Title: New anti-trypanosomal active tetracyclic irridoid isolated from *Morinda lucida* Benth.

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ABSTRACT

Human African trypanosomiasis (HAT), commonly known as sleeping sickness has remained a serious health problem in many African countries with thousands of new infected cases annually. Chemotherapy, which is the main form of control against HAT has been characterized lately by the viewpoints of toxicity and drug resistance issues. Recently, there have been a lot of emphases on the use of medicinal plants world-wide. *Morinda lucida* Benth. is one of the most popular medicinal plants widely distributed in Africa and several groups have reported on its anti-protozoa activities. In this study, we have isolated one novel tetracyclic irridoid, named as molucidin, from the CHCl₃ fraction of the *M. lucida* leaves by bioassay-guided fractionation and purification. Molucidin was structurally elucidated by ¹H and ¹³C NMR including HMQC, HMBC, H-H COSY and NOESY resulting in tetracyclic irridoid skeleton, and its absolute configuration was determined. We have further demonstrated that molucidin presented a strong anti-trypanosomal activity, indicating an IC₅₀ value of 1.27 μM. The cytotoxicity study using human normal and cancer cell lines indicated that molucidin exhibited selectivity index (SI) against two normal fibroblasts greater than 4.73. Furthermore, structure-activity relationship (SAR) study was undertaken with molucidin and oregonin, which is identical to anti-trypanosomal active components of *Alnus japonica*. Overlapping analysis of the lowest energy conformation of molucidin with oregonin suggested a certain similarities of aromatic rings of both oregonin and molucidin. These results contribute to the future drug design studies for HAT.

Keywords: *Morinda lucida*, Tetracyclic irridoid, Molucidin, Anti-trypanosomal activity, Medicinal plants

Human African trypanosomiasis (HAT), commonly known as sleeping sickness has remained a serious health problem in many African countries with thousands of new infected cases

annually. Although millions of people are under threat of HAT in Africa, it is known as one of neglected diseases which lacks the necessary resources to bring new compounds to market for possible drug development.^{1,2} HAT is caused by a protozoa belonging to the genus *Trypanosoma*, transmitted through the bites of tsetse flies. In Africa, there are mainly two species responsible for the disease; *T. brucei gambiense* and *T. b. rhodesiense*. A third species known as *T. b. brucei* causes animal trypanosomiasis popularly known as Nagana.

Chemotherapy against HAT relies on four drugs namely; suramin, pentamidine, melarsoprol and eflornithine were developed in 1921, 1941, 1949 and 1990, respectively that cause frequent and occasionally severe side effects, require lengthy parenteral administration, lack efficacy and are unaffordable for most of the patients.^{1,3,4} In addition, there has been an upsurge in the number of patients failing to respond to melarsoprol because of drug resistance.^{5,6} The increase in drug resistance, coupled with antiquated and toxic drugs available to treat the disease, urges the need for the discovery of new chemotherapeutic agents against HAT.

Recently there have been a lot of emphases on the use of medicinal plants world-wide.^{7,8} *Morinda lucida* Benth. (Rubiaceae), evergreen medium-sized trees with dark-shiny leaves on the upper surface, is one of the most popular medicinal plants widely distributed in Africa.⁹ Phytochemical researchers found that *M. lucida* is a natural resource rich in anthraquinones like oruwal, 3-hydroxyanthraquinone-2-carboxyaldehyde, 1,3-dihydroxy-2-methylanthraquinone, 1,3-dihydroxyanthraquinone-2-carboxyaldehyde and so on.¹⁰⁻¹³ It is used among traditional healers to treat fever, dysentery, abdominal colic and intestinal worm infestation. Several groups have reported on its anti-protozoa activities and some active compounds isolated from *M. lucida*.¹⁴⁻¹⁶ For example, it was reported that anthraquinones isolated from *M. lucida* has anti-leishmanial and anti-malarial activities.¹⁷ Three active compounds purified from *M. lucida* were also reported to have high activities against *Plasmodium falciparum*.¹⁴ Recently, several groups have revealed

anti-trypanosome activities of *M. lucida*. Asuzu and Chineme found the methanolic extract of *M. lucida* leaves possess anti-trypanosome activity *in vivo*.¹⁸ Work done by Nweze also showed that the crude methanol extract and 3 fractions from the leaves of *M. lucida* possess anti-trypanosomal activities against *T. b. brucei* S427 blood stream form *in vitro*.¹⁹

In our ongoing study on anti-trypanosome active compounds,²⁰ we performed bioassay guided fractionation and purification of constituents from *M. lucida* leaves on anti-trypanosome activity and isolated a novel component named as molucidin having tetracyclic irridoid skeleton from the CHCl₃ fraction of the *M. lucida* leaves. Selectivity index (SI) against human cells including normal fibroblast cells revealed that molucidin is a promising candidate for the new drug development for HAT.

This study was started to carry out the screening of anti-trypanosomal activity for selected 100 medicinal plants grown in Ghana according to the traditional knowledge. We found that *M. lucida* had the strongest anti-trypanosomal activity among them (data not shown).

The 50% ethanol extract obtained from the leaves of *M. lucida* was suspended in water and partitioned with hexane, CHCl₃, EtOAc, and n-BuOH, successively (Supporting information). Bioassay-guided column chromatography resulted in the isolation of novel tetracyclic irridoid and named as molucidin (Fig. 1).

The structure of molucidin has been elucidated by HR-ESI-MS experiment, NMR data and comprehensive analysis of the HMQC, HMBC, H-H COSY, and NOESY spectra permitted complete assignments of its proton and carbon signals. The relative configuration including *E*-geometry of C-11–C-13 double bond and, especially, absolute configuration of its spiro lactone tetracyclic irridoid skeleton was assigned as (1*R*,5*S*,8*S*,9*S*,10*S*) based on the extensive NMR spectra, optical rotation value, and circular dichroism (CD) spectrum. As shown in the CD

spectrum, the Cotton effects at 235 nm (positive) and 250 (negative), which were consistent with the literature,²¹ further confirmed the stereochemistry as (1*R*,5*S*,8*S*,9*S*,10*S*) (Supporting information). The general name of molucidin is (3*aS*,4*aR*,4*a*¹*S*,7*aS*,9*aS*,3*E*)-methyl 3-(4-hydroxy-3-methoxybenzylidene)-2-oxo-2,3,3*a*,4*a*,4*a*¹,7*a*-hexahydro-1,4,5-trioxadicyclopenta[*a,hi*]indene-7-carboxylate. It is interesting that molucidin is (-)-oruwacin, which is the enantiomer of oruwacin (Fig. 1) isolated also from *M. lucida* previously.^{22,23}

It is known that both enantiomers were separately isolated from different plant species like lignan from *Arctium lappa*²⁴ and from *Forsythia suspense*,²⁵ moreover, a set of enantiomer of naphthoquinones, shikonin and alkannin were isolated from *Lithospermum erythrorhizon*²⁶ and *Alkanna tinctoria*²⁷, respectively. In the study of enzymatical synthesis of enantiomers we confirmed that a marijuana compound, cannabichromenic acid can be biosynthesized from cannabigerolic acid having no asymmetric carbon. In this case a mixture of enantiomers was obtained because a geranyl group is enzymatically cyclized to prepare chromen framework possessing an asymmetric center on flexible carbon.²⁸ In contrast tetrahydrocannabinolic acid having an asymmetric carbon at cyclic rigid framework can be biosynthesized from the above precursor, cannabigerolic acid to give an enantiomer.^{29,30} From above evidences it is quite noteworthy that both enantiomers, molucidin and oruwacin had been isolated from the same plant species although the biosynthetic pathway of both enantiomers might be impossible by corresponding enzymes because the structures are quite rigid depending on a tetracyclic framework including a spiro carbon in a molecule.

Anti-trypanosomal activity was determined by Alamar blue assay after 48 hours incubation of bloodstream form of trypanosoma cells with or without molucidin (Supporting information). Molucidin showed a strong anti-trypanosomal activity with IC₅₀ values of 1.27 μM (Table 1).

Furthermore, we investigated the toxicity of molucidin against human cells ([Supporting information](#)). As shown in Table 1, totally 10 human cell lines including 2 normal fibroblasts, NB1RGB and HF-19, were treated with or without molucidin for 48 hours. Molucidin showed toxicity and IC_{50} values were less than 10 μ M in most of cancer cell lines we tested. Especially, IC_{50} values in LoVo and KATO III were low (0.11 and 0.21 μ M respectively). On the other hand, IC_{50} values of molucidin against 2 normal fibroblasts, NB1RGB and HF-19, are comparatively higher among the cells we tested. The selectivity index value (SI), which represents how specifically the compound inhibits the growth of parasite, showed that molucidin showed 4.73 and 9.54 of bigger SI values in NB1RGB and HF-19, respectively.

We next focused on the structure-activity relationship (SAR) of molucidin. There were three reasons. Firstly molucidin is reasonably potent (IC_{50} = ca. 1 μ M). Secondly molucidin has an aryl propanone moiety as a part of the molecule in [Fig. 2](#), which was identical with anti-trypanosomal active components of *Alnus japonica*, oregonin and hirsutanone.²⁰ Lastly irridoid molucidin is not flexible, so that the three dimensional structure is easily generated with a reliable manner. Indeed, previously we were unable to produce a 3D pharmacophore map using oregonin and hirsutanone mainly due to the flexibility of the molecules. This time we overcame this issue employing the rigid molecule, molucidin as a reference molecule. Conformation Search was carried out using the Force Field calculation MMFF94x and selected the lowest energy conformation ([Supporting information](#)). These data demonstrated a certain similarities of aromatic rings of both oregonin and molucidin ([Fig. 3](#)). Thus this would be a potential anti-trypanosomal active conformation of molucidin, if both of oregonin and molucidin bind to a same target. In addition, future analyses of the activity comparison between oruwacin and molucidin will promote a deeper understanding of their anti-trypanosomal property.

In summary, molucidin has been isolated as the first strong trypanosomal active constituent possessing a spirolactone tetracyclic irridoid skeleton from *M. lucida*. Cytotoxicity analysis using two normal fibroblasts demonstrated that molucidin showed SI values higher than 4.73. Moreover, overlapping analysis of the lowest energy conformation of molucidin with oregonin suggested a certain similarities of aromatic rings of both oregonin and molucidin. From this evidence molucidin could be an excellent lead compound against *Trypanosoma*. Future study is needed to elucidate the molecular mechanism of molucidin in their activities against *Trypanosoma*.

Acknowledgments

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FIGURE REGENDS

Figure 1. Structures of two enantiomers, molucidin and oruwacin

Figure 2. Comparison of structures of molucidin and anti-trypanosomal compounds; oregonin, hirsutanone, and caffeic acid with their respective IC_{50} values.

Figure 3. Overlapping picture of oregonin (blue) with molucidin (red).

(1*R*,5*S*,8*S*,9*S*,10*S*)-**Molucidin**

(1*S*,5*R*,8*R*,9*R*,10*R*)-**Oruwacin**

Figure 1

Figure 2.

Figure 3.

Table 1. Cytotoxicity and SI of molucidin against 2 human fibroblasts and 8 cancer cell lines.

		IC ₅₀ (μM), 48 h	SI
IC₅₀ of anti-trypanosomal activity		1.27	–
Cytotoxicity			
Normal fibroblast	NB1RGB	6.01	4.73
	HF-19	12.11	9.54
Colon cancer	Caco2	1.35	1.06
	LoVo	0.11	0.09
	HCT-15	16.04	12.63
Stomach cancer	KATO III	0.21	0.17
Leukemia	Jurkat	2.45	1.93
	U937	4.08	3.21
	HL-60	3.87	3.05
	THP-1	6.98	5.50

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Morinda lucida Benth.

CHCl_3 fraction of
the *M. lucida* leaves

(1*R*,5*S*,8*S*,9*S*,10*S*)

Molucidin

Anti-trypanosomal Activity

$\text{IC}_{50} = 1.27 \mu\text{M}$

Untreated

Treated