Protective Effects of *Bacopa Monnieri* on Hydrogen Peroxide and Staurosporine: Induced Damage of Human Neuroblastoma SH-SY5Y Cells

Authors **Maciej Łojewski**[1], **Bartosz Pomierny**[2], **Bożena Muszyńska**[1], **Weronika Krzyżanowska**[2], **Bogusława Budziszewska**[2], **Agnieszka Szewczyk**[1]

Affiliations
[1] Department of Pharmaceutical Botany, Faculty of Pharmacy, Jagiellonian University Collegium Medicum, Kraków, Poland
[2] Department of Biochemical Toxicology, Faculty of Pharmacy, Jagiellonian University Collegium Medicum, Kraków, Poland

Key words
- *Bacopa monnieri*
- Plantaginaceae
- apoptosis
- *in vitro* culture
- caspase-3
- neuroprotection
- oxidative stress
- SH-SY5Y cells

Abstract

Many herbs, and recently their biomass from *in vitro* cultures, are essential for the treatment of diseases. The aim of this study was to determine the optimal growth of *Bacopa monnieri* (water hyssop) in an *in vitro* culture and to examine if extracts of the *B. monnieri* biomass from the *in vitro* culture would affect hydrogen peroxide- and staurosporine-induced injury of the human neuroblastoma SH-SY5Y cell line. It has been found that *B. monnieri* at concentrations of 25, 50, and 100 µg/mL inhibited both hydrogen peroxide-induced efflux of lactate dehydrogenase from damaged cells to culture medium and increased cell viability determined by an MTT assay. Moreover, *B. monnieri* at concentrations of 10, 25, and 50 µg/mL decreased staurosporine-induced activity of an executive apoptotic enzyme-caspase-3 and protected mitochondrial membrane potential. The obtained data indicate that the biomass from the *in vitro* culture of *B. monnieri* prevented SH-SY5Y cell damage related to oxidative stress and had the ability to inhibit the apoptotic process. Thus, this study supports the traditional use of *B. monnieri* as a neuroprotective therapy, and further *in vivo* studies on the effects of this preparation on morphology and function of nerve cells could lead to its wider application.

Introduction

Many raw materials of natural origin are used in the treatment of both common health problems and serious diseases. *Bacopa monnieri* L. (Pennell) (Plantaginaceae), known commonly as water hyssop and in India as "Brahmi" or "Jalanimba," is one of the most important plants in the traditional Indian medicine Ayurveda [1]. *B. monnieri* is used in traditional medicine to improve cognitive function and reduce anxiety [2]. Several studies have shown that extracts of this plant exerted a memory-enhancing effect both in experimental animals and in humans [3–5]. It has also been found that *B. monnieri* has anti-inflammatory, antimicrobial, and antidepressant properties [6] and antioxidant, antipyretic, antiulcer, cardioprotective, cooling, laxative, and adsorbing effects [7]. Compounds attributing to the abovementioned actions are bacosides and triterpenoids belonging to the saponins [1–7]. The demonstration of the beneficial effects of the plant's extract led researchers to study the potential neuroprotective effect of this preparation. It was found that the administration of *B. monnieri* for 30 days attenuated acrylamide-induced oxidative damage in the brain cortex and liver [8]. In prepubertal male mice, *B. monnieri* extract reduced oxidative stress markers in several brain structures induced by 3-nitropropionic acid or rotenone [9–11]. The treatment with *B. monnieri* has also been found to ameliorate cognitive deficits in mice due to transient occlusion of two vessels [5]. Several *in vitro* studies have demonstrated the neuroprotective effect of *B. monnieri* on beta-amyloid-induced cell death in primary cortical culture [12], on N27 cell culture damage exerted by acrylamide [8], and on cell injury in organotypic hippocampal slice cultures induced by oxygen and glucose deprivation [5]. So far, the scientific data indicate that the beneficial effects of *B. monnieri* may result from its antioxidant properties, such as metal ion chelation, free radical scavenging, inhibition of lipid peroxidation, or upregulation of antioxidative enzymes. Most of the existing data suggest the effectiveness of *B. monnieri* in Alzheimer's disease because it was demonstrated that the administration of Brahmi extract reduced the beta-amyloid concentration in the animal model of Alzheimer's disease [13], and in *in vitro* conditions, it inhibited

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Correspondence
Dr. hab. Bożena Muszyńska
Faculty of Pharmacy
Jagiellonian University
Collegium Medicum
Department of Pharmaceutical Botany
ul. Medyczna 9
Kraków 30–688
Poland
Phone: + 48 1 26 20 54 30
Fax: + 48 1 26 20 54 05
muchon@poczta.fm

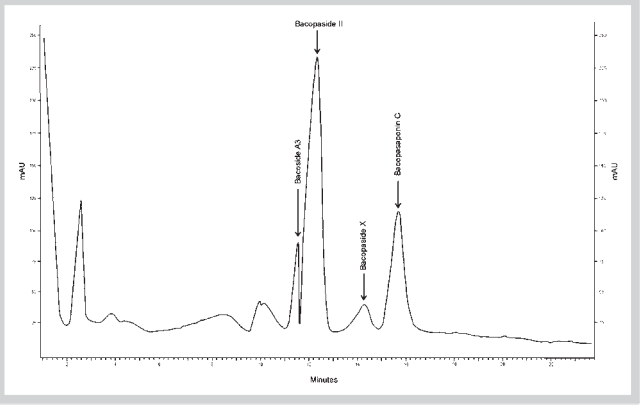

Fig. 1 RP-HPLC chromatogram of bacoside A (bacoside A3, bacopaside II, bacopaside X, bacopasaponin C) in the methanolic extract of *B. monnieri* biomass from an *in vitro* culture.

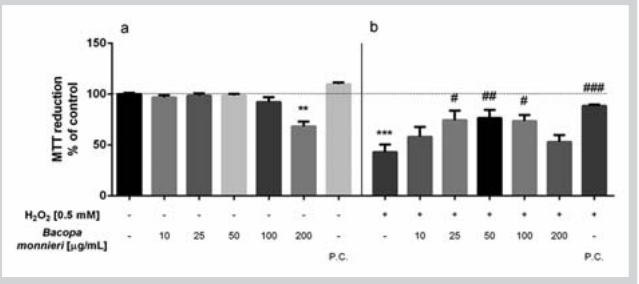

Fig. 2 The influence of *B. monnieri* extract on the basal (**a**) and H_2O_2-induced (**b**) LDH release in the SH-SY5Y human neuroblastoma cell line. Results are shown as a percentage of control cells not exposed to the *B. monnieri* extract and H_2O_2 (**a**) or in the presence of H_2O_2 alone (**b**). The results are expressed as means ± SEM. The significance of differences between the means was evaluated by the Bonferroni post hoc test following a one-way ANOVA analysis of variance (***$p < 0.001$ vs. control cells; #$p < 0.05$, ###$p < 0.001$ vs. cells exposed to H_2O_2 alone; n = 12). P. C.: positive control.

the cell damage induced by beta-amyloid, but not by glutamate [12]. To date, the effects of *B. monnieri* on nerve cell damage induced by oxidative stress have not been studied, despite the demonstration of antioxidant potency of this preparation. Current data report that oxidative stress and an increased production of reactive oxygen species contribute to the pathogenesis of various neurodegenerative diseases [14, 15]. The brain is characterized by weak antioxidant protection because of the low levels of glutathione and the moderate activity of catalase, superoxide dismutase, and glutathione peroxidase. Furthermore, the metabolism of some neurotransmitters, particularly dopamine and nitric oxide, and microglial activation lead to the formation of an increased amount of reactive oxygen or nitrogen species. The generally accepted model of oxidative stress *in vitro* is the addition of hydrogen peroxide to the culture medium. In the present research, an optimal growth of *B. monnieri* biomass in an *in vitro* shoot culture was obtained. *In vitro* culture allows for having constant access to the material regardless of environmental conditions. It allows the experiments and research to be carried out under controlled conditions and permits the possibility of supplementing the biomass with the desired micronutrients and organic compounds to obtain potentially better pharmaceutical material. Our aim in this study was to determine if the methanol extract of the *B. monnieri* biomass from an *in vitro* culture would affect H_2O_2-induced injury of SH-SY5Y cells. Much of the data reported so far indicate that in neurodegenerative diseases, the cells die mainly by apoptosis, and the first apoptotic changes appear many years before the clinical symptoms of these diseases [16]. In the experimental studies, since apoptosis is induced mainly by staurosporine, the effect of *B. monnieri* biomass from *in vitro* cultures on the staurosporine-induced activity of an executive enzyme of this process, i.e., caspase-3 and mitochondrial membrane potential, were also determined.

Results

It was established that biomass growth of *B. monnieri* could be obtained in stationary liquid cultures on modified MS medium at 25 ± 2 °C under 24 h lighting with 4 W/m^2. A 12- to 14-fold growth in liquid cultures was obtained within a typical 28-day growth cycle. The average increase of the *B. monnieri* biomass from *in vitro* cultures was 12 g dry weight/L of medium.

Bacoside A3, bacopaside II, bacopaside X, and bacopasaponin C were determined in the methanolic extracts of the *B. monnieri* biomass by an RP-HPLC method (**Fig. 1**). The total content of bacoside A (bacoside A3, bacopaside II, bacopaside X, bacopasaponin C) in the methanolic extract of the plant material was established by an RP-HPLC method and amounted to 8.73 mg/g dry weight.

The biomass from an *in vitro* culture of *B. monnieri* given alone at a concentration ranging from 25 to 200 µg/mL for 28 h did not affect lactate dehydrogenase (LDH) activity in the culture medium; however, the highest concentration tested, 200 µg/mL, tended to increase the activity of these enzymes (**Fig. 2**). An approximate 24 h incubation of the cells with 0.5 mM hydrogen peroxide increased LDH efflux by ca. fourfold. The biomass from the *in vitro* culture of *B. monnieri* at concentrations of 25, 50, and 100 µg/mL inhibited about twofold the hydrogen peroxide-evoked LDH release, whereas after the addition of 200 µg/mL, only a tendency to reduce cell damage was observed. Butylated hydroxyanisole [(BHA), a synthetic antioxidant used as positive control] present in the medium alone at a concentration of 20 µg/mL did not change LDH release, but significantly reduced H_2O_2-induced damage.

The addition of biomass from an *in vitro* culture of *B. monnieri* at a concentration of 100 µg/mL to the control culture did not affect the ability of the cells to reduce MTT, while this compound at the highest concentration tested (200 µg/mL) significantly reduced cell viability (**Fig. 3**). The incubation of cells with 0.5 mM hydrogen peroxide inhibited about a 30 % reduction of MTT, indicating reduced SH-SY5Y cell viability. Biomass from an *in vitro* culture of *B. monnieri* given at concentrations ranging from 25 to 100 µg/mL for 28 h significantly attenuated the adverse effects of H_2O_2, while the lowest (10 µg/mL) and the highest (200 µg/mL) concentrations of the test preparation had no effect on the hydrogen peroxide-induced reduction in cell viability. BHA had no effect on MTT reduction in the control cells, but inhibited hydrogen peroxide action.

The incubation of cells with biomass from an *in vitro* culture of *B. monnieri* (10–100 µg/mL) alone did not change basal caspase-3 activity (**Fig. 4**). The exposure of cells to 1 µM staurosporine for 24 h ca. six times enhanced the activity of this enzyme. Staur-

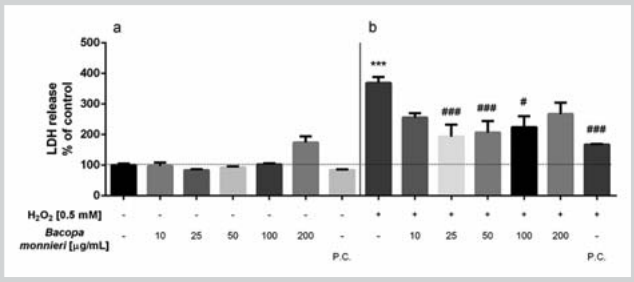

Fig. 3 The influence of *B. monnieri* extract on the basal (**a**) and H_2O_2-induced (**b**) MTT reduction in the SH-SY5Y human neuroblastoma cell line. Results are shown as a percentage of control cells not exposed to the *B. monnieri* extract and H_2O_2 (**a**) or in the presence of H_2O_2 alone (**b**). The results are expressed as means ± SEM. The significance of differences between the means was evaluated by the Bonferroni post hoc test following a one-way ANOVA analysis of variance (**p < 0.01, ***p < 0.001 vs. control cells; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. cells exposed to H_2O_2 alone; n = 12). P. C.: positive control.

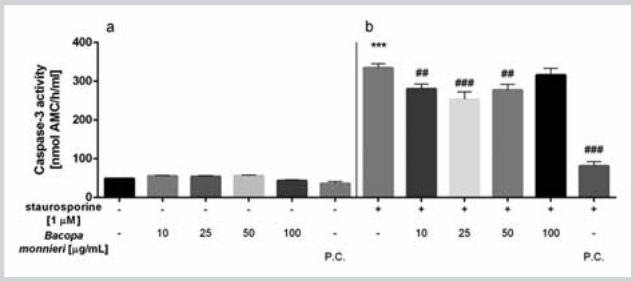

Fig. 4 The effect of *B. monnieri* extract on the basal (**a**) and staurosporine-induced (**b**) caspase-3 activity in the SH-SY5Y human neuroblastoma cell line. Results are presented as a concentration of AMC, the product of caspase-3 enzymatic activity, after 1 h reaction per 1 ml of the reaction volume in non-induced cells (**a**) and in staurosporine-treated cells (**b**). The results are expressed as means ± SEM. The significance of differences between the means was evaluated by the Bonferroni post hoc test following a one-way ANOVA analysis of variance (***p < 0.001 vs. non-treated control cells; ##p < 0.01, ###p < 0.001 vs. staurosporine-treated cells without *B. monnieri* extract; n = 12). P. C.: positive control.

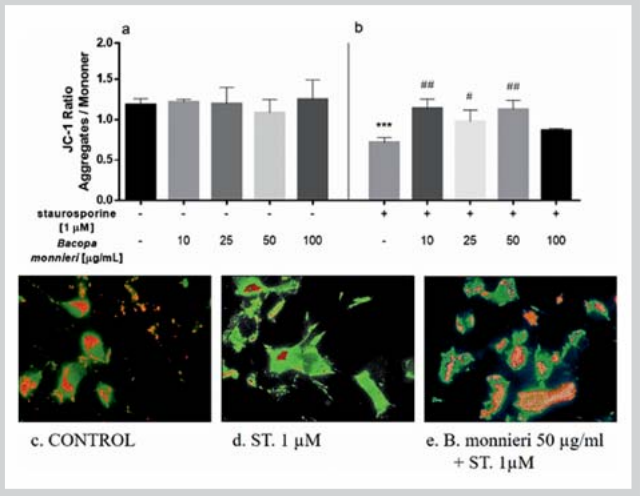

Fig. 5 The effect of *B. monnieri* extract on the basal (**a**) and staurosporine-induced (**b**) mitochondrial membrane potential in the SH-SY5Y human neuroblastoma cell line. Results are presented as a ratio of JC-1 aggregate fluorescence (red) to JC-1 monomer fluorescence (green) in non-induced cells (**a**) and in staurosporine-treated (ST.) cells (**b**). Fluorescent pictures present colocalization of aggregated and monomeric forms of the JC-1 dye in the control cells not exposed to ST. or *B. monnieri* (**c**), staurosporine-treated cells (**d**), and cells treated with staurosporine and pretreated with *B. monnieri* at a concentration of 50 µg/mL (**e**). The results are expressed as means ± SEM. The significance of differences between the means was evaluated by the Bonferroni post hoc test following a one-way ANOVA analysis of variance (***p < 0.001 vs. non-treated control cells; #p < 0.05, ##p < 0.01 vs. staurosporine-treated cells without *B. monnieri* extract; n = 12). (Color figure available online only.)

osporine is a widely accepted tool for inducing apoptosis in the majority of cellular models. Biomass from an *in vitro* culture of *B. monnieri* at concentrations ranging from 10 to 50 µg/mL attenuated the effects of staurosporine on caspase-3 activity in a statistically significant manner, whereas a higher concentration (100 µg/mL) had no effect. The exposure of SH-SY5Y cells to a specific inhibitor of caspase-3 (Ac-DEVD-CHO) had no effect on the activity of this enzyme in the control culture, but it strongly inhibited the staurosporine-induced increase.

It has been found that staurosporine (at the concentration of 1 µM for 24 h) caused a 2-fold drop in mitochondrial membrane potential (**Fig. 5**). *B. monnieri* extracts given alone at concentrations from 10 to 100 µg/mL for 24 h did not distract membrane potential. However, extracts from the concentrations 10 µg/mL to 50 µg/mL attenuated the effects of saturosporine on mitochondrial membrane potential. The highest tested concentration of 100 µg/mL had no effect.

Discussion

The obtained results show that methanolic extracts of the biomass of *B. monnieri* from *in vitro* cultures exerted protective effects in the *in vitro* model of oxidative stress-induced cell damage. The tested extract inhibited both the hydrogen peroxide-induced damage of cell membranes and the H_2O_2-induced reduction in cell viability. In addition to the effect on the necrosis, these extracts of *B. monnieri* also diminished the activity of caspase-3 increased by staurosporine and a change in mitochondrial membrane potential, suggesting a beneficial effect of this preparation on the apoptotic process also.

This *in vitro* study shows that methanolic extracts of the biomass from *in vitro* cultures of *B. monnieri* present in cell cultures in concentrations up to 100 µg/mL neither damaged cell membrane, as evidenced by the LDH release, nor evoked mitochondrial dysfunction, as assessed by the MTT reduction. These data are consistent with previous results that showed that Brahmi extract in the 100 µg/mL concentration, present in cultures for 24 and 72 h, does not affect the viability of primary cortical cell cultures [12]. In contrast, Brahmi extract in higher concentrations damaged both primary cortical neurons as well as the human SH-SY5Y neuroblastoma cell line. The SH-SY5Y cell line of human origin with a dopaminergic phenotype used in the present study is a commonly used cell culture model in studies related to neurodegenerative disorders, neurotoxicity, and oxidative stress [17]. Similarly, the addition of hydrogen peroxide to the cell medium, as a source of detrimental reactive oxygen species, is a well-validated and frequently used model for oxidative stress [18–20]. As in our previous experiments, the addition of 0.5 mM H_2O_2 en-

hanced LDH release from the cytosol to the medium and decreased activity of mitochondria dehydrogenase enzymes [20, 21]. Cell death induced by hydrogen peroxide is thought to progress mainly via the necrotic mechanism, thus demonstrating that *B. monnieri* weakened the effect of this toxic factor, the evidence of which is in its neuroprotective effect. These data confirmed and extended previous reports on the neuroprotective action of Brahmi extract on beta-amyloid-induced cell death in a primary cortical culture, on the cytotoxic effects of 3-nitropropionic acid and acrylamide in the rat dopaminergic cell line (N27), and on cell damage in organotypic hippocampal cultures induced by glucose and oxygen deprivation [5,8,9,12]. These data suggest that *B. monnieri* can exert a protective effect on neuronal damage evoked by many factors, which are important in the pathogenesis of various neurodegenerative diseases. In Alzheimer's and Huntington's diseases, neuronal damage is evoked not only by beta-amyloid and inhibitors of mitochondrial complex II, respectively, but also by the factors intensifying the formation of reactive oxygen and nitrogen species, which play an important role. The role of oxidative stress in the development of Parkinson's disease is particularly well documented. Furthermore, it has also been established that this factor initiates or increases neuronal damage in other neurodegenerative diseases as well [14–16,22]. In the present study, *B. monnieri* exerted a protective effect on human SH-SY5Y cells when it was added at concentrations between 10 and 100 µg/mL, the same as in the primary cortical culture [12], but in a higher concentration than in N27 cells [9–11]. These differences may be due to the varying sensitivity of the tested cells and/or from the composition and content of active substance in the used *B. monnieri* extracts. According to Rastogi et al. [23], bacosides are considered to be the main component of *B. monnieri* and these compounds are responsible for plant neuroprotective activity. Also, Pandareesh and Anand [24] indicates bacosides as the main active ingredient of the extract; however, they showed that the extract contained phenolic acids and flavonoids, which act as primary antioxidants and free radical terminators and contribute to the antioxidant property of the extract.

In addition to demonstrating a protective effect of *B. monnieri* on the viability of neurons, this study showed, for the first time, that this extract can weaken the apoptotic process. *B. monnieri* at a lower concentration than in the case of H_2O_2-induced LDH release and MTT reduction decreased staurosporine-induced caspase-3 activity and prevented a drop of mitochondrial membrane potential caused by staurosporine. Caspase-3 is the executive enzyme in the apoptotic process and is activated by both intrinsic (mitochondrial) and extrinsic (ligand-dependent) pathways [25,26]. It should be noted, however, that several studies showed that caspase-3 activation initiated but did not complete apoptosis and that activation of this enzyme did not always lead to apoptotic neuronal cell death because of the involvement of the caspase family in other processes such as migration, proliferation, and differentiation of brain cells [27]. Thus, determination of mitochondrial membrane potential was performed. Decreased membrane potential is one of the initial stages of the active process of apoptosis. This marker reflects the malfunction of the mitochondria due to exposure to harmful and/or proapoptotic compounds. In our experiment, *B. monnieri* extracts in the concentration range between 10 and 50 µg/mL protected mitochondria from depolarization induced by staurosporine. Together with the results of caspase-3 activity, these data suggest that *B. monnieri* has antiapoptotic activity and should be subjected to further research towards its neuroprotective activity.

Although the neuroprotective action of *B. monnieri* in *in vitro* experiments is very promising, only a confirmation of such action *in vivo* may be evidence for its potential use in the prevention or treatment of neurodegenerative diseases. So far, the antioxidant potency of *B. monnieri* was observed only under *in vivo* conditions [5,8–11], so, further studies on the effects of this preparation on apoptotic markers and on the morphological and functional parameters of nerve cells are urgently needed. In summary, the obtained data indicate that the biomass from *in vitro* cultures of *B. monnieri* prevented SH-SY5Y cell damage related to oxidative processes and possibly inhibited the apoptotic process. Since oxidative stress is involved in the pathogenesis of neurodegenerative diseases, the confirmation of such a beneficial effect *in vivo* and the possible identification of active compounds in the biomass from *in vitro* cultures of *B. monnieri* may lead to the development of a promising neuroprotective procedure in the future.

Materials and Methods

Chemicals and biochemicals

The growth regulators 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), nicotinic acid, DMSO, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT), streptomycin, staurosporine, BHA (purity > 98%), and caspase-3 inhibitor (Ac-DEVD-CHO; purity > 95%) were purchased from Sigma-Aldrich. Vitamin B_1 was obtained from TEVA pharmaceuticals. Myo-inositol was purchased from Fluka. Hydrogen peroxide, methanol, acetonitrile, phosphoric acid, and petroleum ether were obtained from Merck (purity > 98% by HPLC). Quadruple-distilled water with a conductivity of less than 1 µS/cm was achieved using an S2–97A2 distillation apparatus, ChemL. Dulbecco's modified Eagle's medium and 10% fetal bovine serum were purchased from FBS, Gibco-BRL. The standard of bacoside A (bacoside A3, bacopaside II, bacopaside X, bacopasaponin C) was purchased from Sigma-Aldrich.

Plant material

The *in vitro* cultures of *B. monnieri* were established from commercially available *in vitro* cultures of this plant. This material was identified by Associate Professor B. Muszyńska, Department of Pharmaceutical Botany UJ CM. Shoots were cut into small pieces and placed in Erlenmeyer flasks with liquid medium according to Murashige & Skoog [28] and with our modifications consisting of the addition of nicotinic acid (0.5 mL/L), myo-inositol (100 mg/L), vitamin B_1 (4.0 mL/L), and growth regulators BAP 1.0 mg/L and NAA 0.2 mg/L; the pH was adjusted to 5.7–5.8 before autoclaving [29].

Cultures were grown under constant artificial light (4 W/m^2, Philips Lighting LF-40 W lamp, daylight) at 25 ± 2 °C for 4 weeks (representative samples of *B. monnieri* cultures were deposited at the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum, Kraków, Poland; voucher specimen number: BF 196). After this period, the fresh biomass was frozen and lyophilized (lyophilizer Freezone 4.5, Labconco, temp: − 40 °C).

The lyophilized biomass (5 g of each) was ground in a mortar and then subjected to extraction with petroleum ether (2 × 100 mL) to remove lipids. Defatted material was extracted in a glass percolator by methanol (7 × 100 mL, 3 h each at room temperature). The obtained extract was evaporated (Rotavapor® R-210; *Büchi Labortechnik AG*) under pressure of 200 mPa at 40 °C down to dry-

ness [30,31]. The dry extract (0.5 mg) was quantitatively dissolved in 1.5 mL of methanol and was subjected to RP-HPLC analysis of bacoside.

Reversed-phase high-performance liquid chromatography analysis of bacoside

RP-HPLC analysis was conducted according to Pratibha and the European Pharmacopeia described elsewhere [32,33] with our modifications on a Merck-Hitachi liquid chromatograph (LaChrom Elite) equipped with a DAD detector L-2455 and Purospher® RP-18e (250 × 4 mm/5 µm) column. Analyses were carried out at 25 °C, with a mobile phase consisting of A-acetonitrile, B-acetonitrile: 0.5% phosphoric acid 0.01 mol/L 35: 65 (v/v), gradient elution: A (0:100%), B (100:0%) at a flow rate of 1 mL/min, λ = 205 nm. The identification was done by comparing the retention times of the peaks with an authentic reference compound and cochromatography with the standard. Quantification was done by measuring the peak area with reference to the standard of the bacoside A (bacoside A3, bacopaside II, bacopaside X, bacopasaponin C) curve derived from five concentrations (0.1875 to 3 mg/mL).

Cell culture

The SH-SY5Y neuroblastoma cell line was obtained from the American Type Culture Corporation (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin and were kept in a humidified atmosphere of 5% CO_2/95% O_2 at 37 °C. After reaching 80% confluence, cells were seeded at a density of 5×10^5 cells per well onto 96-well plates to determine the LDH release and the MTT reduction at a density of 8×10^5 cells per well on 96-well plates for the caspase-3 activity assay.

Treatment of cells

Dry extracts of *B. monnieri* biomass from *in vitro* cultures were prepared by dissolving ddH_2O (v/v, 2:3) in a small volume of ethanol and then adding them to SH-SY5Y cells at final concentrations ranging from 10 to 200 µg/mL (the final concentration of ethanol in the cell cultures did not exceed 1%). Hydrogen peroxide (0.5 mM) and staurosporine (1 µM) were added 4 h later, and the cells were cultured for the next 24 h. Staurosporine was dissolved in DMSO (the final concentration of DMSO in the cell cultures did not exceed 0.1%). The control cultures were supplemented with an appropriate amount of a vehicle. For LDH release and MTT reduction assays as a positive control, BHA, a synthetic phenol derivative compound of known antioxidant activity, in concentration of 20 µg/mL, was used. In the caspase-3 activity assay as a positive control, a selective inhibitor of the enzyme Ac-DEVD-CHO (Acetyl-Asp-Glu-Val-Asp-al) in a concentration of 1 µM was applied.

Measurement of lactate dehydrogenase release

Hydrogen peroxide toxicity was quantified by measuring the efflux of LDH into the culture media 24 h after hydrogen peroxide treatment. LDH activity was determined in medium using the colorimetric method (Cytotoxicity Detection Kit, Roche Diagnostic GmbH), according to which the amount of colored hydrazone, formed in the reaction of pyruvic acid with 2,4-dinitrophenylhydrazine, was inversely proportional to the LDH activity in the sample and could be quantified by measuring the absorbance at 400–550 nm. The results are expressed as a percentage of the control cells incubated in the absence of *B. monnieri* biomass from *in vitro* cultures and without hydrogen peroxide.

3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide reduction assay

Cell viability was measured, as described previously in Ref. [21], by determining the cellular reducing capacity, estimated through the extent of MTT reduction to the insoluble intracellular formazan, which depends on the activity of intracellular dehydrogenases and is independent of changes in the integrity of the plasma membrane. Briefly, culture medium was removed and SH-SY5Y cells were incubated with MTT for 3 h at 37 °C. MTT was prepared in PBS and added at a final concentration of 0.15 mg/mL. Then, the crystals of formazan were dissolved in DMSO, and the absorbance of each sample was measured at 570 nm in a Multiscan plate reader (Labsystem). The results are expressed as a percentage of the control cells incubated in the absence of *B. monnieri* biomass from *in vitro* cultures and without H_2O_2.

Caspase-3 activity assay

After 24 h treatment with staurosporine, the cells were lysed in lysis buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM $MgCl_2$, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, and 10 µg/mL of both leupeptin and pepstatin A] by shaking on ice for 25 min, and the supernatants were used to determine the caspase-3 activity. The activity of caspase-3 was measured using a fluorometric caspase-3 assay kit (Sigma Aldrich), according to which fluorochrome 7-amino-4-methylcoumarin (AMC) is released from the substrate Ac-DEVD-AMC (caspase-3 fluorogenic substrate) upon cleavage by caspase-3-like enzymes. A yellow-green fluorescence produced by free AMC is proportional to the caspase-3 activity present in the sample. Cell lysates were incubated with Ac-DEVD-AMC (50 µM) for 60 min at 30 °C in the absence and presence of a specific caspase-3 inhibitor (Ac-DEVD-CHO; 50 µM), and the fluorescence was measured with a fluorescence plate reader at 360 nm excitation and 460 nm emission wavelengths. Caspase-3 activity was calculated from the standard curve and presented as nmol of AMC/h/mL.

Mitochondrial membrane potential – JC-1 assay

The mitochondrial membrane potential was determined based on Menon et al. [34] method. The principal of this assay is the ability of the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) cationic dye to enter and accumulate in the mitochondria. In healthy cells with a high mitochondrial membrane potential, JC-1 dye forms aggregates with an intense red fluorescence (excitation 550 nm, emission 600 nm). In apoptotic cells with a decreased mitochondrial membrane potential, JC-1 remains in the monomeric form, exhibiting a green fluorescence (excitation 485 nm, emission 535 nm). The ratio of red to green fluorescence indicates the severity of apoptosis.

In this experiment, after a 24-hour treatment with staurosporine, cells were harvested and incubated with JC-1 solution at 37 °C for 15 min. Next, cells were washed and the fluorescence was measured at both 600 and 535 nm using a fluorescence plate reader (Fluoroscan, Ascent, Thermo Labsystem). The results are presented as a red/green fluorescence ratio.

In the JC-1 microplate assay, it was not possible to use any positive control due to the lack of reference compounds specifically protecting and/or increasing the mitochondrial membrane po-

tential. However, to additionally confirm the acquired results, the analogical experiment was conducted on cells seeded on glass microscopic slides (Millicell EZ slide, Millipore) at a concentration of 60 000 cells per well. After the incubation period with the JC-1 dye solution, cells were washed and analyzed using a fluorescence microscope (Leica Microscope DM IL LED FLUO). Digital photos were taken by means of a digital camera (Leica DFC 3000). The JC-1 slide assay results were comparable to the microplate assay results.

Statistical analysis

The data are presented as the means ± SEM of three independent experiments, and the significance of differences between the means was evaluated by the Bonferroni post hoc test following one-way analysis of variance.

Conflict of Interest

▼

The authors declare no conflict of interest.

References

1 *Anonymous.* The Ayurvedic Pharmacopoeia of India (API). 1st edition. Part 1, Volume 2. New Delhi: Government of India, Ministry of Health and Family Welfare, Departments of ISM and H; 1999; 190–191
2 *Calabrese C.* Effects of a standardized *Bacopa monnieri* extract on cognitive performance, anxiety, and depression in the elderly: a randomized, double-blind, placebo-controlled trial. J Altern Complement Med 2008; 14: 707–713
3 *Stough C, Lloyd J, Clarke J, Downey LA, Hutchison CW, Rodgers T, Nathan PJ.* The chronic effects of an extract of *Bacopa monniera* (Brahmi) on cognitive function in healthy human subjects. Psychopharmacology (Berl) 2001; 156: 481–484
4 *Roodenrys S, Booth D, Bulzomi S, Phipps A, Micallef C, Smoker J.* Chronic effects of Brahmi (*Bacopa monnieri*) on human memory. Neuropsychopharmacology 2002; 27: 279–281
5 *Le XT, Nguyet Pham HT, Van Nguyen T, Minh Nguyen K, Tanaka K, Fujiwara H, Matsumoto K.* Protective effects of *Bacopa monnieri* on ischemia-induced cognitive deficits in mice: the possible contribution of bacopaside I and underlying mechanism. J Ethnopharmacol, advance online publication 7 February 2015; DOI: 10.1016/j.jep.2015.01.041
6 *Priyanka HP, Singh RV, Mishra M, ThyagaRajan S.* Diverse age-related effects of *Bacopa monnieri* and donepezil *in vitro* on cytokine production, antioxidant enzyme activities, and intracellular targets in splenocytes of F344 male rats. Int Immunopharmacol 2013; 15: 260–274
7 *Biswas SK.* Evaluation of antinociceptive and antioxidant activities of whole plant extract of *Bacopa monniera*. J Med Plants Res 2012; 6: 607–614
8 *Shinomol GK, Raghunath N, Bharath MM, Muralidhara.* Prophylaxis with *Bacopa monnieri* attenuates acrylamide induced neurotoxicity and oxidative damage via elevated antioxidant function. Cent Nerv Syst Agents Med Chem 2013; 13: 3–12
9 *Shinomol GK, Bharath MM, Muralidhara.* Neuromodulatory propensity of *Bacopa monnieri* leaf extract against 3-nitropropionic acid-induced oxidative stress: *in vitro* and *in vivo* evidences. Neurotox Res 2012; 22: 102–114
10 *Shinomol GK, Bharath MM, Muralidhara.* Pretreatment with *Bacopa monnieri* extract offsets 3-nitropropionic acid induced mitochondrial oxidative stress and dysfunctions in the striatum of prepubertal mouse brain. Can J Physiol Pharmacol 2012; 90: 595–606
11 *Shinomol GK, Mythri RB, Srinivas Bharath MM, Muralidhara. Bacopa monnieri* extract offsets rotenone-induced cytotoxicity in dopaminergic cells and oxidative impairments in mice brain. Cell Mol Neurobiol 2012; 32: 455–465
12 *Limpeanchob N, Jaipan S, Rattanakaruna S, Phrompittayarat W, Ingkaninan K.* Neuroprotective effect of *Bacopa monnieri* on beta-amyloid-induced cell death in primary cortical culture. J Ethnopharmacol 2008; 120: 112–117
13 *Holcomb LA, Dhanasekaran M, Hitt AR, Young KA, Riggs M, Manyam BV. Bacopa monniera* extract reduces amyloid levels in PSAPP mice. J Alzheimers Dis 2006; 9: 243–251
14 *Olanow CW.* A radical hypothesis for neurodegeneration. Trends Neurosci 1993; 16: 439–444
15 *Halliwell B.* Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 2001; 18: 685–716
16 *Tatton WG, Olanow CW.* Apoptosis in neurodegenerative diseases: the role of mitochondria. Biochim Biophys Acta 1999; 1410: 195–213
17 *Krishna A, Biryukov M, Trefois C, Antony PM, Hussong R, Lin J, Heinäniemi M, Glusman G, Koeglsberger S, Boyd O.* Systems genomics evaluation of the SH-SY5Y neuroblastoma cell line as a model for Parkinson's disease. BMC Genomics 2014; 15: 1154
18 *Leśkiewicz M, Regulska M, Budziszewska B, Jantas D, Jaworska-Feil L, Basta-Kaim A, Kubera M, Lasoń W.* Neurosteroids enhance the viability of staurosporine and doxorubicin treated differentiated human neuroblastoma SH-SY5Y cells. Pharmacol Rep 2008; 60: 685–691
19 *Leśkiewicz M, Jantas D, Budziszewska B, Lason W.* Excitatory neurosteroids attenuate apoptotic and excitotoxic cell death in primary cortical neurons. J Physiol Pharmacol 2008; 59: 457–475
20 *Jaworska-Feil L, Jantas D, Leskiewicz M, Budziszewska B, Kubera M, Basta-Kaim A, Lipkowski AW, Lason W.* Protective effects of TRH and its analogues against various cytotoxic agents in retinoic acid (RA)-differentiated human neuroblastoma SH-SY5Y cells. Neuropeptides 2010; 44: 495–508
21 *Regulska M, Pomierny B, Basta-Kaim A, Starek A, Filip M, Lasoń W, Budziszewska B.* Effects of ethylene glycol ethers on cell viability in the human neuroblastoma SH-SY5Y cell line. Pharmacol Rep 2010; 62: 1243–1249
22 *Seet RC, Lee CY, Lim EC, Tan JJ, Quek AM, Chong WL, Looi WF, Huang SH, Wang H, Chan YH, Halliwell B.* Oxidative damage in Parkinson disease: measurement using accurate biomarkers. Free Radic Biol Med 2010; 48: 560–566
23 *Rastogi M, Ojha RP, Prabu PC, Devi BP, Agrawal A, Dubey GP.* Prevention of age-associated neurodegeneration and promotion of healthy brain ageing in female Wistar rats by long term use of bacosides. Biogerontology 2012; 13: 183–195
24 *Pandareesh MD, Anand T.* Neuroprotective and anti-apoptotic propensity of *Bacopa monniera* extract against sodium nitroprusside induced activation of iNOS, heat shock proteins and apoptotic markers in PC12 Cells. Neurochem Res 2014; 39: 800–814
25 *Porter AG, Jänicke RU.* Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999; 6: 99–104
26 *Jantas D, Szymańska M, Budziszewska B, Lasoń W.* An involvement of BDNF and PI3-K/Akt in the anti-apoptotic effect of memantine on staurosporine-evoked cell death in primary cortical neurons. Apoptosis 2009; 14: 900–912
27 *Garnier P, Prigent-Tessier A, Van Hoecke M, Bertrand N, Demougeot C, Sordet O, Swanson RA, Marie C, Beley A.* Hypoxia induces caspase-9 and caspase-3 activation without neuronal death in gerbil brains. Eur J Neurosci 2004; 20: 937–946
28 *Murashige T, Skoog F.* A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962; 15: 473–497
29 *Łojewski M, Muszyńska B, Smalec A, Reczyński W, Sułkowska-Ziaja K, Opoka W.* Development of optimal medium content for bioelements accumulation in *Bacopa monnieri* (L.) *in vitro* culture. Appl Biochem Biotechnol 2014; 174: 1535–1547
30 *Muszyńska B, Sułkowska-Ziaja K, Ekiert H.* Indole compounds in fruiting bodies of some selected Macromycetes species and in their mycelia cultured *in vitro*. Pharmazie 2009; 64: 479–480
31 *Muszyńska B, Sułkowska-Ziaja K.* Analysis of indole compounds in edible Basidiomycota species after thermal processing. Food Chem 2012; 132: 455–459
32 *Pratibha S.* Stability studies of crude plant material of *Bacopa monnieri* and quantitative determination of bacopaside I and bacoside A by HPLC. Phytochem Anal 2012; 23: 502–507
33 *The European Pharmacopoeia Commission.* European Pharmacopoeia 8.0, Vol. 2. Strasbourg: Council of Europe; 2013: 2850
34 *Menon B, Singh M, Ross RS, Johnson JN, Singh K.* β-Adrenergic receptor-stimulated apoptosis in adult cardiac myocytes involves MMP-2-mediated disruption of β1 integrin signaling and mitochondrial pathway. Am J Physiol Cell Physiol 2006; 290: C254–C261