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Synergistic anti-inflammatory activities of a new flavone and other flavonoids from *Tephrosia hildebrandtii* vatke

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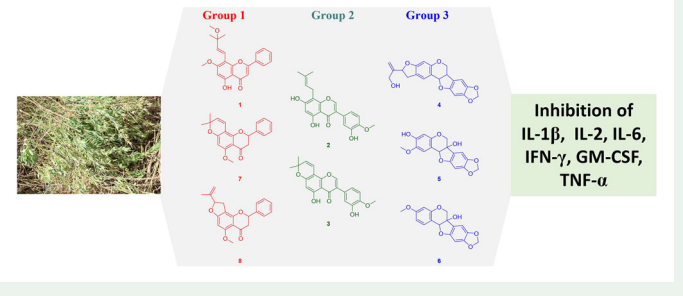
ABSTRACT

A new flavone, named hildeflavone (**1**) along with 7 other known flavonoids were isolated from the aerial parts of *Tephrosia hildebrandtii* Vatke. Their characterisation was based on NMR and MS data analysis. The anti-inflammatory properties of the crude extract, isolated compounds and combination of the compounds were investigated in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs). Treatment of the LPS-stimulated PBMCs with the isolated flavonoids at a concentration of 100 μM significantly reduced the production of interleukins (IL-1β, IL-2 and IL-6), interferon-gamma (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-CSF) and tumour necrosis factor-alpha (TNF-α). It was also found that the combination of a flavone and flavanones exhibited remarkable synergistic anti-inflammatory effects on the production of the cytokines.



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1. Introduction

Tephrosia hildebrandtii Vatke (Fabaceae) is a tropical perennial herb that is widely distributed in the shrublands and grasslands of Ethiopia, Kenya and Tanzania (Beentje 1998; Agnew 2013). Previous phytochemical investigations on the roots of *T. hildebrandtii* Vatke resulted in the isolation of various classes of flavonoids such as pterocarpans (Lwande et al. 1987), flavones (Lwande et al. 1986), flavans (Monache et al. 1986) and flavanones (Monache et al. 1986). Flavonoids have diverse biological activities including anti-inflammatory activity (Touqeer et al. 2013; Leyva-López et al. 2016).

Inflammation is an immune response to infections or irritation (Hofseth 2011) and is controlled by key mediators such as prostaglandins and cytokines (Abdulkhaleq et al. 2018). However, it has been suggested that unregulated inflammation results in the onset and development of diseases including cancer, obesity, diabetes, atherosclerosis and arthritis (Sugimoto et al. 2016; Chen et al. 2017; Muniandy et al. 2018; Yiu et al. 2018). The classical anti-inflammatory drugs target prostaglandin biosynthesis (Dhikav et al. 2003; Dinarello 2010). However, these drugs are associated with adverse effects like gastrointestinal bleeding and peptic ulcer (Lichtenstein et al. 1995; Drini 2017; Wong 2019). Anti-inflammatory drugs targeting the cytokines such as interleukins (IL-1β, IL-2 and IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) have been pursued as alternatives in the treatment of inflammation and its associated diseases (Aggarwal et al. 2009; Leyva-López et al. 2016).

Numerous studies have described the anti-inflammatory properties of flavonoids that decrease the production of pro-inflammatory cytokines (García-Lafuente et al. 2009; Leyva-López et al. 2016; Chen et al. 2019). Although the phytochemical investigations of *Tephrosia hildebrandtii* Vatke have been previously reported, the anti-inflammatory properties of the compounds have not been explored. This paper describes the isolation and structural elucidation of a new flavone along with 7 other known flavonoids as well as their anti-inflammatory effects on the production of cytokines (IL-1β, IL-2, IL-6, IFN-γ, GM-CSF and TNF-α) from lipopolysaccharide-induced peripheral blood mononuclear cells (PBMCs). The synergistic anti-inflammatory effects of the flavonoids were also investigated.

2. Results and discussion

The dichloromethane-methanol (1:1) extract of the aerial parts of *T. hildebrandtii* was subjected to silica gel and Sephadex LH-20 column chromatography followed by preparative reverse phase HPLC to give a new flavone along with 7 known compounds (Figure 1). By comparison of the NMR data with literature, the known compounds were identified as 5,7,3′-trihydroxy-4′-methoxy-8-prenylisoflavone (**2**) (Souza et al., 2017), 5,3′-dihdroxy-4′-methoxy-2″,2″-dimethylpyrano[5″,6″:8,7]isoflavone (**3**) (Souza et al., 2013), 4′-hydroxyemoroidocarpan (**4**) (Harinantenaina et al., 2010), hildecarpin (**5**) (Lwande et al., 1985), pisatin (**6**) (Ingham and Markham, 1980), pongachin (**7**) (Andrei et al., 2000) and emoroidenone (**8**) (Machocho et al., 1995).

Compound **1** was isolated as a yellow paste with UV λ_{max} at 228 and 268 nm. Its molecular formula was established as $C_{22}H_{22}O_5$ from the HRESIMS molecular ion

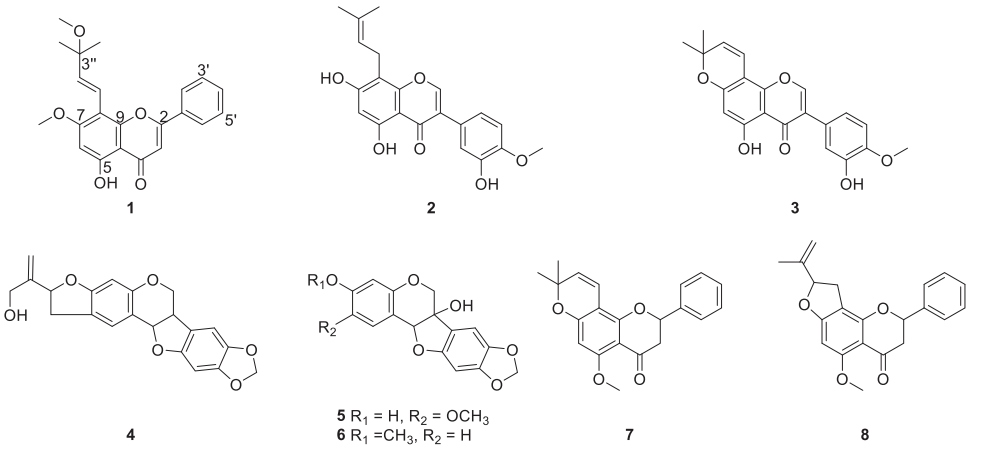

Figure 1. Structure of isolated compounds.

$[M+H]^+$ at *m/z* 367.1540 (calcd 367.1540) and $[M+Na]^+$ at *m/z* 389.1361 (calcd 389.1359) together with NMR data (Table S1 and Figure S1-8, Supplementary Material). The presence of a 5-hydroxyflavone skeleton was evident from the NMR data (δ_H 6.86 (*s*, H-3) and 13.26 (5-OH); δ_C 165.2 (C-2), 105.6 (C-3), 183.8 (C-4)) (Agrawal 1989). A detailed analysis of its NMR data showed a close similarity with *trans*-tephrostachin (Khalid and Waterman 1981) and (*E*)−5-hydroxytephrostachin (Atilaw et al. 2017), Table S1. The key difference is the presence of a 3′′-methoxyprenyl (δ_H 3.23 (δ_C 50.5)) substituent in compound 1 instead of a 3′′-hydroxyprenyl. This is further supported by the loss of CH_2O from the molecular ion to give *m/z* 337 as observed in MS^2 (Figure S2, Supplementary Material). The prenyl substituent was established as *trans*-oriented-3′′-methoxy-3′′-methylbut-1′′-enyl based on the AX spin system of *trans*-olefinic protons at δ_H 6.54 and 6.80 (*d,* 1H, $J = 16.7$ Hz). Three sets of mutually coupled protons resonating at δ_H 8.11 (*m*), δ_H 7.63 (*m*) and δ_H 7.65 (*m*) were attributed to an unsubstituted flavone B-ring. The methoxy proton at δ_H 4.02 showed HMBC correlation with δ_C 164.3 allowing its placement at C-7. Attachment of the *trans*-oriented-3′′-methoxy-3′′-methylbut-1′′-enyl moiety to C-8 (δ_C 106.3) was based on HMBC correlation of H-1′′ with C-9 (δ_C 155.0), C-8 (δ_C 106.3) and C-7 (δ_C 164.3). Therefore, this new compound was characterized as 5-hydroxy-7-methoxy-8-(*E*-3-methoxy-3-methylbut-1-enyl)flavone for which the trivial name hildeflavone was suggested.

The anti-inflammatory activities of the crude extract of *T. hildebrandtii*, as well as the isolated compounds, were evaluated by measuring the levels of IL-1β, IL-2, IL-6, IFN-γ, GM-CSF and TNF-α released from LPS stimulated PBMCs. In this study, the anti-inflammatory drug, ibuprofen was used as a positive control. As shown in Figure S9, the cells treated with LPS showed an increase in the production of the cytokines compared to the untreated control cells. It was also noted that all the cytokines were reduced in the presence of ibuprofen except for IL-1β which was increased in comparison to the LPS control. Similar studies have reported a reduction in the production of cytokines (Sayın et al. 2013; Bessler et al. 2017) while others have found an increase in secretion of cytokines induced by ibuprofen (Sirota et al. 2001; Lee and Chuang

2010). The crude extract suppressed the release of IFN-γ, GM-CSF, and TNF-α, but it did not have any effect on IL-6. However, the crude extract stimulated the production of IL-1β and IL-2 to 139% and 106%, respectively, as compared to the LPS control (Table S2, Supplementary Material).

As shown in Figure S9 and Table S2, all the compounds decreased the release of IL-2, IL-6, IFN-γ, GM-CSF, and TNF-α. The strongest inhibition of IL-6 release was observed in compound **3**, an isoflavone that attenuated the production to 0.6% in compareison to LPS. Similarly, compound **3** attenuated the production of TNF-α to 0.4% in comparison to LPS. The pterocarpans, compounds **5** and **6**, exhibited the highest reduction in the production of IFN-γ to 2.7% and GM-CSF to 11.0%, respectively, as compared to LPS. Almost all the compounds showed strong inhibition for the IL-2 production with the exception of compounds **3**, **7** and **8**. While compounds **1**, **3**, **4**, **6** and **8** suppressed the production of IL-1β compounds **2**, **5** and **7** stimulated its production. The strongest inhibition was exhibited by compound **6** which decreased IL-1β production to 20.2% in comparison to LPS control.

The synergistic anti-inflammatory effects of the different classes of flavonoids were also investigated. The flavonoids were combined and grouped as follows; group 1 (flavone and flavanones; **1**, **7** and **8**), group 2 (isoflavones; **2** and **3**) and group 3 (pterocarpans; **4**, **5** and **6**). All the groups reduced the production of IL-1β, IL-2, IFN-γ, GM-CSF and TNF-α (Figure S10 and Table S2, Supplementary Material) except group 2 which provoked the production of GM-CSF by 184% compared to LPS control. Group 1 provoked the production of IL-6 to 129% while groups 2 and 3 did not affect its productions. Group 1 exhibited significantly synergistic effects. The increased production of GM-CSF by group 2 is attributed to antagonist effects (Zhang et al. 2019)

3. Experimental

3.1. General experimental procedures

Column chromatography was performed using Merck silica gel 60 (70-230 mesh) and Sephadex LH-20. Semi-preparative HPLC was performed on Shimadzu LC-20AP system equipped with DGU 20A5R degassing unit, SPD-M20A detector, SIL-20ACHT autosampler and a Phenomenex, 10 μm C18, 110 Å pore size column (250 × 10 mm) using Labsolution software system. The separation was achieved using MeOH-H_2O (0.1% formic acid) solvent system. NMR spectra were recorded on a Bruker Advance III spectrometer. IR spectra were measured on a Bruker Tensor 27 FT-IR spectrometer (cricket, Harrick Scientific). High-resolution electrospray ionization mass spectrometry (ESI-HRMS) was done on an LTQ orbitrap spectrometer (Thermo Scientific, USA) with a HESI-II source. The spectrometer was equipped with an Agilent 1200 HPLC system (Santa Clara, USA) with pump, PDA detector and autosampler. All MS^n experiments were performed with collision-induced dissociation at 35 arbitrary units.

3.2. Plant material

T. hildebrandtii was collected from Thika (S01°03′19.2″, E037°14′10.4″), Kenya in February 2017. The plant was authenticated by Mr. Patrick Mutiso of the University

Herbarium, School of Biological Science, University of Nairobi, where a voucher specimen, ORO-2017/07 was deposited.

3.3. Extraction and isolation

The air-dried and ground aerial parts of *T. hildebrandtii* (1.0 kg) was extracted three times using dichloromethane-methanol (1:1, 3 L) at room temperature to yield 82.4 g of crude extract. A portion of the extract (42.9 g) was subjected to column chromatography on silica gel eluting with cyclohexane-ethyl acetate in increasing polarity. The fraction that eluted with 30% ethyl acetate (EtOAc) in cyclohexane was purified on Sephadex LH-20 using dichloromethane-methanol (1:1) followed by preparative HPLC (20:80, MeOH/H_2O-100% MeOH gradient elution for 50 min with flow rate of 4 mL/min to give **2**, **3**, **4**, **7** and **8**. Similarly, the 50% EtOAc in cyclohexane fraction was purified using Sephadex LH-20 followed Prep.-HPLC to give **1**, **5** and **6**.

5-Hydroxy-7-methoxy-8-(E-3′′-methoxy-3′′-methylbut-1′′-enyl)flavone, hildeflavone (**1**). Yellow paste, UV (methanol), λ_{max} 228 and 268 nm. IR (neat) ν_{max}: 3071, 1665, and 1578 cm^{-1}. HRESIMS: $[M+H]^+$ *m/z* 367.1538 (calcd 367.1540) and $[M+Na]^+$ at *m/z* 389.1361 (calcd 389.1359). ^1H and ^{13}C NMR see Table S1.

3.4. Biological study

3.4.1. Test solution and concentration

The crude extract, isolated flavonoids and ibuprofen were dissolved in dimethyl sulfoxide (DMSO). All samples were tested at a concentration of 100 μM except for the crude which was tested at a concentration of 100 μg/mL. The final DMSO concentration was 0.5% in all samples. The positive control ibuprofen was also used at 100 μM and all samples were co-incubated with 10 μg/mL LPS.

3.4.2. Assay procedure

The anti-inflammatory tests were conducted by Pharmacelsus, Saarbrücken, Germany. The inflammatory cytokines were assessed using Immunospot (ePBMC®-Uncharacterized Cryopreserved Human PBMC) purchased from Cellular Technologies Limited (C.T.L., Ohio, USA, Figure S11 Supplementary Material) (http://www.immunospot.com/CatalogueRetrieve.aspx?ProductID=10537096&A=SearchResult&SearchID=10324581&ObjectID=10537096&ObjectType=27). Blood samples were obtained from 3 healthy donors (ages 34, 38 and 38 years). Human cryopreserved PBMC were thawed according to the manufacturer's instructions and cells from different donors were pooled. Cells were washed, resuspended in RPMI 1640 containing 10% FBS, plated in 96-well round-bottom plates at 100,000 PBMC/cell and exposed to the test items at different concentrations. The dilution of test items was done in a 96-well plate and transferred to the PBMCs containing wells. The cells were incubated for 24 hours at 37 °C and 5% CO_2. The plates were then centrifuged for 3 min at 350 gyrations without brake and the cell-free supernatant was collected and forwarded for cytokine bead-array assay. The bead-array assay was conducted according to the manufacturer's instructions and read in a MagPix reader. For the dose-response

relationship, absolute concentrations were calculated by the MagPix software using two separate calibration series as provided by the manufacturer. As a negative control, cells were incubated only with cell culture medium. As a positive control for inflammation, cells were incubated with 10 μg/mL LPS and as a positive control for anti-inflammation, cells were co-incubated with 10 μg/mL LPS and 100 μM ibuprofen.

4. Conclusion

In summary, eight flavonoids that inhibited the production of IL-1β, IL-2, IL-6, IFN-γ, GM-CSF and TNF-α in LPS stimulated PMBCs were isolated from *T. hildebrandtii*. Significant inhibitions were exhibited by compound **3** on IL-6 and TNF-α, compound **6** on IL-1β and GM-CSF secretion and compound **5** on IFN-γ production. In the synergistic study, a combination of a flavone and flavanones (group 1) exhibited remarkable anti-inflammatory effects on the production of IL-1β, IL-2, IFN-γ, GM-CSF and TNF-α. The inhibitions of these cytokines may indicate that these flavonoids may serve as potential anti-inflammatory agents.

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Disclosure statement

No potential conflict of interest was reported by the authors

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