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The Acetylation-based synthesis of 3,3',4',5,5',7-hexaacetate myricetin and evaluation of its anti-inflammatory activities in lipopolysaccharide-induced RAW264.7 mouse macrophage cells

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Abstract Recent studies have highlighted the link between diseases and inflammation across our lifespan. Our sedentary lifestyle, high-calorie diet, chronic stress, chronic infections, and exposure to pollutants and xenobiotics, collectively intensify the course and recurrence of infections and inflammation in our bodies, promoting the prevalence of chronic diseases and aging. Given such phenomena and considering additional factors such as the frequency of prescription, and easy access to over-the-counter drugs, the need for anti-inflammatory therapeutics is ever-increasing. However, the readily available anti-inflammatory treatment option comes with a greater risk of side effects or high cost (biologics). Therefore in this growing competition of discovering and developing new potent anti-inflammatory drugs, we focused on utilizing the established knowledge of traditional medicine to find lead compounds. Since lead optimization is an indispensable step toward drug development, we applied this concept for the production of potent anti-inflammatory compounds achieved by structural modification of flavonoids. The derivative obtained through acetylation of myricetin, 3,3',4',5,5',7-hexaacetate myricetin, showed a greater inhibitory effect in the production of pro-inflammatory mediators such as nitric oxide, Prostaglandin E_2, and pro-inflammatory cytokines like interleukin-6, interleukin-1β, in lipopolysaccharide-stimulated RAW264.7 mouse macrophage cells compared to myricetin. The increased potency of inhibition was in conjunction with an increased inhibitory effect on inducible nitric oxide synthase and cyclooxygenase-2 proteins. Through such measures, this study supports lead optimization for well-established lead compounds from traditional medicine using a simpler and greener chemistry approach for the purpose of designing and developing potent anti-inflammatory therapeutics with possibly fewer side effects and increased bioavailability.

Keywords Acetylation · Anti-inflammatory activity · Cyclooxygenase-2 · Inducible nitric oxide synthase · Lipopolysaccharide · Myricetin hexaacetate · RAW 264.7 cell

Introduction

Inflammation is a condition that involves a complex biological response of the body to stimuli such as foreign objects, pathogens (bacteria, viruses, or fungi), toxins or chemicals, or external and internal injuries that cause damage to the tissues. These responses are characterized by five major cardinal signs: redness, heat, swelling, pain, and loss of function (movement disability, impaired senses, etc.) [1]. The mechanism lies in a cascade event of cellular signaling, humoral secretions, and cellular migration, causing changes in the micro-circulatory system (vasodilation and increased vascular permeability with infiltration of immune cells) of the injured site [2,3]. The initial phase of inflammation is described by the release of immune mediators, such as histamines, cytokines, nitric oxide (NO), or prostaglandins (PGEs) by the native cells, mast cells, and macrophages in response to the

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stimulus [3]. The pro-inflammatory cytokines associated with lipopolysaccharide (LPS) stimulation are interleukin-6 (IL-6), interleukin-1β (IL-1β), and Tumor necrosis factor α (TNFα). IL-6, IL-1β, and TNFα, each with their distinct receptors, act by receptor-mediated signal transduction activating several transcription factors, including NF-κB and AP-1, thereby increasing the expression of proteins such as type II phospholipase (PL) A2, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) and endothelial adhesion molecules as well as chemokines [4]. The increased expression of PLA2 and COX-2 initiates the conversion of phospholipids to arachidonic acid and subsequently to PGE_2 [5]. PGE_2 is mainly associated with pain in inflamed tissues brought about by excitation of nociceptors and stimulation of other pain mediators [6]. On the other hand, enhanced expression and synthesis of iNOS increase the rate of catalysis of oxidation of L-arginine to L-citrulline with the simultaneous release of NO. This increase in the production of NO, a free radical, acts as a cytotoxic agent and eventually damages biomolecules in cells [7]. These events in cumulation culminate in inflammation.

Being part of the host defense system, inflammation plays a critical role in alerting, aiding as well as regulating the immune system for the elimination of foreign matters or healing of tissues towards resolution. However, some infections like Human Immunodeficiency Virus, Cytomegalovirus, *Yersinia pseudotuberculosis*, *Helicobacter pylori*, and severe cases of COVID-19 [8] as well as autoimmune diseases are associated with persistent progression of inflammation and immune response [9-11]. Also, it has been argued that the condition of chronic, low-grade inflammation could be a major bridge in gapping the relation between aging and age-related diseases such as atherosclerosis, arthritis, cancer, diabetes, osteoporosis, dementia, vascular diseases, obesity, and metabolic syndrome [12]. And even though therapeutic options such as (i) Glucocorticoids, (ii) Nonsteroidal anti-inflammatory drugs (NSAIDs) [13,14], and the more recently developed ones, (iii) Biological therapeutics [15,16], are available, a greater risk of side effects [17-19], and the high cost (for Biologics) [15] are associated with the use of such products. This calls forth alternatives with a lower risk of side effects and better cost-effectiveness.

About 35 percent of the global pharmaceuticals comprise directly or indirectly of natural products out of which 25% is plant-based natural products [20]. 13 out of 53 drugs falling under the category of anti-inflammatory agents, were natural product derivatives [21]. This growing and renewed interest in natural products are based on their chemically diverse structures that provide unique structural templates for further modification and derivation. Myricetin (structure as shown in Fig. 1), a flavonol class of plant flavonoid, having hydroxyl substitution at the 3, 5, 7, 3', 4' and 5' positions, is an antioxidant and free radical scavenger with well-known anti-inflammatory property. Various *in vitro* and *in vivo* studies point to its great potential as therapeutics [22-27]. However, there haven't been many reports on actual therapeutic use of myricetin.

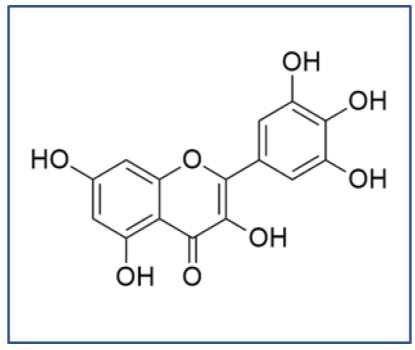

Fig. 1 Structure of myricetin

The problem comes down to its poor bioavailability. According to an article on flavonoids and polyphenols [28], the oral bioavailability of polyphenols and flavonoids in animal ranges from 2-20% which can be even more diverse and lower in humans who are more genetically complex. The attributing factors to the bioavailability of a compound are influenced by their rate of absorption, distribution, metabolism, and excretion of a body. Since flavonoids have a low solubility index it can cause slow dissolution rates which in turn retard the absorption and distribution rates. Moreover, biotransformation of flavonoids into conjugated forms by metabolic enzymes [29] might hinder the biological activity of the flavonoid rendering them inactive or might facilitate their degradation, increasing their rate of elimination. Also, researches on physicochemical properties of myricetin has shown it to be less stable at high temperature and pH [30] decreasing its pharmacological activity. Therefore, it is required to increase the bioavailability for flavonoids to be considered for use in therapeutics.

Lead optimization is a crucial part of developing bioactive compounds as a prodrug. It involves structural modifications of a lead compound to improve its pharmacokinetics and pharmaco-dynamic profiles, which increase its bioavailability, reduce cytotoxicity, and enhance bioactivity. For example, the development of synthetic derivatives of the antimalarial natural compound, artemisinin, and a class of macrolide antibiotics, Erythromycin, with increased potency, solubility in water and pH stability, was made possible by tuning its structure while maintaining an intact pharmacophore [31,32]. In fact, one of the most commonly used anti-inflammatory, antipyretic drugs and pain killers, aspirin, was also developed through chemical modification of salicylic acid [13]. The addition of an acetyl group alleviated the bitter taste of salicylic acid with increased potency as a cyclooxygenases (COXs) inhibitor [13,33]. Similar attempts of chemical modification of flavonoids have also been made [34,35]. After semi-synthesis of acetylated derivatives of chrysin, quercetin, kaempferol etc., evaluation of their bioactivity reported increased anticancer, antimicrobial and anti-inflammatory activities in comparison to

the flavonoids [35-37]. These approaches to designing and developing bioactive compounds as a prodrug has increased the probability of drug candidacy for natural products.

In an attempt to design and develop anti-inflammatory agents for possible therapeutic use by applying the principle of lead optimization, structural modification of a well-established bioactive natural product was carried out. The lead compound chosen for structural modification in this study was myricetin, a plant flavonoid with well-known biological activities. The hypothesis that substitution of the six hydroxyl groups by acetyl moieties might increase its anti-inflammatory activity and possibly its bioavailability led to this study. Therefore, in this study, we tried to explore the anti-inflammatory activity of an acetyl derivative synthesized through acetylation reaction of myricetin. To our knowledge, this study reports for the first time the anti-inflammatory activity of six acetyl substituted myricetin and its comparison to myricetin in LPS-induced RAW 264.7 mouse macrophage cell line, an *in vitro* inflammation model.

Materials and Methods

Chemical Reagents

Myricetin (cat. no. 70050, purity ≥96.0% HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). For cell culture, Dulbecco's modified Eagle's medium (DMEM; cat. no. LM001-05), fetal bovine serum (FBS; cat. no. S001-01) and 100X, Penicillin-streptomycin (P/S; cat. no. 15140122) were bought from Welgene (Gyeongsan-si, South Korea). LPS (*Salmonella enterica* serotype enteritidis; cat. no. L7770), Griess reagent (cat. no. G4410), and dimethyl sulfoxide (DMSO; cat. no. D4540) were purchased from Sigma-Aldrich. Thiazolyl Blue Tetrazolium Bromide (MTT) Ultra Pure (cat. no. 0793) was bought from VWR Chemicals (Solon, OH, USA). Antibodies against β-actin (cat. no. VMA00048), inducible nitric oxide synthase (iNOS) (cat. no. AHP2399) were purchased from Bio-Rad (Hercules, CA, USA), and anti- Cyclooxygenase-2 (COX-2) antibodies (cat. no. 100-401-226) from Rockland (Limerick, PA, USA). Radio-immunoprecipitation acid (RIPA) buffer (cat. no. R2002) was purchased from Biosesang (Seongnam-si, South Korea). Protease inhibitor cocktail was purchased from Sigma-Aldrich (cat. no. P8340). The IL-1β (cat. no. MLB00C) and PGE_2 (cat. no. KGE004B) enzyme-linked immunosorbent assay kits were purchased from R&D Systems (Minneapolis, MN, USA). On the other hand, TNF-α (cat. no. BMS607-3) and IL-6 (cat. no. 550950) ELISA kits were purchased from Thermo Fisher Scientific (Vienna, Austria) and BD Biosciences (San Diego, CA, USA), respectively. HPLC grade water was purchased from Thermo Fisher Scientific. HPLC grade acetonitrile and Trifluoroacetic acid (TFA) (cat. no. T0672) were purchased from Samchun (Pyeongtaek-si, South Korea).

Acetylation of myricetin (MYR)

Acetylation was carried out according to Picq et al. and Ohta & Yagashita [38,39] with some modifications. MYR was reacted with an excess of acetic anhydride in the presence of pyridine over high temperature. In more detail, a quantity of MYR (10 mg, 0.03 mmol) was dissolved in pyridine (1 mL) in a round bottom flask. Then, acetic anhydride (1.5 mL, 15.9 mmol) was added to the mixture and kept for reaction at 90 °C under constant stirring for 1 h. After cooling at room temperature, the mixture product was obtained by evaporation of solvent under reduced pressure using a rotary evaporator at 40 °C.

HPLC analysis and Purification

Preliminary analysis for the formation of the acetylated derivative was carried out through high-performance liquid chromatography using a Shimadzu SpectroMonitor, 3200 digital Photodiode-Array detection recording UV spectra from 190 to 800 nm. The column used was a C18 Shim-pack GIS with inner diameter parameter of 250×4.6 mm. The packing material contained Octadecyl-silica with a particle size of 5 µm. For analysis, the flow rate was maintained at 1.0 mL min^{-1} and oven temperature set to 40 °C. The mobile phase consisted of Solvent A as water containing 0.1% TFA and Solvent B as acetonitrile. A gradient method was applied with a gradual increment of Solvent B from 10 to 100% within a time of 30 min.

A purified form of the product was collected as a fraction of the acetylation reaction mixture by preparative HPLC. A Shimadzu SPD-20A prominence UV/Vis detector equipped with a Phenomenex Luna C18 column (250×10 mm, 5 µm) was used. The solvent system and method were the same as the analytical HPLC. The sample injection volume was 500 µL with a flow rate of 5 mL min^{-1}.

Identification of derivative

LC/MS analysis was performed to identify the compound on the basis of their relative mass to charge ratio using the Agilent InfinityLab LC/MSD with 1260 Infinity LCSystem, equipped with an electrospray interface operating in positive ion mode. Ion mass spectrums were taken in the range of *m/z* 200-700. Two scanning modes were operated, SCAN mode for the total ion chromatogram and SIM mode for a particular ion *m/z*. The spectral data were evaluated by OpenLab CDS ChemStation Edition software, version C.01.10.

High-resolution quadrupole-time-of-flight electrospray ionization-mass spectrometry was achieved in positive ion mode using the ACQUITY system UPLC equipped with a SYNAPT G2-Si column (Waters Corporation, Miliford, MA, USA). Using MassLynx version 4.1, data was processed and extracted between the mass range of 300 to 650.

Using a VNMRS system (Agilent technology, Santa Clara, CA, USA) ^{1}H-NMR spectral data was obtained at 500 MHz. Deuterated

chloroform ($CDCl_3$) was used to as the solvent showing up a reference peak signal (δ^1H 7.26 s). The obtained NMR data was compared to references and literatures for confirmation of the structure.

Cell culture and treatment

Macrophage cell line of murine origin RAW264.7 was obtained from Korean Cell Line Bank (Seoul, South Korea). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, maintaining a humidified atmosphere of 5% CO_2 at 37 °C. Passing of the cells was performed every two to three days or at around 70% confluence.

Cells were seeded at density of 8.0×10^4 cells per well in a 24 well culture plate and incubated at 37 °C under 5% CO_2 until 50-60% confluence. Next, treatment with extract at doses of 100 µg/mL, 200 and 400 µg/mL and simultaneous stimulation by LPS (1 µg/mL) was performed for additional 24 h.

Cytotoxicity assessment

In 24 well culture plate cells were seeded at the density of 6.0×10^4 cells per well and incubated to reach 50-60% confluency. Then, cells were pretreated with MYR or MYR-HA for 2 hours following LPS treated for additional 24 h. MTT (2 mg/mL) was added to each well and incubated at 37 °C for 2 h. After careful aspirations of the culture supernatant, the formazan crystals were dissolved with DMSO and the absorbance of each well was recorded at 570 nm with the help of a microplate reader.

Nitric oxide (NO) determination

RAW264.7 cells were plated in 24 well plate and treated with extract and LPS for 24 h. The cell culture soup was transferred to a 96-well plate. The level of nitric oxide in the soups were quantified with the help of Griess reagent as described in the manual provided by the manufacturer. Briefly, To 100 µL of culture soup, equal amount (100 µL) of Griess reagent (I & II) was added and incubated at room temperature in for about 10 min and then, the absorbance was measured at 540 nm.

ELISA for PGE_2, IL-1β, IL-6 and TNF-α

As described previously, to the 24 well plate cell culture, extract was added followed by LPS stimulation for additional 24 h incubation. From each well, cell culture soups were collected. The levels of PGE2, IL-6, IL-1β and TNFα in the soups were measured with the help of ELISA kits (Mouse TNF alpha ELISA Kit; Invitrogen, Carlsbad, CA, USA; Mouse IL-6 ELISA Kit, BD Biosciences; Mouse IL-1β/IL-1F2, R&D Systems; Mouse Prostaglandin E_2, R&D Systems) following instructions provided in the manual.

Protein extraction, quantification, and sample preparation

Cells were cultured in a 12 well plate seeded at the density of 2×10^5 cells/well and incubated at 37 °C in a humidified incubator supplied with 5% CO_2. The cells were grown for 24 h or at 80% confluency and pretreated with MYR or MYR-HA followed by LPS induction for 18 h. The cells were harvested and lysed with the addition of proteinase-inhibitor cocktail mix in RIPA buffer. The cell lysate obtained was subjected to protein quantification using the Pierce BCA Protein Assay Kit by Thermo Fisher. Protein concentration was obtained using a standard curve of bovine serum albumin as instructed in the manual. The amount of protein in each sample was equated by mixing required volume of cell lysate and RIPA buffer. To this, equal volume of 2X Laemmli sample buffer (Bio-Rad) and 5% of 2-mercaptoethanol were added and boiled for 5 min and loaded in each well of acrylamide gel which were then subjected to electrophoresis.

Western blotting

Cells were lysed in RIPA buffer containing Protease inhibitor cocktail mix in an ice-bath. The cell debris were separated from the supernatant by centrifugation at 13,000 rpm under 4 °C. The obtained cell lysate was subjected to protein quantification by bicinchoninic acid assay (BCA) using Pierce BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific). Then, about 20 µg of protein was loaded and resolved by SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride membrane in a semi-dry transfer system Trans-Blot Turbo (Bio-Rad). After blocking with 5% skim milk powder dissolved in tris-buffered saline (20 mM Tris base, 0.5 M NaCl, pH 7.5) with 0.1% Tween-20 for 1 h, at room temperature and subsequent washing steps, the membrane was incubated overnight at 4 °C in a shaker with primary antibodies as, iNOS antibody (1:1000) and COX-2 antibody (1:1000). The second stage of incubation with peroxidase-conjugated secondary antibody (1:1000) (HRP Anti-Rabbit IgG (H&L), Rockland Immunochemicals, Inc., (Pottstown, PA USA), was for 1 h at room temperature in a shaker. The membrane was exposed with ECL substrate mix (Bio-Rad) and visualized on an Imager (Fujifilm LAS 4000 mini, GE Healthcare Japan Corp., Tokyo, Japan). Image densitometry created by ImageJ was represented in the bar graph as the percentage of control.

Statistical analysis

All experimental data were expressed as mean ± standard deviation (SD) of measured values. Statistical significance of the data was evaluated by one-way analysis of variance (ANOVA)-Tukey-Kramer test (Microsoft 365 Excel). The p-values were set as $p<0.01$.

Results and Discussion

Synthesis, analysis, and identification of the acetylation product

Acetylation reaction was carried out using acetic anhydride as the

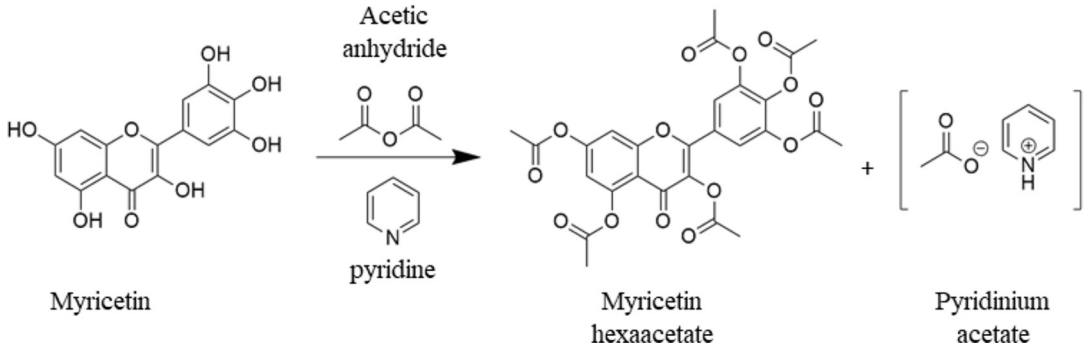

Fig. 2 Acetylation reaction of myricetin using acetic anhydride and pyridine

acyl donor and pyridine as the base catalyst. Figure 2 shows the possible mechanism through which the chemical reaction proceeded. Reversed-phase HPLC analysis was performed to monitor the changes in functional group of myricetin by analyzing the reaction mixture. From the chromatograms, we were able to obtain distinct peaks for the reaction mixture at different retention time from the original compound, myricetin. Likewise, apparent differences in spectral characteristics of the two compounds was seen. In our findings, the UV-VIS spectral analysis of MYR showed maximum absorption at around 254 nm (Band II) and 373 nm (Band I) which was relatively consistent with the data from the literatures [40-42]. In contrast, reaction product demonstrated maximum absorption at around 254 nm (Band II) and 294 nm (Band I). It seemed that there was a hypsochromic shift in the absorption spectrum as similar trend was observed for acetylated derivative of luteolin [43], thus supporting the hypothesis that acetylation reaction led to the modification in the structure of myricetin.

Mass spectrometry is a useful technique for structure elucidation of molecules, such as myricetin hexaacetate. In this technique, ions of the compound are generated and detected based on their mass-to-charge ratio. In positive ion mode, the exact mass of a hydrogen ion is added to the compound's molecular weight. The molecular weight of myricetin is 318.23 g/mol [44], and in our findings it was detected as a peak at *m/z* 319.23. The acetylated product gave a $[M+H]^+$ at *m/z* 571, which matches with the MW of 3,3',4',5,5',7-myricetin hexaacetate (570.5 g/mol) [45]. Additionally, according to HR-QTOF-ESI-MS (supplementary Fig. 1), the peak corresponding to *m/z* 571.1087 is likely the protonated molecular ion fragment of MYR-HA, and the commonly observed sodium adduct formation, explained by the *m/z* value of 593.0910. The hydroxyl group substitution by an acetyl group increases the mass of compounds by 42.011 u in the spectrum. The mass difference of 571.1087 and 319.0450 divided by 42.011 gives the value 6, implying the substitution by six acetyl groups. In accordance with this, we could find in the figure between *m/z* 319.0450 and *m/z* 571.1087, that there are five distinct peaks each obtained at a mass difference of ~42.011 from each other. During ionization, the least stable bonds are most likely to be cleaved making the ester bonds between the flavan and acetyl moieties the most vulnerable. So, cleavage of each of the six ester bonds from MYR-HA gives five fragment ions with neutral loss fragments of the acetyl groups. These five fragment ions are detected as *m/z* 529.0977 [M+H-acetyl moiety]$^+$, 487.0869 [M+H-2acetyl moiety]$^+$, 445.0760 [M+H-3acetyl moiety]$^+$, 403.0656 [M+H-4acetyl moiety]$^+$, 361.0555 [M+H-5acetyl moiety]$^+$.

Insight into the actual structural information of our product was provided by ^{1}H-NMR spectrum. The chemical shifts observed in ^{1}H-NMR spectra of our product sample was compared to that of the reference data of Myricetin [46] and Myricetin hexaacetate [47]. Aromatic protons are known to exhibit chemical shift between 6.5-8 ppm. As for our product, chemical shifts were observed between two distinct ranges. One range exhibited between 6.9-7.63 ppm corresponded to the hydrogens at the A and B rings. Since Ring A constitute a higher electron density, protons in this region are more shielded therefore appear up-field in the spectra than in comparison to the hydrogens in Ring B. So, the peak at 7.63 (most downfield) was designated C2' and C6' protons and 7.36 and 6.90 as C8 and C6 respectively. The chemical shift observed in the other range, between 2.35-2.46 ppm was atypical of a flavonol. As seen in the table MYR do not exhibit chemical shift at this range whereas in the case of MYR-HA, similar pattern in chemical shift can be observed, in fact the pattern almost match. This behavior in chemical shift is promoted by the "methyl-hydrogens" in the acetyl moiety of MYR-HA. Methyl-hydrogens at the carbonyl end of the ester bond show a characteristic chemical shift in the range of 2.1-2.6 ppm. A copy of original spectrum is available as supplementary materials.

Product sample: ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.63 (s, 2H, C2' and C6'), 7.36 (d, J=2.2 Hz, 1H, C8), 6.90 (d, J=2.2 Hz, 1H, C6), 2.46 (s, 3H), 2.39 (s, 3H), 2.37 (s, 3H), 2.36 (s, 3H), 2.35 (s, 6H)

Myricetin [46]: ^{1}H-NMR (CD_3OD, 400MHz): δ 7.34 (s, 2H, C2' and C6'), 6.36 (d, J=2.0 Hz, 1H, C8), 6.16 (d, J=2.0 Hz, 1H, C6)

Myricetin hexaacetate [47]: ^{1}H-NMR ($CDCl_3$, 400MHz): δ 7.62 (s, 2H, C2' and C6'), 7.34 (d, J=2.0 Hz, 1H, C8), 6.88 (d,

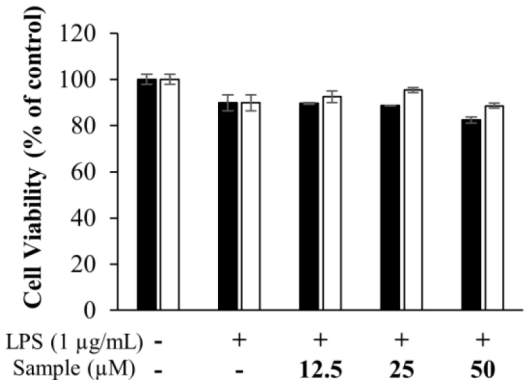

Fig. 3 Effects of MYR and MYR-HA on cell viability in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cell viability was assessed in cells that were not stimulated (-) or stimulated (+) with LPS (1 µg/mL) in the presence of test compounds for 24 h

J =3.2 Hz, 1H, C6), 2.44 (s, 3H), 2.37 (s, 3H), 2.35 (s, 3H), 2.34 (s, 3H), 2.33 (s, 6H)

Such is how we were able to successfully produce an acetyl derivative of myricetin through acetylation reaction which has six hydroxyl groups of myricetin substituted with an acetyl group, 3,3',4',5,5',7-myricetin hexaacetate (MYR-HA).

Cytotoxicity assessment of myricetin and myricetin hexaacetate on RAW 264.7 macrophage cells

Toxicological assessment of a compound is performed in order to evaluate its effect on normal physiology of cells. For a compound to be considered a drug candidate, not only should it have therapeutic effects, but it should also not cause damage to healthy or normal cells. Therefore, cell damage or cytotoxicity assay is also required to evaluate the overall efficacy of a compound for therapeutic use. In this study, cytotoxicity assessment of myricetin (MYR) and myricetin hexaacetate (MYR-HA) was performed on RAW 264.7 macrophage cell line using the MTT assay. The ability of viable cells with active metabolism to convert MTT into a purple-colored compound called formazan, helps in qualitative measurement of cell toxicity of compound by measuring its absorption intensity i.e., the more the intensity of purple colored formazan obtained the more viable the cells are and the less toxic the tested compound is to the cells and vice versa. In Fig. 3, we have represented the percentage of absorption intensity obtained from the cells treated with MYR and MYR-HA at different concentrations ranging from 12.5 to 100 µM, with respect to the no-treatment control. The cell viability remained above 80% for concentration up to 50 µM whereas at 100 µM concentration the viability decreased to about 70% for both of the compounds (data not shown). In this regard, MYR and its derivative, MYR-HA, had similar toxicological effect in LPS-induced RAW 264.7 cells. And since 100 µM concentration showed toxic effect on the cell, further tests were performed only for concentration of 12.5, 25, and 50 µM.

Effect of myricetin and myricetin hexaacetate in production of pro-inflammatory mediators like nitric oxide (NO) and Prostaglandin E_2 (PGE_2)

The pro-inflammatory mediators, such as NO and PGE_2 produced by macrophage cells in response to pathogen-associated molecular patterns (PAMPs) like LPS, are one of the key indicators of the inflammatory response. Determination of the level of these molecules in the system will help elucidate whether the inflammatory response is progressing or not. An effective anti-inflammatory compound possesses the ability to inhibit the production of these mediators, repressing the progression of the inflammatory cascade response. Thus, the assessment of the ability of MYR and MYR-HA to inhibit the production of NO and PGE_2 in LPS-induced RAW264.7 cells took place.

Figure 4A shows the percentage of nitric oxide produced in the cells induced by LPS and treated with MYR and MYR-HA with respect to LPS-treated control. We can see that nitric oxide production decreased in a dose-dependent manner for both MYR and MYR-HA. However, in comparison, treatment of MYR-HA at 50 µM concentration showed significantly lowered nitric oxide production than MYR at the same concentration. This may imply that MYR-HA has an increased inhibitory effect on nitric oxide production than myricetin does. In addition, a similar trend was observed in the case of PGE_2 production, Fig. 4B. In this case, a significant difference in PGE_2 production can be seen between MYR and MYR-HA starting from the dose of 12.5 µM, indicating the effectiveness of MYR-HA even at lower doses than MYR in inhibiting the production of PGE_2.

Impact of myricetin and myricetin hexaacetate in iNOS and COX-2 protein expression

It is well known that the production of nitric oxide and prostaglandin E_2 are mediated by iNOS and COX-2 and most glucocorticoids and NSAIDs possess their anti-inflammatory activity through inhibition of COX-2 expression. To elucidate whether or not MYR and MYR-HA work through similar mechanism of action, further tests were performed to check for their inhibitory role against iNOS and COX-2 protein expression. Western blot analysis in Fig. 5 shows the expression of COX-2 (A) and iNOS (B) protein in LPS induced RAW 264.7 cells treated with MYR and MYR-HA with varying concentration. Consistent with the result of NO and PGE_2 production, MYR and MYR-HA treatment resulted in dose dependent decrease in iNOS and COX-2 protein expression implying the inhibitory effect of NO and PGE_2 production was due to the downregulation of iNOS and COX-2 protein expression. An important feature of the result, again corresponding to NO and PGE_2 production, was that MYR-HA showed a greater inhibitory effect in the expression of both proteins than compared to myricetin, i.e., MYR HA was a more potent inhibitor of iNOS, and COX-2 protein expression.

Moreover, we found that the inhibitory effect in the production of COX-2 and PGE_2 were slightly different similar to other reports

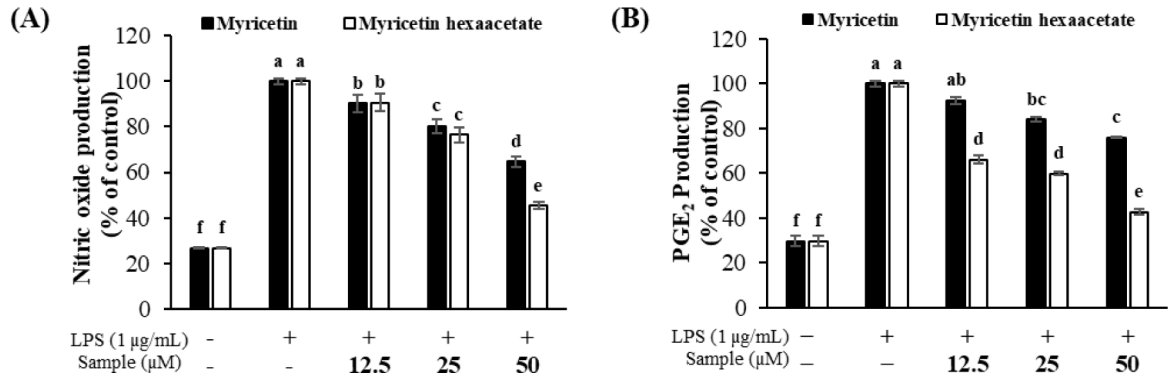

Fig. 4 Effects of MYR and MYR-HA on nitric oxide (A) and Prostaglandin E_2 (PGE_2) (B) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Means not sharing a common superscript are significantly different (ANOVA, $p < 0.01$). Values are mean ± SD. For post-hoc analysis, Tukey-Kramer test was performed

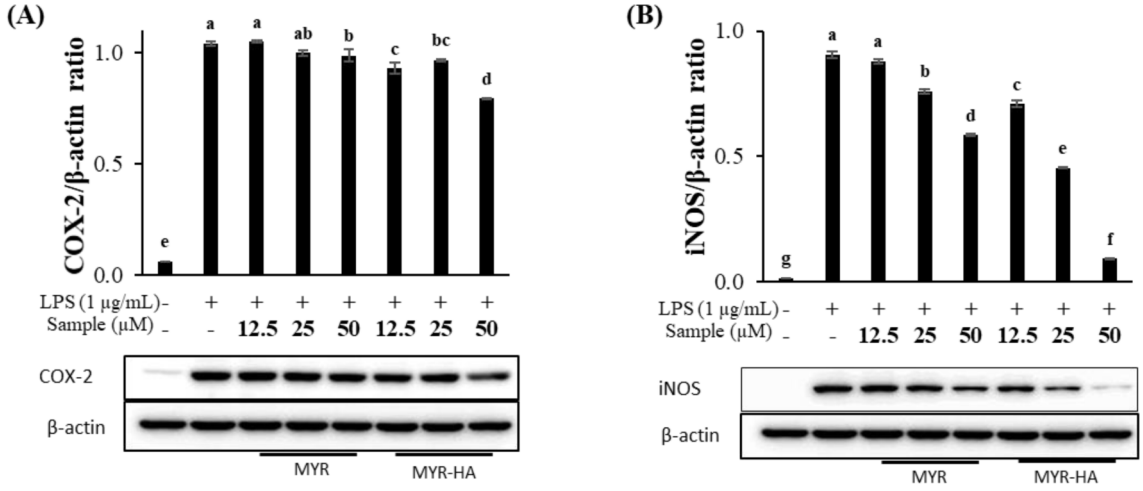

Fig. 5 Effects of MYR and MYR-HA on the protein expression levels of COX-2 (A) and iNOS (B) in LPS-stimulated RAW 264.7 cells determined by western blot and quantified using ImageJ. Means not sharing a common superscript are significantly different (ANOVA, $p < 0.01$). Values are mean ± SD. For post-hoc analysis, Tukey-Kramer test was per-formed

[48,49]. Since COX-2 is an enzyme, its activity may be regulated at distinct phases such as transcription (mRNA expression), translation (protein expression) as well as enzyme level. We believe that the difference in our result was because of cumulative effect of both expression and enzyme inhibition of COX-2 resulting in greater impact on suppression of PGE_2.

Effect of myricetin and myricetin hexaacetate in production of pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α

The pro-inflammatory cytokines play a regulatory function by the activation of immune cells to produce various pro-inflammatory mediators. This transactivating nature of cytokines makes them an important target for anti-inflammatory agents to alleviate the cascade response. Therapeutics targeted to inhibit TNFα have been successful in the treatment of rheumatoid arthritis, psoriatic arthritis, inflammatory bowel syndrome, psoriasis, etc. Thus, it was necessary to check the efficacy of MYR-HA in decreasing the production of TNFα, IL-6 and IL-1β in LPS-treated RAW 264.7 macrophages. It is clearly observed from Fig. 6 that the treatment of macrophages with MYR and MYR-HA resulted in decrement in the production of TNFα, IL-6 and IL-1β. Higher inhibitory effect in the production was seen when MYR-HA was introduced to the LPS-stimulated macrophage cells. Out of the three cytokines tested, production of TNFα and IL-6 was greatly compromised in the cells treated with myricetin hexaacetate, implying its higher potency of inhibition in comparison to myricetin.

The current need for anti-inflammatory agents with a lower risk of side effects and better cost-effectiveness has driven studies and research towards enhancing and optimizing the pharmacological property of natural products that have a pre-established history of uses in traditional medicine. This approach of using chemical structures of natural product as the therapeutic template while combining the principles of combinatorial chemistry has been the basis for the production of most pharmaceuticals in the market.

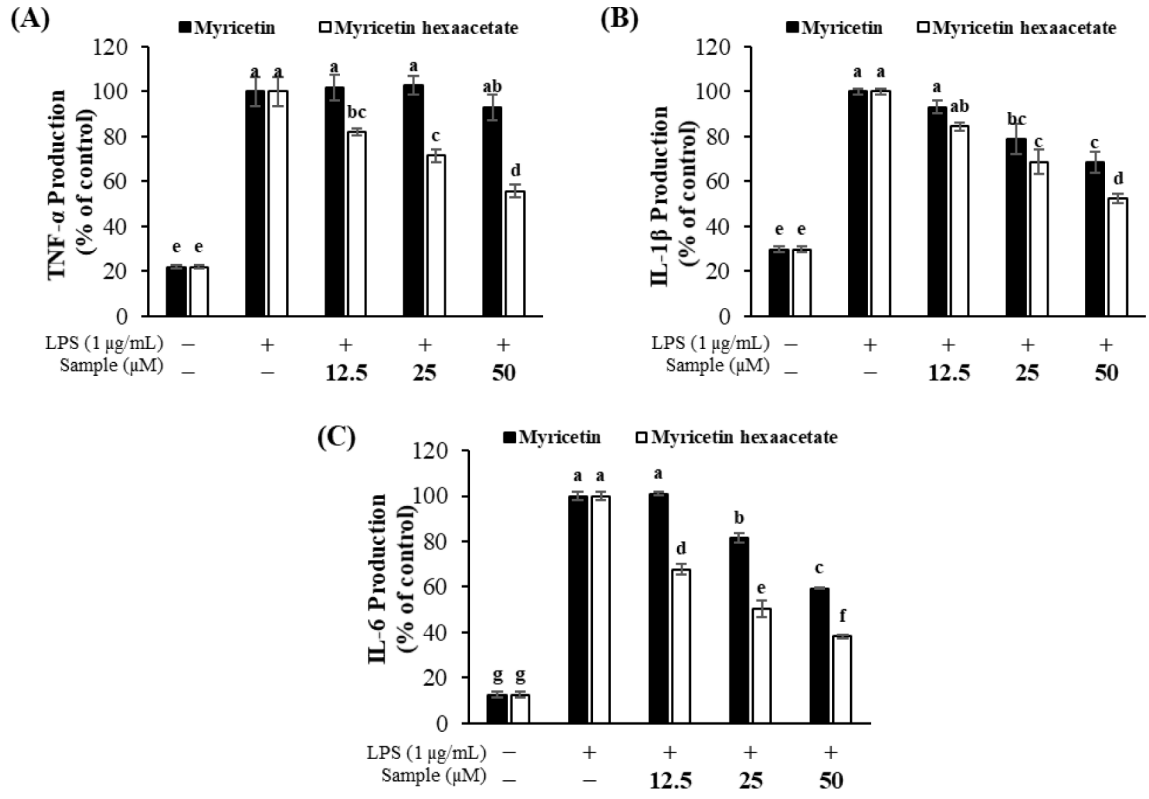

Fig. 6 Effect of MYR and MYR-HA on TNF-α (A), IL-1β (B) and IL-6 (C) production in LPS -stimulated RAW 264.7 cells. Cells were stimulated with 1 μg/mL of LPS only or with LPS along with varying concentrations (50, 100 and 200 μM) of test compounds for 24 h. Their production was determined by ELISA. Means not sharing a common superscript are significantly different (ANOVA, p <0.01). Values are mean ± SD. For post-hoc analysis, Tukey-Kramer test was performed

This study was conducted for the same purpose, employing chemical modification (hydroxyl group substitution with an acetyl group) of a plant flavonoid myricetin for a potential enhancement of its anti-inflammatory properties. Applying green chemistry principle, myricetin derivative, myricetin hexaacetate (MYR-HA) was successfully obtained and correctly characterized, according to the data described in the literatures. Comparative analysis of anti-inflammatory activities of myricetin (MYR) and myricetin hexaacetate (MYR-HA) showed that MYR-HA was a more potent inhibitor of COX-2 and iNOS protein expression which was the major contributing factor in lowering the production of nitric oxide, PGE_2, IL-6, IL-1β and TNFα in LPS-induced RAW264.7 macrophage cells.

The major limitation of plant flavonoids for therapeutic use comes down to its low bioavailability provided by its poor water solubility, higher rate of metabolism and enzymatic degradation. Understanding the metabolic fate of compounds can assist the improvement of their bioavailability through the tailoring of their chemical structures in a way so they will not easily undergo degradation. According to an article on bioavailability and metabolism of flavonoids [50], flavonoid metabolism in the body involves conjugation of glucuronide, sulfate and methyl or acyl groups which facilitate their subsequent degradation and excretion from the body, ultimately decreasing their retention time in the plasma. Interaction between these types of conjugating and hydrolyzing enzymes and flavonoids promoted by hydrogen bonds between the hydroxyl groups of both structures led to their subsequent decrease in bioavailability. Such phenomenon provides a reason for the presence of methyl groups in some flavonoids retarding their excretion rate, providing better bioavailability [51]. Even though we could not perform in vivo experiments to actually test if acetyl group substitution resulted in increased bioavailability of myricetin, it could be considered for future works.

Aside from that, reports have been made that the presence of acetyl side chains in flavonoids enhances its lipophilicity so that it has better interactions with cell membranes and is easily taken up inside the cells [52-54]. The XLOGP3 and XLOGP3-AA value of myricetin and myricetin hexaacetate are 1.2 and 1.9 respectively [44,45], meaning that myricetin hexaacetate is more lipophilic than Myricetin, which could be one contributing factor for the increased anti-inflammatory effect of MYR-HA compared to MYR.

In a study that sought to elucidate the underlying mechanism of anti-inflammatory activity of chrysin derivatives, *in vitro* enzyme activity study of the key pro-inflammatory enzymes led to the discovery that only the acetyl derivative, 5,7-diacetyl chrysin (Ch-

4) had a direct inhibitory effect in the COX-2 activity. The discovery was further supported by docking analysis of COX-2 with Ch-4 which highlighted the hydrogen bonding between the oxygen of the ketone group at the 7-position of Ch-4 and the hydroxyl group ofTyr355 [37]. Similar studies could be conducted to check if MYR and MYR-HA also inhibit the enzyme activity of iNOS and COX-2. Another study [55] conducted in mouse macrophages highlights the role of flavonoids as activators of peroxisome proliferator-activated receptor-γ in suppression of inducible cyclooxygenase and nitric oxide synthase. In addition to this, influence of flavonoids on upstream signaling pathway has been linked to their anti-inflammatory properties.

When PAMPs, from pathogens, like LPS, is detected through pattern recognition receptors (PRRs) present in the membrane surface/endosomes or cytoplasm of cells like mast cells and macrophages, it triggers signaling pathways responsible for inducing pro-inflammatory response [56]. In the case of macrophages, expressing the Toll-like receptors (TLRs) family of PRRs, LPS recognition is mediated through TLR4 whose engagement results in activation of the signaling pathways including (i) nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), (ii) mitogen-activated protein kinases (MAPKs), and (iii) IFN regulatory factors. Studying if the increased ability to suppress the production of pro-inflammatory mediators of MYR-HA was due to its greater influence on these signaling pathways than MYR, will provide in-depth understanding of the role of acetyl moieties in enhancing anti-inflammatory activity. For that purpose, future study will be followed by a comparative study of MYR and MYR-HA involving their influence on inflammatory signaling pathways.

This study allowed for a successful synthesis of an acetyl derivative of myricetin, 3,3',4',5,5',7-myricetin hexaacetate, with improved *in vitro* anti-inflammatory activity, supported by its higher inhibitory effect against production of pro-inflammatory mediators and expression of iNOS and COX-2 protein expression compared to myricetin. This approach could be a step towards designing more potent anti-inflammatory agents for therapeutical use of flavonoids.

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Appendix. Supplementary data Supplementary data to this article can be found at

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