Madecassoside activates anti-neuroinflammatory mechanisms by inhibiting lipopolysaccharide-induced microglial inflammation

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Abstract. Neurodegeneration is typically preceded by neuroinflammation generated by the nervous system to protect itself from tissue damage, however, excess neuro-inflammation may inadvertently cause more harm to the surrounding tissues. Attenuating neuroinflammation with non-steroidal anti-inflammatory drugs can inhibit neuro-degeneration. However, such treatments induce chronic side effects, including stomach ulcers. Madecassoside, a triterpene derived from *Centella asiatica*, is considered to be an alternative treatment of inflammation. In the present study, the anti-neuroinflammatory properties of madecassoside were assessed in BV2 microglia cells, which were pre-treated with madecassoside at a maximum non-toxic dose (MNTD) of 9.50 μg/ml and a ½ MNTD of 4.75 μg/ml for 3 h and stimulated with 0.1 μg/ml lipopolysaccharide (LPS). The effect of madecassoside was assessed by determining reactive oxygen species (ROS) levels in all groups. Furthermore, the expression of pro- and anti-neuroinflammatory genes and proteins were analyzed using reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The results demonstrated that ROS levels in cells treated with the MNTD of madecassoside were significantly reduced compared with cells treated with LPS alone (P<0.05). The expression of pro-neuroinflammatory genes, including inducible nitric oxide synthase, cyclooxygenase-2, signal transducer and activator of transcription 1 and nuclear factor-κB, were significantly downregulated in a dose-independent manner following treatment with madecassoside. Conversely, the anti-neuroinflammatory component heme oxygenase 1 was significantly upregulated by 175.22% in the MNTD-treated group, compared with cells treated with LPS alone (P<0.05). The gene expression profiles of pro- and anti-inflammatory genes were also consistent with the results of western blotting. The results of the present study suggest that madecassoside may be a potent anti-neuroinflammatory agent. The antioxida-tive properties of madecassoside, which serve a major role in anti-neuroinflammation, indicate that this compound may be a functional natural anti-neuroinflammatory agent, therefore, further *in vivo* or molecular studies are required.

Introduction

Neurodegeneration consists of various cellular processes whereby neuronal cells progressively deteriorate, lose function and ultimately die. In 2014, Alzheimer's disease (AD) and Parkinson's disease (PD), were the seventh and 14th leading causes of mortality, respectively, in the US (1). Therefore, more research is required to understand the intricate molecular components of these diseases and their risk factors, including neuroinflammation. This may enable the development of novel therapeutic strategies to treat neurodegenerative diseases. Unlike associated genetic risk factors, including polygluta-mine (2) and glucocerebrocidase (3), neuroinflammation is a major risk factor of neurodegeneration as it progressively develops throughout life (4). Coupled with increasing age, a build-up of reactive oxygen species (ROS) levels (5) and protein misfolding (6), neuroinflammation may act as one of the various triggers of neurodegeneration. Previous studies have suggested that excessive neuroinflammation worsens the symptoms of AD and PD by promoting the excessive genera-tion of amyloid β plaques and destruction of dopaminergic neurons (4). Although the nervous system is known to be an immunologically privileged site, it still hosts astrocytes and glial cells that express major histocompatibility complexes, which propagate neuroinflammation (7). Despite the ability of immune cells associated with the nervous system, such as microglia, to subdue imminent infections and damages, this process typically causes excessive neuroinflammation that harms the surrounding tissue (7).

To produce a model of neuroinflammation, the present study utilized lipopolysaccharide (LPS), which triggers the activation

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of toll-like receptor 4 that is ubiquitously present on the surface of microglia, subsequently triggering neuroinflammation (8). LPS also elicits prolonged responses *in vivo* by upregulating the expression of the following pro-inflammatory components: Tumor necrosis factor α; cyclooxygenase 2 (COX)-2, which is coded by the gene prostaglandin-endoperoxide synthase 2 (PTGS)-2; inducible nitric oxide synthase (iNOS), which is coded by the gene nitric oxide synthase 2 (NOS2); and nuclear factor (NF)-κB, which is coded by the NF-κB subunit 1 gene (9). These pro-inflammatory components form a negative feedback system with superoxide dismutase (SOD) and heme oxygenase 1 (HO-1) (10) to produce anti-neuroinflammatory components, including interleukin (IL)-10 (11). However, in the majority of biological systems, pro-neuroinflammatory signaling pathways typically outweigh anti-neuroinflammatory signaling pathways (10).

Various compounds that trigger anti-neuroinflammatory signaling pathways may therefore help to treat neuroinflammation. Out of the several natural compounds that have been screened, madecassoside, a triterpenoid saponin extractable from *Centella asiatica,* known locally as Pegaga (12), may be considered as a potential method of treating excessive neuroinflammation. Various studies have suggested that this compound may exhibit anti-inflammatory, antioxidant and anti-cancer effects (12,13). Furthermore, madecassoside induces the modulation of inflammatory cytokines, translocation of NF-κB and regulation of COX-2 and iNOS (14,15). This compound, which is commonly used in cosmetology, has also been assessed in neuronal settings (10,16). Our preliminary study profiled the production of pro- and anti-neuroinflammatory cytokines in LPS-stimulated BV2 microglia (data not published), however, the molecular activity of madecassoside has yet to be fully elucidated. Subsequently, the objective of the present study was to analyze the effects of madecassoside on ROS generation and to determine the molecular effects of madecassoside at the genomic and proteomic levels.

Materials and methods

Preparation of madecassoside and indomethacin stock solution. Madecassoside powder (≥95% purity) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). A stock solution of 50 μg/μl was prepared in molecular grade dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). To obtain the maximum non-toxic dose (MNTD) of 9.50 μg/ml and ½ MNTD of 4.75 μg/ml, as determined by Mohan (17), the stock solution was diluted with Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The positive control indomethacin (Sigma-Aldrich; Merck KGaA), a type of non-steroidal anti-inflammatory drug known to elicit anti-inflammatory effects (18), was dissolved in DMSO to a concentration of 10 mM, which was further diluted to 25 μM working solution with fresh DMEM.

Cell culture. BV2 microglia cells were provided by Dr Sharmili Vidyadaran from Universiti Putra Malaysia (Selangor, Malaysia) and subsequently cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, 0.1% fungizone and 0.1% gentamycin (all

Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified incubator containing 5% CO_2 at 37°C. To determine the intracellular ROS levels, cells were seeded at a desired concentration of $5x10^5$ cells/well in 24-well tissue culture plates (Corning Incorporated, Corning, NY, USA) and the trypan blue exclusion method (19) was then performed using 0.4% trypan blue staining (Gibco; Thermo Fisher Scientific, Inc.) to calculate the number of trypsinized cells, while for RNA and protein extraction, $1x10^6$ cells/well were seeded in 60-mm tissue culture dishes (Corning Incorporated).

Treatment with madecassoside and LPS stimulation. Culture medium was aspirated from the culture vessel and BV2 cells were treated with madecassoside at the MNTD (9.50 μg/ml) and ½ MNTD (4.75 μg/ml), or they were treated with indomethacin (25 μM). Untreated cells were used as the control. Cells were subsequently incubated in a humidified incubator containing 5% CO_2 at 37°C for 3 h and were thoroughly mixed at 30 min intervals. Following 3 h incubation, 0.1 μg/ml LPS (Sigma-Aldrich; Merck KGaA) (20,21) was introduced into treatment groups as indicated (Table I). Cells were then further incubated for 24 h within a humidified incubator containing 5% CO_2 at 37°C.

Determination of intracellular ROS levels. DMEM was removed, cells were subjected to trypsinization and the pellet was resuspended in phosphate-buffered saline (PBS). Cell suspension (cell density, $1x10^5$/well) and non-sterile PBS were transferred into 96-well plates (Corning Incorporated). Subsequently, 40 μM 2',7'-dichlorofluorescin diacetate (Sigma-Aldrich; Merck KGaA) was added. Following 30 min incubation at 37°C, fluorescence was measured using SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at excitation and emissions wavelengths of 485 and 538 nm, respectively. The number of cells in each treatment group was determined using the trypan blue exclusion method and values were used to calculate the relative fluorescence unit of each treatment group.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA extraction and purification of BV2 cells was performed using a PureLink RNA Mini kit (Ambion; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Lysis buffer solution was prepared by adding 50 μl mercaptoethanol to 5 ml lysis buffer from the kit. Subsequently, cell dislodgment was performed using a cell scraper (Techno Plastic Products AG, Trasadingen, Switzerland). Scraped off cells were transferred into a sterile 1.5-ml microcentrifuge tube on ice. The contents of the microcentrifuge tubes were passed through an 18-gauge syringe needle 5-10 times. Subsequently, the lysate was subjected to the PureLink RNA Mini kit protocol and extracted RNA was stored in volumes of 35 μl at -30°C. To analyse RNA purity, a Tecan Infinite 200 PRO microplate reader was utilized at 260/280 nm on a NanoQuant plate (both Tecan Group Ltd., Mannedorf, Switzerland).

RNA samples were normalized to 2 mg/ml and converted to cDNA using the qPCRBIO cDNA Synthesis kit (PCR Biosystems Ltd., London, UK) following the manufacturer's

Table I. Treatment groups used in the present study.

Treatment groups	Components
Control	Untreated BV2 cells
LPS	LPS (0.1 μg/ml)
MNTD+LPS	MNTD (9.5 μg/ml)+LPS (0.1 μg/ml)
½ MNTD+LPS	½ MNTD (4.75 μg/ml)+LPS (0.1 μg/ml)
Indo+LPS	Indomethacin (25 μM)+LPS (0.1 μg/ml)

LPS, lipopolysaccharide; MNTD, maximum non-toxic dose; Indo, Indomethacin; Control, untreated BV2 cells.

protocol and an MJ Research PTC-100 Thermal Cycler (GMI, Ramsey, MN, USA). qPCR was performed in a 15-μl reaction mixture containing 2X qPCRBIO SyGreen Mix (qPCRBIO SyGreen Mix with Fluorescein kit; PCR Biosystems Ltd.) and primers (First Base Laboratories Sdn Bhd, Selangor, Malaysia), the sequences of which are listed in Table II. qPCR results were analyzed using iQ5 Optical System Software version 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 1 initial denaturation cycle at 95˚C for 2 min, followed by 40 cycles of denaturation 95˚C for 5 sec, annealing at 60˚C for 30 sec and final elongation at 72˚C for 30 sec. Relative gene expression values were normalized to β-actin and fold changes of each gene were calculated using the $2^{-\Delta\Delta Cq}$ method (22), whereby the baseline of PCR efficiencies were set at 90%.

Western blotting. Proteins were extracted using radioimmuno-precipiation lysis buffer consisting of 23.145 mg dithioethreitol (Bio-Rad Laboratories, Inc.), 30 μl proteinase inhibitor and 1.5 ml lysis buffer (Ambion; Thermo Fisher Scientific, Inc.) with the aid of a cell scraper (Techno Plastic Products AG, Switzerland). Lysates were centrifuged at 20,000 x g for 20 min in 4˚C. Protein concentration was determined using the Bradford assay standard curve of bovine serum albumin (BSA; Nacalai Tesque, Inc., Kyoto, Japan) analyzed using a 96-well plate (Corning Incorporated) administered with Bradford Reagent (Bio-Rad Laboratories, Inc.).

Protein samples at 100 mg/ml were loaded and separated using 12% SDS-PAGE and a PowerPac Basic Machine (Bio-Rad Laboratories, Inc.). Samples were transferred to 0.45-μm polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) and membranes were blocked with 5% BSA in Tris-buffered saline with Tween-20 (TBS-T) at room temperature for 1 h. Membranes were subsequently washed three times with TBS-T, incubated overnight with primary antibodies against iNOS (cat. no., sc-8310), COX-2 (cat. no., sc-23984), HO-1 (cat no., sc-10789), signal transducer and activator of transcription 1 (STAT1; cat. no., sc-271661), NF-κB (cat. no., sc-372) and β-actin (cat., no. sc-130656; all 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C. Following this, membranes were washed three times with TBS-T and incubated with secondary antibodies (1:5,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) against rabbit (cat. no., sc-2004), goat (cat. no., sc-2020) and mouse (cat. no., sc-358914) primary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Proteins were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) on a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data were generated in triplicate from three independent repeats and presented as the mean ± standard deviation. SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA) was used to assess differences between groups using one way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results and discussion

Determination of intracellular ROS levels. ROS production is associated with inflammation, including neuroinflammation (23). The production of various ROS, including superoxide anions and its derivatives, such as hydroxyl radicals, is required in normal physiological conditions, including during the modulation of synaptic and non-synaptic communication, and conditions whereby antimicrobial defence is required (23). Notably, exogenous ROS attained from pollutants or xenobiotics (24), coupled with excess endogenous ROS production, which occurs primarily via NADPH oxidase (25), may cause excessive neuroinflammation.

In the present study, madecassoside significantly reduced ROS levels in BV2 cells by 56.84% in the MNTD+LPS group compared with the group stimulated with LPS alone (P<0.05; Fig. 1). These results were in accordance with the results of a previous study (26). The MNTD+LPS treatment group also exhibited superior ROS reduction compared with the Indo+LPS group, which only caused a 34.62% reduction in ROS levels compared with LPS-treated cells. It has been demonstrated that trypsinization at high concentrations (U/ml) within cell culture produces a certain level of ROS (27); therefore, for more accurate measurement, the results from the treatment groups were all normalized to those of the control group, which consisted of untreated BV2 cells.

Nurlaily *et al* (14) indicated that madecassoside has antioxidative effects. Notably, madecassoside has been studied within the neuronal and other bodily systems, and similar trends have been indicated regarding the reduction of ROS levels. Al Mamun *et al* (26) utilized madecassoside to observe its neuroprotective effects against amyloid β plaques within *in vivo* and *in vitro* models of AD. It has been demonstrated that the plaques propagate excess oxidative stress in the form of ROS and reactive nitrogen species (RNS) (2), which may react with each other to form the toxic product peroxynitrite (10,28). Following exposure to madecassoside there was a significant reduction in lipid peroxidation (LPO), which is indicative of oxidative stress, and ROS levels, *in vivo* and *in vitro*, amongst other pro-neuroinflammatory components (26,28). This suggests that madecassoside may mitigate the worsening symptoms of neurodegenerative diseases, including AD. In addition, Li *et al* (15) indicated that madecassoside may regulate ROS levels by generating SOD and increasing catalase activity to inhibit the production of destructive free radicals and superoxide anions.

In the present study, although this difference was not significant, the reduction of ROS in the ½ MNTD+LPS group

Table II. Forward and reverse sequences of the primers utilized for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')	Amplicon size (bps)
iNOS	TTGCCACGGACGAGACGGATAGG	GGGCACATGCAAGGAAGGGAACTC	131
COX-2	TGGGTGTGAAAGGAAATAAGGA	GAAGTGCTGGGCAAAGAATG	128
HO-1	AGAGTTTCCGCCTCCAACCA	CGGGACTGGGCTAGTTCAGG	107
STAT1	CTGAATATTTCCCTCCTGGG	TCCCGTACAGATGTCCATGAT	103
NF-κB	CTGGTGGACACATACAGGAAGAC	ATAGGCACTGTCTTCTTTCACCTC	198
β-actin	TCCTCCTGAGCGCAAGTACTCT	GCTCAGTAACAGTCCGCCTA	153

iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; STAT1, signal transducer and activator of transcription 1; NF-κB, nuclear factor-κB; HO-1, heme oxygenase 1.
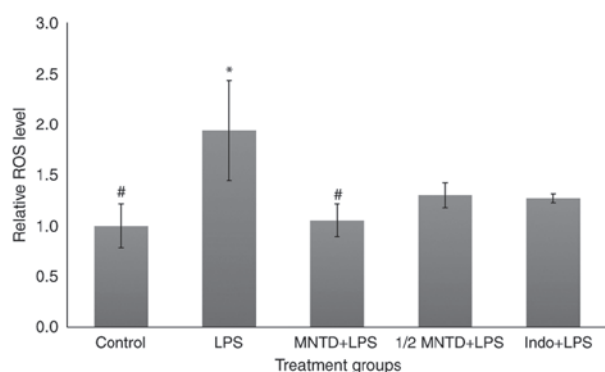

Figure 1. Relative reactive oxygen species levels in LPS-stimulated BV2 cells following treatment with madecassoside. Data are presented as the mean ± standard deviation. *$P<0.05$ vs. Control; #$P<0.05$ vs. LPS. LPS, lipopolysaccharide; MNTD, maximum non-toxic dose; Control, untreated BV2 cells; Indo, indomethacin.

was 32.96% compared with the LPS-treated group; similar to what was observed in the Indo+LPS group (Fig. 1). Despite the well-known anti-inflammatory properties of NSAIDs, including ibuprofen and indomethacin, the results of certain studies have suggested that NSAIDs may cause more harm than good following chronic use and may result in nephrotoxicity (29) or gastric ulcers (30). This suggests that the identification of alternative compounds capable of effectively reducing ROS levels produced by neuroinflammation that also induce minimal side effects may be beneficial.

Determination of pro-inflammatory iNOS gene expression. The expression of the inducible form of NOS is heavily upregulated following inflammation (10). As a widely-acknowledged downstream process of transcription factors, including NF-κB and STAT1 (10,31), the expression of iNOS is commonly upregulated in response to various stimuli, including infection, ischemia or mechanical injury (32). During NO production, the iNOS enzyme will behave as a RNS and may react with ROS to form harmful intermediates (7,29). NO also causes endothelial vasculature damage by upregulating interferon regulatory transcription factor 1, which mediates cellular apoptosis and pro-neuroinflammation (33). Notably, Olivera *et al* (34) indicated that iNOS-deficient mice had a higher degree of Trypanosoma infection; this suggests that, despite having

a negative effect with respect to inflammation, the balanced presence of iNOS is required to maintain the integrity of the blood brain barrier and blood vessels.

Within the present study, the expression of iNOS was significantly downregulated at the MNTD and ½ MNTD of madecassoside (79.81 and 74.21%, respectively) compared with the LPS group (both $P<0.05$; Fig. 2A). These results suggest that madecassoside may modulate anti-neuroinflammatory signalling pathways and their subsequent downstream activities, as iNOS expression was significantly reduced compared the control group ($P<0.05$). The results of the present study are in accordance with those of previous studies that identified the anti-inflammatory effects of other triterpenoid saponins, including tormentic acid (35) and madecassic acid (36). Madecassoside alone is also able to downregulate iNOS amongst various other pro-inflammatory components in a nephrotoxic *in vivo* setting (13). The results of the present study demonstrated the potent effect of madecassoside in reducing NO expression by downregulating iNOS expression in a neuronal setting.

Determination of COX-2 expression. The PTGS2 gene codes for the COX-2 enzyme, which is involved in the production of prostanoids and has been identified at high quantities during inflammation, resulting in subsequent tissue damage (37). The conversion of prostaglandin H2 to the active PGE2 yields pro-inflammatory ROS by-products (38). As indicated in Fig. 2B, significant downregulation of COX-2 expression following treatment with madecassoside and in the Indo+LPS group was observed compared with the LPS group ($P<0.05$). The MNTD and ½ MNTD groups exhibited a reduction of 70.85 and 66.65% in COX-2 expression, respectively, which was similar to the results obtained regarding iNOS expression. These results indicated that the downregulation of COX-2 and iNOS expression was not particularly dose-dependent. Although various studies have not clarified whether changes in the expression of pro-inflammatory factors including iNOS and COX-2 are dose-dependent or -independent, a number of studies using RAW 264.7 macrophages yielded downregulatory trends, similar to the results of the present study. Furthermore, the results of previous studies indicated that, although iNOS is a signaling pathway activated by NF-κB and STAT1, COX-2 expression is downstream of NF-κB but is inhibited by the activation of the STAT1 pro-inflammatory component (39,40).
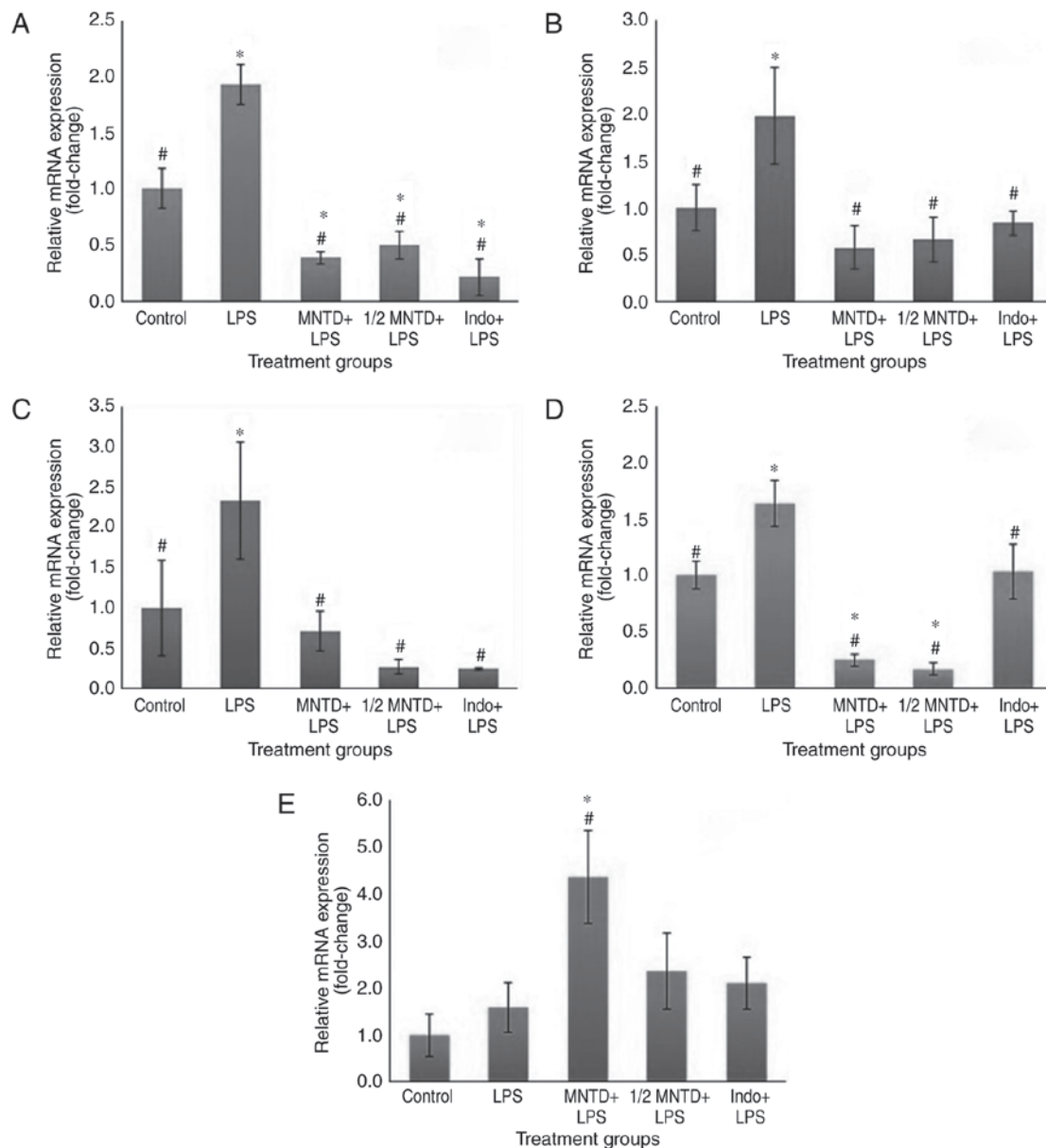
Figure 2. The expression of neuroinflammatory-associated genes following normalization to β-actin. (A) Inducible nitric oxide synthase, (B) cyclooxygenase 2, (C) signal transducer and activator of transcription 1, (D) nuclear factor-κB and (E) heme oxygenase 1 mRNA expression was determined using reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation. *$P<0.05$ vs. Control; #$P<0.05$ vs. LPS. LPS, lipopolysaccharide; MNTD, maximum non-toxic dose; Control, untreated BV2 cells; Indo, indomethacin.

Notably, in the present study, madecassoside treatment significantly downregulated NF-κB and STAT1 gene expression, compared with the LPS group ($P<0.05$; Fig. 2B and C).

Determination of pro-inflammatory STAT1 expression. STAT1 upregulation has been indicated to promote interferon-γ expression during inflammation and STAT1 induces inflammation-induced apoptosis by activating apoptosis-stimulating of p53 protein 2 (ASPP2) (41). Under normal physiological conditions, ASPP2 monitors and keeps neuroinflammation in check; however, following the upregulation of STAT1, ASPP2 mediates cell death (41). Other functions of STAT1 in mediating tissue damage during inflammation include modulating the expression of receptor-interacting serine/threonine-protein kinase 3, which stimulates macrophages during inflammation to increase cell death (42). In the present study, a significant

reduction of STAT1 gene expression within LPS-stimulated BV2 cells was observed in MNTD and ½ MNTD treated groups, but only the latter group exhibited downregulated STAT1 expression to the same extent as in the Indo+LPS group ($P<0.05$; Fig. 2C). These results suggest that the utilization of madecassoside may potentially mitigate the 'cytokine storm' by downregulating the JAK/STAT signaling pathway. Triterpenes, including avicin D (43) and betulinic acid (44) are also able to downregulate the JAK/STAT1 signaling pathway, which is in accordance with the results of the present study.

Determination of pro-inflammatory NF-κB expression. Considered to be the 'master transcription factor', particularly with regards to inflammation, it has been demonstrated that NF-κB is upregulated during inflammation and

specifically following stimulation with LPS (45). Following its localization to the nucleus, NF-κB activates downstream pro-neuroinflammatory components, including iNOS, COX-2 and NADPH oxidase, which elevates ROS levels (10) and the mediator of arachidonic acid, lipoxygenase (46). As well as its pro-inflammatory properties, NF-κB activation also regulates the anti-inflammatory components during inflammation, including HO-1 expression, which is coded by HMOX1 and thioredoxin-1 (TRX1) (10). However, during neuroinflammation, there is an imbalance between the pro- and anti-inflammatory components, which may cause excessive neuroinflammation (10).

As indicated in Fig. 2D, NF-κB expression was significantly downregulated in the MNTD and ½ MNTD-treated groups compared with the LPS group (84.78 and 89.73%, respectively; P<0.05). Notably, this effect was greater in MNTD and ½ MNTD-treated groups compared with the Indo+LPS group. NF-κB expression did not decrease significantly in the Indo+LPS group compared with the LPS group. This may be due to the fact that NSAIDs such as indomethacin commonly block the downstream processes involved in inflammation, including COX-2 and its subsequent prostanoid production, but may not affect NF-κB expression (47). Previous studies investigating indomethacin have unveiled that its mechanism differs from aspirin, as it was demonstrated that indomethacin-induced COX-2 inhibition did not downregulate upstream NF-κB processes (48,49). The effects of madecassoside on NF-κB expression were previously reported by Su *et al* (13) and Patil *et al* (50). These results suggest that the downregulation of NF-κB may be caused by the inhibitory properties of madecassoside on essential complexes within the cascade, including the various subunits of inhibitory κB kinase.

Determination of anti-neuroinflammatory HO-1 gene expression. The gene HMOX1 codes for the antioxidative enzyme HO-1, a key enzyme associated with various antioxidative signaling pathways (51). NF-κB activates many downstream transcription factors (10); a few of which being HO-1 and few other antioxidative enzymes, including SOD and TRX1.In the context of neuroinflammation during multiple sclerosis, the results of a previous study provided an interesting outlook as to how the HMOX1 gene behaves and its effects on excessive neuroinflammation (52). Activation of HO-1, which is associated with upstream antioxidative signalling pathways, including nuclear factor like 2 (Nrf2), produces components including biliverdin, carbon monoxide, iron and IL-10, all of which are antioxidants and function to prevent inflammation at varying stages. These antioxidative elements are potent enough to protect cells against apoptosis during oxidative stress (53).

As indicated in Fig. 2E, HO-1 gene expression in the MNTD+LPS group were significantly upregulated (by 175.22%) compared with BV2 cells treated with LPS alone (P<0.05). This response was significantly greater in the MNTD+LPS group (P<0.05) compared with the positive control (P<0.05). These results suggest that madecassoside has the potential to upregulate antioxidative signaling pathways. Unlike the downregulation of the pro-neuroinflammatory components discussed previously, the upregulation of HO-1 seems to be dose-dependent (Fig. 2E). Compared with the gene expression profile of upstream NF-κB presented in Fig. 2D, the
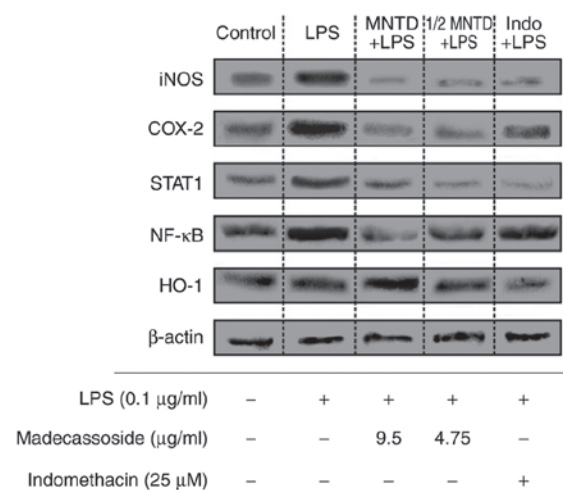

Figure 3. Western blotting of pro- and anti-neuroinflammatory proteins from cell lysates of LPS-induced BV2 microglial cells following pre-treatment with madecassoside or indomethacin. The expression of the pro-neuroinflammatory components iNOS, COX-2, STAT1 and NF-κB and the anti-neuroinflammatory protein HO-1 were assessed using western blotting. β-actin was used as housekeeping protein. LPS, lipopolysaccharide; MNTD, maximum non-toxic dose; Control, untreated BV2 cells; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; STAT1, signal transducer and activator of transcription 1; NF-κB, nuclear factor-κB; HO-1, heme oxygenase 1.

upregulation of HO-1 activity seems to be independent from the transcription factors previously assessed in the current study, further strengthening the hypothesis that HO-1 activity may be mediated by the Nrf2 signaling pathway.

Nrf2 signaling pathways may mediate gain-of-function mutations in squamous cell carcinomas, which provide cancer cells with the necessary protection against treatments and thus may confer a pro-tumorigenic property (54). Similar to results obtained regarding STAT1, a limited number of studies have analyzed the HMOX1 and HO-1 molecular profile of triterpenoid saponins, including madecassoside, asiatic acid and boswellic acid. The results of these studies indicate that HO-1 expression is increased due to upregulation of the Nrf2 pathway and therefore protects cells from apoptosis and oxidative damage (55,56).

Modulation of neuroinflammatory pathway-associated protein expression. The expression of neuroinflammation-associated proteins was determined using western blotting to confirm the gene expression profiles. As indicated in Fig. 3, the immunoblots of these proteins from the cell lysates of LPS-induced BV2 microglial cells following pre-treatment with madecassoside were assessed. The results indicated that the protein expression of the pro-neuroinflammatory components iNOS, COX-2, STAT1 and NF-κB was markedly increased in cells treated with LPS alone compared with the control group, whereas in madecassoside-treated groups, the expression of pro-inflammatory proteins was decreased in the MTND and ½ MNTD groups. Furthermore, the expression pattern of NF-κB protein in the Indo+LPS group was consistent with its gene expression. Furthermore, the protein expression of the anti-neuroinflammatory component HO-1 was notably increased in the MNTD+LPS group, which was also in accordance with
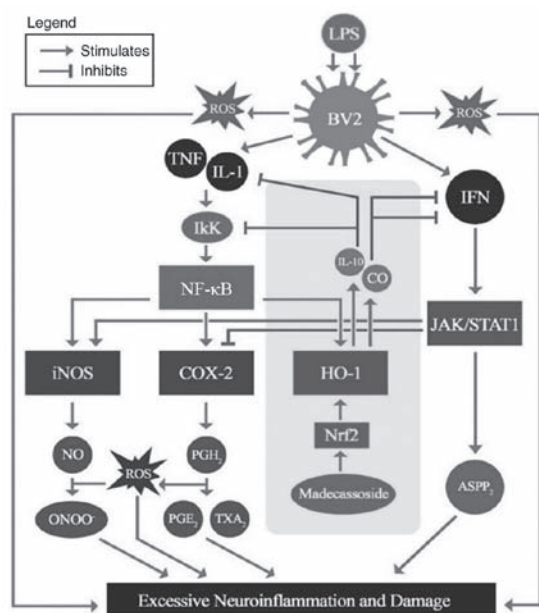
Figure 4. Summary of the primary findings and likely anti-neuroinflammatory mechanism of madecassoside. Shaded in the grey rectangular section are the pathways that madecassoside activated and thus subsequently down-regulated other pro-neuroinflammatory signaling pathways, and upregulated antioxidants and its associated signaling pathways. $ASPP_2$, apoptosis-stimulating of p53 protein 2; CO, carbon monoxide; HO-1, heme oxygenase 1; iNOS, inducible nitric oxide synthase; IFN, interferon; IL, interleukin; JAK/STAT1, janus kinase/signal transducer and activator of transcription 1; LPS, lipopolysaccharide; ROS, reactive oxygen species; NO, nitric oxide; NF-κB, nuclear factor κB; Nrf2, nuclear factor-like 2; ONOO⁻, peroxynitrite; PG, prostaglandin; TXA_2, thromboxane A_2; TNF, tumor necrosis factor.

its gene expression profile. In addition, indomethacin treatment also had similar effects to madecassoside on the protein expression of iNOS, COX-2, STAT1 and HO-1. The increase in NF-kB protein expression in the Indo+LPS group increased, which is consistent with the changes in its gene expression. Notably, the results of a previous study demonstrated the effects of madecassoside on inflammatory-associated signaling pathways, including iNOS and NF-κB, in murine kidneys, which indicated similar results to those demonstrated in a previous study (13). Furthermore, the anti-inflammatory effects of other triterpenoids extracted from *Centella asiatica*, including asiatic acid, have been demonstrated in a study utilizing murine liver samples (57), which further support the anti-neuroinflammatory abilities of madecassoside.

In conclusion, the present study indicated that madecassoside, a triterpenoid saponin, is a potent anti-neuroinflammatory agent, as it is able to reduce intracellular ROS levels and influence genomic and proteomic components that have been implicated in neuroinflammation. The results of the present study suggest that madecassoside successfully and significantly downregulated the gene and protein expression of iNOS, COX-2, STAT1 and NF-κB, all of which are pro-neuroinflammatory components. Furthermore, madecassoside at the MNTD significantly increased the gene expression of the anti-inflammatory component HO-1 by 175.22% compared with the LPS group. The primary results and the likely anti-neuroinflammatory mechanisms of madecassoside are presented in Fig. 4, whereby protein expression of pro-neuroinflammatory components were reduced

while the anti-neuroinflammatory HO-1 was increased following madecassoside treatment. The antioxidative nature of madecassoside, which has been suggested to activate the Nrf2/HO-1 signaling pathways at the gene and protein levels, suggests that this compound may be an ideal treatment for neuroinflammation. The results of the present study warrant further *in vivo* studies investigating the effects of madecassoside to treat neuroinflammation, as it may be developed as a novel anti-neuroinflammatory agent.

Competing interests

The authors declare that they have no competing interests.

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