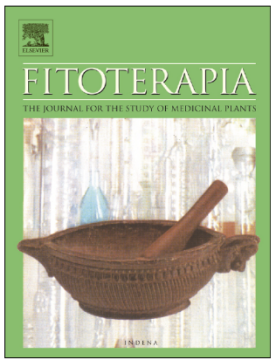

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Antiplasmodial and Antileishmanial Flavonoids from *Mundulea sericea*

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Abstract: A new flavanonol, 3-hydroxyerythrisenegalone (**1**), and four known compounds (**2**-**5**) were isolated from the extract of *Mundulea sericea* leaves. Investigation of the roots of this plant afforded an additional three known compounds (**6**-**8**). The structures were elucidated using NMR spectroscopic and mass spectrometric analyses. The absolute configuration of **1** was established using ECD spectroscopy. In an antiplasmodial activity assay, compound **1** showed good activity with an IC_{50} of 2.0 μM against chloroquine resistant W2, and 6.6 μM against the chloroquine-sensitive 3D7 strains of *Plasmodium falciparum*. Some of the compounds were also tested for antileishmanial activity. Dehydrolupinifolinol (**2**) and sericetin (**5**) were active against drug-sensitive *Leishmania donovani* (MHOM/IN/83/AG83) with IC_{50} values of 9.0 and 5.0 μM, respectively. In a cytotoxicity assay, erythrisenegalone (**3**) showed significant activity on BEAS-2B (IC_{50} 4.9 μM) and to HePG2 (IC_{50} 10.8 μM) human cell lines. All the other compounds showed low cytotoxicity (IC_{50} > 30 μM) against human lung adenocarcinoma cells (A549), human liver cancer cells (HepG2), lung/bronchus cells (epithelial virus transformed) (BEAS-2B) and immortal human hepatocytes (LO2).

Keywords: *Mundulea sericea;* Leguminosae; flavanonol; flavonol; antiplasmodial; antileishmanial; cytotoxicity.

1. Introduction

Protozoan infections are responsible for serious human diseases, such as amoebiasis, Chaga's disease, malaria, African sleeping sickness, leishmaniosis, and toxoplasmosis [1] that cause more than a million deaths annually [2]. The protozoan parasites that cause these diseases live in human blood or tissue, and are transmitted via blood probing insect vectors, mosquitoes or sand flies [3]. These diseases constitute the major health challenges for sub-Saharan countries including Kenya and Indian subcontinents [4–6]. In 2018, 228 million malaria cases occurred with 405 000 deaths worldwide. Leishmaniosis is a disease complex (visceral, cutaneous and mucocutaneous form) with estimated 0.7 - 1 million new cases annually. In 2018, more than 95% of new visceral leishmaniosis occurred in Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan, with an estimated 85% of new cutaneous leishmaniosis cases added from Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, the Syrian Arab Republic and Tunisia. Due to the development of resistance to current drugs [5,7,8], there is an urgent need to find alternative leads to fight malaria and leishmaniosis.

The plant genus *Mundulea* (family Leguminosae) is known for wide uses in traditional medicinal practices [9–12]. This family is a source of flavonoids and isoflavonoids, which have shown anticancer, [13,14] antimicrobial [15], antioxidant and antiplasmodial [16,17] activities. We report the isolation and characterization of a new flavonoid along with seven known compounds from the leaves and roots of *Mundulea sericea*. The antiplasmodial and antileishmanial activities, the cytotoxicity, and the induction of nitric oxide (NO) production in murine cells, *in vitro* (indicator of antileishmanial activity) have been determined for selected compounds.

2. Results and Discussion

The air dried and powdered leaves of *Mundulea sericea* were extracted with CH_2Cl_2/MeOH (1:1). The extract was subjected to a combination of chromatographic separations that yielded five compounds (**1**-**5**, Figure 1) of which one is new. These were characterized using NMR spectroscopy and mass

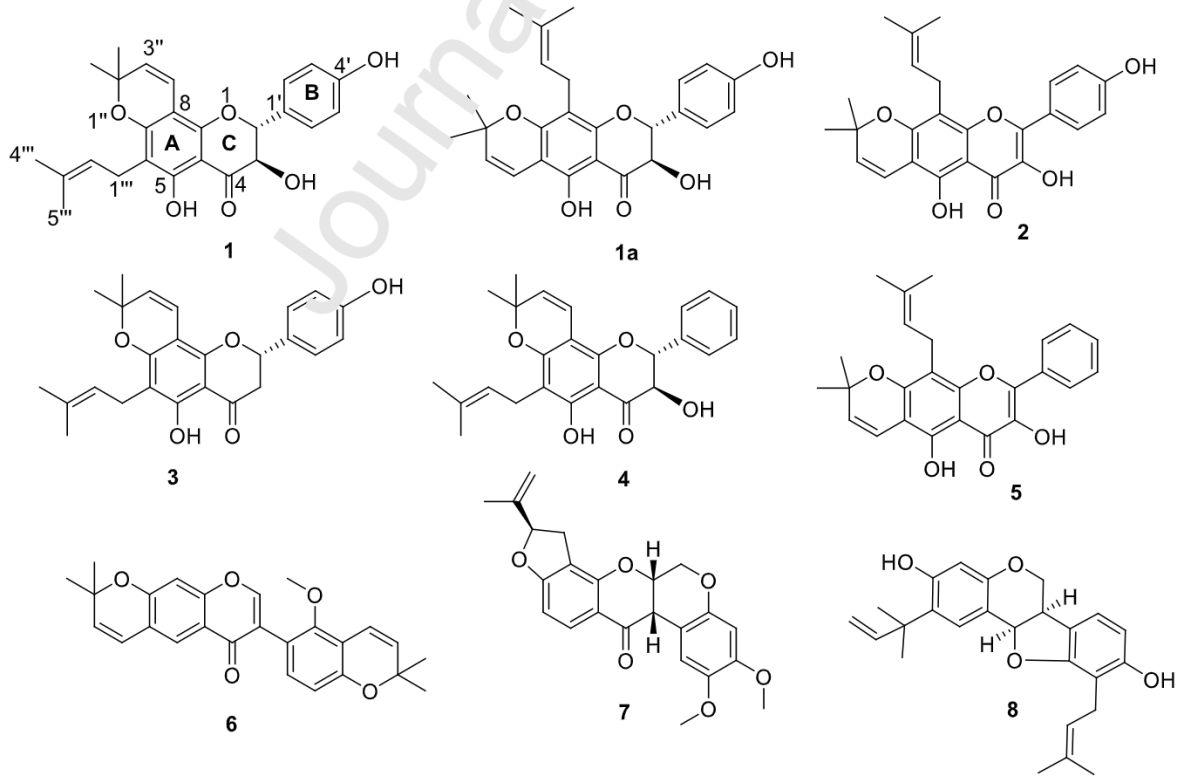

Figure 1. Structures of isolated compounds. Structure **1a** was considered as an alternate structure for compound **1** but was ruled out based HMBC NMR data.

spectrometry, and the new compound was determined to be a flavanonol, 3-hydroxyerythrisenegalone (**1**). The known compounds were identified as dehydrolupinifolinol (**2**) [18] erythrisenegalone (**3**) [19], isomundulinol (**4**) [13] and sericetin (**5**) [20] by comparison of their spectroscopic data with the corresponding data in the literature. Similar investigation of the roots of this plant led to the isolation of three known compounds, namely mutenone (**6**) [14], rotenone (**7**) [21] and striatine (**8**) [22].

Compound **1** was obtained as a white amorphous solid. Its molecular formula, $C_{25}H_{26}O_6$, was determined by HREI-MS, which showed a $[M+H]^+$ peak at *m/z* 423.1807. The UV (λ_{max} 260, 320 nm) and NMR spectral data (Table 1) are consistent with the presence of a flavanonol skeleton [23]. The ^{1}H NMR spectrum showed typical signals for ring C protons of a flavanonol at δ_H 4.98 (H-2), 4.51 (H-3) and 3.60 (3-OH). In agreement with this, the ^{13}C NMR spectrum showed signals at δ_C 83.0 (C-2), δ_C 72.6 (C-3) and δ_C 196.2 (C-4). The nature of ring C was confirmed by the HMBC correlations of H-2 with C-3, C-4 and C-1', as well as by those of H-3 with C-2, C-4 and C-1' (Table 1).

In ring B, the ^{1}H NMR spectrum showed an AA'XX' spin system at δ_H 6.84 (H-3'/5') and δ_H 7.40 (H-2'/6') with corresponding carbon resonances at δ_C 115.6 (C-3'/5') and 128.1 (C-6'/2'), and with oxygenation at C-4' (δ_C 156.4). Ring A is fully substituted with a hydroxy group at C-5 (δ_H 11.37), a 2,2-dimethylpyrano ring, and γ,γ-dimethylallyl moiety. Considering that the C-5 and C-7 positions of flavononols are expected to be oxygenated on the basis of biosynthesis, there are two possible structures (**1** and **1a**) differing in the placement of the pyrano ring and the prenyl unit. One of the olefinic protons of the pyran ring, H-4" (δ_H 6.64), showed HMBC correlation with C-8 (δ_C 103.3), indicating the location of this group at C-7/C-8, and hence the location of the prenyl group is at C-6. Moreover, H-1"' of the prenyl group showed HMBC correlation with C-5 (δ_C 159.5) and C-7 (δ_C 160.9). Based on the above spectroscopic evidence, the gross structure of compound **1** was characterized as (2*R*,3*R*)-3,5-dihydroxy-2-(4-hydroxyphenyl)-8,8-dimethyl-6-(3-methylbut-2-en-1-yl)-2,3-dihydro-4H,8H-pyrano[2,3-f]chromen-4-one, and named 3-hydroxyerythrisenegalone.

Its relative configuration at C-2/C-3 was determined as *trans* from the large vicinal coupling constant (*J* = 11.9 Hz) between H-2 (δ_H 4.98, *d*) and H-3 (δ_H 4.51, *d*), suggesting a 1,2-diaxial relationship of these protons. Hence, two absolute configurations (2*R*,3*R*) and (2*S*,3*S*), were possible [23]. The electronic circular dichroism (ECD) spectrum (Figure 2) showed a negative Cotton effect within the range of the $\pi\rightarrow\pi^*$ transitions (*ca*. 300–340 nm), consistent with the (2*R*,3*R*) absolute configuration of **1** [23].

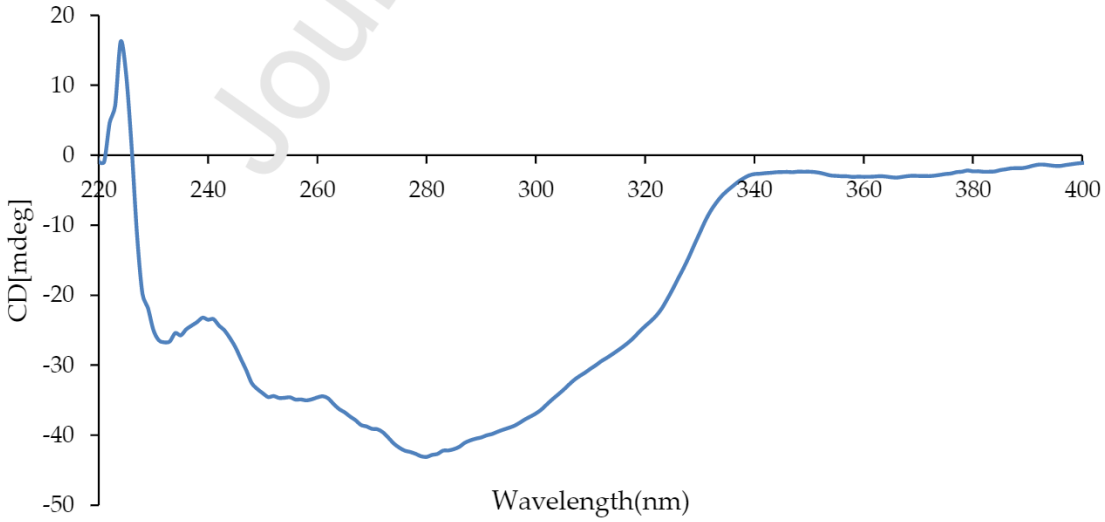

Figure 2. ECD spectrum of **1** (in methanol, 0.01 M).

Table 1. ^{1}H (800 MHz) and ^{13}C (200 MHz) NMR data for **1**, in $CDCl_3$

Position	δ_C	δ_H, mult. (*J* in Hz)	HMBC (^2J, ^3J)
2	83.0	4.98 *d* (11.9)	C-3, C-4, C-1'
3	72.6	4.52 *dd* (11.9, 1.6)	C-2, C-4, C-1'
4	196.2		
4a	100.4		
5	159.5		
6	109.4		
7	160.9		
8	103.3		
8a	156.4		
1'	128.8		
2'/6'	129.1	7.40 AA'	C-2, C-1', C-3'/5', C-4'
3'/5'	115.6	6.84 XX'	C-1', C-2'/6', C-4'
4'	156.4		
2"	78.6		
3"	126.5	5.53 *d* (10.1)	C-8, C-2"
4"	115.5	6.64 *d* (10.0)	C-7, C-8, C-8a, C-2"
2"-$(CH_3)_2$	28.5	1.45	C-2", C-3", C-4"
1'''	21.4	3.19 *m*	C-5, C-7, C-2''', C-3''',
2'''	122.2	5.12 *m*	C-1''', C-4''', C-5'''
3'''	131.5		
4'''-CH_3	26.0	1.64 br s	C-2''', C-3''', C-5'''
5'''-CH_3	17.9	1.60 br s	C-2''', C-3''', C-4'''
5-OH		11.37 *s*	C-4a, C-5, C-8, C-8a

Flavonoids have previously been reported to be effective antimalarial and antileishmanial agents both *in vitro* and *in vivo* [24,25], prompting the evaluation of the bioactivities of the crude extract and isolated compounds in this study. The crude extract of the roots of *Mundulea sericea* was tested for antiplasmodial activity against chloroquine-resistant (W2) and chloroquine-sensitive (3D7) strains of *Plasmodium falciparum* using an established protocol (for details see Supplementary Information) [26]. The extract showed antiplasmodial activity with IC_{50} values of 0.6 µg/ml and 1.8 µg/ml, against W2 and 3D7 strains, respectively. Some of the isolated compounds from this plant were also tested for antiplasmodial activity (Table 2). Compound **1** showed antiplasmodial activity with IC_{50} value of 2.0 µM against the W2 and 6.6 µM against the 3D7 strains, while isomundulinol (**4**) showed IC_{50} of 5.9 µM. Selected compounds were also evaluated for antileishmanial activity against *L. donovani* using an antimony-sensitive (MHOM/IN/83/AG83) and an antimony-resistant *strain* (MHOM/IN/89/GE1) (Table 2). Sericetin (**5**) showed antileishmanial activity against the antimony-sensitive (IC_{50} 5.0 µM), and 38.0 µM against antimony-resistant (IC_{50} 38.0 µM) strains. Dehydrolupinifolinol (**2**) was also active (IC_{50} 9.0 µM) against the antimony-sensitive strain.

Nitric oxide (NO) is considered to be a crucial host anti-leishmanial defense substance. Compounds **2** and **5** showed visible increases in NO production in a cell culture with respect to a control in an amastigote assay. Compound **2** induced the highest NO production (3.3-fold) in the test cells, conferring a stronger NO-mediated protection.

Table 2. Antiplasmodial, and antileishmanial activities together with cytotoxicity of compounds isolated from *Mundulea sericea*

	Antiplasmodial Activity (IC_{50}, µM)		Antileishmanial Activity (IC_{50}, µM)		Nitric Oxide Gene-ration	Cytotoxicity (IC_{50}, µM)				
Sample	W2	3D7	MHOM/IN/83/AG83	MHOM/IN/89/GE1		RAW 264.7	A549	HeP-G2	LO2	BEAS-2B
1	2.0	6.6	>100	>100	NT	NT	45.7	45.2	39.7	36.6
2	NT	NT	9.0	>100	3.3*	40.9	>100	>100	>100	>100
3	12.1	3.6	>100	>100	NT	NT	98.8	10.8	36.6	4.9
4	5.9	2.4	>100	>100	NT	NT	>100	>100	>100	>100
5	NT	NT	5.0	38.0	1.0*	31.4	>100	>100	>100	>100
CQ	0.08	0.008	NT	NT	NT	NT	NT	NT	NT	NT
MF	NT	NT	5.5	6.7	NT	19.85	NT	NT	NT	NT
PT	NT	NT	NT	NT	NT	NT	0.0033	0.19	<0.1	<0.1

MF: Miltefosine; PT: Paclitaxel; NT: Not Tested; CQ: chloroquine; W2: chloroquine-resistant strain of *P. falciparum;* 3D7: chloroquine-sensitive strain of *P. falciparum;* MHOM/IN/83/AG83: antimony-sensitive *L. donovani;* MHOM/IN/89/GE1: antimony-resistant *L. donovani;* RAW 264.7 = Abelson murine leukemia virus-induced tumor; A549 = human lung cancer cells; HePG2 = human liver cancer cells; LO2 = human hepatocytes normal cells; BEAS-2B = human hepatocytes normal cells
*values indicate the number of fold change with respect to control

The isolated compounds were also evaluated for their cytotoxicity against human lung adenocarcinoma (A549), human liver cancer (HepG2), human lung/bronchus cells (epithelial virus transformed, BEAS-2B) and immortal human hepatocytes (LO2) (Table 2). Dehydrolupinifolinol **(2),** isomundulinol (**4**) and sericetin (**5**) did not show significant toxicity against any of the cell lines (IC_{50} >100 µM). 3- Hydroxyerythrisenegalone (**1**) was moderately cytotoxic to the normal cells BEAS-2B (IC_{50} 36.6 µM) and LO2 (IC_{50} 39.7 µM), while erythrisenegalone (**3**) was moderately cytotoxic to LO2 (IC_{50} 36.6 µM) and strongly cytotoxicity to BEAS-2B (IC_{50} 4.9 µM) (Table 2).

3. Materials and Methods

3.1. General Experimental Procedures

The solvents used in extraction and chromatography were distilled in glass apparatus and the extracts concentrated *in vacuo* on a rotary evaporator. Analytical TLC was performed on Merck pre-coated silica gel 60 F_{254} plates. Column chromatography (CC) was run on silica gel 60 (70-230 mesh) and Sephadex LH-20. Structures of the metabolites were determined using mass spectrometry and NMR spectroscopy. CD experiments were run on a JASCO J-810 spectropolarimeter. UV spectroscopy was performed on a Shimadzu spectrophotometer.

3.2. Plant materials

Mundulea sericea was collected from a coastal area in Kenya in July 2017. The plant was authenticated by Pactrick C. Mutiso of the University Herbarium, School of Biological Sciences, University of Nairobi, where a voucher specimen (PCM. 2017/23) was deposited.

3.3. Extraction and isolation

3.3.1. Isolation of compounds from the leaves of *Mundulea sericea*

Air dried leaves of *Mundulea sericea* (835 g) were extracted (4 x 4 L) with CH_2Cl_2 /MeOH (1:1) at room temperature to yield a crude extract after the solvent was evaporated (113.8 g). The crude extract was adsorbed on silica gel, loaded onto a 500 g silica gel column, and eluted with *n*-hexane containing increasing amounts of EtOAc (1 to 80% v/v). The eluents were pulled into 20 fractions. From the fraction that was eluted with 2% EtOAc in *n*-hexane compound **1** (20 mg) was isolated as white amorphous solids after further purification on a silica gel column (50 g), and eluted with *n*-hexane containing increasing amounts of CH_2Cl_2 (1 to 99% v/v). The fraction eluted with 3% EtOAc in *n*-hexane was washing with *n*-hexane and gave compound **4** (16 mg) as a yellow paste. The fraction eluted with 4% EtOAc in *n*-hexane afforded compound **5** (17 mg) as yellow crystals from CH_2Cl_2/*n*-hexane. The fraction eluted with 10% EtOAc in *n*-hexane yielded compound **3** (22 mg) as a white amorphous solid after further purification on a silica gel (50 g) column, eluting with *n*-hexane containing increasing amounts of CH_2Cl_2 (1 to 99% v/v). The fraction eluted with 75% EtOAc in *n*-hexane gave compound **2** (10 mg) as yellow amorphous solid after further purification on a Sephadex LH 20 column, eluting with CH_2Cl_2 /MeOH (1:1) as the eluent.

3-Hydroxyerythrisenegalone (1). White amorphous solid; UV (MeOH, λ_{max}): 260, 320 nm; ECD (MeOH): Figure 2; ^{1}H NMR (800 MHz, $CDCl_3$): Table 1; ^{13}C NMR (200 MHz, $CDCl_3$): Table 1; HRMS found: 423.1807 calculated for $[M+H]^+$ $C_{25}H_{27}O_6$: 423.1808.

3.3.2. Isolation of compounds from the roots of *Mundulea sericea*

Air dried roots of *Mundulea sericea* (965 g) were extracted (4 x 4 L) with CH_2Cl_2 /MeOH (1:1) at room temperature and the solvent removed under reduced pressure. The crude extract (91.2 g). was adsorbed on silica gel, loaded onto silica gel (500 g) column, and eluted with *n*-hexane containing increasing amounts of EtOAc (1 to 99% v/v). The eluents were then pulled into 24 fractions. The fraction eluted with 6% EtOAc in *n*-hexane gave compound **6** (30 mg) as colourless crystals after

further purification on a silica gel (50 g) column eluting with *n*-hexane containing increasing amounts of CH_2Cl_2 (1 to 99% v/v). The fraction eluted with 6% EtOAc in *n*-hexane afforded compound **7** (17 mg) as white solids after further purification on Preparative TLC with *n*-hexane/EtOAc (7:3) as eluent. The fraction eluted with 6% EtOAc in *n*-hexane afforded compound **8** (50 mg) as yellow paste after further purification on centrifugal TLC, eluting with *n*-hexane/EtOAc (7:3).

3.4. In vitro antiplasmodial activity assay

The antimalarial reference drugs alongside pure compounds and crude extracts were tested for antiplasmodial activity against chloroquine resistant clone (W2) and chloroquine sensitive (3D7) using established protocol [3,26] to raise fluorescence of stained nucleic acids and enhance assay sensitivity. Briefly, the components of the lysis buffer were adjusted and the amount of the SYBR Green stain increased as described by Cheruiyot and others [27].

3.5. Antileishmanial activity assay

Antileishmanial activity of pure compounds were evaluated against antimony-sensitive *L. donovani* (MHOM/IN/83/AG83) and antimony-resistant *L. donovani* (MHOM/IN/89/GE1), originally obtained from the Indian Institute of Chemical Biology, Jadavpur, Kolkata, and maintained in BALB/c mice in the animal facility of West Bengal State University, (WBSU) as per the guidelines of Animal Ethics Committee, WBSU, Barasat, West Bengal, India [28]. The anti-proliferative effect of the selected compounds were estimated on *L. donovani* AG83 (MHOM/IN/1983/AG83) and *L. donovani* GEI (MHOM/IN/89/GEI) promastigotes as per the guidelines of Biosafety Committee, WBSU, Barasat, West Bengal, India [28]. For AG83, promastigotes were transformed from intracellular amastigotes taken from infected-spleen of BALB/c mice in complete M199 medium (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% FCS (GIBCO) at required temperature (22-24 °C). To estimate the percentage of inhibition, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micro method was used [28–30]. Briefly, the cultures of promastigotes were incubated without (control) or with the increasing concentrations of selected compounds in complete M199 medium for 48h (96-well plate, 200 µL per well, BD Falcon).. Equal volume of DMSO was added in control experiments. After 48h incubation, MTT (5 mg/mL, 20 µL per well) was added to each well and the plate was incubated for another 4h at 37 °C. The reaction was then stopped with acidic isopropanol (0.4 mL 10 N HCl in 100 mL isopropanol, 100 µL per well), and the absorbance was measured at 595 nm in a microplate reader (Bio-Rad, USA). The 50% inhibitory concentrations were determined from the plots of percent inhibition against increasing concentrations. Cytotoxic effect of the selected active compounds was also evaluated on RAW 264.7 cells in comparison to the reference drug Miltefosine. Nitric Oxide generation from RAW 264.7 cells was assayed by using Griess reagent [31]. Briefly, cells supernatants were collected (at 60h), Nitric Oxide generation was assayed by using Griess reagent [31], briefly, for the estimation of nitric oxide (NO) in RAW 264.7 cells, cells supernatants were collected and distributed (100 µL per well) in 96-well plates, and an equal volume of Griess reagent was added to each well, incubated for 15 min at 37^{o}C, and the absorbance was taken at 540 nm by an microplate reader (Bio-Rad, USA) [32]. Three or more independent experiments were performed in triplicate for each compound. Statistical analyses for all experiments were performed by one-way ANOVA followed by post hoc Holm-Sidak test with Sigma Plot software (version 11.0) [30].

3.6. Cell Culture

A549, HepG2, and non-tumor cells were all purchased from ATCC. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics penicillin (50 U/mL) and streptomycin (50 µg/mL; Invitrogen, Paisley, Scotland, UK). All cell cultures were incubated at 37°C in a 5% humidified CO_2 incubator

3.6. Cytotoxicity assay

Cytotoxicity of the isolated compounds were evaluated against human lung adenocarcinoma cell line (A549), human liver cancer cell line (HepG2), lung/bronchus cell line (epithelial virus transformed) (BEAS-2B) and immortal human hepatocytes (LO2). All tested compounds were dissolved in DMSO at a final concentration of 50 mmol/L and stored at −20°C before use. Cytotoxicity was assessed by using the MTT (5.0 mg/mL) assay as previously described [33]. Briefly, 4×10^3 cells were seeded in 96-well plates per well and then culture for overnight. The cells were then exposed to various concentrations of selected compounds with dosage ranged from 0.039–100 µmol/L for another 72 h. Cells without drug treatment were used as the control. Subsequently, MTT (10 µL) solution was added to each well and incubated at 37°C for 4h followed by the addition of 100 µL solubilization buffer (10% SDS in 0.01 mol/L HCl) and overnight incubation. A570 nm was then determined in each well on the next day. The percentage of cell viability was calculated using the following formula: Cell viability (%) $=A_{treated}/A_{control}$ ×100. Data were obtained from three independent experiments and the standard error was calculated.

5. Conclusions

The discovery of natural products and the evaluation of their bioactivity contribute to the development of new pharmaceutical treatments for diseases, such as malaria and leishmaniasis. In this study, five compounds were isolated from the leaves of *M. sericea*, including one new compound, 3-hydroxyerythrisenegalone (**1**). An additional three known compounds were isolated from the roots of this species. 3-Hydroxyerythrisenegalone (**1**) showed good activity against the W2 and the 3D7 strains of *Plasmodium falciparum*. Dehydrolupinifolinol (**2**) and sericetin (**5**) showed activity against *L. donovani* strains, with low or no cytotoxicity against different cell-lines, while 3-hydroxyerythrisenegalone (**1**) showed varying cytotoxicity against the tested cell lines. Compound **3** showed moderate anticancer activity and good selectivity index towards the liver HepG2 cancer cell line. These results show that prenylated flavonoids can potentially be used in the development of new treatments for malaria and leishmaniosis.

Supplementary Materials: The following are available online at www…: NMR and MS spectra for the new compound **1** and spectral data for the known compounds. The original FIDs for compounds **1**–**8**, and the NMReDATA file for the new compound **1** is available, free of charge open access, at Zenodo with DOI:10.5281/zenodo.3902746.

Author Contributions: The authors contributed to this work as follows. Extraction and isolation of compounds was performed by C.C. and P.J.O. under the supervision of A.Y., A.N. and S.D.; NMR analyses was performed with the help of M.H. and M.E.; Spectroscopic characterization of the compounds was carried out by C.C., A.Y., M.H. and M.E.; antileishmanial tests and NO induction assay were performed by B.S. and A.H. under the supervision of C.P.; antiplasmodial tests were done by H.M.A; cytotoxicity assays were performed by P.C. and L.J.Y. under the supervision of V.K.W.W. All authors contributed to the preparation of the manuscript.

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