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Suppressive effects of acetone extract from the stem bark of three *Acacia* species on nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophage cells

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ABSTRACT

Objective: To compare the inhibitory effects of acetone extracts from the stem bark of three *Acacia* species (*Acacia dealbata*, *Acacia ferruginea* and *Acacia leucophloea*) on nitric oxide production.

Methods: The lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were used to investigate the regulatory effect of acetone extracts of three *Acacia* stem barks on nitric oxide production and the expression of inducible nitric oxide synthase, cyclooxygenase-2 and tumor necrosis factor-α. Further, the phenolic profile of acetone extracts from the *Acacia* barks was determined by liquid chromatography-mass spectrometry/mass spectrometry analysis.

Results: All the three extracts significantly decreased LPS-induced NO production as well as the expression of inducible nitric oxide synthase, cyclooxygenase-2 and tumor necrosis factor-α in a concentration dependent manner (25, 50 and 75 μg/mL). In the liquid chromatography-mass spectrometry/mass spectrometry analysis, acetone extract of *Acacia ferruginea* bark revealed the presence of 12 different phenolic components including quercetin, catechin, ellagic acid and rosmanol. However, *Acacia dealbata* and *Acacia leucophloea* barks each contained 6 different phenolic components.

Conclusions: The acetone extracts of three *Acacia* species effectively inhibited the NO production in LPS-stimulated RAW 264.7 cells and the presence of different phenolic components in the bark extracts might be responsible for reducing the NO level in cells.

1. Introduction

Inflammation is the normal physiological and beneficial host response to tissue injury caused by foreign agents (physical or noxious chemical stimuli or microbiological toxins) that eventually leads to the restoration of normal tissue structure and function [1]. Macrophages play a key role in the immune system by releasing various pro-inflammatory cytokines and mediators such as interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2) in response to various harmful stimuli [2,3]. Among them, NO is produced enormously in macrophages by the iNOS. Prostaglandin is also an important pro-inflammatory mediator, and produced from arachidonic acid by COX-1 and 2 enzymes [4]. Under normal physiological conditions, the short-lived biomolecule, NO mediates many biological functions such as host defense, platelet aggregation, vasoregulation, and neurotransmission [5]. However, excessive production of NO and other mediators has been concerned in the development of many diseases, such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and atherosclerosis [6,7].

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Accordingly, the regulation of these pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated macrophage cell line is an effective therapeutic strategy for the development of novel anti-inflammatory agents.

In recent times, phenolic components from plants have attracted much attention because of their large distribution with a variety of biological properties [8]. The genus *Acacia* belongs to the family of Mimosaceae, comprises more than 1 350 species, and mainly occurs in tropical and some temperate countries including Australia, Africa, India and America. In the traditional systems of medicine, different parts of *Acacia* plants are widely used as indigenous drugs to treat various ailments [9]. The acetone and methanol extracts from the bark of *Acacia leucophloea* (*A. leucophloea*), *Acacia ferruginea* (*A. ferruginea*), *Acacia dealbata* (*A. dealbata*), and *Acacia pennata* showed remarkable antioxidant properties [10]. Sowndhararajan *et al.* [11] reported that the acetone extract from the stem bark of *A. leucophloea*, *A. ferruginea* and *A. dealbata* significantly up-regulated the expression of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) in hydrogen peroxide-induced human hepatoma (HepG2) cells. In Indian traditional systems of medicine, the stem bark of *A. leucophloea* is used to treat a variety of disorders including inflammation, bronchitis, wounds, ulcers, diarrhea, intermittent fevers, leprosy, toothache, *etc*. The bark of *A. ferruginea* is used traditionally for the treatments of itching, leucoderma, ulcers, stomatitis, and diseases of the blood. The bark of *A. dealbata* produces a gum, resembling gum arabic, which is used to treat bronchial disorders [10,11]. Sowndhararajan and Kang investigated the effect of ethyl acetate fraction from the acetone extract of *A. ferruginea* stem bark on ethanol-induced gastric ulcer in rats [12]. With this background, the present study was carried out to investigate anti-inflammatory potential of acetone extract from the stem bark of *A. leucophloea*, *A. ferruginea* and *A. dealbata* by measuring its ability to inhibit NO production and expression of iNOS, COX-2 and TNF-α in LPS-simulated RAW 264.7 cells.

2. Materials and methods

2.1. Chemicals

3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin–streptomycin solution and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline were from HyClone Laboratories, Inc. (South Logan, UT, USA). RNeasy Mini kit and SYBR green master mix were purchased from Qiagen-GmbH (Hilden, Germany). SuperScript III First-Strand synthesis system was purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of the highest available purity analytical grade.

2.2. Plant materials and preparation of extracts

Fresh stem barks of *A. leucophloea*, *A. ferruginea* and *A. dealbata* were collected from Coimbatore, Tamil Nadu state, India. The authenticated plant specimens were deposited in the Botany Herbarium, Bharathiar University with voucher numbers: BUBH-6140, BUBH-6141, and BUBH-6142, respectively [10]. The air-dried and powdered bark samples were extracted with petroleum ether (for disposing lipid and pigments), followed by acetone using Soxhlet apparatus. Each extract was then filtered before being dried by rotary evaporation (RE300, Yamato, Tokyo, Japan) at 50 °C and the remaining water was removed by lyophilization (4KBTXL-75, VirTis Benchtop K, NY, USA).

2.3. Cell culture

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 g/L streptomycin, and 100 IU/mL penicillin at 37 °C in a 5% CO_2 atmosphere (HERAcell 150, Thermo Electron Corp. Waltham, MA, USA).

2.4. MTT cell viability assay

Cell viability of RAW 264.7 cells was determined by MTT assay. Briefly, RAW 264.7 cells were seeded at the density of 5×10^4 cells/well in 96-well culture plates and incubated for 24 h (at 37 °C and 5% CO_2), followed by the pre-treatment with different concentrations of the extracts (0–100 μg/mL). Twenty four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h. The medium was then removed and the formazan precipitate was solubilized in dimethyl sulfoxide. The absorbance was measured at 550 nm on a microplate reader (Biotek, Winooski, VT, USA).

2.5. Inhibition of NO production

NO production was determined by measuring the level of nitrite in the culture supernatant of RAW 264.7 cells. The RAW 264.7 cells were seeded at a density of 5×10^5 cells/well in 24 well plates for 12 h at 37 °C and 5% CO_2. Then, the cells were treated with different concentrations of acetone extracts of *Acacia* barks (12.5–75.0 μg/mL, prepared in FBS-free DMEM). After 1 h treatment, cells were stimulated with 1 μg/mL of LPS for 24 h. The presence of nitrite was determined in cell culture media using commercial NO detection kit (iNtRON, Sungnam, South Korea). Briefly, 100 μL of cell culture medium with an equal volume of Griess reagent in a 96-well plate was incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve.

2.6. RNA isolation and first-strand cDNA synthesis

Total cellular RNA was isolated from cells by using a commercial kit (RNeasy Mini kit, Qiagen) as described by the manufacturer. An amount of 1 μg of total RNA was reverse-transcribed using oligo (dT) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA)

synthesis was carried out according to the manufacturer's instructions, and the resulting cDNA was stored at −20 °C.

2.7. Quantification of mRNA levels by real-time PCR

Using cDNAs as the template, quantitative real-time PCR was carried out using the SYBR Green PCR Master Mix (Qiagen) in a real-time PCR (Rotor-gene Q, Qiagen), according to the manufacturer's instructions, using specific oligonucleotide primers for iNOS, COX-2 and TNF-α (Table 1). β-Actin cDNA was used as an internal control. A dissociation cycle was performed after each run to check for non-specific amplification or contamination. After initial denaturation (95 °C for 5 min), 40 PCR cycles were performed using the following conditions: 95 °C, 5 s; 60 °C, 10 s at the end of PCR reaction, samples were subjected to a temperature ramp (from 60 to 95 °C, 1 °C/s) with continuous fluorescence monitoring. For each PCR product, a single narrow peak was obtained by melting curve analysis at specific temperature [13]. Analysis was performed with relative quantification software (Rotor-Gene Q series 2.0.3 software). The expression of iNOS, COX-2 and TNF-α activity was expressed as percentages of the control (100%).

Table 1
Sequences of PCR primers used for quantitative real-time PCR.

Gene	Primer	Sequence
iNOS	Sense	5′-GAAGCTGAGGCCCAGGAGGA-3′
	Antisense	5′-GAACAAGGTGGCCAGGTCCC-3′
COX-2	Sense	5′-CATCCCCCTCCTGCGAAGTT-3′
	Antisense	5′-TCTGCTACGGGAGGAAGGGC-3′
TNF-α	Sense	5′-GATCGGTCCCCAAAGGGATG-3′
	Antisense	5′-TCAGCCACTCCAGCTGCTCC-3′
β-Actin	Sense	5′-CTGGTCGTCGACAACGGCTC-3′
	Antisense	5′-TCTTGCTCTGGGCCTCGTCA-3′

2.8. Liquid chromatography-mass spectrometry (LC-MS) analysis of acetone extracts

The phenolic profile of acetone extracts from the stem bark of *Acacia* species was identified by LC-MS/MS method using the instrument Perkin Elmer Flexar FX15 UPLC coupled to AB Sciex 3200 QTRAP hybrid trap triple quadrupole mass spectrometer (MA, USA). The negative ion mass spectra were obtained with LC QTrap MS/MS detector in full ion scan mode (100–1 500 *m/z* for full scan and 50–1 200 *m/z* for MS/MS scan) at a scan rate of 0.5 Hz. The system was supported with mass spectrometry software and a spectral library provided by Advanced Chemistry Development labs (Toronto, ON, Canada). Analyte separation was performed on a Agilent Zorbax C18 (150 mm × 4.6 mm × 5 μm) with a gradient mobile phase consisting of water (solvent A) and methanol with 1% acetonitrile (solvent B), each containing 0.1% formic acid and 5 mmol/L ammonium formate. The gradient program was 10% solvent B to 90% solvent B from 0.01 min to 8.0 min, held for 3 min and back to 10% B in 0.1 min and re-equilibrated for 5 min at a flow rate of 0.4 mL/min. The sample injection volume was 20 μL. All chromatographic procedures were performed at ambient temperature, and the corresponding peaks from the QTrap LC-MS/MS analysis of all the samples were identified by comparison with the literature/Advanced Chemistry Development labs mass spectral library.

2.9. Statistical analysis

The values expressed were means of three replicate determinations ± SD. All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) software package. The statistical analysis was carried out by ANOVA followed by Tukey's test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Inhibition of NO production in LPS-stimulated RAW 264.7 cells

To determine the cytotoxic concentrations of acetone extracts of *Acacia* barks, MTT assay was evaluated. The viability of RAW 264.7 cells was not significantly decreased by 24 h incubation with up to 75 μg/mL (cell viability > 90%) of acetone extracts (Figure 1). Based on the results, the concentrations of acetone extracts of *Acacia* barks, 12.5, 25.0, 50.0, and 75.0 μg/mL were selected for further experiments. Figure 2 shows the effect of acetone extracts of *Acacia* barks on NO production stimulated by LPS in RAW 264.7 cells. In un-stimulated RAW 264.7 cells, the nitrite level was at baseline [(0.56 ± 0.10) μmol/L]. However, the level of nitrite in the LPS-stimulated cells was significantly higher (88.33 μmol/L). When compared to LPS control, the pre-treated cells stimulated with LPS released a lower level of NO in the culture medium. All the three acetone extracts significantly inhibited ($P < 0.05$) the nitrite accumulation in LPS-stimulated RAW 264.7 cells in a concentration dependent manner, suggesting the presence of NO inhibitory components in the acetone extracts. Among the three *Acacia* species, *A. leucophloea* and *A. ferruginea* significantly reduced the nitrite level to 29.43 and 28.01 μmol/L (at 75.0 μg/mL), respectively. The IC_{50} values of acetone extracts of *A. dealbata*, *A. leucophloea* and *A. ferruginea* were 84.63, 55.83 and 51.87 μg/mL, respectively.

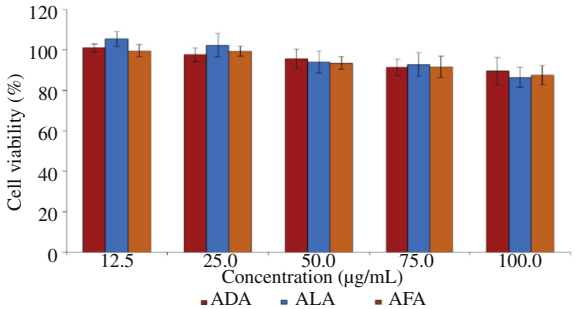

Figure 1. The effect of acetone extracts of stem bark of *Acacia* species on the cell viability of RAW 264.7 cells.
ADA: *A. dealbata*; ALA: *A. leucophloea*; AFA: *A. ferruginea*. Values are mean ± SD ($n = 3$).

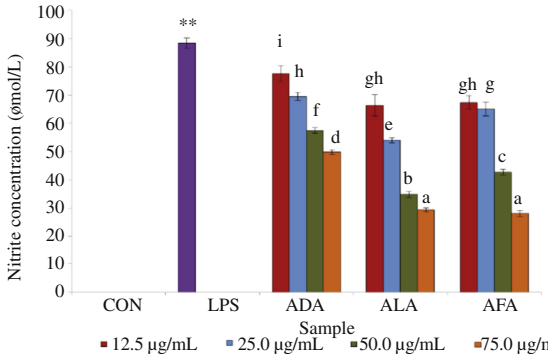

Figure 2. Inhibitory effect of acetone extracts of stem bark of *Acacia* species on NO production in LPS-stimulated RAW 264.7 macrophages.
CON: Control; LPS: LPS control; ADA: *A. dealbata*; ALA: *A. leucophloea*; AFA: *A. ferruginea*. Values are mean ± SD ($n = 3$). **: $P < 0.01$ compared to vehicle control; bars having different letters are significantly different ($P < 0.05$, compared to LPS control).

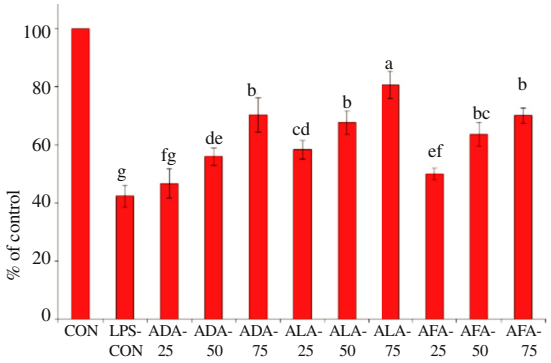

Figure 4. Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of stem bark of *Acacia* species on COX-2 activity in LPS-stimulated RAW 264.7 cells.
ADA: *A. dealbata*; ALA: *A. leucophloea*; AFA: *A. ferruginea*. Values are mean ± SD ($n = 3$). Bars having different letters are significantly different ($P < 0.05$).

3.2. Effect of acetone extracts on iNOS, COX-2 and TNF-α expression

The macrophage RAW 264.7 cells were treated with different concentrations of acetone extracts (25, 50 and 75 µg/mL) to determine the expression of pro-inflammatory mediators stimulated by LPS. As depicted in Figures 3–5, the un-stimulated RAW 264.7 cells expressed very low mRNA levels of iNOS, COX-2 and TNF-α. Whereas mRNA expressions were strongly expressed after the RAW 264.7 cells were stimulated with LPS (1 µg/mL). However, pre-treatment with acetone extracts from all the three *Acacia* species significantly ($P < 0.05$) suppressed the expression of iNOS, COX-2 and TNF-α in LPS-stimulated RAW 264.7 cells. All the three *Acacia* extracts effectively suppressed the expression of iNOS (70.67%–81.00%), COX-2 (70.00%–80.67%) and TNF-α (67.66%–76.67%) at the concentration of 75 µg/mL.

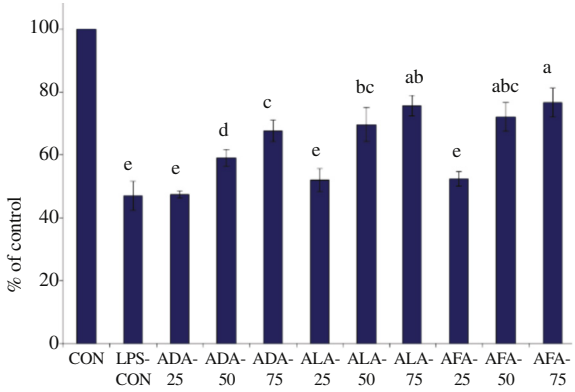

Figure 5. Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of stem bark of *Acacia* species on TNF-α activity in LPS-stimulated RAW 264.7 cells.
ADA: *A. dealbata*; ALA: *A. leucophloea*; AFA: *A. ferruginea*. Values are mean ± SD ($n = 3$). Bars having different letters are significantly different ($P < 0.05$).

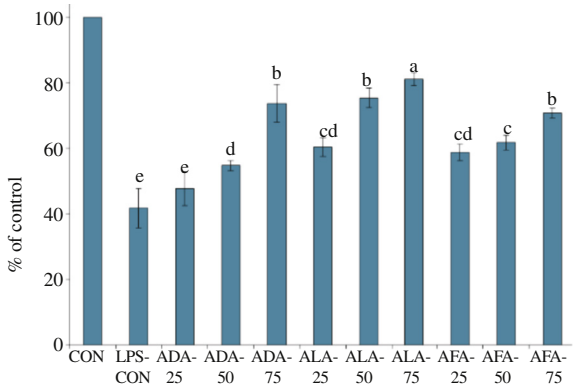

Figure 3. Effect of different concentrations (25, 50 and 75 µg/mL) of acetone extracts of stem bark of *Acacia* species on iNOS activity in LPS-stimulated RAW 264.7 cells.
ADA: *A. dealbata*; ALA: *A. leucophloea*; AFA: *A. ferruginea*. Values are mean ± SD ($n = 3$). Bars having different letters are significantly different ($P < 0.05$).

3.3. Phenolic profile of acetone extracts using LC-MS/MS analysis

In the LC-MS/MS analysis, acetone extract of *A. ferruginea* bark was found to contain higher number of components (12) than other two species. As shown in Tables 2–4, there were 6 (caffeoyl glucose, procyanidin B1, ellagic acid, ellagic acid isomer, rosmanol and an unknown component), 6 (mainly proanthocyanidin derivatives, epiafzelechin–epicatechin and ellagic acid) and 12 (including catechin, procyanidin B1, quercetin, ellagic acid, rosmanol) components detected by LC-MS/MS analysis from the acetone extracts of *A. dealbata*, *A. leucophloea* and *A. ferruginea* barks, respectively. The molecular mass and fragments of the identified components are presented in Tables 2–4. Among the components, ellagic acid has been detected from all the three *Acacia* species. In addition, the component rosmanol was detected in *A. dealbata* and *A. ferruginea*.

Table 2
Phenolic profile of acetone extract from the stem bark of *A. dealbata*.

Peak	Tentative identification	Retention time (min)	Molecular mass	MS/MS fragments (*m/z*)	References
1	Caffeoyl glucose	0.479	342	179, 135, 58	[14]
2	Procyanidin B1	1.200	577	407, 289, 245, 161	[15]
3	Ellagic acid	2.159	302	284, 255, 229, 185	[16]
4	Ellagic acid isomer	2.759	302	284, 255, 229, 185	[16]
5	Unknown	4.940	346	189, 132	–
6	Rosmanol	5.521	346	301, 175, 148, 136, 119, 106	[17]

Table 3
Phenolic profile of acetone extract from the stem bark of *A. leucophloea*.

Peak	Tentative identification	Retention time (min)	Molecular mass	MS/MS fragments (*m/z*)	References
1	Epiafzelechin–epicatechin	0.480	561	543, 289, 245, 161	[18]
2	Procyanidin dimer B4	0.959	577	425, 407, 289	[19]
3	Proanthocyanidin derivatives	1.060	642	561, 289, 245, 205, 158	[20]
4	Proanthocyanidin derivatives	1.670	764	562, 543, 289, 245, 206, 159	[20]
5	Epiafzelechin–epiafzelechin–epicatechin	1.950	833	561, 543, 289, 271, 161	[18]
6	Ellagic acid	3.600	301	284, 229, 185	[21]

Table 4
Phenolic profile of acetone extract from the stem bark of *A. ferruginea*.

Peak	Tentative identification	Retention time (min)	Molecular mass	MS/MS fragments (*m/z*)	References
1	Unknown	0.719	205	161, 114, 98, 71	–
2	Catechin	1.318	290	245, 205, 179, 137, 123, 108	[15]
3	Procyanidin B1	1.198	577	407, 289, 245, 161	[15]
4	Quercetin	1.678	302	179, 149, 122, 93	[15]
5	3-O-methylquercetin	1.910	316	300, 271, 255, 243	[22]
6	Genistein	2.519	270	269, 195, 133	[23]
7	Dihydroxy chalcone compound	2.880	271	135, 119	[23]
8	Ellagic acid	4.203	302	301, 255, 201, 173	[16]
9	Feruloylquinic acid	4.805	367	191, 134	[24]
10	N-feruloylglycine	5.646	251	249, 205, 191, 177, 149	[25]
11	N-feruloylglycine isomer	5.887	251	250, 206, 192, 178, 149	[25,26]
12	Rosmanol	6.128	346	301, 175, 133, 119	[17]

4. Discussion

The present study was undertaken to elucidate the anti-inflammatory potential of three different *Acacia* species (*A. dealbata*, *A. leucophloea* and *A. ferruginea*). Previously, many studies have reported that the inhibition of NO production in LPS-stimulated RAW 264.7 macrophage cell line provides an outstanding strategy for screening the anti-inflammatory drugs [4,27,28]. In general, phenolic components from natural products have been demonstrated to elicit potent anti-inflammatory properties [29]. Therefore, we prepared phenolic-rich acetone extracts from *Acacia* barks to test their effect on the production of NO in the LPS-stimulated RAW 264.7 cells [10]. The RAW 264.7 cells were activated by LPS, and NO production was measured as nitrite concentration in the culture supernatant using Griess reagent [27]. The inhibitory effect of these *Acacia* extracts on NO production was not due to cell toxicity (viability > 90%) as determined in MTT cell viability assay (Figure 1).

In our earlier study, acetone and methanol extracts of barks of *A. leucophloea*, *A. ferruginea*, *A. dealbata*, and *Acacia pennata* registered higher level of total phenolic and flavonoid contents [10]. Further, the acetone extracts of barks showed higher antioxidant and free radical scavenging activities than methanol extracts. In addition, melanoxetin from the heartwood of *Acacia confusa* (*A. confusa*) markedly suppressed LPS-induced NO and PGE2 productions [30]. Similar to our report, the barks from the stem and root of *A. confusa* exhibited excellent antioxidant activity [31]. Previously, Yang *et al.* [32] studied the effect of 260 alcoholic extracts from Jeju plants on inhibitory activity against NO production in LPS-stimulated macrophages. Out of 260 extracts, 122 extracts exhibited potent inhibitory activity against NO production by more than 25% (at 100 μg/mL). NO is a signaling molecule involved in inflammation via iNOS upregulation. Therefore, the inhibitory activity of plant extracts on NO production may be due to the suppression of iNOS expression [1,8].

The LPS-stimulated macrophages up-regulate the expression of iNOS, COX-2, and other inflammatory mediators, which ultimately results in uncontrolled production of NO and PGE2. Since the acetone extracts from *Acacia* barks were shown to have potent inhibitory activity against NO production in LPS-stimulated cells, we performed quantitative RT-PCR to determine whether these extracts can suppressed the expression of iNOS and COX-2 and TNF-α. In the anti-inflammatory screening study, previous studies have suggested that many phytochemicals can selectively inhibit the iNOS and COX-2

expression. In addition, nuclear factor kappa B is a significant transcription factor in the gene expression of iNOS and COX-2 in LPS-stimulated macrophage cell lines [33–35].

In the present study, the cells pre-treated with *Acacia* extracts down-regulated the iNOS, COX-2 and TNF-α gene expression, which was in agreement with the observed inhibition of NO production. Further, the fresh aerial parts of *A. ferruginea* possess immunomodulatory and tumor inhibitory activities by regulating the inflammatory mediators such as TNF-α, iNOS, COX-2, IL-1β, IL-6, and IL-2 [36]. Similarly, *Acacia* species such as *Acacia victoriae*, and *A. confusa* significantly suppressed the expression of various inflammatory mediators [30,37]. The results revealed that the inhibition of NO production in LPS-stimulated RAW 264.7 cells by the acetone extracts of *Acacia* barks is attributed to the suppression of iNOS, COX-2 and TNF-α expression. The suppression of these mRNA expressions is a promising chemotherapeutic strategy to prevent the potentially harmful pro-inflammatory activity of macrophages. The results of the present study indicate that all the three extracts effectively decreased the nitrite accumulation through down-regulating the expression of these mRNA.

Components of plant origin from different chemical classes, especially phenolic groups, have been demonstrated to have anti-inflammatory activity. Ellagic acid is identified as an active metabolite in strawberry that regulates LPS-induced inflammation [38]. Ellagic acid is the main condensed tannin present in the extract of Moroccan *Acacia mollissima* bark [39]. Hydrolyzed ethyl acetate fraction from the green pod of *Acacia nilotica* contains higher level of ellagic acid [40]. Proanthocyanidin-rich fractions from the wild blackberry showed the highest inhibition of iNOS expression [41]. Another important component, rosmanol significantly suppressed the expression of iNOS and COX-2 by inhibiting the activation of nuclear factor kappa B and signal transducer and activator of transcription-3 [42]. Together with these components, well known bioactive components such as catechin and quercetin were also identified.

The presence of various phenolic components in the acetone extracts of *Acacia* species may be attributed to the inhibitory activity on NO production in LPS-stimulated RAW 264.6 cells through the suppression of iNOS, COX-2 and TNF-α gene expression. The results may support the use of phenolic components towards the development of new anti-inflammatory agents. Further, the present study leads to the isolation of bioactive phenolic components from various *Acacia* species.

Acetone extracts from all the three *Acacia* species effectively inhibited the production of NO and suppressed the expressions of COX-2, iNOS and TNF-α in a concentration dependent manner. The present study suggests a potential use of *Acacia* barks as a source of anti-inflammatory agents by suppressing the expression of pro-inflammatory mediators. Further studies are warranted in relation to the mechanism of action and isolation of bioactive components from the acetone extracts of these *Acacia* barks.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References

[1] Kim HY, Hwang KW, Park SY. Extracts of *Actinidia arguta* stems inhibited LPS-induced inflammatory responses through nuclear factor–κB pathway in Raw 264.7 cells. *Nutr Res* 2014; **34**: 1008-16.
[2] Lee HS, Ryu DS, Lee GS, Lee DS. Anti-inflammatory effects of dichloromethane fraction from *Orostachys japonicas* in RAW 264.7 cells: suppression of NF-κB activation and MAPK signaling. *J Ethnopharmacol* 2012; **140**: 271-6.
[3] Joung EJ, Lee B, Gwon WG, Shin T, Jung BM, Yoon NY, et al. Sargaquinoic acid attenuates inflammatory responses by regulating NF-κB and Nrf2 pathways in lipopolysaccharide-stimulated RAW 264.7 cells. *Int Immunopharmacol* 2015; **29**: 693-700.
[4] Shin SY, Shin MC, Shin JS, Lee KT, Lee YS. Synthesis of aurones and their inhibitory effects on nitric oxide and PGE2 productions in LPS-induced RAW 264.7 cells. *Bioorg Med Chem Lett* 2011; **21**: 4520-3.
[5] Nguyen PH, Zhao BT, Lee JH, Kim YH, Min BS, Woo MH. Isolation of benzoic and cinnamic acid derivatives from the grains of *Sorghum bicolor* and their inhibition of lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells. *Food Chem* 2015; **168**: 512-9.
[6] Gurkan AS, Karabay AZ, Buyukbingol Z, Buyukbingol E. Synthesis and effects of some novel tetrahydronaphthalene derivatives on proliferation and nitric oxide production in lipopolysaccharide activated Raw 264.7 macrophages. *Eur J Med Chem* 2011; **46**: 468-79.
[7] Thao NP, Luyen BT, Tai BH, Cuong NM, Kim YC, Minh CV, et al. Chemical constituents of *Miliusa balansae* leaves and inhibition of nitric oxide production in lipopolysaccharide-induced RAW 264.7 cells. *Bioorg Med Chem Lett* 2015; **25**: 3859-63.
[8] Nahar PP, Driscoll MV, Li L, Slitt AL, Seeram NP. Phenolic mediated anti-inflammatory properties of a maple syrup extract in RAW 264.7 murine macrophages. *J Funct Foods* 2014; **6**: 126-36.
[9] Kipkore W, Wanjohi B, Rono H, Kigen G. A study of the medicinal plants used by the Marakwet Community in Kenya. *J Ethnobiol Ethnomed* 2014; **10**: 24.
[10] Sowndhararajan K, Joseph JM, Manian S. Antioxidant and free radical scavenging activities of Indian *Acacias*: *Acacia leucophloea* (Roxb.) Willd., *Acacia ferruginea* DC., *Acacia dealbata* Link. and *Acacia pennata* (L.) Willd. *Int J Food Prop* 2013; **16**: 1717-29.
[11] Sowndhararajan K, Hong S, Jhoo JW, Kim S, Chin NL. Effect of acetone extract from stem bark of *Acacia* species (*A. dealbata*, *A. ferruginea* and *A. leucophloea*) on antioxidant enzymes status in hydrogen peroxide-induced HepG2 cells. *Saudi J Biol Sci* 2015; **22**: 685-91.
[12] Sowndhararajan K, Kang SC. Protective effect of ethyl acetate fraction of *Acacia ferruginea* DC. against ethanol-induced gastric ulcer in rats. *J Ethnopharmacol* 2013; **148**: 175-81.
[13] Hong S, Joo T, Jhoo JW. Antioxidant and anti-inflammatory activities of 3,5-dicaffeoylquinic acid isolated from *Ligularia fischeri* leaves. *Food Sci Biotechnol* 2015; **24**: 257-63.
[14] Sulaiman CT, Arun A, Anandan EM, Sandhya CR, Balachandran I. Isolation and identification of phytoestrogens and flavonoids in an Ayurvedic proprietary medicine using chromatographic and mass spectroscopic analysis. *Asian Pac J Reprod* 2015; **4**: 153-6.
[15] Sun J, Liang F, Bin Y, Li P, Duan C. Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules* 2007; **12**: 679-93.
[16] Abu-Reidah IM, Ali-Shtayeh MS, Jamous RM, Arráez-Román D, Segura-Carretero A. HPLC-DAD-ESI-MS/MS screening of bioactive components from *Rhus coriaria* L. (Sumac) fruits. *Food Chem* 2015; **166**: 179-91.
[17] Borrás-Linares I, Stojanović Z, Quirantes-Piné R, Arráez-Román D, Švarc-Gajić J, Fernández-Gutiérrez A, et al. *Rosmarinus*

officinalis leaves as a natural source of bioactive compounds. *Int J Mol Sci* 2014; **15**: 20585-606.

[18] Falleh H, Oueslati S, Guyot S, Dali AB, Magné C, Abdelly C, et al. LC/ESI-MS/MS characterisation of procyanidins and propelargonidins responsible for the strong antioxidant activity of the edible halophyte *Mesembryanthemum edule* L. *Food Chem* 2011; **127**: 1732-8.

[19] Tsang C, Auger C, Mullen W, Bornet A, Rouanet JM, Crozier A, et al. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br J Nutr* 2005; **94**: 170-81.

[20] Omar MH, Mullen W, Crozier A. Identification of proanthocyanidin dimers and trimers, flavone *C*-glycosides, and antioxidants in *Ficus deltoidea*, a Malaysian herbal tea. *J Agric Food Chem* 2011; **59**: 1363-9.

[21] Sun J, Chen P. UHPLC/HRMS analysis of African mango (*Irvingia gabonensis*) seeds, extract and related dietary supplements. *J Agric Food Chem* 2013; **60**: 8703-9.

[22] Schwingel LC, Schwingel GO, Storch N, Barreto F, Bassan VL. 3-*O*-methylquercetin from organic *Nicotiana tabacum* L. trichomes: influence of the variety, cultivation and extraction parameters. *Ind Crops Prod* 2014; **55**: 56-62.

[23] Ye M, Yang WZ, Liu KD, Qiao X, Li BJ, Cheng J, et al. Characterization of flavonoids in *Millettia nitida* var. *hirsutissima* by HPLC/DAD/ESI-MSn. *J Pharm Anal* 2012; **2**: 35-42.

[24] Stalmach A, Mullen W, Nagai C, Crozier A. On-line HPLC analysis of the antioxidant activity of phenolic compounds in brewed, paper-filtered coffee. *Braz J Plant Physiol* 2006; **18**: 253-62.

[25] Stalmach A, Mullen W, Barron D, Uchida K, Yokota T, Cavin C. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metab Dispos* 2009; **37**: 1749-58.

[26] Pekkinen J, Rosa NN, Savolainen OI, Keski-Rahkonen P, Mykkänen H, Poutanen K, et al. Disintegration of wheat aleurone structure has an impact on the bioavailability of phenolic compounds and other phytochemicals as evidenced by altered urinary metabolite profile of diet-induced obese mice. *Nutr Metab (Lond)* 2014; **11**: 1.

[27] Ma JH, Wang Y, Liu Y, Gao SY, Ding LQ, Zhao F, et al. Four new sesquiterpenes from the rhizomes of *Curcuma phaeocaulis* and their iNOS inhibitory activities. *J Asian Nat Prod Res* 2015; **17**: 532-40.

[28] Rebelo MJ, Sousa C, Valentão P, Rego R, Andrade PB. Phenolic profile of Douro wines and evaluation of their NO scavenging capacity in LPS-stimulated RAW 264.7 macrophages. *Food Chem* 2014; **163**: 16-22.

[29] Bearaa I, Živković J, Lesjaka M, Ristić J, Šavikin K, Maksimović Z, et al. Phenolic profile and anti-inflammatory activity of three *Veronica* species. *Ind Crops Prod* 2015; **63**: 276-80.

[30] Wu JH, Tung YT, Chien SC, Wang SY, Kuo YH, Shyur LF, et al. Effect of phytocompounds from the heartwood of *Acacia confusa* on inflammatory mediator production. *J Agric Food Chem* 2008; **56**: 1567-73.

[31] Wei SD, Zhou HC, Lin YM, Liao MM, Chai WM. MALDI-TOF MS analysis of condensed tannins with potent antioxidant activity from the leaf, stem bark and root bark of *Acacia confusa*. *Molecules* 2010; **15**: 4369-81.

[32] Yang EJ, Yim EY, Song G, Kim GO, Hyun CG. Inhibition of nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages by Jeju plant extracts. *Interdiscip Toxicol* 2009; **2**: 245-9.

[33] Kim DH, An BJ, Kim SG, Park TS, Park GH, Son JH. Anti-inflammatory effect of *Ligularia fischeri*, *Solidago virga-aurea* and *Aruncus dioicus* complex extracts in Raw 264.7 cells. *J Life Sci* 2011; **21**: 678-83.

[34] Kwon DJ, Ju SM, Youn GS, Choi SY, Park J. Suppression of iNOS and COX-2 expression by flavokawain A via blockade of NF-κB and AP-1 activation in RAW 264.7 macrophages. *Food Chem Toxicol* 2013; **58**: 479-86.

[35] Liu YW, Mei HC, Su YW, Fan HT, Chen CC, Tsai YC. Inhibitory effects of *Pleurotus tuber-regium* mycelia and bioactive constituents on LPS-treated RAW 264.7 cells. *J Funct Foods* 2014; **7**: 662-70.

[36] Sakthivel KM, Guruvayoorappan C. *Acacia ferruginea* inhibits tumor progression by regulating inflammatory mediators-(TNF-a, iNOS, COX-2, IL-1β, IL-6, IFN-γ, IL-2, GM-CSF) and pro-angiogenic growth factor-VEGF. *Asian Pac J Cancer Prev* 2013; **14**: 3909-19.

[37] Haridas V, Arntzen CJ, Gutterman JU. Avicins, a family of triterpenoid saponins from *Acacia victoriae* (Bentham), inhibit activation of nuclear factor-κB by inhibiting both its nuclear localization and ability to bind DNA. *Proc Natl Acad Sci USA* 2001; **98**: 11557-62.

[38] Lee J, Kim S, Namgung H, Jo YH, Bao C, Choi HK, et al. Ellagic acid identified through metabolomic analysis is an active metabolite in strawberry ('Seolhyang') regulating lipopolysaccharide-induced inflammation. *J Agric Food Chem* 2014; **62**: 3954-62.

[39] Rhazi N, Hannache H, Oumam M, Sesbou A, Charrier B, Pizzi A, et al. Green extraction process of tannins obtained from Moroccan *Acacia mollissima* barks by microwave: modeling and optimization of the process using the response surface methodology RSM. *Arab J Chem* 2015; http://dx.doi.org/10.1016/j.arabjc.2015.04.032.

[40] Singh BN, Singh BR, Singh RL, Prakash D, Sarma BK, Singh HB. Antioxidant and anti-quorum sensing activities of green pod of *Acacia nilotica* L. *Food Chem Toxicol* 2009; **47**: 778-86.

[41] Cuevas-Rodríguez EO, Dia VP, Yousef GG, García-Saucedo PA, López-Medina J, Paredes-López O, et al. Inhibition of pro-inflammatory responses and antioxidant capacity of mexican blackberry (*Rubus* spp.) extracts. *J Agric Food Chem* 2010; **58**: 9542-8.

[42] Lai CS, Lee JH, Ho CT, Liu CB, Wang JM, Wang YJ, et al. Rosmanol potently inhibits lipopolysaccharide-induced iNOS and COX-2 expression through down regulating MAPK, NF-kappaB, STAT3 and C/EBP signaling pathways. *J Agric Food Chem* 2009; **57**: 10990-8.