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HEALTH SCIENCES

Antimycobacterial and anti-inflammatory activities of fractions and substances from *Erythrina verna* Vell focusing on dual severe TB treatment approach

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Abstract: Tuberculosis remains a major health problem worldwide. Drug-resistant and hypervirulent *Mycobacterium tuberculosis* (Mtb) strains can lead to a hyperinflammatory response and necrotic pathology in hyper-reactive individuals that require adjunctive treatment. Plant-derived substances have been investigated for TB treatment, among which flavonoids stand out. We evaluate the anti-Mtb, anti-inflammatory and cytotoxicity activities of fractions and substances **1**, **2** and **3** isolated from *Erythrina verna* through a bioassay guided fractionation. Seven fractions (**1**, **3**-**5** and **7**-**9**) obtained from dichloromethane *E. verna* extract inhibited NO production (IC_{50} ≤ 15 µg/mL) with none or poor cytotoxic effect, while the fractions **4** and **5** notably reduced TNF-a production. Fractions **4**, **6** and **9** suppressed *Mycobacterium* growth with MIC_{50} ≤ 20 µg/mL. Fraction 4 was the most potent due to dual biological activities. Erythratidinone and alpinumisoflavone inhibited the growth of Mtb H37Rv and hypervirulent strain in bacterial cultures (MIC_{50} ≤ 20 µg/mL), with erythratidinone standing out in reducing intracellular growth of Mtb H37Rv (5.8 ± 1.1 µg/mL). Alpinumisoflavone and erythratidinone were capable of inhibiting NO and TNF-α production besides showing significant inhibitory effects against *Mycobacterium tuberculosis* strains with low toxicity in macrophages. Both substances are promising for further studies focusing on an anti-TB dual treatment approach.

Key words: Alpinumisoflavone, erythratidinone, inflammation, *Mycobacterium tuberculosis*, tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (Mtb) and the leading cause of death from a single infectious disease agent worldwide (WHO 2020). The emergence of multidrug/extensively resistant TB (MDR/XDR-TB) is the major threat to TB control in the world, which still put on this disease as a serious public health problem (Furin et al. 2019). Delay or non-adherence of patients to treatment may also lead to development of Mtb strains resistant to currently used drugs encouraging the search for more effective anti-TB substances (Knight et al. 2019).

Host immune status and bacterial virulence have an important role in the course and outcome of TB disease (Almeida et al. 2017, Young et al. 2019). Severe pulmonary TB can evolve toward the development of a hyperinflammatory response and extensive lung tissue damage in hyper-reactive individuals.

Persistent and excessive immune response leads to uncontrolled production of pro-inflammatory mediators, such as nitric oxide (NO) and tumor necrosis factor-α (TNF-α) (Kaufmann & Dorhoi, 2013).

In this context, adjunct therapy directed at the host immune response is required to reduce or limit exacerbated inflammation and to increase the success of TB treatment (WHO 2019). Steroidal or non-steroidal anti-inflammatory (NSAID) combined with antimycobacterial activity has shown beneficial effects in severe TB cases reducing mortality in patients with hyperinflammatory phenotype (Kadhiravan & Deepanjali 2010, Maitra et al. 2016, Xie et al. 2017). Drug candidates that exhibit both activities, anti-TB and anti-inflammatory, and that are less toxic, can represent a differential in TB therapy increasing its efficacy and benefiting the patient.

Natural products play an important role in the pharmaceutical industry providing many active substances used to treat several diseases. In this context many plant extracts are being investigated in the search for active substances with antimycobacterial activity (Nguta et al. 2015).

Erythrina verna Vell. (Fabaceae) is an endemic Brazilian tree which occurs in north, northeastern and southeastern Brazil. This species is popularly known as "mulungu" and occurs in gardens and urban landscape due to exuberant flowering. (Gilbert & Favoreto 2012).

Species of this genus are known to produce substances as erythrina alkaloids, pterocarpan and isoflavones, being the last two classes attributed to the anti-mycobacterial, anti-inflammatory and antioxidant activities. Previous investigations with pterocarpan and isoflavones had shown the antimycobacterial activity against *M. tuberculosis* and *M. smegmatis* (Rukachaisirikul et al. 2007, Rukachaisirikul 2008).

Considering the presence of potential substances with antimycobacterial activity in *Erythrina* genus and the already published phytochemical and bioactivity works in literature for the species *E. verna*, the background of this work was to guide the extract fractionation to obtain fractions and substances with dual activities which includes the inhibition of mycobacterial growth in bacterial cultures and in infected macrophages and inhibition of cellular inflammatory mediators production (NO and TNF-α) , while showing low cytotoxic effects on macrophages (RAW 264.7).

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) from serotype 0111: B4 *Escherichia coli*, rifampicin (cod. R7382), N^G-Monomethyl-L-arginine acetate salt (L-*N*MMA) (cod. M7033), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cell culture reagents (DMEM-F12/ Dulbecco's modified Eagle's medium, FBS/Fetal Bovine Serum and gentamicin) were purchased from Gibco/Invitrogen (Grand Island, NY, USA). Mycobacterial culture media (Middlebrook 7H9 and 7H10) were obtained from Difco (Detroit, MI, USA); and OADC (oleic acid-albumin-dextrose catalase) and ADC (albumin dextrose complex) supplements were from BD Biosciences (BD, Sparks, MD, USA). Rifampicin and samples were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich); while the LPS and L-*N*MMA were dissolved in sterile phosphate buffered saline (PBS) and sterilized by passage through 0.22 mm nylon filters (Corning Inc., Wilkes-Barre, PA,USA).

Apparatus

Counter-current separation was performed in a Dynamic Extractions Spectrum HPCCC equipped

with 142 mL multilayer coil columns (1.6 mm i.d.). The β-value ranges from 0.52 to 0.86 and revolution speed can be set up to 1600 rpm. The system comprises two Knauer Smartline 100 V5010 pumps, a Knauer Smartline 2500 V7604 UV absorbance detector, a Büchi C-660 fraction collector, a LabTech Smart H-150-1500 chiller and a HiChrom 5 mL manual injection valve.

^{1}H and ^{13}C NMR and two-dimensional NMR spectra (HMQC and HMBC) were acquired on a Brucker Ascend 500 (500 MHz for 1H and 125 MHz for ^{13}C), using chloroform-d as the solvent. The chemical shifts (δ) are given in ppm relative to tetramethylsilane (TMS).

High resolution mass spectra (HR-MS) were acquired on a Bruker MicrOTOF-Q II Time-of-Flight equipped with an electrospray interface (ESI). Analysis parameters were set using the positive ionization to alkaloid and negative ionization to isoflavones, with spectra acquired over a mass range from m/z 50 to 1400. Automatic MS/MS experiments were performed adjusting the collision-energy values as follows: m/z 500, 35 eV (negative mode) and 40 eV (positive mode), using N_2 as collision gas.

Preparation of plant material

Erythrina verna stem barks (3.8 kg) were collected in São José dos Calçados- ES in September 2014, SisGen number: AC4650E. A voucher specimen is deposited at Universidade Estadual do Norte Fluminense herbarium under registration HUENF11242 (Campos dos Goytacazes/Rio de Janeiro-RJ). Plant material was dried at 45°C, crushed in hammer mill, and extracted with dichloromethane in Soxhlet apparatus for 4 h. The obtained extract was concentrated to dryness in vacuum rotary evaporator, with yield 1.29% of the dried plant material (3.8 kg).

An aliquot of 5.0 g of the dried extract was submitted to acid-base extraction using hydrochloric acid solution (5% v/v, hydrochloric acid ACS reagent, 37% in water). First the extract was solubilized in 100 mL of dichloromethane and percolated with 200 mL of hydrochloric acid solution. The obtained aqueous solution was then alkalinized with ammonium hydroxide until pH 10 and extracted with 100 mL of dichloromethane. TLC investigation of organic washing product revealed a single compound which reacted positive to Dragendorff reagent. Further NMR and mass spectrometry analyses (Supplementary Material - Figures S1, S4 to S7 and Table SI and figure S15) led to the identification of the alkaloid erythratidinone (1, 49.5 mg) and the NMR data were compared with the literature (Callejon et al. 2014).

The dichloromethane extract (30.0 g) was also fractionated by adsorption chromatography using silica-gel 60 (Merck) and binary solvent mixtures starting with dichloromethane up to dichloromethane: methanol (1:1 v/v). The fractions were grouped according to their chemical profile observed by TLC. All 9 fractions were evaluated for Antimycobacterial, anti-inflammatory and cytotoxic tests to reveal the most promising fractions. Once fraction 4 showed the best results on biological investigations was selected for further fractionation by high performance counter-current chromatography (HPCCC) using the solvent system hexane: ethyl acetate: methanol: water (4:1:4:1, v/v). The coil was first filled with the stationary phase (lower phase) and rotation was started while the mobile phase (upper phase) was pumped at 1.5 mL/min. Once hydrodynamic equilibrium was established (stationary retention 87.3%) the sample (0.91 g) was injected, and the eluate was collected in 4.5 mL fractions. After 270 mL the rotation was stopped, and the coil content was washed-off using methanol: water (7:3 v/v). The 146 fractions were grouped according to their TLC profile and the resulting 9 fractions were submitted to pharmacological investigations.

Fraction 4.8 (19.8 mg) was analyzed by NMR (Figure S2, S8 to S11 and Table SII and figure S16) leading to identification of alpinumisoflavone (2) (Stewart et al. 2000). Fraction 4.9 (135.0 mg) was submitted to a further purification step by HPCCC. Purification of fraction 4.9 was performed using the upper phase of the solvent system hexane: ethyl acetate: methanol: water (1:1:1:1, v/v) as the stationary phase. After reaching hydrodynamic equilibrium (SF retention - 77.4%) the sample was injected and the eluate was collected in 6.0 mL fractions. After 256 mL the rotation was stopped and the coil content was washed off using methanol: water (7:3 v/v). Ninety-seven fractions were obtained and grouped in 15 new fractions according to their TLC profile. Fraction 4.9.12 (3.4 mg) was leading pure compound identified as erysenegalensein M (3) by NMR analysis (Figure S3, S12 to S14 and Table SIII and figure S17) in agreement with the literature data (Wandji et al. 1995). The complete spectral data of compounds 1, 2 and 3 (Figure 1) are reporting in the Supplementary Material tables SI to SII and figures S1 to S17.

(1) erythratidinone- 2,11,12-trimethoxy-1,2,5,6,8,9-hexahydro-3H-indolo[7a,1-a] isoquinolin-3-one. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 6.72 (1H, s, H-17), 6.53 (1H, s, H-14), 6.19 (1H, *m*, H-1), 4.00 (1H, *dd*, *J*= 12.9 and 5.4 Hz, H-3), 3.80 (3H, *s*, CH_3O-16), 3.70 (3H, *s*, CH_3O-15), 3.56 and 3.31 (2H, *m*, 2H-10), 3.40 (3H, *s*, CH_3O-3), 3.23 and 2.88 (2H, *m*, 2H-8), 3.06 and 2.75 (2H, *m*, 2H-11), 2.79 and 2.56 (2H, *m*, 2H-7), 2.60 and 2.41 (2H, *m*, 2H-4). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 197.0 (C-2), 165.0 (C-6), 149.0 (C-16), 147.0 (C-15), 124.8 (C-12), 123.7 (C-1), 122.9 (C-13), 112.6 (C-17), 109.1 (C-14), 76.2 (C-3), 65.7 (C-5), 58.3 (CH_3O-3), 56.1 (CH_3O-15), 55.6 (CH_3O-16), 45.9 (C-8), 40.7 (C-4), 39.9 (C-10), 27.6 (C-7), 21.3 (C-11). HR-ESI-MS m/z 330.3874 $[M + H]^+$; MS/MS (% abundance) 330 (62.6), 314(32.3), 298 (100), 282 (88.8), 270 (36.0) 254 (46.4), 242 (60.9), 229 (74.5) and 215 (72.5).

(2) alpinumisoflavone- 5-hydroxy-7-(4-hydroxyphenyl)- 2,2-dimethyl-2H,6H-pyrano[3,2-g]chromen-6-one. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.83 (1H, *s*, H-2), 7.40 (2H, *d*, *J*= 8.6 Hz, H-2'and H-6'), 6.92 (2H, *d*, *J*= 8.6 Hz, H-3'and H-5'), 6.74 (1H, *d*, *J*= 10.0 Hz, H-4"), 6.35 (1H, *s*, H-8), 5.64 (1H, *d*, *J*= 10.0 Hz, H-3"), 1.49 (3H, s, 3H-5"), 1.49 (3H, s, 3H-6"). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 180.9 (C-4), 159.5 (C-7), 157.3 (C-8a), 156.9 (C-5), 156.1 (C-4'), 152.5 (C-2), 130.3 (C-2' and C-6'), 128.2 (C-3"), 123.5 (C-3), 122.9 (C-1'), 115.6 (C-3' and C-5'), 115.5 (C-4"), 106.1(C-4a), 105.6 (C-6), 94.9 (C-8), 78.1 (C-2"), 28.3 (C-5"), 28.3 (C-6"). HR-ESI-MS m/z 335.0952 $[M - H]^-$; MS/MS (% abundance) 335 (100) 317 (25.1), 301(23.4) and 241 (46.7).

(3) erysenegalensein M- 5-hydroxy-10-(2-hydroxy-3-methylbut-3-em-1-yl)-7-(4-hydroxyphenyl)-2,2-dimethyl-2H,6H-pyrano[3,2-g]chromen-6-one. ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 8.37 (1H, *s*, H-2), 7.37 (2H, *d*, *J*= 8.8 Hz, H-2'and H-6'), 6.81 (2H, *d*, *J*= 8.7 Hz, H-3'and H-5'), 6.60 (1H, *d*, *J*= 10.0 Hz, H-4"), 5.79 (1H, *d*, *J*= 10.0 Hz,

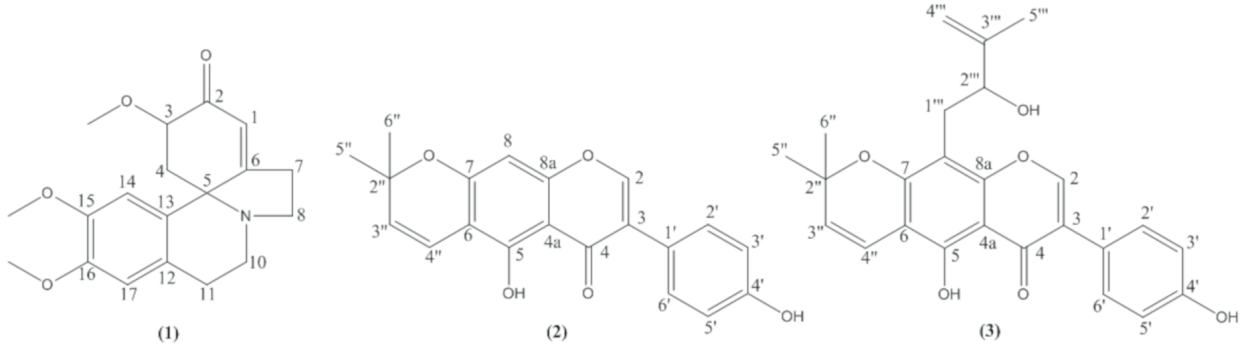

Figure 1. Chemical structures of compounds 1 (erythratidinone), 2 (alpinumisoflavone) and 3 (erysenegalensein M).

H-3"), 1.42 (3H, s, 3H-5"), 1.42 (3H, s, 3H-6"), 2.84 (1H, *dd*, *J*= 13.2 and 6.7 Hz, H-1a'") 2.78 (1H, *dd*, *J*= 13.2 and 7.6 Hz, H-1b'"), 4.17 (1H, *dd*, *J*= 7.6 and 6.7 Hz, H-2'"), 4.62 (2H, s, 2H-4'"), 1.75 (3H, s, 3H-5'"). ^{13}C NMR (125 MHz, DMSO-d_6): δ_C 180.9 (C-4), 156.8 (C-7), 154.9 (C-8a), 154.3 (C-5), 157.4 (C-4'), 154,2 (C-2), 147.8 (C-3'"), 130.1 (C-2' and C-6'), 129.0 (C-3"), 122.0 (C-3), 121.2 (C-1'), 115.2 (C-3' and C-5'), 114.9 (C-4"), 110.3 (C-4'"), 105.1(C-4a), 105.0 (C-8), 104.6 (C-6), 78.0 (C-2"), 74.2 (C-2'"), 28.2 (C-5"), 28.2 (C-6"), 28.6 (C-1'"), 17.9 (C-5'"). HR-ESI-MS m/z 419.1499 [M - H]$^-$; MS/MS (% abundance) 335 (100).

Cell Culture and Treatments

Murine RAW 264.7 macrophage cell line (American Type Culture Collection, Manassas, VA, USA), was cultured in DMEM-F12 supplemented with 10% FBS and gentamicin (50 µg/mL) in 5% CO_2 atmosphere at 37°C. RAW 264.7 cells were seeded in flat bottom 96-well tissue culture plates in the presence or absence of fractions and isolated substances (100, 20, 4 and 0.8 µg/mL) from *Erythrina verna* and/or LPS (1 µg/mL) and incubated for 24 h in 5% CO_2 atmosphere at 37°C. L-*N*MMA (20 µg/mL) was also used as positive control to inhibit NO production. The *Erythrina verna* samples and rifampicin were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) and sterilized by passage through 0.22 µm nylon filters (Corning Inc., Wilkes-Barre, PA, USA). Final DMSO concentration was less than 0.5 % (v/v) in all experiments and the negative controls were culture medium plus vehicle (DMSO) at maximum test concentration.

Quantification of NO and TNF-α

Cell culture supernatants were analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kit to measure TNF-α (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and a standard curve of recombinant murine TNF-α was used as reference for cytokine concentration. Nitrite, a stable NO metabolite, was determined by the Griess method and the absorbance was measured at 570 nm (Ventura et al. 2015).

Cytotoxicity Assay

Cytotoxic effects of fractions and isolated substances from *Erythrina verna* on RAW 264.7 cell viability in cultures stimulated with LPS were determined using MTT method. After 24 h of incubation with samples, 10 µL of MTT reagent (5 mg/mL) was added and incubated for additional 2 h at 37°C in 5% CO_2. The MTT solution was removed and 100 µL of isopropanol was added to solubilize the crystal formazan formed. The absorbance was measured at 570 nm and the results were used to obtain cell cytotoxicity percentage in relation to the concentrations of the samples (Moodley et al. 2014).

Mycobacterial Culture and Evaluation of Bacterial Growth

Three mycobacterial strains were used in this study: avirulent *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) Moreau strain, *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and highly virulent Mtb Beijing strain M299 isolated from TB patient in Mozambique (Ribeiro et al. 2014). Mycobacterial strains were grown in suspension in 7H9 Middlebrook broth containing 10% ADC, 0.5% glycerol and 0.05% Tween-80 at 37°C under Biosecurity level 3 containment conditions. The suspensions densities at 600 nm were previously determined for each strain using serial dilution plating on Middlebrook 7H10 agar plates supplemented with 0.5% glycerol and 10% OADC. To study the antimycobacterial activity of the samples we employed the MTT assay to quantify bacterial growth in a liquid medium (Moodley et al. 2014). The bacterial suspensions were plated (1×10^6 CFU/well in

96-well plate) and incubated in the presence of fractions or isolated substances from *E. verna* (100, 20, 4 and 0.8 µg/mL) or rifampicin (ranging from 0.0011-0.03 µg/mL for *M. bovis* BCG; from 0.00032-1 µg/mL for Mtb H37Rv and from 0.008-10 µg/mL for clinical isolate Mtb M299). After 7 days at 37°C in 5% CO_2 for *M. bovis* BCG or 5 days for *M. tuberculosis* strains, MTT solution (5 mg/mL) were added for 3h to bacterial cultures then subsequently treated with lysis buffer (20% w/v SDS/50% dimethylformamide in distilled H_2O, pH 4.7) for 18 h. Optical densities was spectrophotometrically measured at 570 nm. Untreated bacterial suspensions were used to control spontaneous growth of bacteria.

Intracellular Assay

RAW 264.7 macrophages (1×10^5 cells/well) were seeded in 96-well plates with DMEM-F12 supplemented with 10% FBS without antibiotic. After 24 h, macrophage cultures were infected at a multiplicity of infection (MOI) of 1:1 (macrophage: bacterium) by *M. tuberculosis* H37Rv (optical density adjusted to 0.1) and the phagocytosis was allowed to progress for 3 h. After washing off the extracellular Mtb H37Rv with PBS 1X, the cells were treated for 4 days with samples. On day 4 after infection, cells were lysed with 1% saponin and lysate aliquots were diluted 10-fold in PBS, plated in triplicates on 7H10 agar plates and incubated at 37°C. After 21 days, total CFU (Colony Forming Units) were counted (Ventura et al. 2015).

Statistical Analysis

The data were reported as mean ± standard error of the mean (SEM) and were analyzed by (ANOVA) one-way analysis and Tukey procedure for multiple range tests, using Prism 4 software (GraphPad) to assess statistical significance between groups. $P < 0.05$ was significant. IC_{50}, CC_{50} and MIC_{50} values were calculated by nonlinear regression analysis. Selectivity index (SI) was determined as the ratio of median cytotoxic concentration to a macrophage culture (CC_{50}) and the lowest concentration inhibiting mycobacterial growth (i.e., CC_{50}/MIC50).

RESULTS

Initially, the anti-inflammatory potential to inhibit NO and TNF-α production and the cytotoxic effects of the fractions 1-9 obtained from the dichloromethane extract of *Erythrina verna* stem barks were conducted on LPS-stimulated RAW 264.7 macrophages. Fractions 1 to 9 were able to inhibit NO production ($IC_{50} \leq 15$ µg/mL) with greater potential when compared to N^G-methyl-L-arginine acetate, a known nonselective iNOS inhibitor used as control (IC_{50} 27.1 ± 2.6 µg/mL). Although most of these fractions exhibited NO inhibition effects, only fraction 2, 4 and 5 showed TNF-α inhibitory activity with IC_{50} of 4.7 ± 1.6 µg/mL, 24.2 ± 1.2 µg/mL and 13.4 ± 1.0 µg/mL respectively. The other fractions failed to inhibit NO production showing IC_{50} value higher than 89 µg/mL. Regarding the evaluation of cytotoxic effects, most of the fractions did not show cytotoxic effect on macrophages showing IC_{50} higher than 91.3µg/mL (Fractions 1, 3-5 and 7-9). On the other hand, fractions 2 and 6 were toxic to cultured macrophages showing low IC_{50} values, such as18.2 ± 1.3 µg/mL and 4.3 ± 0.2 µg/mL, respectively (Table I).

As a part of the initial screening, the antimycobacterial potential of the fractions 1-9 were evaluated firstly against the mycobacterial strains Mbv BCG and Mtb H37Rv. Fractions 4, 6 and 9 showed significant growth inhibitory capacity of these strains with MIC_{50} of: 14.6 ± 2.3 µg/mL, 5.4 ± 0.2 µg/mL and 6.1 ± 0.1 µg/mL against Mbv BCG, respectively; and 15.8 ± 1.8 µg/mL, 20.1 ± 2.3 µg/mL and 10.1 ± 2.1 µg/mL against Mtb H37Rv, respectively. It should be observed

Table I. Inhibitory effects of *Erythrina verna* fractions on production of NO and TNF-α by LPS-stimulated RAW 264.7 macrophages, on growth of *M. bovis* BCG, *M. tuberculosis* H37Rv and clinical isolate *M. tuberculosis* M299 in culture and evaluation of cytotoxicity by MTT test.

Fractions *E.verna*	IC_{50} (µg/mL)	CC_{50} (µg/mL)		MIC_{50} (µg/mL)		
	NO	TNF-α	MTT	Mbv BCG	Mtb H37Rv	Mtb M299
1	12.8 ± 2.3 [a,b]	>100	>100	> 100	> 100	XX
2	5.1 ± 0.4 [c,d]	4.7 ± 1.6 a	18.2 ± 1.3	67.8 ± 1.1	82.6 ± 1.2 [a]	XX
3	4.8 ± 1.6 [c,d]	>100	91.1 ± 0.8 [a]	54.3 ± 0.6	78.9 ± 1.1 [a]	XX
4	9.2 ± 1.9 [b]	24.7 ± 1.2	>100	14.6 ± 2.3	15.8 ± 1.8 [b]	XX
5	2.5 ± 0.2 [d]	13.4 ± 1.0	>100	31.7 ± 1.9	48.6 ± 0.9	XX
6	3.4 ± 1.2 [c,d]	89.7 ± 2.3	4.3 ± 0.2	5.4 ± 0.2 a	20.1 ± 2.3 [b]	XX
7	3.5 ± 1.0 [c,d]	>100	91.3 ± 1.2 [a]	72.9 ± 1.1	> 100	XX
8	4.1 ± 0.9 [c,d]	>100	>100	21.5 ± 0.5	> 100	XX
9	2.4 ± 0.1 [d]	>100	>100	6.1 ± 0.1 [a]	10.1 ± 2.1 [c]	XX
4.1	18.1 ± 0.3	>100	>100	85.2 ± 1.3	>100	>100
4.2	12.4 ± 1.3 [a,b]	17.7 ± 0.3	>100	90.1 ± 2.2	>100	>100
4.3	>100	>100	>100	> 100	>100	>100
4.4	>100	>100	>100	> 100	>100	>100
4.5	>100	>100	>100	> 100	>100	>100
4.6	>100	>100	>100	99.1 ± 2.4	>100	>100
4.7	37.2 ± 1.9	>100	>100	2.5 ± 0.4 [a,b]	16.8 ± 0.1 [b]	20.5 ± 0.5
4.9	6.8 ± 1.5 [d]	5.3 ± 0.7 [a]	42.8 ± 1.2	2.6 ± 1.8 [a,b]	2.7 ± 1.7 [d]	2.8 ± 1.7 [a]
L-*N*MMA[1]	27.1 ± 2.6	XX	XX	XX	XX	XX
Rifampicin [2]	XX	XX	XX	0.01 ± 0.03 [b]	0.2 ± 0.01 [d]	3.3 ± 0.2 [a]

[1]Standard nitric oxide inhibitor. [2]Standard antimycobacterial drug. XX—not defined. Values in the same column with different superscript letters (a–d) are significantly different ($p < 0.05$, $p < 0.01$or $p < 0.001$); determined in Tukey test. Values are reported as mean value ± SD (n = 3).

that most of the fractions exhibited MIC_{50} higher than rifampicin, a first line anti-TB drug used as a positive control. Fraction 4 was notably the most potent when compared to other fractions regarding dual biological activities, inhibition of *Mycobacterium* growth, TNF-α and NO production and toxic activity selective for mycobacteria (Table I).

Purification of fraction 4 led to 9 new fractions (4.1-4.9) that were submitted to anti-inflammatory, cytotoxic and antimycobacterial tests. Four fractions (4.1, 4.2, 4.7 and 4.9) were able to inhibit NO production (IC_{50} values ranged of 6.8 - 37.2 µg/mL) without toxicity interference for macrophages. Notwithstanding, only fractions 4.2 and 4.9 also inhibited TNF-α production (Table I). At this stage of the study, the antimycobacterial potential of these fractions also was verified against a highly virulent *M. tuberculosis* strain of the Beijing genotype (strain M299). Fractions 4.7 and 4.9 inhibited the mycobacterial growth of both strains utilized. Fraction 4.9 standing out against the clinical isolate hypervirulent M299 with MIC_{50} of 2.8 ± 1.7 µg/mL and selective index (SI) of 15.3 (Table I). This fraction showed comparable potential to rifampicin, and better

pharmacological results for dual activities being selected for further separations in CCC.

From the fractionation of F4 two isoflavones were isolated: alpinumisoflavone (Fraction 4.8) and erysenegalensein M (Fraction 4.9.12) whereas erythratidinone was obtained from the alkaloidal fraction obtained by acid-base extraction. These substances were assayed for their anti-inflammatory and antimycobacterial properties.

More pronounced effects of inhibitory activity to inhibit NO and TNF-α production were presented by the alkaloid erythratidinone (1) and isoflavone erysenegalensein M (3), although the alpinumisoflavone (2) has also reduced the production of both pro-inflammmatory mediators at the highest concentrations tested (Figure 2a and b). When compared to L-*N*MMA (IC_{50} 27.1 ± 2.6 µg/mL), the substances presented higher inhibitory activity against NO production, although alpinumisoflavone (2) has showed similar potential. However, erysenegalensein M exhibited worse toxicity profiles on macrophages culture (Table II).

The three substances exhibited high capability to inhibit mycobacterial growth of Mtb H37Rv (Figure 3a) and hypervirulent Mtb M299 (Figure 3b) highlighting erythratidinone (1) with equivalent profiles to rifampicin against both Mtb strains and SI of 38.43 for Mtb M299 (Table II).

All three isolated compounds were evaluated for their potential to inhibit intracellular Mtb H37Rv growth in infected macrophages. The substance erysenegalensein M (9.5 ± 2.6 µM) and erythratidinone (17.6 ± 3.3 µM) were able to significantly reduce the intracellular growth of Mtb (75-92% of inhibition) at the highest concentration tested. On the other hand, the alpinumisoflavone exhibited activity at a 100 µg/mL with MIC_{50} of 169.5 ± 3.3 µM. It should be noted that erythratidinone exhibited selectivity for Mtb with SI value of 17.25 (Figure 4).

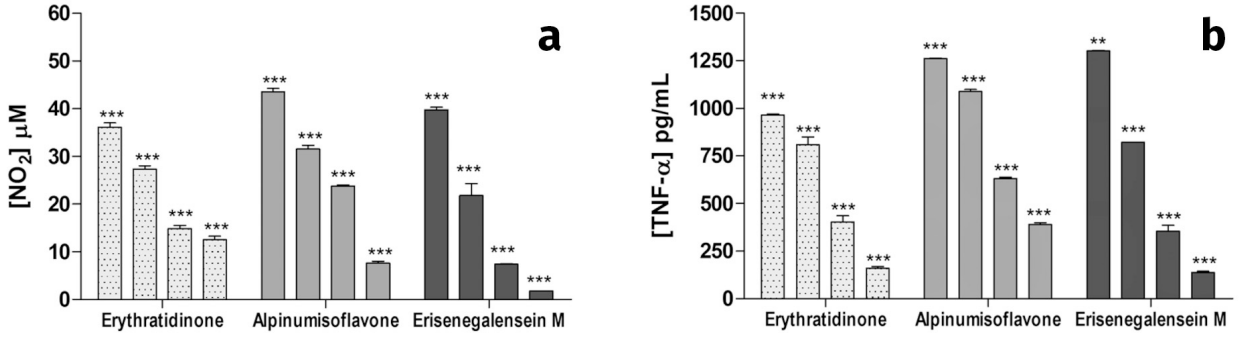

Figure 2. Effect of isolated substances from *Erythrina verna* on production of proinflammatory mediators (NO and TNF-α) by LPS-stimulated RAW 264.7 macrophages. Macrophages RAW 264.7 were plated and stimulated with 1.0 µg/mL de LPS for 24 h at 37 ºC with or without isolated substances from *E. verna* (0.8, 4, 20 and 100 µg/mL). (a) Nitrite was determined by the method of Griess reaction. Macrophages not treated and not stimulated with LPS were used as negative control; and macrophages stimulated with 1 µg/mL LPS (56.1 ± 1.5 µg/mL) and not treated as positive control. L-*N*MMA was also used as positive control at 20 µg/mL inhibiting 51.4 ± 2.4 % NO production. (b) In the TNF-α assay, TNF-α values determined in untreated and only LPS-treated cell cultures were 1.1 ± 0.1 pg/mL and 1428.6 ± 6.6 pg/mL, respectively. The results presented are mean values obtained over three experiments, each done in triplicate. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared to untreated group (0 µg/mL) determined by Tukey test.

Table II. Inhibitory effects of isolated substances from *Erythrina verna* on production of NO and TNF-α by LPS-stimulated RAW 264.7 macrophages, evaluation of cytotoxicity and on growth of the laboratory *M. tuberculosis* strain H37Rv and clinical isolate *M. tuberculosis* M299.

Substances	IC_{50} (μg/mL)		CC_{50} (μg/mL)	MIC_{50} (μg/mL)	
	NO	TNF-α	MTT	Mtb H37Rv	Mtb M299
Erythratidinone	5.2 ± 1.1[a]	5.9 ± 1.2[a]	>100	3.4 ± 1.2	2.6 ± 1.2[a]
Alpinumisoflavone	24.2 ± 1.0	17.7 ± 1.1	95.9 ± 1.1	9.7 ± 1.1	16.8 ± 1.1[b]
Erisenegalensein M	4.6 ± 1.0[a]	5.9 ± 1.0[a]	11.3 ± 1.2	6.5 ± 1.0	18.3 ± 1.1[b]
L-*N*MMA[1]	27.1 ± 2.6	XX	XX	XX	XX
Rifampicin[2]	XX	XX	XX	0.2 ± 0.01	1.0 ± 0.2[a]

[1]**Standard antimycobacterial drug. Statistical analyses were calculated and values in the same column with different superscript letters (a–b) are significantly different ($p < 0.05$, $p < 0.01$or $p < 0.001$); determined in Tukey test. Values are reported as mean value ± SD (n = 3).**

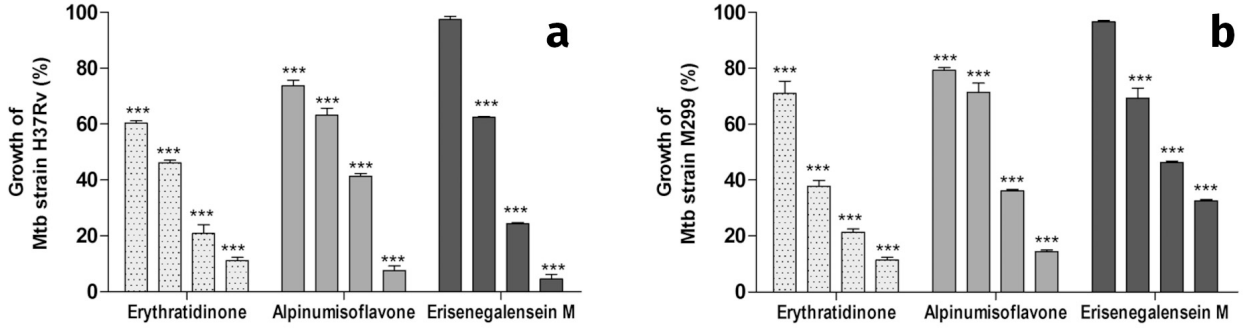

Figure 3. Effect of isolated substances from *Erythrina verna* on *M. tuberculosis* growth in bacterial culture. Bacterial suspensions (1×10^6 CFU/well) of *M. tuberculosis* strain H37Rv (a) and clinical isolate M299 (b) were treated or untreated with substances (0.8, 4, 20 and 100 μg/mL) or rifampicin (0.00032, 0.0016, 0.008, 1 μg/mL for the strain H37Rv and 0.008, 0.04, 0.2 and 10 μg/mL for the strain M299) for 5 days. Bacterial growth in the resulted cultures was quantified by MTT test. Data are presented as a percentage of bacterial growth of each treated culture compared to the growth of corresponding untreated culture (100%). The bars for each substance refer to concentrations tested in ascending order. The results presented are mean values obtained over three experiments, each done in triplicate. *** $p < 0.001$ compared to untreated bacterial suspension (0 μg/mL) determined by Tukey test. Bacterial suspensions treated with antibiotic rifampicin were used as a positive control.

DISCUSSION

The development of new drugs is needed for TB treatment due to increasing drug resistance and toxicity of the current TB drug regimens (WHO 2020). TB disease presents a dynamic spectrum of formats and diverse components of host immune response to mycobacteria have complex and determinate role in the pathology of TB (Young et al. 2019, Martino et al. 2019). Severe TB forms associated with exacerbated pulmonary inflammation presents pathological complications that causes clinical aggravation and may lead patients to death (Zumla et al. 2015, Melo et al. 2019). Therapies capable of reducing pulmonary pathology in TB can improve treatment outcomes (Palucci & Delogu 2018, Young et al. 2019). The search for bioactive substances which can act simultaneously as

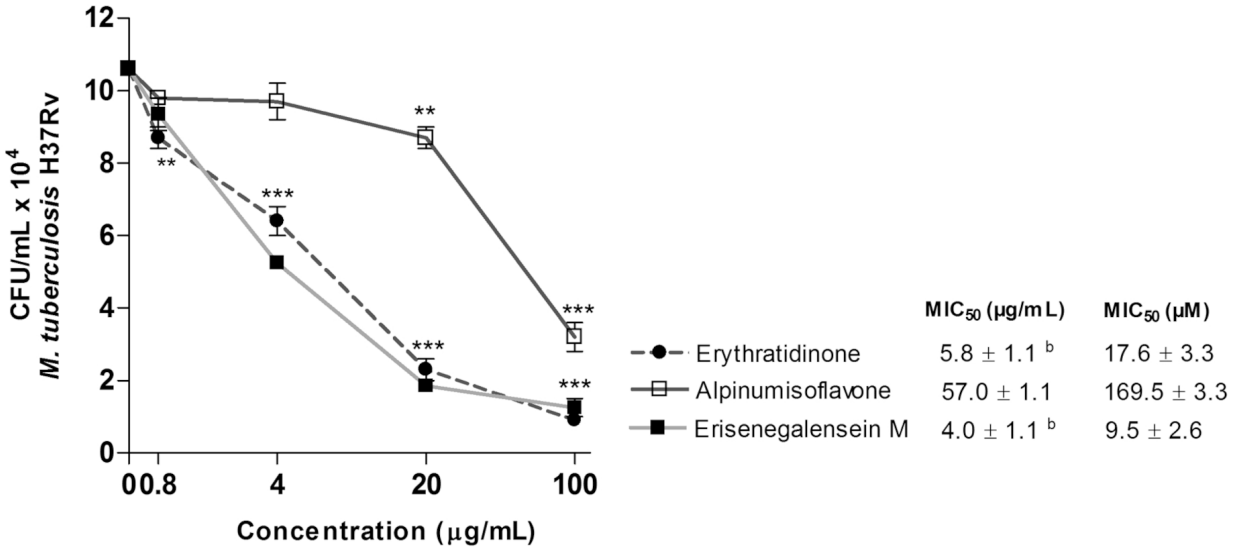

Figure 4. Effect of isolated substances and most active fractions from *Erythrina verna* on *M. tuberculosis* growth in macrophages. RAW 264.7 macrophages were infected with *M. tuberculosis* strain H37Rv at the infection ratio of 1:1(macrophage:mycobacteria) and treated with samples for 4 days. Bacterial intracellular viability after the treatment was determined by bacterial CFU counts. Statistical analyses were calculated and compared to untreated group (* $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$). Minimum inhibitory concentrations of samples on *M. tuberculosis* growth in macrophages were calculated and values in the same column with different superscript letters (a-c) are significantly different ($p < 0.05$ or $p < 0.001$; determined by Tukey test). Values are reported as mean value ± SD (n = 3).**

anti-inflammatory and antimycobacterial is a strategy for severe pulmonary TB treatment and their implications.

The background of this work was to guide the extract fractionation to obtain fractions and substances with dual activities which includes the inhibition of mycobacterial growth in bacterial cultures and in infected macrophages and inhibition of cellular inflammatory mediators production (NO and TNF-α), while showing low cytotoxic effects on macrophages. Considering the anti-inflammatory and cytotoxic results, the fractions were most active to inhibit NO production when compared to the potential to inhibit TNF-α production. It should be noted that TNFα-gene expression and production is regulated at the transcriptional level by several factors (such as NF-κB) and initiate many signaling pathways from binding two receptors (Zia et al. 2020). Thus, the results suggested that the substances present in the fractions 4 and 5, but not in others fractions are able to act on this complex interface.

Most fractions showed inhibitory activity upon either single or both *Mycobacterium* strains highlighting the fractions 4, 6 and 9 as the most active for both strains with $MIC_{50} \leq 20$ μg/mL. Nguta et al. (2015) described that the use of different mycobacterial species is important for the quantification of the inhibitory potential of crude extracts and fractions and selection of novel candidates anti-TB agents. Taking together the results of all biological assays, fraction 4 was elected as candidates to chromatographic fractionation due better inhibitory effects. It can be noted that fraction 4 exhibited a very similar inhibition potential between both prospected activities.

The new fractions obtained from F4 were submitted to the same biological tests, with

inclusion of assay against a hypervirulent *Mycobacterium* strain (Mtb M299). *M. tuberculosis* strain M299 was identified as highly virulent clinical isolate (Ribeiro et al. 2014) that induced exacerbated inflammatory response in the murine model of pulmonary TB, a common aspect of severe TB (Almeida et al. 2017). The evaluation of new anti-TB drugs from natural products in screening approaches that includes high-virulence Mtb strains is very incentivized and have relevance in the field of drug discovery and development (Orme 2011, Nguta et al. 2015). Among the new fractions, only 4.9 inhibited both inflammatory response and the hypervirulent strain growth. Although fraction 4.9 showed moderate cytotoxic effect, the selectivity index (SI) calculated was greater than 10 in relation to anti-Mtb specificity and thus it was further purified due to the excellent inhibitory effect against Mtb H37Rv and Mtb M299 strains (MIC_{50} 2.7 ± 1.7 and 2.8 ± 1.7, respectively) with similar potential to rifampicin despite hypervirulent Mtb, that required at least a ten-fold increase in concentrations used. Values of SI > 10 are considered higher selectivity against microorganisms (Hartkoorn et al. 2007, Nguta et al. 2015). It should be noted that new fractionation process allowed obtaining a fraction 4.9 more potent ($p<0.01$) than F.4 for all prospected activities, although it has also enabled the concentration and/or interaction of substances that result in the presence of toxicity.

Erysenegalensein M was isolated by these procedures (F 4.9) and showed to inhibit Mtb H37Rv and Mtb M299 growth (MIC_{50} 6.5 ± 1.0 and 18.3 ± 1.1, respectively). However, it exhibited toxicity for macrophage culture interfering with NO and TNF-α production. The inhibitory effect of this compound on Mtb growth was not better than fraction 4.9 and citotoxicity values were higher for erysenegalensein M.

Fraction 4.8 was identified as alpinumisoflavone and showed high inhibition of the inflammatory mediator's production with IC_{50} 24.2 ± 1.0 µg/mL for NO and IC_{50} 6.9 ± 1.3 µg/mL for TNF-α. Moreover, alpinumisoflavone did not show cytotoxic effect on macrophages cultures. This substance showed active to *Mycobacterium* strains including against Mtb M299 with MIC_{50} 16.8 ± 1.1 µg/mL. Comparable effects of bacterial growth inhibition were observed for both identified isoflavones. Interestingly, the toxic effects of alpinumisoflavone were selective for Mtb strains.

Substances able to inhibit intracellular bacilli growing in infected macrophages are promising candidates for further *in vivo* pharmacological evaluations once macrophages are main innate defense cells and the first niche for Mtb replication (Martino et al. 2019). Of the three tested substances, erythratidinone and erysenegalensein M showed to be the most active, even though the latter showed significant cytotoxic effects on macrophages which had disqualified this substance as a possible antimycobacterial drug candidate.

As mentioned elsewhere isoflavones are known to possess antimycobacterial activity against *M. tuberculosis* strain in suspension. Mutai et al. (2015) studied pharmacophore interactions of synthetic analogues of formononetin, an isoflavone with hydroxyl group at C7 and methoxyl group at C4'. In this work, the authors synthesized twenty analogues modifying the substituents on ring A and B. The results suggested that hydroxyl group in the chromene is important for the activity due to possible hydrogen bond interaction. The other important characteristic observed was the presence of hydrophobic group such as methoxyl group at C4' which ease the drug entrance in the mycobacterial cell (Mutai et al. 2015). Alpinumisoflavone and erysenegalensein

M possess this same characteristic. Both compounds have a hydroxyl group at C4' and an isoprene group at C6 which forms a pyran ring by attaching with the oxygen atom at C7. The structural difference between alpinumisoflavone and erysenegalensein M is at 2-hydroxy isoprenyl group at C8 which is possibly responsible for the higher cytotoxic effect observed for macrophage.

Previous studies reported anti-inflammatory activity of alpinumisoflavone in LPS stimulated-macrophage and against lung inflammation in mice associated with reduction of pro-inflammatory mediators (Li et al. 2018, Ateba et al. 2019). Antimycobacterial activity of alpinumisoflavone against *M. smegmatis* was described for San-Martin et al. (2015), whereas no potential was observed against Mtb H37Rv (Kuete et al. 2008, San-Martin et al. 2015). In our study, alpinumisoflavone showed an inhibitory effect against *M. tuberculosis* strains used in tests.

Erythratidinone was effective at all evaluated concentrations showing significant inhibitory activity with no cytotoxicity observed on macrophage cultures (Figure 4). Additionally, the substance showed inhibitory activity in mycobacterial suspension against both strains, including higher levels of antimycobacterial activity against hypervirulent Mtb strain, and reduced bacterial viability when macrophage culture was infected with Mtb H37Rv strain. These data suggest that erythratidinone could be a candidate anti-TB with good effectiveness profile.

Thitima Rukachaisirikul et al. (2008) evaluated antimycobacterial activity of the erythrina alkaloids named 10,11-dioxoerythratine and 10,11-dioxoepierythratidine. In their assays the erythrina alkaloids did not show significant inhibitory effects against *Mycobacterium tuberculosis* H37Ra strain (Rukachaisirikul et al. 2007). In contrast, in our study erythratidinone showed excellent antimycobacterial activity. Structural comparison of the above-mentioned alkaloids with erythratidinone reveals the absence of ketone group at C10 and C11 and oxidation of hydroxyl at C2 to a ketone. These structural characteristics seem to be relevant for the antimycobacterial activity observed and may need further evaluations dedicated to pharmacophoric modelling.

In summary, the three substances were isolated from *E. verna* and two of them showed both effects as anti-inflammatory and antimycobacterial inhibitor. The alpinumisoflavone and erythratidinone were also able to modulate inflammation by inhibiting NO and TNF-α production besides showing significant inhibitory effects against *Mycobacterium tuberculosis* strains with different levels of virulence (H37Rv and M299). Erysenegalensein M exhibited cytotoxic effects that affected macrophages functions.

Erythratidinone also can inhibit mycobacterial intracellular growth in infected macrophages without cytotoxic effects in macrophages culture. Erythratidinone is a promising substance for further studies aimed at the search for new anti-TB drugs as it seems to be a good candidate to be used as adjunct on TB therapy where exacerbated inflammation takes place.

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SUPPLEMENTARY MATERIAL

Figure S1. UPLC-UV chromatograms at 280 nm erythratidinone (1). Peak purity index 0,9831.
Figure S2. UPLC-UV chromatograms at 280 nm alpinumisoflavone (2). Peak purity index 0.9589.
Figure S3. UPLC-UV chromatograms at 280 nm erysenegalensein M (3). Peak purity index 0,873243.
Figure S4. ^{1}H NMR spectrum of erythratidinone in $CDCl_3$.
Figure S5. ^{13}C NMR spectrum of erythratidinone in $CDCl_3$.
Figure S6. 2D HSQC experiment of erythratidinone in $CDCl_3$.
Figure S7. 2D HMBC experiment of erythratidinone in $CDCl_3$.
Figure S8. ^{1}H NMR spectrum of alpinumisoflavone in $CDCl_3$.
Figure S9. ^{13}C NMR spectrum of alpinumisoflavone in $CDCl_3$.
Figure S10. 2D HSQC experiment of alpinumisoflavone in $CDCl_3$.
Figure S11. 2D HMBC experiment of alpinumisoflavone in $CDCl_3$.
Figure S12. ^{1}H NMR spectrum of erisenegalensein M in DMSO-d_6.
Figure S13. 2D HSQC experiment of erisenegalensein M in DMSO-d_6
Figure S14. 2D HMBC experiment of erisenegalensein M in DMSO-d_6.
Table SI. 1H (500 MHz) and 13C (125 MHz) NMR spectral data of erythratidinone, in CDCl3 (δ in ppm; J in Hz).

Table SII. 1H (500 MHz) and 13C (125 MHz) NMR spectral data of alpinumisoflavone, in CDCl3(δ in ppm; J in Hz).

Table SIII. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of erisenegalensein M, in DMSO-d_6 (δ in ppm; J in Hz).

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