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Comparative Study of Young and Mature *Dendropanax morbifera* Leaves: Superior Neuroprotective Efficacy of Young Leaves Through Enhanced Anti-Inflammatory and Metabolic Modulation

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Abstract

Neuroinflammation, driven by microglial activation and oxidative stress, is a key pathological feature of various neurodegenerative diseases. *Dendropanax morbifera Léveille* (DM) is a medicinal plant known for its diverse pharmacological activities; however, the influence of leaf developmental stage on its neuroprotective potential remains poorly understood. In this study, we compared the phytochemical profiles of young DM (YDM) and mature DM leaves and evaluated their effects on neuronal metabolism and microglia-mediated neuroinflammation. HPLC analysis revealed that YDM contained approximately 2.4-fold higher levels of chlorogenic acid than DM, while DM exhibited higher quercetin content. In differentiated N2A neuronal cells, YDM treatment significantly upregulated the expression of key metabolic and mitochondrial regulators, including PGC-1α, PPARγ, and CPT2, suggesting an enhanced capacity for mitochondrial biogenesis and fatty acid β-oxidation. Under inflammatory conditions, YDM more potently suppressed the secretion of pro-inflammatory cytokines (IL-6 and TNF-α) in LPS-stimulated BV2 microglia compared to DM. Furthermore, in N2A cells treated with a BV2–conditioned medium, both extracts effectively mitigated reactive oxygen species production and restored brain-derived neurotrophic factor expression. These findings demonstrate that leaf age is a critical determinant of the phytochemical composition and biological activity of DM. Our results suggest that chlorogenic acid-rich YDM preparations may offer superior therapeutic advantages in targeting neuroinflammatory and metabolic dysregulation in the central nervous system.

Keywords: *Dendropanax morbifera*; leaf age; chlorogenic acid; neuroinflammation; microglia; BDNF

1. Introduction

Neuroinflammation is a hallmark feature of various neurodegenerative and neuropsychiatric disorders, including Alzheimer's disease, Parkinson's disease, and major depressive disorder, and is closely associated with microglial activation and oxidative stress [1,2]. Once activated, microglia secrete inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). These mediators impair neuronal mitochondrial function and suppress brain-derived neurotrophic factor (BDNF) signaling—a critical factor for neuroprotection—ultimately leading to reduced neuronal survival and synaptic plasticity [1–3].

Accumulating evidence suggests that preserving metabolic homeostasis, mitochondrial biogenesis, and antioxidant defense systems in neurons is crucial for maintaining resilience against microglia-derived neuroinflammatory damage [4]. Key regulators of these processes include AMPK, SIRT1, and PGC-1α, which coordinately regulate mitochondrial biogenesis and fatty acid oxidation. In particular, PGC-1α acts as a coactivator for the transcription factors PPARα and PPARγ, inducing the expression of mitochondrial fatty acid β-oxidation enzymes such as CPT1A and CPT2. This induction promotes the oxidation of long-chain fatty acids and energy production, thereby contributing to the restoration of cellular metabolic homeostasis. Furthermore, NRF2, recognized as the master regulator of the antioxidant response, interacts with these pathways to simultaneously enhance mitochondrial function and antioxidant defenses [5–7].

Dendropanax morbifera Léveille (DM), an evergreen tree belonging to the family *Araliaceae*, is an endemic species native to the Republic of Korea and has been utilized traditionally as a medicinal plant. Various *in vitro* and *in vivo* studies have reported that DM leaf extracts exhibit a broad spectrum of pharmacological activities, including antioxidant, anti-inflammatory, and neuroprotective effects [8–10].

Phytochemical analyses reveal that DM leaves are rich in polyphenols and flavonoids, with chlorogenic acid, rutin, and quercetin identified as the primary bioactive compounds [11]. The concentrations of these secondary metabolites vary depending on the season, plant part, and extraction conditions. Notably, higher contents of chlorogenic acid and rutin have been correlated with enhanced antioxidant and radical scavenging activities [12,13].

Chlorogenic acid is one of the predominant phenolic acids in DM leaves and has been widely reported to exert anti-inflammatory and neuroprotective effects across various experimental models. These effects are achieved by inhibiting microglial activation, reducing the secretion of pro-inflammatory cytokines, and mitigating oxidative stress [12–14]. In both microglia and brain tissues, chlorogenic acid has been shown to suppress the LPS-induced production of NO and cytokines, while also ameliorating neurobehavioral deficits in rodent models of neuroinflammation and neurotoxicity [15–17]. Furthermore, chlorogenic acid is reported to regulate mitochondrial function and energy metabolism [18], suggesting that its neuroprotective efficacy may be potentially linked to these mechanisms.

However, little is known regarding how the concentrations of these key bioactive compounds differ between young and mature leaves of DM. Furthermore, it remains largely unexplored whether such variations lead to distinct neuroprotective profiles under conditions of microglia-mediated neuroinflammation. Although the developmental stage of a plant is a critical determinant of its secondary metabolite composition, the influence of leaf age on the bioactivity of DM has received little attention. Given that many medicinal plants exhibit significant fluctuations in their chemical profiles according to leaf age [19–21], it is highly probable that young DM (YDM) and mature DM leaves possess distinct concentrations of key bioactive compounds. Consequently, such variations may lead to differential levels of anti-inflammatory and neuroprotective activities.

In the present study, we focused on chlorogenic acid, rutin, and quercetin—the primary bioactive constituents reported in DM—to compare their concentrations in young and mature DM leaf extracts. We further evaluated their effects on neuronal metabolism and the expression of antioxidant-related proteins under physiological conditions, as well as their neuroprotective efficacy within a microglia-mediated neuroinflammatory environment.

Specifically, we investigated the impact of YDM and DM on metabolic and antioxidant protein expression in differentiated N2A cells. Furthermore, we assessed their ability to suppress LPS-induced NO and pro-inflammatory cytokine secretion in BV2 microglia. Finally, using a BV2 microglia–conditioned medium model designed to mimic microglia-derived neuroinflammatory states, we evaluated the effects of these extracts on reactive oxygen species (ROS) production and BDNF expression.

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2. Results

2.1. Young Dendropanax Morbifera Leaf Extracts Show A Higher Concentration of Chlorogenic Acid Compared to Mature Leaf Extracts.

Previous studies have reported that chlorogenic acid, rutin, and quercetin are the major constituents of DM leaves [12,22]. To investigate whether there are differences in these key components between YDM and DM, HPLC analysis was performed (Figure 1). Both YDM and DM contained all three compounds; however, their concentrations differed. The chlorogenic acid content was approximately 2.4-fold higher in YDM than in DM (72.18 vs. 29.96 μg/mL), while rutin levels were comparable between the two groups (139.18 vs. 126.57 μg/mL). In contrast, quercetin was approximately 2-fold higher in DM than in YDM (3.44 vs. 1.66 μg/mL). Chlorogenic acid has been reported to attenuate neuroinflammation by reducing microglial activation and the release of pro-inflammatory cytokines in various models [14,17,23]. Therefore, in this study, we evaluated whether YDM, which is richer in chlorogenic acid, exerts a greater anti-neuroinflammatory effect than DM.

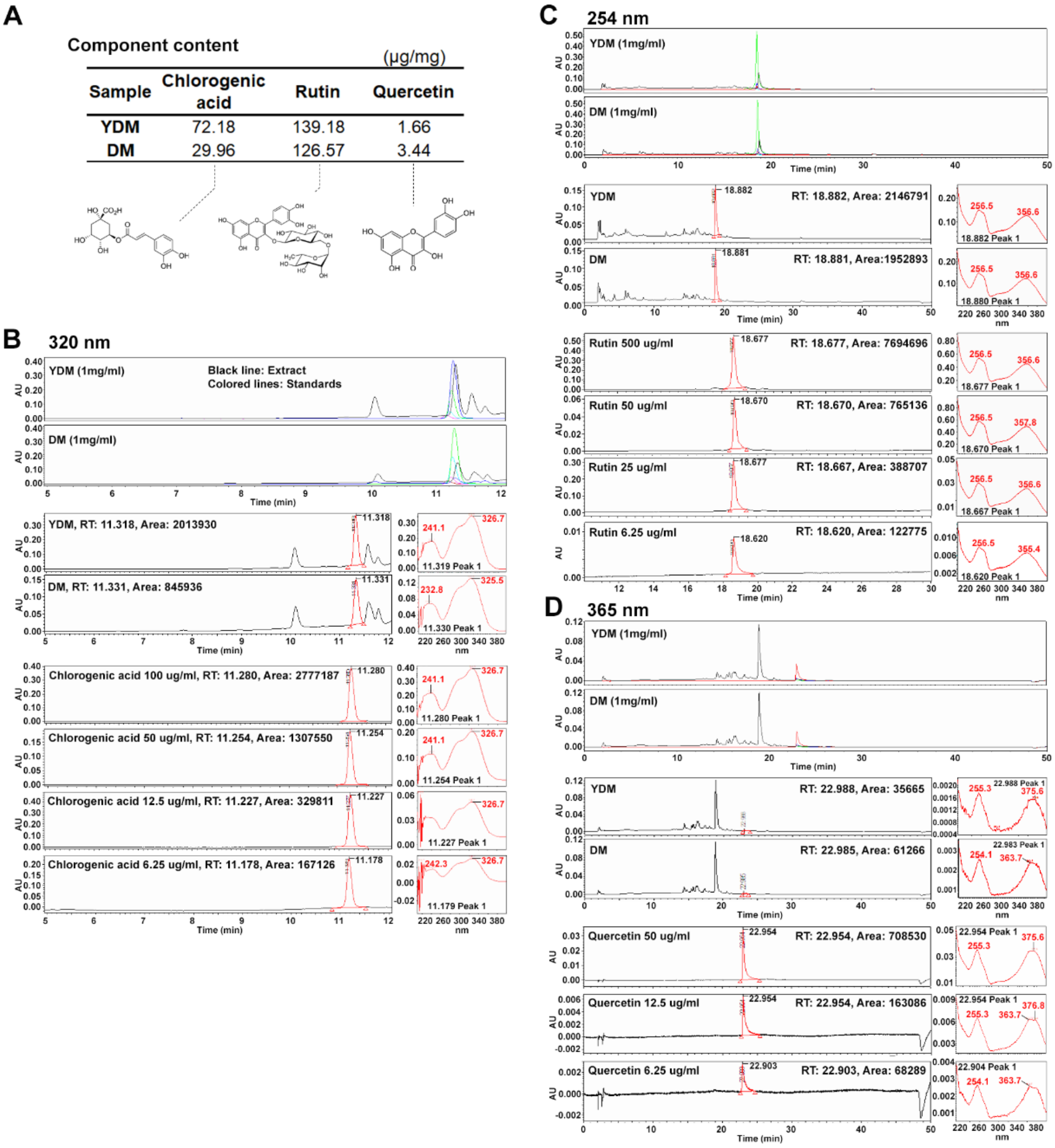
2.2. Young Dendropanax Morbifera Leaf Extracts Enhance the Expression of Metabolic and Mitochondrial-related Factors in Neuronal Cells.

Under neuroinflammatory conditions, neuronal energy metabolism and mitochondrial function play a critical role in neuronal protection. In addition, activation of antioxidant defense systems is essential for alleviating oxidative stress induced by neuroinflammation [24]. Accordingly, using differentiated N2A cells as a model, we investigated the effects of YDM and DM on the expression of proteins related to metabolism and antioxidant responses in neuronal cells.

Cell viability remained above approximately 80% following treatment with YDM and DM at concentrations of 1, 10, and 100 μg/mL for 24 h (Figure 2A). Based on these results, differentiated N2A cells were treated with these three concentrations, and changes in the expression of metabolism-related factors were analyzed by Western blotting (Figure 2B–C). The following markers were examined: upstream metabolic regulators (p-AMPK, SIRT1, PGC-1α), mitochondrial fatty acid oxidation (PPARα, PPARγ, CPT1A, CPT2), and antioxidant response (NRF2).

While DM treatment had no effect on PGC-1α, PPARγ, and CPT2, YDM treatment significantly increased their expression; specifically, PPARγ and CPT2 were upregulated from 1 μg/mL, whereas PGC-1α showed significant induction starting at 10 μg/mL. Although a significant main effect of YDM treatment was observed for p-AMPK/AMPK and PPARα compared to DM ($P < 0.05$), post-hoc analysis revealed no statistically significant differences between specific groups. NRF2 expression did not differ between the two extracts. In contrast, SIRT1 and CPT1A expression decreased following DM treatment, resulting in significantly higher levels in the YDM group compared to DM (Figure 2C).

PGC-1α is a master regulator of mitochondrial biogenesis and is known to promote mitochondrial biogenesis and oxidative metabolism through interaction with PPARγ. CPT2, located in the inner mitochondrial membrane, is a key enzyme regulating the β-oxidation of long-chain fatty acids [5,24–27]. Therefore, the upregulation of these proteins by YDM suggests a potential increase in mitochondrial biogenesis. To test this, a mitochondrial biogenesis assay was performed, which revealed a significant difference among groups by one-way ANOVA (P = 0.023). Notably, YDM showed slightly higher mean values than DM at all concentrations (Figure 2D).



Component content (μg/mg)

Sample	Chlorogenic acid	Rutin	Quercetin
YDM	72.18	139.18	1.66
DM	29.96	126.57	3.44

Figure 1. HPLC analysis of three major components in young and mature *Dendropanax morbifera* leaf extracts. (A) Contents of chlorogenic acid, rutin, and quercetin in young and mature *Dendropanax morbifera* leaf extracts (YDM and DM, respectively). (B) HPLC chromatograms of YDM and DM extracts (1 mg/mL) and chlorogenic acid standards (6.25–100 μg/mL) detected at 320 nm. (C) HPLC chromatograms of YDM and DM extracts (1 mg/mL) and rutin standards (6.25–100 μg/mL) detected at 254 nm. (D) HPLC chromatograms of YDM and DM extracts (1 mg/mL) and quercetin standards (6.25–100 μg/mL) detected at 365 nm.

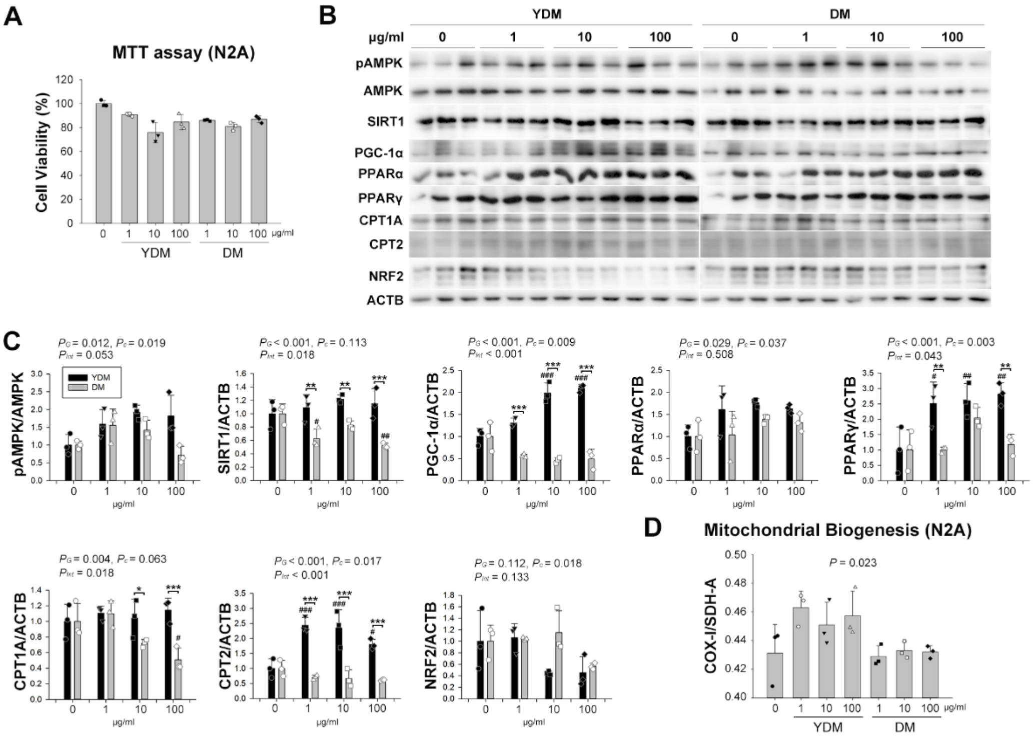


Figure 2. Enhanced expression of metabolism-related genes by young *Dendropanax morbiferus* leaf extracts compared to mature leaf extracts in differentiated neuronal N2A cells. (A) Cell viability (%) of N2A cells treated with YDM or DM at the indicated concentrations for 24 h, determined by MTT assay. (B) Western blot analysis of factors related to upstream metabolic regulators (p-AMPK, SIRT1, PGC-1α), mitochondrial fatty acid oxidation (PPARα, PPARγ, CPT1A, CPT2), and antioxidant response (NRF2) in differentiated N2A cells treated with YDM or DM for 48 h. (C) Quantitative analysis of the western blot results shown in (B). (D) Mitochondrial biogenesis. N = 3 independent cultures. Data were analyzed using two-way ANOVA followed by the Holm-Sidak method for multiple comparisons (C) and one-way ANOVA (D). P_G, P_C, and P_{INT} represent the P-values for group, concentration, and their interaction, respectively. For comparisons between groups at each concentration: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. For comparisons between the control (0 μg/mL) and other concentrations: $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$. Bars indicate mean ± SD.

2.3. Young Dendropanax Morbifera Leaf Extracts Suppresses LPS-Induced Pro-Inflammatory Cytokine Production More Strongly Than Mature Leaf Extracts in BV2 Cells.

Next, we evaluated whether YDM more effectively attenuates microglia-mediated neuroinflammation compared to DM. BV2 cells were pre-incubated with YDM or DM, followed by LPS stimulation to assess nitrite levels and the secretion of pro-inflammatory cytokines (IL-6 and TNF-α). Subsequently, the conditioned media were applied to differentiated N2A cells to examine changes in ROS production.

Cell viability was maintained above approximately 80% at concentrations up to ~250 μg/mL in both BV2 and N2A cells (Figure 3A–B). In the NO assay, both YDM and DM reduced LPS-induced nitrite production in a dose-dependent manner (Figure 3C). Notably, analysis of representative pro-inflammatory cytokines revealed that YDM more markedly suppressed LPS-induced IL-6 and TNF-α secretion compared to DM (Figure 3D, E).

In experiments measuring ROS levels in N2A cells treated with BV2-conditioned media, both YDM and DM dose-dependently attenuated ROS production (Figure 3F). Taken together, YDM more effectively inhibits pro-inflammatory cytokine secretion than DM, while both extracts share a common antioxidant protective effect against microglia-derived oxidative stress.

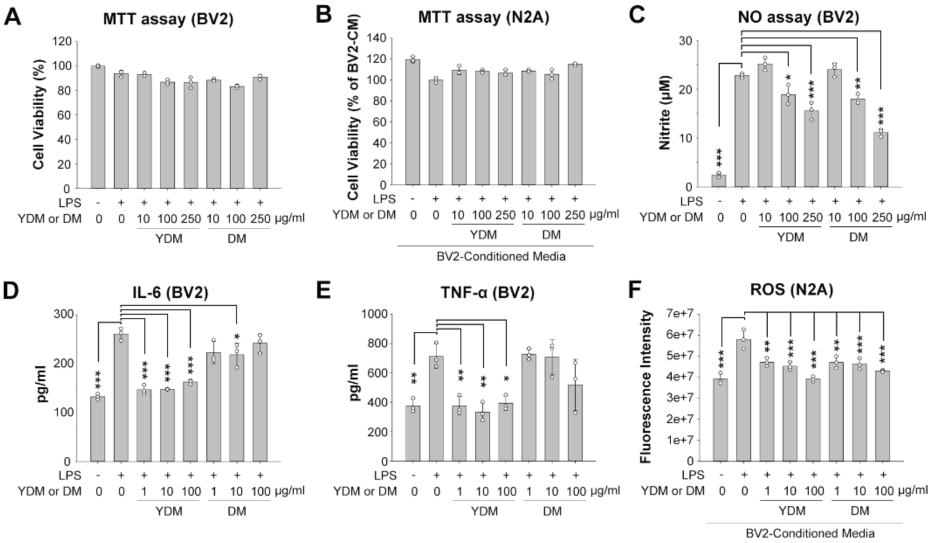


Figure 3. Alleviating effects of young versus mature *Dendropanax morbifera* leaf extracts on neuroinflammation-mediated neurotoxicity. BV2 cells were pre-incubated with vehicle, YDM or DM for 1 h, then stimulated with LPS (final concentration: 100 ng/mL) for 24 h (A, C) or 72 h (D, E). The resulting supernatant (conditioned media, CM) was used for the indicated assays (C–E) and for treating differentiated N2A cells for an additional 24 h (B, F). (A) Cell viability (%) of BV2 cells treated with YDM or DM at the indicated concentrations for 24 h. (B) Cell viability (%) of N2A cells treated with BV2-CM. (C) Nitrite levels (μM) in the supernatant of BV2 cells. (D) IL-6 and (E) TNF-α levels (pg/mL) in the supernatant of BV2 cells. (F) ROS levels in N2A cells treated with BV2-CM. N = 3 independent cultures. Data were analyzed using one-way ANOVA followed by the Holm-Sidak method. For comparisons between LPS-only and other conditions: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bars indicate mean ± SD.

2.4. Young Dendropanax Morbifera Leaf Extracts More Effectively Restores LPS-Induced Reductions in BDNF.

In neuroinflammatory conditions, excessive microglial activation impairs BDNF signaling [28,29]. As shown earlier, under physiological conditions, YDM increased PGC-1α and PPARγ expression in N2A cells and showed differential effects on CPT1A expression compared to DM (Figure 2C). Based on this, we evaluated the effects of YDM and DM on BDNF and metabolism-related protein expression under pathological conditions involving microglia–neuron interactions.

BV2 cells were pretreated with YDM or DM, followed by LPS stimulation, and the resulting conditioned media were applied to differentiated N2A cells to assess changes in protein expression of BDNF, PGC-1α, and CPT1A (Figure 4A–B). LPS treatment slightly reduced the mean level of BDNF expression in N2A cells, which was significantly restored by both DM and YDM. Although the recovery was mildly pronounced with YDM than with DM, it was not significantly different. In addition, no significant changes were observed in PGC-1α or CPT1A expression under these conditions (Figure 4B).

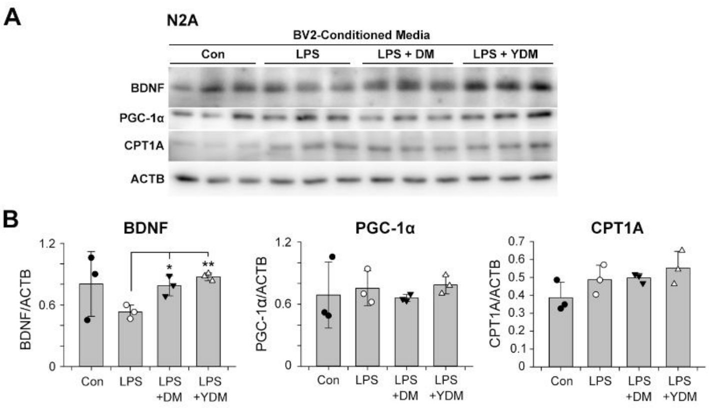


Figure 4. Recovery of BDNF protein expression by young and mature *Dendropanax morbifera* leaf extracts. BV2 cells were pre-incubated with vehicle, YDM, or DM for 1 h, then stimulated with LPS (100 ng/mL) for 72 h. N2A cells were treated with the BV2-conditioned media for 24 h, and then collected for protein analysis. (A) Representative western blot images of the indicated proteins in N2A cells. (B) Densitometric quantification of the western blots shown in (A). The concentration of DM and YDM used was 100 μg/mL. N = 3 independent cultures. Data were analyzed using Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$. Bars indicate mean ± SD.

3. Discussion

The present study demonstrates that YDM, characterized by a higher chlorogenic acid content compared to DM, offers additive benefits in protecting against neuroinflammation in vitro. Compared to DM, YDM more potently upregulated the expression of proteins related to mitochondrial biogenesis and fatty acid oxidation in neuronal cells and more effectively suppressed LPS-induced pro-inflammatory cytokine secretion in microglia. Conversely, both extracts exhibited comparable efficacy in inhibiting NO production in BV2 cells and in restoring ROS levels and BDNF expression in differentiated N2A cells challenged with LPS-stimulated BV2-conditioned media.

These findings suggest that the age-dependent variations in the phytochemical composition of DM leaves are functionally significant. Furthermore, the superior benefits provided by YDM indicate that it may serve as a more promising candidate for targeting neuroinflammatory pathways.

Our phytochemical analysis revealed that while both YDM and DM contain chlorogenic acid, rutin, and quercetin, their quantitative distribution patterns differ significantly. The concentration of chlorogenic acid was higher in YDM than in DM. Consistent with our findings, most coffee species exhibit substantially higher chlorogenic acid content in young leaves compared to mature ones [30]. Similarly, in the medicinal plant *Vaccinium dunalianum*, chlorogenic acid levels were found to be higher in younger leaves, a phenomenon attributed to the upregulation of genes involved in chlorogenic acid biosynthesis during early development [31]. This accumulation may be an adaptive response, as young leaf tissues are typically more vulnerable to oxidative stress and pathogen infection, necessitating the active synthesis of defensive phenolic acids.

Chlorogenic acid has been repeatedly identified as a key mediator of anti-inflammatory and neuroprotective activities, including the inhibition of microglial activation, reduction of NO and pro-inflammatory cytokines, and mitigation of oxidative damage in neurons [12–17]. Consequently, the elevated chlorogenic acid content in YDM likely serves as a primary factor explaining, at least in part, the enhanced biological activities observed in this study—specifically the superior suppression of cytokine secretion and the increased expression of metabolism-related proteins in neurons. In contrast, the higher quercetin content in DM did not translate into similar potent effects under the present experimental conditions.

At the neuronal level, unlike DM, YDM significantly increased the expression of PGC-1α, PPARγ, and CPT2 across most concentrations tested in differentiated N2A cells. This suggests that YDM possesses a distinct ability to promote mitochondrial biogenesis and mitochondrial fatty acid β-oxidation. PGC-1α is a well-recognized master regulator that modulates mitochondrial biogenesis and oxidative metabolism, acting in part through interactions with nuclear receptors such as PPARγ. Furthermore, CPT2, localized in the inner mitochondrial membrane, plays a critical role in the β-oxidation of long-chain fatty acids [24,32]. Given the simultaneous upregulation of these proteins, coupled with the modest but consistent increase in mitochondrial biogenesis observed in our analysis, it can be interpreted that YDM fosters a metabolic environment conducive to enhanced mitochondrial function and energy production in neurons. Such metabolic reinforcement is particularly crucial in neuroinflammatory contexts, where mitochondrial dysfunction and impaired ATP production contribute to neuronal vulnerability.

Interestingly, while the p-AMPK/AMPK ratio and PPARα levels tended to be higher in the YDM-treated group compared to the DM group, these differences did not reach statistical significance, and NRF2 levels remained comparable between the two. In contrast, the expression of SIRT1 and CPT1A was significantly reduced following DM treatment, resulting in a marked disparity between YDM and DM. Given that AMPK, SIRT1, and PGC-1α constitute a core signaling axis that promotes mitochondrial biogenesis and oxidative metabolism [5], the preservation of SIRT1 and the upregulation of PGC-1α by YDM may be particularly vital for maintaining neuronal energy homeostasis under stressful conditions. Collectively, these data suggest that YDM actively enhances key components of the mitochondrial biogenesis and fatty acid oxidation pathways.

Regarding microglial activation, both YDM and DM reduced LPS-induced nitrite production in BV2 cells in a concentration-dependent manner, confirming that both extracts possess anti-inflammatory properties consistent with previous reports on *D. morbifera* leaf preparations [9,10]. Notably, however, YDM exerted a more potent inhibitory effect on the secretion of IL-6 and TNF-α than DM, highlighting a qualitative difference between the two extracts in modulating inflammatory cytokine responses. Given that IL-6 and TNF-α are key mediators of microglia-mediated neurotoxicity [33,34], the superior cytokine-suppressing efficacy of YDM may serve as a central mechanism underlying its enhanced neuroprotective profile.

BDNF plays a central role in neuronal survival, synaptic plasticity, and cognitive function, and its suppression via microglial activation is a hallmark of neuroinflammatory states [35]. Previous studies have demonstrated that DM leaf extracts exert beneficial effects on neurotrophic pathways in vivo, alleviating mercury-induced spatial memory impairment and reduced hippocampal neurogenesis [36]. Our findings extend these prior reports by showing that both quercetin-rich DM and chlorogenic acid-rich YDM preparations can reverse inflammation-induced neuronal BDNF depletion, suggesting potential modulation of microglial-mediated pathways. Although PGC-1α and CPT1A levels did not change significantly under BV2–conditioned media conditions, the basal enhancement of metabolic and mitochondrial-related proteins by YDM under physiological conditions suggests that it may provide a metabolic buffer. This baseline fortification likely supports BDNF-dependent neuroplasticity during inflammatory challenges.

Several limitations of the present study warrant consideration. First, as this research was conducted using immortalized cell lines (BV2 and N2A), it only partially reflects the intricate complexity of primary microglia–neuron interactions *in vivo*. Second, our analysis focused on a limited scope of phytochemicals (chlorogenic acid, rutin, and quercetin); since other individual constituents were not systematically quantified or manipulated, it is difficult to definitively attribute the observed effects to a single specific compound.

Nonetheless, the strong correlation between elevated chlorogenic acid content and enhanced anti-inflammatory and mitochondrial regulatory effects, combined with established evidence regarding chlorogenic acid-mediated neuroprotection, supports the interpretation that this constituent plays a central role in the superior activity of YDM. Future studies involving experiments with purified chlorogenic acid, loss-of-function approaches, and *in vivo* models of neuroinflammation or neurodegenerative diseases are required to confirm this causal relationship and to determine whether YDM provides measurable benefits for cognitive and emotional behaviors.

In conclusion, our data demonstrate that YDM, which are enriched with chlorogenic acid compared to DM, offer distinct advantages in promoting the expression of metabolic and

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mitochondrial-related proteins in neurons under physiological conditions. Furthermore, YDM significantly suppresses microglial pro-inflammatory cytokine production under LPS-induced inflammatory conditions. These findings emphasize that leaf age is a critical factor in determining the neuroprotective potential of DM. Our results suggest that chlorogenic acid-rich YDM preparations may serve as promising candidates for the development of plant-based interventions targeting neuroinflammation and neurotrophic dysfunction.

4. Materials and Methods

4.1. Plant Materials and Extraction

The young and mature leaves of *Dendropanax morbiferus* were collected in June 2022 from 9-year-old trees cultivated in Buhwang-ri, Bogil-myeon, Wando-gun, Republic of Korea. The plant material was registered at the Warm-Temperate and Subtropical Forest Research Center under the resource management number WFRC 10034298 and collection number 71531. The harvested leaves were air-dried and subsequently extracted with distilled water at 80°C for 8 h. After natural cooling to room temperature, the extracts were filtered and freeze-dried for further analysis.

4.2. HPLC Analysis

Quantitative analysis of bioactive compounds was performed using a high-performance liquid chromatography (HPLC) system equipped with a UV detector. Chromatographic separation was carried out on a Kromasil 100-3.5-C18 column (4.6 × 250 mm, 5 µm particle size). The flow rate was maintained at 1 mL/min, and the injection volume was set at 10 µL for all analyses. The column temperature was kept at room temperature. The mobile phase composition and detection wavelengths were optimized for each compound as follows:

For chlorogenic acid, the mobile phase consisted of (A) acetonitrile and (B) 1% acetic acid in water. The gradient elution program was as follows: 0–5 min, 0–10% A; 5–10 min, 10–25% A; 10–15 min, 25–65% A; and 15–20 min, 65–100% A. Detection was performed at 320 nm. For quercetin and rutin, the mobile phase consisted of (A) methanol and (B) water, with the following gradient program: 0–20 min, 10–65% A; 20–40 min, 65–100% A; 40–45 min, 100% A; 45–47 min, 100–10% A; and 47–50 min, 10% A. Detection wavelengths were set at 365 nm for quercetin and 254 nm for rutin.

4.3. Cell Culture and Treatment

The murine microglial cell line BV2 and N2A neuroblastoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were confirmed to be mycoplasma-free prior to use. For differentiation, N2A cells were cultured in DMEM supplemented with 1% FBS and 20 µM retinoic acid.

To induce inflammation, BV2 cells were pretreated with YDM, DM, or vehicle for 1 h, followed by stimulation with 100 ng/mL lipopolysaccharide (LPS) for 72 h. The culture supernatants were collected for the measurement of IL-6 and TNF-*α* levels. For the neurotoxicity model, N2A cells were treated with BV2-conditioned medium for an additional 24 h to evaluate neuroinflammation-mediated toxicity and to collect samples for Western blot analysis.

4.4. Measurement of Inflammatory Cytokines, NO, Cell Viability, and ROS

The levels of secreted TNF-*α* and IL-6 in culture supernatants were quantified using Mouse DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. NO production was measured using the Griess reaction. Equal volumes of conditioned medium and Griess reagent (composed of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride, diluted 1:1 with distilled water) were mixed and incubated. The absorbance was then measured at 570 nm using a microplate reader. Cell viability was evaluated using an MTT-based assay according to the manufacturer's instructions (EZ-Cytox kit, DogenBio). Intracellular ROS levels were measured using a ROS assay kit (ab113851, Abcam) according to the manufacturere's protocol. Mitochondrial biogenesis was assessed using a commercial assay kit (ab110217, Abcam) following the manufacturer's instructions.

4.5. Western Blot Analysis

Cells were lysed on ice using chilled RIPA buffer (WSE-7420, ATTO, DAWINBIO Inc., Hanam, Korea), followed by centrifugation at 15,000 rpm for 15 min at 4°C. The resulting supernatant was collected and protein concentrations were quantified. Equal amounts of protein were mixed with sample buffer, denatured by boiling, and separated via SDS-PAGE before being transferred onto PVDF membranes. The membranes were blocked with EZBlock Chemi (AE-1475, ATTO, Tokyo, Japan) for 30 min at room temperature and subsequently incubated with specific primary antibodies overnight at 4°C. After thorough washing, the membranes were incubated with secondary antibodies for 1 h at room temperature. Protein bands were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (34580, Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescent signals were captured using an Amersham Imager 600 (GE Healthcare, Chicago, IL, USA), and the intensity of the bands was quantified using ImageJ software (NIH, Bethesda, MD, USA).

4.6. Antibodies and Reagents

Rabbit primary antibodies were obtained from the following suppliers: Cell Signaling Technology [phospho-AMPK (2531), AMPK (2532), BDNF(47808)]; Bioss [ACTB (bs-0061R), CPT1A (bs-2047R), CPT2 (bs-5047R)]; Proteintech [SIRT1 (13161-1-AP), PPARγ (16643-1-AP)]; Novus Biologicals [PGC-1α (NBP1-04676SS), PPARα (NB600-636SS)]; and Cusabio [NRF2 (CSB-PA003481)]. LPS (L2630) was purchased from Sigma-Aldrich.

4.7. Statistical Analysis

Data are presented as the mean ± standard deviation (SD). Statistical significance was evaluated using one- or two-way analysis of variance (ANOVA), followed by the Holm-Sidak post-hoc test for multiple comparisons. Comparisons between two groups were performed using Student's *t*-test. All statistical analyses were conducted using SigmaPlot software (version 14.0; Systat Software, Inc.). A *P*-values < 0.05 was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: Original western blot images of figure 2. Figure S2: Original western blot images of figure 4.

Author Contributions: Funding acquisition/Project administration/Resources: D. J.; Conceptualization/Supervision/Writing: H.Y.; Data curation/Formal analysis/Investigation/Methodology/Validation/Visualization: H.Y., A.L., D.K.

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Data Availability Statement: All data generated or analysed during this study are included in this published article and its supplementary information files.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

NO, nitric oxide; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; BDNF, brain-derived neurotrophic factor; DM, *Dendropanax morbifera Léveille*; YDM, young DM; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; SD, standard deviation; ANOVA, analysis of variance.

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