The extracts of *Lobaria pindarensis*, an edible lichen from Himalayas, suppress inflammatory response and fibrillation of amyloid β-protein in cultured microglial cells: Active depsidones, norstictic acid and stictic acid, are responsible for the functions

Mei-Xia Yang\(^a,b\), Edwin Hok-Chi Cheng\(^a,b\), Dusadee Ospandpant\(^a,b\), Ka-Ki Tung\(^c\), Li-Song Wang\(^d\), Sheng-Ying Lin\(^a,b\), Queenie Wing-Sze Lai\(^a,b\), Qi-Yun Wu\(^a,b\), Ying-Jie Xia\(^a,b\), Ka-Wing Leung\(^a,b\), Ran Duan\(^a,b\), Tina Ting-Xia Dong\(^a,b\), Karl Wah-Keung Tsim\(^a,b\)*

\(^a\) Division of Life Science and Center for Chinese Medicine, The Hong Kong University of Science and Technology, Hong Kong, China
\(^b\) Shenzhen Key Laboratory of Edible and Medicinal Bioresources, HKUST Shenzhen Research Institute, Shenzhen, China
\(^c\) Department of Chemistry, The Hong Kong University of Science and Technology, Hong Kong, China
\(^d\) Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Yunnan, China

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ABSTRACT

*Lobaria pindarensis* Raasänen is an edible and medicinal lichen found in the Himalayas that has been used as ethnomedicine for years to combat inflammation. The study aimed to evaluate the ability of *L. pindarensis* extracts and its main depsidones to suppress inflammation and Aβ fibril formation in cultured cells induced by LPS or Aβ\(_{1-42}\). The mRNA and protein expressions of inflammatory cytokines were declined significantly by the applied herbal extracts and depsidones in the LPS/Aβ-treated immune cells (p < 0.05 to p < 0.001). In addition, the extracts and depsidones of *L. pindarensis* showed enhanced phagocytic function of microglia and dis-assembling aggregated Aβ fibrils in dose-dependent manners (p < 0.05 to p < 0.001). The study presents the first evidence that *L. pindarensis* extracts have the potential to suppress inflammation and Aβ fibril protein aggregation, making it a promising candidate for developing health food supplements for Alzheimer’s patients.

1. Introduction

Neurodegenerative diseases are characterized by progressive neurological deterioration, decrease in functionality and personal independence (Abril et al., 2004). Alzheimer’s disease (AD) is the most prevalent form of dementia, which is a complex, multifactorial, progressive neurodegenerative disease primarily affecting elderly population (Eratne et al., 2018). Two major neuropathological characteristics of AD are: (i) the accumulation of neurofibrillary tangles (NFTs); and (ii) cerebrovascular deposition of fibrillar amyloid β-protein (Aβ) (Domingues et al., 2017). Indeed, the cerebral amyloid angiopathy is a prominent pathological feature of AD, as well as in other dementia. Microglial cells, the principle resident immune cells, serve as the first line of defence during brain injury or disease, and this cell type has been extensively studied in considering the prevention of AD. The immortalized mouse microglial cell line BV2 has been evaluated to be a valuable substitute for primary microglia, which secretes vast amounts of pro-inflammatory mediators, e.g., tumour necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), when activated, mimicking to neuroinflammation and/or neuronal damage in the brain (Querfurth &...
The complicated pathophysiology of AD is primarily attributed to excessive production and subsequent aggregation of Aβ oligomers, which is a major cause of a series of neuronal death and tissue damage (Adlard et al., 2014). Similarly, lipopolysaccharide (LPS), a bacterial toxin, causes the pathogenesis of AD by provoking neuroinflammation (Abarshi et al., 2016; Saadat et al., 2019). In parallel, increased levels of proinflammatory markers, including IL-6, IL-1β and TNF-α, were found in blood and cerebrospinal fluid of AD patients (Humpel & Hochstrasser, 2011). These results further suggest the role of neuroinflammation in developing AD.

Lichens are unique symbiosis between a fungus belonging to Ascomycota and Basidiomycota phylum (mycobiont) and a chlorophyll-containing partner (photobiont), which could be an algal or a cyanobacterium. Lichens are being applied in a wide range of medical treatments throughout the world, especially in Himalayas region, mainly as traditional or folk medicine, to treat wounds and skin disorders, or respiratory and digestive problems (Crawford, 2019; Yang et al., 2021).

The symbiosis between a fungus and a photosynthetic partner gives lichen having a production of distinct lichenic metabolites (Fernandez-Moriano et al. 2017). A growing interest in lichens, as sources of innovative pharmacologically active chemicals, has been targeted in its activities of anti-bacterial, anti-inflammatory, and cytotoxicity (Gomez-Serranillos et al., 2014). Depsidones are the most abundant metabolites being produced by lichens with known pharmacological properties, e.g., fumarprotocetraric acid, lobaric acid, norstictic acid, physodic acid, salazinic acid and stictic acid compounds (Urena-Vacas et al., 2021).

However, the neuroprotective properties of these depsidones remain unknown.

Several species of Lobaria are frequently used as traditional medicines in treating pneumonia. Lichens, as ethnomedicines, are extremely widespread across Himalayas region (Devkota et al., 2017; Yang et al., 2022). As one of the most abundant, edible, and medicinal lichens in Himalayas region, Lobaria pindarensis Räsänen (Lobaria (Schreb.) Hoffm, Peltigeraceae) is an endemic species, growing on deciduous and coniferous trees and shrubs in the sub-alpine forests at 2,000–4,000 m. Having the high abundance within the lichen, the major depsidones of L. pindarensis are norstictic acid and stictic acid (Yoshimura, 1971); these depsidones have been shown to be effective in treating cancers (Ebrahim et al., 2016). The pharmacological properties of L. pindarensis extract on inflammation and Aβ fibril in the brain have never been characterized. Here, the possible interactions between inflammatory responses and L. pindarensis extract, as well as its main depsidones, were investigated by evaluating its effects in suppressing LPS/Aβ-induced inflammation in cultured microglial BV2 and macrophagic RAW264.7 cells. The results pave a direction to develop L. pindarensis as a health food supplement for patients suffering from AD.

2. Materials and methods

2.1. Raw material preparation

The dried herbs of L. pindarensis were purchased from Yunnan market (Yunnan, China). The voucher specimens of L. pindarensis were deposited in the Herbarium, Kunming Institute of Botany, CAS and Centre for Chinese Medicine of HKUST. Stictic acid (>95%) and norstictic acid (>95%) were obtained from the Cayman chemical (Ann Arbor, MI). All chemicals were dissolved in Dimethyl sulfoxide (DMSO) at 30 mM and methanol at 1 mg/mL as stock solutions. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), lipopolysaccharide (LPS) and dexamethasone (DEX) were purchased from Sigma-Aldrich (St. Louis, MO). LY294002, rabbit antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5174S) were purchased from Cell Signalling Technologies (Danvers, MA). Ultrapure water was prepared from a Milli-Q purification system (Millipore, Molsheim, France). COX-2 antibody (sc1745) and goat anti-rabbit IgG-HRP (sc2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All culture reagents were from Thermo Fisher Scientific (Waltham, MA). All chemicals used were of analytical grade (AR) or HPLC grade.

2.2. Herbal extract preparation

The powder of L. pindarensis (10.0 g) was placed in a 250-mL round-bottomed flask and dissolved in 100 mL 90% ethanol or distilled water. The solutions were refluxed for 1 h before being filtered through a paper filter (Advantec, Tokyo, Japan). The extract was then evaporated to dryness with a rotary evaporator to yield final ethanol (named as LPEthOH) and water (named as LPeTH) extracts of 2.05 g and 4.16 g, respectively.

2.3. High resolution LC-MS-MS analysis

High resolution Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) analysis was performed on an Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled with a Xevo G2 Q-TOF mass spectrometer with a standard ESI interface (Waters, Milford, MA). Ten microfilters of samples were injected into a GraceSmart VisionHT C18 HL 3µ column (100 × 2.1 mm, 3 µm) eluting at a flow rate of 0.3 mL/min, 0.1% formic acid in water (v/v) (A) and acetonitrile (B) were selected as the mobile phase. The gradient was from 5% B and hold for 1 min before linearly increase to 100% B in 9 min, then hold for 4 min before preconditioning for 3 min. The liquid chromatography (LC) eluate from 1 to 10 min was diverted to mass spectrometer for analysis. The mass spectrometer was operated in negative ion mode with capillary and cone voltages, setting at 1.5 kV and 60 V, respectively. Norstictic and stictic acid were monitored by using the product ion scan mode, MS/MS spectra of [M−H]- were acquired by using 0.75 s scan time and 15 eV collision energy. Both acids in aqueous and ethanol extracts were quantified by standard addition method, the analyte signals were extracted by the exact mass of the most abundance fragment (NA: m/z 327.05, SA: m/z 341.07) with mass window of 0.01 Da. Standard calibration curves were established by plotting the peak area against the concentration of standard added.

2.4. Cell culture

Cultured cell line RAW 264.7 was purchased and shipped from American Type Culture Collection (ATCC account number: TIB-71; Manassas, VA). RAW264.7 cells were cultured in Dulbecco’s modified Eagles medium supplemented with 100 IU/mL penicillin supplied with 100 μg/mL of streptomycin and 10% heat-in-active fetal bovine serum (FBS). BV2, a microglial cell line, was purchased from PUMC (Peking, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (10 000 units and 10 000 μg/mL). The cells were cultured at 37 °C in a water-saturated 5% CO2 incubator, and the subculture was performed every other day. The cell viability was determined, as described previously (Xia et al., 2022).

2.5. Phagocytosis assay

The phagocytosis of BV2 cells was determined by FluoSpheresTM carboxylate-modified microspheres (F8816, Thermo Fisher Scientific). Briefly, BV2 cells were seeded onto 35-mm glass bottom confocal dishes for 24 h, then LPeTH and LPeTH (1, 5, 10, 25, 50, 75 μg/mL, respectively), norstictic acid or stictic acid (30 μM), and dexamethasone (10 μM), were pre-treating the cultures for 3 h before LPS (100 ng/mL) challenge. Afterwards, the cultures were incubated 12 h under 5% CO2 at 37 °C for the following experiments. The latex beads (V/V, 1:5000,
Ex/Em: 625/645) were added into the culture medium and incubated for 3 h for the engulfment. The cells were washed by PBS 3 times and fixed with 4% paraformaldehyde for 15 min before being blocked by 1% BSA for 30 min. Cells were then stained F-actin by incubating cells with rhodamine-phalloidin (V/V, 1:4000, Ex/Em: 540:565 nm) for 20 min. After 3 times of PBS washing, the cells were detected under a SP8 confocal microscope (Leica Microsystems). Three different views were counted under a Leica LAS X software platform. The phagocytic rate was calculated by dividing the number of cells that had phagocytosed at least one bead by the total number of cells per capture. The phagocytic index was corresponding to the average number of beads being phagocytosed per cell. For quantification of phagocytosis, at least 5 views, was examined per experimental condition.

2.6. Preparation of ApH2 fibrils

Synthetic ApH2 fibrils (GL Biochem, Shanghai, China) was dissolved in 100% hexafluoro isopropanol and sonicated for 20 min at 25 °C. The ApH2 monomer solution at a stock concentration of 1 mM was aliquoted and dried in a fume hood overnight for complete removal of hexafluoro isopropanol and then stored at −20 °C. For ApH2 fibril formation, the dried ApH2 peptide film was resuspended in 20 μL dimethyl sulfoxide dissolved in 10 mM HCl, diluted to a final concentration of 100 μM, and vortexed for 1 min. The solution of the ApH2 peptide was incubated at 37 °C for 6 days to form fibrils before further analysis.

2.7. Immunofluorescence and confocal microscopy

BV2 cells were seeded onto coverslips for immunostaining for 24 h, then Lp_water and LpBion (1, 5, 10, 25, 50, 75 μg/mL, respectively), norstictic acid and stictic acid (30 μM), and dexamethasone (10 μM) were pre-treating for 3 h before ApH2 (10 μM) challenge. Afterwards, the cultures were incubated 12 h under 5% CO2 at 37 °C for the following experiments. Afterwards, the cells were washed by PBS before being fixed with 4% paraformaldehyde in PBS for 15 min. Then, the cells were blocked by 5% BSA with 0.1% Triton X-100 for another 1 h. After blocking, cells were washed three times by PBS and followed by being stained with anti-amyloid fibrils (rabbit polyclonal) antibody at 1:500 overnight at 4 °C. Next, cells were labelled by secondary antibody (Alexa Fluor 488-conjugated anti-rabbit antibody) and 4',6-diamidino-2-phenylindole (DAPI, 1:500) for 2 h. The cells were examined by an SP8 confocal microscope (Leica Microsystems, Germany).

2.8. Quantitative real-time PCR

The quantitative real-time PCR was determined as described previously (Xia et al., 2022). Primers, employed in qRT-PCR, were as follows: TNF-α forward, 5′-AGT GAC AAG CCT GTA GCC-3′ and reverse, 5′-AGG TTG ACT TTC TCC TGG-3′; IL-1β forward, 5′-GTG GTA TTC ATG AGC TT-3′ and reverse, 5′-TTG ATA ACA CAG GAC AGG TA-3′; IL-6: forward, 5′-GGA GTA CCA TAG CTATCC GG-3′ and reverse, 5′-CTA GGT TTG CCG AGT AGA TC-3′; iNOS forward, 5′-CGG TAC AAT ATC CTT GAG-3′ and reverse, 5′-ACA TTT TGG TGC TGG AAA CTA-3′; GAPDH: forward, 5′-AAG GGA TTT GGC CGT ATT GG-3′ and reverse, 5′-CTT CCC GTT CAG CTC TGG G-3′.

2.9. Western blot

Cell protein lysis was subjected to SDS-PAGE for revealing the target gene translational activities. After transferring the target proteins to membranes, the membranes were incubated with anti-COX-2 (CST) at 1: 1,000 dilutions, and anti-GAPDH (Santa Cruz, Dallas, TX) at 1: 1,000 dilutions at 4 °C for overnight. The phosphorylation of target proteins was determined by Western blot assay. Cultures were serum-starved for at least 3 h before the drug treatment. After drug treatment for 30 min, the cultures were collected and lysate immediately in lysis buffer for Western blot assay. Following incubation in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies in 1: 2,000 dilutions for 3 h at room temperature, the immune-complexes were visualized by the enhanced chemiluminescence (ECL) method (Amersham Biosciences, Piscataway, NJ). The band intensities of all the samples were run on the same gel and under strictly standardized ECL conditions. The bands were analysed by using Image Lab (version 6.1) software.

2.10. Molecular docking

Chemical structures of molecules were downloaded from Pubchem (https://pubchem.ncbi.nlm.nih.gov/), while the protein structures were downloaded from Protein Data Bank (PDB, https://www.rcsb.org/). Virtual screening was performed on SEESAR software (https://www.biolsolveit.de/) following the procedures as below: (i) The binding site was defined according to the residues composing the identified druggable pocket. Ligand states including protonation and tautomeric forms were automatically assessed in the model using the ProTons method, which subsequently generated the most accessible hydrogen positions based on an optimal hydrogen bonding network; (ii) Docking simulation was performed on “Compute Lead IT Docking” mode by using the FlexX algorithm. Ten binding conformations for each ligand were generated; and (iii) The “assess affinity with hydrogen bond and dehydration (HYDE) in SEESAR” node produced refined binding free energy (i.e., ΔG) and estimated HYDE affinity (KHYDE) for each ligand pose using the HYDE rescoring function (Spagnolli et al., 2021).

2.11. Statistical analysis and other assays

The statistical analyses were performed by using OriginPro 2022. Data are represented as mean ± SEM. The significance of difference was determined by one-way analysis of variance (ANOVA). The p < 0.05 was considered statistically significant.

3. Results

3.1. The extracts of L. pindarensis and its possible active ingredients

L. pindarensis is being found in Himalayas regions nearby the boundary of China (Fig. 1). This species of lichen has a board range of usage, historically, and which is known to be safe for human consumption. The 90% ethanol extract and water extracts of L. pindarensis were named as LpBion and Lp_water, and the extract yield (% w/w) ± S. D.) are 15.3 ± 0.1 and 14.5 ± 0.2, respectively. Two main depsidones in LpBion and Lp_water were identified and validated by UPLC-MS/MS analysis. By the retention times and MS/MS spectra, norstictic acid and stictic acid were identified in LpBion and Lp_water (Supplementary Fig. S1). The contents of norstictic acid and stictic acid in LpBion and Lp_water were determined (Table 1). The amounts of norstictic acid in Lp_water and LpBion were rather similar, ranging from 0.638 to 0.878 μg/mg. Stictic acid in Lp_water was 0.466 μg/mg, which was much higher in LpBion having 10.450 μg/mg. In general, LpBion contained higher amounts of depsidones than Lp_water. The cytotoxicity of L. pindarensis extracts and its depsidones in immune cells, i.e., RAW264.7 and BV2 cells, were analysed. The concentrations (1–75 μg/mL) of applied extracts and below 30 μM of the two depsidones did not show cytotoxicity after 2 days of treatments (Supplementary Fig. S2).

Traditionally, L. pindarensis has been used for treatment under inflammatory diseases. To probe the possible inflammatory responses of this lichen, molecular docking was performed on the possible interactions between inflammatory biomarkers (IL-6, IL-1β, iNOS and TNF-α) with the major depsidones (norstictic acid and stictic acid). The possible binding sites of IL-1β (PDB: 1ITB), iNOS (PDB: 3EBF), IL-6 (PDB: 1L6), and TNF-α (PDB: 2AZ5) were selected, as reported in the literatures (Veverka et al., 2012; Halim et al., 2015; Cinelli et al., 2020; Zia et al., 2020). IL-6, IL-1β, iNOS, TNF-α and COX2 are the import
inflammatory factors, playing critical roles in development of inflammatory medications (Gong et al., 2017). Docking studies were conducted utilising SEESAR software (https://www.biosolveit.de/, version 12.0). The Norstictic acid and stictic acid were proposed binding with IL-1α, TNF-α with good binding energies < 6.0 (Supplementary Fig. S3). Having the virtual analysis, the potential anti-inflammatory activities of L. pindarensis and its depsidones were subsequently determined in cell cultures.

3.2. The extract of L. pindarensis regulates LPS-induced expression of pro-inflammation cytokines

Here, the lipopolysaccharide (LPS)-stimulated cells served as the model in mimicking the chronic inflammatory status. In cultured RAW264.7 (macrophage) and BV2 (microglia) cells, the application of LPS induced robust activation, from 10 to 20 folds, of the expressions of inflammatory markers, including IL-6, IL-1α, TNF-α, NO, and IFN-γ in RAW264.7 cells (Fig. 2). Dexamethasone served as a positive control, suppressed the LPS-treated cultures in expressing inflammatory markers. These parameters indicated proper establishment of the cell model. Under the treatment of norstictic acid, stictic acid and L. pindarensis extracts in cultured LPS-stimulated macrophages, the mRNA levels of IL-6, IL-1α, iNOS and TNF-α were markedly suppressed (p < 0.05 to p < 0.001). Norstictic acid and stictic acid at 30 μM showed a full suppression on the induced mRNA expressions (Fig. 2), as that of dexamethasone. In parallel, the extracts of L. pindarensis, LPwater, and LPEtOH, showed the suppression in dose-dependent manners. The application of LPEtOH declined the mRNA expressions of the inflammatory markers, better than that of LPwater in all scenarios: the IC50 was similar in suppressing the cytokines from about 10 μg/mL of the extracts (Fig. 2). In parallel, the protein expression of COX-2 (~65 kDa) was determined in the LPS-treated macrophages, which was markedly up regulated by ~ 10 folds (p < 0.05) (Fig. 3A-B). Upon the treatments of norstictic acid, stictic acid, LPwater, and LPEtOH, the expression of COX-2, as induced by LPS, was markedly reduced. In addition, the effect of LPwater was more robust than that of LPwater.

Microglial inflammatory response could be a parameter in developing Alzheimer’s disease. The roles of L. pindarensis extracts and its depsidones were tested in cultured microglial cell line BV2. Similar to the situation in macrophage, the L. pindarensis extracts and its depsidones showed robust suppression on the LPS-induced expressions of inflammatory markers, i.e., IL-6, IL-1β, iNOS, TNF-α, NO, and IFN-γ (p < 0.05 to p < 0.001) (Fig. 4), as well as the protein expression of COX-2 (p < 0.05) (Fig. 5A-B). The suppressive effects of L. pindarensis extracts and its depsidones however were less robust as that of the cultured macrophage. Besides, the effect of LPEtOH was more robust than that of LPwater in all cases of analyses.

The microglial phagocytosis is an essential process to maintain brain homeostasis and clearance of potential toxic factors. Especially, the phagocytic function of microglia plays a critical role in the engulfment during the AD pathological process (Li and Barres, 2018). Here, we determined the anti-inflammatory response of L. pindarensis extracts and its depsidones in LPS-induced phagocytosis of cultured BV2 cells. Using the carboxylate-modified fluorescent microspheres, the ability of bead uptake was determined in BV2 cells after different treatments (Fig. 6A). The result showed that the uptake of fluorescent beads by BV2 cells was revealed obviously under LPS challenge. The LPS-induced phagocytosis was markedly suppressed by norstictic acid, stictic acid, LPwater, and LPwater, similar to that of dexamethasone (p < 0.05 to p < 0.01) (Fig. 6B).
3.3. The extracts of L. pindarensis regulate Aβ42-induced neuroinflammatory response

In cultured BV2 cells, application of Aβ42 was able to induce the mRNA expressions of inflammatory markers, i.e., IL-6, IL-1β, iNOS, TNF-α, to 3 to 4 folds by analyses of qRT-PCR, similar to the scenario of LPS application (Fig. 7). The Aβ42-induced inflammatory responses were markedly suppressed by the applied LP_EOH and LP_water in dose-dependent manners (p < 0.05 to p < 0.001). LP_EOH and LP_water, at 25 to 50 μg/mL, were able to suppress most of the induced inflammation. As expected, LP_EOH showed better effect than that of LP_water. In parallel, the depsidones of L. pindarensis, norstictic acid and stictic acid, showed similar suppression on inflammatory responses (Fig. 7). Dexamethasone serving as a positive control blocked the induced inflammatory response.

Further to understand the effect of fibrillation of amyloid under the treatment of L. pindarensis extracts and its depsidones in cultured BV2 cells. The formation of Aβ42 fibrils in cultures were identified by immunofluorescence assay. The number of amyloid fibrils significantly decreased, almost back to control level, after treatments of LP_EOH, LP_water, norstictic acid and stictic acid (p < 0.05) (Fig. 8A-B). In order to account the roles of norstictic acid and stictic acid in L. pindarensis extracts, the efficacy of anti-inflammatory responses was compared between LP_EOH and the amount of depsidones within the extract. The amounts of norstictic acid and stictic acid in 1, 10 and 50 μg/mL of LP_EOH were determined, i.e., norstictic acid from 0.878 to 43.90 ng and stictic acid from 10.45 to 522.50 ng, as shown in Supplementary Table S1. The suppression of LPS-induced inflammatory cytokine expressions could be significantly represented by norstictic acid and stictic acid at different concentrations (p < 0.05 to p < 0.001) (Fig. 9). The result suggests the major contribution of depsidones in L. pindarensis extract responsible for anti-inflammation.

4. Discussion

Inflammation is a import physiological response against a myriad of factors, such as infection, trauma, and various disease (Gholamnezhad et al. 2022; Mahomoodally et al., 2018; Mollica et al., 2021). The pathology of Aβ loading is closely associated with inflammatory response in the brain, and indeed the activated microglial cells are situated in vicinity of the plaques (Bagyinszky et al., 2017). The aggregation of Aβ and their deposition in the brain have been proposed to be one of the causes of AD (Chen et al., 2017). The blockade of Aβ aggregation is one of the several therapeutic strategies for AD treatment, e.g., the new drug of AD, aducanumab, in reducing Aβ plaques. Biochemically, the activated microglia producing inflammatory cytokines could inhibit neural precursor activity in hippocampus of rodents (Perea et al., 2020). TNF-α, IL-1β, IL-6, iNOS and COX2 are among the pro-inflammatory factors, such as infection, trauma, and various disease (Gholamnezhad et al. 2022; Mahomoodally et al., 2018; Mollica et al., 2021). The pathology of Aβ loading is closely associated with inflammatory response in the brain, and indeed the activated microglial cells are situated in vicinity of the plaques (Bagyinszky et al., 2017). The aggregation of Aβ and their deposition in the brain have been proposed to be one of the causes of AD (Chen et al., 2017). The blockade of Aβ aggregation is one of the several therapeutic strategies for AD treatment, e.g., the new drug of AD, aducanumab, in reducing Aβ plaques. Biochemically, the activated microglia producing inflammatory cytokines could inhibit neural precursor activity in hippocampus of rodents (Perea et al., 2020). TNF-α, IL-1β, IL-6, iNOS and COX2 are among the pro-inflammatory factors having over production during AD development. Natural products are considered as an important source of bioactive compounds that are believed to have minimal side effects (Pagano et al., 2020). Finding novel regulators, or drug targets, from the natural products to regulate pro-inflammatory and analgesic drugs on the market, their side effects and ineffectiveness in some conditions require the continuous search for new drug candidates.

Lobaria, as a representative medicinal lichen in Himalayas region, produces unique bioactive secondary metabolites, such as depsidones. Today, the peoples in Himalayas continue their age-old usage of lichens as food and/or medicine. Within the last decade, the market sale of lichen for folk medicine, especially for those health-promoting food products, has increased remarkably: because Himalayas has become a popular region for domestic tourism. However, the low productivity of natural Lobaria means that over-harvesting is a concern today. In
Fig. 3. LP_{water}, LP_{EtOH} and the depsidones regulate COX2 expression in LPS-induced inflammatory response in RAW 264.7 cells. Culture condition and treatment were same as in Fig. 2. The expression of COX2 (~72 kDa) was determined by Western blotting (left). GAPDH served as an internal control. (A): LP_{water} and (B): LP_{EtOH} (from 1 to 75 μg/mL), norstictic acid (NA) or stictic acid (SA) (both at 30 μM), and dexamethasone (DEX; 10 μM) are shown here. Values are presented in relative amount having control level as 1, in Mean ± SEM, n = 4, ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.

Fig. 4. LP_{water}, LP_{EtOH} and the depsidones inhibit the LPS-induced inflammatory response in BV2 cells. Cultured BV2 cells were treated with LP_{water} and LP_{EtOH} (from 1 to 75 μg/mL), norstictic acid (NA) or stictic acid (SA) (both at 30 μM), and dexamethasone (DEX; 10 μM), as indicated, were pre-treating the cultures for 3 h before LPS (100 ng/mL) challenge for another 12 h. Total mRNA was extracted to perform relative qRT-PCR analysis for pro-inflammatory cytokines, IL-6, IL-1β, TNF-α, and the enzyme iNOS. GAPDH, a housekeeping gene, served to perform the relative quantification. Values are presented in fold of change (x basal) compared to the control, in Mean ± SEM, n = 4, ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.

Fig. 5. LP_{water}, LP_{EtOH} and the depsidones regulate COX2 expression in LPS-induced inflammatory response in BV2 cells. Culture condition and treatment are same as in Fig. 4. The expression of COX2 (~72 kDa) was determined by Western blotting (left). GAPDH served as an internal control. (A): LP_{water} and (B): LP_{EtOH} (from 1 to 75 μg/mL), norstictic acid or stictic acid (30 μM), and dexamethasone (10 μM) are shown here. Values are presented in relative amount having control level as 1, in Mean ± SEM, n = 4, ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.
Fig. 6. LP_{water} and LP_{EtOH} and the depsidones regulate phagocytosis in LPS-induced inflammatory response in BV2 cells. Cultured BV2 cells were treated with LP_{water} and LP_{EtOH} (both at 75 μg/mL), norstictic acid (NA) or stictic acid (SA) (both at 30 μM), and dexamethasone (DEX; 10 μM), as indicated, were pre-treating the cultures for 3 h before LPS (100 ng/mL) challenge for another 12 h. Then, FluoSpheres™ carbohydrate-modified microspheres (diameter 1 μm, Ex/Em: 625/645 nm, showed by Green) were included in the culture medium (V/V, 1: 2,000) for 3 h. After bead engulfment, the cells were washed by PBS and stained F-actin by rhodamine-phalloidin (V/V, 1:4000, Ex/Em: 540/565 nm, showed by Red). Different views were randomly captured under a Leica SP8 confocal microscope. (A): The representative images are shown for each group. (B): The phagocytic rate was calculated, as the percentage of control (LPS-treated cells) taking up >1 bead per cell, in Mean ± SEM, n = 4. ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.

Fig. 7. LP_{water} and LP_{EtOH} and the depsidones inhibit the Aβ_{42}-induced inflammatory response in BV2 cells. Cultured BV2 cells were treated with LP_{water} and LP_{EtOH} (from 1 to 75 μg/mL), norstictic acid (NA) or stictic acid (SA) (both at 30 μM), and dexamethasone (DEX; 10 μM), as indicated, were pre-treating the cultures for 3 h before Aβ_{42} (10 μM) challenge for another 12 h. Total mRNA was extracted to perform relative qRT-PCR analysis for pro-inflammatory cytokines, IL-6, IL-1β, TNF-α, and the enzyme iNOS. GAPDH, a housekeeping gene, served to perform the relative quantification. Values are presented in fold of change (x basal) compared to the control, in Mean ± SEM, n = 4. ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.
L. pindarensis, an endemic lichen species and having a rich resource in Himalayas region, which contains the high abundance of depsidones (norstictic acid and stictic acid). Therefore, our study in clarifying the pharmacological properties of L. pindarensis using molecular analysis is important and urgent, which may pave a direction of development on L. pindarensis, instead of other species, as a health supplement for AD patients.

5. Conclusion

In our study, we have shown the establishment of inflammatory cell models both in LPS-exposed RAW 264.7 cell and Aβ42-exposed microglial cells, as evidenced by excessive expression of TNF-α, IL-1β, IL-6, iNOS and COX2. More important, the inflammatory responses were dramatically alleviated by the treatments of L. pindarensis extracts (LPwater and LPEtOH), and its main depsidones (norstictic acid and stictic acid). Our results found that the treatment of LPwater in cultured immune cells could decline the mRNA levels of IL-6, IL-1β, iNOS and TNF-α, and protein expression of COX2. The effect of LPwater is better than that of LPEtOH, which may be due to the fact that LPwater is containing higher amount of depsidones than that of LPEtOH, especially, the content of stictic acid. Supporting this notion, the mRNA and protein expressions of inflammatory cytokines were declined significantly by treating depsidones of L. pindarensis in cultures. Besides, the extracts and the depsidones of L. pindarensis showed phagocytotic enhancement of microglia and disassembled aggregated Aβ42 fibril in dose-dependent manners. Therefore, the results demonstrated the capacity of extracts and depsidones of L. pindarensis in having anti-inflammation and anti-Aβ42 fibril formation, and which had a high biocompatibility, as assessed on microglial BV2 and macrophage RAW264.7 cells under LPS and Aβ42 treatments. These results indicate the molecular mechanism being associated with the cognitive improvement. Furthermore, the inhibiting activities of LPwater, comparing with the defined amount of depsidones on LPS-induced neuroinflammatory BV2 cell model, were conducted. The result revealed that the depsidones (norstictic acid and stictic acid) of L. pindarensis have shown their ability to achieve the anti-inflammatory activities. The herbal extract has better activity than that of two depsidones, probably because there are possible other active compounds within L. pindarensis, or the effects could be enhanced by the lichens in Himalayas region, comparing to other lichen species, and importantly which has a good record of safety as well. Besides, L. pindarensis is an endemic lichen species and having a rich resource in Himalayas region, and which contains the high abundance of depsidones (norstictic acid and stictic acid). Therefore, our study in clarifying the pharmacological properties of L. pindarensis using molecular analysis is important and urgent, which may pave a direction of development on L. pindarensis, instead of other species, as a health supplement for AD patients.

Fig. 8. Co-localization of amyloid fibrils with Aβ42 in BV2 cells. Cultured BV2 cells were treated with Aβ42 (10 μM) for 12 h together with or without dexamethasone (DEX; 10 μM) or norstictic acid (NA) or stictic acid (SA) (both at 30 μM), LPwater (75 μg/ml), LPwater (75 μg/ml). Amyloid fibrils were visualized by the polyclonal antibody. The specific staining of fibril was seen clearly in the green channel. The cells were stained by DAPI (blue). Different views were randomly captured per sample under a Leica SP8 confocal microscope. (A): The representative images are shown for each group. (B): The aggregation rate of Aβ42 was calculated by fluorescence proportion. Data are expressed as a percentage. The aggregation was calculated as the percentage to control (Aβ42-treated cells), in Mean ± SEM, n = 4. ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.

Fig. 9. Norstictic acid and stictic acid account for the activity of L. pindarensis extract. Cultured BV2 cells were treated with LPwater (from 1 to 50 μg/ml), norstictic acid (NA) or stictic acid (SA) (at corresponding concentrations within the defined LPwater see Supplementary Table 1), and dexamethasone (DEX; 10 μM), as indicated, were pre-treating the cultures for 3 h before LPS (100 ng/ml) challenge for another 12 h. Total mRNA was extracted to perform relative qRT-PCR analysis for pro-inflammatory cytokines, IL-6, IL-1β, TNF-α, and the enzyme iNOS. GAPDH, a housekeeping gene, served to perform the relative quantification. Values are presented in fold of change (x basal) compared to the control, in Mean ± SEM, n = 3. ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA.

addition, a growing interest in lichens, as sources of innovative pharmacologically active chemicals, has been targeted in its activities. L. pindarensis is one of the most common folk edible and medicinal
synergism, that may occur among the depsidones.

In summary, the result clarified, for the first time, the capacity in acting anti-inflammatory and anti-Aβ fibrils of extracts and main depsidones of L. pindaresis. Meanwhile, previous report has shown that the mixture of acetyl depsidones having moderate inhibitory activity against acetylcholinesterase was isolated from L. pulmonaria (Pejin et al., 2012). These findings deepened the potential pharmacological activities of lichen species, suggesting the novel anti-acetylcholinesterase together with anti-inflammatory property. Thus, the depsidone of Lobaria genus could be further developed. Indeed, the findings could lead to the identification of novel Lobaria extract, or depsidone fraction, in suppressing inflammation, fibrillar Aβ formation and anti-acetylcholinesterase activity. However, the cell study is not sufficient for its generalization, a series of in vivo studies should be conducted in the future. Additionally, we are aiming to extract more active ingredients from lichens and to explore ways obtaining the required effective ingredients through artificial culturing or fermentation, as to reduce the dependence on limited natural resources.

Ethics statement

The research did not include any human subjects and animal experiments.

Data availability

Data will be made available on request.

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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