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In vitro Mechanistic Assays of Tetracyclic Iridoid Compounds Isolated from *Morinda lucida* Benth in *Leishmania* species

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MO, FAA, MAB and KKD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FAA and MO reviewed and structured first draft of manuscript. Authors TU, FA, AAA and YS prepared and authenticated the compounds. Authors TMG, TT, ANA, MT-T, GID, NO and SI managed the analyses of the study and performed final review input and proofreading of manuscript. Authors FAA and MO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study investigates the activity of tetracyclic iridoid compounds against *Leishmania spp.* and the mechanism(s) of action.
Study Design: An experimental study.
Place and Duration: Department of Parasitology, Noguchi Memorial Institute for Medical Research, between September 2017 and July 2018.
Methodology: The 50 % inhibitory concentration (IC_{50}) of compounds against *Leishmania donovani* and *L. major* promastigotes were determined after 48 hours of incubation using the Alamar blue. Cytotoxicity of compounds was determined against cell lines using MTT assay. The anti-amastigote activity of compounds was further assessed by DAPI (4′,6-diamidino-2-phenylindole) staining. The mechanism of cell death induced by compounds was determined using nexin assay. Mitosis, cytokinesis and morphometry were monitored by DAPI and Kinetoplastid Membrane Protein (KMP) staining. Cell cycle arrest induced by compounds was analyzed by FACS.
Results: Molucidin and ML-F52 inhibited the growth of promastigote in *L. donovani* (Molucidin; IC_{50} = 2.94±0.60 μM, ML-F52; IC_{50} = 0.91±0.50 μM) and *L. major* (Molucidin; IC_{50} = 1.85± 0.20 μM, ML-F52; IC_{50} = 1.77± 0.20 μM). ML-F52 had a 10-fold cytotoxic effect on parasites relative to normal cell lines. Against intracellular forms, Molucidin and ML-F52 inhibited intracellular amastigote replication and infectivity. Amphotericin B, Molucidin and ML-F52, induced a dose-dependent apoptotic effect on promastigotes. Although no change in KMP-11 expression was observed, iridoids inhibited cell division and morphological changes in promastigote cultures. Molucidin and ML-F52 induced apoptotic mechanism of cell death, inhibited cytokinesis and induced phenotypic changes in promastigotes. Molucidin further induced ''nectomonad-like'' forms and loss of kDNA, ML-F52 induced 'cell-rounding' with loss of flagellum. Molucidin also induced cell growth arrest at G_2-M phase (54.5 %). A significant induction of apoptosis (*P = .05*) was shown by an enhanced peak in the sub-G_1 confirming the apoptotic inducing properties of iridoids.
Conclusion: This study shows the anti-*leishmania* activity of tetracyclic iridoids which could be further investigated for the development of new chemotherapy against Leishmaniasis.

Keywords: Leishmania donovani; Leishmania major; In vitro screening; medicinal plants; Tetracyclic iridoids; Morinda lucida; Apoptosis.

ABBREVIATIONS

Fluorescent Activated Cell Sorting	*: FACS*
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	*: MTT*
Selectivity Index	*: S.I.*
4',6-diamidino-2-phenylindole	*: DAPI*
Phosphate Buffered Saline	*: PBS*
Phosphatidylserine,	*: PS*
Kinetoplastid Membrane Protein	*: KMP*
Fetal Bovine Serum	*: FBS*
Dimethylsulfoxife	*: DMSO*
Medium M199	*: M199*
Phosphate Buffered Saline/Triton X	*: PBST*
IC_{50}	*: 50 % inhibitory concentration*
$IC_{50}s$	*: 50 % inhibitory concentrations*

1. INTRODUCTION

Leishmaniasis remains a significant health issue due to its global presence and socioeconomic impact on both human and animal health [1]. It is a disease classified among the neglected tropical diseases and is caused by protozoans of the genus *Leishmania*. The main vector of transmission is the female phlebotomine sand fly. It is estimated that about 350 million people are at risk of infection with 12 million currently living with the condition across the globe [2]. Leishmaniasis is endemic in the tropical, subtropical, Southern Europe, and in settings

such as the rainforests of the Americas to deserts in Western Asia. Parasites within this genus have two major developmental life stages, the promastigote stage in the sand fly and the intracellular amastigote forms; a host related stage where they can be found engulfed by the host macrophages. Several clinical syndromes are subsumed under the term Leishmaniasis; the more disseminating presentation being the visceral (more disseminating form), the mucosal infections and the self-curing cutaneous forms. However, clinical presentations are dependent on the species of *Leishmania* [3], which when poorly managed, can lead to death.

Due to the devastating nature of the condition, some control measures to ameliorate the infections include early diagnosis and treatment. In the past decade, antimonials have been used as the first line of treatment [4]. Despite recent advances in drug development, the control of Leishmaniasis is complicated by the absence of vaccines, resistance to current drugs, drug toxicity and also the continuous presence of the vector and reservoirs. There is, therefore, the need to identify alternative chemotherapy against Leishmaniasis.

It is estimated that about 80% people living in developing countries use traditional medicinal plants as sources for treating tropical diseases. Moreover, there is an increase in selecting these plants for the development of novel drugs in modern medicine [5]. Some medicinal plants have already demonstrated activity against *Plasmodium, Trypanosoma* and *Leishmania* species [6,7]. Our group previously identified three novel tetracyclic iridoids compounds, Molucidin, ML-2-3 and ML-F52, isolated from the leaves of *Morinda lucida,* to have anti-protozoan and anti-microbial activity[8-11]. ML-2-3 and ML-F52 have shown to suppress the expression of paraflagellar rod protein subunit 2 (PFR-2), resulted in the cell cycle alterations and apoptosis-like cell death in the bloodstream form of *Trypanosoma* parasites. This current study therefore aimed at determining the anti-*Leishmania* properties of these and the mechanism of action.

2. MATERIALS AND METHODS

2.1 Plant Materials and Compound Preparations

Leaves of *M. lucida* Benth were collected by the Centre for Plant Medicine Research, Mampong. The isolation and purification of compounds was done by the Centre for Plant Medicine Research and the Faculty of Pharmaceutical Sciences, Nagasaki International University. The structural elucidation of compounds was as described previously [10,12]. Briefly, plant material was collected from the Centre for Plant Medicine Research, Mampong and air-dried. The ground leaves of *Morinda lucida* was weighed (1,550 g) and extracted with 50% aqueous ethyl alcohol (EtOH) at 40°C under sonication. The residue was obtained after evaporation of the solvent which was further suspended in 1.0 litre and partitioned with hexane, $CHCl_3$, and ethyl acetate (EtOAc) [10,12]. Tetracyclic iridoids, ML-2-3, Molucidin, and ML-F52, were purified by subjecting the $CHCl_3$ fraction to a silica gel column (45 by 350 mm) fractionation with hexane-EtOAc (2:1, vol/vol) as the mobile phase [10,12]. Fractions obtained were rechromatographed over a reversed-phase (RP) column with methanol (MeOH)-H_2O (10:1, vol/vol) to yield compounds as described previously [10,12].

The distinct chemical structure of tetracyclic iridoids, Molucidin, ML-2-3 and ML-F52 are shown in Fig. 1 varying only in the side chain (-R-group). DMSO was used as the solvent system in the dissolution and preparations of stock concentrations of compounds. Working concentrations were then prepared from the stock volume using Medium 199 (M199: from Sigma-Aldrich) for compound dilutions and assays (final concentration of DMSO = 0.1%).

2.2 Parasite and Cell Culture Conditions

In vitro cultures of *L. donovani* (D10 strain) and *L. major* (NR48815) parasites were maintained in M199 medium supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS), gentamycin and BME vitamins, and passaged every 72 hours at a mean density of 1.0 x 10^6 cells/ml in fresh medium as described previously [13].

Murine macrophages (RAW 264.7) were seeded at a mean cell density of 1.0 x 10^6 cells/ml in M199 medium (pH 7.4). The medium was supplemented with 10 % heat-inactivated FBS cells cultured for 48-72 hours at 37 °C in 5 % CO_2. Confluent RAW 264.7 cells were diluted in the fresh M199 medium at a mean density of 1.0 x 10^6 cells/ml for assays.

2.3 *In vitro* Anti-leishmanial Activity of Compounds on Promastigote of *Leishmania spp*

The Alamar blue cell viability assay was performed and half-maximal inhibitory concentration (IC_{50}) was determined as previously established [14]. The IC_{50} value is the concentration that inhibits 50 percent of parasite growth *in vitro*. Briefly, the assay was performed in triplicates with promastigotes seeded at cell density of 3 x 10^6 cells/ml in 96-well plates and incubated for 48 hours at 28ºC with or without compounds (< 50 μM) in two-fold dilutions. Amphotericin B was the reference drug. 10% of alamar blue reagent was added 4 hours prior to the end of treatment and absorbance was read at 540nM using a microtitre-plate reader (Tecan Sunrise Wako spectrophotometer). IC_{50}s were determined by graphical extrapolation after plotting the mean percentage absorbance versus the log concentration of the compounds using the GraphPad Prism (v.7). IC_{50}s were reported as mean ± standard error of means (SEM).

2.4 *In vitro* Toxicological Study of Compounds

The cytotoxicity of compounds was assessed on four human cell lines including lung, skin fibroblast, lung fibroblast and Chang Liver. Lung and skin fibroblast were maintained in Minimum Essential Medium- (MEM-) supplemented with 10 % FBS and 1 % penicillin-streptomycin. Chang liver and lung were grown in Eagle's minimum essential medium (EMEM) and RPMI 1640, respectively, with supplement same as above. The cells (0.5 x 10^4 cells/ml in 96-well plates) were treated with compounds at two-fold concentrations (> 50 μM) for 48 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and incubated in the dark for 4 hours and then measured at a wavelength of 595 nm using the microplate reader (Immuno Mini NJ-2300; Nihon InterMed, Tokyo, Japan). Cytotoxicity was determined as a percentage of live cells relative to the untreated control. The selectivity index (SI) was expressed as the ratio of the IC_{50} obtained for mammalian cells and the IC_{50} against *Leishmania*.

2.5 Anti-*Leishmania* Activity of Compounds on Intracellular Amastigotes

2 x 10^5 cells/ml of murine macrophages (RAW264.7) were plated in M199 medium (10 % FBS, complete medium) in 8-chamber well plates and incubated at 37 °C in 5 % CO_2 overnight. Attached macrophages were infected with stationary-phase promastigotes at a ratio of 10:1 (parasites: macrophages) for 4-10 hours. Promastigotes not internalized were washed off gently and intracellular amastigotes treated with compounds (> 25μM) for 24 hours. Media and compounds were then washed off, fixed in 4 % PFA, DAPI-stained (5 μg/mL) and observed under the fluorescence microscope. The percentage inhibition of infected macrophages was determined using the formula shown below [15]. The IC_{50} values were then extrapolated from a graph plot using the GraphPad Prism [16].

$$\% \text{ inhibition} = 100 - \frac{\% \text{ macrophages infected in treated cultures}}{\text{mean } \% \text{ macrophages infected in untreated controls}} X100$$

and

$$\% \text{ Inhibition} = 100 - \frac{PT}{PC} X \, 100$$

where PT = average number of amastigotes/macrophages in treated wells and PC= average number of amastigotes/ macrophage in control wells.

2.6 Assessment of Apoptosis and Cell Cycle

Apoptotic inducing properties of the tetracyclic iridoids were determined by assessing the externalization of phosphatidylserine (PS) on the outer membrane of compound-treated promastigotes. Promastigotes were treated with compounds at half-IC_{50}, IC_{50} and 2 x IC_{50} for 48 hours. 7-AAD and Annexin-V were added. Absorbance was read using the FACS machine after 30 minutes' incubation.

Cell cycle assay was performed as previously established by [17]. Briefly, 3 x 10^6 cells/ml mid-log promastigotes were incubated with compounds (< 50 μM) for 48 hours at 37 °C under 5 % CO_2. Parasites were then centrifuged and pellets re-suspended in ethanol (70 % ice-cold) dropwise, refrigerated at 4 °C for an hour and then analyzed using the Flow analyzer (BD LSRFortessa™ X-20). Algorithms obtained were analyzed using the FlowJo (v.10). Amphotericin B was used as a reference drug for the apoptosis induction property [18].

2.7 KMP Expression and Morphological Analysis of Compounds Treated Promastigotes

Promastigote parasites treated with Molucidin or ML-F52 were assessed their effect on the KMP

protein expression with their morphology. Promastigotes were incubated with compounds at half-IC_{50}, IC_{50} and 2 x IC_{50} concentrations for 48 hours. Parasites were then fixed with 4 % PFA on a polylysine slides and applied for the immunohistochemistry using anti-KMP primary antibody (MyBioSource) and secondary antibody: goat-anti-mouse FITC (Alexafluor) with DNA staining by 4′,6-diamidino-2-phenylindole, DAPI (5µg/mL: SIGMA). Untreated parasites were set as negative control. Promastigote cellular morphology, nuclei and kDNA division, and flagella were monitored under fluorescent and phase-contrast objectives.

2.8 Statistical Analysis

Compound activity was computed as IC_{50}s using GraphPad Prism (Version 7.0). Sigmoidal dose-response curves were generated by fitting a non-linear regression curve to the absorbance computed. IC_{50}s were then extrapolated from the dose-response curves reported as the mean ± (SEM) of three independent assays set up in triplicate.

2.9 Ethics

This study was approved by the Ethics Committee of Noguchi Memorial Institute for Medical Research Review Board (NMIMR-IRB:16/15-16).

3. RESULTS AND DISCUSSION

3.1 Tetracyclic Iridoids Isolation and Structures

The three compounds ML-2-3, Molucidin and ML-F52 were isolated by a bioassay-guided column chromatography described previously [10,12]. These compounds from the NMR and HR-ESI-MS analysis shared a distinct tetracyclic iridoid skeleton. Details of NMR and HR-ESI-MS of compounds isolated are as follows [10,12]:

Molucidin. Colorless crystal; [α] ^{25}D-188.5° (*c* 1.0, $CHCl_3$); HR-ESI-MS *m/z*, 399.1084 $(M+H)^+$ (calculated for $C_{21}H_{19}O_8$, 399.1080); ^{1} H-NMR ($CDCl_3$, 400 MHz) δ, 3.58 (1H, dd, *J* = 10.0, 6.0 Hz, H-9), 3.78 (3H, s, 14-$COOCH_3$), 3.96 (3'H, s, 3'-OCH_3), 4.05 (1H, dt, *J* = 10.0, 2.0 Hz, H-5), 5.22 (1H, s, H-10), 5.63 (1H, dd, *J* = 6.4, 2.4 Hz, H-7), 5.64 (1H, d, *J* = 5.6, H-1), 6.03 (1H, dd, *J* = 6.4, 2.0 Hz, H-6), 6.99 (1H, d, *J* = 8.0 Hz, H-5'), 7.26 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.43 (1H, d, *J* = 2.0 Hz, H-2'), 7.46 (1H, s, H-3), 7.78 (1H, s, H-13); and^{13}C-NMR ($CDCl_3$, 100 MHz) δ, 102.4 (C-1), 153.0 (C-3), 109.6 (C-4), 38.5 (C-5), 141.1 (C-6), 125.9 (C-7), 104.4 (C-8), 54.3 (C-9), 82.2 (C-10), 120.1 (C-11), 170.0 (C-12), 144.9 (C-13), 166.7 (C-14), 51.7 (14-$COOCH_3$), 126.5 (C-1'), 112.4 (C-2'), 149.1 (C-3'), 147.0 (C-4'), 115.1 (C-5'), 125.9 (C-6'), 56.0 (3'-OCH_3) [10,12].

ML-2-3. Colorless crystal; [α]^{25}D-89.2° (*c* 0.35, $CHCl_3$); HR-ESI-MS *m/z*, 385.0925 $(M+H)^+$ (calculated for $C_{20}H_{17}O_8$, 385.0923); ^{1}H-NMR (CDCl3,400 MHz) , 3.60 (1H, dd, *J* = 10.0, 6.0 Hz, H-9), 3.95 (3H, s, 3'-OCH_3), 4.05 (1H, dt, *J* = 10.0, 2.0 Hz, H-5), 5.28 (1H, s, H-10), 5.67 (1H, dd, *J* = 6.4, 2.4 Hz, H-7), 5.68 (1H, d, *J* = 5.6, H-1), 6.06 (1H, dd, *J* = 6.4, 2.0 Hz, H-6), 6.92 (1H, d, *J* = 8.0 Hz, H-5'), 7.25 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.49 (1H, d, *J* = 2.0 Hz, H-2'), 7.50 (1H, s, H-3), 7.75 (1H, s, H-13); and 13C-NMR ($CDCl_3$, 100 MHz) , 103.6 (C-1), 153.9 (C-3), 110.2 (C-4), 39.2 (C-5), 141.9 (C-6), 126.9 (C-7), 105.7 (C-8), 54.9 (C-9), 83.0 (C-10), 120.0 (C-11), 169.2 (C-12), 145.9 (C-13), 171.7 (C-14), 127.2 (C-1'), 113.7 (C-2'), 151.1 (C-3'), 148.8 (C-4'), 116.2 (C-5'), 126.0 (C-6'), 56.1 (3'-OCH_3) [10,12].

The NMR spectra and optical rotation value of ML-F52 was as described by [10,12]. White amorphous powder; [α]^{25}D- 62° (*c* 0.33, $CHCl_3$); HR-ESI-MS *m/z*, 413.1249 $(M+H)^+$ (calculated for $C_{22}H_{21}O_8$, 413.1236); 1H-NMR ($CDCl_3$, 400 MHz) , 1.31 (3H, t, *J* = 7.2 Hz, -OCH_2CH_3), 3.56 (1H, dd, *J* = 9.6, 6.0 Hz, H-9), 3.96 (3H, s, 3'-OCH3), 4.06 (1H, dt, *J* = 9.6, 2.0 Hz, H-5), 4.24 (2H, q, *J* = 3.6 Hz, -OCH_2CH_3), 5.22 (1H, s, H-10), 5.63 (1H, dd, *J* = 6.4, 2.4 Hz, H-7), 5.64 (1H, d, *J* = 5.6, H-1), 6.03 (1H, dd, *J* = 6.4, 2.0 Hz, H-6), 7.00 (1H, d, *J* = 8.0 Hz, H-5'), 7.25 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.43 (1H, d, *J* = 2.0 Hz, H-2'), 7.46 (1H, s, H-3), 7.77 (1H, s, H-13); and 13C-NMR ($CDCl_3$, 100 MHz) , 102.3 (C-1), 152.7 (C-3), 109.8 (C-4), 38.5 (C-5), 141.1 (C-6), 126.4 (C-7), 104.4 (C-8), 54.3 (C-9), 82.2 (C-10), 120.2 (C-11), 170.0 (C-12), 144.8 (C-13), 166.3 (C-14), 60.5 (-OCH_2CH_3), 14.3 (-OCH_2CH_3), 126.4 (C-1'), 112.4 (C-2'), 149.1 (C-3'), 147.0 (C- 4'), 115.1 (C-5'), 126.0 (C-6'), 56.0 (3'-OCH_3) [10,12].

3.2 Anti-*leishmania* Activity of Tetracyclic Iridoids against *Leishmania* Promastigote

For the assessment of the leishmanial activity of three compounds, ML-2-3, Molucidin and ML-F52, promastigote parasites of two strains, *L. donovani* (D10) and *L. major* (NR48815) were

used. Molucidin and ML-F52 had significant anti-proliferative effect (*P* = *.05*) on the parasites treated for 48 hours with IC_{50} values of 2.94±0.60 µM and 0.91±0.50 µM against *L. donovani*, and 1.85±0.20 µM and 1.77±0.20 µM against *L. major* respectively, while ML-2-3 showed no observed activity against either species of the *Leishmania* tested (IC_{50}>50 µM). Although there was no significant difference in IC_{50}s (susceptibility, *P* > *.05*) in both species of *Leishmania*, *Leishmania* parasites were more susceptible to ML-F52 relative to Molucidin.

In previous studies, we demonstrated the anti-protozoan and anti-microbial properties of tetracyclic iridoids, Molucidin, ML-2-3, and ML-F52 [8-11]. All three compounds showed significant anti-*trypanosomal* activities. However, in this study, Molucidin and ML-F52 showed significant anti-*leishmania* activity against *L. donovani* and *L. major*. In addition, the susceptibility of MLF52 to *L. donovani* and *L. major* was higher than Molucidin (*P* = *.05*). The anti-leishmanial effect of these iridoid compounds isolated from *Morinda lucida* supports previous studies done on other iridoids isolated from the bark of some plants including *Peperomia galoides*, grifolin and piperogalin which showed similar anti-*Leishmania* activity against *L. donovani* and other species by causing promastigote total lysis with a concentration of 100 µg/ml [19]. Increased efficacy of compounds on *Leishmania* was observed with the increasing carbon chain of the side group suggesting a possible link between the activity and the R group. This association has been previously identified in other compounds regarding the structure/activity relationships in naphthoquinones [20]. The effect of tetracyclic iridoids and the addition of carbon to the side group chain (-R-) may be associated with parasite susceptibility. However, this observation does not preclude other factors associated with a compound activity such as its solubility, absorption, and stability in the parasite.

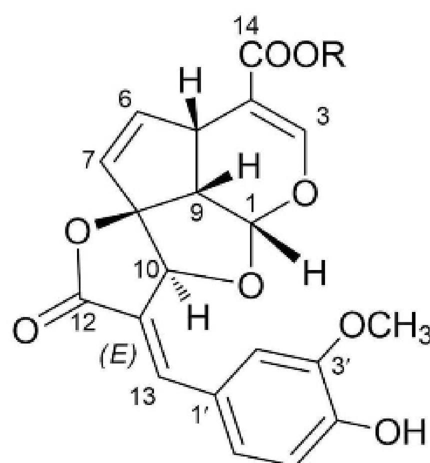


Fig. 1. Chemical structure of compounds isolated from *M. lucida*. Tetracyclic spirolactone iridoids, ML-2-3 (R= H), Molucidin (R= CH_3), and ML-F52 (R= CH_2CH_3).

3.3 Cytotoxicity of Tetracyclic Iridoids against Cell Lines

We also determined the cytotoxic effect of compounds on normal human cell lines and murine macrophages (RAW 264.7) by MTT Assay. Compounds were screened against normal lung, skin fibroblast, lung fibroblast and Chang Liver. The IC_{50}s obtained against each cell line were shown (table 2), extrapolated from dose-response sigmoidal curves using the GraphPad Prism (v.10). The toxicity of compounds was computed using selectivity indices (SI) which is the ratio of the IC_{50} determined for toxicity of compounds using normal cell lines versus the IC_{50} obtained against promastigotes. The SI for Molucidin and ML-F52 were determined to less than 5 for Molucidin and relatively above 10 for ML-F52. This showed that ML-F52 had a significantly lower (*P* = *.05*) toxicity profile against normal cell lines relative to Molucidin.

Table 1. *In vitro* anti-proliferative activity of compounds against *Leishmania donovani* and *L. major* promastigotes

Promastigotes	IC_{50} (µM)			
	Molucidin	ML-F52	ML-2-3	Amphotericin B
L. donovani	2.94 ± 0.60	0.91± 0.50	>1000	0.43± 0.40
L. major	1.85± 0.20	1.77± 0.20	>1000	0.32± 0.20

Mean ± S.E.M = Mean values ± Standard error of means of three experiments

Table 2. IC_{50} (μM) values and selectivity indices of compounds screened against cell lines

Cell type	Cell line	IC_{50} (μM)			Selectivity Index, (SI)		
		Molucidin (2.94)	ML-F52 (0.91)	ML-2-3 (>1000)	Molucidin	ML-F52	ML-2-3
Normal Skin fibroblasts	NBIRGB	7.11	4.74	>50	2.42	5.21	>20
Normal lung fibroblasts	HF-19	14.24	10.94	>50	4.84	12.02	>20
Normal lung	Hs-888Lu	9.29	8.89	>50	3.16	9.77	>20
Normal liver	Chang Liver	9.34	18.13	>50	3.18	19.92	>20
Macrophage	Raw 264.7	6.54	9.80	37.06	2.22	10.77	>20

3.4 Anti-amastigote Activity of Tetracyclic Iridoids

Intracellular amastigote forms of *L. donovani* was established using murine macrophages (RAW264.7) and activity of the compounds was assessed. Intracellular amastigotes were treated for 48 hours with or without varied concentrations of the compounds. After DAPI staining, parasitemia was counted under the phase contrast and fluorescence objectives using two counting methods and then each IC_{50} was extrapolated from dose-response curve. The number of infected macrophages per field was assessed to find the inhibitory effect on the parasite secondary infection in other macrophages, and a number of parasites per 100 infected macrophages were counted to determine the effect on parasite growth inside infected macrophages. Respectively, Molucidin was determined to have an IC_{50} of 1.418±0.26 μM and 4.654±0.46 μM, and ML-F52 an IC_{50} of 0.627±0.20 μM and1.928±0.19 μM for a number of parasites/macrophages and number of infected macrophages/field respectively, comparable to Amphotericin B with 0.177 μM and 2.251μM (Table 3). Coherent with the promastigote forms, no activity was observed with ML-2-3 treatment (IC_{50} > 100 μM). The S.I. values of ML-F52 were generally >10 (about 10-fold), suggesting significant (*P* = *.05*) lower toxicity and higher selectivity against parasites relative to normal cell lines. Molucidin though active against promastigotes also showed significant toxic effect on cell lines.

The effect of Molucidin and ML-F52 on the inhibition of intracellular amastigotes in macrophages as well as the infection of new macrophages was determined using the two methods described earlier by [15]. Corroborating these methods, both Molucidin and ML-F52 showed anti-proliferative activity against intracellular amastigotes by inhibiting the multiplication of amastigotes and the infection of macrophages.

3.5 Tetracyclic Iridoids Induced Apoptotic-like Cell Death in *Leishmania* spp.

A characteristic feature of apoptosis in *Leishmania* is the externalization of Phosphatidyl Serine (PS) from the inner to the outer plasma membrane layer [16]. Therefore, by staining treated parasites with Nexin reagent (SIGMA), which contained 7-AAD and annexin-V, parasites were examined by FACS. Flow data were analyzed by gating healthy non-apoptotic cells (in the lower left quadrant), early apoptotic cells (in the lower right quadrant), late apoptotic cells (in the upper right quadrant) and necrotizing cells (in the left upper quadrant) shown in Fig. 2a. Molucidin and ML-F52 significantly (*P* = *.05*) induced cell death reminiscent of apoptotic-like mechanism when observed after 48 hours of treatment. Molucidin induced apoptosis-like effect on parasites the most with 38.9 % of the late stage of apoptotic cells, whereas ML-F52 showed moderate apoptotic induction with 12.8 % of late stage of apoptosis. Dose-dependent

Table 3. IC_{50} values of compounds on intracellular amastigotes of *L. donovani*

Amastigotes	IC_{50} (μM)		
	Molucidin	ML-F52	Amphotericin B
No. of parasites/macrophage	1.418 ± 0.26	0.627 ± 0.02	0.177 ± 0.08
No. of infected macrophages/field	4.654 ± 0.46	1.928 ± 0.19	2.251 ± 0.62

RAW 264.7 macrophages were infected with stationary phage promastigotes in an infectivity ratio of 10:1 (parasites/macrophage).

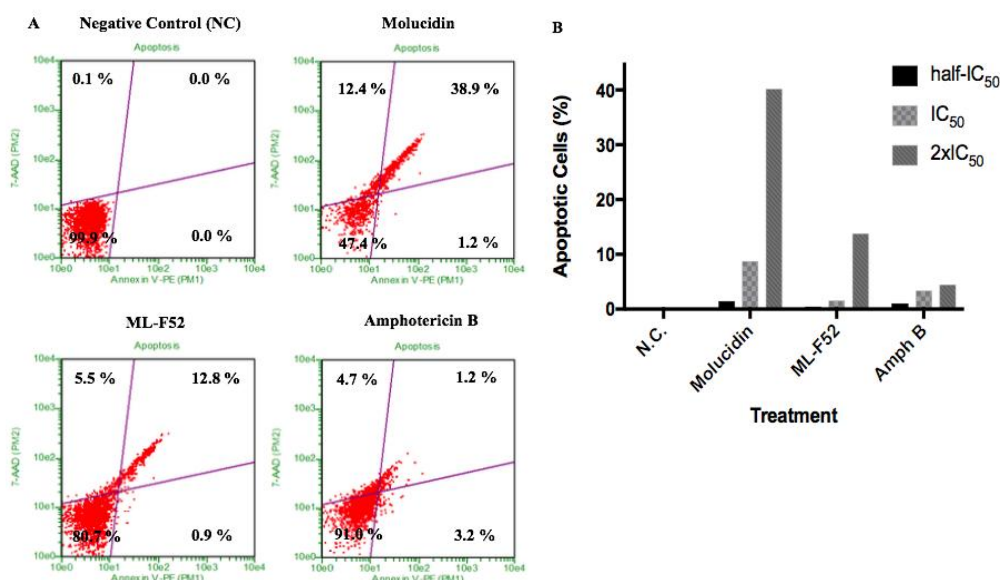


Fig. 2. (A) Dot plot of Nexin Apoptosis Assay. Active compounds, Molucidin induced strong apoptotic effect (38.9 %) relative to ML-F52 (12.8 %) at 2x IC_{50}s after 48 hours' treatment (B). Bar graph plot of a dose-dependent apoptotic-effect of compounds on promastigotes

apoptotic induction was observed in the treated group with increasing concentration of compounds around the range of IC_{50} (Fig. 2B). *L. donovani*, like in many unicellular organisms, is known to show typical features of apoptotic cell death such as the shrinkage of the cell, condensation of the nuclear and DNA fragmentation triggered by changes in mitochondrion potential. Molucidin and ML-F52 induced significant apoptotic-like cell death in *L. donovani* (P = .05) relative to the reference drug amphotericin B which has been shown to induce apoptosis in *Leishmania*. Agents capable of precipitating apoptosis in *Leishmania* parasites include leishmanial drugs such as pentamidine, antimony, amphotericin B, and miltefosine.

3.6 Cell Cycle Analysis of Promastigote Parasite Treated with Tetracyclic Iridoids Compounds

Cell cycle analysis was performed on treated and untreated (48 hours) promastigotes of *L. donovani*. Four peaks were observed in both the treated and untreated cell cycle representing four cell replication phases of parasites (Fig. 2). The two high peaks observed in each group are the G_0-G_1 and G_2-M phases. The S phase was a small population in between these peaks and a sub-G_1 population (apoptotic/damaged DNA of cells) adjacent the G_0-G_1 as labeled (Fig 2). Molucidin and ML-F52 induced an increase in sub-G_1 (damaged DNA content) cells which indicates induction of apoptosis in *L. donovani*. Molucidin and ML-F52, however, appeared to cause different effects on the other phases of cell cycle of *L. donovani* (P = *.05*) (Fig. 3). Molucidin induced cell cycle arrest at S phase indicated by the significantly higher peak relative to negative control (P = *.05*). On the other hand, ML-F52 was observed to induce significant cell cycle arrest at the G_2-M relative to negative control (P = *.05*). *In vitro* studies on *Leishmani*a cell cycle have shown promastigotes to replicate nuclear DNA first, followed by growth of a second flagellum before the onset of mitosis [21]. During mitosis, the kinetoplast segregates after the onset of nuclear anaphase and then there is cytokinesis at the end of mitosis [21]. Treatment with tetracyclic iridoids was carried out to determine the growth arrest phase induced by compounds. However, the analysis of cell cycle after treatment with tetracyclic iridoids showed an increase of cell population at the subG_1 phase indicating damaged DNA confirming the apoptotic induction properties of Molucidin and ML-F52 compounds. An increase in the peak of S phase cells was observed with Molucidin, while ML-F52 triggered an increase of G_2-M phase cells, suggesting differences in the effect of compounds on cellular events of *Leishmania*. This difference in the mechanism was also suggested by the immunohistochemistry study

where Molucidin induced a significant accumulation of slender elongated nectomonad-like forms and loss of kinetoplast DNA, whereas ML-F52 induced mid-mitotic cells (*P* = *.05*). An increase of G_2-M phase cells by ML-F52 can be attributed to the inhibition of subsequent cytokinesis after doubling of DNA in the parasite cell cycle, which is consistent with the fact that increased concentration of ML-F52 induced accumulation of incomplete-mitotic parasites (Fig 5B, 5D). The increased cell count in the S phase (18.7) by Molucidin was confirmed by a significant increase (*P* = *.05*) in the number of mid-mitotic and rounded parasites at 2x IC_{50}, shown in Fig 5A & 5C. These events further suggest possible differences in the mechanisms of action between Molucidin and ML-F52.

3.6 KMP Immunohistochemistry Analysis

Cell cycle analysis indicated that the parasites in the cycle of S and G_2-M phase were affected by Molucidin and ML-F52, respectively. Gene-knockout studies of the Kinetoplastid Membrane Protein (KMP) showed inhibition of basal body segregation, accumulation of multi-unequal sized nuclei and inhibition of cytokinesis [22]. Therefore, we assessed the effect of Molucidin and ML-F52 on the expression of KMP using anti-KMP-11 antibody and DAPI. Anti-KMP stained the basal body of promastigotes and DAPI stained both the nucleus and kinetoplast. They allowed the evaluation of phenotypic features and cellular events within parasites, respectively. Although no change in the

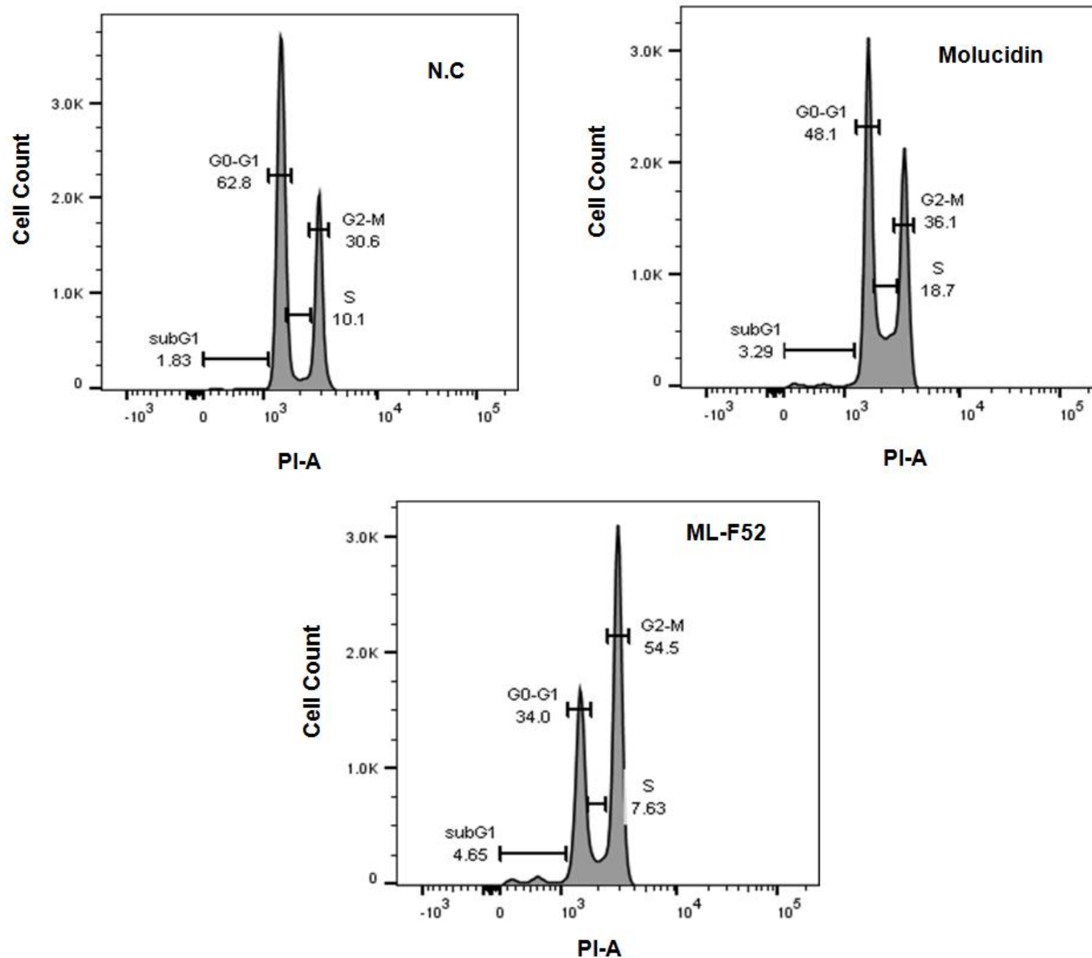


Fig. 3. Cell Cycle Analysis of promastigotes after 48 hours of incubation with tetracyclic iridoids. The quantification of apoptotic-like parasites was monitored as the percentage of cells in the sub-G_1 phase. ML-F52 induced cell cycle arrest at G_2-M Phase. Molucidin caused an enhanced peak at S-phase relative to the negative control (NC), *P = .05.*

expression level of KMP protein was observed, lucid transformations in the phenotype of promastigotes were observed (Fig. 4). Applying the morphological definitions for promastigote transformation as described by [23], the procyclic promastigote (body length 6.5-11.5 μm) with flagellum shorter than cell body, the slender elongated nectomonad-like promastigote (cell body longer than 12 μm), mid-mitotic cells with 2N:2K configuration. We also observed condensed round cells with loss of kDNA, and shortened or loss of flagellum in the treated groups. Interestingly, there was a significant increase (*P* = *.05*) in the population of slender elongated nectomonad-like cells with Molucidin treatment with half-IC_{50}. Increasing the concentration of Molucidin (IC_{50} and 2x IC_{50}) induced mid-mitosis and condensed round cells. However, ML-F52 induced condensed round cells (at IC_{50} and 2x IC_{50}, *P* = *.05*). Treatment at a moderate concentration (half-IC_{50}) with ML-F52 showed no effect on the cell phenotype.

ML-F52 induced accumulation of incomplete mitotic parasites shown in immunohistochemistry data (Fig. 4) confirming the growth arrest at the G_2-M phase shown in Fig. 3. Molucidin and ML-F52 induced loss of kDNA in promastigotes (rounded forms, Fig. 4) as had been reported in previous studies using DNA-intercalating drugs such as acriflavine and berenil. These compounds were shown to inhibit parasite motility and cytokinesis [24]. Acriflavine and Berenil have also been shown in *T. cruzi* epimastigote forms to act preferentially on and interfering with the kinetoplast DNA replication, thus inhibiting cell proliferation, ultrastructure, and mitochondrial activity [24,25].

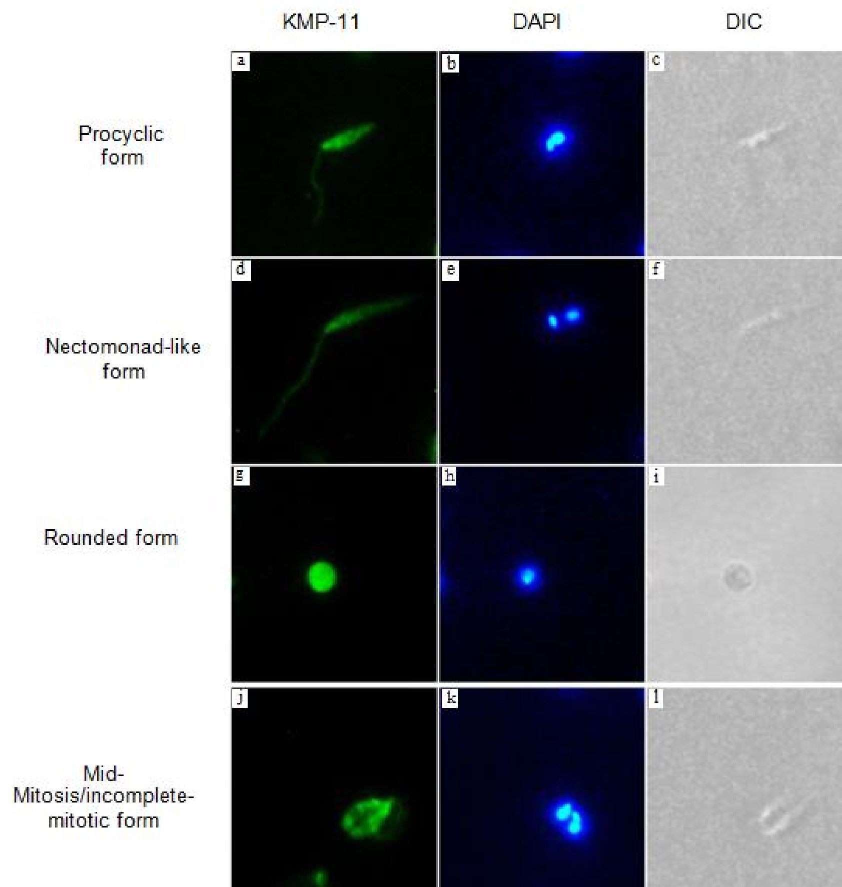
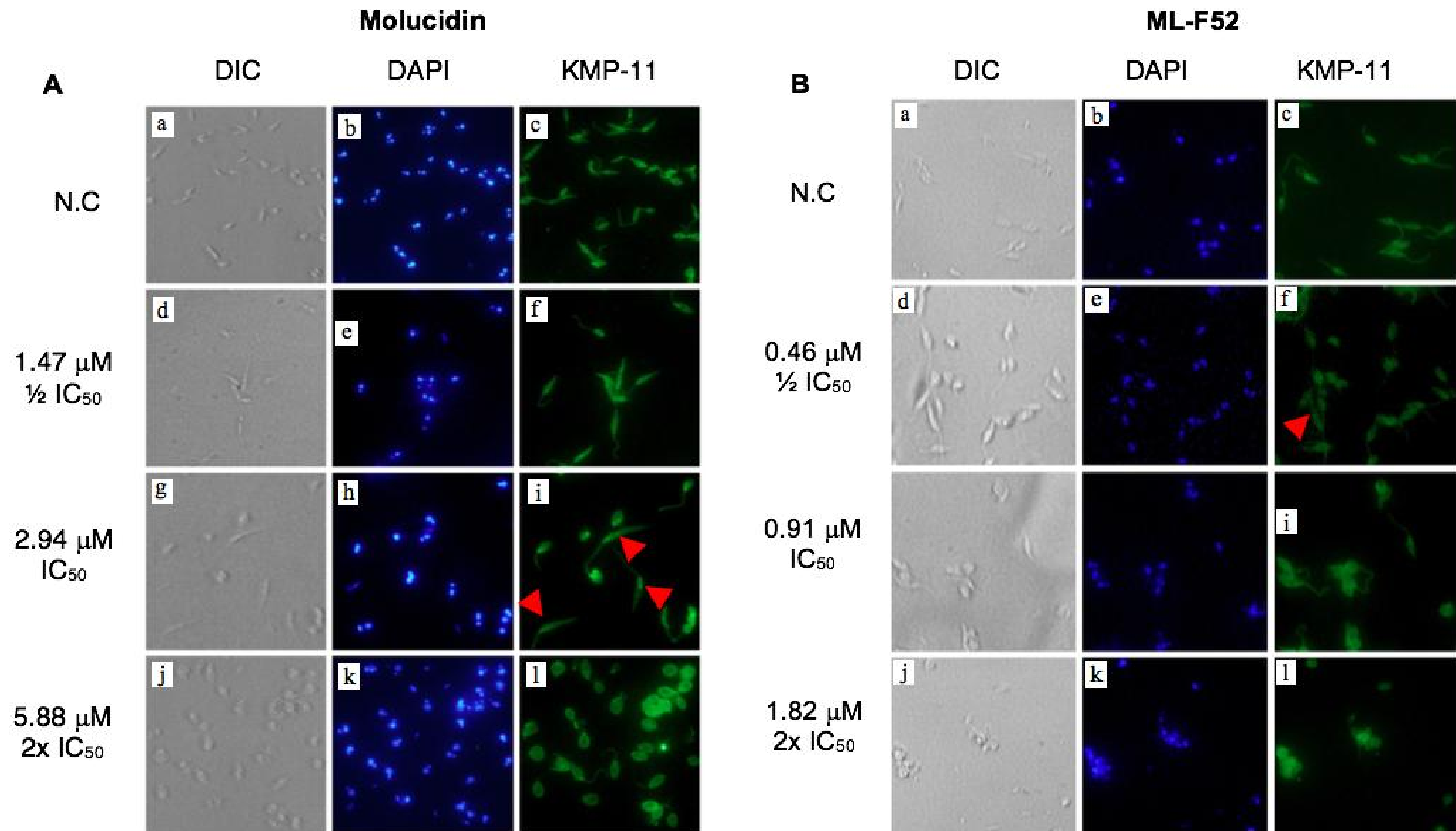


Fig. 4. Asymmetries in division of *L. donovani* promastigote. a–l, Micrographs of cells observed in assays with tetracyclic iridoids. Configuration of cellular events is nuclear division of parent cell followed by kinetoplast DNA and complete segregation of two daughter cells.


Molucidin
ML-F52
A
B
DIC
DAPI
KMP-11
N.C
1.47 μM
½ IC50
2.94 μM
IC50
5.88 μM
2x IC50
0.46 μM
½ IC50
0.91 μM
IC50
1.82 μM
2x IC50
a
b
c
d
e
f
g
h
i
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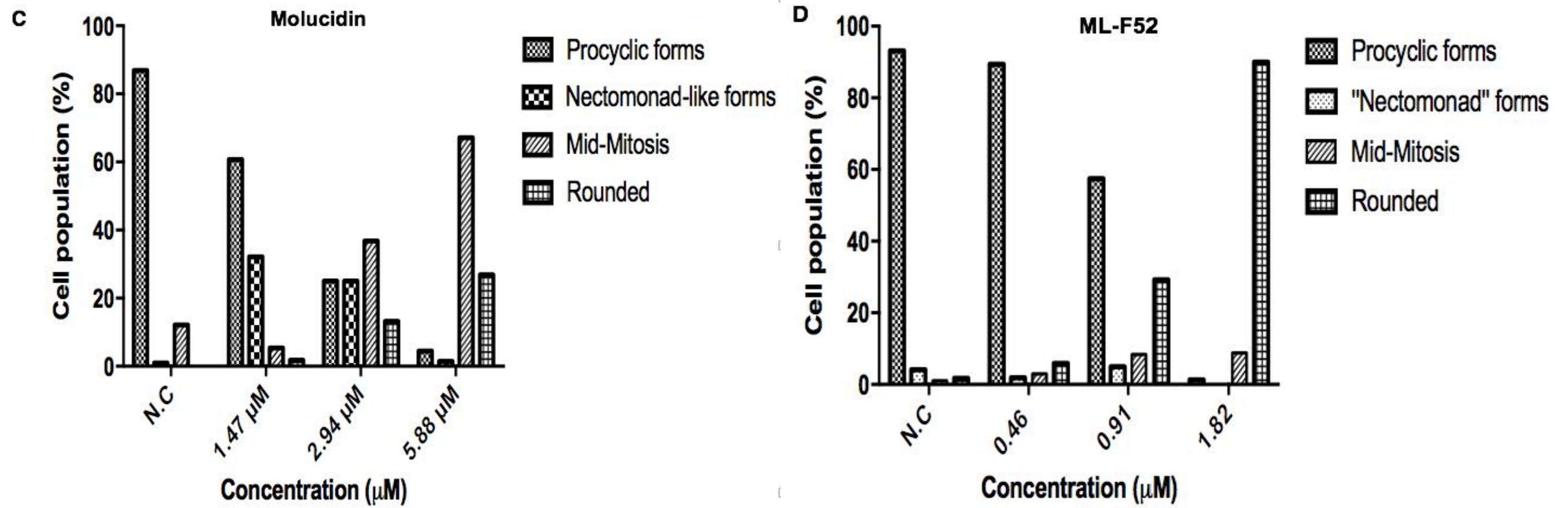

Fig. 5. (A & B) Cellular events in treated and control cultures. Increasing concentration of compounds induces varying phenotypes in cultures. Tetracyclic iridoids inhibit cell cycle progress inducing accumulation of incomplete-mitotic cells. Panel c-k fluorescence stain view, and panel a-j, differential interference contrast, (DIC) view. (C & D) shows a plot of different cell population against iridoid concentration

Although kinetoplastid membrane protein is essential for parasites' cell division and basal body segregation in *Leishmania* [22], immunohistochemistry analysis in this study did not show any effect of iridoids on the expression of KMP protein. However, this study showed the potency of Molucidin and ML-F52 to induce apoptosis and also found to halt cellular events within promastigote forms of *Leishmania donovani*. With further studies on these tetracyclic iridoid compounds, potential therapeutic target molecules of Molucidin and ML-F52 could be identified. These findings suggest that Molucidin, ML-2-3 and ML-F52 could be promising leads against *Leishmania* and other protozoan parasites.

4. CONCLUSION

In the present study, apoptosis induced by tetracyclic iridoids, ML-F52 and Molucidin were shown. These findings, taken together with the different presentations of apoptotic markers validated, supports the apoptotic inducing potential and the anti-amastigote activity of Molucidin and ML-F52 on *Leishmania spp.* Our findings also showed the different effects of iridoids on the morphology, and cellular events suggesting a different mechanism of action against *Leishmania.* This study, therefore, demonstrates *Morinda lucida* as a beneficial source of potential candidate leads in anti-*Leishmania* chemotherapy development.

CONSENT

It is not applicable.

ETHICAL APPROVAL

IRB approval was sought from the Noguchi Memorial Institute for Medical Research, Ghana, IRB board before the start of the project.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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