PHARMACIA

Pharmacia 72: 1–8
DOI 10.3897/pharmacia.72.e175172

Research Article

In vivo evaluation of purified methanolic extract and medicagenic acid saponin from *Chenopodium bonus-henricus* L. roots in a rotenone-induced Parkinson's disease model

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Received 16 October 2025 ♦ **Accepted** 5 November 2025 ♦ **Published** 1 December 2025

Citation: Kondeva-Burdina M, Panayotova D, Sharkov M, Popov G, Manov V, Nedialkov PT, Kokanova-Nedialkova Z (2025) *In vivo* evaluation of purified methanolic extract and medicagenic acid saponin from *Chenopodium bonus-henricus* L. roots in a rotenone-induced Parkinson's disease model. Pharmacia 72: 1–8. https://doi.org/10.3897/pharmacia.72.e175172

Abstract

A purified methanolic extract (PME) and compound **S** [3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester], isolated from the roots of *Chenopodium bonus-henricus* L., were investigated in vivo using a rotenone-induced model of Parkinson's disease. Biochemical parameters, including malondialdehyde (MDA) levels and reduced glutathione (GSH) content, were measured in brain homogenates of treated mice. Rotenone administration alone induced significant neurotoxicity, evidenced by a 50% reduction in GSH levels and a 150% increase in MDA levels compared with the control group (untreated animals). Co-administration with PME preserved GSH levels by 50% and decreased MDA production by 52%, while compound **S** preserved GSH levels by 60% and reduced MDA levels by 48% compared with animals treated with pure rotenone. Additionally, preservation of brain histoarchitecture was observed in groups co-treated with rotenone and either the purified MeOH extract or compound **S**. These results demonstrate that both PME and compound **S** exert statistically significant neuroprotective effects against rotenone-induced neurotoxicity. This protective activity is likely attributable to their antioxidant properties, including the mitigation of oxidative stress and the neutralization of free radicals. Overall, the findings highlight the critical role of oxidative stress in rotenone-induced toxicity and support the potential of antioxidant-based therapies for Parkinson's disease.

Keywords

Chenopodium bonus-henricus, in vivo, neuroprotection, rotenone-induced model, saponins

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PENSOFT

Introduction

The genus *Chenopodium* (Amaranthaceae) includes over 200 species with a global distribution, found on all continents except Antarctica. It also occurs on remote islands like the Juan Fernández Islands, New Zealand, and Hawaii (Nedialkov and Kokanova-Nedialkova 2021). *Chenopodium bonus-henricus* L. is a perennial herb mainly found in Bulgaria's mountainous regions (Grozeva 2011). In Bulgarian folk medicine, the roots of *C. bonus-henricus* ("chuven") are traditionally used to treat ailments such as bronchitis, laryngitis, rheumatism, gout, constipation, and skin conditions. Additionally, a decoction prepared from the roots is utilized in the food industry for the production of traditional products such as tahin and white halva (Kokanova-Nedialkova et al. 2019). Recent phytochemical studies on the roots of *C. bonus-henricus* have led to the isolation of six saponins based on the aglycones phytolaccagenin, 2β-hydroxyoleanoic acid, bayogenin, 2β-hydroxygypsogenin, and medicagenic acid. Both the methanolic (MeOH) extract and the isolated saponins exhibited moderate to marginal cytotoxic activity against five human leukemic cell lines (HL-60, SKW-3, Jurkat E6-1, BV-173, and K-562). Furthermore, they demonstrated stimulatory effects on interleukin-2 (IL-2) production in PHA/PMA-stimulated Jurkat E6-1 cells (Kokanova-Nedialkova et al. 2019). A UHPLC-HRMS-based profiling of a purified methanolic (MeOH) extract from the roots of *C. bonus-henricus* tentatively identified 15 saponins derived from six different sapogenins. In addition, both the MeOH extract and the isolated saponins exhibited hepatoprotective and antioxidant activities in in vivo and in vitro models of CCl_4-induced liver damage, showing effects comparable to those of silymarin (Kokanova-Nedialkova et al. 2019a). In a related study, the saponins isolated from the title plant were evaluated for their neuroprotective potential. All tested saponins (at a concentration of 10 μM) demonstrated statistically significant neuroprotective effects on isolated rat brain synaptosomes in an in vitro model using 6-hydroxydopamine. They preserved synaptosome viability as well as the reduced glutathione level (Kokanova-Nedialkova et al. 2021a). Furthermore, these compounds exhibited anti-α-glucosidase and prolipase activities, as determined by measuring the levels of the released 4-nitrophenol using LC-MS (Kokanova-Nedialkova et al. 2021a; Kokanova-Nedialkova and Nedialkov 2022). Additionally, a UHPLC-HRMS method was developed and validated for the simultaneous quantification of the saponins isolated from the roots of *C. bonus-henricus* L. The saponins of medicagenic acid, 2β-hydroxygypsogenin, and bayogenin were the predominant compounds and reached 15.01%, 3.87%, and 2.41% in the crude EtOH extract and 43.69%, 16.16%, and 10.07% in the purified EtOH extract, respectively (Kokanova-Nedialkova and Nedialkov 2021b).

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with cases expected to reach 12 million by 2040 (Dorsey and Bloem 2018). PD is characterized by the abnormal formation of Lewy neurites and Lewy bodies, consisting of intraneuronal protein aggregates (α-synuclein), and the loss of dopaminergic neurons in the substantia nigra, leading to dopamine deficiency (Raza et al. 2019; Brás and Outeiro 2021). PD is primarily diagnosed based on motor symptoms, including tremor, rigidity, postural instability, and gait disturbances, which substantially impair daily functioning. These motor features are frequently accompanied by a range of non-motor symptoms, such as constipation, depression, excessive daytime sleepiness, and rapid eye movement (REM) sleep disturbances (Kalia and Lang 2015). The most commonly used experimental models of Parkinson's disease involve the administration of neurotoxic compounds designed to replicate dopaminergic neuron degeneration and dopamine deficiency. Recently, rotenone has attracted considerable attention as a neurotoxic agent in experimental models of Parkinson's disease. It is a well-known inhibitor of mitochondrial complex I. In rats, rotenone induces progressive degeneration of dopaminergic neurons, promotes the formation of α-synuclein inclusions, and leads to microtubule destabilization (Ibarra-Gutiérrez et al. 2023).

As a continuation of our pharmacological studies, particularly in the area of neuroprotection, the purified MeOH extract and medicagenic acid saponi**n** isolated from the roots of *C. bonus-henricus* were investigated in vivo in a rotenone-induced Parkinson's disease model.

Materials and methods

Apparatus, materials, and chemicals

UHPLC-HRMS analysis was performed using a Thermo Scientific Dionex Ultimate 3000 RSLC (Germering, Germany) consisting of a 6-channel degasser SRD-3600, a high-pressure gradient pump HPG-3400RS, an autosampler WPS-3000TRS, and a column compartment TCC-3000RS coupled to a Thermo Scientific Q Exactive Plus (Bremen, Germany) mass spectrometer. All solvents were HPLC grade and purchased from Merck or Sigma-Aldrich (Taufkirchen, Germany). Column chromatography (CC) was carried out with Diaion HP-20 (Supelco, USA). Dry column vacuum chromatography (DCVC) was performed on silica gel (15–40 μm, Merck, Germany). Flash chromatography was performed using a Büchi Reveleris X2 flash system on a Büchi FlashPure EcoFlex C18 120 g column (Flawil, Switzerland) at 40 mL min^{-1}. TLC was performed on silica gel 60 plates (Merck, Germany) using EtOAc–HCOOH–AcOH-H_2O (16:1:1:3) as the mobile phase. The chromatograms were visualized by spraying with anisaldehyde–sulfuric acid reagent [H_2SO_4–AcOH–MeOH–anisaldehyde (5:10:85:0.5, v/v/v/v)] and heating at 110 °C for 2–3 min. Rotenone, DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)], trichloroacetic acid, and thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, USA).

Plant material

The roots of *Chenopodium bonus-henricus* L. were collected at altitudes of 1600 m from Beglica, Western Rhodopes, Bulgaria, in June 2020 and were identified by Z. Kokanova-Nedialkova. A voucher specimen (No. SOM-178268) was deposited at the National Herbarium, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Preparation of MeOH extract and isolation of 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester

The roots of *C. bonus-henricus* were dried in the shade, and the powdered plant material (105 g) was subsequently extracted with MeOH (1.5 L) and 80% aqueous MeOH (1.8 L) by percolation. The MeOH extracts were combined, and the solvent was evaporated under reduced pressure to yield a 62 g white-yellow residue. For further purification, the dried MeOH extract was dissolved in 200 mL of H_2O and subjected to column chromatography (CC) over Diaion HP-20 (15 × 8 cm), using H_2O (3 L) followed by 90% aqueous MeOH (1.5 L) as eluents. The 90% aqueous MeOH eluate was evaporated to dryness using a rotary evaporator, yielding 25 g of a whitish to creamy powder, referred to as PME (purified MeOH extract). This PME was further subjected to column chromatography separation and in vivo experiments.

The resulting PME (20 g) was further purified using DCVC on silica gel 60 (0.015–0.040 mm; 15 × 8 cm) with A:B (90:10 → 60:40) as the mobile phase system, where A = MeOH:H_2O (75:25) and B = EtOAc. A total of 151 fractions (100 mL each) were collected and combined into six pooled fractions (I–VI) based on TLC analysis. For further purification, fraction V (2.83 g, B–A (65:30 → 60:40)) was subjected to flash chromatography using a Reveleris X2 flash system. The mobile phase was a binary gradient of solution A (0.025% $C_2HF_3O_2$ in 20% MeCN) and B (0.025% $C_2HF_3O_2$ in 80% MeCN), and the elution was performed according to the following gradient program: 0–2 min, 100% A; 2–3 min, 100 → 75% A and 0 → 15% B; 3–28 min, 75% A and 15% B; 28–43 min, 75 → 0% A and 15 → 100% B; 43–53 min, 100% B. Fifty-five fractions were collected and combined into five pooled subfractions (A–E) based on their UHPLC-HRMS similarities. Subfraction C yielded pure compound **S** (1.9249 g), which was identified according to literature data as 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester (Kokanova-Nedialkova et al. 2019). Compound **S** was further subjected to in vivo experiments.

Rotenone model toxicity

Animals

Male mice, line H, with body weight 20–30 g, purchased from the National Breeding Center, Sofia, Bulgaria, were used for all experiments. The experiments were carried out in compliance with Ordinance No. 15 on the Minimum Requirements for the Protection and Welfare of Experimental Animals (SG No. 17, 2006), the European regulations concerning the handling of experimental animals, and under Permission No. 304, valid until 28 June 2026, issued by the Bulgarian Food Safety Agency.

Design of in vivo *experiment*

A PD model was developed in mice through the intraperitoneal administration of 3 mg/kg/day body weight rotenone for 30 consecutive days (Salama et al. 2017). The animals were divided into six groups of six animals. Group 1 served as a normal control and received saline water (0.5 mL/g, i.p.) for a period of 30 days. Group 2 served as animals treated with purified MeOH extract from the root of *C. bonus-henricus* (100 mg/kg p.o., 21 days) for a period of 30 days. The dose represents 1/20 of the LD_{50} (for mice) = 2000 mg/kg. Group 3 served as animals treated with 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester (compound **S**), isolated from the root of *C. bonus-henricus* (100 mg/kg p.o., 21 days) for a period of 30 days. The dose represents 1/20 of the LD_{50} (for mice), which is 2000 mg/kg. Group 4 served as animals treated with pure rotenone (3 mg/kg i.p.) daily for 30 days. Group 5 was treated simultaneously with rotenone (3 mg/kg i.p.) and purified MeOH extract (100 mg/kg p.o.) for a period of 30 days. Group 6 was treated simultaneously with rotenone (3 mg/kg i.p.) and 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester (compound **S**) (100 mg/kg p.o.) for a period of 30 days.

Behavioral evaluation

Behavioral assessment

To assess the potential therapeutic effects of purified MeOH extract and 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester, a neurobehavioral study was conducted on all animals by observing their overall activity levels, as well as the occurrence of tremors, akinesia, and catalepsy.

Tremors

Tremors were observed immediately following the administration of rotenone. Three experienced evaluators assessed the animals, rating the intensity of the tremors using a scale ranging from 0 to 5.

Determination of malondialdehyde (MDA) in brain homogenate

The brain homogenate was prepared by homogenizing the brain in 0.1 M potassium-sodium phosphate buffer, pH 7.4. To the resulting homogenate, 25% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) were added. The samples were then heated at 100 °C for 20 min, after which they were centrifuged at 4000 rpm for 20 min. After cooling,

MDA was determined spectrophotometrically at λ = 532 nm (Bai et al. 2001). The concentration of MDA was calculated using the molar extinction coefficient of the MDA–TBA complex and was expressed in nmol/g of fresh tissue.

Determination of reduced glutathione (GSH) in brain homogenate

The brain was homogenized with 5% trichloroacetic acid. The homogenate was centrifuged at 4000 rpm for 20 min. To the supernatant, 0.1 M potassium-sodium phosphate buffer (pH 8) and DTNB (Ellman's reagent) solution were added. After cooling, GSH was determined spectrophotometrically at λ = 412 nm (Bai et al. 2001). The concentration of GSH was calculated using the molar extinction coefficient and was expressed in nmol/g of fresh tissue.

Histological examination

Histological brain samples from the mice were fixed in a 10% buffered neutral formalin solution. The fixed materials were dehydrated in ascending grades of alcohol: 50°, 60°, 70°, 80°, 90°, 96°, and absolute alcohol. After dehydration, the samples were cleared in xylene, embedded in paraffin blocks, and cut with a rotary microtome with a section thickness of 5 μm. The sections were attached to slides using histological glue, processed with xylene, and then rehydrated through a descending alcohol series—absolute alcohol, 96°, 90°, 80°, 70°, 60°, 50°—and finally stained with hematoxylin and eosin (Bancroft and Gamble 2008). Microscopic examination and photography were performed with a Levenhuk D740T light microscope with an integrated camera.

Statistical analysis

The results of the conducted experiments were statistically processed using the statistical software MEDCALC, applying the nonparametric Mann–Whitney method at significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Results and discussion

In the present study, a phytochemical investigation of the roots of *C. bonus-henricus* (Fig. 1) led to the preparation of a purified MeOH extract (PME), from which the pure compound **S** was obtained through further fractionation by dry column vacuum chromatography (DCVC) and flash chromatography. This compound was previously identified as 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester (Kokanova-Nedialkova et al. 2019) (Fig. 2). Both the PME and compound **S** were investigated in vivo in a rotenone-induced Parkinson's disease model.

Rotenone is a widely used neurotoxin in animal models designed to mimic the pathology and behavioral symptoms associated with Parkinson's disease. It not only induces dopaminergic degeneration but also replicates



Figure 1. *Chenopodium bonus-henricus* L.

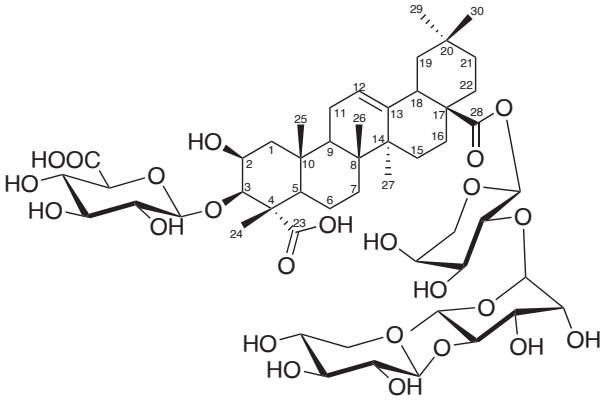


Figure 2. Compound **S**: 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester.

oxidative stress, mitochondrial complex I dysfunction, Lewy body formation, and α-synuclein aggregation. In both cell cultures and animal models, rotenone disrupts the electron transport chain, leading to increased production of reactive oxygen species (ROS), oxidative stress, and inhibition of mitochondrial respiration. Therefore, chronic exposure to rotenone is believed to cause dopaminergic neuron degeneration primarily through cumulative oxidative damage (Sherer et al. 2003; Cannon and Greenamyre 2010; Sanders and Greenamyre 2013).

The effect of PME and compound **S** on the behavior of the treated animals was evaluated against that of mice treated only with rotenone. Upon the administration of PME/compound **S** in combination with rotenone, animal salivation increased, without convulsions or mortality. The results show a positive locomotor effect—namely, the maximum tremor intensity was only up to a score of 3 (from scale 0–5); the maximum tremor duration was 15 min, and the intensity peak occurred at 7 min. Moreover, administration of the combination led to rare tremors in the animals, the intensity of which hardly reached a score of 1.

When applied individually, the PME and compound **S**, isolated from the roots of *C. bonus-henricus*, did not exhibit a statistically significant neurotoxic effect compared

with the control group (untreated animals). They did not alter the levels of reduced glutathione (GSH) or the production of malondialdehyde (MDA) (Fig. 3).

In a rotenone-induced Parkinson's disease model in mice, the PME and compound **S** demonstrated statistically significant neuroprotective and antioxidant effects relative to animals treated with the neurotoxic agent rotenone (Fig. 3). When administered alone, rotenone induced a neurotoxic effect by decreasing GSH levels by 50% and increasing MDA production by 150% compared with the control group (untreated animals) (Fig. 3).

In combination with rotenone, the PME preserved GSH levels by 50% and reduced MDA production by 52%, while compound **S** preserved GSH levels by 60% and decreased MDA production by 48% compared with animals treated with pure rotenone (Fig. 3).

In previous studies, it was established that the PME contained saponins of six sapogenins (phytolaccagenin, bayogenin, medicagenic acid, 2β-hydroxygypsogenin, 2β-hydroxyoleanoic acid, and oleanolic acid), as well as very small amounts of flavonoid glycosides with aglycones 6-methoxykaempferol, isorhamnetin, spinacetin, and patuletin (Kokanova-Nedialkova et al. 2015, 2019, 2019a; Kokanova-Nedialkova and Nedialkov 2022). Studies on the quantitative content of the main saponins in the PME show that compound **S** [3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester] is the main component, and its amount is approximately 40% (Kokanova-Nedialkova and Nedialkov 2021b).

The results showed that compound **S** was more effective at preserving GSH (60% preservation compared with 50% for the methanolic extract), suggesting that it might offer stronger protection against oxidative stress at the cellular level. This could be due to its specific molecular structure, which may interact more effectively with cellular antioxidant mechanisms. On the other hand, the methanolic extract, although slightly less effective in preserving GSH, demonstrated a more significant reduction in MDA (52% vs. 48% for compound **S**). This suggests that the extract is also very effective at reducing lipid peroxidation and cellular damage, likely due to a combination of active compounds present, including saponins and flavonoids.

The experiments conducted on this in vivo model demonstrated that PME and compound **S** are capable of reducing the concentration of stable free radicals, exhibiting a statistically significant neuroprotective effect against rotenone-induced neurotoxicity. These neuroprotective effects are linked to both preventing cellular damage from oxidative stress (via MDA reduction) and promoting the neutralization of free radicals (via GSH preservation). This combination helps maintain overall cellular health and function, particularly in the context of neurodegenerative diseases such as Parkinson's disease or other conditions characterized by oxidative stress.

The histological cerebral and cerebellar structures were examined by light microscopy. In the control group, no microscopically visible changes were found in the ganglion and glial cells of the brain structures (Fig. 4A). The cerebral cortex had a preserved layered appearance. The cortical neurons were characterized by normal histological features, including oval nuclei and pale cytoplasm. Astrocytes were found in the neuropil without evidence of pathological changes. Histologically, no alterations were found in the cerebellar area. The three layers of the cerebellar cortex—the outer molecular layer, the middle Purkinje layer, and the inner granular layer—were well differentiated and without visible pathological changes. A similar microscopic finding, without visible morphological changes, was also found in the animals treated alone with purified MeOH extract and compound **S**, isolated from the roots of *C. bonus-henricus* L. (Fig. 4B, C).

In different cerebral areas of animals treated with rotenone, obvious degenerative and necrotic changes were found. Pyknotic changes were detected in a significant number of ganglion cells, characterized by shrinkage, reduced size, and dark staining of the nuclei. In a few places in the cerebral cortex, loss of integrity, granular appearance, and vacuolated neuropil were found, and

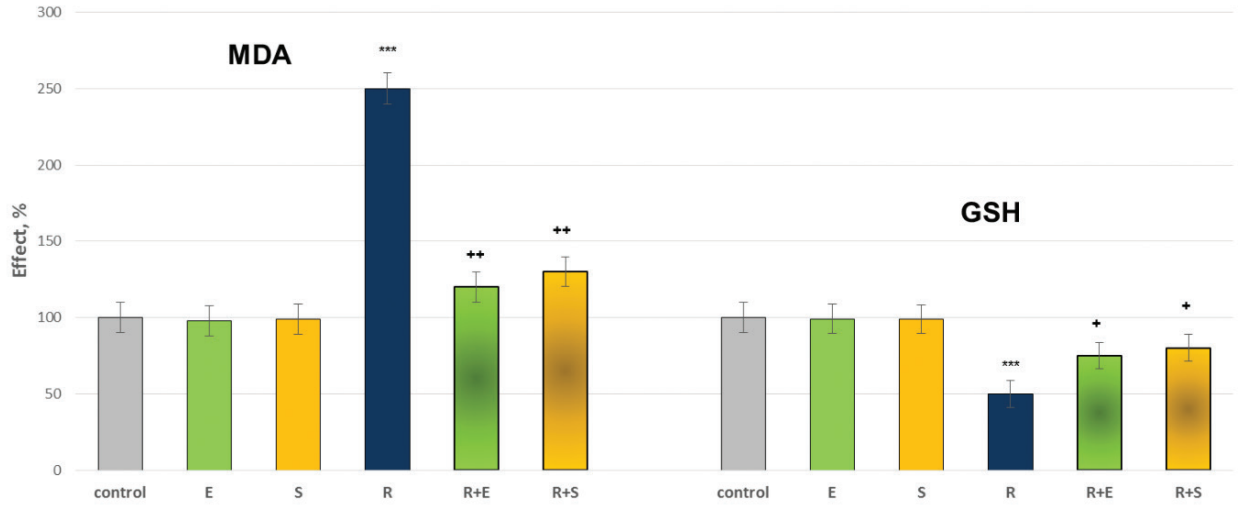


Figure 3. In vivo effects of the purified MeOH extract (E) and compound **S** (S) isolated from the roots of *Chenopodium bonus-henricus* L., administered alone and in a rotenone-induced Parkinsonism model. *** $p < 0.001$ compared with the control group (untreated animals). +$p < 0.05$; ++$p < 0.01$ compared with animals treated with rotenone (R).

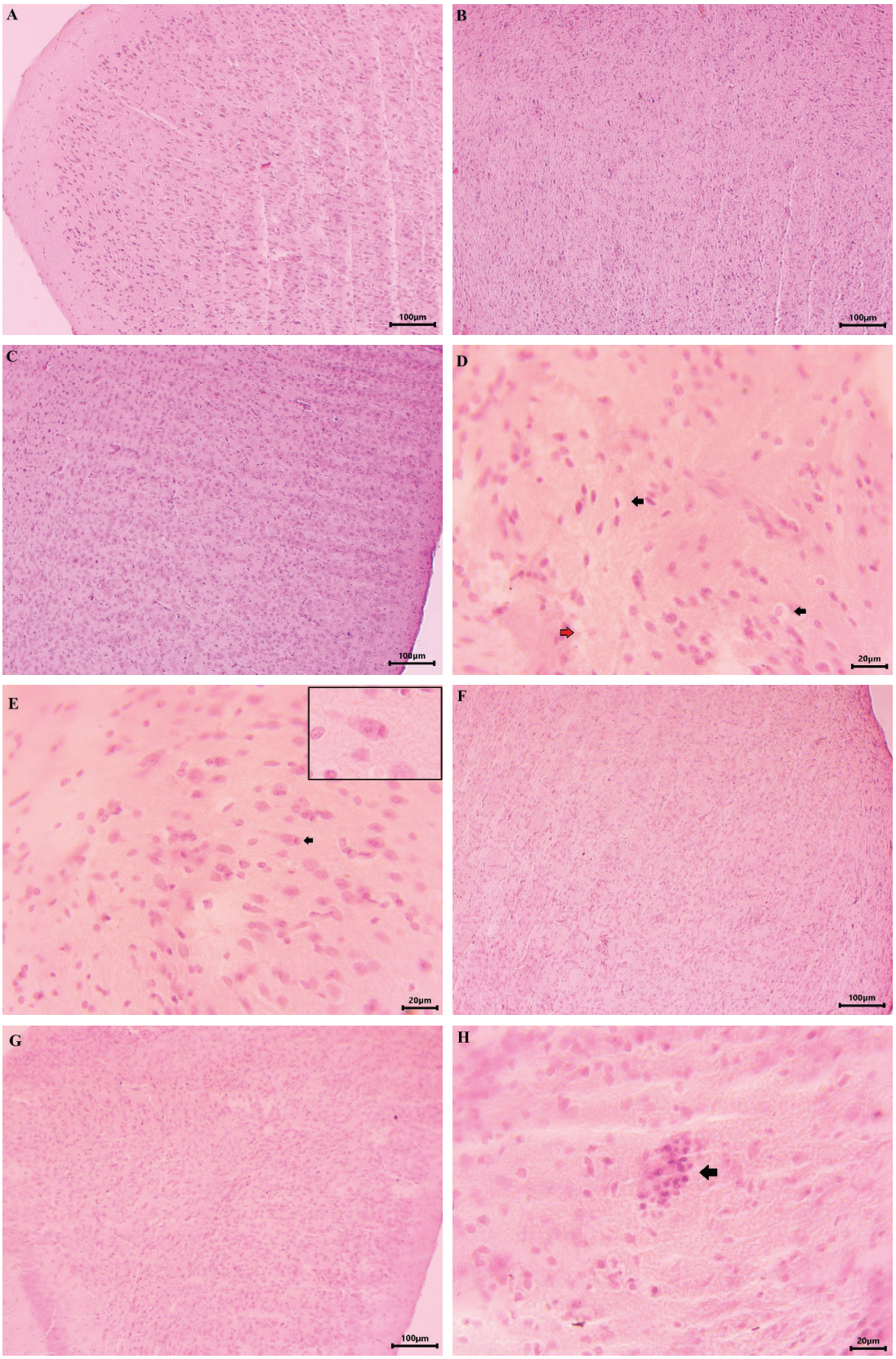


Figure 4. Histological examination of mice brains (H&E). **A.** Control group of animals: normal histoarchitecture of the brain structure (100×); **B.** Animal treated with PME alone: no visible changes in the brain tissue (100×); **C.** Animal treated with compound **S** alone: no visible changes in the brain tissue (100×); **D.** Animal treated with rotenone: presence of pericellular edema (black arrow) and loss of integrity with vacuolization of the neuropil (red arrow) (400×); **E.** Animal treated with rotenone alone: presence of eosinophilic protein deposits (Lewy bodies) (black arrow, high magnification) in cells near the degenerative areas (400×); **F.** Animal treated with rotenone + PME: unaltered microscopic structure of the brain tissue (100×); **G.** Animal treated with rotenone + compound **S**: preserved microscopic structure of the brain tissue (100×); **H.** Animal treated with rotenone + compound **S**: presence of glial nodule (arrow) (400×).

pericellular edema was observed around some of the cellular structures (Fig. 4D). In the cytoplasm of some of the neuronal cells, well-defined protein deposits were found, forming Lewy bodies (Fig. 4E). In separate areas of the cerebral cortex, aggregations of glial cells were observed, forming glial nodules. In the cerebellar area, degenerative changes were found in the layer of Purkinje cells, expressed as shrinkage and loss of the characteristic pyriform shape of the cells, dark staining with hardly noticeable nuclei, and lack of regular linear cellular arrangement.

In the brains of mice treated with rotenone in combination with PME, preserved histoarchitecture was found (Fig. 4F). No microscopically visible alterations in ganglion and glial cells were observed. In animals treated with rotenone in combination with compound **S**, preserved morphological appearance of the brain tissue was found (Fig. 4G). However, in separate areas, a fine-grained appearance of the neuropil was found, and single glial nodules were detected in the cerebral cortex (Fig. 4H).

The results show that both treatments with rotenone combined with either the PME or compound **S** generally preserved the brain tissue structure in mice. Additionally, the PME provides better neuroprotection compared with the isolated compound **S**, despite the latter being the main saponin in its composition. This suggests that synergism among different saponins, as well as the possible involvement of flavonoids, significantly contributes to the overall protective effect on brain tissue. However, the slight microscopic changes observed following treatment with compound **S** indicate the need for more in-depth studies of its mechanisms.

Conclusion

The experiments on this in vivo model have shown that the purified MeOH extract and compound **S** exhibit a statistically significant neuroprotective effect against rotenone-induced neurotoxicity. This is most likely due to both the prevention of cellular damage from oxidative stress (via MDA reduction) and the promotion of free radical neutralization (via GSH preservation). Based on the established histological findings, it can be concluded that PME and compound **S** have a pronounced neuroprotective effect in rotenone-induced Parkinsonism in mice, demonstrated by the preservation of midbrain histoarchitectonics and the absence of degenerative-necrotic changes. However, the slight microscopic changes observed following treatment with compound **S** indicate the need for more in-depth studies of its mechanisms.

Acknowledgments

This research was funded by the European Union—NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, contract number BG-RRP-2.004-0004-C01.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: The experiments were carried out in compliance with Permission No. 304, valid until June 28, 2026, issued by the Bulgarian Food Safety Agency.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Use of AI

No use of AI was reported.

Funding

This research was funded by the European Union—NextGenerationEU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, contract number BG-RRP-2.004-0004-C01.

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Data availability

All of the data that support the findings of this study are available in the main text.

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