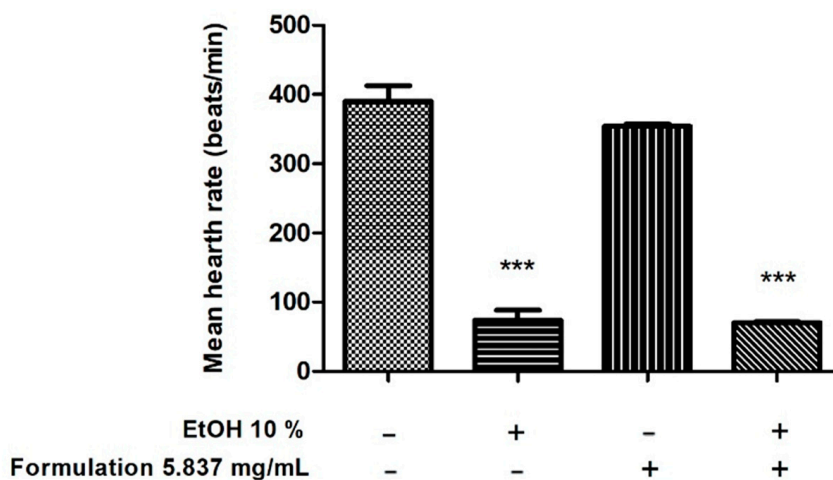


Figure 3. Acute exposure to the formulation at 5.837 mg/mL showed no cardiotoxic effect, and no cardioprotective effect was observed when co-treated with 10% ethanol as a cardiotoxic stimulus. Data are reported as means ± SEM. ANOVA, $p < 0.0001$. *** $p < 0.001$ vs. negative ctrl group.

3.5. Ex Vivo Neuroprotective Effects

Considering the results of *A. salina* and *D. magna* toxicity assays, a concentration at least five-fold lower (1000 µg/mL) was selected in order to evaluate the neuroprotective effects of the formulation on isolated mouse cortex specimens exposed to K⁺ 60 mM Krebs–Ringer buffer. K⁺ 60 mM represents a neurotoxic stimulus able to induce neuroinflammation and lead to serotonin depletion, as previously demonstrated [24,34]. In this context, the formulation blunted the K⁺ 60 mM-induced PRLR and ESR1 receptors (Figure 4A,B), deeply involved in anxiety and depression [61,62]. The evaluation of the receptor gene expression was also evaluated in comparison with single extracts (Figures S6 and S7, Supplementary Material) and permitted to confirm the effectiveness of the selected concentration of the formulation for the following gene expression evaluations. The formulation was effective in preventing the K⁺ 60 mM-induced gene expression of IL-6 and COX-2 (also known as PTGS2) (Figure 4C,D), two well-established biomarkers of neuroinflammation [62]. In parallel the formulation increased the gene expression of the anti-inflammatory cytokine IL-10 [63]. This showed anti-neuroinflammatory effects (Figure 4E) that also contributed to recent studies pointing to the strict relationships between neuroinflammation and PMS [5]. The anti-neuroinflammatory effects induced by the formulation could also be related, albeit partially, to the antiradical properties of the phenolic compounds (Tables 3 and 4) [64]. It has been shown that reactive oxygen species (ROS) are capable of inducing COX-2 expression [65]. Furthermore, pro-oxidant stimuli are well-known to increase the gene expression of COX-2, in neurons, and this effect is blunted by phenolic compounds [38]. The formulation was also able to increase the gene expression of BDNF (Figure 4F). This further suggested neuroprotective and anti-depressant effects [66] that were also supported by the decrease in the gene expression of SERT (Figure 4G). This membrane transport protein is involved in 5-HT reuptake from neurons and represents a key target in pharmacological anti-depressant therapy [67]. The abovementioned effects could be related to the content in crocins and safranal [44], agnuside [38], but also phenols and flavonoids, especially the hydroxycinnamic acids (gentisic acid, caftaric acid, rosmarinic acid). These phytochemicals are known not only to exert anti-neuroinflammatory effects, but also to improve neurotransmission, with particular regard to BDNF and serotonergic targets, including SERT and monoamine oxidases (MAOs) inhibition [39,68–70].

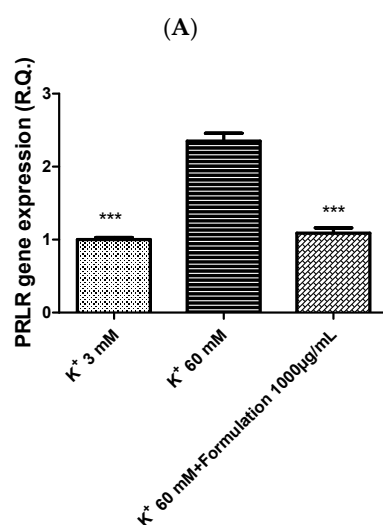


Figure 4. Cont.

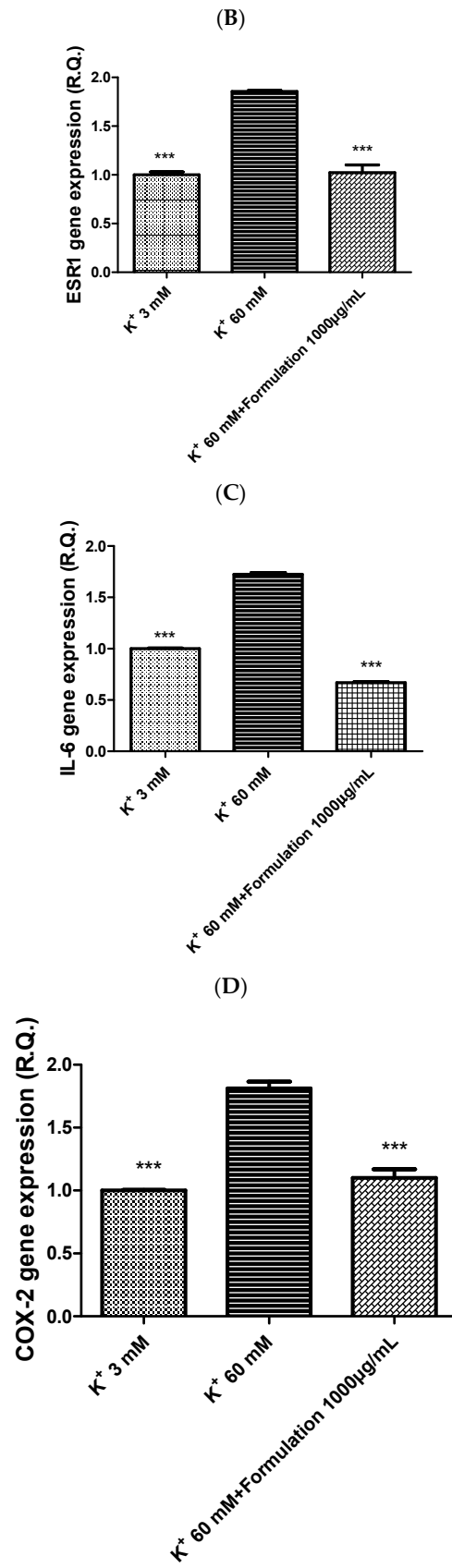


Figure 4. Cont.

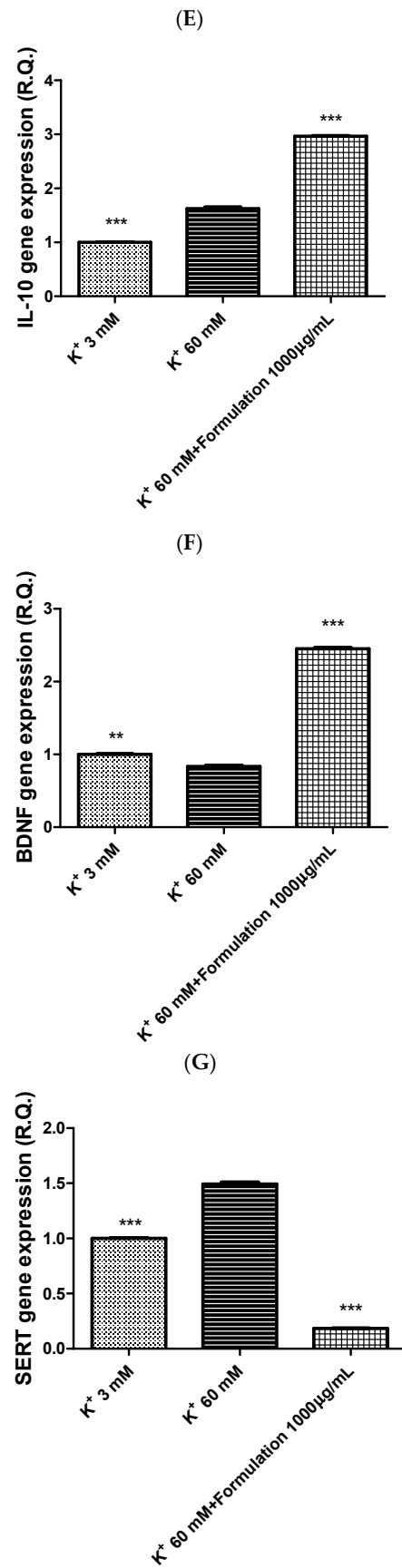


Figure 4. (A) Inhibitory effect induced by the formulation (1000 g/mL) on PRLR gene expression in isolated mouse cortex specimens exposed to K⁺ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$;

*** $p < 0.001$ vs. K^+ 60 mM group. (B) Inhibitory effect induced by the formulation (1000 g/mL) on ESR1 gene expression in isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$; *** $p < 0.001$ vs. K^+ 60 mM group. (C) Inhibitory effect induced by the formulation (1000 g/mL) on IL-6 gene expression in isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$; *** $p < 0.001$ vs. K^+ 60 mM group. (D) Inhibitory effect induced by the formulation (1000 g/mL) on COX-2 gene expression in isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$; *** $p < 0.001$ vs. K^+ 60 mM group. (E) Stimulatory effect induced by the formulation (1000 g/mL) on IL-10 gene expression in isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$; *** $p < 0.001$ vs. K^+ 60 mM group. (F) Stimulatory effect induced by the formulation (1000 g/mL) on BDNF gene expression in isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$; ** $p < 0.01$, *** $p < 0.001$ vs. K^+ 60 mM group. (G) Inhibitory effect induced by the formulation (1000 g/mL) on SERT gene expression in isolated mouse cortex specimens exposed to K^+ 60 mM Krebs–Ringer buffer. ANOVA, $p < 0.0001$; *** $p < 0.001$ vs. K^+ 60 mM group.

3.6. Bioinformatics

The phenolic compounds present in the formulation could also contribute to the prevention of the K^+ 60 mM-induced gene expression of ESR1 and PRLR through direct interactions with these receptors. The components–targets analysis conducted on the platform SwissTargetPrediction predicted putative direct interactions between ESR1 with caffeic acid, chicoric acid, p-coumaric acid, resveratrol, rosmarinic acid, hesperetin, and kaempferol (Figure 5). Moreover, indirect mechanisms could be involved. Indeed, the bioinformatics analysis carried out on the platform STRING suggested putative interactions between all assayed proteins. A prominent position is played by BDNF, ESR1, and IL-6 in the scenario of the predicted protein–protein interactions (Figure 6). These interactions could be at the basis of the simultaneous inhibitory effects exerted by the formulation on the neurotoxic stimulus-induced gene expression alteration of all tested proteins, in isolated cortex. This is also consistent with an anti-depressant role that further supports the rational use of the formulation in PMS. Other than phenolic compounds, we must consider the role played by Mg and Vitamin B6, as neuroprotective agents, that could influence the overall effect of the formulation [24,71].

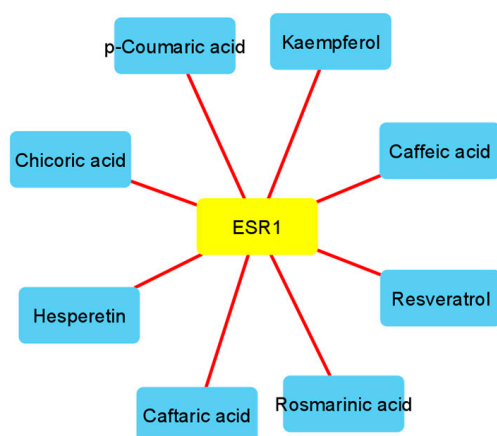


Figure 5. Components–targets analysis pointing to putative direct interactions between ESR1 and the following phenolic compounds present in the formulation: caffeic acid, chicoric acid, p-coumaric acid, resveratrol, rosmarinic acid, hesperetin, and kaempferol.

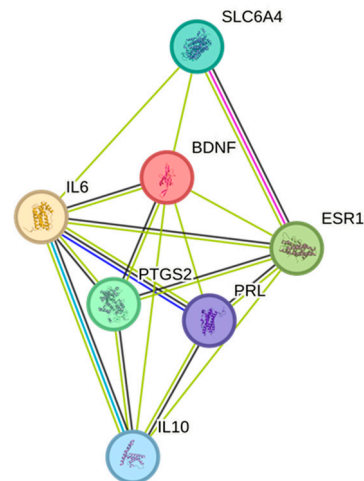


Figure 6. Protein–protein interactions predicted by the platform STRING. A prominent position is played by BDNF, ESR1, and IL-6 in the scenario of the predicted protein–protein interactions.

4. Conclusions

In the present study, an innovative formulation based on Mg, Vitamin B6, and water extracts from *Vitex agnus-castus* L., *Crocus sativus* L., *Melissa officinalis* L., *Betula pendula* Roth, and *Betula pubescens* Ehrh. was investigated in order to unravel the efficacy in facing the burden of inflammation and the neurotransmission deficit, especially the serotonergic and BDNF pathways, in premenstrual syndrome (PMS). Specifically, the formulation reduced the gene expression of COX-2, IL-6, SERT, ESR1, and PRLR, and increased the gene expression of BDNF and IL-10. These effects could be related, albeit in part, to the richness in specialized metabolites, including carotenoid (crocins), monoterpene (safranal), iridoid (agnuside), hydroxycinnamic acid (rosmarinic acid, gentisic acid, caftaric acid), and flavonoid content. The presence of these compounds corroborated its rational use as an innovative tool to contrast neuroinflammation, oxidative stress, and neurotransmitter impairment associated with PMS. The limitations of the present study include the lack of behavioral evaluations that could derive from animals, but above all clinical studies. In this context, future clinical investigations will be conducted on this formulation taking into consideration the accurate evaluation of efficacy and biocompatibility provided by the present work.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nutraceuticals5010005/s1>, Table S1. Gradient elution condition for chromatographic determination (HPLC-UV) analysis of phenolic compounds; Figure S1. Tested formulation effect (0.62 to 10 mg/mL) on *Cichorium intybus* relative germination rate; Figure S2. Tested formulation effect (0.62 to 10 mg/mL) on *Dichondra repens* relative germination rate; Figure S3. Tested formulation effect (0.62 to 10 mg/mL) on *Raphanus sativus* relative germination rate; Figure S4. Tested formulation effect (concentration range: 0.62 to 10 mg/mL) on *Secale cereale* relative germination rate; Figure S5. Tested formulation effect (0.62 to 10 mg/mL) on *Avena sativa* relative germination rate; Figure S6. Inhibitory effect induced by the single herbal extracts and formulation (1000 µg/mL) on ESR1 gene expression in isolated mouse cortex specimens exposed to K⁺ 60 mM Krebs-Ringer buffer; Figure S7. Inhibitory effect induced by the single herbal extracts and formulation (1000 µg/mL) on PRLR gene expression in isolated mouse cortex specimens exposed to K⁺ 60 mM Krebs-Ringer buffer.

Author Contributions: A.A.: methodology, formal analysis, investigation, writing—original draft. S.C.D.S.: methodology, formal analysis, investigation, writing—original draft. P.A.: methodology, formal analysis, investigation, writing—original draft. G.A.F.: methodology, formal analysis, investigation. M.L.L.: methodology, formal analysis, investigation. A.C.: methodology, formal analysis,

investigation. L.R.: methodology, formal analysis, investigation. S.L.: methodology, formal analysis, investigation. N.N.: methodology, formal analysis, investigation. G.C.: methodology, formal analysis, investigation. F.T.: methodology, formal analysis, investigation. G.O.: methodology, formal analysis, investigation, writing—original draft. G.Z.: methodology, formal analysis, investigation, writing—original draft. L.M.: methodology, formal analysis, investigation, writing—original draft. C.F.: methodology, formal analysis, investigation, writing—original draft. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author.

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