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# Neuroprotective effects of lignan 7-hydroxymatairesinol (HMR/lignan) in a rodent model of Parkinson's disease

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ABSTRACT

*Objectives:* Parkinson's disease (PD) is a neurodegenerative disease characterized by loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc). The proinflammatory response can occur early in the disease, contributing to nigrostriatal degeneration. Identification of the new molecules, which are able to slow down the degenerative process associated with PD, represents one of the main interests. Recently, natural polyphenols, especially lignans, have raised attention for their anti-inflammatory, antioxidant, and estrogenic activity at a peripheral level. The aim of this study was to evaluate the central effects of chronic treatment with lignan 7-hydroxymatairesinol (HMR/lignan) on neurodegenerative, neuroinflammatory processes and motor deficits induced by a unilateral intrastriatal injection of 6-hydroxydopamine (6-OHDA) in rats to evaluate the potential neuroprotective properties of this compound.
*Methods:* Sprague-Dawley male rats underwent lignan (10 mg/kg) or vehicle treatment (oral) for 4 wk starting from the day of 6-OHDA injection. The degree of nigrostriatal damage was evaluated by immunohistochemistry. Moreover, we performed a quantitative and qualitative assessment of neuroinflammatory process, including phenotypic polarization of microglia and astrocytes. The motor performance was assessed by behavioral tests.
*Results:* We demonstrated that chronic treatment with HMR/lignan was able to slow down the progression of degeneration of striatal dopaminergic terminals in a rat model of PD, with a consequent improvement in motor performance. Nevertheless, the anti-inflammatory effect of HMR/lignan observed in SNc was not sufficient to protect dopaminergic cells bodies.
*Conclusion:* These results suggest intriguing properties of HMR/lignan at neuroprotective and symptomatic levels in the context of PD.



## Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc) and consequent dopaminergic denervation of the striatum, the main recipient of SNc neuron projections. PD is characterized by a combination of motor symptoms including resting tremor, bradykinesia, rigidity, and postural instability that appear only when the dopaminergic neuron loss reaches 50% to 60% [1]. Several biological mechanisms are likely involved in the pathogenesis of PD, with an increasingly recognized role for neuroinflammation [2] mostly sustained by microglia and astroglia activation in the brain parenchyma accompanied by augmented levels of cytokines, chemokines, prostaglandins, and reactive species of oxygen and nitrogen [3,4]. Glial activation manifest not only with an increase in the number of these cells, but also through their morphologic and functional modification, a mechanism known as *polarization*. After activation, microglia or astroglia can be converted into two phenotypes with opposite characteristics—M1-A1, cytotoxic, or M2-A2, neuroprotective—depending on the modulatory effects of the molecules released into extracellular space and consequently on the microenvironment shared with neurons [4–6].

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M1 or A1 cell release proinflammatory cytokines and cytotoxic molecules promoting inflammation and cytotoxic responses. In contrast, M2 or A2 cell secrete anti-inflammatory gene products and trophic factors that promote repair, regeneration, and restore homeostasis [2]. The importance of the equilibrium between cytotoxic and cytoprotective glial cell in the chronic neuroinflammation that characterizes PD is attracting increasing attention [2,4,7,8]. Therefore, the use of compounds that are able to positively modulate glial cell activity may represent a valuable innovation in the treatment of PD. Current pharmacologic treatments for PD are mainly symptomatic and directed at compensating the loss of dopamine by administration of its direct precursor L-dopa. L-dopa considerably improves PD motor symptoms, but its prolonged use is hampered by side effects, termed *motor complications*, that considerably worsen the quality of life of these patients. The development of pharmacologic treatments that may modify PD progression is therefore the main priority in the PD field.

Various substances exhibiting anti-inflammatory, antioxidant, and metal-chelating activity in the central nervous system have been tested to facilitate the management of PD [9–11]. In particular, it has been demonstrated that natural products, such as plant extracts and their secondary metabolites, may have neuroprotective activity in PD [12–14]. In this context, natural polyphenols (PPH) have raised much attention in the recent decade.

PPH have been reported to inhibit the formation of α-synuclein misfolded aggregates and to reduce mitochondrial dysfunction-induced oxidative stress [15,16]. It has also been shown that PPH can modulate neuroinflammation by inhibiting the expression of inflammatory genes [17,18]. PPH can exert their neuroprotective effects by targeting different molecular mechanisms. Lin et al. demonstrated that luteolin, a food-derived flavonoid, induces neurite outgrowth and causes augmentation of the cellular antioxidant defense capacity through the activation of the extracellular signal-regulated kinase signaling pathway [19]. Accordingly, it was demonstrated that PPH can activate phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase [20,21]. Furthermore, PPH are able to suppress the activation of microglia and to affect the inflammatory redox signaling pathways via modulation of the proinflammatory gene expression, mainly acting through nuclear factor-κB [22–26].

Lignans are a class of PPH commonly found in whole grains, seeds, nuts, legumes. and vegetables. In the past decade these plant metabolites have been actively studied as potential therapeutics for several neuropathologic conditions and neurodegenerative diseases like PD and Alzheimer's disease (AD) [27–30]. Indeed, lignans were shown to have considerable neuroprotective, anti-inflammatory, antioxidant, and immunomodulatory activities [27,31–33]. It was demonstrated that treatment with lignans extracted from Schisandra chinensis fruits reduced the levels of amyloid β (Aβ) peptide$_{1-42}$, attenuated A$\beta_{1-42}$-induced neuronal cell injury by enhancing the activities of superoxide dismutase and glutathione peroxidase and decreasing the levels of lactate dehydrogenase, malondialdehyde, and reactive oxygen species [28,29]. Moreover, these lignans inhibited cell apoptosis by reducing the caspase-8, caspase-3, and Bcl-2–like protein 4 expressions and upregulating Bcl-2 [29]. The lignan Sesamin, derived from Asiasari Radix was shown to have in vitro neuroprotective properties against 6-OHDA–induced cytotoxicity by means of the transient phosphorylation of extracellular signal-regulated kinase 1/2 and Bad and the inhibition of sustained phosphorylation of p38 mitogen-activated protein and c-Jun N-terminal kinases [30].

In this study, we evaluated the potential neuroprotective properties of the lignan 7-hydroxymatairesinol (HMR/lignan, Linnea SA, Riazzino [Locarno], Switzerland) in a PD model. HMR/lignan was extracted from the heartwood of the Norway spruce [34] and was shown to act as a precursor of the mammalian lignan enterolactone (ENL), which has strong antioxidant properties [32,35]. Antioxidant and anti-inflammatory effects of HMR/lignan have been demonstrated in studies on cardiovascular diseases and tumors [36–38].

The effects of a 4-wk oral treatment with HMR/lignan on neurodegenerative and neuroinflammatory processes and motor deficits were evaluated using a classic cytotoxic model of PD generated by unilateral intrastriatal injection of 6-OHDA in rats [39]. The procedure causes a slowly evolving lesion of nigrostriatal dopaminergic neurons—associated with a neuroinflammatory response—starting at the terminal level and progressing backward toward the cell bodies residing in the SNc. We observed a significant reduction in the loss of striatal terminals and associated neuroinflammatory response within the SNc in the animals that received HMR/lignan, which also showed marked improvement at the behavioral tests.

## Materials and methods

### *Extraction and characteristics of HMR/lignan*

HMR/lignan is a purified plant lignan, 7-hydroxymatairesinol (7-HMR), naturally occurring in the Norwegian spruce knotwood (Picea abies, L., H. Karst.). The lignan 7-HMR was identified and extracted from the heartwood of the Norway spruce, Picea abies [40]. The spruce knots, that are part of the branches embedded in the stem, consist of 6% to 16% of lignans, and HMR represents 65% to 80% of the total lignan content [41]. 7-HMR potassium acetate complex (HMR/lignan) was prepared by Linnea (Locarno, CH) by ethanol extraction followed by co-crystallization of the concentrated Picea abies extract (78–82%) and potassium acetate (18–22%). The potassium acetate complex of 7-HMR is generated during the process of purification from the plant extract to obtain a final product with the purity of min 70% of 7-HMR. The balance to 100% is potassium acetate and water.

Animal standard food (Charles River) was enriched with HMR/lignan (10 mg/kg) and with 5% sucrose to increase its palatability. This concentration of was chosen according to the 50 mg 7-HMR daily dosage in human. Pellets containing only 5% sucrose were used to feed the control group of animals.

### *Blood–brain barrier permeability study*

To study the ability of HMR/lignan and its metabolites to cross the blood–brain barrier (BBB) in vivo, a group of animals (N = 16) received HMR/lignan orally via gavage (10 mg/kg, dissolved in saline containing 5% DMSO). After 5 d of the treatment, rats were sacrificed (in deep anesthesia with 150 mg/kg of Zoletil) at different time points after the last gavage (24 h and 30, 60, 120 min), and their brains and blood were collected for the evaluation of brain and plasma levels of 7-HMR, ENL, and their glucoronides (7-HMR-g and ENL-g) by mass spectrometry (API 4500 Qtrap AB Sciex). Blood was collected into tubes containing EDTA; the plasma was separated by centrifugation (3000g, 10 min) and stored at −80°C until analysis. Brains were rapidly removed, washed in saline, dried on tissue paper, weighted, and immediately frozen at −80°C.

Stock solutions of 7-HMR and ENL were prepared in MeOH at the concentration of 1 mg/mL. An initial mix working solution of the two analytes (250 μg/mL) was prepared from the stock solutions in acetonitrile, and working solutions were prepared by sequential dilution in acetonitrile. Forty-five μL of plasma and 5 μL of working solution were added to 300 μL of acetonitrile solution containing the Internal Standard Diclofenac (5 ng/mL). Supernatant was evaporated under nitrogen and resuspended in 100 μL of water/acetonitrile 1/1. Calibration range was from 100 to 0.5 ng/mL for both analytes. After mixing, samples were centrifuged for 5 min at 3000g (5°C) and injected into liquid chromatography with tandem mass spectrometry. Brain samples were homogenized in Ammonium Formiate buffer 10 mM 1:10 w/volume. Samples were prepared as previously described from 50 μL of homogenate.

Solution of β-glucuronidase from Escherichia coli - Type IX-A at 52 500 U/mL was prepared in 0.1% sodium acetate (pH brought to 5.0 with acetic acid 1%) for β-glucoronidases reaction [42]. Plasma and brain samples (100 μL) were incubated with 1 μL of this solution (10 500 U/mL plasma) at 37°C for 3 h. After the incubation, samples (50 μL) were processed as previously described.

Samples were analyzed for pre- and postglucuronides hydrolysis in the same run to avoid possible instability during the freeze and thaw process or longer upkeep in the freezer. Concentrations of 7-HRM-g and ENL-g were calculated as difference between the amount of the two analytes before and after hydrolysis. Samples were analyzed on an ultra-fast LC Prominence Shimadzu interfaced with the mass spectrometer API 4500 Qtrap AB Sciex. The column Kinetex 2.6 μ C18 100 A 75 × 3 mm was used at 35°C with the mobile phases 0.2% formic acid in water and 2% formic acid in acetonitrile with a 0.4 mL/min flow rate. The chromatographic gradient started from 2% B at 0.5 min to 60% B in 2.5 min followed by an isocratic step up to 4.5 min and by a final gradient up to 98% B in 0.5 min and a final isocratic step at 98% B up to 6 min. The acquisition was in electrospray ionization-negative mode and the multiple reaction monitoring transitions are reported in Table 1.

**Table 1**
MRM transitions and parameters

| Compound | Parent ion | Product ion | Time (msec) | DP (V) | CE (eV) |
|---|---|---|---|---|---|
| 7-HMR_1 | 373.3 | 296.2 | 150 | −73 | −29 |
| 7-HMR_2 | 373.3 | 230 | 150 | −68 | −28 |
| 7-HMR-glucuronide | 549.3 | 373.3 | 150 | −67 | −30 |
| Enterolactone 1 | 297.2 | 252.7 | 150 | −78 | −27 |
| Enterolactone 2 | 297.2 | 189.6 | 150 | −78 | −28 |
| Diclofenac | 293.9 | 245.1 | 150 | −63 | −21 |

CE, collision energy; DP, declastering potential; HMR, hydroxymatairesinol.

*Animal model*

Male Sprague-Dawley rats (Charles River, Calco, LC, Italy), weighing 200 to 225 g at the beginning of the experiment, were housed at the Centralized Animal Facility of the University of Pavia one per cage at 20 to22°C on a 12-h light/dark cycle, with food and water was available ad libitum. Animals were left in the housing facilities for ≥1 wk before initiating the experiments. Animals were anesthetized with Zoletil (50 mg/kg; Virbac) and placed in a stereotaxic frame (Stoelting) with the incisor bar positioned 3.3 mm below the interaural line. Animals received a unilateral injection of 6-OHDA (20 μg/3 μL in saline/0.02% ascorbic acid; Sigma) into the right striatum (1 mm anterior, 3 mm lateral, and 5 mm ventral, with respect to bregma and dura) at the speed 1 μL/min using a Hamilton 10 μL syringe with a 26-gauge needle. The needle was left in place for 5 min before being retracted to allow complete diffusion of the medium and wounds were clipped [39].

All procedures were conducted in accordance with the European Communities Council Directive and were approved by the local Animal Care Committee.

*Experimental design*

The treatment of rats with food containing HMR/lignan (10 mg/kg daily, n = 32) or vehicle (sucrose 5%, control, n = 32) started on the day of surgery (6-OHDA injection) and continued for 28 d. The day before surgery and the day before sacrifice, all animals underwent behavioral evaluation of their motor abilities in the cylinder test (baseline versus treatment); the apomorphine-induced rotation test was used only once before sacrifice. After sacrifice, brain tissue and blood samples were collected for further biochemical evaluations and molecular biology analysis. At the time of sacrifice, animals destined to biochemical analysis were deeply anesthetized with 150 mg/kg of Zoletil and transcardially perfused with saline and ice-cold 4% paraformaldehyde (Merck). Brains were rapidly removed, postfixed for 24 h in the same fixative and subsequently transferred in solutions of sucrose at increasing concentrations (≥30%). Brains were then cut in serial coronal sections (25 μm) containing both the striatum and the SNc using a microtome (Leica SM 2000 R) and underwent immunohistochemical staining. For molecular biology analyses, animals were deeply anesthetized with 150 mg/kg of Zoletil underwent cardiac puncture blood sampling, decapitation, and rapid removal of brain areas (striatum and midbrain), which were frozen immediately at −80°C.

*Immunohistochemical staining for evaluating nigrostriatal damage and neuroinflammation*

The nigrostriatal lesion was assessed by immunohistochemistry for the dopaminergic marker tyrosine hydroxylase (TH) on coronal sections of both SNc and striatum. Sections were processed with a rabbit anti-TH primary antibody (1:2000, Chemicon AB152) and a biotinylated antirabbit immunoglobulin G secondary antibody (1:500, Vector Laboratories) and revealed using a commercial kit based on the avidin-biotin technique (Vectastain ABC Elite kit, Vector Laboratories). Reaction products were developed using nickel-intensified 3′-3′-diaminobenzedine tetra-hydrochloride for 2 min (DAB Substrate Kit for Peroxidase, Vector Laboratories). Immunofluorescent staining with mouse anti cluster of differentiation molecule 11 b (CD11 b) (1:300, Serotec MCA275 R) primary antibody (microglia) or mouse anti-glial fibrillary acidic protein (GFAP; 1:1000, Sigma G3893) primary antibody (astrocytes) was performed to assess glial activation and density in the SNc, as previously described [43]. Anti-TH primary antibody was added for SNc localization (1:500, Chemicon). Microglia and astrocytes polarization toward cytotoxic (M1-A1) or neuroprotective (M2-A2) phenotypes was performed using cluster of differentiation molecule 32 (CD32) or CD206 (1:300, Santa Cruz sc-28842, sc-48758) antibodies, respectively [44]. Goat anti-mouse AlexaFluor488 and goat anti-rabbit AlexaFluor594 (1:300, Life Sciences) were used as secondary antibodies.

*Image analysis*

Image analysis was performed using an AxioSkop2 microscope, equipped with Apotome.2, connected to a computerized image analysis system (AxioCam MR5) with dedicated software (AxioVision Rel 4.2). Densitometric analysis was performed on the lesioned striatum to evaluate the loss of dopaminergic terminals. The striatal degeneration was evaluated and expressed as the percentage of striatal volume deprived of TH immunoreactivity, with respect to the entire striatal volume. The number of TH-positive neurons in the SNc was counted bilaterally on every four sections throughout the entire nucleus using the unbiased stereologic optical fractionator method (Stereo Investigator System, Microbrightfield Inc.). Results are expressed as the percentage of TH-positive neurons in the lesioned SNc compared with the intact hemisphere. Cell count for microglia and astroglia was performed by analyzing three different SNc sections, chosen according to rostro-caudal coordinates. Cell density was assessed by counting CD11 b- or GFAP-positive cells from a stack of 16 pictures (in a 0.04 $mm^2$ frame, 1 μm-thick, 40 × magnification) taken from three discrete areas of the same SNc section. The analysis of microglia or astrocytes polarization was performed by evaluating the percentage of CD32-positive (M1-A1) and CD206-positive cells (M2-A2) of the total microglia cells or astrocytes from a stack of 16 pictures (in a 0.04 $mm^2$ frame, 1 μm-thick, 40 × magnification) taken from three discrete areas of the same SNc section.

*Quantitative real-time reverse transcription polymerase chain reaction analysis*

The mRNA expression levels of proinflammatory mediators (M1-A1;tumor necrosis factor [TNF]-α) and inducible nitric oxide synthase [iNOS]) or anti-inflammatory mediators (M2-A2) (transforming growth factor [TGF]-β) and CD206) were detected using cDNAs from lesioned and non-lesioned side SNc of control and HMR/lignan group isolated 28 d after the 6-OHDA injections, as reported in a previous study [45]. Quantitative real-time polymerase chain reaction analysis was performed using the forward and reverse primers for selected genes were reported in Table 2.

*Behavioral evaluation*

The effects of chronic treatment with HMR/lignan on the motor behavior of rats with 6-OHDA lesion were evaluated by cylinder test and apomorphine-induced rotational test. Tests were performed during the light phase (1000−1600), in full compliance with the directive of the European community.

*Cylinder test*

The test evaluates the asymmetry in the use of the front limbs to support the weight of the body against the walls of a cylindrical container, during the exploratory behavior of the animal [46]. In the presence of a unilateral nigrostriatal lesion, the animal will prefer using the forepaw ipsilateral to the lesioned side over the contralateral, "parkinsonized" forepaw. To perform this test, rats were placed individually in a glass cylinder (21 cm in diameter, 34 cm in height) for 5 min, and their behavior was recorded by video camera for further evaluation. The number of contacts made by left or right forepaw with the cylinder wall was counted; preference of paw use was calculated by the following equation: [Ipsilateral / (ipsilateral + contralateral) − (contralateral / ipsilateral + contralateral)].

*Apomorphine-induced rotational test*

This test is widely used in literature as a functional index of nigrostriatal lesion in animal models of unilateral infusion of 6-OHDA and based on the functional imbalance between the striatum of the injured side, where dopaminergic denervation causes development of hypersensitivity to dopaminergic agonists and the non-injured one [47]. One day before sacrifice, the animals were injected with apomorphine (0.5 mg/kg dissolved in saline with 0.2% ascorbic acid, i.p.) to induce sensitization of dopamine receptors to apomorphine (priming). Twenty-four hours after priming, the animals were injected again with apomorphine (the same dose of priming), which induces a rotational motor response toward the opposite side to that of the injury. This response was evaluated using an automatic rotameter connected to the rat by means of an elastic belt. A sensor sensitive to rotation allowed us to count the number of complete turns for 30 min, starting from the first minute after the injection of apomorphine. The response was calculated by subtracting the total number of ipsilateral rotations from the total number of contralateral rotations.

**Table 2**
Forward and reverse primers used in real-time polymerase chain reaction for mRNA expression levels of pro- and anti-inflammatory mediators investigation

| Gene | Forward primer (5′−3′) | Reverse primer (5′−3′) |
|---|---|---|
| TNF-α (M1-A1) | TTCCCAAATGGGCTCCCTCT | GTGGGCTACGGGCTTGTCAC |
| iNOS (M1-A1) | CCTGGTGCAAGGGATCTTGG | GAGGGCTTGCCTGAGTGAGC |
| TGF-β (M2-A2) | TGGCGTTACCTTGGTAACC | GGTGTTGAGCCCTTTCCAG |
| CD206 (M2-A2) | GGTTCCGGTTTGTGGAGCAG | TCCGTTTGCATTGCCCAGTA |

CD, cluster of differentiation molecule; iNOS, inducible nitric oxide synthase; TGF, transforming growth factor; TNF, tumor necrosis factor.

*Statistical analysis*

The results are express as mean ± SEM. Statistical analysis was performed using GraphPad Prism 3 (GraphPad software, San Diego, CA, USA). Comparisons between groups were made using Student's *t* test for paired data. Statistical significance was set at $P < 0.05$.

## Results

*HMR/lignan administration*

Animals were monitored daily for food intake and body weight gain. No differences between rats in HMR group were detected, either in food intake or weight gain, during the experimental time course (data not shown). Therefore, we expect that all animals received more or less the same amount of HMR/lignan with their food.

*Evaluation of HMR/lignan and enterolactone concentration in plasma and brain*

It was previously shown [34] that 7-HMR is rapidly metabolized into its glucuronide and ENL. Animals treated with HMR/lignan by oral gavage for 5 d showed high plasma levels of 7-HMR-g at 30 min, with a peak at 60 min followed by a substantial reduction at 120 min. Conversely, 7-HMR and ENL-g showed low plasma levels at all time points. No ENL was detected in plasma (Fig. 1A). Regarding brain concentrations, 7-HMR-g showed low levels at all time points, whereas 7-HMR concentration increased between 60 and 120 min with a peak at 120 min. No ENL or its glucuronide were detected in brain tissue (Fig. 1B).

*Evaluation of nigrostriatal lesion*

The intrastriatal injection of 6-OHDA induced considerable loss of TH-positive terminals and cell bodies in the ipsilateral nigrostriatal pathway of animals of both experimental groups (Fig. 2). However, animals treated with HMR/lignan showed a 30% significant reduction of dopaminergic terminal loss with respect to untreated controls ($P = 0.005$). Conversely, no differences attributable to HMR/lignan treatment were found in the SNc, where both groups showed similar cell body loss (Fig. 2). Animals in the control group with the lesion <30% were excluded from the analysis, whereas for rats in the treated group no exclusion criteria was applied since any variability of the lesion size could be caused by the treatment.

*Microglia and astrocyte activation and polarization*

Infusion of 6-OHDA induced substantial microglial and astrocyte activation in the SNc of the injured side compared with the non-injected side. A significant reduction in the density of CD11b+ (−21%; $P = 0.001$; Fig. 3A and C) or GFAP+ (−19%; $P = 0.003$; Fig. 4A and C) cells in the SNc was observed in animals treated with HMR/lignan with respect to control animals. A significant reduction of microglia polarization toward the cytotoxic M1 (CD11b+/CD32+) phenotype was also observed in animals treated with HMR/lignan compared with controls (−25%; $P = 0.043$), whereas a slight non-significant increase was observed in the number of microglia cells expressing the M2 phenotype (CD11b+/CD206+) in HMR-treated animals (Fig. 3B and D). No differences in astrocytes polarization toward the A1 (GFAP+/CD32+) or A2 (GFAP+/CD206+) phenotype were observed in animals treated with HMR/lignan compared with controls (Fig. 4B and D).

The quantitative real-time polymerase chain reaction analysis showed moderate, non-significant reductions in gene transcription of proinflammatory mediators TNF-α and iNOS in the SNc of HMR-treated animals (Fig. 5A and B), associated with a general increase in the transcription of anti-inflammatory mediators TGF-β and CD206, which reached statistical significance for the latter ($P = 0.042$; Fig. 5C and D).

*Behavioral evaluation*

*Cylinder test*

Analysis of baseline motor activity did not reveal any preference in the use of forepaws in animals of both groups. After 28 d from the surgery, animals in the control group showed a marked preference in the use of the right forepaw, ipsilateral to the lesioned hemisphere, over the left, "parkinsonized" forepaw. In contrast, animals treated with HMR/lignan showed a significantly lower preference in the use of the ipsilateral limb than control rats ($P = 0.027$), with a 47% increase in the use of the left forepaw (Fig. 6A).

*Apomorphine-induced rotational test*

Control animals showed marked rotational response to apomorphine 28 d after surgery, contralateral to the lesioned side. This response was reduced in the animals treated with HMR/lignan (−54%; $P = 0.049$ versus control animals; Fig. 6B).

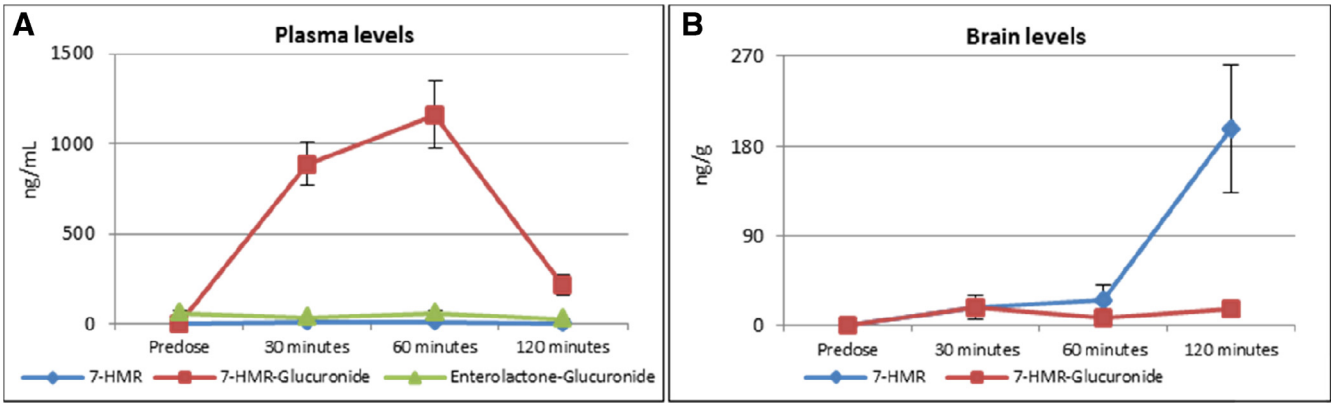


**Fig. 1.** Blood–brain barrier permeability study. Profiles of 7-HMR, 7-HMR-g, and ENL-g in plasma and brain tissue of rats after 5 d of daily HMR/lignan oral administration (10 mg/kg) by gavage. **(A)** Higher levels of 7-HMR-g were detected in plasma compared with all other metabolites, at all time points, with a peak at 60 min post-gavage. 7-HMR and ENL-g showed very low plasma levels at all time points, whereas ENL was undetectable. In the brain **(B)**, 7-HMR-g showed low levels for all time points; whereas 7-HMR concentration increased between 60 and 120 min with a peak at 120 min. No ENL or its glucuronide were detected in brain tissue. Results are expressed as mean ± SEM. 7-HMR, 7-hydroxymatairesinol; 7-HMR-g, 7-hydroxymatairesinol glucuronide; ENL, enterolactone; ENL-g, enterolactone glucuronide.

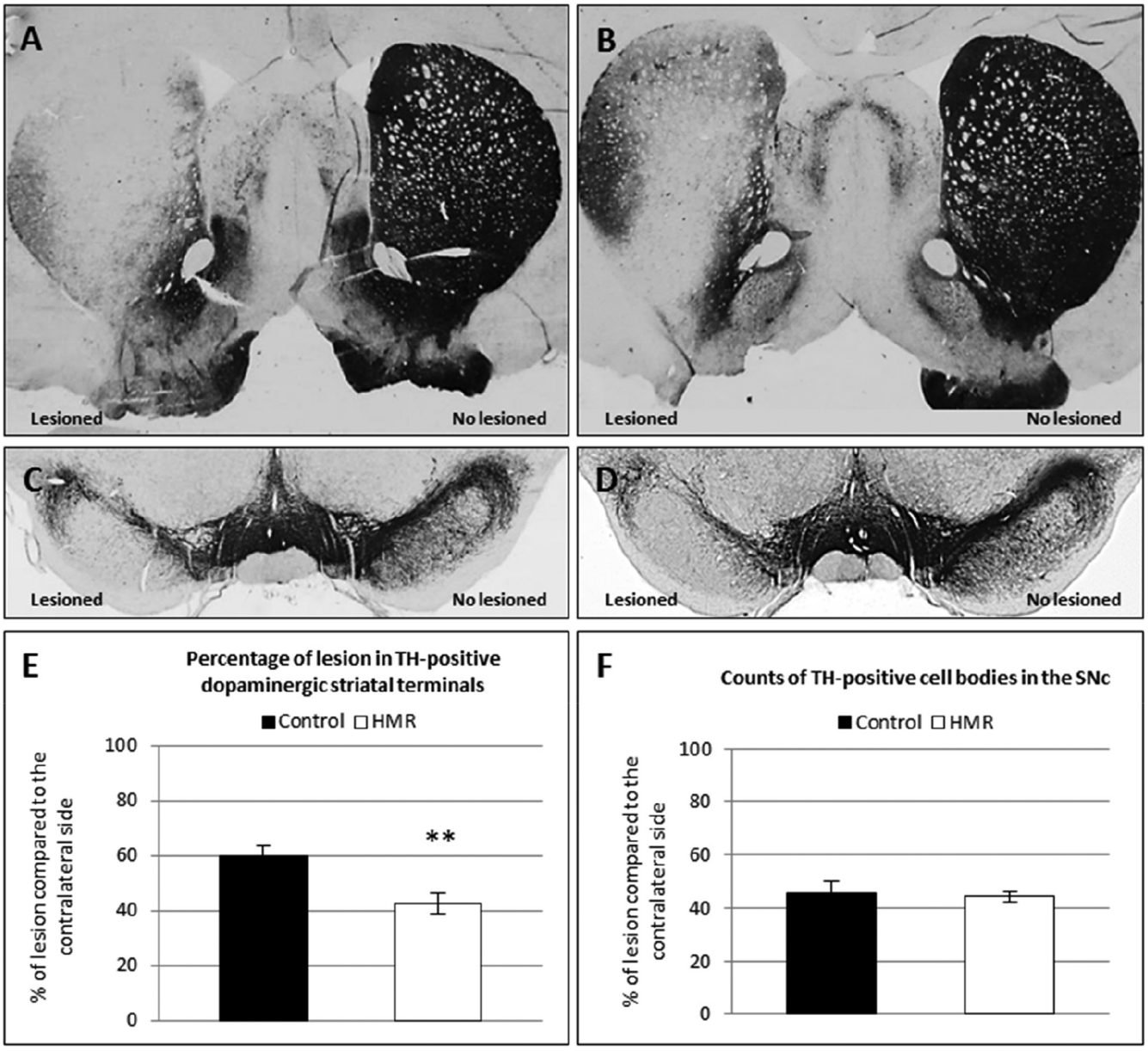


**Fig. 2.** Effect of treatment with HMR/lignan on the neurodegenerative process. (**A**–**D**) Representative images of the nigrostriatal damage in both experimental groups. In the striatum (**E**), the loss of TH+ terminals caused by 6-OHDA was reduced by 30% at day 28 after surgery in the HMR/lignan group (**B**) compared with the control group (**A**). In the lesioned SNc (**F**), no differences were observed in cell body loss between animals treated with HMR/lignan (**D**) and controls (**C**). Results are expressed as mean ± SEM. $^{*}P = 0.005$ vs control. 6-OHDA, 6-hydroxydopamine; HMR, hydroxymatairesinol; SNc, Substantia Nigra pars compacta; Th, tyrosine hydroxylase.

## Discussion

Current pharmacologic strategies for PD are mainly symptomatic and aim to compensate the lack of dopamine in midbrain circuits without affecting the pathologic mechanisms and therefore being ineffective in preventing the disease progression. In this context, various substances exhibiting anti-inflammatory, antioxidant, and metal-chelating activity in the central nervous system may represent a valuable innovative tool for the management of PD [9–11].

PPH represent an exciting example of natural compounds with potential neuroprotective properties [13–15]. In vitro and in vivo studies have demonstrated that PPH are able to decrease neuroinflammation and slow down α-synuclein accumulation and oxidative stress [15,16]. HMR/lignan is a typical PPH with strong antioxidant and anti-inflammatory properties [36–38], making it a promising candidate as a potential therapeutic agent able to counteract the pathologic processes implicated in the progression of PD. In this study, we evaluated the neuroprotective efficacy of HMR/lignan in a 6-OHDA rat model of PD to support its potential application for PD therapy.

### *BBB permeability to HMR/lignan and its metabolites*

It has been previously demonstrated that certain PPH derived from fruits and vegetables (phenolic acids, flavonoids, and lignans) can cross the BBB [48–50] and reduce or block neuronal death in animal models of neurodegeneration [12–14]. None of these properties has been proposed for HMR/lignan thus far. To the best of our knowledge, there is no evidence that HMR/lignan or its active metabolites can consistently reach the brain parenchyma; their

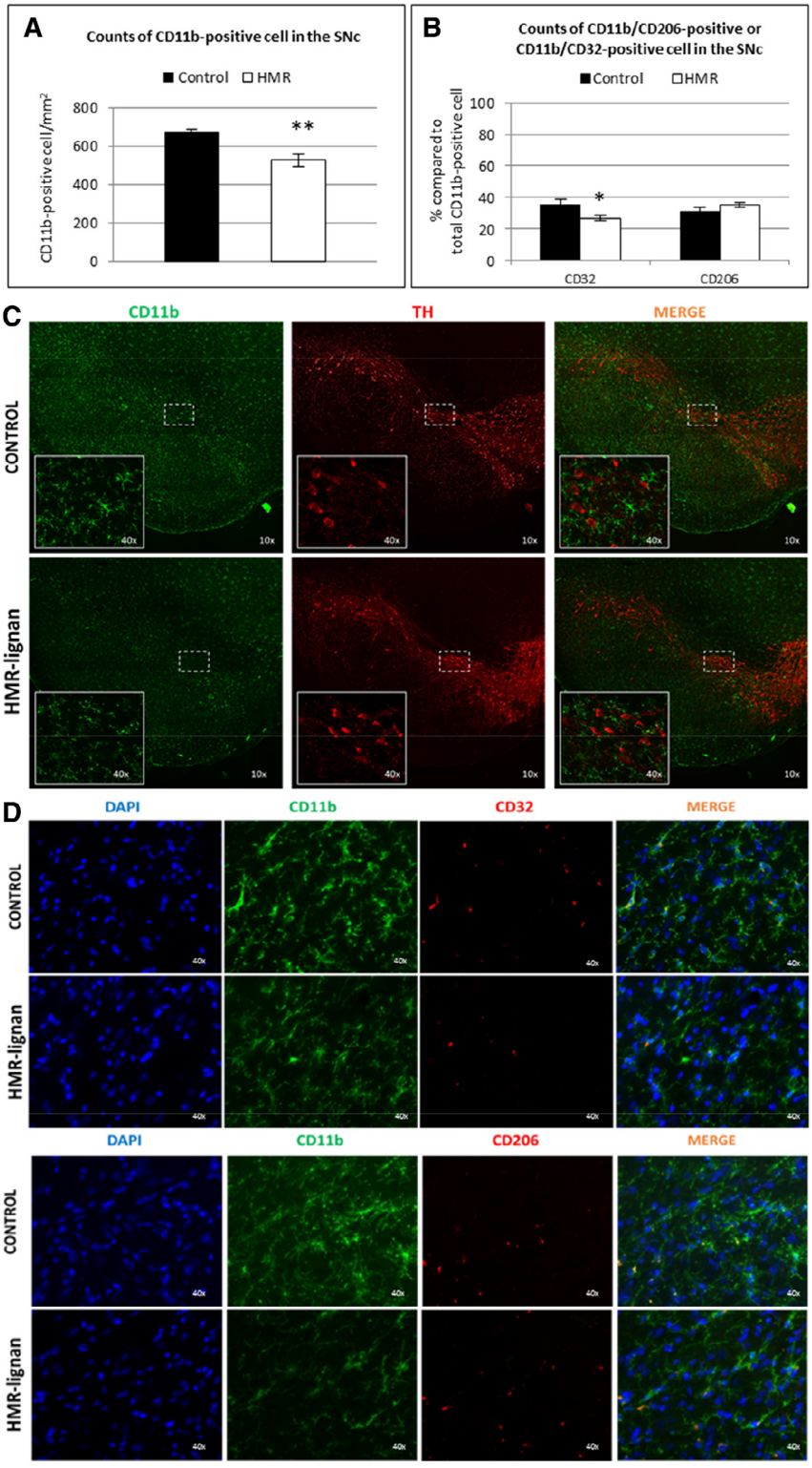


**Fig. 3.** Immunomodulatory effect of chronic treatment with HMR/lignan on microglial activation and polarization in the SNc. (**A**) Comparison of CD11b+ cell density in the right (lesioned) SNc between animals that received HMR/lignan and the control group. (**B**) Comparison of microglia polarization in the right (lesioned) SNc between animals that received HMR/lignan and the control group. Animals treated with HMR/lignan showed significant reduction of microglia activation and polarization toward the cytotoxic M1 (CD11b+/CD32+) phenotype compared with the control group. No statistically significant differences were observed for microglia polarization toward the neuroprotective M2 (CD11b+/CD206+) phenotype between the two groups. Results are expressed as mean ± SEM. (**C**) Immunofluorescent images of the right (lesioned) SNc, in control and HMR/lignan-treated animals. Green signal: CD11b+ cells (microglia); red signal: TH+ cells (dopaminergic neurons). (**D**) Representative images of microglia polarization in the lesioned SNc. Blu signal: Nuclei; green signal: CD11b+ cells; red signal: CD32+/CD206+ cells, respectively M1 and M2 phenotype. $^{*}P = 0.001$. $^{\dagger}P = 0.043$. CD, cluster of differentiation molecule; HMR, hydroxymatairesinol; SNc, Substantia Nigra pars compacta; Th, tyrosine hydroxylase.

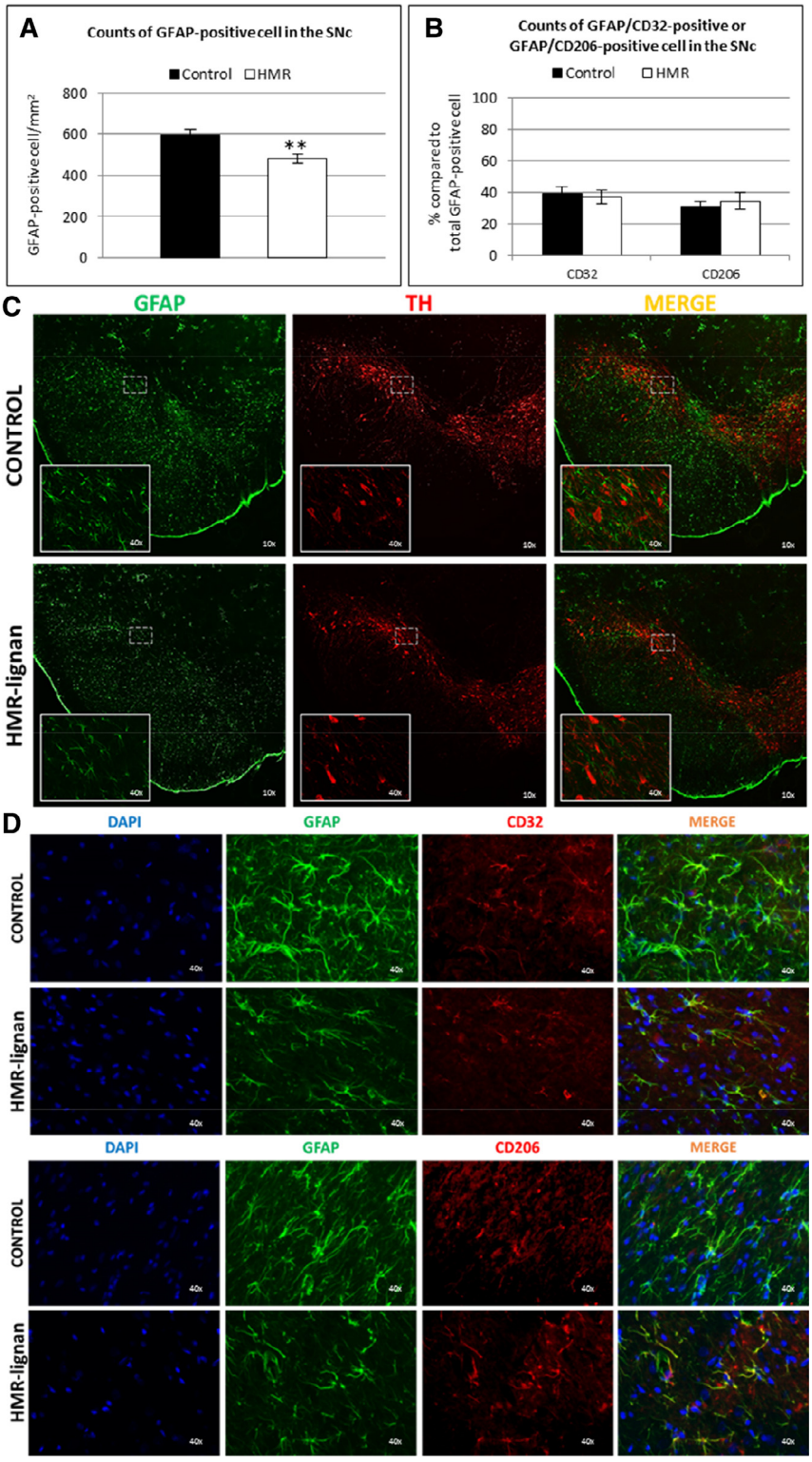


**Fig. 4.** Immunomodulatory effect of chronic treatment with HMR/lignan on astrocyte activation and polarization in the SNc. (**A**) Comparison of GFAP+ cell density in the right (lesioned) SNc between animals that received HMR/lignin and the control group. (**B**) Comparison of astrocyte polarization in the right (lesioned) SNc between animals that received HMR/lignan and the control group. No differences in astrocyte polarization toward the A1 (GFAP+/CD32+) or A2 (GFAP+/CD206+) phenotype were observed in animals treated with HMR/lignan compared with controls. Results are expressed as mean ± SEM. (**C**) Immunofluorescent images of the right (lesioned) SNc, in control and HMR-treated animals. Green signal: GFAP+ cells (astrocytes); red signal: TH+ cells (dopaminergic neurons). (**D**) Representative images of astrocytes polarization in the lesioned SNc. Blu signal: Nuclei; green signal: GFAP+ cells; red signal: CD32+ /CD206+ cells, respectively A1 and A2 phenotype. $^{*}P = 0.003$. CD, cluster of differentiation molecule; GFAP, glial fibrillary acidic protein; HMR, hydroxymatairesinol; SNc, Substantia Nigra pars compacta; Th, tyrosine hydroxylase.

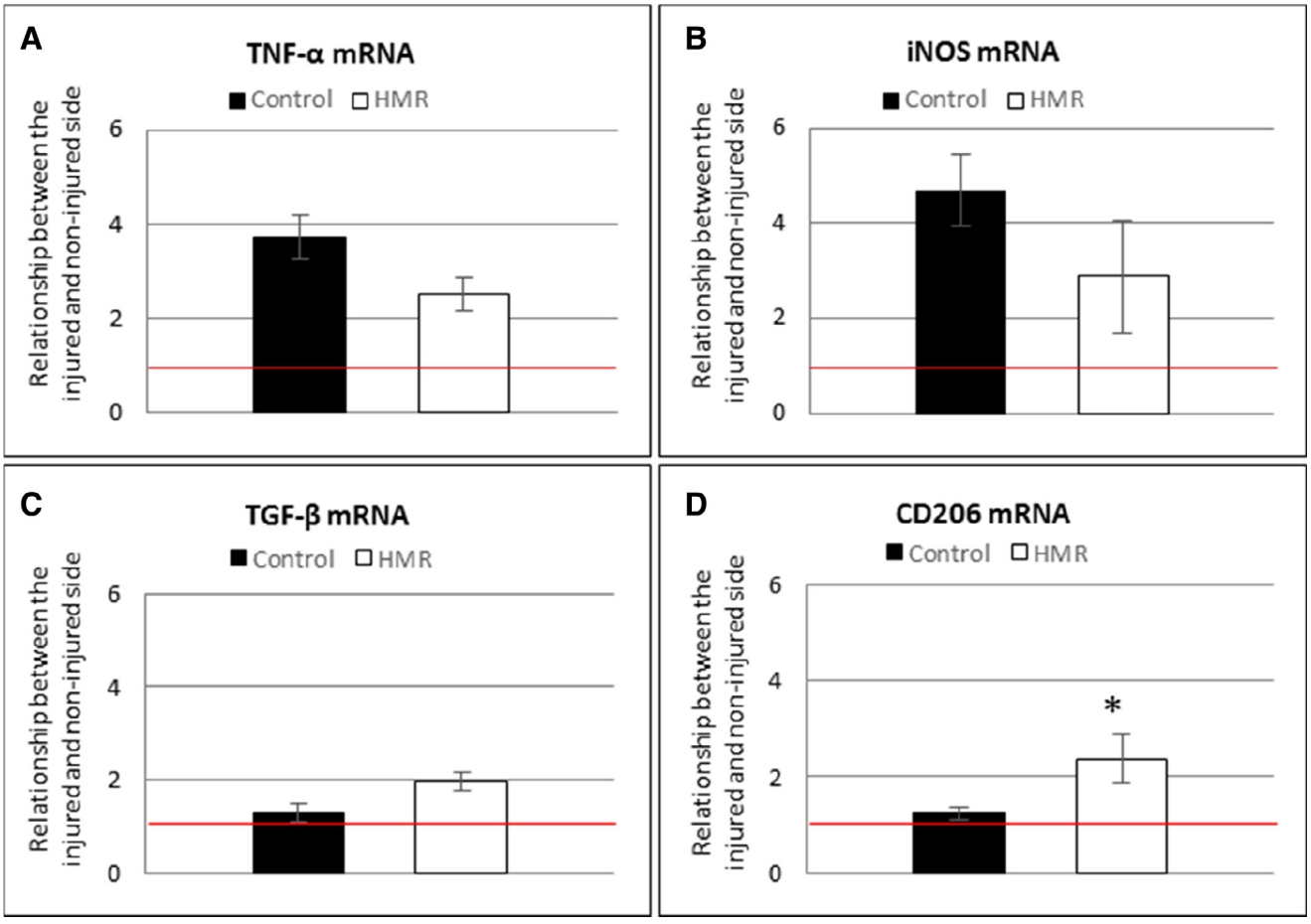


**Fig. 5.** Effect of chronic treatment with HMR/lignan on the gene transcription of pro- or anti-inflammatory mediators in the SNc after injection of 6-OHDA. Animals treated with HMR/lignan showed moderate reduction in gene transcription of proinflammatory mediators TNF-α (**A**) and iNOS (**B**) in comparison with the control group. On the contrary, increased mRNA levels of anti-inflammatory mediators TGF-β (**C**) and CD206 (**D**) were observed in HMR-treated animals, a statistically significant difference between groups being detected for CD206. The red line indicates the expression basal levels of these mRNAs. Results are expressed as mean ± SEM. $^{*}P = 0.0042$. 6-OHDA, 6-hydroxydopamine; CD, cluster of differentiation molecule; HMR, hydroxymatairesinol; iNOS, inducible nitric oxide synthase; SNc, Substantia Nigra pars compacta; TGF, transforming growth factor; TNF, tumor necrosis factor.

anticarcinogenic and estrogenic activity have been tested only in peripheral tissues [49,51]. For this reason, our first objective was to verify whether HMR/lignan or its metabolites could cross the BBB and exert their potential protective properties at the central level. Indeed, HMR/lignan has been previously shown to have anti-inflammatory and immunomodulatory properties [35,52,53] and antioxidant effects on endothelial cells in cardiovascular diseases and tumors [36,38]. In the present study, determination of 7-HMR

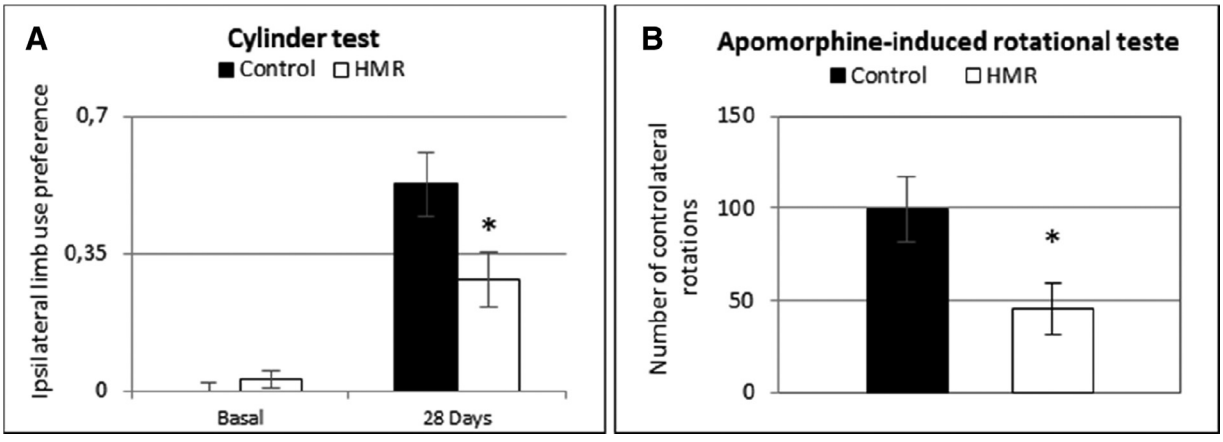


**Fig. 6.** Effects of chronic treatment with HMR/lignan on motor behavior of 6-OHDA lesioned rats. (**A**) Cylinder test: Animals treated with HMR/lignan showed significantly lower preference in the use of the ipsilateral anterior limb compared with the animals of the control group. (**B**) Apomorphine-induced rotational test: Animals treated with HMR/lignan showed a significant reduction of the number of contralateral rotations induced by apomorphine compared to the control group. Results are expressed as mean ± SEM. $^{*}P = 0.027$. $^{\dagger}P = 0.049$. 6-OHDA, 6-hydroxydopamine; HMR, hydroxymatairesinol.

and its related metabolites in plasma and brain samples after 5 d of oral HMR/lignan (10 mg/kg daily) proved that both 7-HMR and 7-HMR-g reach the brain tissue, with a substantial increase in concentration over time for the former. Conversely, neither ENL nor ENL-g were detected within the brain parenchyma. Taking this into consideration, it is safe to assume that all effects detected at the nigrostriatal level in our experimental model and described further in detail were exclusively caused by HMR/lignan.

*Effects of HMR/lignan in 6-OHDA rat model of PD*

We used a 6-OHDA rat model of PD to investigate the potential neuroprotective effects of HMR/lignan on the neurodegenerative process and motor deficits associated with the disease.

The unilateral intrastriatal injection of the neurotoxin 6-OHDA causes a partial progressive lesion of the nigrostriatal pathway, associated with neuroinflammatory response. The neurodegenerative process starts at the terminal level in striatum, progresses backward toward the cell bodies residing in the SNc and reaches its completion within 4 to 6 wk [39]. This gradual progression of the lesion creates a "4-wk concept," defining a therapeutic time window that can be exploited to evaluate the effectiveness of treatments aimed to counteract or modify the progression of nigrostriatal damage and related motor deficits [47]. For this reason, we establish a chronic treatment with oral HMR/lignan (10 mg/kg daily) for 28-d (4 wk) starting on the first day after 6-OHDA infusion.

The present data demonstrated that treatment with HMR/lignan induced significant reduction of the striatal damage, with a 30% increased survival of striatal dopaminergic terminals. Various mechanisms may explain this finding. The antioxidant properties previously proposed for HMR/lignan [36] may play a role given that the mechanism of action of 6-OHDA is centered on its powerful prooxidant activity. Under canonical experimental conditions, however, the prooxidant effect of 6-OHDA is extremely rapid and localized to the injected area. The drug selectively accumulates within the cytosol of dopaminergic neurons, where it immediately undergoes autooxidation causing massive intracellular formation of reactive oxygen species and cell death associated with pronounced glial activation [47]. Therefore, it is unlikely that the antioxidant properties of HMR/lignan, given the day after the injection of 6-OHDA, may have interfered with the instantaneous and profound oxidative damage caused by the toxin. Additional confirmation of the neuroprotective action of HMR/lignan at the striatal level comes from the evaluation of the motor deficit in the behavioral tests, which are correlated to the level of striatal dopaminergic denervation. Animals treated with HMR/lignan showed significant amelioration at both cylinder and apomorphine tests, with similar degrees of improvement (~50%) over untreated animals. This observation is even more interesting than the anatomic finding per se because it points to a symptomatic effect of HMR/lignan administration that exceeds the pure neuroprotective effect and will deserve further investigation.

Surprisingly, unlike the striatum, no changes in neuronal survival were observed at the level of dopaminergic cell bodies in the SNc, whereas the inflammatory process in this area was significantly reduced. Because it is widely discussed in literature, neuroinflammation plays a dominant role in the neurodegenerative disorders, particularly in PD, mostly through the activation of microglia and astrocytes [54–56]. An excessive inflammatory response accompanied with additional pathologic process in the brain can be a source of further damage to the neurons. Neuroinflammatory process has been known to accompany the nigrostriatal degeneration in 6-OHDA and 1-methyl-4-phenyl1,2,3,6,-tetrahydropyridine (MPTP) animal models of PD [2,5,54–56]. Depending on the trigger stimuli and their duration, activated glial cells (microglia or astroglia) can be polarized and driven toward a cytotoxic (M1-A1) or a cytoprotective (M2-A2) phenotype [5]. Such a dynamic balance between these phenotypes can affect the different levels of neuroinflammation and intensity of the associated neurodegeneration [44].

In our experiments, chronic treatment with HMR/lignan affected the complex neuroinflammatory response at the level of SNc that typically accompanies the 6-OHDA–induced neurodegenerative process and significantly reduced the number of CD11b (marker of microglia) and GFAP-positive (marker of astrocyte) cells. Furthermore, detailed analysis of the microglia phenotypes revealed a significant reduction in the number of microglial cells polarized toward the cytotoxic phenotype M1. At the transcriptional level, treatment with HMR/lignan induced a moderate reduction of proinflammatory mediators such as TNF-α and iNOS, whereas mRNA levels for anti-inflammatory mediators TGF-β and CD206 was increased. Liddelow et al. [4] showed in vitro that cytotoxic M1 microglia cells were able to induce the activation of resting astroglial cells, promoting in turn their polarization toward A1 cytotoxic phenotype by the release of proinflammatory factors such as TNF-α, C1 q, interleukin-1α. Furthermore, in this research it was also demonstrated that in conditions free from the proinflammatory factors, A1 astrocytes did not revert back to the resting astrocytes. When the same conditions were coupled with an increase of anti-inflammatory factors (TGF-β or fibroblast growth factor), transcript levels of A1 astrocytes was significantly decreased. One potential explanation for this anti-inflammatory effect of HMR/lignan might be connected to its proestrogen activity. Cosentino et al. [32] demonstrated that HMR/lignan possesses an estrogen-like activity, and its effects were significantly reduced in the presence of estrogen receptor (ER) antagonist tamoxifen [32]. Estrogens are known to act as anti-inflammatory agents by binding ERβ, broadly expressed both on microglial and astrocytes cells [57–60]. In addition, we recently demonstrated that estrogen supplementation to ovariectomized mice with 6-OHDA lesions reduced microglia activation while favoring expression of the M2 phenotype, resulting in a reduction of the neuronal damage [61]. Taking this into account, we suppose that central anti-inflammatory effects of the compound may be related to its interaction with ERs expressed by activated microglia. This would decrease proinflammatory factors released by microglia, in turn reducing activation of the astroglial cells and dampening their cytotoxic response. In addition, we cannot exclude that effects of HMR/lignan are attributable to the simultaneous activation of ERs expressed both by microglial and astroglial cells.

However, despite a significant decrease of neuroinflammation in SNc, the beneficial effect of HMR/lignan on neuronal survival in this nucleus was apparently lower than in the striatum. One potential explanation for this apparent discrepancy might be due to the dosage used, which was not sufficient to cause neuroprotection at this level in the chosen time span. It is known that the chronic release of proinflammatory factors from microglia and astrocytes in the extracellular environment favors the degeneration of surrounding dopaminergic neurons [62], and therefore a reduction of inflammation should be beneficial against the neuronal damage [2]. The 6-OHDA rat model used in this study is characterized by a crucial period of neuronal loss in SNc lasting from 7 to 14 d after neurotoxin injection, when the propagation of the neurodegeneration at this nucleus accelerates dramatically and becomes irreversible [44]. Over the same time span, the neuroinflammatory response for the neurotoxic stimuli reaches its maximum, causing profound microglial and astroglial activation. Taking this into account, we hypothesize that the beneficial anti-inflammatory effect in the SNc of HMR/lignan 10 mg/kg treatment was not developing fast enough to mitigate the complex of pathologic processes in nigrostriatal pathway, which was arising in response to neurotoxin injection. To support our hypothesis, previous studies in rat models of PD also have reported a dose-dependent neuroprotective

and anti-inflammatory effect of polyphenols [62,63]. All these findings led us to suppose that chronic treatment with HMR/lignan at higher doses could have more efficacy on neuroinflammatory processes, thus also providing neuroprotection at the level of dopaminergic neurons in the SNc.

## Conclusion

Overall, the findings from the present study suggest intriguing neuroprotective and symptomatic properties of HMR/lignan and provide some insights into its potential application in the treatment of PD. Further studies are needed to corroborate these data and to deeply investigate the additional features of this polyphenol.

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