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* (Rubiacae) 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 (IC50 of 0.96 and 0.80 μM, CC50 of 1.02 and 0.88 μM, and SI of 1.06 and 1.10, respectively) was shown, while new iridoids 1 and 2 and pinoresinol (5) displayed moderate activity (IC50 of 40.9, 20.6, and 24.2 μM) without cytotoxicity (CC50 > 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.

Keywords *Morinda morindoides* · Iridoid · Phenylpropanoid conjugated iridoid · Malaria · *Plasmodium falciparum*

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. (Rubiacae) (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 was 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 $\delta$.

![Fig. 1 Structure of isolated (1–11) and detected (12) compounds from *M. morindoides*](image-url)
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\textsubscript{18} RS column (150 × 2.0 mm, S-3 μm, 8 nm) and a gradient solvent system using CH\textsubscript{3}CN + 0.1% formic acid/ H\textsubscript{2}O + 0.1% formic acid were 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™ Spectra Systems using Biotage® SNAP Ultra C\textsubscript{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\textsubscript{3}CN + 0.1% formic acid)-(H\textsubscript{2}O + 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\textsubscript{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 \textit{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 \textit{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 \textit{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\textsubscript{3}CN 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, \textit{t}\textsubscript{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\textsubscript{3}CN with 0.1% formic acid; gradient elution 15% B (0–10 min), 50% B (30 min), 100% B (58–60 min), UV detection at 254 nm] to yield Frs. B1 (181.5 mg, \textit{t}\textsubscript{R} 0–28.2 min), B2 (6.0 mg, \textit{t}\textsubscript{R} 28.2–31.5 min), B3 (6.3 mg, \textit{t}\textsubscript{R} 31.5–33.3 min), B4 (10.3 mg, \textit{t}\textsubscript{R} 33.3–37.5 min), B5 (16.4 mg, \textit{t}\textsubscript{R} 37.5–45.3 min), and B6 (8.3 mg, \textit{t}\textsubscript{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), CH\textsubscript{2}Cl\textsubscript{2} (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), CH\textsubscript{2}Cl\textsubscript{2}- (20.2 g), EtOAc- (6.9 g), 1-BuOH- (39.2 g), and water- (60.6 g) soluble fractions, respectively. A portion of CH\textsubscript{2}Cl\textsubscript{2}-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. 1–8).

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\textsubscript{18} 120 g cartridge) eluted with a gradient of 0–100% MeOH in water to obtain 17 fractions (Frs. 5–1 to
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 (3.7 g) was subjected to ODS MPLC (SNAP Ultra C18 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 C18 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–H2O (7:3), to yield 7 (2.1 mg). A portion of Fr. 12 (1.1 g) was rechromatographed on ODS MPLC (SNAP Ultra C18 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) $\lambda_{max}$ nm (log $\varepsilon$) 231 (1.1); ECD (c 2.6 x 10^{-4} M, MeOH) $\lambda_{max}$ nm ($\Delta \varepsilon$) 245 (+3.5) and 222 (−1.7); IR (KBr) $\nu_{max}$ 3410, 2953, 1739, 1700, 1636, and 1614 cm^{-1}; 1H NMR and 13C NMR data in CD3OD, see Table 1 and supplementary data; HRESIMS $m/z$ 369.0954 [M - H2O + H]+ (calcd for C20H17O7, 369.0969, Δ−1.5 mmu) and $m/z$ 385.0935 [M−H]− (calcd for C20H17O8, 385.0929, Δ+0.6 mmu).

Morindoidin (2): pale-yellow amorphous; $[\alpha]_D^{25} + 469$ (c 1.7, MeOH); UV (MeOH) $\lambda_{max}$ nm (log $\varepsilon$) 239 (1.2); ECD (c 1.6 x 10^{-4} M, MeOH) $\lambda_{max}$ nm ($\Delta \varepsilon$) 235 (+27.8), 217 (+4.4), and 203 (+22.7); IR (KBr) $\nu_{max}$ 3428, 2954, 1771, 1697, 1645, and 1615 cm^{-1}; 1H NMR and 13C NMR data in CD3OD, see Table 1 and supplementary data; HRESIMS $m/z$ 369.0995 [M−H2O + H]+ (calcd for C20H17O7, 369.0969, Δ+2.6 mmu), $m/z$ 431.0969 [M + HCOO]− (calcd for C21H19O10, 431.0984, Δ−1.5 mmu), and $m/z$ 385.0952 [M−H]− (calcd for C20H17O8, 385.0929, Δ+2.3 mmu).

Molucidin (3): $[\alpha]_D^{22} – 319$ (c 0.3, CHCl3); ECD (c 2.0 x 10^{-4} M, MeOH) $\lambda_{max}$ nm ($\Delta \varepsilon$) 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 x 10^{-4} M, MeOH) $\lambda_{max}$ nm ($\Delta \varepsilon$) 325 (−12.7), 262 (−3.4), 232 (+3.7), and 216 (+5.8).

| Position | 1 | 2 |
|----------|---|---|
| 1 | 4.93, d (4.5) | 92.8 | CH | 5.51, d (5.7) | 103.2 | CH |
| 3 | 7.50, brs | 153.3 | CH | 7.39, brs | 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, (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 |

Table 1 1H (500 MHz) and 13C NMR (125 MHz) data in CD3OD of 1 and 2.
Culture of *P. falciparum* and primary adult mouse brain cells

The *P. falciparum* chloroquine–mefloquine-sensitive (3D7) and -resistant (Dd2) strains were maintained in RPMI-1640-based complete media 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 μM hypoxanthine at 37 °C under mixed gas (5% CO₂, 5% O₂, and 90% N₂) 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% CO₂. 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 was 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). The concentration of sample required to reduce cell viability by 50% (CC₅₀) was calculated using the following equation:

\[
CC_{50} = 10^{\frac{\log \left( \frac{A}{B} \right) \times \frac{C-D}{A-C} + \log \left( B \right)}{2}}
\]

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

The IC₅₀ and CC₅₀ 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 CC₅₀ value by the IC₅₀ 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 2). The data are presented as anti-malarial activity (IC₅₀) values, and cytotoxicity (CC₅₀) 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 IC₅₀ 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 2). The IC50 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 IC50 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, IC50 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 IC50 of 24.2 μg/mL, while other fractions did not show significant activity (IC50 > 300 μg/mL) (Table 2). This result indicated that active compounds may exist in Fr. A4. Next, to separate the sub-fractions 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 2). Thus, the peaks with activity potential were estimated.

### Isolation and identification of compounds

Next, to isolate active compounds, the aqueous alcohol extract of the leaves was partitioned sequentially with hexane, CHCl3, EtOAc, 1-BuOH, and water. Because active peaks in Frs. B5 and B6 were present in the CHCl3- and EtOAc-soluble fractions, fractionations of the CHCl3- 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, (+)-(6R, 9S)-blumenol C (8) [38] and (+)-(6S, 9R)-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).

#### Table 2 Anti-malarial activities (IC50) and cytotoxicities (CC50) of the extracts and crude fractions

| Sample          | IC50 (μg/mL)a | CC50 (μg/mL)b | SF   | 3D7 | Dd2 | AMB | 3D7 | Dd2 |
|-----------------|---------------|---------------|------|-----|-----|-----|-----|-----|
| MeOH extract    | 66.54         | 94.38         | 487.68c | 7.33 | 5.17 |
| EtOH extract    | 53.96         | 62.95         | 274.28d | 5.08 | 4.36 |
| Hot water-soaking extract | > 500 > 500 > 500d | NDf | NDf | | |
| Boiled water extract | > 250 > 250 > 250d | NDf | NDf | | |
| Artesunate      | 0.0039        | 0.0047        | 1.21d  | 311.25 | 255.90 |
| Chloroquine     | 0.0044        | 0.0508        | ntf   | 4.24 | 4.07 |
| Aqueous alcohol extract | 87.62 91.27 371.06 | 4.24 | 4.07 | | |

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

a50% inhibitory concentration (IC50) against 3D7 or Dd2 strain

b50% cytotoxic concentration (CC50) using AMB cells

cSelectivity index (SI) was obtained by dividing CC50 value by IC50 value

dCC50 values are the mean of single experiment performed in duplicate

eNot tested

fNot determined
\(^{13}\text{C} \) NMR spectra coupled with HMQC indicated the presence of two ester carbonyl carbons (\( \delta_{\text{C}} 172.5 \) and 168.7), eight \( \text{sp}^2 \) methines (\( \delta_{\text{C}} 116.2 \) (2C), 129.6 (2C), 129.9, 141.5, 150.2, and 153.3), four \( \text{sp}^2 \) quaternary carbons (\( \delta_{\text{C}} 110.0, 133.2, 137.8, \) and 158.6), an oxygenated \( \text{sp}^3 \) quaternary carbon (\( \delta_{\text{C}} 98.1 \)), two \( \text{sp}^3 \) oxymethines (\( \delta_{\text{C}} 69.9 \) and 92.8), a methoxy (\( \delta_{\text{C}} 51.9 \)), and two \( \text{sp}^3 \) methines (\( \delta_{\text{C}} 39.9 \) and 52.2) (Table 1). Consideration of unsaturation degree suggested the presence of four other rings in \( 1 \), since 8 out of 12 unsaturation degrees were accounted for.

The \( ^1\text{H} \) and \( ^{13}\text{C} \) 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 \( ^1\text{H}–^1\text{H} \) COSY and HMBC data (Fig. 3) 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 \( ^1\text{H} \) and \( ^{13}\text{C} \) NMR chemical shift values, including coupling pattern and biosynthetic considerations. The NOESY correlations of H-1/H-10 and H-7/H-10 indicated \( \alpha \)-orientation of H-1 and 8R configuration, and correlations of H-5/H-9 indicated that H-5 and H-9 were \( \beta \)-oriented. Finally, the configuration of C-13 in \( 1 \) was determined to be \( \text{S} \) by the similarity of ECD spectra with negative and positive Cotton effects in MeOH at 222 and 245 nm, respectively [\( \lambda_{\text{max}} \) nm (\( \Delta\varepsilon \))] 223 (−12.9) and 264 (+3.1) in MeOH for gaertneroside \([44]\).

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 (\( \text{m/z} \) 385.0952, calcd for C\(_{20}\)H\(_{17}\)O\(_8\) [M − H]−, \( \Delta +2.3 \) mmu). The \( ^1\text{H} \) and \( ^{13}\text{C} \) NMR chemical shift values (Table 1) at C-1–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 (\( \delta_{\text{H}} 5.51 \)) and C-10 (\( \delta_{\text{C}} 85.0 \)). Similarity of the chemical shift values of 2 ascribed to C-13 and \( p \)-hydroxybenzene moiety (C-1’–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 \( \text{sp}^3 \) methine [\( \delta_{\text{H}} 3.06, \text{d} (J=2.6 \text{ Hz})/\delta_{\text{C}} 57.7 \)] 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 \( \text{sp}^2 \) quaternary carbon at C-11, the chemical shift value of H-10 was \( \delta_{\text{H}} 5.26 \), the higher field-shifted chemical shift value of H-10 (\( \delta_{\text{H}} 4.51 \text{ s} \)) was observed in 2, supporting the presence of \( \text{sp}^3 \) 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 8R configuration, \( \alpha \)-orientation of H-10 and H-11, and \( \beta \)-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 IC₅₀ of 0.96 and 0.80 μM, CC₅₀ 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 IC₅₀ 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 IC₅₀ of 32.8 μM.

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

| Compound | IC₅₀ (μM)a | CC₅₀ (μM)b | SIc |
|----------|------------|------------|-----|
| 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.

a50% inhibitory concentration (IC₅₀) against 3D7 strain
b50% cytotoxic concentration (CC₅₀) using AMB cells
cSelectivity index (SI) was obtained by dividing CC₅₀ value by IC₅₀ value
dNot tested
eNot determined
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 and 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.

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**Declarations**

**Conflict of interest** KT has received a research grant from Kobayashi Foundation.

**References**

1. WHO (2020) World malaria report 2020. 20 years of global progress and challenges. Global malaria programme. WHO, Geneva, pp 1–65
2. Sachs J, Malaney P (2002) The economic and social burden of malaria. Nature 415:680–685. https://doi.org/10.1038/415680a
3. Winstanley PA (2000) Chemotherapy for falciparum malaria: the armoury, the problems and the prospects. Parasitol Today 16:146–153. https://doi.org/10.1016/s1079-2564(00)00045-6
4. Murakami N, Umezome T, Mahmoud T, Sugimoto M, Kobayashi M, Wataya Y, Kim H-S (1998) Anti-malarial activities of acetylated bruceolide derivatives. Bioorg Med Chem Lett 8:459–462. https://doi.org/10.1016/S0960-894X(98)00045-6
5. Kim H-S, Shibata Y, Ko N, Ikemoto N, Ishizuka Y, Murakami N, Sugimoto M, Kobayashi M, Wataya Y (2000) Potent in vivo anti-malarial activity of 3,15-di-O-acetyltbruceolide against Plasmodium berghei infection in mice. Parasitol Int 48:271–274. https://doi.org/10.1016/s1383-5769(99)00023-9
6. Murakami N, Mostaquil HM, Tamura S, Itagaki S, Horii T, Kobayashi M (2001) New anti-malarial flavonol glycoside from hydrangeae dulcis folium. Bioorg Med Chem Lett 11:2445–2447. https://doi.org/10.1016/S0960-894X(01)00467-X
7. Murakami N, Sugimoto M, Kawanishi M, Tamura S, Kim H-S, Begum K, Wataya Y, Kobayashi M (2003) New semisynthetic quassinoids with in vivo anti-malarial activity. J Med Chem 46:638–641. https://doi.org/10.1021/jm020197l
8. Kerharo J, Adam JG (1974) La pharmacopée sénégalaise traditionnelle. Plantes médicinales et toxiques. J Agric Trop Bot Appl 21:76–77
9. Tona L, Cimanga RK, Mesia K, Musuamba CT, De Bruyne T, Apers S, Hernans N, Van Miert S, Pieters L, Totté J, Vlietinck AJ (2004) In vitro antiplasmodial activity of extracts and fractions from seven medicinal plants used in the Democratic Republic of Congo. J Ethnopharmacol 93:27–32. https://doi.org/10.1016/j.jep.2004.02.022
10. Abdoulaye T, Souleymane M, Howele O, Rene SY, Joseph DA, Adama C (2017) Comparative antimicrobial effectiveness of two medicated soaps with herbal soap from Morinda morindoides (Rubiaceae) against skin pathogens. Am J Microbiol Res 5:74–77. https://doi.org/10.12691/ajmrr-5-4-1
11. Touré A, Bahi C, Ouattara K, Djama JA, Coulibaly A (2011) Phytochemical screening and in vitro antifungal activities of extracts of leaves of Morinda morindoides (Morinda, Rubiaceae). J Med Plant Res 5:6780–6786. https://doi.org/10.5897/JMPR11.1116
41. Han J-T, Bang M-H, Chun O-K, Kim D-O, Lee C-Y, Baek N-I (2004) Flavonol glycosides from the aerial parts of *Aceriphyllum rossii* and their antioxidant activities. Arch Pharm Res 27:390–395. https://doi.org/10.1007/bf02980079

42. Habila JD, Shode FO, Ndukwe GI, Amupitan JO, Nok AJ (2011) Novel antimalarial agent (cinnamic 3β-hydroxyolean-12-en-28-carboxylic anhydride): synthesis, characterization and in vivo studies. Afr J Pharm Pharmacol 5:2667–2675. https://doi.org/10.5897/AJPP11.589

43. Cimanga RK, Tona GL, Mesia GK, Kambu OK, Bakana DP, Kalenda PDT, Penge AO, Muyembe J-JT, Totté J, Pieters L, Vlietinck AJ (2006) Bioassay-guided isolation of antimalarial triterpenoid acids from the leaves of *Morinda lucida*. Pharm Biol 44:677–681. https://doi.org/10.1080/13880200601009123

44. Takenaka Y, Okazaki N, Nishi T, Nagakura N, Tanahashi T (2020) Iridoid glucosides from *Linociera sangda*. Heterocycles 101:186–194. https://doi.org/10.3987/COM-19-S(F)10

45. Ohta T, Tikanont T, Ayertey F, Nakagawa M, Tung NH, Bolah P, Jnr HB, Appiah AA, Ocloo A, Ohashi M, Tanoue K, Yamaguchi Y, Ohta N, Yamaoka S, Iwanaga S, Uto T, Shoyama Y (2019) Establishment of a quantitative and qualitative analysis and isolation method for tetracyclic iridoids from *Morinda lucida* Bentham leaves. J Pharm Biomed Anal 164:475–480. https://doi.org/10.1016/j.jpba.2018.10.044

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