

Research Article

Nigella sativa* L. Seed Extract Modulates the Neurotransmitter Amino Acids Release in Cultured Neurons *In Vitro

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Nigella sativa L. (NS) has been used for medicinal purposes since ancient times. This study aimed to investigate the cytotoxicity of NS dry methanolic extract on cultured cortical neurons and its influence on neurotransmitter release, as well as the presence of excitatory (glutamate and aspartate) and inhibitory amino acids (gamma-aminobutyric acid—GABA— and glycine) in NS extract. Cultured rat cortical neurons were exposed to different times and concentrations of NS dry methanolic extract and cell viability was then determined by a quantitative colorimetric method. NS did not induce any toxicity. The secretion of different amino acids was studied in primary cultured cortical neurons by high-performance liquid chromatography (HPLC) using a derivation before injection with dansyl chloride. NS modulated amino acid release in cultured neurons; GABA was significantly increased whereas secretion of glutamate, aspartate, and glycine were decreased. The *in vitro* findings support the hypothesis that the sedative and depressive effects of NS observed *in vivo* could be based on changes of inhibitory/excitatory amino acids levels.

1. Introduction

Nigella sativa L. (NS) is a vegetal specie of the Ranunculaceae family, commonly known as “black cumin seed”, “neguilla” or “ajemuz”, that is widely cultivated in the Mediterranean region. Its seeds have played an important role over the years in ancient Islamic system of herbal medicine and in Spain, where they have been traditionally used in folk medicine. NS seeds have shown several therapeutic effects such as prevention of cancer [1], antihypertensive effect [2], anti-inflammatory, analgesic [3], and antihistaminic [4] actions. These properties have been proved and documented by recent research [5–10].

The volatile oil from this plant presents a relaxant action on different smooth muscles [11] and tracheal muscles of guinea pigs [12]. There is evidence of anticonvulsant and antioxidant effects against pentylentetrazol-induced kindling in mice [13]. In addition, it is more potent as an

anticonvulsant agent than valproate [13]. Likewise, aqueous extract of this plant suppresses penicillin-induced epileptic activity in rats. This anticonvulsant effect is a consequence of selectively altering the monoamine level in different brain regions [14].

In recent works, histopathological changes of neurodegeneration in the frontal cortex and brain stem in neurons after exposition to toluene have been observed [15, 16]. The administration of NS extract and thymoquinone (major component from NS volatile oil) causes morphologic improvement over apoptosis and indicates that NS therapy is useful as a potential treatment of neurodegeneration prevention.

NS seeds composition includes nutritional components such as carbohydrates (glucose, xylose, rhamnose, and arabinose), vitamins as thiamine, riboflavin, pyridoxine, niacin and folic acid [5], mineral elements, and proteins. The NS

seeds are also a source of calcium, iron and potassium, alkaloids (nigellidine, nigellimine, and nigellicine) [17], 36%–38% fixed oil and 0.4%–2.5% essential oil [18]. The fixed oil is mainly composed of unsaturated and essential fatty acids (linoleic acid, followed by oleic acid) [19] whereas the volatile oil has been shown to contain 18.4–24% thymoquinone and 46% monoterpenes such as *p*-cymene and pinene [5, 20]. Thymoquinone, as indicated above, is thought to be the main active component of NS seeds [18] and suppresses itself epileptic seizures in rats [21], while a monodesmosidic triterpene saponin, α -hederin, has also been isolated from the extract of NS seeds and proved to exert antitumoral activity [22].

Other active principles are nigellone and nigellidine which contains an indazol nucleus [23]. Three flavonoid glycosides and triterpene saponins were also identified from *Nigella sativa*, together with four phospholipid classes: phosphatidylcholine phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol [24, 25]. In previous studies we demonstrated that aqueous and methanolic extracts of NS seeds exert a potent sedative and depressive effect on CNS and induce analgesia [26]. The effect on CNS resulted in a significant reduction of spontaneous motility, a decrease in normal body temperature and significant analgesic action against hot-plate and pressure tests. Body temperature reduction can be interpreted as an index of alteration of various central neurotransmitters; anxiety and sedation are mainly mediated by the GABA-A receptor in the CNS. Since depressant action was confirmed specially for the methanolic extract, we decided to study whether the addition of this methanolic dry extract in cortical neurons culture could exert any influence on the secretion of the excitatory amino acids aspartate (Asp) and glutamate (Glu), and the inhibitory amino acids GABA and glycine (Gly), as well as the presence of these amino acid neurotransmitters in the extract. We also considered its effect on the amino acids secretion when stimulated by a depolarizing agent and its effect on cultured neurons viability.

2. Results

2.1. Cell Viability. The viability of the cultured neurons after exposition to NS extract concentrations 2.5, 25, and 250 $\mu\text{g}/\text{mL}$ during 15 and 60 minutes are shown in Figure 1. No significant changes were observed on neuronal viability at any assayed concentration. Results are expressed as a percentage with respect to control value (100% viability).

2.2. Amino Acids Determination

2.2.1. Amino Acids Determination in the NS Extract. The amino acids GABA, Gly, Glu, and Asp were measured in NS methanolic extract by HPLC. The results are expressed as pmoles/mg of NS extract (Table 1). The results showed Gly as the most abundant, followed by GABA, Glu, and Asp, respectively. The amino acids content in the extract was subtracted from the total amino acid content measured in the

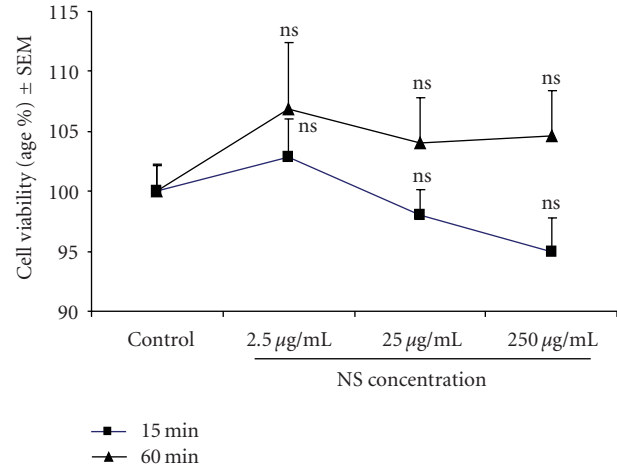


FIGURE 1: Effect of *Nigella sativa* methanolic extract on neuronal viability measured by MTT assay. The cells were stimulated for 15 and 60 minutes with different concentrations of this extract (2.5, 25 and 250 $\mu\text{g}/\text{mL}$, final concentration). The results are presented as \pm SEM of three independent experiments and statistical analysis resulted in no significant differences when compared with the control group.

cellular medium after neuronal stimulation with NS extract so we can state that the final amino acids content in cell culture is a direct consequence of cell release.

2.2.2. Amino Acids Release in Presence of NS Extract. The release of the four amino acids after stimulation with the chosen nontoxic concentrations of NS extract (2.5, 25, and 250 $\mu\text{g}/\text{mL}$) showed a diminished secretion response that was statistically significant ($P < .05$ for Gly and Glu; $P < .01$ for GABA; $P < .001$ for Asp) after 15 minutes of incubation with respect to control (neuronal cells stimulated with normal Locke medium during the same period of time and considered as 100% secretion) (Figures 2, 3, 4, and 5). Gly and Asp release was reduced in a dose dependent manner; Glu and GABA showed a tendency to retrieve control values, although their secretion was lower than control. This fall of aminoacids release is greater for higher extract concentration except for Glu and GABA, which showed a tendency to recuperation to control values at the same time (Figure 2). The HPLC analysis revealed the same behavior for all the amino acids, with the exception of GABA, after treatment with NS extract during 60 minutes. The increased presence of this amino acid was statistically significant for 25 and 250 $\mu\text{g}/\text{mL}$ NS extract.

2.2.3. Amino Acid Release Evoked by 60 mM KCl. In order to know the response to a depolarizing agent, cortical neurons were stimulated with NS extract at the indicated concentrations, during 15 and 60 minutes previous to depolarization with 60 mM KCl (Figures 6 and 7).

The neurons treated with NS extract during 15 minutes and subsequently stimulated with KCl showed a dose-dependent decrease in amino acids secretion with respect

TABLE 1: Amino acids composition of methanolic extract of *Nigella sativa*. The results are expressed as pmoles of each amino acid by mg of methanolic extract, prepared under the conditions indicated in experimental procedures.

Amino acid	Asp	Glu	Gly	GABA
pmoles/mg NS Methanolic extract	234.64 ± 8.03	345.26 ± 11.82	1943.28 ± 51.8	396.24 ± 13.59

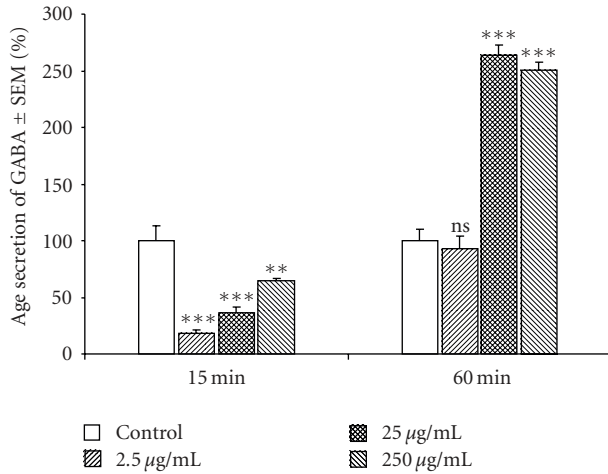


FIGURE 2: Effect produced by different concentrations (2.5, 25, and 250 µg/mL) of methanolic extract of *Nigella sativa* on GABA release from cortical neurons. The cells were stimulated for 15 and 60 minutes with each concentration. The values are expressed as a percentage of secretion with respect to basal value (100%). Statistical significances were calculated with respect to the corresponding control at its respective time points: ** $P < .01$; *** $P < .001$.

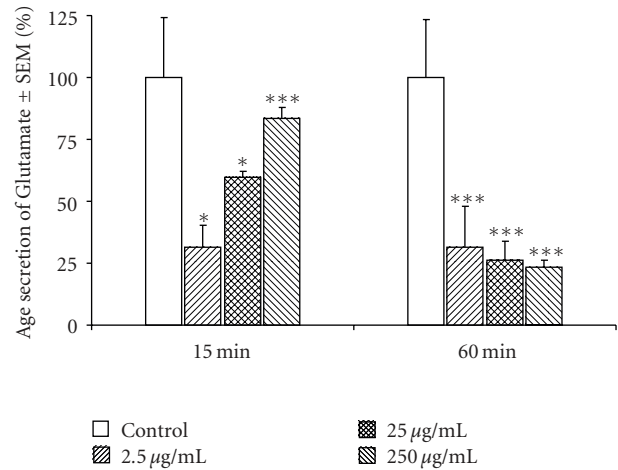


FIGURE 4: Effect produced by different concentrations (2.5, 25, and 250 µg/mL) of methanolic extract of *Nigella sativa* on Glutamate release from cortical neurons. The cells were stimulated for 15 and 60 minutes with each concentration. The values are expressed as a percentage of secretion with respect to basal value (100%). Statistical significances were evaluated with respect to the corresponding control at its respective time points: * $P < .05$ and *** $P < .001$.

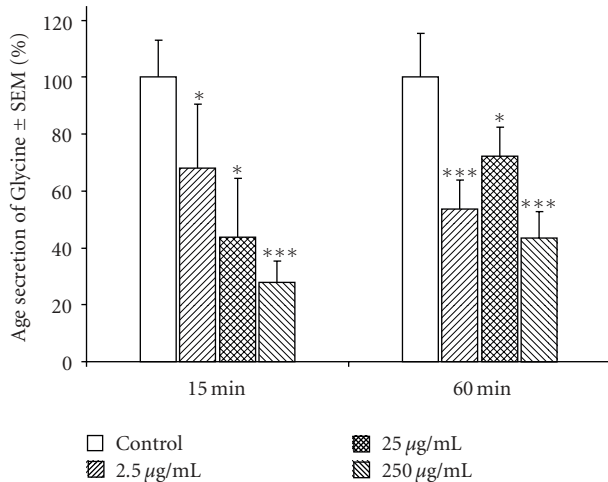


FIGURE 3: Effect produced by different concentrations (2.5, 25, and 250 µg/mL) of methanolic extract of *Nigella sativa* on Glycine release from cortical neurons. The cells were stimulated for 15 and 60 minutes with each concentration. The values are expressed as a percentage of secretion with respect to basal value (100%). Statistical significances were calculated with respect to the corresponding control at its respective time points: * $P < .05$ and *** $P < .001$.

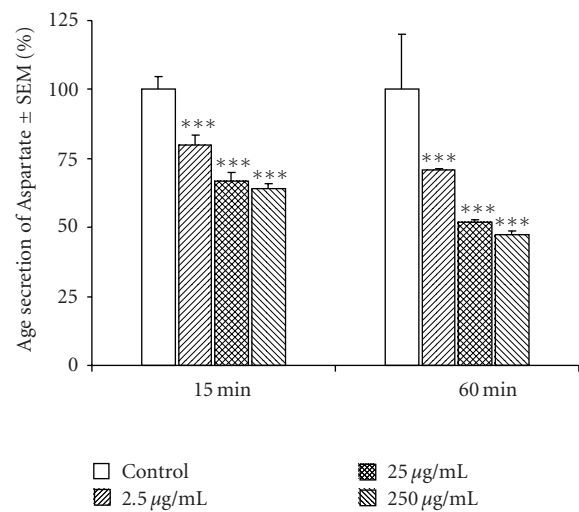


FIGURE 5: Effect produced by different concentrations (2.5, 25, and 250 µg/mL) of methanolic extract of *Nigella sativa* on Aspartate release from cortical neurons. The cells were stimulated for 15 and 60 minutes with each concentration. The values are expressed as a percentage of secretion with respect to basal value (100%). Statistical significances were calculated with respect to the corresponding control at its respective time points: *** $P < .001$.

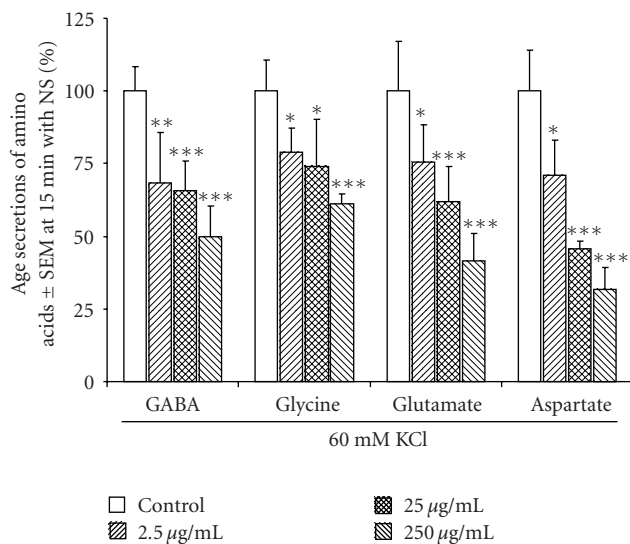


FIGURE 6: Secretion of amino acid neurotransmitters evoked by 60 mM KCl, measured in cortical neurons culture. The cells were previously treated with methanolic extract of *Nigella sativa* for 15 minutes. The values are expressed as a percentage of secretions with respect to basal value (100%). Statistical significances were calculated with respect to the corresponding control at its respective time points: * $P < .05$, ** $P < .01$ and *** $P < .001$.

to control value (neuronal cells stimulated with Locke medium), which was considered as 100%. The observed behaviour was more relevant for Glu and Asp at 25 and 250 µg/mL than for GABA and Gly under the same conditions (Figure 6).

Measurement of secretion mediated by KCl during 60 minutes revealed an inhibition of the liberation of these neurotransmitters. In this case, only GABA and Glu were released in a dose-dependent manner (Figure 7).

3. Discussion

The aim of the study was to determine the effects of NS methanolic extract on the release of neurotransmitter amino acids by measuring their concentrations in the culture media using HPLC precolumn derivatization technique. Three concentrations of NS extract (2.5, 25, and 250 µg/mL) and two time points (15 and 60 min) for the determination of the effects were used. This is a preliminary study which shows that exposure of the cultured neurons have a modulatory effect on the release and contents of these aminoacids.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to estimate the cells viability when neurons were treated with NS extract. The three concentrations of dry methanolic extract used in our study did not affect cellular respiratory capacity at any of the two periods of time considered. These results allowed us to consider the adequate approach to the study of amino acid secretion in the conditions selected and to confirm the innocuous characteristics of the chosen extract concentrations.

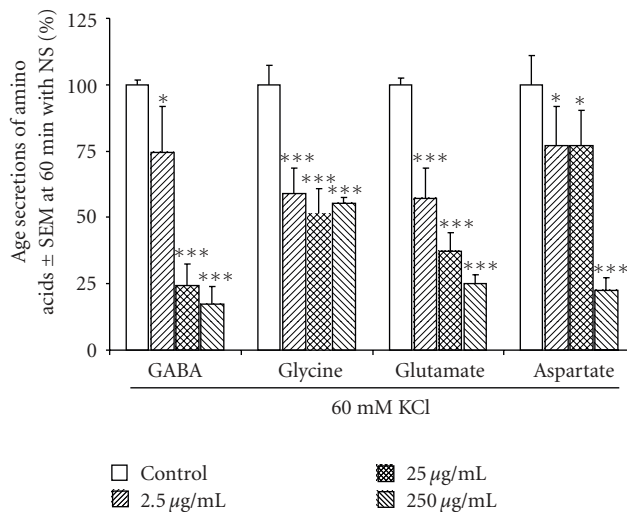


FIGURE 7: Secretion of amino acid neurotransmitters evoked by 60 mM KCl, measured in cortical neurons culture. The cells were previously treated with methanolic extract of *Nigella sativa* for 60 minutes. The values are expressed as a percentage of secretions with respect to basal value (100%). Statistical significances were calculated with respect to the corresponding control at its respective time points: * $P < .05$ and *** $P < .001$.

Our previous *in vitro* findings support the hypothesis that the sedative and depressive effects of *Nigella sativa* (NS) observed *in vivo* [26], could be based on changes of inhibitory/excitatory amino acids levels. Several authors attribute the sedative effects of different plant extracts (*Valeriana officinalis* L., *Scutellaria lateriflora* L.) [27, 28] to its endogenous GABA concentration, although they also hypothesize that there exist other components of the vegetal extracts with benzodiazepine-like effects that may account for their *in vivo* effects. The aim of our research was to determine whether these substances that are present in the NS extract could mediate the specific effects previously observed *in vivo* through the secretion of Asp, Glu, GABA, and Gly.

First of all, the presence and content of the four amino acids in the extract was analyzed by HPLC, showing a major presence of inhibitory amino acids (Gly and GABA) (Table 1).

Then, the cell amino acids release was assayed after addition of several concentrations of NS extract to cultured neurons during two different periods of time (15 and 60 minutes). The aim of this approach was to observe whether neuronal secretion could be modified depending on the exposition time. High secretion of GABA was observed after 60 minutes' contact with NS extract at 25 and 250 µg/mL. This time is close to the one that allowed the maximum effect in our *in vivo* study, close to 40 minutes [26].

Under these conditions, neuronal GABA was liberated to the medium. Similar results have been observed when high GABA concentrations are present in an aqueous extract of *Valeriana officinalis* root which induced *in vitro* liberation

of [^3H]GABA in rat synaptosomes by reversal of the GABA carrier and inhibition of its reuptake [27]. Subsequently, it was described the same behavior for *Scutellaria lateriflora* L. [28]. Both plants are widely prescribed as sedative/anxiolytic ones. As the GABA presence in the methanolic extract of NS was confirmed with our results, it is possible to assume that a high level of GABA in the medium during cellular incubation and a longer period of exposition at this one could exert the same effect above described.

Recent findings suggest that NS protects from induced generalized epilepsy in rats by selectively altering the monoamine level in different brain regions [14]. This study pointed that NS possibly facilitates the inhibitory activity of the GABAergic system through a competitive agonist action in the benzodiazepine (BZD) site of the GABA receptor as well as the involvement of dopaminergic and noradrenergic system. The confirmed presence of GABA in the methanolic extract could be directly related to its conduct over the GABA receptor and this could explain the potent sedative and depressive effect on CNS as previously reported. Moreover, the important presence of Gly in the extract could be also related to its inhibitory action. The binding of both neurotransmitters to their receptors on the neuronal membrane induces hyperpolarization that could be responsible for a significantly lower secretion of amino acids with respect to control values. In addition to this, our results showed a decrease in neuronal excitatory activity derived from a diminished Asp and Glu secretion, specially the second one. These actions could also be concurrent with the *in vivo* observed effects.

Our results show the behavior of the cortical neurons and confirm what we expected from the previous study performed *in vivo*. Likewise the methanolic extract composition is able to mediate in the neuronal amino acids release. In this case, the NS extract had an influence on neuronal transmission because it modifies the neurotransmitter amino acids release.

Depolarization of neuronal cells by application of high K⁺ concentration (60 mM KCl) induced a diminished amino acid release in both periods of time assayed. This diminished release was similar to nondepolarizing secretion except for GABA which also diminished its secretion. Although multiple mechanisms of neurotransmitter release evoked by elevated extracellular K⁺ may be involved [29, 30], we consider that the effect of NS extract over neuronal cells produces a drop in the transmission and is responsible for of the inhibitory effect indicated above. A possible action of NS extract over L-Type Calcium channels or an opening on potassium channels had been suggested by others authors [31–35], and it could contribute to the relaxant activities of this plant. Furthermore, its possible effect over these channels could persist even after the extract was eliminated from the medium before depolarization.

With respect to GABA secretion, the only amino acid which differs in its behavior with respect to nondepolarizing liberation, the diminished secretion may derive from the membrane transporter-reversal for GABA above suggested. In our study, we have considered the possibility that the GABA carrier had been previously affected by the presence

of NS extract during 15 or 60 minutes, inhibiting GABA reuptake and favouring its liberation from neurons. Other authors [30] have concluded that extracellular K⁺ (50 mM KCl) provokes GABA release by reversal transporter of GABA. In our case, we observed a diminished liberation that could be justified by the previous loss of cellular GABA content as the neurons had been treated with the NS extract.

In conclusion, this study suggests a sedative effect of NS methanolic extract by modification of neurotransmitter amino acids release, because the NS extract may induce an important release of GABA and Gly in the cultured neurons medium and therefore, exert an increase in the agonist action over their receptors. The results explain the sedative and depressive effects observed *in vivo* by an increase in inhibitory amino acids at the synaptic terminals. Even more, this effect is complemented with a possible decrease of excitatory transmission, as it has been demonstrated *in vitro* and could contribute to inhibitory response.

4. Experimental Procedures

4.1. Materials. Minimum Essential Eagle's Medium (EMEM) was obtained from Bio-Whittaker, and Foetal Bovine Serum (FBS) and Horse Serum (HS) were procured from Sera-Lab (Sussex, England). Standards of Glutamate, Aspartate, GABA and Glycine were purchased from Sigma (St. Louis, Mo, USA). Reagents and solvents for HPLC were Triethylamine from Sigma (St. Louis, Mo, USA), Acetic acid and Methanol ultra gradient grade from Merck (Darmstadt, Germany). Other chemicals were reagent grade products from Merck (Darmstadt, Germany).

Distilled water used for the preparation of buffers and standards was deionized with Milli-Q purification system. Syringe filters Millex-GV were obtained from Millipore (Milford, MA, USA).

Membrane filters (0.45 μm pore size) from Tecknokra (Barcelona, Spain) were used for filtration of the mobile phase and samples. Cell Proliferation kit II (XTT), colorimetric determination, was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

4.2. Instruments. An HPLC system (Spectra Physics Model SP 4400; San José, Calif., USA) coupled with a photodiode array detector Shimadzu SPD-10A (Izasa, Madrid, Spain) was used for the amino acids isolation and quantification. The analytical system consisted of a Waters ODS Spherisorb 150 \times 4.6 mm I.D.; 5 μm packed column (Teknokroma, Barcelona, Spain) as stationary phase, preceded by a guard column Spherisorb RP-18, 5 μm , 4 mm \times 4 mm.

A microplate fluorescence reader FL600-BioTek spectrofluorimeter was used for the quantification of cell viability at 492 nm.

4.3. *Nigella sativa* Extract. Seeds of *Nigella sativa* L. were supplied by the Medicinal and Aromatic Plants research Institute of Egypt (El Cairo, Egypt). Herbarium samples were authenticated by a taxonomist and a voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy,

Universidad Complutense de Madrid, with voucher number MAF 161043. The methanolic extract of the plant was prepared according to (Science and Technology Program for Development) CYTED protocol for vegetal species from countries that are included in this Program [36]. Seeds were powdered and then extracted in a soxhlet apparatus with hexane. Seeds were then extracted with methanol and kept for further studies. Methanol was completely evaporated in a rotatory evaporator under vacuum. A blackish-brown dry extract was obtained. It was kept at 4°C until use.

4.4. Neuronal Primary Culture. Brain neurons were obtained from foetal rat brains of 19 days of gestation as previously described [37] with minor modifications. Isolated neurons were suspended in Eagle's Minimum Essential Medium (EMEM) containing 0.3 g/L glutamine, 0.6% glucose, 5% Phosphate Buffered Saline (PBS), 5% Horse Serum (HS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells, at a density of 1×10^6 cells/well, were placed on plastic Petri dishes of 24 wells, treated with 10 µg/mL poly-L-lysine to aid attachment. The plates were incubated in a humidified incubator in an atmosphere of 5% CO₂/95% air at 37°C. After 72 hours, nonneuronal cells (contaminating glial cells) were mitotically inhibited by exposure to cytosine arabinoside. The incubation medium was replaced by fresh medium to which cytosine arabinoside was added to a final concentration of 10 µM.

After 3 days, this medium was replaced by fresh medium and experiments were carried out using cultures ranging from 10–15 days. Cell viability was checked by the dye exclusion method. Viability was routinely >95%. Cell purity was checked by both cells staining with cresyl violet to identify neurons and with the specific antiglial fibrillary acidic protein (GFAP) antibody to identify glial cells.

4.5. Measurement of Glial Contamination. Cortical neurons, after 7 days in culture, were detached from the culture plates with trypsin solution (0.25% trypsin and 0.02% EDTA in Dulbecco's buffered saline without calcium and magnesium) and then the cells were fixed (during 30 min) with 2% p-formaldehyde. After two washes with 1 mL of PBS, the cells were treated (during 1 h) with anti-GFAP antirabbit (at dilution of 1/500). Subsequently, the cells were washed with PBS and treated with antirabbit conjugated IgG FITC, at 1/100, for 30 min. Cells were identified by flow cytometry analysis. Under these conditions, the glial cells in the cultures were $8.3 \pm 3.6\%$ of the total (neural + glial cells).

4.6. Assessment of Cell Viability. Assessment of neuronal injury was performed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is used as an index of cell survival or cellular respiratory capacity-based on method of Mossmann [38] and improved by Weislow et al. [39] and Roehm et al. [40]. MTT assay is an indicator of the mitochondrial activity of living cells. The tetrazolium assay is based on the mitochondrial dehydrogenases (MD) activity and their inactivation after

cell death. In live cells, MTT is reduced to a highly water-soluble orange colored product, formazan dye. Neuronal cells were seeded in 96-multiwell plates at a density of 36×10^3 cells/well (in 200 µL medium) and kept in the incubator until 80–90% confluence. After this, the medium was removed and the cells were washed twice with PBS and the NS extract previously dissolved in PBS was added to the wells and incubated for 15 minutes or 1 hour. After each one of the treatment periods (15 or 60 min), the medium was removed and incubated with MTT solution (final concentration 0.3 mg/mL), according to the kit specifications. After 2 h incubation at 37°C in a humidified atmosphere, orange dye solution was spectrophotometrically quantified using an ELISA plate reader at 492 nm. The amount of orange formazan formed, as monitored by the absorbance, directly correlates to the number of living cells. Results are expressed as a percentage with respect to control (untreated cells). All experiments were performed in triplicate.

4.7. Measurement of the Amino Acid Secretion. HPLC analysis of amino acids was performed by a previously described method [41] with minor modifications and with the equipment and conditions that were previously developed for the amino acids determination [42]. Prior to HPLC amino acid secretion analysis cells were washed twice, at 10 min intervals, with 1 mL of Locke medium. After removing the medium, cells were stimulated for 15 min or 1 hour at 37°C with 250 µL fresh Locke medium (control cells) or with 250 µL of Locke medium containing the dry methanolic extract of NS seeds at different concentrations (2.5, 25 and 250 µg/mL).

After stimulation, the wells medium (supernatant S1) was taken for amino acid valuation and cells were then stimulated with 250 µL of 60 mM KCl for 15 min. After this, the cells secretion (supernatant S2) was removed for their valuation and cells were lysated with 250 µL of distilled water for total intracellular amino acids content determination. The different supernatants obtained, S1 and S2, as well as the cell lysated, were lyophilized for their dansylation.

The content of the four amino acids present in the methanolic extract was also determined by HPLC analysis under the same conditions and these results were subtracted from the total amino acids content in cellular medium in presence of NS extract (S1) in order to obtain cellular secretion value itself.

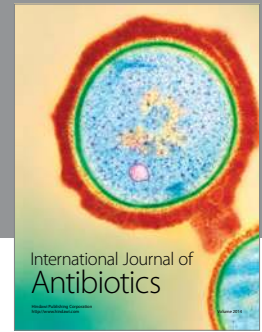
The results were calculated as the amino acid release into the incubation medium with respect to the total amino acid content. These results were expressed as a percentage of secretion with respect to control experiments that were considered as 100%.

4.8. Data Presentation. Data are presented as means \pm SEM of four separated experiments from different cell cultures, each one performed in triplicate with different batches of neuronal cells. Statistical comparisons were made using the Student's *t*-test.

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