Discovery of active components from *Morinda morindoides* toward the development of anti-malarial drug

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Introduction

Background

A decoction of *Morinda morindoides* (Rubiaceae) leaves is a traditional medicine in Africa to treat a number of disorders, including malaria, fever, and diabetes.

Both *in vitro* and *in vivo* anti-malarial activities of the leaf extract of *M. morindoides* have been reported[1, 2]. 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.

Purpose

1. Identification and characterization of the compounds with anti-malarial activity to select suitable leaves for the preparation of safe and effective remedy.
2. Evaluation of anti-malarial activity of isolated compounds against the chloroquine/mefloquine-sensitive (3D7) strains of *P. falciparum*.

Conclusion

1. In our study, two phenylpropanoid conjugated iridoids (**1**, **2**) and three phenylpropanoid conjugated iridoid glucosides (**12**, **13**, and **21**) together with 24 known compounds were isolated and identified from *M. morindoides* leaves.
   This was also first isolation of **3**–**10**, **15**, **16**, **24–26**, **28**, and **29** from this plant.
2. New compounds **1** and **2** showed weak anti-malarial activity. Compounds **3** and **4** exhibited the most potent anti-malarial activity as well as cytotoxicity. 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**–**4** and pinoresinol (**5**) may be promising lead compounds for anti-malarial chemotherapy.
3. This is the first report on the anti-trypanosomal activity of **4** against *Trypanosoma cruzi* (human trypanosoma).
   We found that **3** also had anti-trypanosomal activity against *T. cruzi*.
4. Among the isolated compounds, two phenylpropanoid conjugated iridoids **3** and **4** were shown to have moderate anti-trypanosomal activity against *T. cruzi*, suggesting that both two could be potential lead compounds for anti-Chagas' drugs.
5. Comparing the structures of phenylpropanoid conjugated iridoids (**1**–**4** and **12**–**23**) and their activity suggested that the presence of a double bond between C-11 and C-13 may be essential for the anti-malarial activity as well as anti-trypanosomal activity.

Anti-malarial activity of the extracts

Anti-malarial assay

Plasmodium falciparum chloroquine–mefloquine-sensitive (3D7) and -resistant (Dd2) strains, 96-well
↓ medium of infected human red blood cells , 37℃, 72 h
↓ add samples
↓ 37℃, mixed gas (90% N_2, 5% O_2, 5% CO_2), 48 h
↓ add SYBR Green I 1 h
↓ Fluorescence measuring（485–515 nm）

Cytotoxicity

AMB cells (1 × 10^4 cells/well), 96-well
↓ 37℃, 48 h
↓ add samples
↓ 37℃, 48 h
↓ add10% Alamar Blue solution (100 µL) 2 h
↓ Fluorescence measuring（560–590 nm）

AMB: Adult mouse brain

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

Sample ext.	IC_{50} (µ/mL) 3D7	IC_{50} (µ/mL) Dd2	CC_{50} (µg/mL) AMB	SI[a] 3D7	SI[a] Dd2
KB001 MeOH	4.09	12.31	587.96[b]	143.89	47.77
KB002 MeOH	35.25	nt[c]	>1000[b]	>28.4	ND[d]
KB003 MeOH	49.55	nt[c]	620.27[b]	12.51	ND[d]
KB004					
MeOH	66.54	94.38	487.68[b]	7.33	5.17
EtOH	53.96	62.95	274.28[b]	5.08	4.36
80% EtOH	155.37	144.83	>500[b]	ND[d]	ND[d]
hot water-soaking	>500	>500	>500[b]	ND[d]	ND[d]
boiled water	>250	>250	>250[b]	ND[d]	ND[d]
artesunate	0.0039	0.0047	1.21[b]	311.25	255.90
chloroquine	0.0044	0.0508	nt[c]	ND[d]	ND[d]

[a] selectivity index (SI) = CC_{50}/IC_{50}
[b] CC_{50} values are the mean of single experiment performed in duplicate.
[c] not tested.
[d] not determined

Activity-guided fractionation

KB004 *Morinda morindoides* leaves 178 g
↓ 50% EtOH (2 L, 4 times)
50% EtOH extract 25.97 g (14.6%) (10.08 g)
↓ dissolve in water 500 mL, partitioned *n*-hexane (0.5 L, 4 times)
n-hexane layer 93.7 mg (0.14%) ← $CHCl_3$ (0.5 L, 4 times)
$CHCl_3$ layer 532.4 mg (0.77%) ← EtOAc (0.5 L, 4 times)
EtOAc layer 549.2 mg (0.79%) ← *n*-BuOH (0.5 L, 4 times)
n-BuOH layer 3.38 g (4.8%)
water layer 5.67 g (8.2%)

Anti-malarial activity of the layers

Sample	IC_{50} (µ/mL) 3D7	IC_{50} (µ/mL) Dd2
50% EtOH ext.	233.99	199.81
n-hexane layer	20.99	12.03
$CHCl_3$ layer	4.39	4.72
EtOAc layer	67.53	56.59
n-BuOH layer	446.89	371.65
water layer	>500	>250
artesunate	0.0079	0.0035
chloroquine	0.0215	0.1889

Isolation Procedure

Red : New

Morinda morindoides leaves KB004 1060 g
↓ 80% MeOH (3 L, 16 times), 50% EtOH (6 L, 2 times)
aqueous alcohol ext. 144.87g (140 g)
∗ A=CH_3CN + 0.1% formic acid
∗ D=H_2O + 0.1% formic acid
↓ partition

n-Hexane layer 7.82 g
Chloroform layer 20.23g (18.6 g) → silica gel Hexane:EtOAc, 50% MeOH, 100% MeOH
- Fr. 1 0.42 g
- Fr. 2 0.31 g
- Fr. 3 2.63 g → 1.ODS MPLC Water:MeOH; 2.P-HPLC A:D; 3.Sephadex LH-20 MeOH
  - Fr. 3-1 137
  - Fr. 2 8.6
  - Fr. 3 2.9
  - Fr. 4 4.5
  - Fr. 5 935.4 → 1. P-HPLC A:D; 2. Sephadex LH-20 MeOH
    - **8** 6.9 mg, **5** 2.3 mg
    - **2** 4.4 mg, **4** 2.4 mg
    - **3** 1.7 mg
- Fr. 4 0.21 g
- Fr. 5 2.92 g (2.85 g) → 1.P-HPLC A:D
  - **15** 6.6 mg
  - **18** 7.0 mg
  - **14** 3.9 mg
  - **6** 13.1 mg
- Fr. 6 0.49 g (0.46 g) → Fr. 4 (198 mg) → 1. HPLC A:D; 2. Sephadex LH-20 MeOH
  - **9** 6.4 mg
- Fr. 7 8.41 g
- Fr. 8 0.54 g → Fr. 7 (3.66 g) → 1.ODS MPLC Water:MeOH; 2.P-HPLC A:D
  - **11** 1.8 mg
  - **17** 2.6 mg
  - **19** 3.4 mg

EtOAc layer 6.91 g (6.38 g) → silica gel Hexane:EtOAc, 50% MeOH, 100% MeOH
- Fr. 9 0.01
- Fr. 10 0.03
- Fr. 11 0.89 845 mg → 1.ODS MPLC Water:MeOH; 2.P-HPLC A:D; 3.Sephadex LH-20 MeOH; 4.P-TLC
  - **1** 7.1 mg
  - **7** 2.1 mg
- Fr.12 2.75
- Fr. 13 0.08
- Fr. 14 2.56 g → Fr. 14 (1.1 g) → 1.ODS MPLC Water:MeOH; 2.P-HPLC A:D; 3.P-TLC
  - **20** 22.3 mg
  - **13** 2.3 mg
  - **10** 5.9 mg

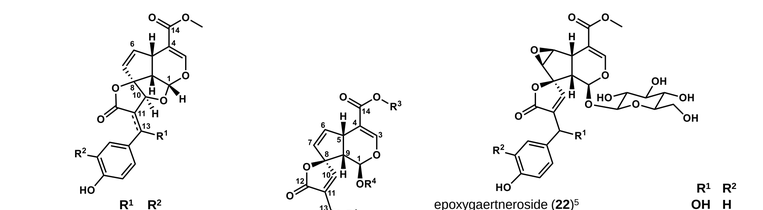
n-BuOH layer 39.23 g (14.5 g) → 1.ODSM PLC Water:MeOH
- Fr. 15 8.92 g → 1.ODS MPLC Water:MeOH; 2.P-HPLC A:D
  - **16** 1.0 mg
- Fr. 16 5.18 g (2.4 g) (2.7 g) → 1.ODS MPLC Water:MeOH; 2.P-HPLC A:D; 3.P-TLC
  - **12** 2.3 mg

Water layer 60.61 g

Morinda morindoides leaves KB004 1.81 kg
↓ 80% MeOH (8 L, 10 times), 50% EtOH (10 L, 3 times)
aqueous alcohol ext. 253.5 g (246.9 g)
∗ A = CH_3CN + 0.1% formic acid
∗ D = H_2O + 0.1% formic acid
↓ partition

n-Hexane layer 8.2 g
EtOAc layer 38.28 g
Water layer 200.0 g (56.0 g) → ODS MPLC Water:MeOH
- Fr. 17 31.9 g
- Fr. 18 11.6 g (11.3 g) → ODS MPLC Water:MeOH
  - Fr. 18-1 5.44 g (2.5 g) → P-HPLC A:D
    - **26** 38.6 mg
    - **25** 10.2 mg
  - Fr. 18-2 1.62 g (1.6 g) → P-HPLC A:D
    - **24** 49.1 mg
  - Fr. 18-3 1.46 g
  - Fr. 18-4 1.29 g
  - Fr. 18-5 0.97 g (0.96 g) → P-HPLC A:D
    - **21** 1.1 mg
- Fr. 19 11.7 g (7.6 g) → P-HPLC A:D
  - Fr. 19-1 1.19 g (1.14 g) → P-HPLC A:D
    - **22** 35.9 mg
    - **28** 10.9 mg
    - **23** 1.7 mg
    - **29** 13.0 mg
  - Fr. 19-2 4.00 g
  - Fr. 18-3 0.84 g (0.83 g) → P-HPLC A:D
    - **27** 17.3 mg
  - Fr. 18-4 0.95 g

Structure of isolated iridoids

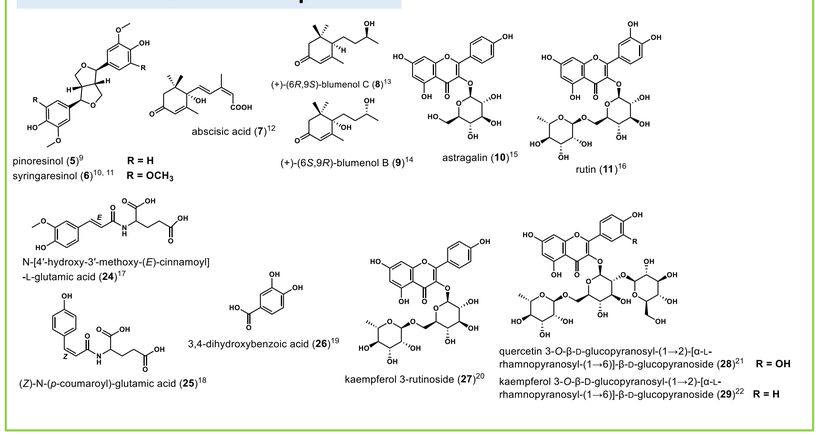


	R[1]	R[2]	
molucidin (**3**)[3]	H	OCH_3	$\Delta^{11\ (13)}$
prismatomerin (**4**)[4]	H	H	$\Delta^{11\ (13)}$
morindoidin (**2**)	OH	H	

	R[1]	R[2]
epoxygaertneroside (**22**)[5]	OH	H
morintoside B (**15**)[6]	=O	H
epoxymethoxygaertneroside (**23**)[5]	OH	OCH_3
dehydroepoxymethoxygaertneroside (**16**)[6-8]	=O	OCH_3

	R[1]	R[2]	R[3]	R[4]
deglucosyl gaertneroside (**1**)	OH	H	CH_3	H
dehydrogaertneric acid (**21**)	=O	H	H	glucosyl
acetylgaertneric acid (**12**)	OH	H	H	6-acetyl-glucosyl
acetylgaertneroside (**14**)[5]	OH	H	CH_3	6-acetyl-glucosyl
acetyldehydrogaertneroside (**13**)	=O	H	CH_3	6-acetyl-glucosyl
gaertneroside (**17**)[5]	OH	H	CH_3	glucosyl
dehydrogaertneroside (**18**)[5]	=O	H	CH_3	glucosyl
methoxygaertneroside (**19**)[5]	OH	OCH_3	CH_3	glucosyl
dehydromethoxygaertneroside (**20**)[5]	=O	OCH_3	CH_3	glucosyl

Structure of isolated compounds

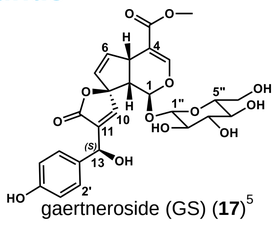


pinoresinol (**5**)[9] R = H
syringaresinol (**6**)[10, 11] R = OCH_3
abscisic acid (**7**)[12]
(+)-(6*R*,9*S*)-blumenol C (**8**)[13]
(+)-(6*S*,9*R*)-blumenol B (**9**)[14]
astragalin (**10**)[15]
rutin (**11**)[16]
N-[4'-hydroxy-3'-methoxy-(*E*)-cinnamoyl] -L-glutamic acid (**24**)[17]
3,4-dihydroxybenzoic acid (**26**)[19]
kaempferol 3-rutinoside (**27**)[20]
quercetin 3-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**28**)[21] R = OH
kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**29**)[22] R = H
(*Z*)-N-(*p*-coumaroyl)-glutamic acid (**25**)[18]

Structure elucidation of new compounds

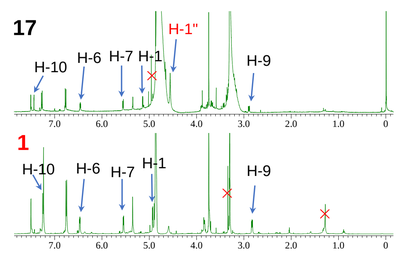
deglucosylgaertneroside (**1**)

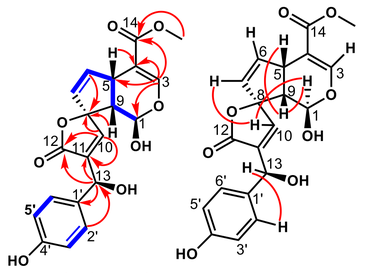
1	gaertneroside (**17**)[5]
brown amorphous	yellow amorphous
$[\alpha]_D^{22.8}$ +190.6 (*c* 3.3, MeOH)	$[\alpha]_D^{25}$ + 24.8 (*c* 0.75, MeOH)
HRESIMS *m/z* 385.0935 $[M-H]^-$ calcd for $C_{20}H_{17}O_8$, Δ +0.6	ES-MS *m/z* 547.144766 $[M-H]^-$ calcd for $C_{26}H_{27}O_{13}$, Δ +2.3
ECD (MeOH) λ_{max} nm (Δε) 245 (+3.5) and 222 (−1.7) **13S**	CD (MeOH) λ_{max} nm (Δε) 264 (+3.1) and 223 (−12.9) **13S**



gaertneroside (GS) (**17**)[5]

^{1}H-NMR comparison (500 MHz in CD_3OD)



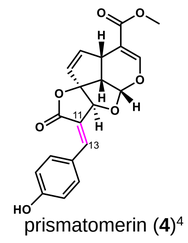


deglucosylgaertneroside (**1**)

COSY, key HMBC, and key NOESY

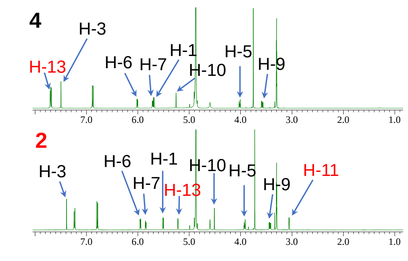
morindoidin (**2**)

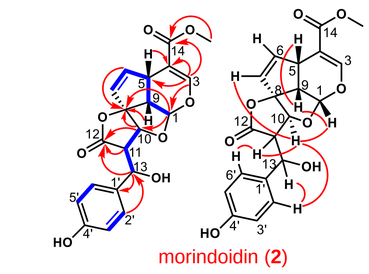
2	prismatomerin (**4**)[4]
pale yellow amorphous	White needles
$[\alpha]_D^{24.6}$ + 469.4 (*c* 1.7, MeOH)	$[\alpha]_D^{25}$ −136 (*c* 0.01, EtOH)
HRESIMS *m/z* 385.0952 $[M-H]^-$ calcd for $C_{20}H_{17}O_8$, Δ +2.3	FABMS *m/z* 369 $[M+H]^-$
ECD (MeOH) λ_{max} nm (Δε) 235 (+27.8), 217 (+4.4), and 203 (+22.7)	ECD (MeOH) λ_{max} nm (Δε) 325 (–12.7), 262 (–3.4), 232 (+3.7), and 216 (+5.8)



prismatomerin (**4**)[4]

^{1}H-NMR comparison (500 MHz in CD_3OD)



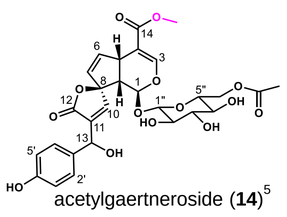


morindoidin (**2**)

COSY, key HMBC, and key NOESY

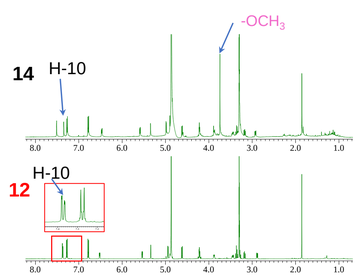
acetylgaertneric acid (**12**)

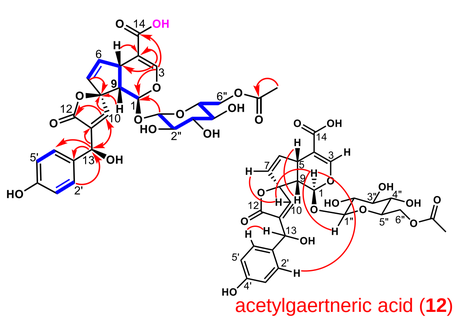
12	acetylgaertneroside (**14**)[5]
pale yellow amorphous	light yellow amorphous
$[\alpha]_D^{23}$ + 45.5 (*c* 1.8, MeOH)	$[\alpha]_D^{25}$ +23.4 (*c* 0.28, MeOH)
HRESIMS *m/z* 575.1407 $[M-H]^-$ calcd for $C_{27}H_{27}O_{14}$, Δ + 0.1	ES-MS *m/z* 589.154922 $[M-H]^+$ calcd for $C_{28}H_{29}O_{14}$, Δ +1.4
ECD (MeOH) λ_{max} nm (Δε) 255 (+4.1), 222 (–13.0), and 203 (+13.6)	ECD (MeOH) λ_{max} nm (Δε) 263 (+1.5) and 229 (–5.5)



acetylgaertneroside (**14**)[5]

^{1}H-NMR comparison (500 MHz in CD_3OD)



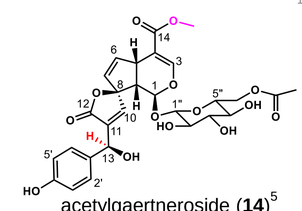


acetylgaertneric acid (**12**)

COSY, key HMBC, and key NOESY

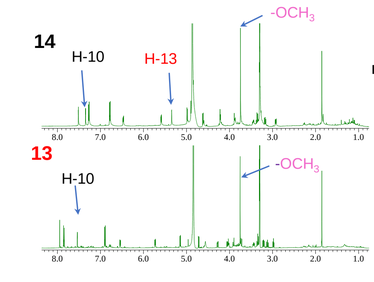
acetyldehydroacetylgaertneroside (**13**)

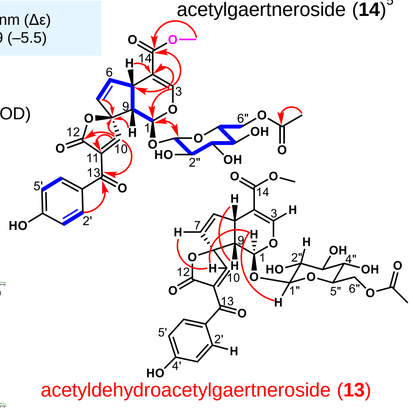
13	acetylgaertneroside (**14**)[5]
pale yellow amorphous	light yellow amorphous
$[\alpha]_D^{23.4}$ −33.1 (*c* 1.55, MeOH)	$[\alpha]_D^{25}$ +23.4 (*c* 0.28, MeOH)
HRESIMS *m/z* 589.1553 $[M+H]^+$ calcd for $C_{28}H_{29}O_{14}$, Δ +0.1	ES-MS *m/z* 589.154922 $[M-H]^+$ calcd for $C_{28}H_{29}O_{14}$, Δ +1.4
ECD (MeOH) λ_{max} nm (Δε) 228 (–8.3)	ECD (MeOH) λ_{max} nm (Δε) 263 (+1.5) and 229 (–5.5)



acetylgaertneroside (**14**)[5]

^{1}H-NMR comparison (500 MHz in CD_3OD)



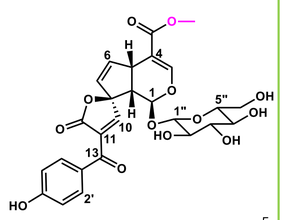


acetyldehydroacetylgaertneroside (**13**)

COSY, key HMBC, and key NOESY

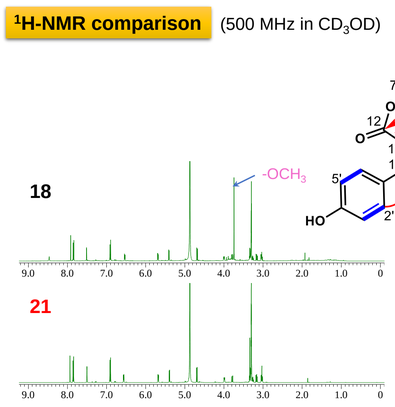
dehydrogaertneric acid (**21**)

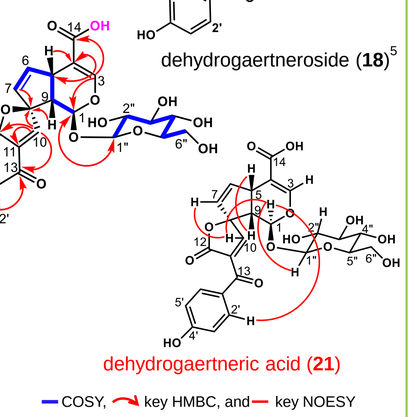
21	dehydrogaertneroside (**18**)[5]
beige amorphous	light yellow amorphous
$[\alpha]_D^{23.3}$ − 15.9 (*c* 1.6, MeOH)	$[\alpha]_D^{23.3}$ − 19.5 (*c* 0.31, MeOH)
HRESIMS *m/z* 531.1146 $[M-H]^-$ calcd for $C_{25}H_{23}O_{13}$, Δ +0.2	ES-MS *m/z* 545.128335 $[M-H]^-$ calcd for $C_{26}H_{25}O_{13}$, Δ +2.2
ECD (MeOH) λ_{max} nm (Δε) 232 (–9.5)	ECD (MeOH) λ_{max} nm (Δε) 227 (–13.0)



dehydrogaertneroside (**18**)[5]

^{1}H-NMR comparison (500 MHz in CD_3OD)





dehydrogaertneric acid (**21**)

COSY, key HMBC, and key NOESY

Anti-malarial activity

Anti-malarial activity of the isolated compounds from *M. morindoides*

compound	IC_{50} (µM)	CC_{50} (µM)	SI[c]
1	40.9	>50	ND[d]
2	20.6	>50	ND[d]
3	0.96	1.02	1.06
4	0.80	0.88	1.10
5	24.2	>50	ND[d]
6–21	>50	>50	ND[d]
22–29	>10	>10	ND[d]
artesunate	0.0037	1.90	518.01
chloroquine	0.0118	>5	

- Molucidin (**3**) had been reported to show potent anti-trypanosomal activity (IC_{50} 1.27 µM) against *Trypanosoma brucei brucei* [23], suggesting that **3** and its related compounds might show similar activity against *T. cruzi*.

[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 determined

Anti-trypanosomal activity

Anti-trypanosomal assay

Trypanosoma cruzi Tulahuen strains (3 × 10^4 cells/well) 96-well
↓ MEM+10% NBCS, 37℃, 72 h
↓ add samples
↓ 37℃, 72 h
↓ add 0.6% NP40 (100 µL)
↓ centrifugation
↓ add luciferin (100 µL)
↓ Luminescence measuring（544–590 nm）

MEM: minimal essential medium
NBCS: new-born calf serum.

Cytotoxicity

NBMH cells (1 × 10^4 cells/well) 96-well
↓ 37℃, 72 h
↓ add samples
↓ 37℃, 72 h
↓ add10% Alamar Blue solution (100 µL) 4 h
↓ Fluorescence measuring（544–590 nm）

NBMH: New-born mouse heart fibroblast

Anti-trypanosomal activity of the isolated compounds from *M. morindoides*

compound	IC_{50} (µM)[a]	CC_{50} (µM)[b]	SI[c]
1–2, **5–21**	>10	>10	ND[d]
3	4.67	2.76	0.56
4	5.70	3.22	0.57
22–29	>50	>50	ND[d]
benznidazole	2.73	>40	ND[d]

Values are the mean from two independent experiments performed in duplicate.
[a] 50% inhibitory concentration against *T. cruzi* (trypomastigote and amastigote) Tulahuen strains. *T. cruzi*: causative parasite of Chagas' disease.
[b] 50% cytotoxic concentration against NBMH cells.
[c] selectivity index (SI) = CC_{50}/IC_{50}
[d] not determined

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