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Polyphenols Limit Cerebral Endothelial Cell Dysfunction Under Inflammatory Conditions Related to Oral and Gut Microbiota

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Abstract

Background/Objectives: During oral and gut microbiota dysbiosis, lipopolysaccharides (LPSs) of major bacteria, such as *Porphyromonas gingivalis* and *Escherichia coli*, translocate into the bloodstream and lead to endotoxemia. Cerebral endothelial cells are targets of LPSs that may aggravate inflammation and cerebrovascular disorders. This study aimed to evaluate the protective role of the characterized polyphenol-rich extract of the *Dodonaea viscosa* medicinal plant and a predominant component, epicatechin, on murine bEnd.3 cerebral endothelial cells exposed to *P. gingivalis* or *E. coli* LPSs. **Methods:** The effects of LPSs and polyphenols were assessed on cell viability (MTT, trypan blue exclusion assays) and inflammatory, redox, vasoactive and permeability markers (RT-qPCR, Western blot, ELISA, FITC-Dextran test). **Results:** The data show that LPSs activated the TLR2-4/NFκB signaling pathway and promoted IL-1β, IL-6, TNF-α, MCP-1, COX-2, iNOS, ICAM-1, VCAM-1 and E-selectin production without affecting cell viability. LPSs induced oxidative stress by elevating intracellular ROS levels and altering the expression of genes encoding NOX2-4, SOD, catalase, GPx, HO-1 and Nrf2. LPSs imbalanced NO vasodilator and ET-1 vasoconstrictor levels and reduced the production of occludin and ZO-1 tight junction proteins. Meanwhile, LPSs raised the permeability to FITC-Dextran, suggesting cell integrity loss. The extent of endothelial dysfunction caused by LPSs depended on their bacterial origin. Importantly, plant polyphenols and epicatechin exerted anti-inflammatory and antioxidant effects, and attenuated LPSs' deleterious action on vasoactive and permeability markers. **Conclusions:** This study shows that polyphenols limit cerebral endothelial cell dysfunction under inflammatory conditions mediated by LPSs, highlighting their therapeutic potential in protecting brain homeostasis during oral and gut microbiota dysbiosis.

Keywords: polyphenols; epicatechin; cerebral endothelial cell; inflammation; oxidative stress; LPSs; microbiota dysbiosis

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1. Introduction

Cerebrovascular dysfunction occurring during stroke is a major contributor to brain homeostasis disruption and subsequent neurological damage. Ischemic stroke, caused by reduced cerebral blood flow following arterial occlusion, remains a leading cause of death and disability worldwide [1]. One key consequence of stroke is the disruption of the blood–brain barrier (BBB), which plays a critical role in maintaining cerebral homeostasis by regulating exchanges between the blood and the brain [2]. The BBB is primarily formed by

cerebral endothelial cells interconnected by tight junctions, including occludin, claudin-5, and zona occludens (ZO) proteins, which are essential for controlling paracellular permeability [3,4]. In pathological conditions such as stroke, inflammation-induced endothelial dysfunction leads to altered tight junction integrity and increased BBB permeability, contributing to edema and secondary brain injury. Identifying the mechanisms involved in BBB-associated endothelial dysfunction is therefore crucial.

Although traditional risk factors of stroke such as hypertension, dyslipidemia and diabetes are well-established, the literature data indicate an emerging association between stroke and the dysbiosis of oral and gut microbiota [1,5–7] that may aggravate the cerebral endothelial cell dysfunction. Oral microbiota dysbiosis mainly occurs during periodontitis, which is a common dental infection causing a persistent inflammation of the periodontium, the tissue supporting the teeth. Several epidemiological studies have demonstrated a link between periodontitis and ischemic stroke [8,9]. Recent data showing that regular dental care is associated with a 23% relative reduction in the risk of ischemic stroke reinforce the strength of this association. *Porphyromonas gingivalis* is one of the major bacterial species of the oral cavity associated with the pathogenesis of periodontitis [10]. Gingival ulceration of periodontal pockets during dental activities induces bleeding and leads to bacterial translocation into the bloodstream [11]. Once in circulation, periodontal bacteria and their endotoxins such as lipopolysaccharides (LPSs) can spread to distant sites in the body, explaining increased circulating LPSs' levels, as well as worsening damaged tissue [12–15]. It was reported that ischemic stroke patients exhibit higher plasma levels of LPSs compared to healthy controls, that correlate with worsening disability [16,17]. Higher LPSs' circulating levels characterizing "endotoxemia" also occur during the deregulation of the ecology of gut bacteria such as *Escherichia coli*. This gut microbiota dysbiosis is particularly observed during obesity and results from the intestinal barrier "leaking", contributing to the blood translocation of LPSs [18].

During endotoxemic conditions related to oral and gut microbiota dysbiosis, LPSs promote a pro-inflammatory response of targeted tissues. Indeed, the lipid A moiety of LPSs is the main microbe-associated molecular pattern (MAMP) that acts on the innate immunity Toll-like receptors (TLRs), and the number of acyl chains in lipid A is a key determinant of immune activation by the LPS-TLRs system. Once activated by LPSs, TLRs mediate signaling cascades involving the nuclear factor kappa B (NFκB) transcriptional factor and mitogen-activated protein kinases (MAPKs), leading to an increase in pro-inflammatory chemokines/cytokines such as monocyte chemoattractant protein-1 (MCP-1), interleukins-1 beta and 6 (IL-1β, IL-6) and tumor necrosis factor-alpha (TNF-α). It is established that these pro-inflammatory mediators promote oxidative stress [19–21], and the crosstalk between oxidative stress and inflammation may exacerbate endothelial cell dysfunction [22,23]. The *E. coli* LPSs' condition used to mimic metabolic endotoxemia associated with metabolic diseases can induce inflammation, metabolic defect activation, and contribute to atherosclerotic damage via TLR4-mediated oxidative stress [5,24,25]. In parallel, the *P. gingivalis* LPSs have been shown to aggravate chronic inflammation [26]. Meanwhile, imbalances of oral commensal nitrate-reducing bacteria have been associated with a decreased level of nitric oxide (NO), causing endothelial dysfunction [27]. Thus, in the context of oral and gut microbiota dysbiosis, an elevation in circulating LPSs may target cerebral endothelial cells constituting the BBB, worsening inflammation and oxidative stress, and aggravating the BBB disruption in stroke condition. Nevertheless, to the best of our knowledge, no studies have compared how structural differences in the lipid A moiety of LPSs from different bacterial origins may contribute to cerebral endothelial cell dysfunction.

Understanding principal mediators and molecular mechanisms involved in cerebral endothelial cell dysfunction is crucial for the development of novel therapeutics. Interestingly, polyphenols provided by the human diet [28] exert antioxidant and anti-inflammatory properties that may help to improve endothelial cell dysfunction and BBB disruption during cerebrovascular disorders related to stroke [29–32]. Polyphenols constitute a large family of micronutrients abundant in fruits, vegetables and plant-derived beverages such as tea, coffee and herbal preparations [28]. Among the polyphenols commonly consumed, flavonoids such as quercetin, catechin, epigallocatechin gallate and resveratrol have been shown to enhance NO synthesis, reduce the production of enzymes generating reactive oxygen species (ROS) such as NADPH oxidases (NOXs) and attenuate pro-inflammatory signaling, thereby preserving endothelial function and limiting risk factors associated with cardiovascular diseases [33]. Other dietary phenolics like caffeic acid, chlorogenic acid, gallic acid and hydroxytyrosol can mitigate pro-adhesive endothelial cell responses under stress conditions, highlighting the vasoprotective roles of diverse polyphenols through the modulation of the oxidative, inflammatory and adhesion pathways [34,35]. Previously, we showed that polyphenols extracted from the *Dodonaea viscosa* medicinal plant referenced in the *French Pharmacopeia* for antidiabetic properties attenuate inflammation and oxidative stress as well as the deregulation of vasoactive markers and the permeability of cerebral endothelial cells during hyperglycemia [32,35].

Given that the mechanisms whereby specific LPSs linked to oral and gut microbiota dysbiosis may alter cerebral endothelial cells remain unclear, as well as the possible protective role of plant polyphenols, the present study aimed (1) to evaluate the effects of specific LPSs from *P. gingivalis* and *E. coli* on cerebral endothelial cell dysfunction markers, and (2) to examine the ability of *D. viscosa* polyphenolic extract and a predominant component, epicatechin, to improve endothelial function under LPSs' conditions.

2. Materials and Methods

2.1. Characterization of D. viscosa Polyphenolic Extract Composition and Antioxidant Capacity

The medicinal plant *D. viscosa* collected in Réunion Island (France) was botanically identified with voucher number (Sapindaceae, TCN-P028) at the University of Réunion Island. To extract the polyphenols from the leaves dried with an airflow at 45 °C, 2 g of plant material were added to 10 mL of aqueous acetone solution (70%, *v*/*v*, Sigma-Aldrich, St. Louis, MO, USA), and the mixture was incubated at 4 °C for 90 min. After centrifugation at 1400× *g* for 20 min at 4 °C, the supernatant corresponding to the polyphenol-rich extract was collected and stored at −80 °C until analysis. A Folin–Ciocalteu assay [36] was performed to determine the total polyphenol content of *D. viscosa* extract. Then, the polyphenols present in the plant extract were analyzed by ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS-MS, Agilent Technologies, Les Ulis, France) and described in our published study [32]. To assess the free radical-scavenging and reducing capacity of *D. viscosa* extract, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed, according to the method previously described [32]. Epicatechin (St-Louis, MO, USA) was used as a positive control given its identification as a predominant polyphenol present in *D. viscosa* extract [32]. Briefly, 40 µL of the polyphenolic extract of *D. viscosa* or epicatechin prepared at different concentrations (5–10–20–40–80–160 µM) were placed in a 96-well plate. Then, 200 µL of 0.25 mM DPPH radicals (Sigma-Aldrich, St. Louis, MO, USA) diluted in methanol were added to each well, and the mixture was incubated at 25 °C. After 25 min, the absorbance was measured at 517 nm (FLUOstar Optima, Bmg Labtech, Cambridge, UK). The percentage of reduced DPPH was used to calculate the antioxidant capacity.

2.2. Cerebral Endothelial Cell Culture

Immortalized murine bEnd3 cerebral endothelial cells obtained from the American Type Culture Collection (ATCC, CRL-2299, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose, 10% heat-inactivated fetal bovine serum, 5 mM L-glutamine, 50 µU/mL penicillin and 2 µg/mL streptomycin (Pan Biotech, Aidenbach, Germany). To generate the bEnd3-Blue cell line model, bEnd3 cells were transfected with the plasmid pNiFty-secreted alkaline phosphatase (pNiFty2-SEAP) using Lipofectamine 3000 (ThermoFisher Scientific, Les Ulis, France) and Zeocin™ at 200 µg/mL (Invivogen, Toulouse, France) for 3 weeks, according to the previously published protocol [37]. The pNiFty2-SEAP plasmid contains a SEAP reporter gene, an endothelial cell–leukocyte adhesion molecule proximal promoter, and five distinct NFκB repeat transcription factor binding sites, helping to investigate the transcriptional activity of NFκB in experimental conditions. The growth medium used for bEnd3-Blue cells contained 200 µg/mL Zeocin™. Both cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. Here, the choice of the immortalized bEnd3 cell line model is in line with our previous studies [32,35] and the literature data shows its suitability for a mimetic system of the BBB's permeability, with respect to the production of tight junction proteins and a variety of transporters such as the efflux transporters P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP) as well as glucose transporter-type 1 (GLUT-1) [38,39]. This provides evidence for the possible translatability of the bEnd3 model to mimic cerebral endothelial cells constituting the BBB, keeping in mind that it remains an in vitro model and that the BBB is a complex structure composed of the neurovascular unit with other cellular types [3,4].

2.3. Evaluation of the Cell Viability and Mitochondrial Metabolic Activity

The cells were plated (7.5×10^4 cells/well) in a 24-well plate in DMEM during 24 h. Then, the medium was removed, and cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL) in the presence or absence of the polyphenol-rich plant extract (10 µM gallic acid equivalent, GAE) or epicatechin (10 µM) for 24 h. The selection of LPS dose was based on literature studies using similar or close concentrations to evaluate the impact of LPSs on key endothelial markers, such as tight junction proteins, without causing cytotoxicity [40]. Regarding the dose of 10 µM of polyphenols, it is consistent with the pharmacological doses broadly used in the literature and in our published studies [32,35]. This dose is considered to be close to circulating concentrations reaching less than 10 µM in nutritional situations, given that polyphenols are poorly absorbed through the gut barrier and that their bioavailability depends on their structure and microbial catabolism [28]. Afterwards, the medium was removed, and cells were washed once in phosphate-buffered saline (PBS), separated using trypsin-EDTA (Pan Biotech, Germany) and centrifuged (500× *g*, 4 min, 25 °C). Cells were stained with Trypan blue solution (Sigma-Aldrich) and counted in a Malassez chamber. Regarding the mitochondrial metabolic activity of cells, it was measured by using a 3-(4.5-dimethyl-thiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay, according to a previously published method [36]. Briefly, cells were plated on a 96-well plate (1.0×10^4 cells/well) for 24 h. Then, the medium was removed and cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL) in the presence or absence of the polyphenol-rich plant extract (10 µM GAE) or epicatechin (10 µM) for 24 h. Five hours before the experimental period ended, 20 µL of a solution of 5 mg/mL MTT reagent (Sigma-Aldrich) prepared in PBS were added to each well. After centrifugation (500× *g*, 4 min, 25 °C), the medium was aspirated and dimethyl sulfoxide (DMSO, 200 µL) was added to each well for dissolving formazan crystals formed from the mitochondrial metabolism of MTT reagent by living cells. The absorbance was read at 560 nm (TECAN, Männedorf, Switzerland).

2.4. Evaluation of Gene Expression

The cells were cultured in a 6-well plate (3.5×10^5 cells/well) in DMEM for 24 h. After removing the medium, the cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL) in the presence or absence of the polyphenol-rich plant extract (10 µM GAE) or epicatechin (10 µM) for 24 h. Total RNA was extracted using TRIzol™ (Invitrogen, ThermoFisher Scientific, Dardilly, France) and 4 µg of RNA were subjected to reverse transcription (RT) by using Random Hexamer Primers (Eurogentec, Liège, Belgium) with SuperscriptTM II (Invitrogen, ThermoFisher Scientific, Dardilly, France). The quantitative polymerase chain reaction (qPCR) was made using Fast SYBR green™ Master Mix (Applied Biosystems, ThermoFisher Scientific, Dardilly, France).

The relative expression of the targeted genes was normalized to the expression rate of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene that was reported as a suitable housekeeping gene in similar literature studies conducted on cerebral endothelial cells exposed to LPSs [40] and after we controlled its stability under our experimental conditions. The genes targeted were those coding for inflammatory mediators (TLR2, TLR4, MyD88, NFκB, IL-1β, IL-6, IL 10, TNF-α, MCP-1, iNOS, COX2, ICAM-1, VCAM-1, E-selectin), redox factors (NOX2, NOX4, Cu/ZnSOD, MnSOD, catalase, GPx, HO-1, Nrf2), permeability markers (claudin-5, occludin, ZO-1, ZO-2) and vasoactive molecules (eNOS, ET-1). The primer sequences are listed in Table 1. Data were obtained through analysis conducted with 7500 system SDS software (Applied Biosystems).

Table 1. Primers used for the reverse-transcribed-quantitative polymerase chain reaction (RT-qPCR) analysis.

Mouse Gene Sequence	Forward	Reverse
Catalase	CCT-CCT-CGT-TCA-GGA-TGT-GGT-T	CGA-GGG-TCA-CGA-ACT-GTG-TCA-G
Claudin-5	GCT-GGC-GCT-GGT-GGC-ACT-CTT-TGT	GGC-GAA-CCA-GCA-GAG-CGG-CAC
COX2	TTT-GTT-GAG-TCA-TTC-ACC-AGA-CAG-AT	CAG-TAT-TGA-GGA-GAA-CAG-ATG-GGA-TT
Cu/ZnSOD	GCA-GGG-AAC-CAT-CCA-CTT	TAC-AAC-CTC-TGG-ACC-CGT
eNOS	CTG-TGG-TCT-GGT-GCT-GGT-C	TGG-GGC-AAC-TTG-AAG-AGT-GTG
E-selectin	TCT-GGA-CCT-TTC-CAA-AAT-GG	TGC-AAG-CTA-AAG-CCC-TCA-TT
ET-1	CCC-TTT-GCA-GAA-TGG-ATT-AT	CTG-TAG-TCA-ATG-TGC-TCG-GT
GAPDH	CTT-TGT-CAA-GCT-CAT-TTC-CTG-G	TCT-TGC-TCA-GTG-TCC-TTG-C
GPx	TGC-TCA-TTG-AGA-ATG-TCG-CGT-CTC	AGG-CAT-TCC-GCA-GGA-AGG-TAA-AGA
HO-1	GGT-CAT-GGC-TTC-CTT-GTA-C	AGT-GAG-GCC-CAT-ACC-AGA-AG
ICAM-1	CGA-AGG-TGG-TTC-TTC-TGA-GC	GTC-TGC-TGA-GAC-CCC-TCT-TG
IL-1β	GAC-CTT-CCA-GGA-TGA-GGA-CA	AGC-TCA-TAT-GGG-TCC-GAC-AG
IL-6	CAA-GAG-ACT-TCC-ATC-CAG-TTG-C	TTG-CCG-AGT-AGA-TCT-CAA-AGT-GAC
IL-10	ACC-TCC-TCC-ACT-GCC-TTG-CT	GGT-TGC-CAA-GCC-TTA-TCG-GA
iNOS	GCA-GCC-TGT-GAG-ACC-TTT-G	GCA-TTG-GAA-GTG-AAG-CGT-TTC
MMP-2	GAT-ACC-CCA-AGC-CAC-TGA-CC	AGT-AGC-TAT-GAC-CAC-CAC-CCT
MMP-9	CGT-TCA-TGT-ACC-CGC-TGT-AT	TGT-CTG-CCG-GAC-TCA-AAG-AC
MnSOD	ATG-TTG-TGT-CGG-GCG-GCG	AGG-TAG-TAA-GCG-TGC-TCC-CAC-ACG
MyD88	TCG-AGT-TTG-TGC-AGG-AGA-TG	AGG-CTG-AGT-GCA-AAC-TTG-GT
NFκB	GTG-ATG-GGC-CTT-CAC-ACA-CA	CAT-TTG-AAC-ACT-GCT-TTG-ACT
NOX2	ACC-TTA-CTG-GCT-GGG-ATG-AA	TGC-AAT-GGT-CTT-GAA-CTC-GT
NOX4	GAT-CAC-AGA-AGG-TCC-CTA-GCA-G	GTT-GAG-GGC-ATT-CAC-CAA-GT
Nrf2	TTG-GCA-GAG-ACA-TTC-CCA-T	GCT-GCC-ACC-GTC-ACT-GGG
Occludin	CAC-ACT-TGC-TTG-GGA-CAG-AGG	TGA-GCC-GTA-CAT-AGA-TCC-AGA-AG
TLR2	CGT-TGT-TCC-CTG-TGT-TGC	AAA-GTG-GTT-GTC-GCC-TGC-T
TLR4	TTC-ACC-TCT-GCC-TTC-ACT-ACA	GGG-ACT-TCT-CAA-CCT-TCT-CAA
TNF-α	CTT-CTG-TCT-ACT-GAA-CTT-CGG-G	CAG-GCT-TGT-CAC-TCG-AAT-TTT-G
VCAM-1	TGG-AGG-AAT-GGG-CAT-AAA-G	CAG-GAT-TTT-GGG-AGC-TGG-TA
ZO-1	GCT-GTC-CCT-GTG-AGT-CCT-TC	TGC-CAG-GTT-TTA-GGG-TCA-CA
ZO-2	AGA-AGA-ACC-TCC-GCA-AGA-GC	GCC-TCA-CGG-TAT-TCA-ACC-GA

2.5. Evaluation of NFκB/SEAP Activity

The bEnd3-Blue cells containing NFκB/SEAP reporter gene were cultured in a 96-well black plate (1.8×10^4 cells/well) in DMEM for 24 h. After removing the medium, the cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL) in the presence or absence of the polyphenol-rich plant extract (10 µM GAE) or epicatechin (10 µM) for 1 or 3 h. These short treatments were selected to allow measurement of the early activation of the NFκB transcriptional factor under in vitro cell culture conditions. Next, the Quanti-Blue assay (Invivogen, Toulouse, France) was performed to measure NFκB/SEAP activity. The absorbance was measured at 620–655 nm (FLUOstar Omega, Bmg Labtech, Ortenberg, Germany).

2.6. Quantification of IL-6 and MCP-1 Secretion

The cells were cultured in a 6-well plate (3.5×10^5 cells/well) in DMEM for 24 h. After removing the medium, the cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL) in the presence or absence of the polyphenol-rich plant extract (10 µM GAE) or epicatechin (10 µM) for 24 h. Then, the cell culture medium was collected to measure the secreted levels of IL-6 and MCP-1 by using specific Mouse ELISA kits (eBioscience, ThermoFisher Scientific, Dardilly, France). To extract the cellular proteins, 700 µL of PBS were added per well and the cells were scraped, collected and centrifuged (900× *g* for 4 min at 4 °C). After removing the supernatants, a volume of 200 µL of lysis buffer (pH 8.3, Tris 25 mM, KCl 10 mM, EDTA 1 mM, DTT 1 mM, Triton X-100 1%, protease inhibitors 1 X) was added to resuspend cell pellets. Next, the resuspended cell pellets were centrifuged (900× *g*, 4 min, 4 °C) and the supernatants containing proteins were collected. The BCA assay [41] was used to determine the total cellular protein contents, and the absolute values of IL-6 and MCP-1 secreted levels were normalized to these total protein contents.

2.7. Measurement of Intracellular ROS Levels

To measure the intracellular ROS levels, the fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay was performed, according to a previously published method [36]. Briefly, the cells were plated in a 96-well black plate (1.8×10^4 cells/well) in DMEM for 24 h. Then, the medium was removed, the cells were washed twice with PBS, and a volume of 100 µL PBS containing 10 µM DCFH-DA (Sigma-Aldrich) was added to each well. After the incubation of cells in a humidified environment (5% CO_2, 37 °C) for 45 min, the PBS containing DCFH-DA was removed. The cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL) in the presence or absence of the polyphenol-rich plant extract (10 µM GAE) or epicatechin (10 µM) for 1, 3, 6 or 24 h. The short treatments within this kinetic analysis were selected to allow measurement of the early activation of metabolic reactions producing ROS under in vitro cell culture conditions. Next, the fluorescence was measured at an excitation wavelength of 492 nm and an emission wavelength of 520 nm (FLUOstar Optima).

2.8. Measurement of Intracellular NO Levels

To measure the intracellular NO levels, a fluorogenic probe 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate assay was performed according to a previously published method [42]. Briefly, the cells were cultured in a black 96-well plate (1.8×10^4 cells/well) in DMEM for 24 h. Then, the medium was removed, and the cells were exposed to LPSs from *P. gingivalis* or *E. coli* (10 µg/mL), in the presence or absence of the polyphenol-rich plant extract (10 µM GAE) or epicatechin (10 µM) for 1 or 3 h. These short treatments were selected to allow measurement of the early activation of metabolic reactions producing NO under in vitro cell culture conditions. After removing the medium, the cells were rinsed once with PBS and a volume of 100 µL of a solution of PBS containing

5 μM of DAF-FM (Sigma-Aldrich) was added to each well. After the incubation of cells in a humidified atmosphere (5% CO_2, 37 °C,) for 45 min, the PBS containing DCFH-DA was removed and the cells treated for 2 h with 100 μL of insulin (1 μg/mL, Sigma-Aldrich) to enhance NO production. Next, the fluorescence was measured at an excitation wavelength of 492 nm and an emission wavelength of 520 nm (FLUOstar Optima).

2.9. Western Blot Analysis

The proteins extracted, as described above, from the cells exposed to *P. gingivalis* or *E. coli* LPSs (10 μg/mL) in the presence or absence of the polyphenol-rich plant extract (10 μM GAE) or epicatechin (10 μM) for 24 h, were analyzed by Western blot. Briefly, 20 μg of proteins were separated by using dodecyl sulphate–polyacrylamide gel electrophoresis with 7.5% of acrylamide. After the transfer of the separated proteins onto a nitrocellulose membrane, the blocking of nitrocellulose membrane was achieved by using a solution of 5% (*w*/*v*) non-fat dry milk in Tris-buffer containing 0.1% (*v*/*v*) Tween 20 (TBS-Tween), at room temperature for 1 h. Then, the nitrocellulose membrane was incubated with the primary rabbit antibody against iNOS (1:500, ab283655, Abcam, Paris, France) or α-tubulin (1:1000, ab4074, Abcam) at 4 °C, overnight. The day after, the nitrocellulose membrane was washed with TBS-Tween and incubated for 1 h with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:2000, ab6721, Abcam). Next, a chemiluminescence kit (GE Healthcare Life Sciences, Velizy-Villacoublay, France) and an imager (Amersham Imager 600, GE Healthcare Life Sciences) were used for detecting protein/antibody complex. Data were normalized to α-tubulin protein levels.

2.10. Statistical Analysis

Data were expressed as means ± SEM of three independent experiments (three cellular passages). For each biological parameter evaluated, the statistical analysis was performed through one-way analysis of variance (ANOVA), followed by Bonferroni post hoc correction to account for multiple comparisons, by using GraphPad Prism 6 program (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered statistically significant for a p value < 0.05. Given the number of independent gene expressions and biomarker measurements analyzed, the study was designed as an exploratory investigation and a statistical approach was chosen to provide a conservative assessment of group differences while limiting the risk of type I error at the level of individual parameters.

3. Results

3.1. Free Radical-Scavenging and Reducing Capacity of D. viscosa Polyphenolic Extract and Epicatechin

The composition of the polyphenol-rich extract of *D. viscosa* used in the present study was previously analyzed by UPLC-MS-MS and published by our group [32]. We reported that *D. viscosa* extract exhibits a total polyphenol content of 3 g gallic acid equivalent/100 g dried leaves. It mainly contains polyphenols belonging to the family of procyanidins identified as epicatechin dimer B2, trimer type A and tetramer type A (80% of total polyphenol content), indicating that epicatechin represents a major structural unit in the plant extract. A hexoside derivative of the flavonol quercetin (isorhamnetin–hexoside–rhamnoside) and 5,7-dihydroxy-3,6,4′-trimethoxyflavone (santin) were also detected to a lesser extent (20% of total polyphenol content) [32]. Here, to assess if the presence of polyphenols in the plant extract confers it an antioxidant capacity, DPPH radical-scavenging and reducing capacity assay was performed for different doses ranging from 5 to 160 μM. The results indicate that *D. viscosa* extract dose-dependently scavenged and reduced DPPH radicals (Figure 1). Given the predominance of epicatechin among the plant polyphenols identified, it was

used as a positive control. Similar to *D. viscosa* polyphenolic extract, epicatechin exerted a dose-dependent antioxidant capacity, although this capacity was significantly higher than that of the plant extract for the doses ranging 10–40 µM. Polyphenol concentrations of 10 µM were selected based on their reported bioavailability, with some dietary polyphenols reaching low micromolar plasma levels, and on their widespread use in endothelial cell studies investigating signaling-mediated protective effects.

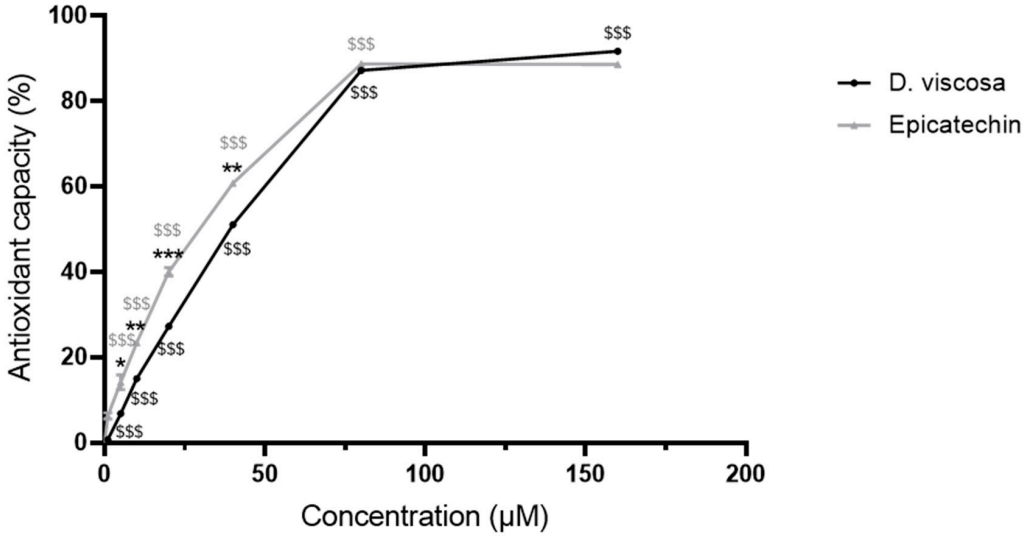

Figure 1. Antioxidant capacity of *D. viscosa* polyphenolic extract and epicatechin. Radical-scavenging and reducing capacity of *D. viscosa* polyphenolic extract and epicatechin at the doses of 0, 5, 10, 20, 40, 80 and 160 µM gallic acid equivalent were measured by the DPPH method, and expressed as % DPPH reduced. Data are means ± SEM of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$ as compared to *D. viscosa*. $$$: $p < 0.005$ as compared to the lower concentration.

3.2. Effect of LPSs and Polyphenols on the Viability of Cerebral Endothelial Cells

The viability of cerebral endothelial cells exposed for 24 h to *E. coli* and *P. gingivalis* LPSs in the presence or absence of *D. viscosa* extract and epicatechin, was assessed by cell counting and mitochondrial metabolic activity measurement. The data show that there was no significant change in the cell number (Figure 2a) and mitochondrial metabolic activity (Figure 2b). This suggests that cellular viability was not altered in our experimental conditions whether or not they were combined with polyphenols.

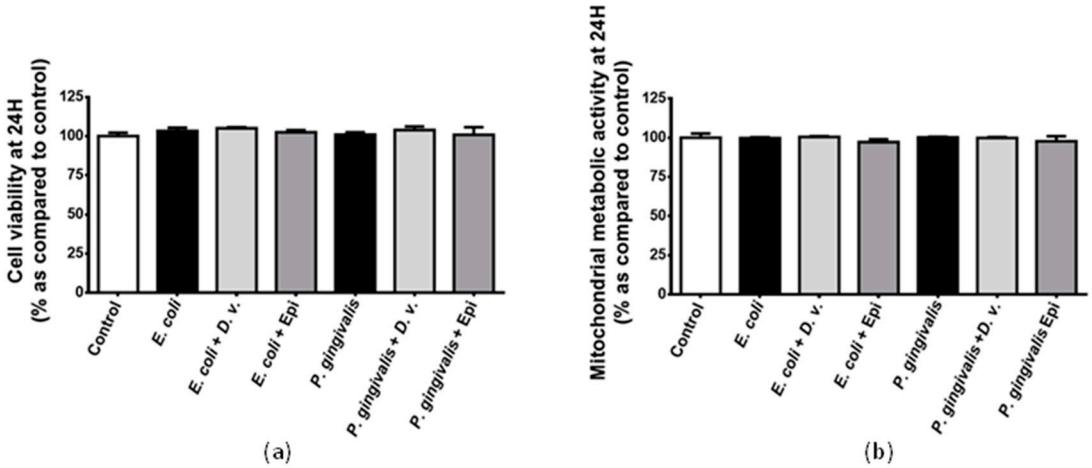

Figure 2. Effect of LPSs and polyphenols on the viability of cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or

absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 24 h. Then, the cell number was counted by using the Trypan blue exclusion method (**a**) and the mitochondrial metabolic activity was determined by using MTT assay (**b**). Data are means ± SEM of three independent experiments.

3.3. Effect of LPSs and Polyphenols on the Inflammatory Response of Cerebral Endothelial Cells

LPSs are known to mediate pro-inflammatory effects via the activation of the pathway linking their innate immunity receptors TLRs to the transcriptional factor NFκB. We evaluated the expression of genes encoding factors involved in this pathway in cerebral endothelial cells exposed for 24 h to *E. coli* and *P. gingivalis* LPSs in the presence or absence of polyphenols. The results show that both LPSs led to an upregulated expression of the *TLR4* gene, whereas only *P. gingivalis* LPSs increased *TLR2* gene expression (Table 2). This suggests a specific ability of *P. gingivalis* LPSs to target TLR2. Both LPSs also enhanced the expression of genes coding for key pro-inflammatory mediators including MyD88, NFκB, IL-1β, IL-6, TNF-α, MCP-1, COX2 and iNOS. Notably, *E. coli* LPSs exerted a significantly more deleterious action on the expression of *TLR4*, *MyD88*, *NFκB*, *IL-6* and *MCP-1* genes compared to *P. gingivalis* LPSs. Conversely, *P. gingivalis* LPSs led to a more detrimental alteration in *TNF-α* and *iNOS* gene expression compared to *E. coli* LPSs. These results underline that the extent of the pro-inflammatory response mediated by LPSs may depend on their bacterial origin, thereby driving their differential capacity to activate the innate immunity system. Interestingly, in *E. coli* LPSs conditions, *D. viscosa* extract and epicatechin limited the deregulation of all targeted genes, except for the *COX2* gene that was not improved by epicatechin. In *P. gingivalis* LPSs conditions, *D. viscosa* extract and epicatechin attenuated the deregulation of the main markers, except for NFκB, which was slightly affected by these LPSs. Additionally, the plant extract did not improve the expression of *IL-6* and *MCP-1* genes that were slightly elevated by *P. gingivalis* LPSs. Notably, if both LPSs significantly downregulated the mRNA level of the anti-inflammatory cytokine IL-10, polyphenols abrogated this effect. These data provide evidence for the anti-inflammatory capacities of *D. viscosa* polyphenolic extract and epicatechin in endotoxemic conditions and suggest the possible contribution of epicatechin to the bioactivity of *D. viscosa* extract.

Table 2. Effect of LPSs and polyphenols on the inflammatory response of cerebral endothelial cells.

Relative Gene Expression	Control	*E. coli*	*E. coli* + *D. v.*	*E. coli* + Epi	*P. gingivalis*	*P. gingivalis* + *D. v.*	*P. gingivalis* + Epi
TLR2	1.00 ± 0.08	1.11 ± 0.11	0.95 ± 0.10	0.92 ± 0.03	1.56 ± 0.09 **§	1.04 ± 0.08 $^{\#\#}$	1.26 ± 0.04 $^{\#}$
TLR4	1.00 ± 0.12	3.96 ± 0.20 ***	1.06 ± 0.02 $^{\$\$\$}$	1.35 ± 0.03 $^{\$\$\$}$	1.66 ± 0.08 **§§§	1.01 ± 0.02 $^{\#\#\#}$	1.00 ± 0.03 $^{\#\#\#}$
MyD88	1.00 ± 0.30	4.17 ± 0.50 *	1.77 ± 0.20 $^{\$\$}$	2.25 ± 0.19 $^{\$}$	1.242 ± 0.03 §	1.040 ± 0.04	0.93 ± 0.10 $^{\#}$
NFκB	1.00 ± 0.02	3.31 ± 0.30 ***	1.68 ± 0.04 $^{\$\$}$	1.52 ± 0.11 $^{\$\$\$}$	1.79 ± 0.09 **§§	1.64 ± 0.20	1.42 ± 0.10
IL-1β	1.00 ± 0.08	2.73 ± 0.04 ***	1.39 ± 0.30 $^{\$\$}$	1.53 ± 0.20 $^{\$\$}$	2.53 ± 0.13 ***	1.04 ± 0.10 $^{\#\#\#}$	1.38 ± 0.13 $^{\#\#}$
IL-6	1.00 ± 0.04	9.73 ± 0.80 ***	4.55 ± 0.18 $^{\$\$\$}$	6.37 ± 0.26 $^{\$\$}$	2.43 ± 0.39 **§§	1.52 ± 0.41	1.18 ± 0.16 $^{\#}$
TNF-α	1.00 ± 0.30	2.43 ± 0.10 ***	1.54 ± 0.09 $^{\$\$}$	1.95 ± 0.11 $^{\$}$	3.27 ± 0.20 ***§	1.70 ± 0.19 $^{\#\#}$	1.74 ± 0.20 $^{\#\#}$
MCP-1	1.00 ± 0.10	11.57 ± 1.00 ***	5.89 ± 0.20 $^{\$\$}$	8.61 ± 0.26 $^{\$}$	1.47 ± 0.05 *§§§	1.29 ± 0.09	1.02 ± 0.09 $^{\#}$
IL-10	1.00 ± 0.23	0.55 ± 0.04 **	1.36 ± 0.40	1.32 ± 0.20 $^{\$}$	0.45 ± 0.01 **	1.69 ± 0.10 $^{\#\#\#}$	1.86 ± 0.06 $^{\#\#\#}$
COX2	1.00 ± 0.12	3.02 ± 0.42 **	1.38 ± 0.18 $^{\$}$	1.89 ± 0.19	2.54 ± 0.10 **	1.30 ± 0.11 $^{\#\#}$	1.30 ± 0.02 $^{\#\#}$
iNOS	1.00 ± 0.04	4.52 ± 0.51 **	2.44 ± 0.14 $^{\$}$	2.33 ± 0.57 $^{\$}$	10.18 ± 0.51 ***§§	0.59 ± 0.14 $^{\#\#\#}$	0.55 ± 0.15 $^{\#\#\#}$

Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 24 h. Then, the expression of genes encoding inflammatory mediators was measured by RT-qPCR and normalized to *GAPDH* gene expression. Data are means ± SEM of three independent experiments. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.005$ as compared to the Control. $: $p < 0.05$, $$: $p < 0.01$ and $$$: $p < 0.005$ as compared to *E. coli*. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.005$ as compared to *P. gingivalis*. §: $p < 0.05$, §§: $p < 0.01$ and §§§: $p < 0.005$ as compared to *E. coli*.

It is well-established that under LPS conditions, the nuclear translocation of NFκB occurs and leads to the regulation of the transcription of targeted genes such as those en-

coding inflammatory mediators. We assessed NFκB transcriptional activity and measured the secreted levels of IL-6 and MCP-1 in a bEnd3-Blue cell model exposed to LPS conditions. The results show that NFκB transcriptional activity was significantly increased in cerebral endothelial cells exposed to LPSs for 1 h (Figure 3a) or 3 h (Figure 3b), as evidenced by the increased release of SEAP protein reporter in the cell culture medium. Meanwhile, both LPSs raised the secretion of IL-6 (Figure 3c) and MCP-1 (Figure 3d). In line with the results described above, the effect of *E. coli* LPSs was significantly more pronounced than that of *P. gingivalis* LPSs concerning IL-6 and MCP-1 secretion. *D. viscosa* polyphenolic extract attenuated the deregulation of NFκB transcriptional activity and the alteration in IL-6 and MCP-1 secretion caused by LPSs. Similarly, epicatechin improved these pro-inflammatory markers, except for MCP-1 in *E. coli* LPSs-exposed cells.

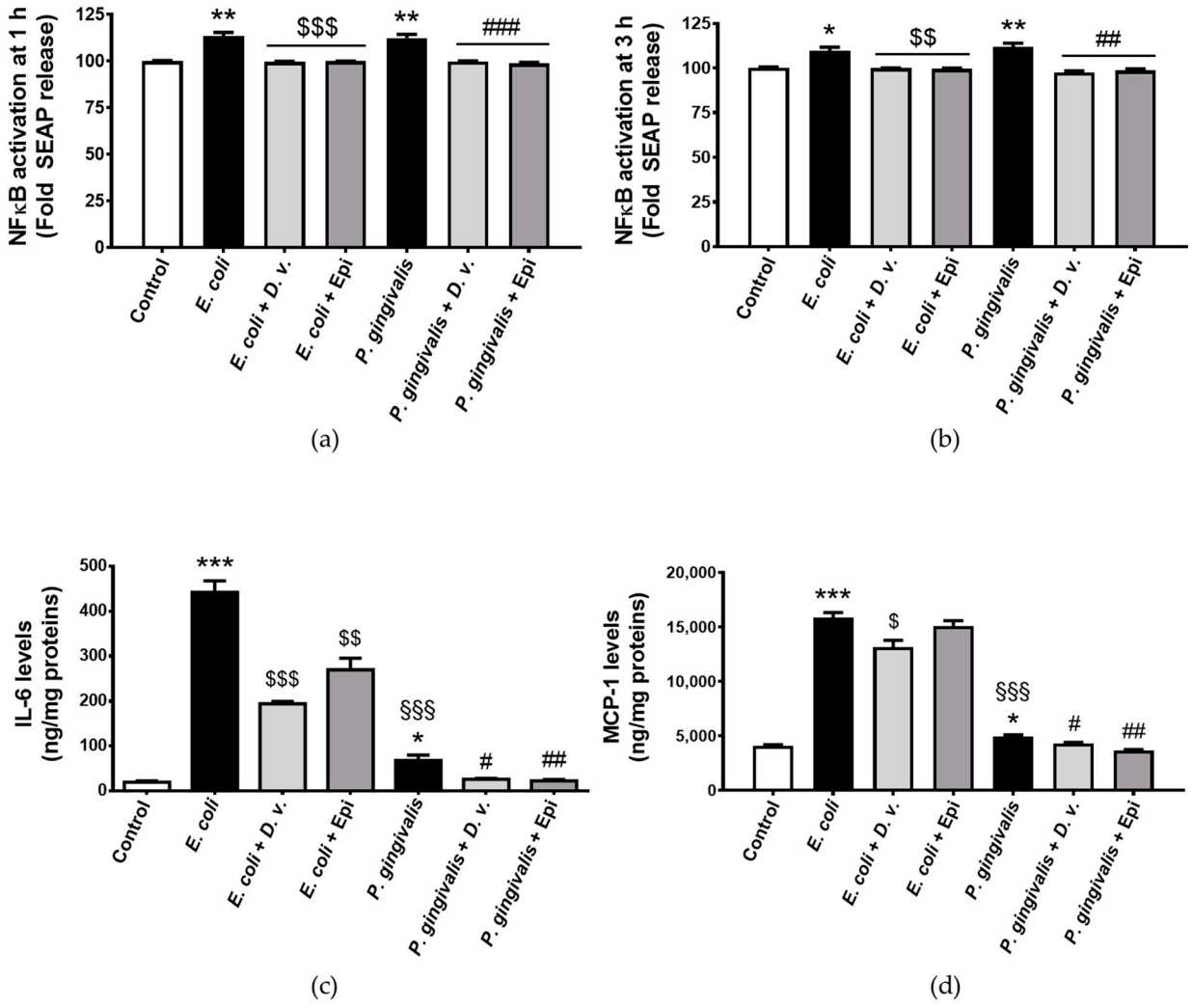

Figure 3. Effect of LPSs and polyphenols on the transcriptional activity of NFκB and the secretion of IL-6 and MCP-1 in cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 μg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 μM GAE) or pure epicatechin (Epi, 10 μM) for 1, 3 and 24 h. Relative NFκB/SEAP transcriptional activity was determined by Quanti-Blue assay at 1 h (**a**) and 3 h (**b**), and expressed as a percentage of the Control. The levels of IL-6 (**c**) and MCP-1 (**d**) secreted in the cell culture medium after 24 h were measured by ELISA kits. Data are means ± SEM of three independent experiments. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.005$ as compared to the Control. $: $p < 0.05$, $$: $p < 0.01$ and $$$: $p < 0.005$ as compared to *E. coli*. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.005$ as compared to *P. gingivalis*. §§§: $p < 0.005$ as compared to *E. coli*.

In inflammatory conditions, endothelial cells play a critical role in facilitating the migration of circulating immune cells through the expression of endothelial-leukocyte adhesion molecules such as ICAM-1, VCAM-1 and E-selectin. Our results indicate that both LPSs induced the expression of genes coding for these adhesion molecules. On the one hand, *E. coli* LPSs exerted a more deleterious effect on *ICAM-1* (Figure 4a) and *VCAM-1* (Figure 4b) genes. On the other hand, *P. gingivalis* LPSs caused a more significant deregulation of the *E-selectin* gene (Figure 4c). *D. viscosa* extract and epicatechin were able to protect against the modification of *ICAM-1* and *VCAM-1* gene expression in *E. coli* LPSs conditions without impact on these genes in *P. gingivalis* LPSs conditions. Regarding *E-selectin* gene expression, it was significantly decreased by the plant extract and epicatechin in cells exposed to *P. gingivalis* LPSs, and by epicatechin during *E. coli* LPSs exposure.

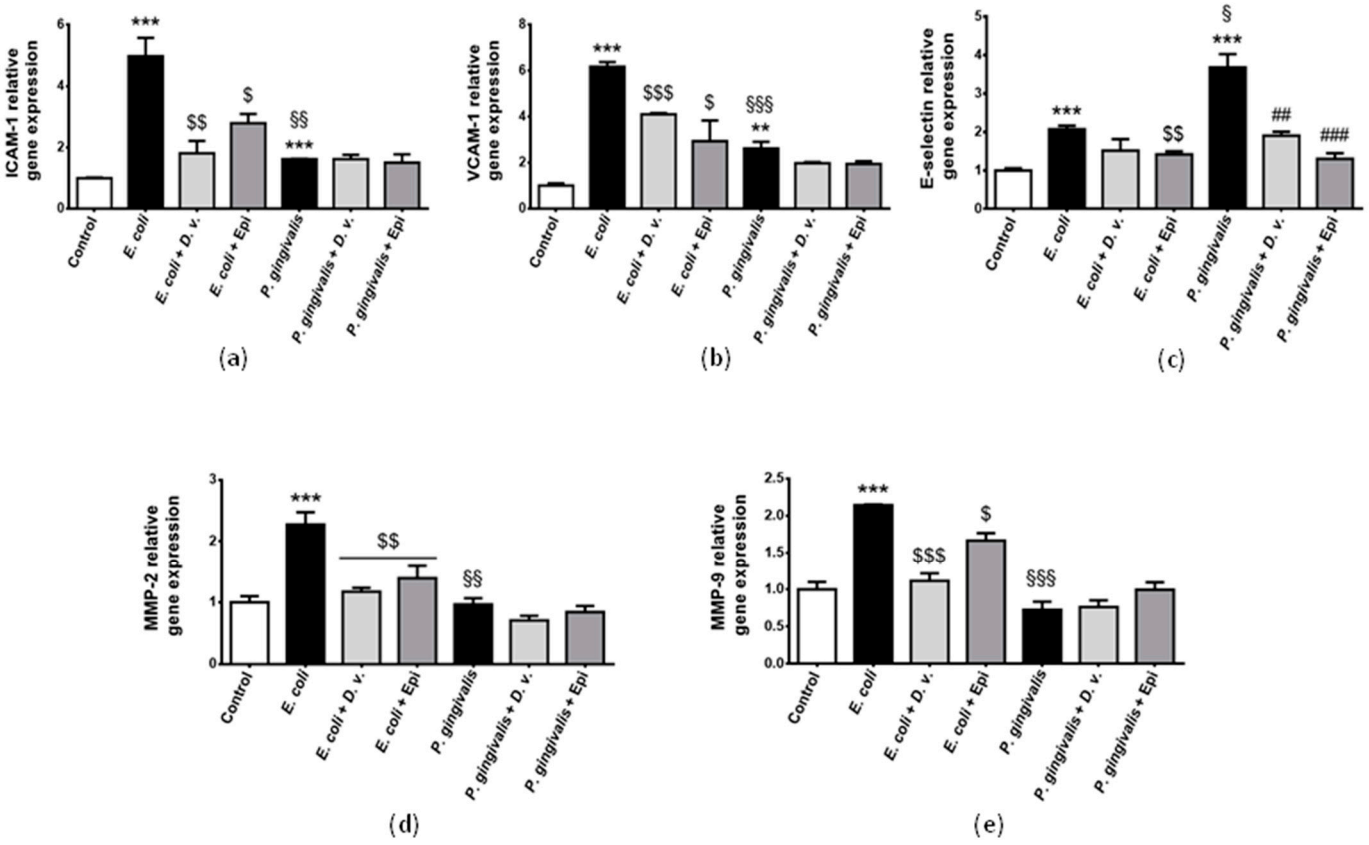

Figure 4. Effect of LPSs and polyphenols on the production of adhesion molecules and matrix-metalloproteinases in cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 24 h. Then, the expression of genes coding for ICAM-1 (**a**), VCAM-1 (**b**), E-selectin (**c**), MMP-2 (**d**) and MMP-9 (**e**) were measured by RT-qPCR and normalized to *GAPDH* gene expression. Data are means ± SEM of three independent experiments. **: $p < 0.01$ and ***: $p < 0.005$ as compared to the Control. $: $p < 0.05$, $$: $p < 0.01$ and $$$: $p < 0.005$ as compared to *E. coli*. ##: $p < 0.01$ and ###: $p < 0.005$ as compared to *P. gingivalis*. §: $p < 0.05$, §§: $p < 0.01$ and §§§: $p < 0.005$ as compared to *E. coli*.

The endothelial–leukocyte interaction that facilitates the migration of immune cells to the site of inflammation is favored by metalloproteinases, including MMP-2 and MMP-9. These enzymes are involved in the degradation of the extracellular matrix and serve as pro-inflammatory biomarkers. Our data indicate that only *E. coli* LPSs significantly enhanced *MMP-2* (Figure 4d) and *MMP-9* (Figure 4e) gene expression. This enhancement was limited by *D. viscosa* extract and epicatechin.

In endothelial cells, iNOS promotes the synthesis of NO, which participates in oxidative stress and eNOS uncoupling, leading to a reduction in vasodilation. Given the increase in *iNOS* gene expression caused by both LPSs, and particularly by *P. gingivalis* LPSs, which upregulated it by a 10-fold factor (Table 2), iNOS protein levels were measured. Western blot analysis shows that both LPSs led to a significant elevation in iNOS protein levels in cerebral endothelial cells, consistent with *iNOS* gene expression increase (Figure 5a,b). *D. viscosa* and epicatechin abolished this alteration in iNOS protein levels under both LPSs conditions, supporting their anti-inflammatory role in endotoxemic conditions.

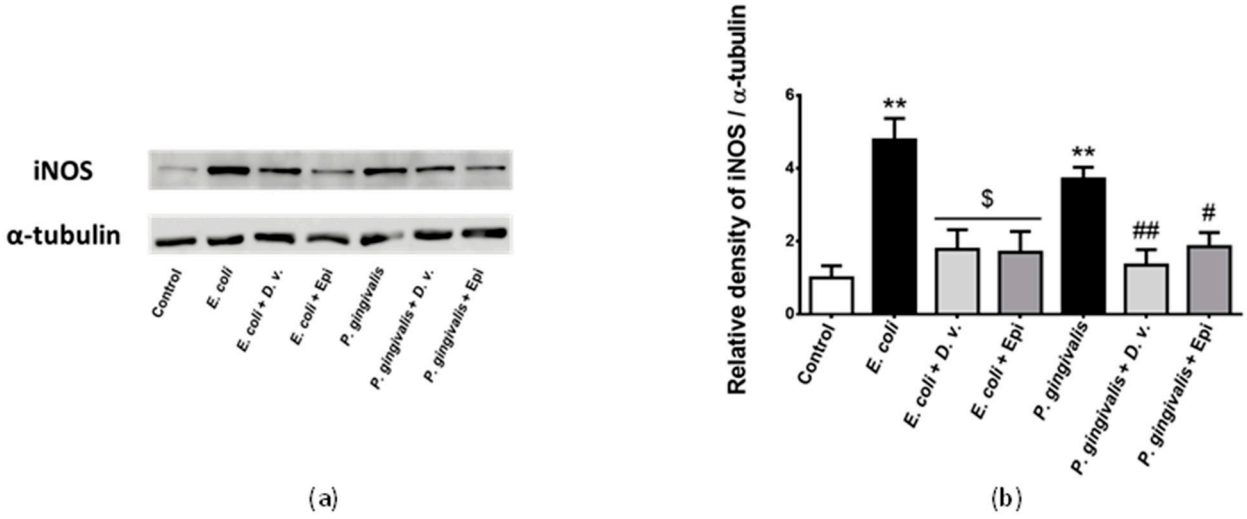

Figure 5. Effect of LPSs and polyphenols on iNOS protein levels in cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 24 h. Then, iNOS protein levels were determined by Western blot analysis (**a**) and normalized to α-tubulin housekeeping protein levels (**b**). Data are means ± SEM of three independent experiments. **: $p < 0.01$ as compared to the Control. $: $p < 0.05$ as compared to *E. coli*. #: $p < 0.05$ and ##: $p < 0.01$ as compared to *P. gingivalis*.

Taken together, these data show that *E. coli* LPSs exerted the most detrimental effects on inflammatory markers, except for TLR2, iNOS and E-selectin, which were more affected by *P. gingivalis* LPSs. Interestingly, *D. viscosa* extract and epicatechin protected cerebral endothelial cells by attenuating LPSs-mediated pro-inflammatory response.

3.4. Effect of LPSs and Polyphenols on Oxidative Stress Markers in Cerebral Endothelial Cells

A close link has been established between inflammation and oxidative stress, notably through the interaction of LPS-binding TLRs and NOX4 which is recognized as a key free radical-producing enzyme at the endothelial level. We evaluated the effect of *E. coli* and *P. gingivalis* LPSs in combination with or without polyphenols on ROS levels in cerebral endothelial cells. It was found that both LPSs led to an increase in intracellular ROS levels after 1 h of exposure (Figure 6a), whereas only *E. coli* LPSs still significantly deregulated ROS levels after 3 h of treatment (Figure 6b). This result shows that LPSs induced oxidative stress, with a time-dependent effect for *P. gingivalis* LPSs. Notably, *D. viscosa* polyphenolic extract and epicatechin limited the deleterious action of LPSs by normalizing ROS levels. Otherwise, both LPSs caused an increase in the mRNA levels of NOX2 and NOX4, with *E. coli* LPSs exerting the more damaging effect on the *NOX4* gene (Table 3). *E. coli* LPSs also upregulated the expression of genes encoding the antioxidant enzymes Cu/ZnSOD, MnSOD, catalase and GPx, but not HO-1, and the sensitive-redox transcription factor Nrf2. In parallel, LPSs of *P. gingivalis* altered the expression of genes coding for MnSOD, catalase,

GPx, HO-1 and Nrf2, without impact on the *Cu/ZnSOD* gene. Collectively, these findings indicate that *E. coli* and *P. gingivalis* LPSs induced oxidative stress in cerebral endothelial cells by affecting the production of ROS and actors of the antioxidant defense system, with *E. coli* LPSs exhibiting the most deleterious action. *D. viscosa* polyphenolic extract and epicatechin exerted antioxidant effects by limiting the alteration in redox markers in both LPSs conditions.

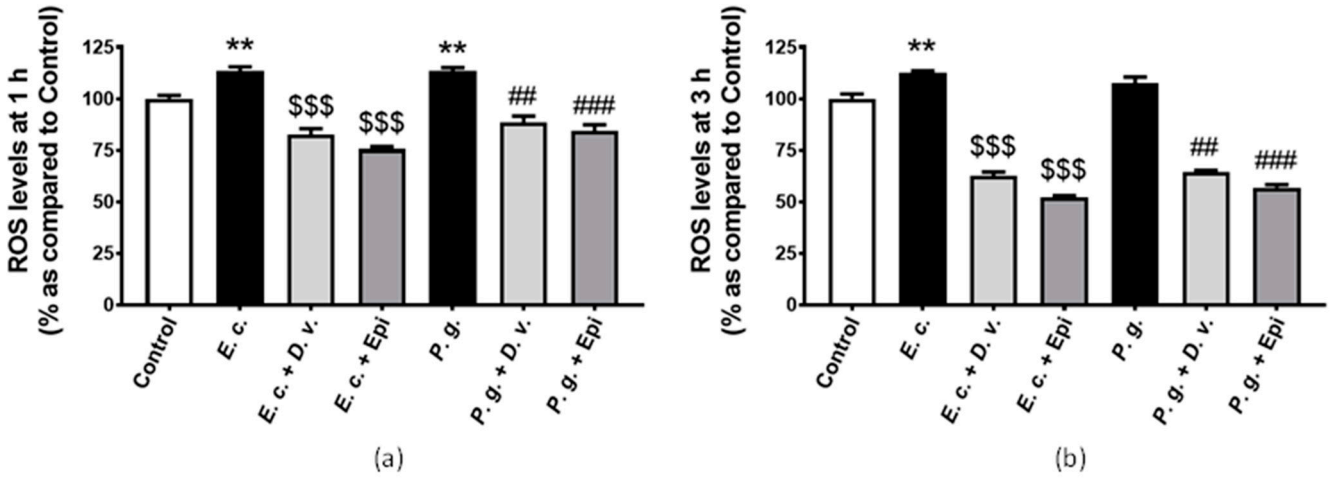

Figure 6. Effect of LPSs and polyphenols on intracellular ROS production in cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 1 h (**a**), 3 h (**b**). Then, intracellular ROS levels were quantified by DCFH-DA assay. **: $p < 0.01$ as compared to the Control. $$$: $p < 0.005$ as compared to *E. coli*. ##: $p < 0.01$ and ###: $p < 0.005$ as compared to *P. gingivalis*.

Table 3. Effect of LPSs and polyphenols on oxidative stress markers in cerebral endothelial cells.

Relative Gene Expression	Control	*E. coli*	*E. coli* + *D. v.*	*E. coli* + Epi	*P. gingivalis*	*P. gingivalis* + *D. v.*	*P. gingivalis* + Epi
NOX2	1.00 ± 0.07	5.08 ± 0.60 ***	1.90 ± 0.09 $	1.60 ± 0.10 $$$	6.61 ± 0.12 ***	0.49 ± 0.10 ###	0.46 ± 0.04 ###
NOX4	1.00 ± 0.09	6.43 ± 1.10 **	2.13 ± 0.14 $$	3.84 ± 0.24 $	3.30 ± 0.40 **§	1.87 ± 0.15 #	1.65 ± 0.04 ##
Cu/ZnSOD	1.00 ± 0.40	2.20 ± 0.10 *	1.30 ± 0.05 $$	1.38 ± 0.20 $$	1.56 ± 0.20 §	3.28 ± 0.03 ###	2.88 ± 0.20 ##
MnSOD	1.00 ± 0.10	2.23 ± 0.20 **	1.64 ± 0.03 $	1.33 ± 0.08 $$	2.11 ± 0.08 ***	1.71 ± 0.20	1.75 ± 0.10
Catalase	1.00 ± 0.09	2.39 ± 0.20 **	1.30 ± 0.10 $$	1.70 ± 0.10 $	0.92 ± 0.05 §§	1.61 ± 0.10 ##	1.88 ± 0.10 ###
GPx	1.00 ± 0.11	3.49 ± 0.40 ***	1.56 ± 0.04 $$	2.15 ± 0.08 $	1.39 ± 0.09 *§§	2.35 ± 0.01 ###	2.98 ± 0.5 ##
HO-1	1.00 ± 0.04	0.81 ± 0.07	1.66 ± 0.07 $$	1.75 ± 0.09 $$	0.63 ± 0.05 **	1.63 ± 0.04 ###	1.30 ± 0.10 ##
Nrf2	1.00 ± 0.10	3.02 ± 0.40 **	1.38 ± 0.11 $	1.89 ± 0.19	2.50 ± 0.20 **	1.29 ± 0.10 ##	1.30 ± 0.02 ##

Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 24 h. Then, the expression of genes coding for factors involved in oxidative stress were measured by RT-qPCR and normalized to *GAPDH* gene expression. Data are means ± SEM of three independent experiments. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.005$ as compared to the Control. $: $p < 0.05$, $$: $p < 0.01$ and $$$: $p < 0.005$ as compared to *E. coli*. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.005$ as compared to P. gingivalis. §: $p < 0.05$ and §§: $p < 0.01$ vs. *E. coli*.

3.5. Effect of LPSs and Polyphenols on Vasoactive Markers in Cerebral Endothelial Cells

Endothelial cells are a major source of the vasodilator NO produced by the enzyme eNOS. Oxidative stress and inflammatory conditions may alter eNOS activity and cause an imbalance between NO vasodilator and ET-1 vasoconstrictor levels. We evaluated the effect of LPSs and polyphenols on the production of these vasoactive markers. Results show that both LPSs caused a slight but significant decrease in intracellular NO levels after 3 h of exposure, without effect after a treatment of 1 h (Figure 7a,b). *D. viscosa* extract and epicatechin upregulated basal NO levels after 1 h treatment while counteracting their deregulation by LPSs after 3 h exposure. This protective effect of polyphenols may

result from their capacity to enhance the production of eNOS in endotoxemic conditions (Figure 7c). Moreover, polyphenols limited the up-regulation of ET-1 production mediated by both LPSs, noting that *E. coli* LPSs' action was more pronounced than that of *P. gingivalis* LPSs (Figure 7d).

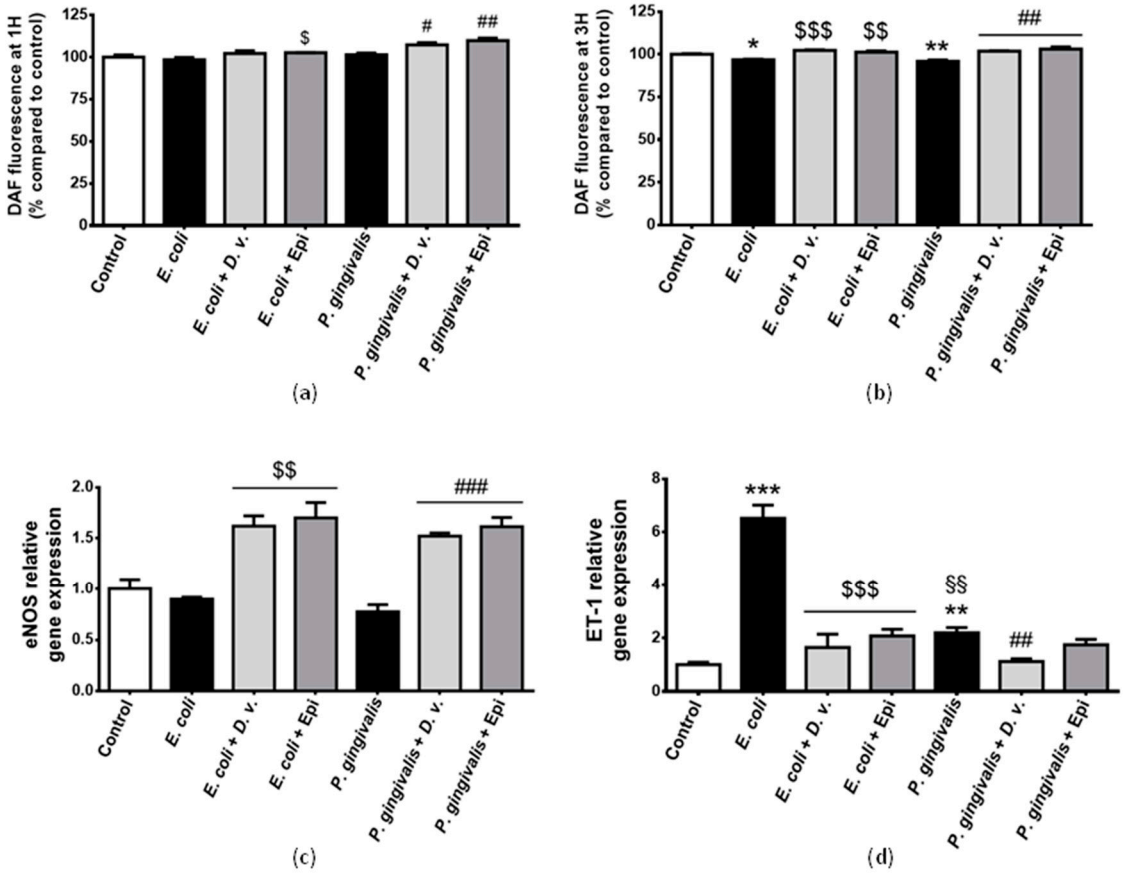

Figure 7. Effect of LPSs and polyphenols on the production of vasoactive markers in cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 μg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 μM GAE) or pure epicatechin (Epi, 10 μM) for 1, 3 and 24 h. Then, the intracellular nitric oxide (NO) levels were measured by DAF-FM assay at 1 h (**a**) and 3 h (**b**). The expression of genes coding for endothelial nitric oxide synthase (eNOS) (**c**) and endothelin-1 (ET-1) (**d**) were measured by RT-qPCR and normalized to *GAPDH* gene expression. Data are means ± SEM of three independent experiments. *: p <0.05, **: $p < 0.01$ and ***: $p < 0.005$ as compared to the Control. $: $p < 0.05$, $$: $p < 0.01$ and $$$: $p < 0.005$ as compared to *E. coli*. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.005$ as compared to *P. gingivalis*. §§: $p < 0.01$ as compared to *E. coli*.

3.6. Effect of LPSs and Polyphenols on Tight Junctions and Permeability of Cerebral Endothelial Cells

The tight junction proteins occludin, claudin-5, ZO-1 and ZO-2 play a pivotal role for maintaining the integrity of cerebral endothelial cells. Our results show that both LPSs led to a decrease in the expression of the gene coding for occludin (Figure 8a). In addition, *P. gingivalis* LPSs caused a decrease in *claudin-5* gene expression (Figure 8b) and *E. coli* LPSs decreased that of *ZO-1* gene (Figure 8c). No change in *ZO-2* gene expression was depicted in LPSs-treated cells (Figure 8d). Concomitantly, both LPSs led to increased FITC-Dextran permeability, suggesting a loss of endothelial cell integrity (Figure 8e). Importantly, *D. viscosa* polyphenolic extract and epicatechin improved the production of the tight junction proteins and attenuated the permeability of cerebral endothelial cells under endotoxemic conditions.

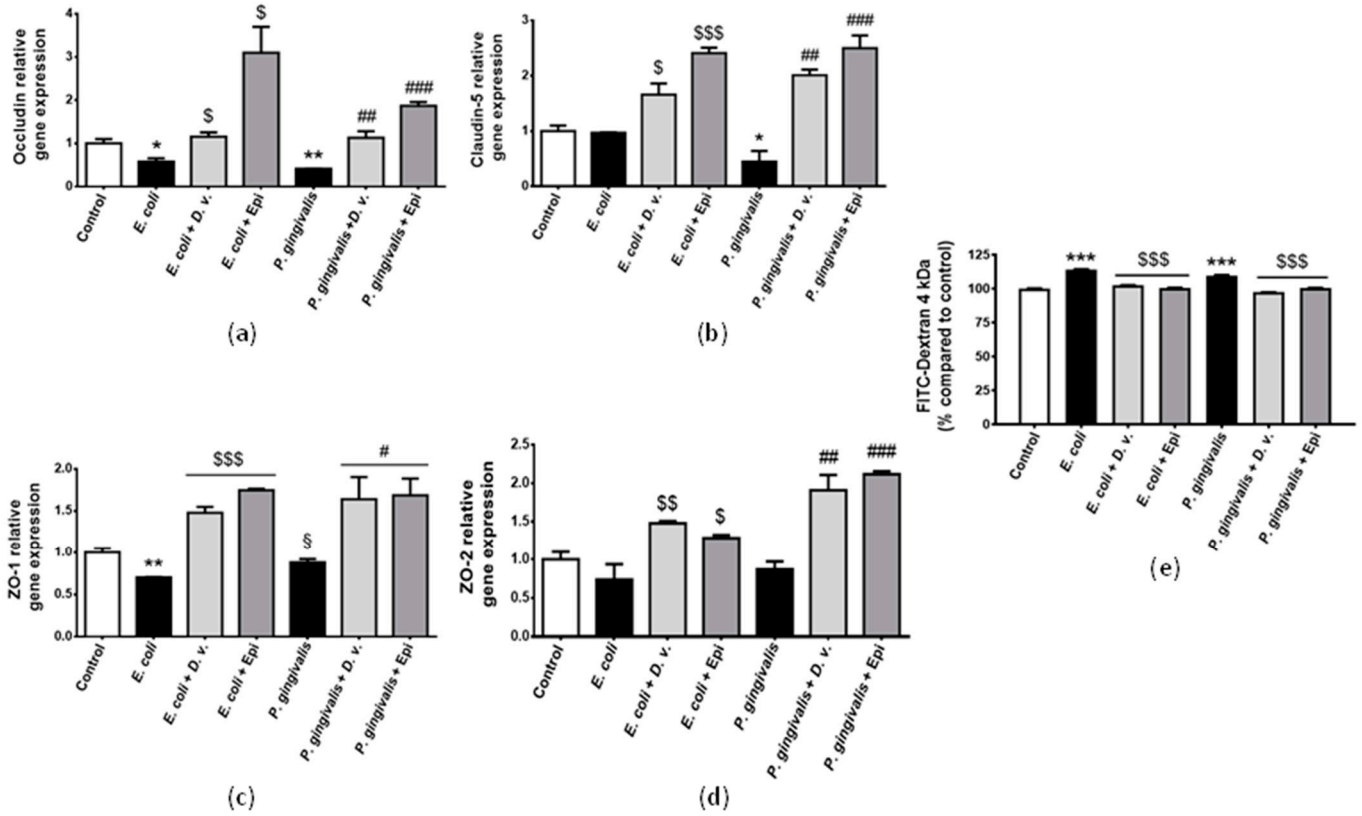

Figure 8. Effect of LPSs and polyphenols on the production of tight junction proteins and permeability in cerebral endothelial cells. Cells were exposed to the vehicle (Control) or LPSs from *E. coli* and *P. gingivalis* (10 µg/mL) in the presence or absence of the polyphenol-rich extract of *D. viscosa* (*D.v.*, 10 µM GAE) or pure epicatechin (Epi, 10 µM) for 24 h. Then, the relative expression of genes coding for claudin-5 (**a**), occludin (**b**), ZO-1 (**c**) and ZO-2 (**d**) was measured by RT-qPCR and normalized to *GAPDH* gene expression. The relative endothelial permeability (**e**) was determined by using FITC-Dextran assay and expressed as the percentage of the Control. Data are means ± SEM of three independent experiments. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.005$ as compared to Control. $: $p < 0.05$, $$: $p < 0.01$ and $$$: $p < 0.005$ as compared to *E. coli*. #: $p < 0.05$, ##: $p < 0.01$ and ###: $p < 0.005$ as compared to *P. gingivalis*. §: $p < 0.05$ vs. *E. coli*.

4. Discussion

The dysfunction of cerebral endothelial cells plays a key role in the BBB disruption and the development of neurological complications in ischemic stroke. Understanding the principal mediators and molecular mechanisms involved in cerebral endothelial cell dysfunction is crucial for the development of novel therapeutics. This study demonstrates (1) the deleterious effects of both *E. coli* and *P. gingivalis* LPSs on the inflammatory and redox status, the production of vasoactive markers and the permeability of cerebral endothelial cells; and (2) the protective role of the polyphenol-rich extract of the *D. viscosa* medicinal plant and its predominant component, epicatechin, against LPSs' detrimental action. Depending on the bacterial origin of LPSs, a differential response of cerebral endothelial cells was depicted.

First, our results provide evidence for the ability of LPSs to affect the inflammatory state of cerebral endothelial cells through the deregulation of key mediators involved in the pro-inflammatory TLRs/NFκB pathway. Globally, LPSs altered the expression of genes coding for TLR4, NFκB, IL-1β, IL-6, TNF-α, MCP-1, IL-10, COX2 and iNOS. More specifically, we found an increase in the expression of the genes encoding the innate immunity receptor TLR4 and its adaptor MyD88 caused by *E. coli* LPSs, while *P. gingivalis* LPSs slightly elevated the expression of the genes encoding both TLR4 and TLR2 receptors but not MyD88.

Notably, the rate of *NFκB*, *IL-6*, and *MCP-1* gene expression was significantly higher in response to *E. coli* LPSs compared to *P. gingivalis* LPSs, whereas this pattern was not observed for *TNF-α* and *iNOS* genes. This result is in accordance with the literature data reporting that *P. gingivalis* LPSs may be able to (i) reach the bloodstream through loss of alveolar bone following periodontitis or through the alimentary bolus due to intestinal leakage induced by gut microbiota dysbiosis, (ii) activate the innate immunity receptors TLR2 and TLR4, and (iii) set on a pro-inflammatory response via NFκB activation [43,44]. It was established that *P. gingivalis* LPSs activate both innate immunity receptors due to their structural affinity. Indeed, the lipid portion (lipid A) structure comprising LPSs modulates their affinity for TLRs. Regarding *E. coli* LPSs, the lipid A is composed of a di-phosphorylated glucosamine disaccharide hexa-acylated with six fatty acids of 12–14 carbon units and specifically activates TLR4. Concerning *P. gingivalis* LPSs, the lipid A is a mono-phosphorylated penta-acylated with five fatty acids of 15–17 carbon units and is able to recruit both TLR2 and TLR4, depending on the cellular types and the ratio of both TLRs. Given these structural differences, it has been reported that the lipid A of *P. gingivalis* LPSs exhibits a weaker endotoxicity than the lipid A of *E. coli* LPSs [45,46]. A number of studies have found that a lipid A structure with a di-phosphorylated, hexa-acylated disaccharide backbone consisting of two β(1-6)-linked glucosamine residues is responsible for the highest toxicity of LPSs, as this configuration allows optimal recognition by receptor molecules in the TLR4 signaling pathway [47–49]. Furthermore, the number of fatty acid chains is a main factor regulating the relationship between lipid A structure and LPSs' toxicity. Lipid A units with six lipid chains often show the best immune-stimulation [50–52], whereas five lipid chains can show a 100-fold reduction in biological toxicity [53]. Notably, altering the inner-core sugars [54–57] or removing phosphate groups [58–60] significantly reduces LPSs' toxicity, resulting in a less active immune response due to reduced cytokine production. This is consistent with our findings on IL-6 and MCP-1 production, which was significantly more affected by *E. coli* LPSs than by *P. gingivalis* LPSs. Besides the control of cytokine synthesis, the NFκB transcriptional factor activated during LPSs conditions is known to enhance the production of enzymes like COX-2 and iNOS, which generate mediators of inflammation. Based on our results, both *E. coli* and *P. gingivalis* LPSs led to an elevation in *COX-2* and *iNOS* gene expression. In particular, *P. gingivalis* LPSs led to a 10-fold increase in *iNOS* gene expression while a 4-fold rise was detected for *E. coli* LPSs compared to control cells. The effect of *P. gingivalis* LPSs was significantly more pronounced than that of *E. coli* LPSs. However, this differential capacity of *P. gingivalis* and *E. coli* LPSs to alter *iNOS* gene expression was not confirmed by the Western blot analysis of iNOS protein, suggesting that the experimental conditions need to be kept in mind. Indeed, given that *iNOS* gene expression was still critically elevated after 24 h exposure to LPSs, it would have been relevant to assess the iNOS protein level beyond 24 h to better describe the differential capacity of both LPSs. Discrepancies between iNOS mRNA and protein levels are well-documented in the literature and reflect the multi-level regulation of iNOS expression. *iNOS* gene transcription is known to be rapidly induced following TLR activation, whereas protein expression is subject to additional regulatory steps at the post-transcriptional and post-translational levels, including modulation of mRNA stability, translation efficiency, and protein turnover [61]. In addition, *P. gingivalis* and *E. coli* LPSs may differentially affect post-transcriptional regulatory pathways, thereby influencing translation or protein stability independently of transcriptional changes. Interestingly, our study demonstrates the ability of *D. viscosa* polyphenolic extract and epicatechin to counteract LPSs-mediated pro-inflammatory response by reducing both TLR2 and TLR4 mRNA levels. However, no significant differences were observed at the magnitude of this reduction between *D. viscosa* extract and epicatechin for both TLRs. These results suggest that the protective effects of *D. viscosa* extract and epicatechin may not be

associated with a preferential inhibition of one TLR over another at the transcriptional level. Interestingly, Chauhan et al. [62] reported that the polyphenol isorhamnetin can directly interact with the hydrophobic binding pocket of myeloid differentiation factor-2 (MD-2), preventing TLR4/MD-2 dimerization and thus inhibiting the TLR4 cascade during *E. coli*-induced sepsis. Further studies would be required to determine whether *D. viscosa* polyphenols and epicatechin differentially modulate TLR2- or TLR4-dependent signaling pathways at the functional level in cerebral endothelial cells under LPSs conditions. The present study also shows that plant polyphenols and epicatechin efficiently reduce LPSs-induced IL-1β, IL-6, TNF-α, MCP-1, COX2 and iNOS production while elevating IL-10 levels. Numerous studies have reported that polyphenols enhance the anti-inflammatory response by reducing the release of pro-inflammatory cytokines in LPSs-treated endothelial cells, via the modulation of NFκB activity and the mitogen-activated protein kinase activation (MAPK) pathway [63–66]. Although there is a lack of data regarding the molecular mechanisms of action of polyphenols in cerebral endothelial cells, the literature data underline that the polyphenol effect may involve cell-surface receptors or transporters. For the polyphenol epigallocatechin gallate, it was demonstrated that it suppresses the LPSs-induced expression of IL-1β, TNF-α and MCP-1 in human cerebral microvascular endothelial cells (hCMECs) through the implication of the 67 kDa laminin receptor, which prevents LPSs-induced NFκB activation [67]. Notably, in the present study, we found that *D. viscosa* extract also contains the polyphenol quercetin, which could contribute to a synergistical effect with epicatechin in the bioactivity of the whole plant extract. Indeed, previously, we reported that quercetin reduces the elevation in NFκB transcriptional activity caused by hyperglycemic conditions in cerebral endothelial cells via the involvement of peroxisome proliferator-activated receptor gamma (PPARγ). The preconditioning of cells with PPARγ antagonist aggravates NFκB transcriptional activity, suggesting a possible regulatory role of polyphenols on the PPARγ/NFκB axis [35]. Moreover, we demonstrated, via a metabolomic analysis of the intracellular contents, the presence of quercetin and its methylated metabolite isorhamnetin in cerebral endothelial cells exposed to quercetin. The preconditioning of cells with an inhibitor of the efflux transporter BCRP changes quercetin and isorhamnetin intracellular levels, while an inhibitor of the Pgp efflux transporter does not affect them [35]. This shows that BCRP may be involved in polyphenol bio-accessibility to cerebral endothelial cells. It will be of high interest to compare quercetin and epicatechin uptake by cerebral endothelial cells to discuss their possible synergistic contributions and the advantage of the use of a mixture of natural polyphenolic compounds contained in the whole plant extract for nutritional strategies. In parallel, the anti-inflammatory properties of *D. viscosa* polyphenolic extract and epicatechin in LPSs-treated cells may result from their ability to attenuate oxidative stress. Indeed, the crosstalk between oxidative stress and inflammation is well-established [68–70]. We found that *D. viscosa* polyphenolic extract and epicatechin exhibited DPPH-radical scavenging and reducing capacities that were dose-dependent, and could have contributed to their capacity to reduce intracellular ROS levels time-dependently in LPSs conditions. According to the literature data, polyphenols, which act as scavengers of ROS, abrogate the nuclear translocation of NFκB by modulating the phosphorylation of the NFκB inhibitor protein (IκB) in human umbilical endothelial cells or ex vivo aortic vessels exposed to oxidative stress conditions [30,71]. Furthermore, antioxidant polyphenols may exert anti-inflammatory properties via their ability to activate the redox-sensitive transcriptional factor Nrf2, which is known to down-regulate the expression of genes coding for NFκB and cytokines [70]. Our data show that polyphenols improve the production of Nrf2 deregulated by LPSs conditions.

In the crosstalk established between oxidative stress and inflammation, oxidative stress is recognized as a contributing element to inflammatory condition, which in turn

may play a role in cellular dysfunction [68–70]. Here, we found that both *E. coli* and *P. gingivalis* LPSs induced oxidative stress by enhancing intracellular ROS levels and the expression of genes coding for the ROS-producing enzymes NOX2 and NOX4. For NOX4 markers, *E. coli* LPSs were more detrimental than *P. gingivalis* LPSs. This could contribute to explain why intracellular ROS levels were time-dependently altered by *P. gingivalis* LPSs at 1 h but returned to baseline by 3 h, while the effects of *E. coli* LPSs were sustained. The normalization of intracellular ROS levels at 3 h under *P. gingivalis* LPSs conditions is also in line with the observed expression of other redox-related genes at 24 h following *P. gingivalis* LPSs exposure, suggesting a more transient oxidative stress response compared with *E. coli* LPSs conditions. Moreover, both LPSs elevated the expression of the gene coding for Nrf2, showing that the endogenous antioxidant defense system was solicited. Interestingly, *P. gingivalis* LPSs had no effect on the expression of the gene encoding the cytosolic antioxidant enzyme Cu/ZnSOD, but significantly increased that of the mitochondrial MnSOD. This is consistent with data showing that *P. gingivalis* LPSs treatment led to an elevation in mitochondrial ROS levels, followed by the production of inflammatory cytokines in human gingival fibroblasts [72]. It has been reported that the protein P53 activated by *P. gingivalis* LPSs formed a feedback loop with ROS, participating together in the inflammatory response, with mitochondrial ROS generation being a key signaling event in LPSs-induced pro-inflammatory response [73,74]. According to our present data, *E. coli* LPSs modulated the expression of genes encoding different antioxidant enzymes, including Cu/ZnSOD, MnSOD, catalase and GPx, but not HO-1. In parallel, *P. gingivalis* LPSs modulated only *GPx* and *HO-1* gene expression. Overall, these results are in agreement with our finding that *P. gingivalis* LPSs do not have the same immunostimulatory effects as *E. coli* LPSs, resulting in a differential activation of the cellular antioxidant system during oxidative stress conditions. Notably, our data show that *P. gingivalis* LPSs significantly downregulated *HO-1* gene expression. This is in agreement with the results from the study of Li et al. [75] reporting that *P. gingivalis* LPSs induce atherogenic effects in macrophages and are associated with a decrease in *HO-1* gene expression. Furthermore, the development of foam cells and exacerbation of atherosclerosis are caused by the absence of HO-1 [76]. Interestingly, our study highlights the antioxidant capacity of *D. viscosa* polyphenolic extract and epicatechin in cerebral endothelial cells exposed to LPSs by lowering intracellular ROS levels triggered by LPSs. Such antioxidant properties may be explained by their ability to scavenge free radicals, as discussed above, and/or preserve the endogenous antioxidant defense system. Consistently, our data show that *D. viscosa* polyphenolic extract and epicatechin limited the alteration in the production of several redox factors comprising Nrf2, MnSOD, catalase, GPx and HO-1 in *E. coli* LPSs-exposed cells. Similarly, polyphenols counteracted Nrf2 change induced by *P. gingivalis* LPSs. Polyphenols enhanced the expression of genes encoding all antioxidant enzymes, except for MnSOD. Notably, *HO-1* gene expression, which was significantly downregulated by LPSs from *P. gingivalis*, was elevated in the presence of plant polyphenols and epicatechin. The literature data demonstrated that HO-1 plays a crucial role in the inflammatory response and oxidative stress. Evidence suggests that HO-1 production can be upregulated through the phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathways, resulting in an anti-inflammatory response [77,78]. Indeed, the enhancement of HO-1 pathway inhibits inflammasome activation and thereby blocks the downstream inflammatory response involving NFκB, IL-1β, IL-6, TNF-α, COX2 and iNOS induced by LPSs [79–81]. Regarding the protective role of polyphenols such as epicatechin on HO-1 gene expression, our data are consistent with the literature supporting a functional link between polyphenols and HO-1 pathway. Using HO-1- and Nrf2-deficient mice exposed to an experimental ischemic stroke, Shah et al. [82] demonstrated that the neuroprotective effects of epicatechin were largely abolished in the absence of either HO-1

or Nrf2. This indicates that HO-1/Nrf2 pathway activation substantially contributes to the beneficial effects of epicatechin. Furthermore, Bao et al. [83] reported that the pharmacological inhibition of HO-1 using zinc protoporphyrin (ZnPP) partially attenuated the anti-inflammatory effects of the polyphenol epigallocatechin gallate during an experimental intracerebral hemorrhage in mice. Taken together, these data support the contribution of HO-1 to the protective phenotype observed in our model, although additional HO-1-independent mechanisms are not excluded. Further experiments using HO-1 inhibition or knockdown strategies would be helpful to determine to what extent the anti-inflammatory effects of plant polyphenols and epicatechin are mediated by HO-1 in our model of cerebral endothelial cells under LPSs conditions.

Under inflammatory conditions, endothelial cells become highly vasoconstrictive, prothrombotic, and pro-adhesive [84]. Upregulated adhesion molecules such as ICAM-1, VCAM-1 and E-selectin by inflammatory stimuli in endothelial cells induce the recruitment of inflammatory cells to the endothelium, ultimately inducing vascular dysfunction [85]. Our results demonstrate that LPSs exposure enhanced the expression of genes encoding adhesion molecules (VCAM-1, ICAM-1 and E-selectin). *P. gingivalis* LPSs significantly affected the expression of the gene encoding E-selectin, suggesting that *P. gingivalis* bacteria may have adapted to improve its cellular adhesion and promote host–pathogen interactions via virulence factor, including LPSs [86]. Crucially, endothelial cells secrete matrix-degrading enzymes such as MMPs in response to inflammatory cytokines. This leads to a decrease in the amount of extracellular matrix proteins, which makes it easier for leukocytes to migrate across the endothelium. According to our data, only *E. coli* LPSs affected the expression of *MMP-2* and *MMP-9* genes. This is probably because *E. coli* LPSs have stronger immunostimulatory properties than *P. gingivalis* LPSs. LPSs have been found to activate activator protein 1 (AP-1) by inducing MMP-9 production and cell migration through the TLR4/PI3K/Akt/p38 MAPK and JNK1/2 pathways [87]. In addition, LPSs can directly activate MMP-2 through the NFκB-dependent pathway [88]. Notably, ROS also affect MMP-2 and MMP-9 activity [89]. The literature data provide evidence for polyphenols' ability to target NFκB and Nrf2 activity, and to improve vascular inflammation. Calabriso et al. [90] demonstrated that polyphenolic extracts downregulated the production of the adhesion molecules ICAM-1, VCAM-1 and E-selectin, as well as MCP-1 at mRNA and protein levels in human endothelial cells [91]. The decrease in endothelial inflammatory gene expression was related to the inhibition of NFκB and AP-1 activation [90]. Li et al. [67] demonstrated that the polyphenol epigallocatechin gallate significantly suppressed the LPSs-induced expression of VCAM-1 and ICAM-1 in hCMECs. Furthermore, the polyphenol resveratrol attenuated endothelial inflammation by reducing ICAM-1 expression and monocyte adhesiveness to TNF-α-treated endothelial cells. This protective effect was mediated partly through the AMPK/p38 MAPK/NFκB pathway [92]. According to Hu et al. [93], phytochemicals with antioxidant and anti-inflammatory properties, such as saponins, may act as an external regulator by activating PI3K/Akt, which in turn activates the Nrf2 antioxidant defense mechanism. To reduce LPSs-induced blood–brain barrier disruption and monocyte adhesion to cerebral endothelial cells, these phytochemicals also block NFκB inflammatory signaling. This could be extrapolated to polyphenols exhibiting anti-inflammatory and antioxidants properties [94,95]. Remarkably, research has shown that the polyphenol quercetin activates Nrf2 and upregulates the expression of antioxidant enzymes, in part through p38 MAPK mediation, thereby suppressing LPSs-induced oxidant generation and the expression of adhesion molecules (E-selectin and ICAM-1) in human aortic endothelial cells. The suppressive effect of quercetin on adhesion molecule expression is not due to the inhibition of NFκB activation, but rather to the antioxidant-independent activities of HO-1 [96]. Endothelial cells isolated from HO-1-deficient mice show higher levels of

intracellular ROS as well as VCAM-1, ICAM-1 and E-selectin during exposure to TNF-α compared to endothelial cells isolated from wild-type mice [97]. Altogether, the literature data indicate that the HO-1 pathway has a dual role in vascular damage prevention, regulating LPSs-induced oxidative stress and inflammation in cerebral endothelial cells via the Nrf2 and/or NFκB pathways. The relative importance of each pathway may differ depending on the cellular type, endotoxic potential of LPSs and polyphenol structure.

Endothelial dysfunction refers to a change in the available levels of NO metabolism or a discrepancy between endothelial-derived relaxing and constrictor molecules, including ET-1. Reduced NO production, oxidative stress-induced ROS and inflammation are all contributors in dysfunctional endothelial vasodilatory responses [98]. Here, we found that LPSs affected NO intracellular levels and the expression of the *ET-1* gene, but did not modulate the expression of the gene coding for eNOS that produces NO. This finding is in contrast to the results of Lee et al. [99], who demonstrated that a treatment with LPSs for 12 h led to a decrease in eNOS mRNA and protein levels via mRNA destabilization. This suggests that NFκB-responsive miRNAs may play a role in controlling eNOS expression even in the absence of promoter activity. Notably, eNOS mRNA half-life is decreased upon exposure to inflammatory stimulants [99–101]. This could explain why we did not find a variation in *eNOS* gene expression during a longer treatment with LPSs for 24 h, while Lee et al. [99] proceeded at 12 h. Our study shows that *P. gingivalis* LPSs had a less pronounced effect on *ET-1* gene than *E. coli* LPSs, which is in line with the inflammatory and redox status of cerebral endothelial cells that were less altered by *P. gingivalis* LPSs. Interestingly, our results demonstrate that *D. viscosa* polyphenolic extract and epicatechin counteracted the LPSs-induced decrease in NO generation. Meanwhile, polyphenols increased the expression of the gene encoding eNOS and prevented LPSs-induced overexpression of *ET-1* gene. The activation of the Nrf2/HO-1 pathway and/or the inhibition of the NFκB pathway is likely the mechanism by which polyphenols protected against altered production of vasoactive markers in LPSs conditions. It was reported that vasoconstriction is reduced and vascular function is improved in diabetic mouse and Nrf2-deficient rat models when Nrf2 is activated. Polyphenols are known to modulate Nrf2 production [102,103], which lowers ROS levels and in turn increases NO generation, suggesting a negative correlation between intracellular ROS levels and NO vasodilator production [32]. The literature data have demonstrated that polyphenols such as resveratrol could improve endothelial function by increasing NO bioavailability and reducing ROS scavenging [104]. Accordingly, other studies showed that polyphenols are able to potently modulate the expression of enzymes such as HO-1 via Nrf2 activation. Increased HO-1 production has been shown to improve metabolic diseases [105]. The association between HO-1 and eNOS is strengthened and the connection between eNOS and its upstream kinase Akt is facilitated by this HO-1 overexpression. This ultimately leads to the increased phosphorylation of eNOS and NO generation [106]. Furthermore, polyphenols may improve the production of eNOS under LPSs conditions by limiting the NFκB pathway, which acts as a suppressor of *eNOS* gene expression [99].

Numerous studies demonstrated that oxidative stress [107–109] and inflammatory mediators [110] compromise the integrity of endothelial tight junctions contributing to BBB dysfunction. Our study demonstrates that both *P. gingivalis* and *E. coli* LPSs modulated the permeability to the FITC-Dextran marker and decreased the production of occludin tight junction protein. While both LPSs did not modulate the production of ZO-2, only *E. coli* LPSs reduced that of ZO-1. In parallel, *P. gingivalis* LPSs specifically altered claudin-5 production, suggesting differential deleterious roles of both LPSs on integrity markers. This is in line with literature studies showing that LPSs may affect tight junction proteins differently depending on the cellular state and the localization of tight junctions [111]. Moreover,

the differential action of both LPSs could be related to the differential pro-inflammatory effects mediated, as we depicted the activation of TLR4 for *E. coli* LPSs and that of both TLR2 and TLR4 for *P. gingivalis* LPSs. This result is in agreement with the literature data demonstrating that inflammatory mediators alter BBB function. Additionally, *P. gingivalis* LPSs are known to be a major virulence factor inducing the inflammatory response in periodontal disease, exerting a strong stimulatory effect on the breakdown of the epithelial barrier and possibly having a specific adverse effect on endothelial cells [43,44]. Our study demonstrates that *D. viscosa* polyphenolic extract and epicatechin were able to abrogate the deleterious effects of LPSs on the production of tight junction proteins and cellular permeability, highlighting their interest for strategies aiming to improve protection against cerebral endothelial dysfunction associated with BBB impairment. In accordance, Li et al. [67] confirmed this finding by showing that the polyphenol epigallocatechin gallate induced the expression of tight junction proteins (occludin and claudin-5) in hCMECs exposed to LPSs. Previous research has also suggested polyphenol potential benefits to preserve BBB integrity and brain homeostasis during cerebrovascular disorders, although their ability to cross the BBB, reach brain cells and exert neuroprotective effects in stroke conditions have not been sufficiently investigated [112–114]. The beneficial effects of polyphenols can be attributed to their ability to activate the HO-1 pathway through Nrf2, along with the PI3k/Akt, MAPK and NFκB pathways, thereby reducing the generation of ROS and cytokines [115].

The limitations of the study warrant mention. Although the present study primarily relies on transcriptional analyses, it should be acknowledged that protein-level validation of adhesion molecules and tight junction components would further strengthen the conclusions. Further studies will aim to confirm these effects at the protein and functional levels using complementary approaches. Given that the biological effects of *D. viscosa* polyphenols and epicatechin may depend on their ability to reach their molecular targets in cerebral endothelial cells, it will be important to assess their bioavailability and cellular accessibility rate. Interestingly, we previously demonstrated the capacity of the polyphenol quercetin to cross the cellular membrane of bEnd3 cerebral endothelial cells with the involvement of the BCRP efflux transporter [35]. We also detected caffeic acid and its methylated metabolite ferulic acid in the infarcted cerebral hemisphere of mice intraperitoneally treated with caffeic acid or the *Antirhea borbonica* medicinal plant's polyphenolic extract during an experimental ischemic stroke [37]. Similarly, other authors reported the detection of polyphenols at nanomolar concentrations in the brain [116], providing evidence for their capacity to pass through the BBB.

5. Conclusions

This study demonstrates that *E. coli* and *P. gingivalis* LPSs led to cerebral endothelial cell dysfunction via the modulation of several molecular targets involved in pro-inflammatory response, oxidative stress, deregulation of vasoactive markers and endothelial permeability. The differential deleterious effects of both LPSs may depend on their bacterial origin. Thus, endotoxemic conditions that occur during oral and gut microbiota dysbiosis may contribute as a critical factor aggravating cerebrovascular damage during stroke. Importantly, our data highlight the protective effects of polyphenols, such as those present in the *D. viscosa* medicinal plant, including epicatechin, against LPSs deleterious action. By showing the potential of polyphenols to attenuate cerebral endothelial cell dysfunction under inflammatory conditions, our research contributes to offer a new perspective for therapeutic strategies aiming to limit neuroinflammation and brain homeostasis loss. Further studies will be needed in order to better explore polyphenol effects on cerebrovascular disorders re-

lated to ischemic stroke with oxygen-glucose deprivation, as well as on post-stroke cerebral reparation during oral and gut microbiota dysbiosis conditions.

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Abbreviations

BBB	Blood–brain barrier
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein
COX	Cyclooxygenase
Cu/ZnSOD	Copper–zinc superoxide dismutase
D. v.	*Dodonaea viscosa*
DAF-FM	4-amino-5-methylamino-2′,7′-difluorofluorescein
DCFH-DA	2′,7′-dichlorofluorescein diacetate
E. c.	*Escherichia coli*
eNOS	Endothelial nitric oxide synthase
Epi	Epicatechin
E-selectin	Endothelial–leukocyte adhesion molecule
ET-1	Endothelin-1
FITC	Fluorescein isothiocyanate
GAE	Gallic acid equivalent
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT-1	Glucose transporter-type 1
hCMEC	Human cardiac microvascular endothelial cells
HO-1	Heme oxygenase-1
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IκB kinase	I kappa B kinase
JNK	c-Jun *N*-terminal kinase
LPSs	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MD-2	Myeloid differentiation factor-2
MMP	Matrix metalloproteinase
MnSOD	Manganese-dependent superoxide dismutase
MTT	3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor κappa B
NO	Nitric oxide
NOX	NADPH oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
P. g.	*Porphyromonas gingivalis*
P-glycoprotein	Pgp
PBS	Phosphate-buffered saline
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SEAP	Secreted alkaline phosphatase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
UPLC-MS-MS	Ultra-performance liquid chromatography coupled to electrospray ionization-tandem mass spectrometry
VCAM-1	Vascular cell adhesion protein-1
ZnPP	Zinc protoporphyrin
ZO	Zona occludens

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