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Vernonia patula (Dryand.) Merr. and Leucas chinensis (Retz.) R. Brown exert anti-inflammatory activities and relieve oxidative stress via Nrf2 activation

Pei-Shan Wu a,1, Jingyueh Jeng b, c, 1, Jeng-Jer Yang d, e, Vivia Kao b, e, Jui-Hung Yen f, Ming-Juan Wu a, b, *

a Department of Biotechnology, Chia Nan University of Pharmacy and Science, Tainan, 717, Taiwan
b Bachelor Program in Cosmeceutical and Biotech Industry, Chia Nan University of Pharmacy and Science, Tainan, 717, Taiwan
c Department of Medicinal Chemistry, Chia Nan University of Pharmacy and Science, Tainan, 717, Taiwan
d Bachelor Program in Pharmaceutical Botanicals & Health Applications, Chia Nan University of Pharmacy and Science, Tainan, 717, Taiwan
e Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan, 717, Taiwan
f Department of Molecular Biology and Human Genetics, Tzu Chi University, Hualien, 970, Taiwan

ARTICLE INFO

Keywords:
Vernonia patula
Leucas chinensis
Nrf2
HO-1
HPLC-MS
Apigenin
Luteolin
Betulinic acid

ABSTRACT

Ethnopharmacological relevance: Vernonia patula (Dryand.) Merr. and Leucas chinensis (Retz.) R. Brown have anti-inflammatory properties and are popularly used as complementary and alternative medicine in Asia.

Aim of the study: To investigate the underlying molecular mechanism and active chemicals in the ethanol extracts of V. patula (VP) and L. chinensis (LC).

Materials and methods: The inhibitory activities of VP and LC on lipopolysaccharide (LPS)-stimulated nitric oxide (NO) and interleukin-6 (IL-6) production were investigated in RAW264.7 macrophages and BV2 microglia. Downregulation of pro-inflammatory genes and upregulation of Nrf2 (NF-E2 p45-related factor 2)-ARE (antioxidant response element) pathway were investigated using RT-Q-PCR and Western blotting. Direct antioxidant capacities were measured using free radical scavenging and Folin-Ciocalteu assays. The flavonoids and triterpenes in VP and LC were identified by HPLC-ESI-MS.

Results: VP and LC inhibited NO and IL-6 production and suppressed iNOS, IL-6, IL-1β and CCL2 gene expression. VP and LC were potent direct antioxidants and effective indirect antioxidants assayed by Nrf2 activation and induction of heme oxygenase (HO)-1, glutamate-cysteine ligase modifier subunit (GCLM) and NAD(P)H quinone oxidoreductase 1 (NQO1). Three flavonoids including apigenin (1), luteolin (2) and chryseriol (3), and one triterpene betulinic acid (4) were found in VP; while compounds 1–4 and oleanolic acid (5) were in LC.

Conclusion: Anti-inflammatory and antioxidant activities of VP and LC may be in great part attributed to the identified Nrf2 activating compounds, which induce expression of Phase II enzymes and attenuate the upregulation of pro-inflammatory genes.

1. Introduction

Inflammation is a complex biological response of the immune system which is triggered by pathogens, damaged cells or toxic compounds (Chen et al., 2017). Macrophages are tissue-resident or infiltrated immune cells critical in the initiation, maintenance, and resolution of acute inflammation (Watanabe et al., 2019). Activated macrophages exert phagocytic activities and secrete inflammatory cytokines and mediators. Uncontrolled acute inflammation may become chronic, and is associated with many diseases, such as asthma, cancer, atherosclerosis, diabetes, and autoimmune diseases (Chen et al., 2017). Emerging evidence suggests that cytokine storm syndrome, caused by an aggressive

Abbreviations: GCLM, glutamate-cysteine ligase modifier subunit; HPLC-ESI-MS, high-performance liquid chromatography electrospray ionization mass spectrometry; HO, heme oxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; LC, Leucas chinensis; LPS, lipopolysaccharide; NO, nitric oxide; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, NF-E2 p45-related factor 2; PMB, polymyxin B sulfate; TIC, total ion chromatograms; VP, Vernonia patula; XIC, extracted ion chromatogram.

* Corresponding author.

E-mail addresses: mingjiuanwu@gmail.com, imwu@mail.cnu.edu.tw (M.-J. Wu).

1 These authors contributed equally to this work.

https://doi.org/10.1016/j.jep.2020.113155
Received 25 February 2020; Received in revised form 26 June 2020; Accepted 2 July 2020
Available online 28 July 2020
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pro-inflammatory response in combination with an insufficient anti-inflammatory response, leads to high morbidity and mortality in COVID-19 (severe coronavirus disease 2019) (Mehta et al., 2020).

Microglial cells are the major brain-resident macrophages and mediate the immune surveillance and homeostasis in the central nervous system (Osman and Kubes, 2012). Several lines of evidence have highlighted the association between microglial activation and neuronal injury and neurodegeneration (Luo and Chen, 2012). Microglia also play an important role in the neuropathic pain development and inhibiting microglial activation reduces hyperalgesia and allodynia (Inoue and Tsuda, 2018; Zhao et al., 2017). Thus, there is increasing interest in therapeutically targeting the inflammatory response and desperate hunt for agents blocking hyperinflammation (Konig et al., 2020; Mehta et al., 2020; Tabas and Glass, 2013).

Nrf2 (NF-E2 p45-related factor 2) signaling is the major cellular defense to relieve oxidative and electrophilic stress (Nguyen et al., 2009). Under oxidative stress, Nrf2-bound Keap1 (Kelch-like ECH-associated protein) is inactivated and, consequently, newly synthesized Nrf2 proteins bypass Keap1 and translocate into the nucleus, bind to the ARE (antioxidant response element) and drive the expression of Nrf2 target genes (Kansanen et al., 2013). The Nrf2 target genes include antioxidant responsive genes, such as heme oxygenase-1 (HO-1) and glutamate cysteine ligase (GCL), and drug metabolism genes, such as NAD(P)H dehydrogenase, quinone 1 (NQO1). Small molecules that activate Nrf2 signaling are under active study for their potential to become anti-inflammatory and cancer preventive agents (Wu et al., 2010). Plants are rich sources of Nrf2 activating phytochemicals; therefore, development of anti-inflammatory Nrf2 activators from plant extracts has attracted attention in biomedical field (Ahmed et al., 2017; Ding et al., 2016; Wu et al., 2015, 2018).

Vernonia patula (Dryand.) Merr. (VP) is a folk medicine claimed to have the properties of antipyretic, detoxication, antibacterial, anti-phlogistic, hypotension, blood stasis removal, hemostasis, expectoration, cough-relieving, antihepatitis and antitumor in Taiwan (Lin et al., 2008). Its ethanol extract was reported to inhibit rat paw edema induced by carrageenan and histamine (Hira et al., 2013). Leucas chinesis (Retz.) R. Brown (LC, also named as L. mollissima Wall.) is a herbal medicine widely available in Southeast Asia for reducing inflammatory symptoms (Ku et al., 2000). The essential oil isolated from LC has antimicrobial, free radical scavenging and antioxidant activities (Mothana et al., 2017). R. Brown (LC, also named as L. mollissima Wall.) is a herbal medicine widely available in Southeast Asia for reducing inflammatory symptoms (Ku et al., 2000). The essential oil isolated from LC has antimicrobial, free radical scavenging and antioxidant activities (Mothana et al., 2017). These two herbs can be used alone or in combination with three other herbs, Euonymus laxiflorus Champ. ex Benth., Justicia procumbens L., and Viola diffusa Ging to produce “Five hong san”, a traditional Taiwanese anti-inflammatory and analgesic decoction (Chang et al., 2011; Kan, 1978; Lee, 2004).

RAW264.7 cells are macrophage cell line established from an ascites of a tumor induced by Abelson leukemia virus transformation in BALB/c mice (Ralph and Nakoinz, 1977). BV2 cell line is generated by transfection of C57BL/6 mice microglial cells with v-raf/v-myc oncogene carrying retrovirus (Blasi et al., 1990). Both cell lines are suitable models for screening anti-inflammatory agents characterized by inhibiting lipopolysaccharide (LPS)-triggered pro-inflammatory cytokines and mediators (Ding et al., 2016; Wu et al., 2004, 2018).

The biologically active components of medicinal herbs are responsible for their efficacy. However, they usually consist of a complex mixture of phytochemicals and the active ingredient content is relatively low and hard to separate for identification. High-performance liquid chromatography with mass spectrometry (HPLC-MS) does not require extensive purification of chemical constituents and is becoming a popular choice for analysis of medicinal herbs, especially on micro- or trace-scale (Kumar, 2017; Li et al., 2011).

Most herbal medicines customarily are extracted with hot water. However, in preliminary experiments, we found that hot water extracts of V. patula (VP) and L. chinesis (LC) exhibited much lower anti-inflammatory activities than ethanol extracts in LPS-stimulated RAW264.7 cells and BV2 cells. Therefore, ethanol was used for extracting the bioactive components to maximize anti-inflammatory activities. The possible involvement of Nrf2-ARE pathways in their anti-inflammatory and antioxidant activities will also be investigated. The active chemical components, with special focus on phenolics and triterpenes, will be identified using HPLC-MS with electrospray ionization.

2. Materials and methods

2.1. Materials

V. patula (VP) and L. chinesis (LC) were purchased from Tien-Yi Herb, Yunlin, Taiwan in July 2017 and authenticated by Dr. J. J. Yang of Chia Nan University of Pharmacy and Science. They were planted in the herbary of Department of Biotechnology. The voucher specimens CNS91070501 for VP and CNS230190112 for LC, were deposited in the Traditional Materia Medica Applied Technology Development Center, Chia Nan University of Pharmacy and Science.

Gries reagent, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), lipopolysaccharide (LPS) from Escherichia coli O111:B4, polymyxin B sulfate (PMB), sodium nitrite, RIPA buffer, reference compounds used in HPLC-MS and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless otherwise indicated.

2.2. Ethanol extraction

The aerial parts of VP and LC were air-dried and cut into small pieces. 300 g sample/each was extracted by reflux in 1.5 L 95% ethanol at 50 °C. After extraction three times, the extraction of ethanol-reflux was combined. Subsequently, the extract solution was filtered and then evaporated to dryness on rotary evaporator. The dried extracts were kept at 4 °C. Before each experiment, the extracts were dissolved in 95% ethanol and sonicated for 2 min at room temperature.

2.3. Culture of RAW264.7 and BV2 cells

RAW264.7 cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific Inc.) with 10% fetal bovine serum (GE Life Sciences), 2 mM glutamine, 1% nonessential amino acid, 1 mM pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific Inc.).

BV2 cells was kindly provided by Professor Shun-Fen Tzeng (National Cheng Kung University) and cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Thermo Fisher Scientific Inc.) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Both cell lines were maintained in a humidified incubator at 37 °C in 5% CO2.

2.4. Measurement of nitric oxide, interleukin-6 and cell viability

Both cell lines (1 × 10⁵/well) were pretreated with antibiotic polymyxin B (PMB) or indicated extract for 30 min prior to LPS treatment in 96-well plates. After 20 h, the culture supernatants were collected for the determination of nitrite (NO2⁻). Nitrite is the primary, stable and nonvolatile breakdown products of nitric oxide (NO). It was analyzed by formation of a red pink azo upon treatment with the Griess reagent and measured spectrophotometrically at 550 nm against a nitrite standard curve (Giustarini et al., 2006). Interleukin-6 (IL-6) released in the supernatant was measured after LPS treatment for 16 h by Mouse IL-6 ELISA Set (BD Biosciences, San Diego, CA) according to the manufacturers’ instructions.

Cell viability in the cultured well was analyzed by treatment with 0.5% MTT (in serum-free cultured medium) and incubated for 3 h in the incubator. MTT solution was then removed and the formazan crystals
produced were dissolved by DMSO and absorbance was measured spectrophotometrically at 550 nm. (Carmichael et al., 1987).

2.5. RNA extraction and reverse transcription real-time PCR

RNA was extracted from BV2 and RAW264.7 cells with the illustra RNA Spin Mini RNA Isolation Kit (GE Healthcare). The reverse transcription was performed using a High-Capacity cDNA Archive kit (Thermo Fisher Scientific). Quantitative PCR was then performed using a Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) in a total volume of 20 μL that contained 0.4 μM of each primer (Table 1). Thermal cycling was performed at 95 °C for 2 min, 40 cycles at 94 °C for 15 s, and 60 °C for 60 s (ABI StepOne Real Time PCR System). The relative mRNA expression was normalized with β-actin expression and then calculated by the 2−ΔΔCT method. Specificity verification was done by melting curve.

2.6. Western blotting analysis

Cell lysates and nuclear extracts were prepared using RIPA lysis buffer and nuclear extraction kit (Cayman), respectively. The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as a standard reference.

Equal amounts of protein were subjected to separate on 8–12% SDS-PAGE. Following electrophoretic separation, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, GE Healthcare) with CAPS buffer (10 mM, pH 10.5, 10% methanol) at 20 V overnight at 4 °C. The membranes were blocked in freshly made blocking buffer (5% skim milk in PBS with 0.05% Tween 20, pH 7.4) for 8 h at room temperature and then probed with specific primary antibody (Table 2) overnight at 4 °C. After rinsing the membrane to remove unbound primary antibody, the membrane was exposed to suitable horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h. The antigen-antibody reaction was detected using enhanced chemiluminescence detection (GE Healthcare).

2.7. DPPH scavenging capacities

DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl, 0.1 mM in methanol) scavenging capacity was analyzed according to the literature (Sharma and Bhat, 2009). IC50 was calculated from dose-inhibition curve.

### Table 1

| Gene  | Primers | Amplicon (bp) |
|-------|---------|---------------|
| β-actin | GCGCTGATTCGCCCTGACCATGC | 154 |
|         | CCAGTGGTACAAATGCCCATGT | |
| iNOS | GTCTTCACGGCCAACATACAGGA | 127 |
| IL-6 | TATGTTCCCTTACCCCAATTTCC | 153 |
|         | CGCATTAGTGGTGGCAGTA | |
| IL-1β | TTCAGGCGGCGCTATACCTC | 75 |
|         | GAAGGGTCAGGGAAGAAGCAC | |
| CCL2 | TTAAAACCTGGATGGGACCAAA | 121 |
|         | GCATTGCTGCATTTGACGCGTT | |
| HO-1 | AAGCCTGAAGATGGTTC | 100 |
|         | GCGCTGATTAGATTTGACGAAGGA | |
| GCLM | AACGACCCAGTGGTGGCCGCG | 141 |
|         | AGTGGGATCGGATTGC | |
| NQO1 | TGTGATAGATGGATGGGACGA | 127 |

2.8. Assay of Trolox equivalent antioxidant capacity (TEAC)

The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazolone-6-sulfonic acid) (ABTS•+) is generated by ABTS/ K2S2O8 prior to addition of tested antioxidant. The results of ABTS•+ radical assays were presented as Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) using Trolox as reference standard. To calculate the TEAC, the slope of concentration-inhibition curve for tested extract is divided by that for Trolox and the results are expressed minigrams of Trolox equivalent per gram of dry weight (mg·TE/g·dw) (Re et al., 1999).

2.9. Folin-Ciocalteu assay

Total phenolic content was determined by the slightly modified Folin-Ciocalteu (F–C) assay (Everette et al., 2010). Absorbance was read at 620 nm and the results are expressed as minigrams of gallic acid equivalent per gram of dry weight (mg·GAE/g·dw).

2.10. Identification of bioactive compounds by HPLC-ESI-MS (high-performance liquid chromatography electrospray ionization mass spectrometry)

VP and LC extracts were subjected to 0.45 μm filtration before analyzed by HPLC-MS using Shimadzu LCMS-8050 triple quadrupole mass spectrometry with electrospray ionization. The column for the HPLC system was a Shim-pack GIS C18 (C18, 4.6 × 250 mm, Shimadzu) at 30 °C. The mobile phase was consists of methanol (solvent A) and 5 mM ammonium formate (solvent B) in a linear gradient (from 15 to 95% of A) for 45 min with a flow rate of 0.8 mL/min. In all analyses, the injected volume was 3 μL.

For identification of the phytochemicals, authentic reference phenolic compounds (morin, fisetin, quercetin, kaempferol, apigenin, luteolin, chrysoeriol, dihydroxyflavone) and triterpenes (betulinic acid and oleanolic acid) were analyzed in the same conditions as described above.

2.11. Statistical analysis

All experiments were repeated at least three times, and the values were expressed as the mean ± SD. The results were analyzed using One-way ANOVA with Dunnett’s post hoc test, and a p value < 0.05 was considered statistically significant. Results

3. Results

3.1. Anti-inflammatory activities of V. patula (VP) and L. chinensis (LC)

Anti-inflammatory activities of ethanol extracts of VP and LC were first measured by inhibition of nitric oxide (NO) production in LPS-treated RAW264.7 and BV2 cells. Nitric oxide (NO) is a signaling molecule that is involved in the pathogenesis of inflammatory disorders and therefore represents an important therapeutic target (Breidt and Snyder, 1994; Sharma et al., 2007). Fig. 1a shows that RAW264.7 cells dramatically increased NO production (from unstimulated 2.37 ± 0.35 μM to 29.53 ± 0.35 μM), 20 h after treatment with LPS (10 ng/mL).
Polymyxin B (PMB), a cyclic amphipathic peptide antibiotic binding to LPS (Duff and Atkins, 1982), was used as a control. PMB (10 μg/mL) completely blocked LPS-induced NO production. In comparison, VP and LC (0.05–0.2 mg/mL) significantly inhibited NO production by 32%–84% in a dose-dependent manner. To investigate the NO-lowered effect was not due to direct cytotoxic activity, MTT assay was employed. Fig. 1 b shows VP (0.2 mg/mL) exhibited about 5% cytotoxicity, while the other treatments did not exert detectable cytotoxicity.

LPS-mediated microglia activation and NO production plays a key role in the development of neuroinflammation and neuropathic pain (Ellis and Bennett, 2013). Therefore, we further investigated the anti-NO effects of VP and LC in LPS-treated BV2 microglia. As shown in Fig. 1 c, BV2 cells exhibited increased production of NO (from 3.12 ± 0.58 μM to 46.84 ± 1.35 μM), 20 h after treatment with LPS (10 ng/mL). VP and LC (0.05–0.2 mg/mL) dose-dependently inhibited LPS-induced NO production in BV2 cells.

Fig. 1 d shows that LPS (10 ng/mL) caused significant BV2 cell death, as compared with the vehicle control (0.1% ethanol), supporting the notion that LPS causes apoptosis in microglia (Liu et al., 2001). PMB (10 μg/mL) completely blocked LPS-caused cell death. VP (0.1 mg/mL) and LC (0.05 mg/mL) slightly mitigated LPS-mediated cytotoxicity; however, higher concentration of LC (0.2 mg/mL) resulted in enhanced cytotoxicity.

Interleukin-6 (IL-6) is a soluble mediator with a pleiotropic effect on inflammation, immune response, and hematopoiesis. Dysregulated synthesis of IL-6 has being implicated in the pathogenesis/pathophysiology of numerous inflammatory diseases (Tanaka et al., 2014). We thus further studied the inhibitory activities of VP and LC, at effective and non-toxic concentrations (0.05 and 0.1 mg/mL), on LPS-mediated IL-6 production in RAW264.7 and BV2 cells. We found that LPS induced high level of IL-6 production in RAW274.7 cells and BV2 cells, and VP and LC (0.05 and 0.1 mg/mL) could inhibit LPS-stimulated IL-6 production significantly (Fig. 2). The dose-dependent effects of VP and LC were more pronounced in BV2 cells than in RAW264.7 cells.

3.2. Effects of VP and LC on the mRNA expression of the pro-inflammatory genes

We further investigated how VP and LC affect pro-inflammatory gene expression in LPS-treated BV2 cells. Production of NO is produced from L-arginine via the action of NO synthase (NOS). Inducible NO synthase (iNOS) is induced by LPS in microglia. We found that LPS (10 ng/mL) significantly upregulated iNOS transcript by 1513-fold in BV2 cells after 4 h treatment (Fig. 3 a). VP and LC (0.05 and 0.1 mg/mL) dose-dependently attenuated iNOS mRNA expression. Western blot analysis (Fig. 4 b) shows that LPS (10 ng/mL) dramatically increased iNOS protein expression after 16 h treatment and VP and LC (0.05 and 0.1 mg/mL) attenuated LPS-mediated iNOS protein expression in dose-dependent manners in parallel to their anti-NO potencies.

The expression and degradation of IL-6 mRNA is regulated transcriptionally and post-transcriptionally by several transcription factors, proteins and microRNAs (Tanaka et al., 2014). We found that LPS strongly stimulated IL-6 mRNA expression by 3012-fold in BV2 cells after 4 h incubation. VP and LC at 0.1 mg/mL, but not at lower concentrations, could significantly reduce LPS-upregulated IL-6 mRNA level (Fig. 3 b). This indicates that the reduction of IL-6 production by VP and LC in BV2 cells (shown in Fig. 2 b) might occur via both transcriptional and translational inhibition.

Interleukin-1 (IL-1) mediates highly inflammatory responses via two cytokine species, IL-1α and IL-1β (Mayer-Barber and Yan, 2017). IL-1β...
mRNA levels rise rapidly and transiently upon LPS stimulation due to short mRNA half-life or the action of microRNA (Dinarello, 2018). Fig. 3c shows that LPS (10 ng/mL) caused a 187-fold increase in IL-1β mRNA expression in BV2 cells after 4 h treatment. VP and LC (0.05 and 0.1 mg/mL) markedly down-regulated LPS-stimulated IL-1β mRNA expression in BV2 cells after 4 h treatment. VP and LC (0.05 and 0.1 mg/mL) markedly down-regulated LPS-stimulated IL-1β mRNA expression in BV2 cells after 4 h treatment.

The main activity of CCL2 (CC chemokine ligand 2)/MCP-1 (monocyte chemoattractant protein-1) is to recruit monocytes, memory T cells, and dendritic cells to the sites of inflammation during inflammation (Bose et al., 2016). A 23-fold induction of CCL2/MCP-1 mRNA expression was observed 4 h after LPS (10 ng/mL) treatment, and co-treatment with VP (0.025–0.1 mg/mL) and LC (0.1 mg/mL) inhibited its expression (Fig. 3d).

From the above data, we conclude that LPS-mediated pro-inflammatory mRNA overexpression, including enzyme: iNOS, cytokines: IL-6 and IL-1β, and chemokine: CCL2/MCP-1, were attenuated by VP and LC dose-dependently in BV2 cells.

In comparison, VP and LC exhibited much weaker efficacy in inhibition of LPS-induced iNOS and IL-6 mRNA expression in RAW264.7 cells than in BV2 cells (Supplemental Fig. 1). These results indicate that downregulation of NO and IL-6 production by VP and LC possibly occurs at steps other than regulating mRNA levels in RAW264.7 cells.

3.3. Direct antioxidant capacities of VP and LC

It is well-known that most of the anti-inflammatory herbal extracts also exert antioxidant activities (Chiou et al., 2015; Ding et al., 2016; Hira et al., 2013; Moita et al., 2013). Direct antioxidant capacity can be defined as scavenging free radical by donating hydrogen or electrons. On the other hand, the indirect antioxidant capacity is involved in mitigating the oxidative stress via the expression of Phase II detoxifying and antioxidant genes (Dinkova-Kostova and Talalay, 2008). We first used DPPH and ABTS•⁺ quenching activities as well as total phenolic content by Folin-Ciocalteu (F-C) assay to analyze the direct antioxidant activities of VP and LC. DPPH and ABTS•⁺ quenching activities are considered as mixed electron transfer and hydrogen atom transfer assays (Apak et al., 2016); while F-C assay is normally described as electron transfer assay (Everette et al., 2010). Table 3 shows that VP had about twice higher phenolic content and exerted three-to-four-fold stronger DPPH and ABTS•⁺ bleaching activities than LC. The differences in antioxidant potentials may result from the fact that F-C measures only the hydrophilic antioxidants, DPPH detects only those soluble in organic solvents, while ABTS•⁺ determines both hydrophilic and hydrophobic samples (Apak et al., 2007; da Silva Oliveira et al., 2019).

3.4. VP and LC induce Nrf2 activation and phase II gene expression

We further investigated indirect antioxidant capacities of VP and LC by measuring Nrf2 transcription factor activation in BV2 cells. Western blot analysis was employed to determine nuclear Nrf2 protein level. The ubiquitously expressed histone deacetylase 2 (HDAC2) was used as a loading control. Fig. 4a shows that LPS (10 ng/mL) induced Nrf2 translocation into nucleus after 2 h insult. Both VP and LC enhanced Nrf2 activation dose-dependently, and in parallel to the direct antioxidant activities shown above, VP activated Nrf2 more prominently than LC.

Heme oxygenase (HO)-1 is one of the Nrf2-driven genes and exhibits antioxidant and anti-inflammatory properties (Kim et al., 2013; Lee et al., 2005; Lin et al., 2003; Wang et al., 2014). Similar to those found for Nrf2 activation, LPS slightly induced HO-1 protein expression after 16 h treatment, while co-treatment with VP and LC enhanced HO-1 upregulation dose-dependently. VP was more potent than LC in HO-1 mRNA and protein expression as glutamate cysteine ligase modifier subunit (GCLM) and NAD(P)H quinone dehydrogenase 1 (NQO1) (Magesh et al., 2012). Fig. 4d and e shows that LPS alone did not significantly induce GCLM or NQO1 transcription; however, co-treatment with VP or LC (0.05 and 0.1 mg/mL) markedly stimulated GCLM and NQO1 mRNA expression in a dose-dependent manner. VP and LC also exerted HO-1 and NQO1 gene induction in RAW264.7 cells (Supplemental Fig. 2). This observation suggests that VP and LC activate Nrf2-ARE signaling pathway so as to repress inflammatory response.
3.5. Determination of phenolic and triterpene compounds in VP and LC

VP and LC may contain lots of flavonoids and triterpenes with similar structures and polarities. We therefore used HPLC-MS to identify their bioactive composition without extensive purification. The identification of chemical constituents was based on their mass-to-charge ratio (m/z) and direct retention time matching with authentic references. Total ion chromatograms (TIC) by negative ion mode electrospray ionization mass spectrometry (ESI/MS) of VP and LC were shown in Fig. 5a. Lots of peaks were separated and the strongest peaks were observed at retention time about 17.0 min and peak clustered between 26 and 27.5 min. The peaks with the same m/z as references were further extracted from TIC as extracted ion chromatogram (XIC). Three flavonoids, apigenin (1), luteolin (2), and chryseriol (luteolin 3′-methyl ether) (3), were identified in both VP and LC via comparing with 8 authentic references (Fig. 5b and c and Table 4). All three identified flavonoids were at retention time around 17 min. Compound x, likely to be diosmetin (luteolin 4′-methyl ether) or other isomer, was also detected at m/z 299.25 at 16.65 min for VP.

Betulinic (BA) and oleanolic (OA) acids are triterpenic acids with multiple therapeutic effects; therefore m/z 455.7 was monitored to identify these two compounds. Fig. 5b and c show that BA was identified in VP, while BA and OA were in LC, and both were at retention time about 27 min. Combination of TIC and XIC data indicate that the identified flavonoids and triterpenic acids may be the major constituents of VP and LC.

4. Discussion

In this research, we report the anti-inflammatory and antioxidant activities and mechanisms of ethanol extracts of *V. patula* (VP) and *L. chinensis* (LC). They inhibited NO and IL-6 production in LPS-treated RAW264.7 macrophages and BV2 microglia. They also inhibited LPS-mediated expression of pro-inflammatory cytokine and pro-inflammatory chemokine, IL-1β and CCL2/MCP-1, in a dose-dependent manner in BV2 cells.

It is well-documented that lots of natural antioxidants also serve as anti-inflammatory agents (Arulselvan et al., 2016). In general, direct antioxidants are redox active, short-lived small molecules that directly scavenge reactive oxygen and/or nitrogen species (Dinkova-Kostova and Talalay, 2008). On the other hand, indirect antioxidants induce Keap1/Nrf2/ARE pathway and target Phase II gene expression resulting in increased antioxidant capacity and long-lived protective effect compared to direct antioxidants (Dinkova-Kostova and Talalay, 2008). We found that VP showed stronger direct antioxidant capacities by exhibiting potent DPPH and ABTS•⁺ free radical scavenging activities and had higher total phenolic contents as compared with LC. In parallel to the direct antioxidant capacity, VP induced Nrf2 activation and Phase II genes expression stronger than LC.

Nrf2 activation is crucial in modulating redox homeostasis and regulating inflammatory conditions via the induction of many stress responsive and cytoprotective enzymes including HO-1, GCLM and NQO1 (Kobayashi and Yamamoto, 2006). It has been reported that HO-1 inhibited LPS-mediated inflammatory responses in RAW264.7 and BV2 cells (Kuhn et al., 2011; Wu et al., 2018). HO catalyzes the degradation of heme to biliverdin/bilirubin, ferrous iron, and carbon monoxide. Carbon monoxide acts as an inhibitor of the NF-κB pathway which leads to the decreased expression of pro-inflammatory cytokines, while bilirubin also acts as antioxidant (Ahmed et al., 2017).

GCL catalyzes the first and rate-limiting step in de novo GSH synthesis. GCL is composed of two subunits, the catalytic subunit GCLC and the modifier subunit GCLM. GSH is not only important as an antioxidant and inhibitor of inflammation but also as a signaling molecule for redox regulation (Ghezzi, 2011). NQO1 catalyzes the reduction of quinones to...
chlorogenic acid, as well as esculetin, and two catechols, caffeic acid and protocatechuic acid have been identified from VP (Lin and Wang, 2002).

Direct antioxidant capacities of VP and LC.

Table 3

| Samples | IC_{50} for DPPH Scavenging (mg/mL) | TEAC (mg TE/g dw) | Total Phenolic Content (mg GAE/g dw) |
|---------|-----------------------------------|------------------|-----------------------------------|
| VP      | 20.95 ± 1.43                      | 190 ± 4          | 78 ± 4                            |
| LC      | 60.27 ± 1.23                      | 50 ± 2           | 41 ± 4                            |
| α-tocopherol | 10.92 ± 2.58                 |                  |                                   |

Data are represented as the mean ± SD (n = 3).

It was reported that there were eight compounds: oleanolic acid 3-acetate, apigenin, apigenin-7-O-β-D- (6′-p-coumaroyl)glucoside, cirsiamartin (5,4′,8-dihydroxy-6,7-dimethoxyflavone), mixture of β-sitostanol and stigmasterol, and mixture of β-sitostanol-3-O-β-D-glucoside and stigmasterol-3-O-β-D-glucoside in the methanolic extract of L. chinensis (Ku et al., 2000). In this report, luteolin (2), chryseriol (3), BA (4), and oleanolic acid (OA) (5) were further identified from LC (Fig. 6).

It was reported that there were eight compounds, oleic acid, zinc, and vitamin D3, which are key components of cellular antioxidant defense system. Several reports establish an inverse correlation between NQO1 and NF-κB activation (Jamshidi et al., 2012). Current study demonstrates that both VP and LC significantly induce the expression of HO-1, GCLM and NQO1, and the upregulation might be responsible for the repression of pro-inflammatory gene expression.

Previously, four flavones, luteolin, tricin, luteolin 4-O-β-D-glucoside and luteolin 7-O-β-D-glucoside, four caffeoylquinates, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, ethyl 3,4-dicaffeoylquininate and chlorogenic acid, as well as esculetin, and two catechols, caffeic acid and protocatechuic acid have been identified from VP (Lin and Wang, 2002). In this report, we further reported the presence of apigenin (1), chryseriol (3), and betulinic acid (BA) (4) in VP (Fig. 6).
OA is a natural triterpenoid widely present in anti-inflammatory and hepatoprotective Chinese medicines (Wu et al., 2004). It has been reported to activate Nrf2 and antioxidant gene expression (Loboda et al., 2012; Wang et al., 2010). In this report we found LC, but not VP, contained OA.

In conclusion, anti-inflammatory and antioxidant activities of VP and LC may be in great part attributed to these identified Nrf2 activators, which induce expression of Phase II enzymes and attenuate transcriptional upregulation of proinflammatory genes. We realized our studies were found only on the in vitro effects, the anti-inflammatory and antioxidant effects of VP and LC in animal and human studies warrant further investigation.

5. Conclusions

The ethanol extracts of V. patula (VP) and L. chinensis (LC) inhibited LPS-induced NO and IL-6 production in both RAW264.7 and BV2 cells. VP and LC also suppressed iNOS, IL-6, IL-1β and CCL2 expression. VP
had stronger DPPH and ABTS•+ bleaching activity and activated greater Nrf2/ARE signaling than LC. Five known compounds: apigenin (1), luteolin (2), chryseriol (3), betulinic acid (4) and oleanolic acid (5) were found in LC; while compounds (1) to (4) were identified in VP by HPLC-MS. The anti-inflammatory and antioxidant effects of VP and LC may be attributed, at least in part, to these identified Nrf2 activating phytochemicals.

### Declaration of competing interest

The authors declare that they have no conflict of interests.

### Acknowledgements

This research was supported by research grant MOST 106-2320-B-041 -006 -MY3 from the Ministry of Science and Technology, R.O.C.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.113155.

### Authors’ contributions

Conceptualization, Jeng-Jer Yang and Jui-Hung Yen; Data curation, Pei-Shan Wu and Jui-Hung Yen; Formal analysis, Pei-Shan Wu; Funding acquisition, Ming-Jiuan Wu; Investigation, Pei-Shan Wu and Jingyueh Jeng; Methodology, Jingyueh Jeng; Project administration, Ming-Jiuan Wu; Resources, Jeng-Jer Yang and Vivia Kao; Supervision, Ming-Jiuan Wu; Validation, Ming-Jiuan Wu; Visualization, Ming-Jiuan Wu; Writing – original draft, Pei-Shan Wu and Jingyueh Jeng; Writing–review & editing, Vivia Kao, Ming-Jiuan Wu.

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