Phytochemical Constituents of *Aquilaria malaccensis* Leaf Extract and Their Anti-Inflammatory Activity against LPS/IFN-γ-Stimulated RAW 264.7 Cell Line

Manar A. Eissa,* Yumi Z. H-Y. Hashim, Saripah S. S. Abdul Azziz, Hamzah Mohd. Salleh, Muhammad Lokman Md. Isa, Nor Malia Abd Warif, Fauziah Abdullah, Eman Ramadan, and Dina M. El-Kersh

**ABSTRACT:** This study aims to identify the major phytochemical constituents in *Aquilaria malaccensis* (Thymelaeaceae) ethanolic leaf extract (ALEX-M) and elucidate their ability to suppress nitric oxide (NO) production from a murine macrophage-like cell line (RAW 264.7) stimulated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ). Dichloromethane (DCM) and ethyl acetate (EtOAc) fractions of ALEX-M were subjected to column chromatography. Eight known compounds were isolated for the first time from this species. Compounds were identified using spectroscopic techniques (IR, UV, HRESIMS, and 1D and 2D NMR). Anti-inflammatory activity of both extract and isolated compounds were investigated in vitro. The fractions offered the isolation of epifriedelanol (1), 5-hydroxy-7,4′-dimethoxyflavone (2), luteolin-7,3′,4′-trimethyl ether (3), luteolin-7,4′-dimethyl ether (4), acacetin (5), aquilarinenside E (6), iriflophenone-2-O-α-L-rhamnopyranoside (7), and iriflophenone-3-C-β-glucoside (8). The findings suggest the pharmacological potential of the crude extract (ALEX-M) and its isolates as natural anti-inflammatory agents, capable of suppressing NO production in RAW 264.7 cells stimulated by LPS/IFN-γ.

1. **INTRODUCTION**

Inflammation has become a major global health issue in recent years. Although inflammation is a normal physiological body response against a noxious stimulus to initiate tissue homeostasis,1,2 it has been recognized among the causal pathophysiology of several chronic progressive diseases including cancer,3 rheumatoid arthritis, atherosclerosis, and diabetes.3 Synthetic medications used for treatment of inflammation such as the nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids may cause intolerable gastrointestinal, hepatic, and cardiovascular side effects.1 Plant-derived products have become safe alternatives for alleviation of inflammation through interference with inflammatory pathways.7

*Aquilaria malaccensis* (family Thymelaeaceae) was used traditionally as a tonic during pregnancy and after giving birth, as a carminative and to control heart palpitations.6 It was also prescribed to alleviate jaundice, ulcer, edema, skin diseases, fever, and body aches.6,7 In addition, the plant was involved in the treatment of central nervous system diseases such as epilepsy and schizophrenia as well as fumigation therapy for sterilization and personal hygiene.8 Previous scientific research on *A. malaccensis* reported its potential pharmacological activities including anticancer,9,10 antimicrobial,10−13 antioxidant,11,13−16 anti-inflammatory,17,18 immunomodulatory,19 antidiabetic,13,20 embryogenesis,21,22 and anti-trypanosomal activity.23

Research on the phytochemical constituents of *Aquilaria* species reported the presence of a number of compounds such as flavonoids, terpenoids, phenolic acids, benzophenones, xanthones, sesquiterpenes, chromones, fatty acids, phytosterols, and lignans.24−26

Previous anti-inflammatory studies have been conducted on *Aquilaria* species. It has been reported that the ethanolic
extract of *Aquilaria sinensis* leaves demonstrated inhibitory effect on NO levels in LPS-induced peritoneal macrophages with an IC_{50} = 80.4 μg/mL. In addition, the flavonoid Aquisiflavoside, which was isolated from *Aquilaria sinensis* leaves, was recognized as a nitric oxide inhibitor in LPS-induced RAW 264.7 cells with an IC_{50} = 39.95 μM. Chen et al. reported the ability of different Aquilarones isolated from the resinous agarwood of *Aquilaria sinensis* to inhibit NO production in LPS-stimulated RAW 264.7 with IC_{50} ranging between 5.95 μM and 22.26 μM. Similarly, other chromones isolated from *Aquilaria sinensis* agarwood demonstrated anti-inflammatory activity against LPS-induced NO production in the RAW 264.7 cell line as reported by several researchers.

Herein, as an extension of the phytochemical profiling studies carried out on *A. malaccensis* species and their pharmacological activities, *A. malaccensis* ethanol leaf extract (ALEX-M) and its structurally identified compounds were investigated for their potential to inhibit NO production from LPS/IFN-γ-stimulated RAW 264.7 in the present study.

2. RESULTS AND DISCUSSION

2.1. Structural Characterization. From the leaves of *A. malaccensis*, eight known compounds (1–8) were obtained and structurally characterized (Figure 1). Through comparing the NMR spectroscopic data of the isolated compounds with literature values, the compounds were recognized as epifriedelanol (1), 5-hydroxy-7,4′-dimethoxyflavone (2), luteolin-7,3′,4′-trimethyl ether (3), luteolin 7,4′-dimethyl ether (4), acacetin (5), aquilarinenside E (6), iriplphenone-2-O-α-L-rhamnopyranoside (7), and Iriplphenone 3-C-β-glucoside (8). The above-mentioned compounds were obtained from the species of *A. malaccensis* for the first time.

Figure 1. Structures of the compounds isolated from *A. malaccensis* leaves.
Compound 1 appeared as white needle crystals, and its molecular formula was assigned as C_{30}H_{35}O with [M + H]^+ ion peak at m/z 429.1163 on HRESIMS (positive mode). The IR spectrum of compound 1 showed absorptions for OH (3467 cm\(^{-1}\)) functional group. The UV spectrum demonstrated maximal absorption at 250 nm. The \(^1\)H and \(^13\)C NMR data were summarized in Table 1. Through interpretation of 1D NMR data of compound 1 and relating its spectroscopic data to literature, compound 1 was identified as epifriedelanol.\(^{33}\)

### Table 1. \(^1\)H (CDCl\(_3\), 500 MHz) and \(^13\)C (CDCl\(_3\), 125 MHz) NMR Data of Isolated Terpenoid (Compound 1)

| position | \(\delta^1\)H (multiplicity, \(J\) in Hz) | \(\delta^13\)C | position | \(\delta^1\)H (multiplicity, \(J\) in Hz) | \(\delta^13\)C |
|----------|---------------------------------|--------------|----------|---------------------------------|--------------|
| 1        | –                               | –            | 2        | 16.0                            | 16            |
| 2        | 35.5                            | –            | 3        | 73.0                            | 18            |
| 3        | 3.71 (br d, \(J = 2.9\))        | –            | 4        | 49.3                            | 19            |
| 5        | 37.3                            | –            | 6        | 41.9                            | 21            |
| 7        | 17.7                            | –            | 8        | 53.4                            | 23            |
| 9        | 38.6                            | 0.93 (s)     | 10       | 61.5                            | 0.83 (s)      |
| 11       | 35.4                            | 0.98 (s)     | 12       | 30.8                            | 0.96 (s)      |
| 13       | 39.9                            | 0.97 (s)     | 14       | 38.0                            | 0.92 (s)      |
| 15       | 33.0                            | 1.14 (s)     |          |                                 |              |

The molecular formula of compound 2 was assigned as C_{17}H_{14}O_{5} by HRESIMS at 297.1225 \([M + H]^+\) ion peak at m/z 325.0777 \([M – H]^–\). The UV spectrum appeared at 254 nm. The IR bands (3350 cm\(^{-1}\), 1652 cm\(^{-1}\)) of compound 3 are comparable to those of 2, indicating the presence of OH and C=O functional groups. The \(^1\)H and \(^13\)C NMR spectra of compound 3 are disclosed in Table 2. COSY, HMQC, and HMBC experiments revealed correlations as exposed in Figure 2. Thus, compound 3 was interpreted to be luteolin-7,3',4'-trimethyl ether based on the comparison of its spectroscopic data with literature.\(^{35}\)

Compound 4 was collected as a pale yellow amorphous powder. The study on HRESIMS of compound 4 recorded \([M – H]^–\) at m/z 313.0703 which agreed with molecular formula of C_{17}H_{14}O_{5}. The UV spectrum demonstrated absorption bands at 270 and 334 nm. Absorption bands of OH group at 3367 cm\(^{-1}\) and C=O group at 1653 cm\(^{-1}\) appeared on the IR spectrum. The \(^1\)H and \(^13\)C NMR spectra of compound 4 are displayed in Table 1, while the 2D NMR correlations are illustrated in Figure 2. Compound 4 was recognized as 5,3'-dihydroxy-7,4'-dimethoxyflavone (luteolin 7,4'-dimethyl ether) by analysis of its spectral data which were in good agreement with spectral data in literature.\(^{37}\)

The molecular formula of compound 5, isolated as yellow amorphous powder, was determined as C_{17}H_{14}O_{5} by HRESIMS and showed a significant molecular ion peak at \(m/z\) 283.0631 \([M – H]^–\). The UV absorption peaks appeared at 268 and 335 nm, meanwhile IR bands corresponding to OH (3252 cm\(^{-1}\)) and C=O (1663 cm\(^{-1}\)) are similar to compounds 2, 3, and 4 which suggests a flavonoid nucleus.\(^{44}\)

### Table 2. \(^1\)H (CDCl\(_3\), 500 MHz) and \(^13\)C (CDCl\(_3\), 125 MHz) NMR Data of the Isolated Flavonoids (Compounds 2, 3, 4, and 5)\(^{35}\)

| position | \(\delta^1\)H | \(\delta^13\)C | \(\delta^1\)H | \(\delta^13\)C | \(\delta^1\)H | \(\delta^13\)C | \(\delta^1\)H | \(\delta^13\)C |
|----------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 1        | –            | –            | 16.4         | –            | 16.4         | –            | 16.4         | –            |
| 2        | 6.55 (s)     | 6.59 (s)     | 104.6        | 6.55 (s)     | 104.6        | 6.79 (s)     | 103.0        |
| 3        | –            | –            | 182.7        | –            | 182.3        | –            | 182.6        |
| 4        | –            | –            | 162.3        | –            | 162.1        | –            | 162.3        |
| 5        | 6.34 (d, \(J = 1.7\)) | 6.37 (d, \(J = 2.3\)) | 98.1         | 6.35 (d, \(J = 1.7\)) | 98.3         | 6.36 (d, \(J = 1.7\)) | 98.0         |
| 6        | 165.6        | –            | 157.9        | –            | 157.6        | –            | 157.8        |
| 7        | 92.8         | 6.49 (d, \(J = 2.3\)) | 92.6         | 6.47 (d, \(J = 1.7\)) | 92.8         | 6.74 (d, \(J = 1.7\)) | 92.7         |
| 8        | 105.7        | –            | 132.3        | –            | 123.9        | –            | 123.5        |
| 9        | 7.83 (d, \(J = 8.6\)) | 7.34 (d, \(J = 2.3\)) | 108.6        | 7.31 (d, \(J = 1.7\)) | 108.5        | 7.94 (d, \(J = 8.6\)) | 128.6        |
| 1        | 149.2        | –            | 152.2        | –            | 149.4        | –            | 161.5        |
| 2        | 114.7        | –            | 6.98 (d, \(J = 8.6\)) | 111.1        | 7.02 (d, \(J = 8.6\)) | 115.1        | 6.94 (d, \(J = 9.2\)) | 116.0        |
| 3        | 7.83 (d, \(J = 8.6\)) | 7.53 (d, \(J = 2.3, 8.6\)) | 120.1        | 7.48 (d, \(J = 1.7, 8.6\)) | 120.9        | 7.94 (d, \(J = 8.6\)) | 128.6        |
| 4        | 3.96 (s)     | 56.1         | 3.98 (s)     | 55.8         | 3.98 (s)     | 56.3         | 3.87 (s)     | 56.1         |
| 5        | 3.87 (s)     | 56.0         | 3.88 (s)     | 56.1         | 3.86 (s)     | 56.0         | –            | –            |

*Assignments were confirmed by COSY, HMQC, and HMBC.*

1664 cm\(^{-1}\), respectively. The UV spectrum revealed maximal absorptions at 270 and 322 nm. The \(^1\)H and \(^13\)C NMR spectra of compound 2 are shown in Table 2. Figure 2 demonstrates \(^1\)H-\(^1\)H COSY, HMQCD, and HMBC correlations in compound 2. The spectral data for compound 2 is in well agreement with 5-hydroxy-7,4'-dimethoxyflavone reported in ref 34.

Compound 3, appearing as pale yellow amorphous powder, exhibited the molecular formula C_{17}H_{14}O_{5} as determined by HRESIMS at m/z 327.0777 \([M – H]^–\). The UV absorption appeared at 254 nm. The IR bands (3350 cm\(^{-1}\), 1652 cm\(^{-1}\)) of compound 3 are comparable to those of 2, indicating the presence of OH and C=O functional groups. The \(^1\)H and \(^13\)C NMR spectra of compound 3 are disclosed in Table 2. COSY, HMQD, and HMBC experiments revealed correlations as exposed in Figure 2. Thus, compound 3 was interpreted to be luteolin-7,3',4'-trimethyl ether based on the comparison of its spectroscopic data with literature.\(^{35}\)
Figure 2. COSY, HMBC, and HMQC correlations of compounds (a) 2, (b) 3, (c) 4, and (d) 5.

Table 3. $^1$H (CDCl$_3$, 500 MHz) and $^{13}$C (CDCl$_3$, 125 MHz) NMR Data of Isolated Benzophenones (Compounds 6, 7, and 8)$^a$

| position | compound 6 | compound 7 | compound 8 |
|----------|------------|------------|------------|
|          | $\delta^1$H | $\delta^{13}$C | $\delta^1$H | $\delta^{13}$C | $\delta^1$H | $\delta^{13}$C |
| 1        | –          | 109.6      | –          | 109.7      | –          | 104.7      |
| 2        | –          | 157.9      | –          | 158.4      | –          | 163.0      |
| 3        | 6.25 (d, $J = 1.7$) | 94.9 | 6.30 (s) | 95.6 | – | 96.4 |
| 4        | –          | 163.0      | –          | 163.0      | –          | 163.0      |
| 5        | 6.08 (d, $J = 1.7$) | 98.1 | 6.07 (s) | 98.0 | 5.96 (s) | 107.3 |
| 6        | –          | 160.4      | –          | 160.4      | –          | 161.5      |
| 7        | –          | 197.7      | –          | 197.7      | –          | 199.9      |
| 8        | –          | 132.9      | –          | 132.7      | –          | 133.3      |
| 9        | 7.60 (d, $J = 8.6$) | 132.8 | 7.62 (d, $J = 8.5$) | 132.9 | 7.61 (d, $J = 8.5$) | 133.0 |
| 10       | 6.82 (d, $J = 8.6$) | 116.3 | 6.82 (d, $J = 8.6$) | 116.2 | 6.78 (d, $J = 8.6$) | 115.6 |
| 1'       | –          | 163.6      | –          | 163.5      | –          | 160.9      |
| 2'       | 6.82 (d, $J = 8.6$) | 116.3 | 6.82 (d, $J = 8.6$) | 116.2 | 6.78 (d, $J = 8.6$) | 115.6 |
| 3'       | 7.60 (d, $J = 8.6$) | 132.8 | 7.62 (d, $J = 8.5$) | 132.9 | 7.61 (d, $J = 8.5$) | 133.0 |
| 4'       | 5.26 (d, $J = 1.7$) | 99.7 | 5.22 (s) | 100.6 | 4.87 (d, $J = 10.1$) | 76.6 |
| 5'       | 3.51 (m) | 71.6 | 3.44 (m) | 71.7 | 3.90 (m) | 73.7 |
| 6'       | 3.08 (m) | 69.8 | 3.14 (m) | 71.9 | 3.45 (m) | 80.0 |
| 7'       | 3.34 (m) | 75.1 | 3.29 (m) | 73.7 | 3.40 (m) | 71.6 |
| 8'       | 3.49 (m) | 68.8 | 3.42 (m) | 70.9 | 3.47 (m) | 82.7 |
| 9'       | 1.06 (d, $J = 6.3$) | 17.9 | 1.20 (d, $J = 6.3$) | 18.1 | 3.75 (dd, $J = 12.5.1$) | 62.6 |
| 10'      | –          | 172.7      | –          | 109.7      | –          | 104.7      |
| 5"       | 2.06 (s) | 21.1 | –          | 158.4      | –          | 163.0      |
| 6"       | –          | 109.6      | 6.30 (s) | 95.6 | –          | 96.4      |
| 6"-CH$_3$ | –          | 157.9      | –          | –          | –          | –          |
| 11"      | 6.25 (d, $J = 1.7$) | 94.9 | –          | –          | –          | –          |
| 2"-CH$_3$ | –          | 163.0      | –          | –          | –          | –          |

$^a$Assignments were confirmed by COSY.
Table 2 demonstrates the $^1$H and $^{13}$C NMR data of compound 5, while Figure 2 illustrates the 2D NMR correlations. Thus, compound 5 was identified as acacetin, and its spectroscopic data are identical with those obtained by. Assignments were confirmed by COSY, HMQC, and HMBC.

Compound 6 was collected as a white amorphous powder. An absorption band appeared at 285 nm on the UV spectrum. Compound 6 showed bands at 3275, 1709, and 1608 cm$^{-1}$ in IR spectrum. The molecular formula of the compound (C$_{21}$H$_{22}$O$_{10}$) was in agreement with the molecular ion peak revealed an [M + H]$^+$ peak at m/z 391.1002 [M + H]$^+$ analyzed using the HRESIMS technique. Based on the spectroscopic data of the compound presented in Table 3, which are similar to aquilarinenside E.40 and flower buds,41 compound 6 is identified as aquilarinenside E.

The HRESIMS spectrum of compound 7 revealed a significant peak at m/z 434.1250 [M + H]$^+$ indicated to the molecular formula of C$_{19}$H$_{20}$O$_{9}$, while the UV spectrum demonstrated a maximum absorption band at 325 nm. IR spectrum revealed absorption bands at 3326, 1635, and 1595 cm$^{-1}$ recognized as OH, C$\equiv$O, and aromatic groups absorptions, respectively. The compound was isolated as white needle crystals. The data of $^1$H and $^{13}$C NMR of compound 7 are presented in Table 3. The comparison of the spectral data obtained with literature recommended that 7 is iriophenone 2-o-α-L-rhamnopyranoside.

Compound 8, isolated as orange crystalline powder, had the molecular formula of C$_{19}$H$_{20}$O$_{9}$ according to the HRESIMS which revealed an [M + H]$^+$ peak at m/z 407.0997. The compound demonstrated strong absorption bands at 295 and 310 nm in the UV, while the IR spectrum exposed absorption bands of OH group (3296 cm$^{-1}$) and C$\equiv$O group (1607 cm$^{-1}$). The $^1$H and $^{13}$C NMR data of compound 8 are displayed in Table 3. Compound 8 was identified as iriophenone 3-C-β-glucoside.42,43

2.2. Cytotoxicity Assay. Before further studies were performed, the extract and the isolated compounds 1–8 were examined for their cytotoxicity against RAW 264.7 cell line using MTT assay. The consequence of the tested samples on the viability of RAW 264.7 is demonstrated in Figure 3. It was noted that the LPS/IFN-γ group showed no effect on cell viability. Safe concentrations of the extract and the compounds that are well tolerated by RAW 264.7 cell line and that showed cell viability above 85% were selected for the subsequent Griess anti-inflammatory assay, as it indicates the inhibition of NO levels is not a result of cell death but due to the inhibitory potential of NO production by the tested substance.46

2.3. Anti-Inflammatory Assay. Macrophages can promote the production and release of nitric oxide (NO) when stimulated by the bacterial endotoxin lipopolysaccharide (LPS).47 The subsidence of the NO levels reflects a potential anti-inflammatory effect. The cell lines were treated with the samples 1 h prior to stimulation with LPS/IFN-γ. After incubation for 12 h, the control group produced a basal level of NO of about 8.28 ± 0.43 μM in the culture medium, while the LPS/IFN-γ-treated group demonstrated a remarkable increase in NO of about 25.03 ± 1.66 μM. The extract and compounds groups demonstrated significant inhibition of NO production at different concentrations ranging between 200 and 6.25 μg/mL (P < 0.05) when compared to the LPS/IFN-γ-stimulated group. Compounds 1–5, and 7 were able to significantly reduce NO production (P < 0.05) at low concentration (6.25 μg/mL). However, compounds 1 and 2 displayed significant toxicity toward RAW 264.7 cells when the concentration reached 100 μg/mL, as demonstrated in Figure 3. The inhibitory concentration that reduces NO production by 50% (IC$_{50}$) of the crude extract and the compounds was calculated and is presented in Table 4. The extract showed a moderate but significant NO inhibitory effect compared to the LPS/IFN-γ-treated cells, while compounds 2, 4, 5, and 7 showed the uppermost suppression of NO in cells stimulated by LPS/IFN-γ, followed by compounds 1 and 3. Compounds 6 and 8 inhibited NO levels by 45.44 ± 4.22% and 40.58 ± 5.55%, respectively, at a dose of 200 μg/mL. Ibuprofen was used as a reference standard in the present study. Figure 4 demonstrates the inhibitory effect on NO production by the extract and the compounds. The results of MTT cell viability assay revealed that the inhibitory effect of the samples was not caused by cell death (viability >85%). This study is an initial discussion of the anti-inflammatory effect of A. malaccensis leaf extract (ALEX-M). The results are...
consistent with a previous study conducted on A. sinensis leaves, where the ethanol extract suppressed NO production in LPS-stimulated RAW264.7 with IC₅₀ = 80.4 μg/mL using hydrocortisone as the positive control (IC₅₀ = 0.1 μM). The inhibitory activities of epifriedelanol (1), 5-hydroxy-7,4′-dimethoxyflavone (2), luteolin-7,3′,4′-trimethyl ether (3), and 5,7-dihydroxy-4′-methoxyflavone (acacetin) (5) were comparable to the results obtained in previous studies with IC₅₀ values of 21.76 μM, 24.5 μM, 23.3 μg/mL, and 7.23 μM, respectively. The anti-inflammatory activity of compound (4) (5,3′-dihydroxy-7,4′-dimethoxyflavone) was observed through its ability to suppress a number of inflammatory mediators in LPS-treated RAW264.7. In this study, the anti-inflammatory activity of aquilarinenside E (6), irirolphenone-2-O-α-L-rhamnopyranoside (7), and irirolphenone-3-C-β-D-glucoside (8) was investigated for the first time, to our knowledge. Compound (7) was abundant in ALEX-M and therefore might be an important contributor to its anti-inflammatory effect.

2.4. Quantitative RT-PCR Analysis. To investigate the effect of ALEX-M on the expression of proinflammatory mediators, namely, IL-6 and TNF-α, in RAW 264.7 cells. The cells were treated with various concentrations of ALEX-M for 6 h in the presence of LPS (0.1 μg/mL) and IFN-γ (10 U/mL). In RT-PCR, ALEX-M noticeably induced the expressions of IL-6 and TNF-α mRNA at a concentration of 50 μg/mL (Figure 5).

3. EXPERIMENTAL SECTION

3.1. Chemicals and Reagents. Analytical grade solvents 95% ethanol (EtOH), methanol (MeOH), n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol, Dulbecco’s modified Eagle’s medium (DMEM) (41965039, Gibco, USA), fetal bovine serum (10082139,GIBCO, USA), penicillin/streptomycin 10,000 U/mL (15140122, Gibco, USA), Griess reagent kit (G4410, Sigma-Aldrich, St. Louis, MO, USA) for NO quantification, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (CT01-5 Sigma-Aldrich), and murine interferon-γ (315-05) (PeproTech, NJ, USA) were generously donated by the British University in Egypt (BUE). Silica gel (SiO₂) and TLC plates were gifted by the National Research Center (NRC) (Cairo, Egypt). GeneJET RNA Purification Kit (Thermo Fisher Scientific, Massachusetts, USA) according to manufacturer’s protocol was used for the RNA extraction. One μg of RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, USA) with random primer according to the manufacturer’s instruction.

3.2. Plant Materials. Fresh leaves were obtained from a noninoculated and healthy A. malaccensis tree located at a local plantation in Semenyih, Selangor in March 2018 and were identified according to their morphology. The herbarium specimens have been consigned at KAED, the herbarium located at the International Islamic University in Malaysia (Voucher specimen: HBL707 [VS-1]).

3.3. Extraction, Isolation, and Purification. EtOH (95%) was used to extract the dried and ground leaves (2.75 kg) at room temperature, and 280 g of crude extract was yielded after complete evaporation of solvent under reduced pressure using rotavapor (Heidolph) at 40 °C. The EtOH extract was sequentially treated with n-hexane, DCM, EtOAc, and n-butanol to give corresponding extracts. The DCM fraction (16.5 g) was exposed to vacuum-liquid chromatography (VLC) on silica gel using elution solvent system of hexane:EtOAc:MeOH. Three fractions were obtained and

![Figure 4](https://doi.org/10.1021/acsomega.2c00439)
were chromatographed on normal column chromatography (silica gel; n-hexane:EtOAc; gradient). Fraction I yielded compound 1 (26.5 mg), eluted with n-hexane:EtOAc (99:1). Fraction II yielded compound 2 (30.2 mg) upon elution with n-hexane:EtOAc (97:3). Two subfractions obtained from fraction III gave compound 3 (8 mg) and compound 4 (14 mg) upon elution with n-hexane:EtOAc (95:5) and (95:10), respectively. The EtOAc fraction (10.8 g) was also fractionated by VLC on silica gel using elution system of DCM:EtOAc:MeOH and yielded three fractions (IV–VI) that were subjected to further separation on normal column chromatography (silica gel; DCM:MeOH (100:30); gradient) took place. Fraction IV yielded compound 5 (71.2 mg) using DCM:MeOH (98:2) as eluent. By using the elution system DCM:MeOH (90:10), fraction V yielded compound 6 (88.5 mg). Compound 7 (1.5 g) and compound 8 (310 mg) were obtained from two subfractions collected from fraction VI when eluted with DCM:MeOH (85:15) and (75:25), respectively. The compounds were observed on SiO2 TLC heated on hot plate after being sprayed with p-anisaldehyde reagent.

3.4. Structural Elucidation. UV spectra were measured on Cary 60 UV–vis spectrometer (Agilent, Santa Clara, CA, USA), and the IR spectra were defined using a NICOLET iS50 FTIR spectrometer (Thermo Fisher Scientific, USA). HRESIMS spectra were obtained using TOF mass spectrometer (PerkinElmer, Norwalk, USA) and were observed using Peakview software (SCIEX, Framingham, MA, USA), whereas the spectra of NMR were run on a JEOL Instrument (JEOL, Peabody, MA, USA) operating at 500 MHz (1H NMR) or 125 MHz (13C NMR). The NMR spectra were processed using JEOL’s native Delta software (Version 4.3.6).

3.5. Cytotoxicity Assay. In accordance with the protocol used by refs 54 and 55, the MTT assay was conducted. Concisely, RAW 264.7 cells were seeded at 1 × 10^5 cells/well into 96-well plates and were incubated for 12 h. After 24 h of exposure to samples dissolved in high glucose phenol red-free DMEM containing DMSO (0.1%) and complemented with 10% v/v FBS and penicillin-streptomycin (1% v/v), the cells were then stained with MTT solution (5 mg/mL) and were incubated for 4 h. The supernatants were afterward drawn, and the formazan crystals were solubilized by adding 100 μL of DMSO to the wells. The absorbance of the purple solution was recorded at λ = 540 nm (Multiskan Sky Microplate Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA). The cell viability was determined using the equation:

\[
\text{cell viability (％) } = \frac{\text{OD treated cells} - \text{OD blank} \times 100}{\text{OD untreated cells} - \text{OD blank}}
\]

where OD is the absorbance at λ = 540 nm.

3.6. Anti-Inflammatory Griess Assay. High-glucose DMEM incorporating FBS (10% v/v) and penicillin-streptomycin (1% v/v) was used to culture the murine RAW 264.7 cells. Approximately, 1 × 10^5 cells/well were seeded into 96-well plates and were incubated at 37 °C overnight. ALEX-M and the compounds (1–8) were dissolved in media containing 0.1% DMSO. After the cells were exposed to different concentrations of samples after 2 h, the NO production was stimulated by adding 0.1 μg/mL of LPS and 10 U/mL of IFN-γ. Based on the results of the cytotoxicity assay, the minimum toxic dose (≥85% viability) was used as the maximum dose in the Griess assay.

Equal amounts (50 μL) of the supernatant and Griess reagent were mixed in another 96-well plate. Subsequent to incubation at room temperature for a duration of 15 min, the absorbance was determined at λ = 540 nm (Multiskan Sky Microplate Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA). The NO inhibition percentage was determined with reference to the standard sodium nitrite as per the following equation:

\[
\text{NO inhibition (％) } = \frac{\text{OD LPS/IFN-γ stimulated cells} - \text{OD test}}{\text{OD LPS/IFN-γ stimulated cells}} \times 100
\]

where OD is the absorbance at λ = 540 nm.

3.7. Quantitative RT-PCR Analysis. One-tenth of the resulting cDNA was used as a template for real-time PCR amplification using Maxima SYBR Green qPCR master mix (Thermo Fisher Scientific, Massachusetts, USA). For each sample, the quantitative real-time PCR was performed in triplicates in StepOne Real-Time PCR System (Applied Bio Systems) using the SYBR green method. Specific primers for
IL6 and TNF-α were used. GAPDH was amplified and used as the endogenous control to normalize for gene expression. A no-template control was used as negative control using DEPC-treated water. The primers used for the amplification of the indicated genes were designed using the IDT Primer Quest Primer Design Tool and are listed in Table 5. Fold changes in gene expression were determined using the 2−ΔΔCt method.58 Thermal cycle parameters were: 1 cycle at 95°C for 15 min followed by 35 cycles at 95°C for 10 s, annealing at 61°C for 20 s and at 72°C for 20 s.

3.8. Statistical Analysis. All experiments were carried out in triplicate (n = 3) independently. Comparisons were carried out by analysis of variance (ANOVA) followed by Tukey’s post-hoc test using p < 0.05 as the level of significance.

### 4. SPECTRAL DATA FOR (COMPOUNDS 1–8)

**Epifriedelanol (1).** White needle crystals; IR νmax (cm−1): 3467 (OH), 2917 (C−H); UV (CHCl3) λmax 250 nm; HRESIMS m/z 429.1163 [M + H]+, 1H NMR (500 MHz, CDCl3) and 13C NMR (125 MHz, CDCl3) data, refer to Table 1.

**5-Hydroxy-7,4′-dimethoxyflavone (2).** Pale yellow amorphous powder; IR νmax (cm−1): 3367 (OH), 1653 (C−O); UV (MeOH) λmax 270, 322 nm; HRESIMS m/z 297.1225 [M − H]+, 1H NMR (500 MHz, CDCl3) and 13C NMR (125 MHz, CDCl3) data, refer to Table 2.

**Luteolin-7,3′,4′-trimethyl Ether (3).** Pale yellow amorphous powder; IR νmax (cm−1): 3350 (OH), 1652 (C=C), 1604 (C=C); UV (CHCl3) λmax 270, 327 nm; HRESIMS m/z 327.0777 [M − H]+, 1H NMR (500 MHz, CDCl3) and 13C NMR (125 MHz, CDCl3) data, refer to Table 2.

**Luteolin-7,4′-dimethyl Ether (4).** Pale yellow amorphous powder; IR νmax (cm−1): 3367 (OH), 1653 (C=C=O); UV (CHCl3) λmax 270, 334 nm; HRESIMS m/z 313.0703 [M − H]+, 1H NMR (500 MHz, CDCl3) and 13C NMR (125 MHz, CDCl3) data, refer to Table 2.

**Acacetin (5).** Yellow amorphous powder; IR νmax (cm−1): 3252 (OH), 1663 (C=O), 2889 and 2816 (C−H); UV (DMSO) λmax 268, 335 nm; HRESIMS m/z 283.0631 [M − H]+, 1H NMR (500 MHz, DMSO-d6) and 13C NMR (125 MHz, DMSO-d6) data, refer to Table 2.

**Aquilarineside E (6).** White amorphous powder; IR νmax (cm−1): 3275 (OH), 1709 (C=O); UV (MeOH) λmax 285 nm; HRESIMS m/z 434.1250 [M − H]+, 1H NMR (500 MHz, CD3OD) and 13C NMR (125 MHz, CD3OD) data, refer to Table 3.

**Iriflophenone-2-O-α-L-rhamnopyranoside E (7).** White needle crystals; IR νmax (cm−1): 3236 (OH), 1635 (C=O); UV (MeOH) λmax 285 nm; HRESIMS m/z 391.1002 [M − H]+, 1H NMR (500 MHz, CD3OD) and 13C NMR (125 MHz, CD3OD) data, refer to Table 3.

### 5. CONCLUSION

The current research resulted in the separation of one terpenoid, four flavonoids, and three benzophenones from noninoculated A. malaccensis leaf for the first time. By using detailed spectroscopic analysis and comparison with previously published data, the structures of the isolated compounds were elucidated. The anti-inflammatory activity of the extract and eight compounds was evaluated, and they demonstrated the ability to suppress NO levels in RAW 264.7 cells induced by LPS/IFN-γ. The study introduced the use of A. malaccensis leaves extract and the isolated bioactive compounds in the treatment of inflammatory-associated disorder. Currently, a study is in progress to elucidate the underlying immunomodulatory mechanisms of the extract. Further studies are ought to be dedicated toward identifying the inhibitory NO pathways of the active compounds.

**ASSOCIATED CONTENT**

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00439.

1D and 2D NMR, UV, IR, and HRESIMS spectroscopic data of compounds 1–8 (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

Manar A. Eissa – International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, 53100 Gombak, Selangor, Malaysia; Center for Drug Research and Development (CDRD), The British University in Egypt (BUE), Cairo 11837, Egypt; orcid.org/0000-0003-3664-7984; Email: manareissa1210@gmail.com

**Authors**

Yumi Z. H-Y. Hashim – International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, 53100 Gombak, Selangor, Malaysia

Saripah S. S. Abdul Aziz – Faculty of Science and Mathematics, Sultan Idris Education University, 35900 Tanjong Malim, Perak, Malaysia

Hamzah Mohd. Salleh – International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, 53100 Gombak, Selangor, Malaysia

Muhammad Lokman Md. Isa – Department of Basic Medical Sciences for Nursing, Kulliyah of Nursing, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

Nor Malia Abd Warif – Biomedical Sciences Program, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia

Fauziah Abdullah – Phytochemistry Program, Natural Products Division, Forest Research Institute Malaysia, 52109 Kepong, Selangor, Malaysia
Eman Ramadan — Department of Pharmacology and Textiology, Faculty of Pharmacy, The British University in Egypt (BUE), Cairo 11837, Egypt; Center for Drug Research and Development (CDRD), The British University in Egypt (BUE), Cairo 11837, Egypt

Dina M. El-Kersh — Pharmacognosy Department, Faculty of Pharmacy, The British University in Egypt (BUE), Cairo 11837, Egypt; Center for Drug Research and Development (CDRD), The British University in Egypt (BUE), Cairo 11837, Egypt

Ramadan conducted RT-PCR assay. D. M. El-Kersh provided review, and editing. F. Abdullah conducted HRESIMS. E. M. L. Md. Isa, N. M. A. Warif provided cosupervision, paper review, and editing. S. S. S. Abdul Azziz provided supervision and funding acquisition, paper review, and editing.

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c00439

Author Contributions
M. Eissa conceived, designed, and performed the experiments, analyzed data, and wrote the paper. Y. Z. H-Y. Hashim provided supervision, funding acquisition, paper review, and editing. S. S. S. Abdul Azziz provided supervision and confirmation of compounds identification. H. Mohd. Salleh, M. L. Md. Isa, N. M. A. Warif provided cosupervision, paper review, and editing. F. Abdullah conducted HRESIMS. E. Ramadan conducted RT-PCR assay. D. M. El-Kersh provided method validation, participated in compounds isolation and purification, paper review, and editing.

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Notes
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