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Red *Panax ginseng* root promotes neuronal plasticity *in vitro* and improves cognitive function in aged animals

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ABSTRACT

Background: Cognitive function declines with aging, primarily due to reduced neuronal plasticity, decreased release of trophic factors, and neuronal stress. Genetic and environmental factors have been shown to impact cognitive function. Diets enriched with neuroactive ingredients have been proposed to support cognitive functions in humans. Several neuroactive compounds, including ginsenosides, have been identified in *Panax ginseng*. Many studies show that the roots of this plant promote neuroprotection and neuroplasticity.

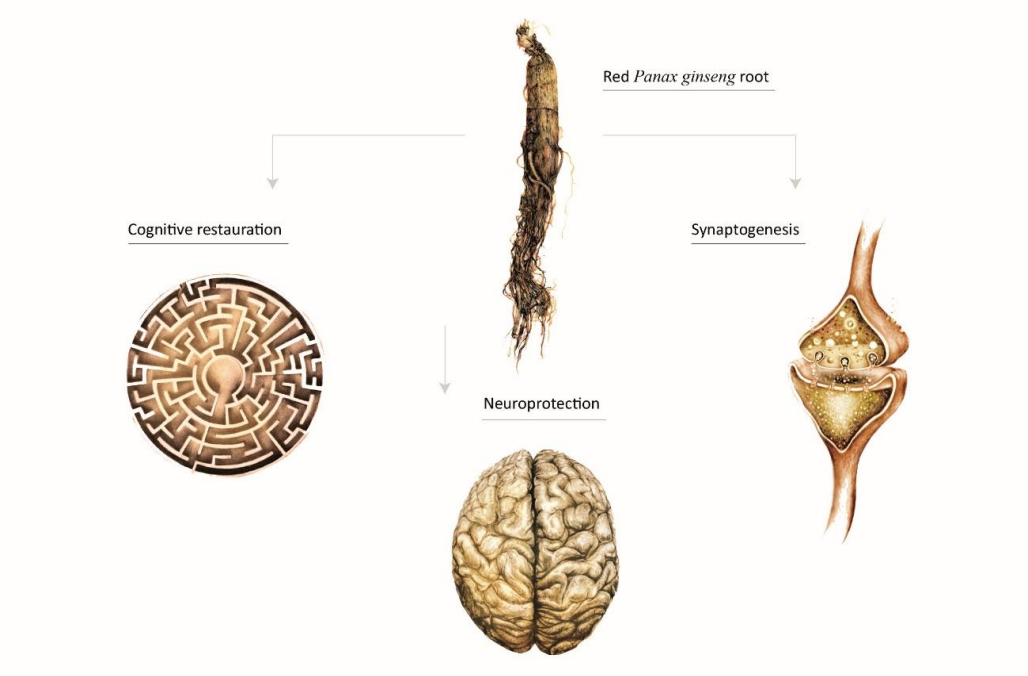
Objective: The aim of this study was to examine the impact of the red *Panax ginseng* roots cultivated in an innovative vertical farming technology. The effects of the red *Panax ginseng* roots were investigated to determine whether they could (1) support neuronal plasticity and neuronal survival during stress *in vitro* and (2) improve cognitive function (short-term memory) *in vivo*.

Methods: An extract of the Botalys red Panax ginseng root powder was tested in vitro on primary hippocampal and cortical neurons, injured or not, with glutamate. Its effects on the neuritogenesis and the synaptogenesis was investigated as well as its neuroprotective efficacy. Moreover, the effect of the red Panax ginseng root powder was investigated in vivo on the cognitive functions of aged animals.

Results: The results demonstrated that prolonged treatment of hippocampal neurons with the red *Panax ginseng* extract significantly prompts synapse formation and neurite elongation in primary cultures of hippocampal neurons. In addition, the red *Panax ginseng* extract protected neurons from glutamate-induced excitotoxicity in a primary culture of cortical neurons. In aging mice, oral administration of red *Panax ginseng* root powder for 7 days significantly improved short-term memory deficit associated with aging.

Conclusion: Altogether, these results indicate that red *Panax ginseng* root promotes neuronal plasticity and synaptogenesis in *in vitro* models and improves short-term memory deficits in *in vivo* models of aging.

Keywords: Panax ginseng, neuronal plasticity, cognitive functions, in vitro, in vivo



Graphical Abstract:

INTRODUCTION

Aging, or senescence, is a process associated with progressive decline in biological functions, such as impaired adaptive neuroplasticity and resilience, aberrant neuronal network activity, dysregulation of neuronal Ca^{2+} homeostasis, and inflammation. These changes render the aging brain more susceptible to strokes, Alzheimer's disease, and Parkinson's disease. Age-related cognitive decline varies among individuals, stemming from genetic and environmental factors (e.g. education). At the cellular level, the ability to preserve existing neural resources and/or to compensate for their loss has a strong influence on cognition [1].

The hippocampus plays a major role in neuroplasticity and is a key structure for memory processing and consolidation. Neurogenesis and formation of new synapses in the hippocampus directly contribute to building and storing new memories. Adult neurogenesis in the dentate gyrus is associated with improvement in acquisition, formation, and maintenance of memory [2].

Cognitive decline is primarily due to weaker neuronal plasticity and progressive neuronal loss. The importance of growth factors and their decline during aging is well-known and well-established. Low levels of growth factors are directly connected to lower neuronal plasticity and neuronal loss, leading to brain atrophy during aging [3]. Various brain regions exhibit continuous dendritic regression with increasing age [4].

Glutamate is an important neurotransmitter that binds to ionotropic (e.g. AMPA, NMDA) and metabotropic (mGluR) receptors, mediating synaptic plasticity by supporting long-term potentiation. During aging, glutamatergic signaling appears dysregulated due to lower re-uptake of synaptic glutamate by

astrocytes, causing increased intracellular calcium concentration and promoting neuronal stress [5]. There is a proposed correlation between weakened memory function and reduced NMDA receptors during senescence [6]. In addition, in aged rats, a decline in glutamate uptake has been linked to extrasynaptic NMDA receptors at the hippocampal CA1 synapse [7]. Recent reports suggest that overexpression is initiated by stimulating these receptors [8]. Given that the GLuN2B subunit is found in extrasynaptic NMDA receptors, it is being explored as a potential target for age-related neurodegenerative disorders like Alzheimer's disease (AD) [9].

Diets enriched with neuroactive ingredients have been proposed to support cognitive functions [10-12]. *Panax ginseng* root, considered a functional food product [13-16], has been utilized in Asia for thousands of years for the treatment of various diseases. Throughout Europe, ginseng preparations are predominantly used as a tonic to alleviate tiredness, weakness, and decreased mental and physical capacity (EMA/HMPC/321232/2012). Many studies suggest that the roots of this plant promote neuroprotection and neuroplasticity [17-18]. Neuroactive compounds identified in *Panax ginseng* extracts typically include ginsenosides (also called panaxosides), ginsan, or gintonin. Ginsenosides are triterpene saponosides grouped into protopanaxadiols (Ra1, Ra2, Ra3, Rb1, Rb3, Rc, Rd, Rg3, Rh2), protopanaxatriols (Rg1, Rg2, Re, Rf, Rh1, Rh3), or oleanane (Ro) [19]. Ginsan is an acidic anti-inflammatory polysaccharide, while gintonin is a glycoprotein known to activate lysophosphatidic acid receptors [20].

Although *Panax ginseng* is widely recognized as an effective treatment for numerous disorders, it often suffers from the general poor notoriety of phytotherapy. Detractors of phytotherapy mainly point out the lack of standardization (i.e., active compound level below the effective dose), leading to a lack of uniformity and replicability in the results of several studies. The solution to this issue lies in guaranteeing a duplicable form of herbal preparations. This was achieved by Botalys (Belgium) through hydroponic cultivation of Red *Panax ginseng* root powder under strictly controlled growing conditions. Moreover, despite extensive research on cognitive decline and interventions to mitigate its effects, the potential of natural compounds, particularly ginseng, remains underexplored in contemporary studies. Previous research has established the cognitive benefits of *Panax ginseng* [17-18], focusing primarily on its effects in general populations and its basic mechanisms of action. However, there is limited understanding of how specific extracts of ginseng, such as the innovative Botalys red *Panax ginseng* root extract, can influence neuronal plasticity and memory functions in aging models.

In this paper, we analyzed the neuroactive effects of the red *Panax ginseng* root powder. An extract of the Botalys red *Panax ginseng* root powder was tested *in vitro* on primary hippocampal and cortical neurons, both injured and uninjured, with glutamate. Its effects on neuritogenesis and synaptogenesis were investigated, as well as its neuroprotective efficacy. Moreover, the effect of the red *Panax ginseng* root powder was investigated *in vivo* on the cognitive functions of aged animals.

MATERIALS AND METHODS

Ethics: Following sanction by the local ethic committee and the Ministry of Higher Education and Research, the experiments were conducted under the authorized investigator (APAFIS#27790). The latest European Union regulations were adhered to (Directive 2010/63/EU), and the experiments were executed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory. Agreement number: A1301337, B1301310.

The red Panax ginseng root

Preparation of the red Panax ginseng root extract: The *Panax ginseng* root powder is processed by Botalys company (Ghislenghien, Belgium). The carefully selected cultivar HRG80 of *Panax ginseng* is hydroponically cultivated in an advanced vertical farming system, maintaining rigid control of growing

conditions (Botalys is FSSC22000 certified). This allows for a reproducible chemical composition of the roots from one batch to another without any contamination, while containing a high content of rare ginsenosides with better bioavailability and bioactivity.

An extract of the red *Panax ginseng* root powder was prepared to make the product available for cell culture. For this purpose, the *Panax ginseng* root powder was extracted in 70% ethanol at 100°C under agitation for over 8 hours. The solution was then filtrated and evaporated to obtain the dry extract.

Analysis of the Panax ginseng compounds (UHPLC): Compounds extraction and analysis were performed as follows. The red *Panax ginseng* root powder or the dry extract (1g) was extracted in 100 mL of methanol 70% in a round flask equipped with condenser (connected to cold water) at 100°C under agitation for over 8 hours. The volume was potentially adjusted at 100 mL after the extraction. The extract solution was filtrated through a 0.45-mm Millipore filter and used for the UHPLC analysis.

Ginsenosides content (total and rare) was quantified using a SHIMADZU UHPLC LC20 ADXR modular system, which consists of a Detector SPD-40V, Autosampler SIL-40C, Pump LC-40B XR, and Column oven CTO-40C with column Shim-pack GIST C18 2 µm (150 x 2.1 mm). The sample injection volume was established at 10 µL, analysis was performed at 40°C, and the detection wavelength was set at 245 nm. Separation was accomplished through elution employing a linear gradient with solvent A (0.1% phosphoric acid solution) and solvent B (acetonitrile). The gradient was as follows: t=0min, 80% A; t=40 min, 10% A; t=45 min, 10% A; and t=46 min, 80% A. The flow rate was modified to 0.25mL/min. In total, 23 ginsenosides were analyzed. Standards of ginsenosides were purchased (Rh1, Rb3, F1, Rd, Rg6, F2, Rh4, Rg3, Rk1, Rg5, Rh3 and 20S-protopanaxtriol from Sigma Aldrich; Rg1, Re, Rf, Rb1, Rc, Rb2 and Rh2 from Extra synthese; F4, Rk2 and Rk3 from Chemfaces; and protopanaxdiol and 20R-protopanaxtriol from VWR), and calibration curves were performed.

In vitro pharmacology

Primary culture of hippocampal neurons: Rat hippocampal neurons were cultivated as illustrated previously [21]. Briefly, pregnant female rats (Wistar) of 17 days of gestation were euthanized by cervical dislocation after deep anesthesia with a CO_2 chamber. Upon collection, fetuses were promptly immersed in ice-cold L15 Leibovitz medium (L15, Pan Biotech, Aidenbach, Germany) containing a 2% penicillin (10,000 U/mL) and streptomycin (10 mg/mL) solution (PS, Pan Biotech), along with 1% bovine serum albumin (BSA, Pan Biotech). Hippocampal areas underwent treatment for 20 min at 37 °C with a trypsin-EDTA solution (Pan Biotech) at a final concentration of 0.05% trypsin and 0.02% EDTA. The dissociation was ceased by introducing Dulbecco's modified Eagle's medium (DMEM, Pan Biotech) with 4.5 g/L of glucose, comprising DNAse I grade II (final concentration 0.5 mg/mL, Pan Biotech) and 10% fetal calf serum (FCS, Invitrogen). Cells experienced mechanical dissociation by three forced passages through the tip of a 10-mL pipette. Subsequently, cells were centrifuged at 515 x g for 10 min at 4 °C. The supernatant was disposed of, and the pellet was resuspended in a specified culture medium comprised of Neurobasal medium (Invitrogen) with a 2% solution of B27 (Invitrogen) supplement, 2 mmol/L of L-glutamine (Pan Biotech), 2% of PS solution, and 10 ng/mL of brain-derived neurotrophic factor (BDNF, pan-biotech, CB-1115002). Viable cells were calculated in a Neubauer cytometer, utilizing the trypan blue exclusion test. The cells were planted at a density of 20,000 per well in 96-well plates (Corning Biocoat) pretreated with poly-L-lysine (Sigma Aldrich) and cultured at 37 °C in an air (95%)-CO2 (5%) incubator. Every 2 days, the medium was regenerated. To prevent any edge effects, the first and last columns, as well as the first and last lines of the plate, were not used. Empty wells were filled with water.

The red *Panax ginseng* root extract was solubilized in DMSO (Pan Biotech) and diluted in culture medium (0.2% DMSO final). On day 3 of culture, the red *Panax ginseng* root extract (from 0.1 µg/mL to

5 µg/mL) or BDNF (50 ng/mL) were incubated with primary hippocampal neurons for 7 days. Every other day, half of the medium was regenerated in presence of the red *Panax ginseng* root extract and BDNF.

Primary culture of cortical neurons: As outlined by [21], rat cortical neurons were propagated with modifications. Briefly, pregnant female rats (Rats Wistar; Janvier Labs France) of 15 days of gestation were euthanized after a deep anesthesia with CO_2 by cervical dislocation. Upon collection, fetuses were promptly positioned in ice-cold L15 with a 2% PS and 1% BSA. Cortexes underwent treatment for 20 min at 37 °C with a trypsin-EDTA solution at a final concentration of 0.05% trypsin and 0.02% EDTA. The dissociation was halted by introducing DMEM with 4.5 g/L of glucose, containing DNAse I grade II (final concentration 0.5 mg/mL) and 10% FCS.

Utilizing the tip of a 10-mL pipette, cells were mechanically separated by three forced passages. Afterwards, cells were centrifuged at 515 x g for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in a specified culture medium comprised of Neurobasal medium with a 2% solution of B27 supplement, 2 mmol/L of L-glutamine (Pan Biotech), 2% of PS solution, and 10 ng/mL of BDNF. Employing the trypan blue exclusion test, viable cells were calculated in a Neubauer cytometer.

The cells were plated at a density of 25,000 per well in 96-well plates pretreated with poly-L-lysine and cultured at 37 °C in an air (95 %)-CO2 (5 %) incubator. The medium was renewed every 2 days. To mitigate any potential edge effects, the first and last columns, as well as the first and last lines of the plate, were not used. Empty wells were filled with water.

The red *Panax ginseng* root extract (from 0.1 µg/mL to 5 µg/mL) was solubilized in DMSO and diluted in culture medium (0.2% DMSO final). On day 13 of culture, the red *Panax ginseng* root extract or BDNF (50 ng/mL) was incubated with primary cortical neurons for 1 hour. After the pre-incubation, cortical neurons were subjected to glutamate (20 µmol/L) for 20 min in presence of the red *Panax ginseng* root extract or BDNF. After 20 min, the medium was discarded, and new culture medium with the red *Panax ginseng* root extract or BDNF was added for an additional 48 hours.

Immunostaining and image analysis: Upon culmination of the culture, primary neurons were fixed by a cold solution of ethanol (95%) and acetic acid (5%) for 5 min at -20 °C. Cells were treated with a solution containing 0.1% saponin (Sigma Aldrich) and 1% FCS for 15 min to permeabilize cell membranes and obstruct unspecific binding sites. Then, cells were incubated for 2 hours with the primary antibodies.

MAP-2 staining was performed with a chicken monoclonal antibody (ab5392, Abcam) anti microtubule-associated-protein 2 (MAP-2), diluted to 1/1000 in PBS, 1% FCS, and 0.1% of saponin.

PSD-95 staining was executed with a mouse monoclonal antibody (ab13552, Abcam), diluted to 1/200 in PBS, 1% FCS, and 0.1% of saponin. These antibodies were detected with the secondary antibodies Alexa Fluor 568 goat anti-chicken IgG (Invitrogen, SAB4600079) diluted to 1/400 and an Alexa Fluor 488 goat anti-mouse IgG (sigma, SAB4600042), diluted to 1/400 in PBS containing 1% FCS, and 0.1% saponin, at room temperature for 1 hour.

Pictures (30 pictures per well and per staining) were taken automatically utilizing ImageXpress (Molecular Devices) at 20x magnification (to assess the number of neurons and the length of the neurite network) or at 40x magnification (for synapse evaluation). Employing the same acquisition parameters, images were automatically generated. Then, from the images, analyses were conducted using MetaXpress® (Molecular Devices).

In vivo pharmacology

Animals: 2-month-old and 18-month-old C57BL6 male mice were supplied by Janvier Labs (France). Mice were

acclimated for 1 week in Neuro-Sys facilities and were sustained in a reversed 12-hour light–dark cycle. The animals were housed in groups (2-4 per cage) and kept in a room with controlled temperature and humidity, with food and water available ad libitum.

Treatment: The red *Panax ginseng* root powder was suspended in 0.5% methylcellulose (MEC) in water) and was administrated once daily per os (gavage) for 8 consecutive days. Experimental groups were constituted by 12 mice. The control group received the vehicle (0.5% MEC). The red *Panax ginseng* root powder was given at a dose of 50 mg/kg/day. Animals were observed daily.

Forced alteration Y-maze test: The Y-maze arena consisted of a light-grey colored polyvinylchloride (PVC) Y-shaped compartment (35 cm arm length x 6 cm x 15 cm arm height) with equal length arms. One day before the first administration of the red *Panax ginseng* root powder, mice were allowed unrestricted exploration of the entire Y-maze arena for 5 minutes for habituation purposes. Seven days after the first administration of the red *Panax ginseng* root powder, mice were tested for short-term memory deficit in the Y-maze test. First, mice were authorized to explore two arms of the Y-maze without restriction for 5 min, and the last arm remained closed. At the end of the 5 min, the animals were allowed to rest for 3 min in an empty cage. The Y-maze was cleaned with acetic acid to neutralize sui generis odor. After the 3-min rest, the mice were then permitted to explore all three open arms of the Y-maze for 5 min. After an intertrial interval, the mouse was expected to remember which arm they had not explored previously and visit this arm more often. Trials were automatically recorded by a video camera using the Ethovision system (Noldus). The time spent in each arm was automatically determined for each animal.

Statistical analysis: All values are presented as the mean ± SEM (standard error of the mean). Graphs and statistical analyses were made using GraphPad Prism version 8.0.2. One-way or two-way ANOVA tests, succeeded by Fisher's test, were utilized to evaluate differences between groups. A p-value of <0.05 was considered significant. Outliers were recognized by Grubb's test (alpha = 0.2) based on abnormal deviation from the mean or abnormal behavior during the Y-maze test (e.g. freezing behavior during the test).

RESULTS

Composition of the red Panax ginseng root extract (used for in vitro experiments) and powder (used for in vivo study): The key active constituents of *Panax ginseng* are ginsenosides, although not all ginsenosides are equally bioactive. The predominant ginsenosides found in commercial ginseng are Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1, which are inadequately absorbed after oral ingestion [22]. Colonic bacteria facilitate the deglycosylation of these ginsenosides, forming intestinal metabolites. These metabolites are the primary compounds detected in systemic circulation after oral administration of ginsenosides and are widely acknowledged for their role in ginseng's pharmacologic activity [22]. Rare ginsenosides, which are less abundant in ginseng, are ginsenoside metabolites and are considered the most pharmacologically active. The transformation of ginsenosides into rare ginsenosides, which involves changing its chemical structure can occur by physical methods (e.g. steaming), chemical methods (acid or alkaline hydrolysis), and biotransformation (bacterial/fungal fermentation) [23]. Red ginseng (i.e. steamed ginseng) amplifies the concentration of rare ginsenosides and was developed to improve oral absorption of *Panax ginseng* components, strengthening its pharmacologic efficacy.

The ginsenosides content of the *Panax ginseng* root powder and the dry extract were analyzed using

UHPLC. Results are compiled in Table 1. The results demonstrate a high level of ginsenosides in both the *Panax ginseng* root powder and the *Panax ginseng* root dry extract. Moreover, the proportion of rare ginsenosides (indicated in brackets in Table 1), such as Rh3, Rh4, Rg3, Rg5, Rk1 and Rk2, is very elevated (>80%).

Table 1. Ginsenosides content (expressed in mg/g of dry matter; except for the total expressed in %) measured in the *Panax ginseng* root powder and in the dry extract. Rare ginsenosides are indicated in brackets.

Ginenoside (mg/g)	The red *Panax ginseng* root (powder)	The red *Panax ginseng* root (extract)
Rg1	1.43	0.93
Re	1.15	4.55
Rf	1.32	3.22
Rb1	0.4	1.83
Rc	3.52	18.86
Rh1 (rare)	1.17	2.82
Rb2	0.41	5.17
Rb3	1.46	1.54
F1 (rare)	0.15	0.19
Rd	1.14	19.04
Rg6 (rare)	1.8	8.68
F4	3.89	17.2
F2 (rare)	2.82	5.24
Rh4 (rare)	19.07	43.56
Rg3 (rare)	14.76	43.07
PPT (rare)	1.29	1.48
Rk1 (rare)	11.63	40.27
Rg5 (rare)	16.33	75.34
Rh2 (rare)	3.18	9.24
Rh3 (rare)	4.91	18.17
PPD (rare)	0	1.5
Rk3 (rare)	1.83	4.14
Rk2 (rare)	36.85	113.57
Total (%)	**13.3%**	**44.2%**
Total of rare ginsenosides	**11.6%**	**36.7%**

The red Panax ginseng root extract promotes synapse formation and neurite elongation in the primary culture of hippocampal neurons: Hippocampal neurons play a central role in acquisition and maintenance of memories because the hippocampus is a major site of synaptic plasticity and neurogenesis. The formation of new synapses in the hippocampus directly contributes to cognitive function [24].

First, the effects of the red *Panax ginseng* root dry extract on neuritogenesis and synaptic plasticity in the primary cultures of hippocampal neurons were determined. For *in vitro* experiments, the red *Panax ginseng* root dry extract was chosen due to its superior solubility in culture medium compared to root powder. Various concentrations of extract were applied to the cultures for 7 days, starting 3 days after seeding. Differentiated neurons, neurite length, and the synaptic network were assessed using immunostaining (Fig. 2).

After 7 days of treatment with red *Panax ginseng* root extract (1 µg/mL), the number of differentiated MAP2-positive neurons significantly increased compared to the control condition, suggesting that the red *Panax ginseng* root dry extract stimulated neural progenitor growth. Other concentrations did not significantly affect the number of differentiated neurons. The total length of the neurite network was significantly greater in the presence of the red *Panax ginseng* root dry extract (0.5 and 1 µg/mL) compared to the control group. Synaptogenesis was assessed by investigating the PSD95 post-synaptic marker, revealing that the red *Panax ginseng* root dry extract (1 µg/mL) significantly increased the synaptic area on differentiated neurons. BDNF, a potent neurotrophic factor, also positively affected the number of MAP2 neurons, the neurite network, and synapse formation. The potency of the red *Panax ginseng* root dry extract at 1 µg/mL was equivalent to that of BDNF at 50 ng/mL. Altogether, these results indicate the positive role of the red *Panax ginseng* root dry extract on neuronal differentiation, neurite elongation, and synaptogenesis.

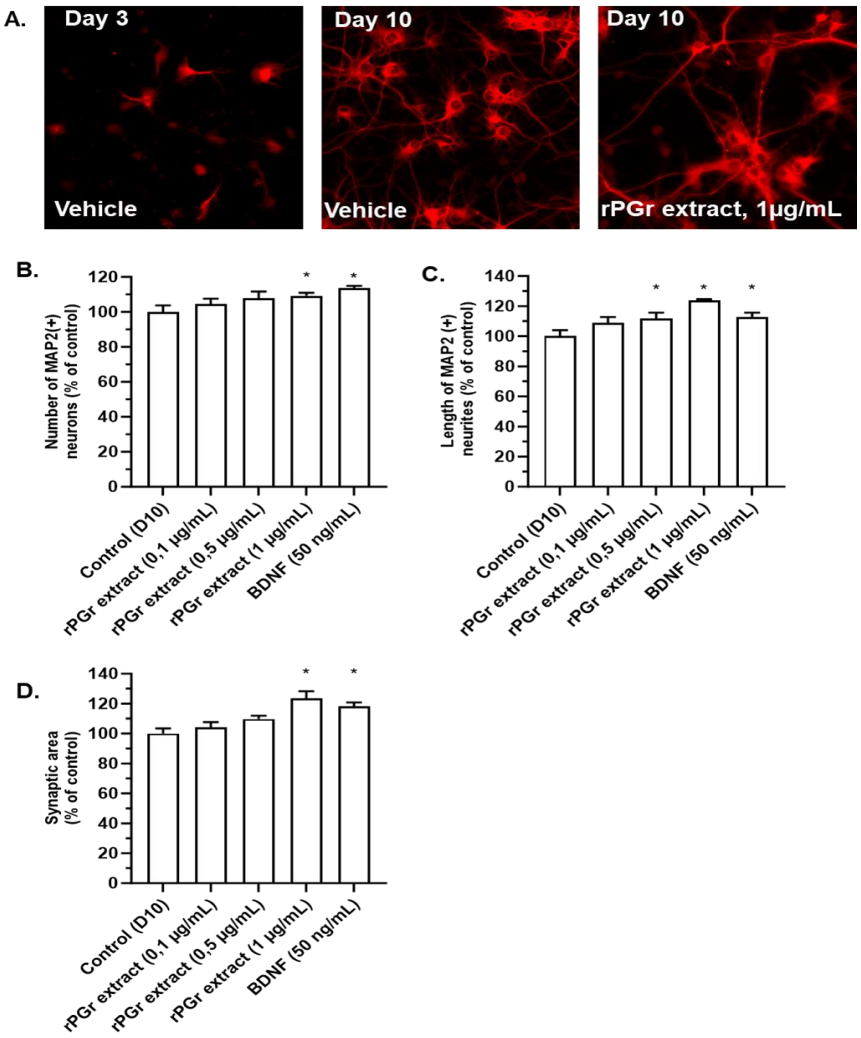


Figure 1. *The red Panax ginseng root (rGPr) dry extract promotes neuronal differentiation, neuritogenesis, and synaptogenesis in primary cultures of hippocampal neurons.* (A) Images of primary hippocampal neuron cultures immunostained for MAP-2 at day 3 (before treatment) and at day 10 (end of treatment, with the vehicle and the red *Panax ginseng* root (rGPr) extract). In these cultures, the red *Panax ginseng*-treated neurons displayed better differentiation. The number of differentiated neurons (B), total neurite network length (C) and total synapse area (D) were measured by immunostaining 7 days after application of the red *Panax ginseng* root dry extract. *P < 0.05, One-way ANOVA followed by Fisher's LSD test. All values show the mean ± SEM (standard error of the mean, n=4-6 per group).

The red Panax ginseng root extract protects mature neurons from glutamate stress in the primary culture of cortical neurons: During aging, excess glutamate around neurons can overstimulate NDMA receptors, leading to neuronal stress, loss of connections, and ultimately neuronal loss. We hypothesized that the red *Panax ginseng* could protect mature neurons from glutamatergic stress.

The red *Panax ginseng* root extract was applied to mature cortical neurons as a preventive treatment one hour before injury. The application of glutamate significantly decreased the number of cortical neurons (Fig. 3A) due to excitotoxicity [25] and strongly reduced the total neurite network length (Fig. 3B). Neurotrophic factors, such as BDNF, are known to improve neuronal survival during various stress [26]. In our experiment, BDNF exerted neuroprotective effects by preventing neuronal loss and protecting the neurite network. The red *Panax ginseng* root extract improved the survival of cortical neurons at a concentration of 5 µg/mL. In addition, after glutamatergic stress, the neurite network was significantly longer in the presence of the red *Panax ginseng* root extract at 5 µg/mL.

Overall, these results indicate that the red *Panax ginseng* root extract, when administrated as a

preventive treatment, provides protective effects to neurons under glutamate stress.

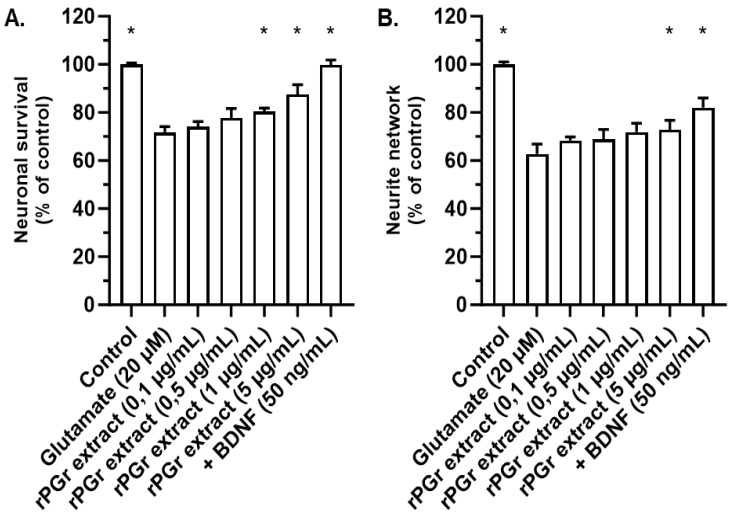


Figure 2. *The red Panax ginseng root (rPGr) extract supports neuronal survival of primary cortical neurons after glutamatergic stress.* In the primary culture of cortical neurons, neuronal survival (A) and the total neurite network length (B) were investigated by immunostaining 48 hours after injury. *P < 0.05, One-way ANOVA followed by Fisher's LSD test. All values show the mean ± SEM (standard error of the mean, n=4-6 per group).

The red Panax ginseng root powder improves short-term memory deficit in aged mice: Cognitive decline, often associated with lower working and short-term memory. This decline is due to factors, such as reduced attention, slower information processing speed, and higher inhibitory control, leading to an inability to suppress non-pertinent information [27]. Impairment in learning and memory are well-characterized in aged humans [28], monkeys [29], rats [30], and mice [31].

The effect of red *Panax ginseng* root powder on the short-term memory of aged animals was investigated using the Y-maze forced alternation test. This test relies on rodents' natural tendency to explore new settings, and good performance in the test indicates functional short-term spatial memory.

The red *Panax ginseng* root powder was administered daily, per os, at doses of 10 and 50 mg/kg/day to 18-month-old mice. Young mice, aged 2 months, were used as cognitively fit controls. No effect of the red *Panax ginseng* root powder was observed on the body mass of the animals after one week of treatment (Fig. 4A). Aging impacted the total distance run, with aged mice covering a shorter distance than younger animals (Fig. 4B), and the red *Panax ginseng* root powder did not show any effect on the distance travelled by aged mice compared to aged controls.

The forced alternation Y-maze test indicated that 18-month-old mice explored the new arm less than 2-month-old mice (Fig. 5D, E). It indicated that they had a significant impairment in short-term spatial memory [31].

Interestingly, 18-month-old mice treated with the red *Panax ginseng* root powder (50 mg/kg/d) explored the new arm of the Y-maze significantly longer. This demonstrates a stronger interest in the novel arm and, hence, improved cognitive performance (Fig. 5D). The performance of aged mice treated with the red *Panax ginseng* root powder was similar to that of cognitively fit young animals.

At the beginning of the test, we also noted that aged mice left the entry arm significantly later than younger mice (Fig. 4C). Apathy, or lack of reactivity, is often found in elderly and has a negative impact on cognitive performance, including working memory [32]. The red *Panax ginseng* root powder (50 mg/kg/d) significantly reduced the latency to leave the entry arm, indicating a higher reactivity. The results

collectively indicate that the red *Panax ginseng* root powder improved short-term memory in aged mice.

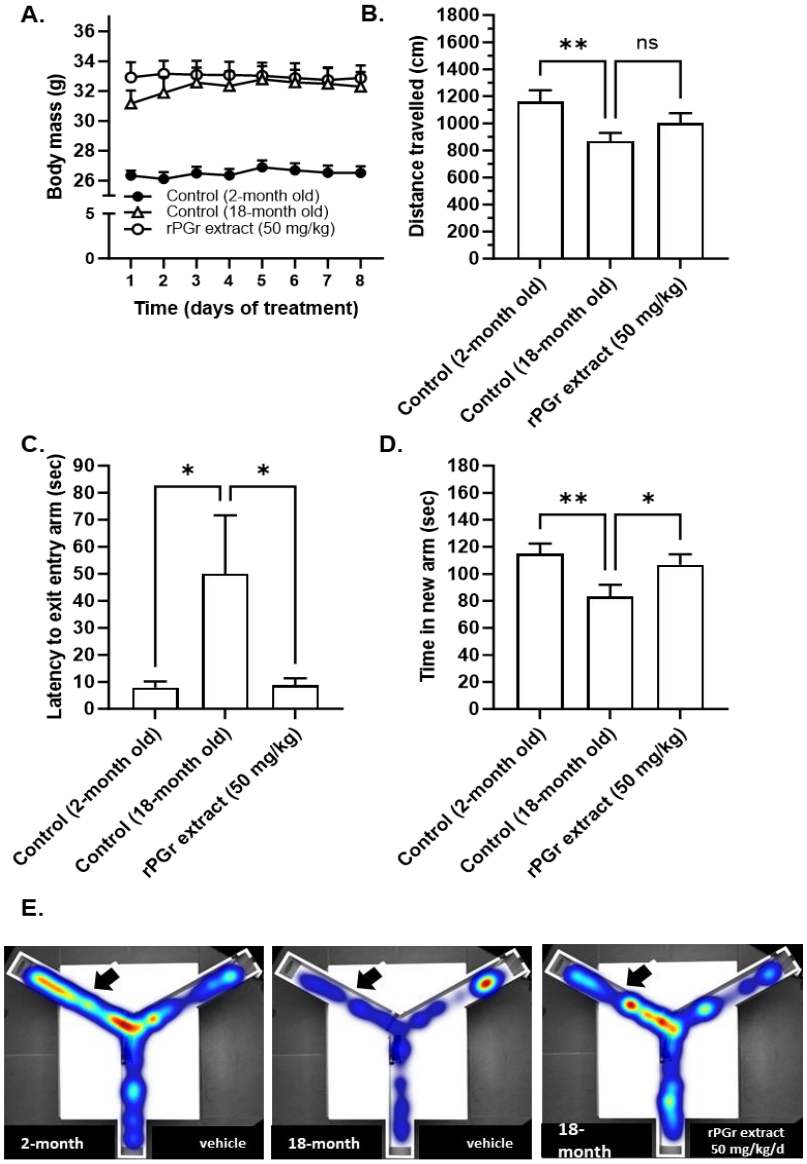


Figure 3. *The red Panax ginseng root powder improves short-term memory deficit in aged mice.* (A) Evolution of body mass of young and aged mice treated with either the vehicle or the red *Panax ginseng* root powder. (B) Total distance run during the Y-maze test. (C) Latency to exit the entry arm by the young and aged mice, used as a proxy for apathy. (D) Representative heat map showing time spent by mice in each arm of the Y-maze, with arrows indicative of the novel arm. (E) Time spent exploring the novel arm by young and aged mice. *, $P < 0.05$, **$P < 0.01$, One-way ANOVA ensued by Fisher's LSD test.

DISCUSSION

Cognitive performance declines with age. Notably, age-associated decline in spatial short-memory was reported [33]. Short-term memory refers to the ability to retain information for restitution after a short period, such as remembering the localization of an object handled a few hours earlier. It relies on the temporal brain area, particularly the hippocampus.

Several factors appear to contribute to variations in the effectiveness of plasticity mechanisms across the lifespan. These include genetic and hormonal factors, diabetes, cancer, infections, traumatic brain injury, toxins, stress, sleep deprivation, substance abuse, insufficient cognitive reserve, nutrition, and sedentary lifestyle. Conversely, certain environmental factors can support cognitive functions and exert a trophic effect on neurons. Physical activity, for example, reinforces neuronal plasticity in the hippocampus and is associated with improved short-term and long-term memory [34]. Many reports also show that specific

dietary habits and nutrients, such as polyphenol intake [35], can support cognitive functions in aging.

Panax ginseng has been used in traditional medicine in East Asia (China, Korea, and Japan) as a dietary supplement. Clinical evidence suggests that it improves cognitive functions in elderly individuals. Recent scientific cohort studies on life prolongation with ginseng consumption support this finding, as those who consumed ginseng for more than 5 years had reduced mortality and cognitive decline compared to non-consumers. Clinical studies have also demonstrated that both acute and long-term intake of ginseng total extract can enhance acute working memory performance or cognitive function in healthy individuals, as well as those with subjective memory impairment (SMI), mild cognitive impairment (MCI), or early Alzheimer's disease (AD) dementia who are on AD medication [36-37].

Ginseng components exhibit additional neuronal activities. Kim and colleagues have shown that gintonin activates the G protein-coupled receptor of the phospholipid lysophosphatidyl choline, which induces a transient Ca^{2+} influx and the release of glutamate, a neurotransmitter key for synaptic plasticity, from astrocytes [38]. Wu and colleagues also reported a pro-differentiation of embryonic stem cells into neurons by Rg1 via the glucocorticoid receptor and the activation of MAPK-Erk1/2 and PI3K/Akt pathways [39]. Positive modulation of neurotrophic signaling is highly relevant for preventing and treating neurodegenerative diseases.

The red *Panax ginseng* root powder evaluated in the present study was cultivated and processed by Botalys using innovative vertical farming technology. The Botalys red *Panax ginseng* root powder contains ginsenosides, such as Rg3 (see Table 1), known to improve cognitive functions. Our results showed that the red *Panax ginseng* root powder improved the cognitive performance of aged animals in a short-term memory test after 7 days of treatment. This suggests that active ingredients in the red *Panax ginseng* root powder successfully reached the CNS to mediate pro-memory activity. This is consistent with a recent clinical study performed with the Botalys red *Panax ginseng* root powder on elderly humans [40]. This study demonstrated that the red *Panax ginseng* root powder (called HRG80 in this paper) strongly influenced electrical brain activity, with effects varying across several brain regions depending on the mental demands during relaxation and cognitive tasks related to memory, attention, and mental performance. These findings suggest that the red *Panax ginseng* root powder has favorable impacts on the cognitive functions of elderly subjects [41].

In addition, our results support a neurotrophic-like effect of the red *Panax ginseng* on *in vitro* culture of primary hippocampal neurons. Treatment with red *Panax ginseng* root extract was associated with a longer neurite network and an increased number of synapses, both of which are negatively impacted with age span. The application of red *Panax ginseng* root extract to the culture also resulted in a higher number of differentiated neurons. Since the red *Panax ginseng* root extract was applied after seeding, it may have promoted the differentiation of neuronal progenitor into mature neurons in the culture. This aspect warrants further investigation. Interestingly, Dimpfel and colleagues recently demonstrated that red *Panax ginseng* root powder (HRG80 cultivar) enhanced LTP in hippocampal sections of rats. This effect was dependent on NMDA and kainite receptors [41]. Moreover, another *in vitro* study showed that Botalys red *Panax ginseng* root extract positively affected gene expression related to neuroinflammation, senescence, apoptosis, and immune response in the hippocampal neuronal cell line HT22, suggesting potential benefits for different disorders [42]. In their article, Panossian and colleagues provide the list of genes whose expression is regulated by the red *Panax ginseng* root extract. Based on their study, we used the online pathway database Reactome (https://reactome.org) to further investigate the effects of the red *Panax ginseng* root extract on pathways relevant to neuronal plasticity. Genes highly regulated by the red *Panax ginseng* root extract at 1 µg/mL were selected (fold

change higher than 200 or lower than -200), as this concentration corresponds to the active concentration in our *in vitro* assay of neuronal plasticity.

According to Reactome, the most regulated genes by the red *Panax ginseng* root extract are associated with the "nervous system development," the "slit-robo pathway," (highly involved in axonal guidance), and "cellular response to stress" (suppl. Table 1). The slit-robo pathway is involved in neurogenesis and neuronal migration during development, axonal guidance, and spinogenesis [43]. The expression of slip and robo genes is found in adult rat brains, particularly in the CA1 and CA3 areas of the hippocampus and in the dendate gyrus. These findings suggest the role of the slit-robo pathway in neuronal plasticity [44] and align with our own observations in primary cultures of hippocampal neurons, where the red *Panax ginseng* root extract promoted axonal elongation and synaptogenesis.

Impaired glutamate and calcium homeostasis cause synaptic dysfunction and mitochondrial stress, directly contributing to neuronal loss in aging and neurodegenerative diseases [5]. Our results demonstrated that the red *Panax ginseng* root extract improved survival of primary cortical neurons injured with high concentrations of glutamate. This neuroprotective effect could be linked to the activation of pro-survival pathways.

Altogether, our results demonstrate that the Botalys red *Panax ginseng* root extract can support neuronal plasticity and short-term memory in models of aging.

CONCLUSION

Our study demonstrates that Botalys red *Panax ginseng* root extract exhibits significant neurotrophic and neuroprotective effects. The extract improves cognitive performance, particularly short-term memory, in aged animal models. It supports neuronal plasticity by enhancing neurite outgrowth, synapse formation, and neuronal differentiation. Additionally, it offers protection against glutamate-induced neurotoxicity. These findings suggest that the active ingredients in the red *Panax ginseng* root extract successfully reach the central nervous system and exert beneficial effects on cognitive functions. This aligns with previous clinical studies indicating cognitive improvements in elderly individuals consuming red *Panax ginseng*. Therefore, the red Panax ginseng root extract holds promise as a potential therapeutic agent for mitigating age-related cognitive decline and promoting brain health. Further research is warranted to explore its mechanisms of action and long-term benefits in human subjects.

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List of Abbreviations: AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA: N-methyl-D-aspartate; mGluR: metabotropic glutamate receptors; AD: Alzheimer's disease; BSA: bovine serum albumin; DMEM: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; BDNF: brain-derived neurotrophic factor; MAP-2: microtubule-associated-protein 2; MEC: methylcellulose; PVC: polyvinylchloride; FSSC: Food Safety System Certification; UHPLC: Ultra high performance liquid chromatography; PS: penicillin and streptomycin solution; DMSO: dimethyl sulfoxide; rGPr: red Panax ginseng root; SEM: standard error of the mean.

Competing interests: CL, AM, NH, SD and PAM are employees at Botalys SA (Ghislenghien, Belgium), producer of Red Panax ginseng roots. The authors declare that there are no known competing financial interests or personal relationships that could have appeared to influence this work.

Authors' Contributions: Conceptualization, CL, SD, AM and PAM; writing, CL, SD and AM; writing—review and

editing, SD; supervision, NH; project administration, PAM. All authors have read and agreed to the published version of the manuscript.

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