Phenylpropanoid conjugated iridoids with anti-malarial activity from the leaves of *Morinda morindoides*

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Abstract

Two phenylpropanoid conjugated iridoids, deglucosyl gaertneroside (**1**) and morindoidin (**2**), were isolated from the leaves of *Morinda morindoides* (Rubiaceae) by activity-guided fractionation using an anti-malarial activity assay. The known related iridoids molucidin (**3**) and prismatomerin (**4**), two lignans, abscisic acid, two megastigmanes, and two flavonol glycosides were also identified. The structures of isolated compounds were elucidated using spectroscopic analysis. The isolated compounds were evaluated for anti-malarial activity against the chloroquine/mefloquine-sensitive strains of *Plasmodium falciparum* together with cytotoxicity against adult mouse brain cells. Potent anti-malarial activity of **3** and **4** (IC_{50} of 0.96 and 0.80 μM, CC_{50} of 1.02 and 0.88 μM, and SI of 1.06 and 1.10 μM, respectively) was shown, while new iridoids **1** and **2** and pinoresinol (**5**) displayed moderate activity (IC_{50} of 40.9, 20.6, and 24.2 μM) without cytotoxicity (CC_{50} > 50 μM). These results indicate that **1**–**5** may be promising lead compounds for anti-malarial drugs. In addition, our results imply the necessity of the quality control of the extract of *M. morindoides* leaves based on the contents of **1**–**5** in terms of the safety and efficacy.

Introduction

Malaria remains a life-threatening parasitic disease and is a serious human health problem in wide areas of tropical and subtropical countries. Transmission of *Plasmodium* parasites through infected female *Anopheles* mosquitoes to humans causes malaria. There were 229 million estimated cases of malaria in 2019, with more than 400,000 deaths. The most susceptible group affected by malaria are children aged under 5 years. Malaria control and therapy are still challenging issues due to parasite resistance to therapeutic drugs and mosquito resistance to insecticides [1]. Although there has been much effort to develop malaria vaccines for decades, no effective vaccine has been approved. Hence, anti-malarial chemotherapy and vector control are the basic options against malaria infection. Therefore, the discovery of therapeutic agents with potential anti-malarial effects is greatly anticipated [2-7].

In the Democratic Republic of Congo, the plant *Morinda morindoides* (Baker) Milne-Redh. (Rubiaceae) (synonym: *Gaertnera morindoides* Baker or *Morinda confusa* Hutch.) is called Nkonga bululu in Tshiluba, and Nkongo bololo or Nkama meso in Lingala and Kikongo. Our field investigation found that a decoction of the leaves has been used as a traditional remedy for several health problems, including infectious disease (intestinal worms, amoebiasis, malaria, and gonorrhea), fever, diabetes, and hemorrhoids. The traditional use of the leaves of this plant in other African countries has been described [8]. The fractions obtained from the leaf extract by solvent partition have been reported to possess anti-plasmodial activity [9], anti-microbial activities against some skin diseases [10], and anti-fungal activity [11] by *in vitro* assay. Extensive phytochemical research on the leaves has identified flavonoids [12], iridoids [13, 14], and anthraquinones [15], and on the roots has identified ketosteroid [16]. Flavonoids from *M. morindoides* have anti-complementary [17], anti-oxidative [18], anti-amoebic [19], and anti-spasmodic activities [20] *in vitro*. Iridoid glucosides from this plant have anti-malarial [21] and anti-amoebic activities [22].

In the course of identifying the responsible bioactive compound in the crude drug materials used in the traditional medicine [23-25], we focused on the leaves of *M. morindoides*. Both *in vitro* and *in vivo* anti-malarial activity of the leaf extract of this plant have been reported [9, 26]. Based on these findings, development of a remedy using the leaf extract could be an effective strategy, because people in African countries have experienced the use of this plant as traditional medicine. Identification and characterization of the compounds with anti-malarial activity are necessary to select both leaves and a suitable solvent for the preparation of safe and effective remedy. We also found anti-malarial activity in methanol (MeOH) and ethanol (EtOH) extracts of the leaves. Several anti-malarial phenylpropanoid conjugated iridoids were reported from this plant, including gaertneroside and dehydromethoxy gaertneroside [21]. In our preliminary study, the presence of new iridoids along with known anti-malarial iridoids in the leaf extract were indicated by LC/MS analysis.

Described herein are the activity-guided isolation and structure elucidation of two new phenylpropanoid conjugated iridoids, named deglucosyl gaertneroside (**1**) and morindoidin (**2**), which were isolated along with nine known compounds (**3**–**11**) from *M. morindoides* leaves (Fig. 1). These isolated compounds were investigated for anti-malarial activity.

Experimental

General experimental procedures

Optical rotations were recorded on a JASCO P-2100 digital polarimeter; ECD and UV spectra were measured on a JASCO J-805 spectropolarimeter. The IR spectra were measured with a JASCO FT/IR-460 Plus spectrophotometer. The ^{1}H (400 and 500 MHz) and ^{13}C (100 and 125 MHz) NMR spectra were recorded using a JEOL ECX400 and ECA500II Delta spectrometer, and chemical shifts are expressed in δ values. The high-resolution electrospray ionization mass spectrometry (HRESIMS) and LC/MS data were obtained on a hybrid ion trap time-of-flight (IT-TOF) mass spectrometer (Shimadzu, Kyoto, Japan). For LC/MS, a YMC-Pack Pro C_{18} RS column (150 × 2.0 mm, S-3 μm, 8 nm) and a gradient solvent system using CH_3CN + 0.1% formic acid/H_2O + 0.1% formic acid was employed. Column chromatography was performed with normal-phase (Wakogel® C-200, Wako Pure Chemical Industries Ltd, Osaka, Japan), and Sephadex LH-20 (GE Healthcare Life Sciences, Uppsala, Sweden). The medium-pressure liquid chromatography (MPLC) was performed on a Biotage Isolera™ One apparatus (Biotage, Uppsala, Sweden) equipped with Isolera™ Spektra Systems by using Biotage® SNAP Ultra C_{18} 30, 60, and 120 g (25 μm) cartridge pack. Preparative HPLC was conducted on a YMC-Pack R and D ODS-A column 250 × 20 mm, S-5 μm, 12 nm, using (CH_3CN + 0.1% formic acid) – (H_2O + 0.1% formic acid) gradient mixture (15–100%), flow rate: 10.0 mL/min, and UV detection at 254 nm equipped with a Waters Delta 600 pump and a Waters 2489 UV/visible detector. Analytical and preparative TLC were carried out on precoated RP-18F_{254}s plates (0.25 Merck KGaA, Darmstadt, Germany). Commercially available oleanolic acid (**12**) (Wako Pure Chemical Industries Ltd) was used for anti-malarial assays. All solvents used for isolation were of analytical grade (Wako Pure Chemical Industries Ltd).

Plant Material

Leaves of *M. morindoides* were collected in the suburbs of Kinshasa, Democratic Republic of Congo, in 2016 and identified by Prof. José Nzunzu Lami, Faculty of Pharmaceutical Sciences, University of Kinshasa. The voucher specimen (KB004) was deposited in the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and fractionation for anti-malarial assay

The air-dried and ground leaves (2.0 g) of *M. morindoides* were extracted with MeOH or EtOH (50 mL, three times) by ultrasonication for 30 min at room temperature. After removal of the solvent, 127 mg of MeOH extract and 70 mg of EtOH extract were obtained.

Hot water (soaking) extract (326.6 mg) was prepared by soaking extraction with hot water (85°C, 50 mL, 30 min, two times) from 2.0 g of ground leaves. Boiled water extract (350 mg) was prepared by extraction with boiling water (60 mL, 30 min) from 2.0 g of ground leaves.

The air-dried and ground leaves of *M. morindoides* (1060 g) were subjected to extraction with 80% MeOH (3 L, 16 times), then residue was extracted with 50% EtOH (6 L, two times) at room temperature. The extracts were combined and concentrated under reduced pressure to yield an aqueous alcohol extract (144.8 g).

A portion of aqueous alcohol extract (600 mg) was subjected to preparative ODS HPLC [YMC-Pack R and D ODS-A column (250 × 20 mm , S-5 μm, 12 nm), flow rate: 10.0 mL/min, solvent A: water with 0.1% formic acid; solvent B: CH_3CN with 0.1% formic acid; gradient elution 15% B (0–10 min), 50% B (30 min), 100% B (45–60 min), UV detection at 254 nm] to yield Frs. A1 (256 mg, t_R 0–11.0 min), A2 (46.8 mg, 11.0–22.5 min), A3 (14.5 mg, 22.5–23.2 min), and A4 (78.5 mg, 23.2–60 min).

A portion of aqueous alcohol extract (360 mg) was subjected to preparative ODS HPLC [YMC-Pack R and D ODS-A column (250 × 20 mm, S-5 μm, 12 nm), flow rate: 10.0 mL/min, solvent A: water with 0.1% formic acid; solvent B: CH_3CN with 0.1% formic acid; gradient elution 15% B (0–13 min), 50% B (55 min), 100% B (58–60 min), UV detection at 254 nm] to yield Frs. B1 (181.5 mg, t_R 0–28.2 min), B2 (6.0 mg, t_R 28.2–31.5 min), B3 (6.3 mg, t_R 31.5–33.3 min), B4 (10.3 mg, t_R 33.3–37.5 min), B5 (16.4 mg, t_R 37.5–45.3 min), and B6 (8.3 mg, t_R 45.3–60 min).

Fractionation for compound isolations

A portion of aqueous alcohol extract (140 g) was suspended in water (1500 mL) and partitioned successively with hexane (seven times, 9.6 L), $CHCl_3$ (10 times, 10.5 L), ethyl acetate (EtOAc) (nine times, 9.2 L), 1-butanol (1-BuOH) (10 times, 10.4 L), and water to give hexane- (7.8 g), $CHCl_3$- (20.2 g), EtOAc- (6.9 g), 1-BuOH- (39.2 g), and water- (60.6 g) soluble fractions, respectively. A portion of $CHCl_3$- soluble

fraction (18.6 g) was subjected to silica gel (300 g) open column chromatography using a hexane–EtOAc gradient mixture (0–100%) followed by 50% MeOH and 100% MeOH to give eight fractions (Frs. 1–Fr. 8).

A portion of Fr. 3 (2.3 g) was subjected to preparative HPLC to give five fractions (Frs. 3-1 to 3-5). A portion of Fr. 3-5 (815 mg) was subjected to preparative HPLC to give 12 fractions (Frs. 3-5-1 to 3-5-12). Fr. 3-5-3 (12 mg) was purified by Sephadex LH-20 column chromatography eluted with MeOH to give **8** (6.9 mg) and **5** (2.3 mg). The Fr. 3-5-8 (9.5 mg), Fr. 3-5-10 (5.8 mg), and Fr. 3-5-11 (4.8 mg) were purified by Sephadex LH-20 column chromatography (MeOH) to give **2** (4.4 mg), **4** (2.4 mg), and **3** (1.7 mg), respectively. A portion of Fr. 4 (198 mg) was subjected to preparative HPLC to give 10 fractions (Frs. 4-1 to 4-10). A portion of Fr. 4-4 (7 mg) was further purified by Sephadex LH-20 column chromatography (MeOH) to give **9** (6.4 mg).

A portion of Fr. 5 (2.9 g) was subjected to ODS MPLC (SNAP Ultra C_{18} 120 g cartridge) eluted with a gradient of 0–100% MeOH in water to obtain 17 fractions (Frs. 5-1 to 5-17). A portion of Fr. 5-9 (67 mg) was subjected to preparative HPLC to give 16 fractions (Frs. 5-9-1 to 5-9-16), then Fr. 5-9-11 (16 mg) was purified by Sephadex LH-20 column chromatography (MeOH) to give **6** (13.1 mg).

A portion of Fr. 7 (3660 mg) was subjected to ODS MPLC (SNAP Ultra C_{18} 120 g cartridge) eluted with a gradient of 0–100% MeOH in water to obtain 12 fractions (Frs. 7-1 to 7-12), then Fr. 7-8 (390 mg) was subjected to preparative HPLC to give **11** (1.8 mg).

A portion of EtOAc- soluble fraction (6.4 g) was subjected to silica gel (350 g) open column chromatography using a hexane–EtOAc gradient mixture (0–100%) followed by 50% MeOH and 100% MeOH to give six fractions (Frs. 9 to 14). A portion of Fr. 11 (845 mg) was subjected to ODS MPLC (SNAP Ultra C_{18} 30 g Cartridge) eluted with a gradient of 0–100% MeOH to give nine fractions (Frs. 11-1 to 11-9). A portion of Fr. 11-9 (302 mg) was subjected to preparative HPLC to give 11 fractions (Frs. 11-9-1 to 11-9-11). A portion of Fr. 11-9-6 (16.5 mg) was purified by Sephadex LH-20 column chromatography (MeOH) to give **1** (7.1 mg). A portion of Fr. 11-9-10 (5 mg) was purified by preparative ODS TLC, using a solvent system of MeOH–H_2O (7:3), to yield **7** (2.1 mg). A portion of Fr. 12 (1.1 g) was rechromatographed on ODS MPLC (SNAP Ultra C_{18} 30 g cartridge) eluted with a gradient of 0–100% MeOH to give 14 fractions (Frs. 12-1 to 12-14). A portion of Fr. 12-10 (46 mg) was subjected to preparative HPLC to give 12 fractions (Frs. 12-10-1 to 12-10-12). A portion of Fr. 12-10-3 (8.5 mg) was purified by preparative HPLC to give **10** (5.9 mg).

Deglucosyl gaertneroside (**1**): light-brown amorphous; $[\alpha]_D^{23}$ +190 (*c* 3.3, MeOH); UV (MeOH) λ_{max} nm (log ε) 231 (1.1); ECD (*c* 2.6 × 10^{-4} M, MeOH) λ_{max} nm (Δε) 245 (+3.5) and 222 (−1.7); IR (KBr) ν_{max} 3410, 2953, 1739, 1700, 1636, and 1614 cm^{-1}; ^1H NMR and ^{13}C NMR data in CD_3OD, see Table 2 and supplementary data; HRESIMS *m/z* 369.0954 $[M-H_2O+H]^+$ (calcd for $C_{20}H_{17}O_7$, 369.0969, Δ −1.5 mmu) and *m/z* 385.0935 $[M-H]^-$ (calcd for $C_{20}H_{17}O_8$, 385.0929, Δ +0.6 mmu).

Morindoidin (**2**): pale-yellow amorphous; $[\alpha]_D^{25}$ +469 (*c* 1.7, MeOH); UV (MeOH) λ_{max} nm (log ε) 239 (1.2); ECD (*c* 1.6 × 10^{-4} M, MeOH) λ_{max} nm (Δε) 235 (+27.8), 217 (+4.4), and 203 (+22.7); IR (KBr) ν_{max} 3428, 2954, 1771, 1697, 1645, and 1615 cm^{-1}; ^1H NMR and ^{13}C NMR data in CD_3OD, see Table 2 and supplementary data; HRESIMS *m/z* 369.0995 $[M-H_2O+H]^+$ (calcd for $C_{20}H_{17}O_7$, 369.0969, Δ +2.6 mmu), *m/z* 431.0969 $[M+HCOO]^-$ (calcd for $C_{21}H_{19}O_{10}$, 431.0984, Δ −1.5 mmu), and *m/z* 385.0952 $[M-H]^-$ (calcd for $C_{20}H_{17}O_8$, 385.0929, Δ +2.3 mmu).

Molucidin (**3**): $[\alpha]_D^{22}$ −319 (*c* 0.3, $CHCl_3$); ECD (*c* 2.0 × 10^{-4} M, MeOH) λ_{max} nm (Δε) 338 (−5.2), 264 (−0.7), 251 (−1.9), 231 (+1.9), and 223 (+1.2).

Prismatomerin (**4**): $[\alpha]_D^{24}$ −107 (*c* 1.4, MeOH); ECD (*c* 2.4 × 10^{-4} M, MeOH) λ_{max} nm (Δε) 325 (−12.7), 262 (−3.4), 232 (+3.7), and 216 (+5.8).

Culture of *P. falciparum* and primaryadult mouse brain cells

The *P. falciparum* chloroquine-mefloquine-sensitive (3D7) and -resistant (Dd2) strains were maintained in RPMI-1640-based complete medium supplemented with 5% AB^+ human serum (prepared from plasma), 2% hematocrit type O^+ red blood cells (RBCs), 0.25% AlbuMax I (Gibco, Waltham, MA, USA), 12.5 μg/mL gentamycin, and 200 mM hypoxanthine at 37°C under mixed gas (5% CO_2, 5% O_2, and 90% N_2) condition using a closed jar [27]. Human plasma and RBCs were obtained from the Japanese Red Cross Society (number: 28J0060). Primary adult mouse brain (AMB) cells were isolated and established in NEKKEN Bio-Resource Center, Institute of Tropical Medicine, Nagasaki University as previously described [28]. After several times of passage for adaptation, the primary cells were cultivated in RPMI-1640 media supplemented with 10% fetal bovine serum, penicillin/streptomycin solution (100 units/mL penicillin G and 100 mg/mL streptomycin sulfate, Wako Pure Chemicals Industries Ltd) at 37°C under 5% CO_2. After three passages, the cells were used for cytotoxicity assay.

Anti-malarial assay

A SYBR Green-based microfluorometric assay was employed for quantification of parasite level [29]. In brief, 50 μL of *P. falciparum* strains in a 96-well plate were incubated with 50 μL of sample solution for 48 h. Then, 100 μL of lysis buffer [20 mM Tris, 10 mM EDTA, 0.01% saponin (wt/vol), and 0.1% Triton X-100 (vol/vol)] containing 1 × the final concentration of SYBR Green-I (Lonza, Rockland, ME, USA) was added to the wells. The plates were then incubated at room temperature for 1 h on a shaker with gentle mixing in darkness. The fluorescence [excitation (Ex) at 485 nm and emission (Em) at 515 nm] was measured by plate reader (ARVO 1430; Perkin Elmer, Waltham, MA, USA) for 0.1 s per exposure.

The 50% inhibitory concentration (IC_{50}) was calculated using the following equation: **see equation 1 in the supplementary files section.**

where A is the lowest concentration value at which the percentage inhibition exceeded 50%, B is the highest concentration value at which percentage inhibition was less than 50%, and C and D are the percentage inhibition values of the sample at concentrations B and A, respectively. Artesunate and chloroquine were used as positive controls, while dimethyl sulfoxide (DMSO) was assigned as negative control. The final concentration of DMSO for all tested samples and negative and positive control was 0.5%.

Cytotoxicity assay

The AMB cells were seeded in a 96-well culture plate (black plate with clear bottom, 1×10^4 cells/100 µL/well) and cultivated at 37°C for 48 h in a CO_2 incubator. Then, test samples, or their positive and negative controls, were added to each well. After 48 h of incubation, 10 µL of Alamar Blue solution (10%, Funakoshi Co., Tokyo, Japan) was added to each well. The plates were incubated for 2 h, and the fluorescence (Ex at 530–560 nm and emission Em at 590 nm) of each well was measured in a multilabel plate reader (ARVO 1430; Perkin Elmer).
The concentration of sample required to reduce cell viability by 50% (CC_{50}) was calculated using the following equation: **see equation 2 in the supplementary files section.**

where A is the lowest concentration value at which the cell viability exceeded 50%, B is the highest concentration value at which cell viability cell was less than 50%, and C and D are the cell viability values of the sample at concentrations B and A, respectively. All assays were repeated three times in duplicate.

The IC_{50} and CC_{50} values were used as an indicator of *in vitro* anti-malarial activity and an indicator of cytotoxicity in AMB cells, respectively. The selectivity index (SI) was obtained by dividing the CC_{50} value by the IC_{50} value.

Results And Discussion

Activity-guided fractionation

The identification and structural characterization of the active compounds is the basis of ensuring the efficacy and safety for an anti-malarial remedy. To identify active compounds in *M. morindoides* leaves, activity-guided fractionation was conducted as follows. The crude extracts and crude fractions were tested for their anti-malarial activity against the chloroquine/mefloquine-sensitive (3D7) and -resistant (Dd2) strains of *Plasmodium falciparum* together with cytotoxicity against mammalian cells using AMB cells (Table 1). The data are presented as anti-malarial activity (IC_{50}) values, and cytotoxicity (CC_{50}) values which represent the 50% inhibitory concentrations.

First, to identify an active extract, four different extracts were prepared using MeOH alone, EtOH alone, hot water (soaking), or boiling water. The MeOH and EtOH extracts of the leaves showed anti-malarial activity with IC_{50} values of 66.5 and 54.0 µg/mL against 3D7 strain, and 94.4 and 63.0 µg/mL against Dd2 strain, respectively; however, the hot water-soaking and boiled water extracts did not show such activity (Table

1). The IC_{50} values against 3D7 and Dd2 strains were almost the same, indicating that the leaf extract of *M. morindoides* was active against both chloroquine/mefloquine-sensitive (3D7) and -resistant (Dd2) strains of *P. falciparum*. In our preliminary study, the MeOH extract prepared from a different batch (KB001) of *M. morindoides* leaves showed potent anti-malarial activity with IC_{50} values of 4.09 μg/mL against 3D7 strain (SI of 143.9), and 12.31 μg/mL against Dd2 strain (SI of 47.8). However, this batch was too small, and so we used another batch (KB004) for the present study. According to previous papers, IC_{50} values for *in vitro* anti-malarial activities of the EtOH extract of *M. morindoides* leaves against chloroquine sensitive strains of *P. falciparum* were 9.8, 11.6, and 94.2 μg/mL [9, 30, 31], suggesting the variation in activity.

Our results suggested that the active compounds had less polar properties; however, the boiled and hot water (soaking) extracts are the traditional methods to use this plant for anti-malarial or other medicinal purposes. The related compounds to active one, even though they do not have activity, could be polar or water soluble. Therefore, to extract the compounds in as wide a range of polarity as possible, aqueous alcohol was used for extraction in further study.

To identify the active fractions, the aqueous alcohol extract was separated into four fractions (Frs. A1–A4) by preparative ODS HPLC (Fig. 2a). Anti-malarial activity was observed in Fr. A4 with IC_{50} of 24.2 μg/mL, while other fractions did not show significant activity (IC_{50} > 300 μg/mL) (Table 1). This result indicated that active compounds may exist in Fr. A4. Next, to separate the subfractions in Fr. A4, the aqueous alcohol extract was separated into six fractions (Frs. B1–B6) by preparative ODS HPLC using gradient solvent in different ratios (Fig. 2b). In this fractionation, the fraction corresponding to Fr. A4 was separated into Frs. B2–B6. Potent or moderate anti-malarial activity was observed in Frs. B5 and B6, while weak activity was observed in Frs. B2–B4 (Table 1). Thus, the peaks with activity potential were estimated.

Sample	IC_{50} (μg/mL)[a]		CC_{50} (μg/mL)[b]	SI[c]	
	3D7	Dd2	AMB	3D7	Dd2
MeOH ext.	66.54	94.38	487.68[d]	7.33	5.17
EtOH ext.	53.96	62.95	274.28[d]	5.08	4.36
hot water-soaking ext.	>500	>500	>500[d]	ND[f]	ND[f]
boiled water ext.	>250	>250	>250[d]	ND[f]	ND[f]
artesunate	0.0039	0.0047	1.21[d]	311.25	255.90
chloroquine	0.0044	0.0508	nt[e]	ND[f]	ND[f]
aqueous alcohol ext.	87.62	91.27	371.06	4.24	4.07
Fr. A1	>500	>500	>500	ND[f]	ND[f]
Fr. A2	413.03	>500	>500	ND[f]	ND[f]
Fr. A3	334.00	>500	>500	ND[f]	ND[f]
Fr. A4	26.14	29.29	124.52	4.76	4.25
artesunate	0.0023	0.0023	0.35	155.55	151.95
chloroquine	0.0034	0.0643	>3.20	ND[f]	ND[f]
Fr. B1	>500	nt[e]	>500	ND[f]	ND[f]
Fr. B2	208.98	nt[e]	>500	ND[f]	ND[f]
Fr. B3	109.97	nt[e]	407.91	3.71	ND[f]
Fr. B4	178.21	nt[e]	>500	ND[f]	ND[f]
Fr. B5	70.70	nt[e]	339.53	4.80	ND[f]
Fr. B6	21.45	nt[e]	70.66	3.29	ND[f]
artesunate	0.0018	nt[e]	0.73	395.47	ND[f]
chloroquine	0.0042	nt[e]	>1.60	ND[f]	ND[f]

Table 1 Anti-malarial activities (IC_{50}) and cytotoxicities (CC_{50}) of the extracts and crude fractions

Values are the means from two or more independent experiments performed in duplicate.

[a] 50% inhibitory concentration against 3D7 or Dd2 strain.

[b] 50% cytotoxic concentration using AMB cells.

[c] selectivity index was obtained by dividing CC_{50} value by IC_{50} value.

[d] CC_{50} values are the mean of single experiment performed in duplicate.

[e] not tested

[f] not determined

Isolation and identification of compounds

Next, to isolate active compounds, the aqueous alcohol extract of the leaves was partitioned sequentially with hexane, $CHCl_3$, EtOAc, 1-BuOH, and water. Because active peaks in Frs. B5 and B6 were present in the $CHCl_3$- and EtOAc-soluble fractions, fractionations of the $CHCl_3$- and EtOAc-soluble fractions using silica gel, MPLC, Sephadex LH-20, and preparative ODS HPLC were conducted to yield two new phenylpropanoid conjugated iridoids, deglucosyl gaertneroside (**1**) and morindoidin (**2**), together with nine known compounds: two iridoids, molucidin (**3**) [32] and prismatomerin (**4**) [33]; two lignans, pinoresinol (**5**) [34] and syringaresinol (**6**) [35, 36]; a sesquiterpenoid abscisic acid (**7**) [37]; two megastigmanes, (+)-(6*R*,9*S*)-blumenol C (**8**) [38] and (+)-(6*S*,9*R*)-blumenol B (**9**) [39]; and two flavonol 3-*O*-glycosides, astragalin (**10**) [40] and rutin (**11**) [41]. Moreover, in our preliminary study, the peak of oleanolic acid (**12**) – for which anti-malarial activity has been reported [42, 43] – was detected (*m/z* 457.3662 $[M+H]^+$ and 455.3554 $[M-H]^-$) in both MeOH and EtOH extracts in the less polar part by LC/MS. The structures of these compounds (**1**–**12**) are summarized in (Fig. 1).

Compound **1** was isolated as light-brown amorphous shown to have the molecular formula $C_{20}H_{18}O_8$ on the basis of its HRESIMS data (*m/z* 385.0935, calcd for $C_{20}H_{17}O_8$ $[M-H]^-$, Δ +0.6 mmu). Analysis of its ^1H and ^{13}C NMR spectra coupled with HMQC indicated the presence of two ester carbonyl carbons (δ_C 172.5 and 168.7), eight sp^2 methines (δ_C 116.2 (2C), 129.6 (2C), 129.9, 141.5, 150.2, and 153.3), four sp^2 quaternary carbons (δ_C 110.0, 133.2, 137.8, and 158.6), an oxygenated sp^3 quaternary carbon (δ_C 98.1), two sp^3 oxymethines (δ_C 69.9 and 92.8), a methoxy (δ_C 51.9), and two sp^3 methines (δ_C 39.9 and 52.2) (Table 2). Consideration of unsaturation degree suggested the presence of four other rings in **1**, since 8 out of 12 degrees of unsaturation degree were accounted for. The ^1H and ^{13}C NMR spectra of **1** were analogous to that of the aglycon part in gaertneroside [13], a phenylpropanoid conjugated iridoid glucoside ($C_{26}H_{28}O_{13}$), except for absence of the signals ascribed to a glucose unit observed in gaertneroside. Detailed analysis of the ^1H–^1H COSY and HMBC data revealed that the planar structure of **1** was identical with the aglycon part of gaertneroside. Thus, **1** was named deglucosyl gaertneroside.

The stereochemistry of **1** was suggested to be the same as in gaertneroside [13], based on the similarity of their ^{1}H and ^{13}C NMR chemical shift values, including coupling pattern and biosynthetic considerations. The NOESY correlations of H-1/H-10 and H-7/H-10 indicated α-orientation of H-1 and 8*R* configuration, and correlations of H-5/H-9 indicated that H-5 and H-9 were β-orientated. Finally, the configuration of C-13 in **1** was determined to be *S* by the similarity of ECD spectra with negative and positive Cotton effects in MeOH at 222 and 245 nm, respectively [λ_{max} nm (Δε) 223 (−12.9) and 264 (+3.1) in MeOH for gaertneroside] [44].

Table 2 ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data in CD_3OD of **1** and **2**

	1			2		
Position	δ_H (*J* in Hz)	δ_C	type	δ_H (*J* in Hz)	δ_C	type
1	4.93, d (4.5)	92.8	CH	5.51, d (5.7)	103.2	CH
3	7.50, br s	153.3	CH	7.39, br s	154.3	CH
4		110.0	C		110.3	C
5	3.84, m	39.9	CH	3.92, dt (11.5, 2.3)	39.4	CH
6	6.47, dd (5.5, 2.9)	141.5	CH	5.95, dd (5.7, 2.3)	141.3	CH
7	5.55, dd (5.5, 1.7)	129.9	CH	5.85, dd (5.7, 2.3)	128.7	CH
8		98.1	C		108.7	C
9	2.83, dd (7.5, 4.6)	52.2	CH	3.43, dd (9.7, 5.7)	55.2	CH
10	7.24, s	150.2	CH	4.51, s	85.0	CH
11		137.8	C	3.06, d (2.6)	57.7	CH
12		172.5	C		178.3	C
13	5.35, brs	69.9	CH	5.22, d (2.6)	72.2	CH
14		168.7	C		168.6	C
1'		133.2	C		134.1	C
2'	7.25, d (8.6)	129.6	CH	7.23, d (8.0)	127.8	CH
3'	6.75, d (8.6)	116.2	CH	6.79, d (8.0)	116.3	CH
4'		158.6	C		158.0	C
5'	6.75, d (8.6)	116.2	CH	6.79, d (8.0)	116.3	CH
6'	7.25, d (8.6)	129.6	CH	7.23, d (8.0)	127.8	CH
14 - OCH_3	3.74, s	51.9	CH_3	3.73, s	52.0	CH_3

Morindoidin (**2**) was isolated as a pale-yellow amorphous compound and its molecular formula was suggested as $C_{20}H_{18}O_8$, the same as that of **1**, using HRESIMS data (*m/z* 385.0952, calcd for $C_{20}H_{17}O_8$ $[M-H]^-$, Δ +2.3 mmu). The ^1H and ^{13}C NMR chemical shift values (Table 2) at C-1 to C-9, C-14, and methoxy at C-14 of **2** were similar to those of **4**, indicating that the iridoid core structure of **2** was the same as for **4**. The presence of an ether bridge between C-1 and C-10 was confirmed by the HMBC

correlation of H-1 (δ_H 5.51)/C-10 (δ_C 85.0). Similarity of the chemical shift values of **2** ascribed to C-13 and *p*-hydroxybenzene moiety (C-1' to C-6') with those of **1** indicated that **2** and **1** shared the same side chain moiety. In NMR data of **2**, the presence of sp^3 methine [δ_H 3.06, d (J = 2.6 Hz)/δ_C 57.7] was observed and the position of this methine was determined at C-11 of the spirolactone ring by COSY correlation of H-10/H-11/H-13 and HMBC correlations (Fig. 3). Although in **4**, which had sp^2 quaternary carbon at C-11, the chemical shift value of H-10 was δ_H 5.26, the higher field-shifted chemical shift value of H-10 (δ_H 4.51 s) was observed in **2**, supporting the presence of sp^3 methine at C-11 in **2**. The NOESY correlation of H-7/H-10, H-10/H-11, H-1/H-9, and H-5/H-9 indicated 8*R* configuration, α-orientation of H-10 and H-11, and β-orientation of H-1, H-5, and H-9. Although we observed the NOESY correlation of H-13/H-10, H-13/H-11, and H-13/H-2' and H-6', the stereochemistry at C-13 was not determined. Thus, the structure of **2** was elucidated as shown. Compound **2** might be biologically synthesized by the hydration of **4** at double bond between C-11 and C-13.

Compounds **3** and **4** were isolated from the leaves of another species in the same genus, *M. lucida* [32], and *Prismatomeris tetrandra* (Rubiaceae) [33], respectively; however, this was the first isolation of these two compounds from *M. morindoides* leaves. This was also the first isolation of **5**–**9** from *M. morindoides* leaves.

Anti-malarial activity of isolated compounds

All isolated compounds were tested for their anti-malarial activity against the chloroquine/mefloquine-sensitive (3D7) strains of *P. falciparum* together with cytotoxicity against mammalian cell using AMB cells (Table 3). Among the compounds tested, **3** and its 3'-methoxy derivative **4** displayed the most potent anti-malarial activity, with IC_{50} of 0.96 and 0.80 μM, CC_{50} of 1.02 and 0.88 μM, and SI of 1.06 and 1.10, respectively. These results indicated that **3** and **4** had strong cytotoxicity to mammalian cells. New compounds deglucosyl gaertneroside (**1**) and morindoidin (**2**), and a known compound pinoresinol (**5**), were shown to have moderate anti-malarial activity, with IC_{50} of 40.9, 20.6, and 24.2 μM, respectively, but they did not show cytotoxicity against AMB cells. Moreover, oleanolic acid (**12**) was shown to have moderate activity with IC_{50} of 32.8 μM.

Table 3 Anti-malarial activity of the isolated compounds

compound	IC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]
1	40.9	>50	ND[e]
2	20.6	>50	ND[e]
3	0.96	1.02	1.06
4	0.80	0.88	1.10
5	24.2	>50	ND[e]
6	>50	>50	ND[e]
7	>50	>50	ND[e]
8	>50	>50	ND[e]
9	>50	>50	ND[e]
10	>50	>50	ND[e]
11	>50	>50	ND[e]
12	32.8	nt[d]	ND[e]
artesunate	0.0037	1.90	518.01
chloroquine	0.0118	>5	ND[e]

Values are the mean from two or three independent experiments performed in duplicate.

[a] 50% inhibitory concentration against 3D7 strain.

[b] 50% cytotoxic concentration using AMB cells.

[c] selectivity index was obtained by dividing CC_{50} value by IC_{50} value.

[d] not tested

[e] not determined

This is the first report on the anti-malarial activity of **1**–**5**, although the anti-trypanosomal activity of **3** against the GUTat 3.1 strain of *Trypanosoma brucei brucei*, a causative parasite of animal trypanosomiasis, and cytotoxicity against several human cell lines, including normal fibroblasts, colon cancer, stomach cancer, and leukemia were reported [32]. Krohn reported the potent cytotoxicity of **4** against several cell lines, including murine connective tissue cell (L-929), human cervix carcinoma (KB-3-

1), human lung carcinoma (A-549), and human colon adenocarcinoma (SW-480) [33]. Our results also showed potent cytotoxicity of **3** and **4** against AMB cells and are in good agreement with the previous reports.

Comparing the structures of **1**–**4** showed that **1** and **2** with moderate activities had a secondary hydroxy group at C-13, while **3** and **4** with potent activity had a double bond between C-11 and C-13. Considering the activity and structural differences, the presence of a double bond between C-11 and C-13 may be important for the anti-malarial activity as well as cytotoxicity. Interestingly, comparison of the structures of **2**–**4**, which share the same carbon skeleton, suggested that the presence of a five-membered ether bridge between C-1 to C-10 may not affect the activity. Although anti-malarial activity of phenylpropanoid conjugated iridoid glucosides [21] and isolation of iridoids without a glucose unit [32, 33] have been previously found, anti-malarial activity of phenylpropanoid conjugated iridoids without a glucose unit is reported here for the first time.

Ohta *et al*. reported seasonal and individual variations in the contents of **3** and its derivatives in *M. lucida*, with old leaves accumulating these compounds [45]. In our preliminary study, the variation in anti-malarial activity of the extract derived from a different batch of *M. morindoides* leaves was observed. These results suggested the contents of anti-malarial compounds varied in *M. morindoides* leaves. To avoid variation in efficacy and safety of any remedy for malaria derived from *M. morindoides* leaves, standardization using marker compounds including **1**–**5** and **12** will be necessary.

In conclusion, the activity-guided isolation of *M. morindoides* leaves yielded two new phenylpropanoid conjugated iridoids and their related two iridoids, along with seven known compounds. New compounds **1** and **2** showed moderate anti-malarial activity. Compounds **3** and **4** exhibited the most potent anti-malarial activity. These results suggested that the *M. morindoides* leaves traditionally used for the remedy of malaria contained anti-malarial compounds. Our results suggest that compounds **1**–**5** may be promising lead compounds for anti-malarial chemotherapy. Moreover, our findings also provide chemical evidence supporting the traditional use of *M. morindoides* leaves for treating malaria. In addition, compounds **3** and **4** showed cytotoxicity against mammalian cells as well as anti-malarial activity. Our results imply the necessity of quality control of the extract of *M. morindoides* leaves based on the contents of **1**–**5** and **12** in terms of the safety and efficacy for further continuous use.

Declarations

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Compliance with ethical standards

Conflict of interest

K.T. has received a research grant from Kobayashi Foundation

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Figures

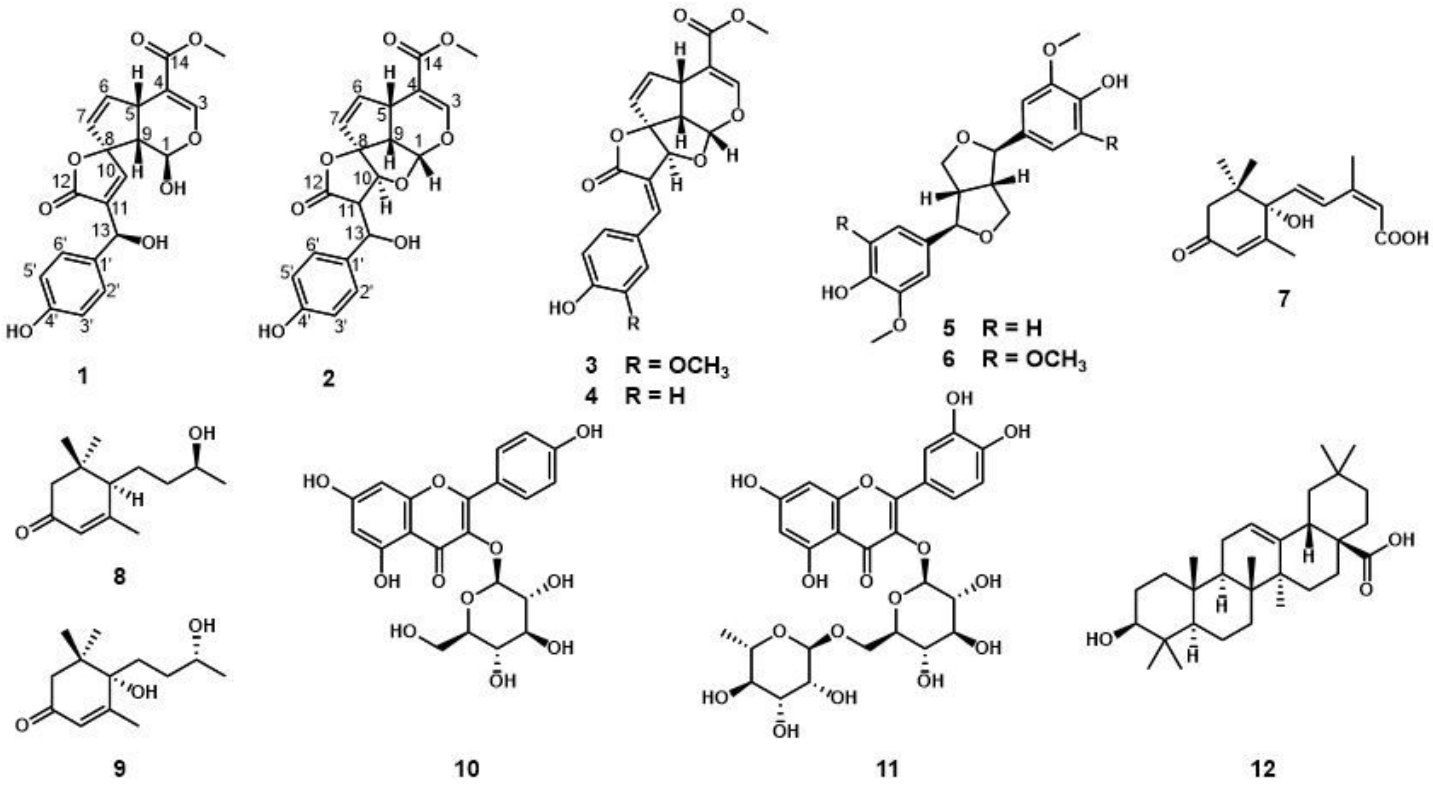

Figure 1

Structure of isolated (1–11) and detected (12) compounds from M. morindoides

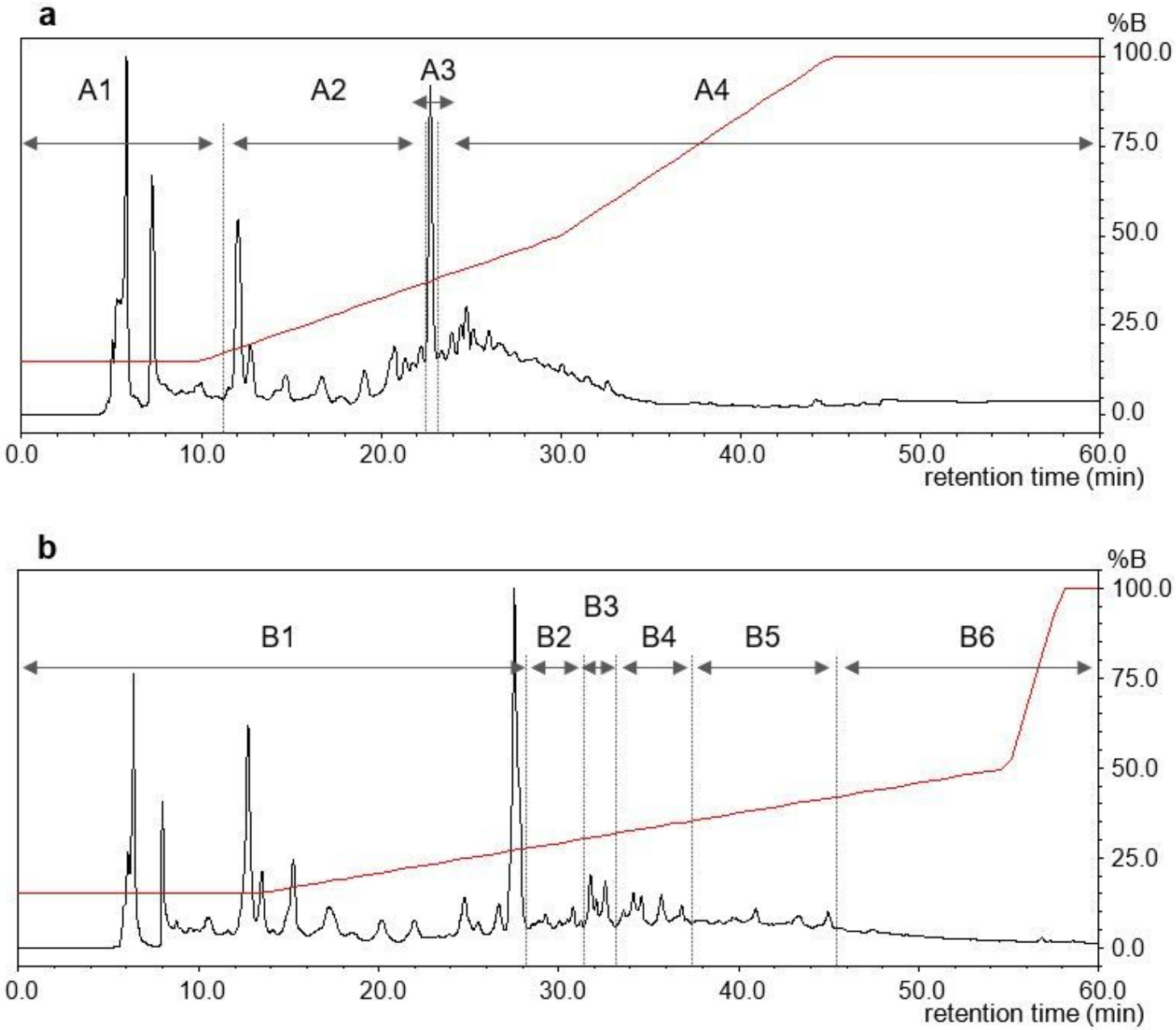

Figure 2

Preparative ODS HPLC chromatogram of aqueous alcohol extract to yield Frs. A1–A4 (a) and B1–B6 (b). (red line shows %B of gradient elution)

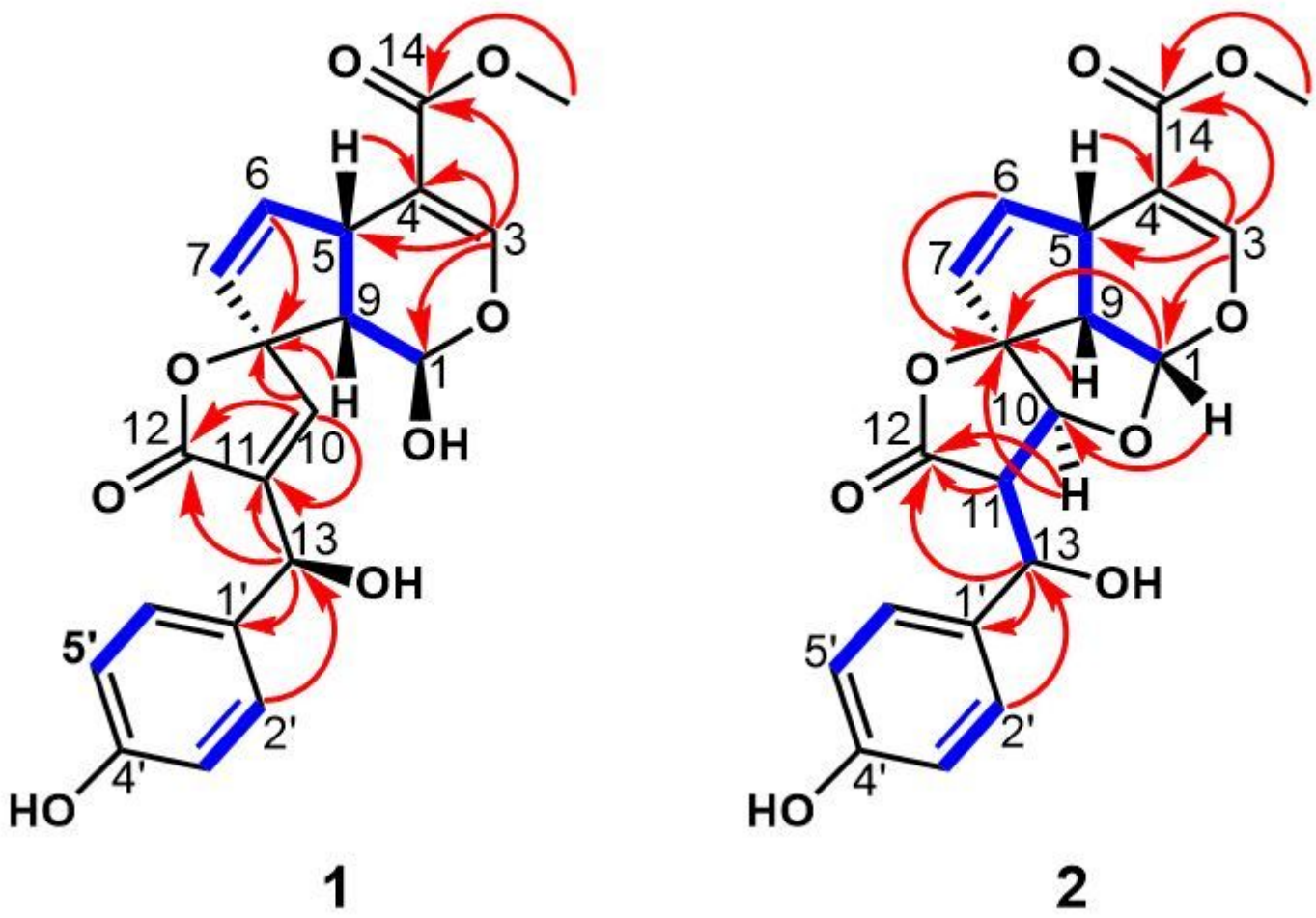

Figure 3

Key COSY (bold lines) and HMBC (arrows) correlations of 1 and 2

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata20210503YA.pdf