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Original Article

Efficacy of netropsin dihydrochloride against the viability, cytopathogenicity and hemolytic activity of *Trichomonas vaginalis* clinical isolates☆

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ABSTRACT

Trichomonas vaginalis (*T. vaginalis*) is a common sexually transmitted infection, affecting the urogenital tract. Trichomoniasis is customarily treated with metronidazole (MTZ). MTZ is known to cause undesirable side effects and there is several reports on MTZ resistant *T. vaginalis*. Thus, the present study aimed to in-vitro evaluate the activity of DNA minor groove binder drug "Netropsin dihydrochloride" against metronidazole-sensitive *T. vaginalis* isolates (G and U isolates) and resistant *T. vaginalis* isolate (ATCC50138) (R isolate). Netropsin was tested at concentrations ranging from 3.5 to 200 μg/ml. It showed effectiveness against all isolates with MLC of 12.5 μg/ml for G and U isolates and of 25 μg/ml for R isolate. Cytotoxicity assay of isolates exposed to the respective MLC of netropsin for 42 h showed a highly significant reduction in the death percentage of MCDK cell line as compared to the effect elicited by drug free controls. The hemolytic activity was evaluated by hemolytic assay and by monitoring the interaction of *T. vaginalis* isolates with human erythrocytes by inverted microscopy and scanning electron microscopy. The hemolytic assay showed (0%) hemolysis of RBCs incubated with *T. vaginalis* isolates treated with the corresponding MLC of netropsin for 24 h. Scanning electron microscopy revealed cytoskeletal deformities of netropsin treated isolates. Taken together, these observations suggest that netropsin is a promising therapy for *T. vaginalis* infection affecting its viability, virulence, cytopathogenic and hemolytic activity with a mechanism of action that might overcome *T. vaginalis* resistance to metronidazole.

1. Introduction

Trichomonas vaginalis (*T. vaginalis*) is a flagellated protozoan that inhabits the human vagina [1]. It is one of the most common STDs, with an estimated incidence of 248 million new cases annually [2,3]. Trichomoniasis usually presented clinically with vaginitis and cystitis [4]. It can be complicated by preterm birth, cervical erosion, and infertility [5]. Additionally, trichomoniasis was reported with HIV acquisition [6]. Trichomoniasis is mostly treated with metronidazole (MTZ) [7]. Which is activated inside the cell by anaerobic reduction forming cytotoxic nitro radical anion [8]. Unfortunately, increased MTZ resistance augments the refractory cases, which are usually treated with higher doses, leading to toxicity in some patients [9,10]. FDA listed MTZ as B risk factor in pregnancy for possible teratogenicity [11]. Also, increased preterm rates in pregnant females were reported with MTZ [12]. The aforementioned, poses many therapeutic dilemmas. Other nitroimidazoles, have been tried, but because of a similar mode of action, resistance has to be considered [13]. Other non-nitroimidazole drugs were tested, but they had limited effect [13,14].

Lately, attention was drawn to minor groove binders (MGBs), they bind to DNA minor groove, inhibiting DNA synthesis [15]. MGBs display a broad spectrum of antiviral, antibacterial, antitumor and antiprotozoal activity [16]. They are divided GC (Guanine-Cytosine) and the AT (Adenine-Thymine) classes [16]. Most of AT-specific MGBs were synthesized based on naturally occurring netropsin [15]. Netropsin binds to specific AT-base sequence; blocking DNA helicase, topoisomerase, endonuclease, interfering

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with transcription and all phases of the cell cycle, with a relative emphasis on the G2 phase [17]. Accordingly, and based on high AT-base pairs content (71%) of *T. vaginalis* genome [16]. The present study was designed to investigate the netropsin dihydrochloride effect against *T. vaginalis*.

2. Materials and methods

2.1. T. vaginalis collection, culture and maintenance

Three isolates were tested in the present study, first isolate is a clinically and in-vitro MTZ resistant isolate [18], reference number (ATCC50138) designated in this study as (R). Two isolates were collected from female patient's vaginal washout, referred to the "Early cancer detection unit", Ain Shams University, Gynecology Hospital, designated as (G) and (U) isolates.

Trophozoites were cultured in modified Diamond's trypticase–yeast extract–maltose (TYM) medium [19]. The log phase was determined. Based on the growth pattern of each isolate, the harvested log phase trophozoites (viability $\geq$ 95%), were adjusted to the standardized inoculum (20×10^4/ml) and sub-cultured every 24 h for the G and U isolates, and every 30 h for the R isolate. The mean generation time (MGT) was calculated [20].

2.2. Drugs

Netropsin dihydrochloride 5 mg (Sigma-Aldrich), was dissolved in 5.0 ml distilled water.

MTZ 250 mg tablets (Sanofi-Aventis), dissolved in 250 ml distilled water. Both kept at − 20 °C.

2.3. In-vitro MTZ-susceptibility assay of G and U isolates

Cultures inoculated with MTZ concentrations (1–128 μg/ml), were assessed at 2, 4, 6, 9, 12, 24 and 48 h' intervals for viability by 0.4% trypan blue dye, respective isolates untreated controls were included.

2.4. In-vitro netropsin susceptibility assay

Netropsin concentrations of (3.5–200ug/ml) were tested, the viability was assessed at 2, 4, 6, 9, 12, 24, 30 and 48 h' intervals by 0.4% trypan blue, respective isolates untreated controls were included.

The growth inhibition percentage was calculated as described by Ref. [21], where, Percent of inhibition = a-b/a x 100, where a = mean number of viable control trophozoites and b = mean number of viable treated trophozoites.

The minimal lethal concentration (MLC) was determined as the lowest concentration of the drug showing no viable organisms and revealing no trophozoites growth after subculture [22].

In-vitro growth inhibitory molar dosages were calculated according to the equation: C = m/V × 1/MW, where C is the molar concentration in mol/L, m is the mass in grams, V is volume in liters and MW is the molecular weight in g/mol.

The IC50 was determined using the online IC50 tool kit program.

2.5. Netropsin effect on T. vaginalis cytopathogenicity

The Madin-Darby-canine-kidney (MDCK) monolayer (purchased from Vacsera Institute, Cairo, Egypt), was used, briefly, confluent MDCK (density of 10^4 cells/well) co-incubated with 8×10^5trophozoites/ml, at varying ratios (1:2, 1:4 and 1:8) for 24 h at 37 °C in 5% CO_2, were observed by inverted microscopy [23,24]. The colorimetric MTT assay, was used to determine cell viability after 24 h of incubation [25], as follows: the MDCK cells were washed twice with PBS, MTT solution was added and the plates were incubated at 37 °C for 4 h. After incubation, MTT solvent was added. The absorbance was measured at wavelength 570 nm after 2 h. The cytotoxicity% was calculated as: 100-cell viability%. The mean of twice repeated triplicate experiments was determined. Data were expressed as a percentage, with the control monolayer given a100% value. For each isolate, untreated and treated trophozoites with the respective MLC of netropsin for 24 h and 42 h were assayed. R isolate treated with 12.5 μg/ml netropsin for 24 and 42 h were included. Also, 4.0 μg/ml metronidazole-treated G and U isolates. Netropsin concentrations were tested against the MDCK, drug free and trophozoites free control wells were included.

2.6. Quantitative assessment of hemolysis

Hemolysis assay was performed by mixing a volume of 50 μl of fresh undiluted erythrocytes of A, B, AB, and O blood groups, with 2.5 ml of HBSS containing 1×10^6 untreated trophozoites of each isolate, incubated for 18 h at 37 °C, centrifuged, the supernatant absorbance was measured at 540 nm, osmotic lysis tubes and HBSS controls were included. The results were expressed as percentage [26]. Hemolysis assay was performed on isolates treated with the respective MLC of netropsin using blood group "O".

2.7. Microscopic observations of isolates hemolytic reactivity

2.7.1. Inverted microscopy

An optimized ratio for inverted microscopy was obtained by trophozoites: erythrocytes ratio of 1:20.

2.7.2. Scanning electron microscopy (SEM)

One hour after erythrocytes and trophozoites interaction, the suspension was fixed with 2.5% glutaraldehyde, pH 7.0 for 2 h at 4 °C. Rinsed with PBS, samples were allowed to adhere to 0.1% poly-L-lysine coated glass cover-slips, then were post-fixed in 1% osmium tetroxide in 0.1 M at room temperature for 2 h. Fixed samples were dehydrated in gradually increasing concentrations of ethanol, the cover-slips were mounted on stubs and gold coated by SPI-Module and examined with a JEOL-JSM scanning electron microscope [27].

All experiments, were carried out under defined, standardized inoculum concentration of (20×10^4/ml). All assays were performed twice in triplicates, in the presence of the respective untreated controls of each isolate.

2.7.3. Statistical analysis

The data was analyzed using Statistical package for Social Science (SPSS 15.0.1).

2.7.4. Ethical considerations

The study was approved by the Research Ethics Committee Faculty of Medicine, Ain Shams University, FMASU-FWA00006444, informed patients consents were approved by the Institutional Review Board.

3. Results

3.1. Growth pattern of T. vaginalis isolates

The growth pattern of all drug free isolates (Fig. 1) and the MGT (Table 1).

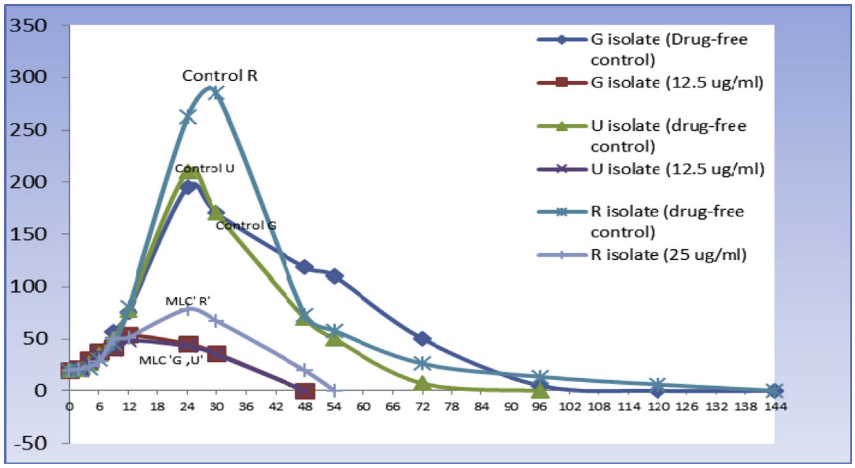

Fig. 1. *T. vaginalis* isolates normal growth pattern and growth inhibition exposure response to corresponding netropsin MLC (12.5 μg/ml) on G isolate, U isolate and (25 μg/ml) on R isolate.

Table 1
Mean generation time of *T. vaginalis* isolates in TYM medium inoculum size (20×10^4/ml).

Isolates	Generation time in hours (GT)
	Mean ± SD
G isolate	7:11 ± 0.049
U isolate	6:56 ± 0.051
R isolate	7:50 ± 0.042

SD: Standard deviation. GT = T log 2/(logN- $\log N_0$) where, N_0: Initial inoculums N: Second count T: Time in hours between the initial and the second count.

3.2. MTZ susceptibility assay of G and U isolates

MTZ effect starts at 6 h for both isolates at concentrations ≥32 μg/ml, the reduction in viability of MTZ treated isolates showed highly significant difference as compared to the respective drug free control, the MLC was 4.0 μg/ml at 48 h (Table 2).

3.3. T. vaginalis-netropsin susceptibility assay

Growth inhibition of the G and U isolates by netropsin concentration of 12.5 μg/ml was 76.9% and 79.5% respectively at 24 h, reaching 100% at 48 h (Tables 3 and 4). Growth inhibition of the R isolate by concentration of 25 μg/ml was 70.04% at 24 h, progressed to complete inhibition at 54 h (Table 5). The MLCs of netropsin was 12.5 μg/ml at 48 h for the G and U isolates and 25 μg/ml at 54 h for the R isolate (Fig. 1).

The estimated IC50 for G, U and R isolates were 5.21 μg/ml, 6.4 μg/ml and 20.43 μg/ml respectively.

3.4. Netropsin effect on T. vaginalis cytopathogenicity

The cytotoxicity of untreated isolates was directly proportional to the ratio of trophozoites: MDCK cells, being highest with the

Table 2
Metronidazole susceptibility assay and Pairwise comparison between respective drug free control and metronidazole treated G and U isolates.

Isolates	Interaction time	Drug free Control	Mean viable count x 10^4/ml ± SD with Metronidazole concentrations								
			1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml	32 μg/ml	64 μg/ml	128 μg/ml	P
G isolate	2 h	24 ± 1.6	22.5 ± 1.1•	23 ± 1.4•	22 ± 1.9•	22 ± 2.5•	22 ± 1.8•	23 ± 0.79•	21 ± 1.6•	20 ± 0.9•	.279*
	4 h	27.5 ± 1.8	28 ± 1.63•	27 ± 1.1•	28 ± 2.9•	27 ± 2.4•	26 ± 1.6•	27 ± 0.52•	24 ± 0.9•	22.5 ± 0.8•	.409*
	6 h	37 ± 0.8	36 ± 0.5•	35.5 ± .78•	34 ± 0.9•	30 ± 1.6•	25.5 ± 1.1••	22.5 ± 0.9•••	20 ± 0.8•••	15 ± 1.6•••	.001***
	9 h	65 ± .7	35 ± 2.8•••	32 ± 0.92•••	33 ± 0.85•••	28 ± 2.8•••	20 ± 1.2•••	13 ± 1•••	12 ± 0.5•••	10 ± 1.4•••	.001***
	12 h	78 ± 2.9	32 ± 1.6•••	30.5 ± 1.1•••	25.5 ± .7•••	20 ± 1.8•••	10 ± 1.22•••	4 ± 0.4•••	3 ± 1.23•••	2.5 ± 1.38•••	.001***
	24 h	185 ± 3.4	23 ± 1.12•••	12 ± 2.4•••	8 ± 1.6•••	3 ± 1.4•••	0•••	0•••	0•••	0•••	.001***
	48 h	120 ± 1.8	6.0 ± 1.4•••	4 ± 1.4•••	0•••	0•••	0•••	0•••	0•••	0•••	.001***
U isolate	Time of interaction	Drug free Control	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml	32 μg/ml	64 μg/ml	128 μg/ml	P
	2 h	21 ± 0.4	22 ± 1.4•	21 ± 1.65•	22 ± 0.8•	22 ± 1.6•	21.5 ± .7•	20.5 ± 0.8•	20.5 ± .7•	20.± 0•	.652*
	4 h	28 ± 1.6	27.5 ± .7•	27 ± 1.8•	28 ± 1.02•	28 ± 2.9•	28.5 ± 0.8•	27.± 0.6•	25.± 0.92•	23.± 3.4•	.215*
	6 h	34.5 ± 0.8	34 ± 3.6•	34 ± 0.8•	31 ± 2.4•	30 ± 3.6•	28.5 ± 0.4•	21 ± 2.9•••	20.5 ± 0.9•••	16.± 1.4•••	.001***
	9 h	50 ± 1.2	33 ± 2.8•••	32 ± 1.4•••	28 ± 2.8•••	29 ± 0.8•••	25.0 ± 1.26•••	14.± 1.38•••	13.± 1.28•••	11.± 1.35•••	.001***
	12 h	80 ± 1.4	29 ± 2.9•••	24.± 1.46•••	21 ± 1.51•••	15.± 1.8•••	9.0 ± 1.1•••	5.0 ± 1.42•••	4.0 ± 1.4•••	3.5 ± 1.6•••	.001***
	24 h	198.5 ± 3.5	20.5 ± 2.4•••	8.5 ± 0.78•••	3.0 ± 1.33•••	2.0 ± 1.4•••	0•••	0•••	0•••	0•••	.001***
	48 h	70 ± 2.2	3 ± 1.34•••	2 ± 1.4•••	0•••	0•••	0•••	0•••	0•••	0•••	.001***

Comparison using ANOVA test where; Mean viable count: mean of triplicate assay for two separate experiments. SD: Standard deviation, hrs: Hours, P: Probability value where; *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference.
Pairwise Comparison between drug free control and different concentrations of metronidazole using Post Hoc test where; •P>0.05 = non-significant difference, ••P < 0.05 = significant difference and •••P < 0.01 = highly significant difference.

Table 3
Susceptibility of the G isolate to netropsin showing viability inhibition percentage.

Duration	Mean viable count x10^4/ml± SD, % of inhibition								P
	Drug free Control	Netropsin concentrations							
		3.5 μg/ml	6.5 μg/ml	12.5 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	
2 hrs	22 ± 1.22	22 ± 1.63, 0	22 ± 1.29, 0	22 ± 1.41, 0	21 ± 1.22, 4.5%	22 ± 1.47, 0	21 ± 1.15, 4.5%	21 ± 2.00, 4.5%	21 ± 1.41, 4.5%	.957*
4 hrs	27.75 ± 1.89	29 ± 2.73, 0	30 ± 2.85, 0	30 ± 2.48, 0	25.50 ± 1.92, 8%	25.75 ± 1.93, 7.2%	25 ± 1.47, 9.9%	24 ± 2.16, 13.5%	24 ± 2.58, 13.5%	.166*
6 hrs	37.5 ± 3.41	38 ± 2.82, 0	38 ± 2.73, 0	38 ± 2.44, 0	36.50 ± 1.77, 2.6%	34 ± 1.29, 9.3%	33 ± 2.38, 12%	32 ± 3.9, 14.6%	31 ± 2.94, 17.3%	.211*
9 hrs	56.5 ± 1.91	53 ± 4.24•, 6.1%	45 ± 3.65•••, 20.3%	42 ± 3.58•••, 25.6%	40.70 ± .95•••, 27.9%	44.50 ± 2.67•••, 21.2%	36 ± 2.48•••, 36.2%	35 ± 2.9•••, 38%	34.5 ± 1.9•••, 38.9%	.001***
12 hrs	75.5 ± 2.38	73 ± 3.51•, 3.3%	56 ± 4.6•••, 25.8%	53.50 ± 4.1•••, 29.1%	48 ± 1.47•••, 36.4%	46 ± 2.04•••, 39%	35 ± 2.85•••, 53.6%	34 ± 2.58•••, 54.9%	32 ± 3.65•••, 57.6%	.001***
24 hrs	195 ± 4.08	198 ± 4.32•, 0	93 ± 3.56•••, 52.3%	45 ± 1.95•••, 76.9%	40.50 ± 2.51•••, 79.2%	40 ± 2.34•••, 79.4%	28 ± 1.41•••, 85.6%	28 ± 2.16•••, 85.6%	25 ± 2.58•••, 87.17%	.001***
30 hrs	170 ± 4.39	169 ± 2.94•, 5.88%	80 ± 3.84•••, 52.9%	36 ± 2.85•••, 78.8%	35 ± 3.55•••, 79.4%	27 ± 1.82•••, 84.11%	18 ± 2.45•••, 89.4%	10 ± 2.94•••, 94.11%	8 ± 2.48•••, 95.29%	.001***
48 hrs	118 ± 3.08	100 ± 3.51•••, 15.25%	6.00 ± 2.32•••, 94.9%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.001***

Comparison between netropsin concentrations using ANOVA test where; Mean viable count: SD: Standard deviation hrs: Hours P: Probability value where; *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference.
Pairwise comparison between drug free control and netropsin concentration using Post Hoc test where; Probability value (P): •P > 0.05 = non-significant difference •• P < 0.05 = significant difference •••P < 0.01 = highly significant difference.

Table 4
Susceptibility of the U isolate to netropsin showing viability inhibition percentage.

Durations	Mean viable count x10^4/ml± SD, % of inhibition								P
	Drug free control	Netropsin concentrations							
		3.5 μg/ml	6.5 μg/ml	12.5 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	
2 hrs	21.00 ± 1.4	21.5 ± 1.3, 0	22 ± 0.8, 0	22 ± 0.8, 0	21.50 ± .4, 0	20.50 ± .5, 2.3%	21 ± 0.9, 0	21 ± 0.8, 0	20 ± .00, 4.76%	.698*
4 hrs	26.50 ± .4	27.5 ± 1.2, 0	28 ± 0.9, 0	27.50 ± 0.4, 0	25 ± 2.4, 5.66%	24.75 ± 1.8, 6.6%	25 ± 2.5, 5.66%	24.5 ± 2.4, 7.5%	24 ± 1.1, 9.4%	.477*
6 hrs	35 ± 2.4	37 ± 1.6, 0	37 ± 1.1, 0	36 ± 1.8, 0	35 ± 1.6, 0	34 ± 1.6, 2.85%	32 ± 2.3, 8.5%	32 ± 1.6, 8.5%	31 ± 0.8, 11.4%	.310*
9 hrs	50 ± 1.6	48 ± 2.4•, 4%	43 ± 2.7•, 14%	40 ± 2.3••, 20%	38 ± 0.9•••, 24%	37 ± 1.1•••, 26%	36 ± 2.9•••, 28%	35 ± 1.8•••, 30%	34 ± 2.3•••, 32%	.005***
12 hrs	78.50 ± 1.7	73 ± 2.92•, 7%	54 ± 2.4•••, 30.7%	48.50 ± .5•••, 38.2%	45 ± 2.9•••, 42.6%	40 ± 2.4•••, 49%	35.50 ± 0.9•••, 54.7%	35 ± 2.5•••, 55.4%	33 ± 1.6•••, 57.9%	.0001***
24 hrs	210 ± 3.4	160 ± 3.4•••, 23.8%	100 ± 2•••, 52.3%	43 ± 2.4•••, 79.5%	37 ± 2.3•••, 82.3%	35 ± 3.4•••, 83.3%	29 ± 1.8•••, 86.1%	28 ± 0.9•••, 86.6%	22.5 ± 2.4•••, 89.2%	.0001***
30 hrs	170 ± 2.4	145 ± 2.4•••, 14.7%	80 ± 3.4•••, 52.9%	35 ± 2.83•••, 79.5%	33 ± 3.4•••, 81%	18 ± 2.9•••, 89.4%	15 ± 1.6•••, 91.17%	7 ± 2.3•••, 95.8%	5 ± 2.9•••, 97%	.0001***
48 hrs	70 ± 2.9	88 ± 1.6•••, 0	10 ± 1.1•••, 85.7%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.00 ± .00•••, 100%	.0001***

Comparison using ANOVA test where; Mean viable count: Mean count of two repeated triplicate assays. SD: Standard deviation hrs: Hours P: Probability value where; *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference.
Pairwise comparison between drug free control and netropsin concentration using Post Hoc test where; Probability value (P): •P > 0.05 = non-significant difference •• P < 0.05 = significant difference •••P < 0.01 = highly significant difference.

ratio of 8:1 and it differs significantly from treated isolates (Tables 6–8).

Netropsin treated isolates gave lower cytotoxicity on MDCK line as compared to the respective untreated controls, additionally the cytotoxic effect of netropsin treated isolates diminished with extending the time of exposure to netropsin (Tables 6–8).

All netropsin treated isolates for 42 h gave lower cytotoxic effect as compared to respective untreated controls and the differences were highly significant (Tables 9–11).

Regarding R isolate, highly significant difference in cytotoxicity was found between netropsin treated trophozoites for 24 and 42 h, no significant difference in the cytotoxicity of trophozoites treated with the concentration of 12.5 μg/ml and 25 μg/ml for 24 and 42 h (Table 11).

As regards G isolate, netropsin treated trophozoites elicited lower cytotoxicity than MTZ treated trophozoites, while netropsin treated U isolate gave higher cytotoxicity than MTZ treated trophozoites (Tables 6 and 7), no significant differences were found between netropsin treated and MTZ treated trophozoites for 42 h concerning both G and U isolates (Tables 9 and 10).

All untreated isolates caused total disappearance of MDCK cell line after 24 h incubation ratio (8:1) (Fig. 2A), netropsin treated isolates for 42 h showed intact cell line (Fig. 2B).

3.5. Hemolytic assay of untreated and netropsin treated isolates

Variable degrees of hemolysis were induced by the untreated isolates on the different blood groups (Table 12). Blood group O was

Table 5
Susceptibility of the R isolate to netropsin showing viability inhibition percentage.

Durations	Mean viable count x10^4/ml± SD, % of inhibition							P
	Drug free control	Netropsin concentrations						
		6.5 μg/ml	12.5 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	
2 hrs	20 ± 0.00	21 ± 1.41, 0	20 ± 0.00, 0	20.75 ± 0.95, 0	20.75 ± 0.95, 0	20 ± 0.00, 0	20 ± 0.00, 0	20 ± 0.00, 0	.974*
4 hrs	22 ± 1.47	22 ± 1.63, 0	22.5 ± 2.38, 0	23.5 ± 1.82, 0	21.5 ± 1, 2.2%	20.5 ± 0.5, 6.8%	20.5 ± 0.5, 6.8%	20.50 ± 1, 6.8%	.475*
6 hrs	30 ± 2.82	28 ± 3.51, 6.6%	27.5 ± 2.73, 8.3%	28 ± 2.8, 6.6%	26 ± 2.48, 13.3%	23 ± 1.87, 23.3%	22 ± 2.44, 26.6%	21.5 ± 1.6, 28.3%	.101*
9 hrs	45 ± 2.38	48 ± 2.48•, 0	49 ± 1.73•, 0	50 ± 3.65•, 0	27.50 ± 1.77•••, 38.8%	30.5 ± 1.73•••, 32.2%	28 ± 1.6•••, 37.7%	26 ± 1.63•••, 42.2%	.0001***
12 hrs	80 ± 3.58	75 ± 2.58•, 6.25%	51 ± 2.94•••, 36.25%	51.5 ± 1.9•••, 35.63%	33.5 ± 3.5•••, 58.1%	28.5 ± 1.5•••, 64.3%	26.5 ± 1.2•••, 66.8%	24.50 ± 1.9•••, 69.4%	.0001***
24 hrs	262 ± 3.55	255 ± 3.51•, 2.67%	248 ± 2.58••, 5.3%	78.5 ± 3.4•••, 70.04%	35.5 ± 2.42•••, 86.45%	16.75 ± 1.55•••, 93.6%	14.5 ± 1.29•••, 94.46%	12 ± 1.41•••, 95.4%	.0001***
30 hrs	285 ± 2.73	279 ± 3.67•, 2.1%	251 ± 3.18••, 11.9%	67.5 ± 1.2•••, 76.37%	17.5 ± 1.29•••, 93.8%	12 ± 1.41•••, 95.7%	8.5 ± 1.9•••, 97%	5.5 ± 1.77•••, 98%	.0001***
48 hrs	73.25 ± 2.21	80 ± 3.26•, 0	95 ± 3.55•••, 0	19.5 ± 3.1•••, 73%	10.5 ± 3.00•••, 85.6%	2.5 ± 1.08•••, 96.58%	1.5 ± 1•••, 97.9%	0•••, 100%	.0001***
54 hrs	58 ± 2.44	63 ± 2.94•, 0	68 ± 2.85•••, 0	0•••, 100%	0•••, 100%	0•••, 100%	0•••, 100%	0•••, 100%	.0001***

Comparison using ANOVA test where; Mean viable count: Mean count of two repeated triplicate assays SD: Standard deviation hrs: Hours P: Probability value where; *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference.
Pairwise comparison between drug free control and netropsin concentration using Post Hoc test where; Probability value (P): •P > 0.05 = non-significant difference •• P < 0.05 = significant difference •••P < 0.01 = highly significant difference.

Table 6
Comparison between cytotoxic effect (MDCK mean cell death %) induced by netropsin treated, metronidazole treated and drug free control of *T. vaginalis* G isolate.

Ratio	Drug-free Control	Netropsin-treated 24 hrs	Metronidazole-treated 24 hrs	Netropsin-treated 42 hrs	Metronidazole-treated 42 hrs	P
	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	
(2:1)	0.07 ± 0.06 (7%)	.00 ± 0.00 (0)	0.083 ± 0.007 (8.33%)	.00 ± 0.00 (0)	0.11 ± 0.011 (11%)	.216*
(4:1)	0.423 ± 0.07 (42.33%)	0.07 ± 0.008 (7%)	0.126 ± 0.002 (12.67%)	.00 ± 0.00 (0)	0.10 ± 0.002 (10%)	.0001***
(8:1)	0.663 ± 0.04 (66.33%)	0.086 ± 0.007 (8.67%)	0.308 ± 0.009 (30.8%)	0.016 ± 0.002 (1.67%)	0.1747 ± 0.004 (17.47%)	.0001***
P value	0.001***	0.191*	0.052*	0.444*	0.409*	

Comparison using ANOVA test where; Mean: Mean optic density of two repeated triplicate assays SD: Standard deviation, hrs: hours P: Probability value *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference Isolate exposed to the respective MLC of netropsin for a specified duration.

Table 7
Comparison between cytotoxic effect (MDCK mean cell death %) induced by netropsin treated, metronidazole treated and drug free control of *T. vaginalis* U isolate.

Ratio	Drug-free Control	Netropsin-treated 24 hrs	Metronidazole-treated 24 hrs	Netropsin-treated 42 hrs	Metronidazole-treated 42 hrs	P Value
	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	
(2:1)	0.509 ± 0.065 (50.93%)	0.178 ± 0.04 (17.80%)	0.123 ± 0.011 (12.33%)	0.113 ± 0.051 (11.33%)	0.006 ± 0.011 (0.67%)	.0001***
(4:1)	0.723 ± 0.015 (72.33%)	0.366 ± 0.01 (36.67%)	0.083 ± 0.035 (8.33%)	0.133 ± 0.066 (13.33%)	0.078 ± 0.071 (7.80%)	.0001***
(8:1)	0.726 ± 0.015 (72.67%)	0.513 ± 0.017 (51.33%)	0.186 ± 0.012 (18.60%)	0.178 ± 0.012 (17.80%)	0.118 ± 0.04 (11.80%)	.0001***
P value	0.006***	0.086*	0.355*	0.721*	0.093*	

Comparison using ANOVA test where; Mean: Mean optic density of two repeated triplicate assays SD: Standard deviation hrs: hours P: Probability value *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference Isolate exposed to the respective MLC of netropsin for a specified duration.

Table 8
Comparison between cytotoxic effect (MDCK mean cell death %) induced by netropsin treated and drug free control of *T. vaginalis* R isolate.

Ratio	Drug-free Control	Netropsin-treated 24 hrs	Netropsin-treated 24 hrs•	Netropsin-treated 42 hrs	Netropsin-treated 42 hrs•	Netropsin-treated 48 hrs	P value
	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	
2:1	.457 ± 0.013 (45.70%)	.18 ± 0.095 (18.00%)	28.8 ± 0.018 (28.80%)	.00 ± 0.00 (0%)	.096 ± 0.084 (9.67%)	.0067 ± 0.011 (0.67%)	.011**
4:1	.73 ± 0.037 (73.07%)	.502 ± 0.029 (50.23%)	.556 ± 0.007 (55.60%)	.125 ± 0.010 (12.57%)	.234 ± 0.029 (23.43%)	.00 ± 0.00 (0%)	.0001***
8:1	.794 ± 0.049 (79.47%)	.631 ± 0.017 (63.10%)	.788 ± 0.016 (78.80%)	.268 ± 0.049 (26.87%)	.426 ± 0.01 5 (42.60%)	.132 ± 0.018 (13.27%)	.0001***
P value	0.128*	0.118*	0.160*	0.110*	0.04**	0.064*	

Comparison using ANOVA test where; Mean: Mean optic density of two repeated triplicate assays, SD: Standard deviation hrs: hours P: Probability value *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference Isolate exposed to the respective MLC of netropsin for a specified duration. •: exposed to 12.5 μg/ml.

Table 9
Pairwise comparison between study groups as regard cytotoxicity "mean cell death %" (8:1) *T. vaginalis* trophozoites: MDCK cell ratio, G isolate.

	Drug-free control	Netropsin 24 hrs	Netropsin 42 hrs	Metronidazole 24 hrs
Netropsin 24 hrs	.000***			
Netropsin 42 hrs	.000***	1.000*		
Metronidazole 24 hrs	.000***	.010***	.001***	
Metronidazole 42 hrs	.000***	1.000*	.087*	.207*

Comparison using Post Hoc Test. Where; Hrs: hours P value: Probability value *P > 0.05 = non-significant difference, ***P < 0.01 = highly significant difference Isolate exposed to the respective MLC of netropsin for the specified duration.

Table 10
Pairwise comparison between study groups as regard cytotoxicity "mean cell death %" at (8:1) *T. vaginalis* trophozoites: MDCK cells ratio for U isolate.

	Drug-free control	Netropsin 24 hrs	Netropsin 42 hrs	Metronidazole 24 hrs
Netropsin 24 hrs	.042**			
Netropsin 42 hrs	.0001***	.004***		
Metronidazole 24 hrs	.0001***	.005***	.932*	
Metronidazole 42 hrs	.0001***	.001***	.526*	.473*

Comparison using Post Hoc Test. Where; Hrs: hours P value: Probability value *P > 0.05 = non-significant difference, **P < 0.05 = significant difference, ***P < 0.01 = highly significant difference. Isolate exposed to the respective MLC of netropsin for the specified duration.

Table 11
Pairwise comparison between study groups as regard cytotoxicity "mean cell death %" at (8:1) *T. vaginalis* trophozoites: MDCK cells ratio for R isolate.

	Drug-free control	Netropsin (25 μg/ml) 24 hrs	Netropsin (25 μg/ml) 42 hrs	Netropsin (25 μg/ml) 48 hrs	Netropsin (12.5 μg/ml) 24 hrs
Netropsin (25 μg/ml) 24 hrs	.531*				
Netropsin (25 μg/ml) 42 hrs	.000***	.003***			
Netropsin (25 μg/ml) 48 hrs	.000***	.000***	1.000*		
Netropsin (12.5 μg/ml) 24 hrs	1.000*	.633*	.000***	.000***	
Netropsin (12.5 μg/ml) 42 hrs	.003***	.176*	.627*	.017**	.003***

Comparison using Post Hoc Test. Where; Hrs: hours P value: Probability value *P > 0.05 = non-significant difference, **P < 0.05 = significant difference, ***P < 0.001 = highly significant difference Isolate exposed to the respective MLC of netropsin for the specified durations and to netropsin 12.5 μg/ml.

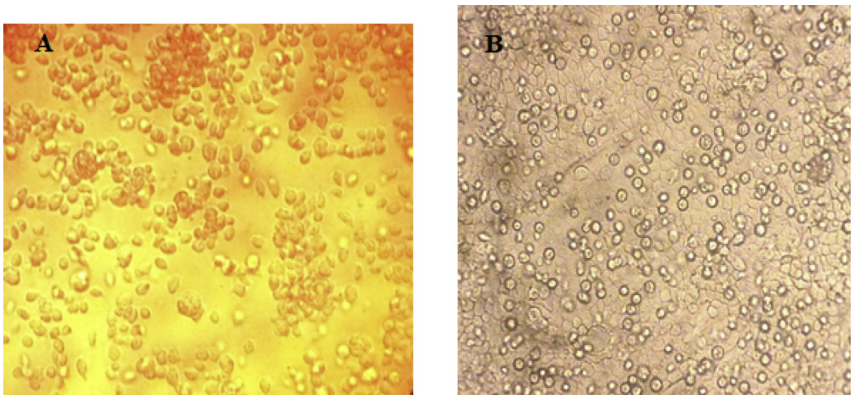

Fig. 2. Interaction between *T. vaginalis* trophozoites and MDCK cell line ratio (8:1) after 24 h incubation. (A) showing untreated isolate with total disappearance of cell line, (B) showing netropsin treated isolate for 42 h, apparent trophozoites ballooning and intact cell line (x400).

Table 12
Quantitative hemolysis of human blood erythrocytes incubated with *T. vaginalis* isolates.

Blood group	G isolate mean ± SD (gm/dl) %	U isolate mean ± SD (gm/dl) %	R isolate mean ± SD (gm/dl) %	Control HBSS Mean (gm/dl)	Total haemolysis Mean (gm/dl)
O	0.2 ± 0.081 (80%)	0.15 ± 0.057 (60%)	0.1 ± 0.00 (40%)	0.00	0.25
B	0.1 ± 0.082 (50%)	0.05 ± 0.0 55 (25%)	0.05 ± 0.057 (25%)	0.00	0.2
AB	0.05 ± 0.05 (12.5%)	0.003 ± 0.006 (0.75%)	0.1 ± 0.0 (25%)	0.00	0.4
A	0.05 ± 0.05 (14.3%)	0.05 ± 0.058 (14.3%)	0.15 ± .057 (42.8%)	0.00	0.35

Mean: Mean optic density of twice repeated triplicate assays SD: Standard deviation. Control HBSS: The optical density obtained due to spontaneous lysis of equivalent amount of RBCs in HBSS (pH 7.0) without organism. Total haemolysis: The optical density obtained from the osmotic lysis of an equivalent amount of RBCs.

Table 13
Quantitative hemolysis of blood group (O) erythrocytes incubated with isolates treated with the respective MIC of netropsin for 24 and 42 hs.

	Drug free control Mean ± SD (%) (gm/dl)	Inoculated with MLC of netropsin for 24 h Mean ± SD (%) (gm/dl)	Inoculated with MLC of netropsin for 42 h Mean ± SD (%) (gm/dl)
G isolate	0.177 ± 0.12 (71%)	0.00 ± 0.00 (0%)	0.00 ± 0.00 (0%)
U isolate	0.13 ± 0.11 (52%)	0.00 ± 0.00 (0%)	0.00 ± 0.00 (0%)
R isolate	0.1 ± 0.00 (40%)	0.00 ± 0.00 (0%)	0.00 ± 0.00 (0%)

SD: standard deviation.

selected to assay the effect of netropsin on the hemolytic activity of the isolates. Netropsin treated isolates showed no hemolysis of RBCs (Table 13).

3.6. Microscopic monitoring of hemolytic reactivity

Untreated trophozoites showed amoeboid transformation and morphological damage of RBCs (Figs. 3A and 4), all netropsin treated isolates for 24 h showed no interaction with erythrocytes (Fig. 3B). U isolate was chosen to elucidate the effect of netropsin on trophozoites ultrastructure's, deformed cytoskeletal structures, ballooning, and axostyle bending was noticed, membrane blebbing and vacuolation, straw-like appearance of flagella (Fig. 5A, B, C).

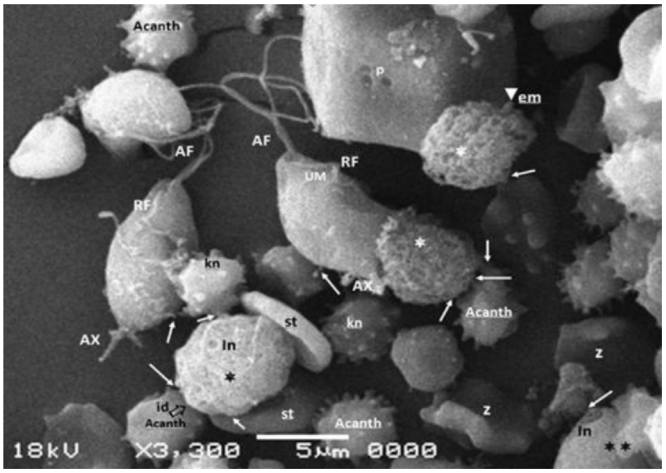

Fig. 4. Scanning electron micrograph showing untreated U isolate interaction with blood group O erythrocytes during the first hour, demonstrating pathological signs of attached and non-attached erythrocytes including knobbing (kn), Knizocytes (z), Stomatocytes (st), and acanthocytes (Acanth). Tiny filose extensions (arrows) mediating attachment are obvious. The tight nature of attachment is discernible by the indentation (id) of the RBC surface at the site of attachment. The image also illustrates compact globular forms with rough convoluted surface showing deep micropores (asterisk), compact form with smooth surface (double asterisks), internalized (In) and emerging (em) flagella of globular forms. The ovoid trophozoites show axostyle (AX), recurrent flagellum (RF) and entrapping of erythrocytes by anterior flagella (AF) (x3300).

4. Discussion

Trichomoniasis is one of the most common STDs [3]. Failures of therapy are mainly associated with metronidazole resistance [28]. Moreover, trichomoniasis connection with HIV acquisition [29], prioritize the need for alternative drug. Netropsin dihydrochloride AT-specific MGB is a promising candidate, it binds to DNA, altering gene transcription, inhibiting cell proliferation, interestingly, netropsin was reported to reduces lymphoma cells proliferation through competing with Epstein–Barr virus in an AT-hook dependent manner [30]. Also, it inhibits *Plasmodium falciparum* ATPase activity [31], and inhibited multidrug resistant Gram negative bacteria [32]. Thus, we investigated the efficacy of "Netropsin" against MTZ-sensitive and -resistant *T. vaginalis* isolates.

In the current study the three tested *T. vaginalis* isolates were susceptible to netropsin, with MLCs of 12.5 μg/ml at 48 h for G and U isolates and 25 μg/ml at 54 h for R isolate, noticeably, higher concentrations of netropsin lead to complete elimination of viable trophozoites after 48 h, the difference in MLCs of netropsin between G, U isolates and R isolate can be explained by the observed difference in peak of growth as G and U isolates showed peak of growth at 24 h, while R isolate peak was at 30 h, using the same inoculum size of 20×10^4/ml. Similarly, trophozoites maximum growth using the same inoculum size at 24 h were reported [33,34]. The MGTs of G, U and R isolates were relatively short 7:11, 6:56 and 7:50 h respectively, as compared to 19:33 h reported by Boulos et al. (2012) [35], different growth peak as well as MGT between different *T. vaginalis* isolates were previously reported [35,36].

The observed inhibitory effect of netropsin in the current study could be attributed to its ability to inhibit some helicases leading to

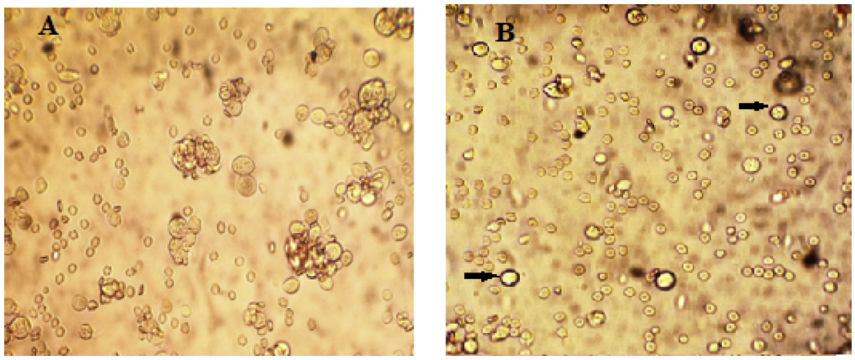

Fig. 3. Inverted microscopy images (**A**) showing interaction between drug free isolate and erythrocytes during the first hour of incubation, attachment and clumping of trophozoites to RBCs. (**B**) showing netropsin treated trophozoites and erythrocytes evident ballooning of trophozoites "arrow" with no attachment to RBCs, normal shape of RBCs (x400).

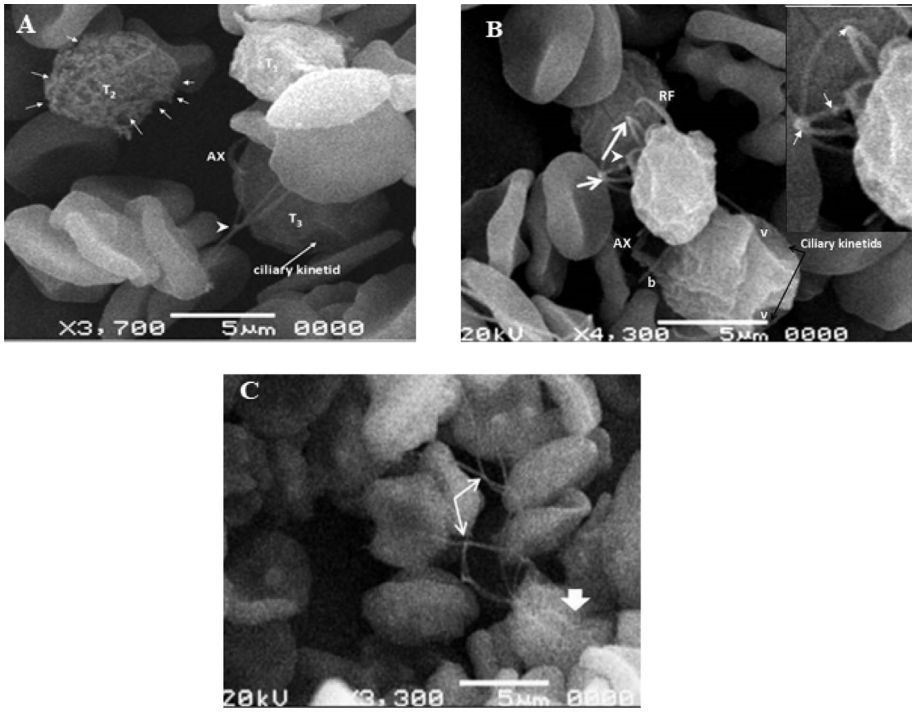

Fig. 5. Scanning electron micrograph of U isolate treated with netropsin (12.5 µg/ml), (**A**) illustrating effect of exposure to netropsin on trophozoites (T) at an ultra-structural level, bushy pellicle (T1), membrane necrosis (arrows) of globular form (T2), pellicular vacuoles around ciliary kinetid (T3), bended axostyle (AX) and broken flagella with pronounced knobs (arrowhead) (x3.700), (**B**) showing rounding up of trophozoites with intense vacuolation (v) of anterior pole, curbing (shortening) and blebbing (b) of the axostyle (AX), recurrent flagellum (RF) and crooked flagella (arrows) articulated with pronounced knobs (arrowhead) (x4300, Insertion: x5000), (**C**) showing misshapen flagella forming straw-like mesh and shrunken trophozoites with bushy hairy rudiments of the pellicle (arrowhead) (x3300) (x4500).

inhibition of DNA-unwinding reaction which indirectly affect some DNA metabolic pathways, leading to inhibition of growth [37,38]. Also, netropsin was reported to inhibit cells growth by affecting specific cell cycle phases and eventually the viability declines with time [39]. Likewise, the time needed for its action could correspond to the length of isolates log phase, this might explain the longer time needed to completely inhibit R isolate growth (54 h), while G and U isolates were completely inhibited after 48 h.

Unpredictably, the R isolate showed decrease in growth inhibition instead of increase when treated with the concentration of 12.5 µg/ml, from 36.25% at 12 h to 5.3% at 24 h, highlighting that 12.5 µg/ml concentration might induce delayed growth not arrested growth, possibly due to G1 phase prolongation caused by netropsin [17]. Likewise, *Physarum* myxamoebae showed arrested growth immediately when using netropsin (40 µg/ml), while at least one cell doubling occurred before complete inhibition with the concentration of 20 µg/ml [40]. Netropsin IC_{50} of G, U and R isolates was 10.34 µM, 12.7 µM and 40.58 µM respectively at 24 h contrasting with the lower netropsin IC_{50} (1.2 µM) that inhibits *Plasmodium falciparum* DNA helicase 60 [37]. Additionally, netropsin IC_{50} (4.7 µM) was needed to inhibit *Plasmodium falciparum* helicase activity, indicating that different IC_{50} values are needed to inhibit specific enzymes in different parasites [39].

Both G and U isolates, were susceptible to MTZ, its effect starts at 6 h with concentrations ≥32 µg/ml, metronidazole MLC was 4 µg/ml at 48 h incubation for both isolates. Similarly, Narcisi and Secor (1996) [41], noticed that metronidazole effect on viability didn't start until after 6 h of incubation even with higher concentrations, affirming the possible lag in metronidazole effect under aerobic condition.

In the present study the MTT assay of untreated isolates, showed increase in cytotoxicity percentage with higher trophozoites: MDCK cell ratio, agreeing with previously reported observations on the cytopathogenic effect of *T. vaginalis* on human vaginal epithelial cells in vitro [23,24]. Concerning netropsin treated isolates, there was no significant difference between the cytotoxic effects of different trophozoites: MDCK cell ratios, indicating that netropsin affects the function of trophozoites even with higher ratios.

A decrease in isolates cytotoxicity were noticed with prolonged incubation time with netropsin, highly significant difference was found between the cytotoxic effect of trophozoites incubated with netropsin for 24 and 42 h concerning U and R isolates. Furthermore, non-significant difference was found between the cytotoxicity of metronidazole and netropsin treated G and U isolates for 42 h, this highlights the comparable effect of netropsin and MTZ. Interestingly, the R isolate showed no significant difference in cytotoxic effect between 12.5 µg/ml and 25 µg/ml (MLC) netropsin treated trophozoites for the same period, even though the 25 µg/ml concentration showed higher viability inhibition percentage, indicating that netropsin disturbs the function prior to the viability especially with prolonged exposure even to lower concentration. Likewise, the same conclusion was reported with non-lethal MTZ concentrations [23]. The inverted microscopy observations of MDCK line exposed to netropsin treated trophozoites mostly showed no cell-line defects, especially with prolongation of trophozoites-netropsin co-incubation time to 42 h, highlighting the reduction of trophozoites' cytotoxic activity induced by netropsin. Additionally, the viability of MDCK monolayer cells was not affected when inoculated with MLCs of netropsin, proposing its safety.

Regarding the hemolytic activity, untreated isolates induced variable percentages of hemolysis, being highest with blood group O, the variations in hemolytic activity with different blood groups demonstrated in the current study were previously reported and explained by differences in surface erythrocytes molecules [26].

Hemolytic activity of netropsin treated trophozoites was abolished (0%) hemolysis, opposing to the reported decline in hemolysis (>50% reduction) with MTZ treated trophozoites [42].

Netropsin treated trophozoites appeared ballooned with disappearance of the pseudopodia-like extensions, which normally appear with untreated trophozoites as observed the current study and previous studies [43,44]. The ballooned form was considered as degenerative, non-proliferative form with arrested growth [4,45]. Likewise, netropsin was reported to induce 4 N cells appearance "cells unable to divide" in *Leishmania* promastigote [46]. Additionally, ballooning of *Tritrichomonas foetus* was reported with benzimidazole (DNA-binding compound) [47], and it was considered as a defense mechanism under stressful conditions even though ending by loss of viability [48].

Moreover, the observed amoeboid transformation of untreated trophozoites when interacting with the RBCs, was totally absent in isolates treated with MLCs of netropsin for 24 and 42 h, together with morphological ultrastructure alterations as revealed by SEM, trophozoites showed membrane blebbing, vacuolation of anterior pole and pellicular vacuoles as signs of necrosis in agreement with Tang et al. (2010) [49], who reported similar deformities when exposing the trophozoites to different drugs. Observed deformities in this study can be explained by the ability of MGBs to cause cytoskeletal structure alteration thus inhibiting cytoskeleton rearrangement and amoeboid transformation that are required for phagocytosis [50,51]. Collectively, our results demonstrated the anti-*T. vaginalis* activity of netropsin, and highlight that netropsin can be a promising alternative to MTZ for treatment of trichomoniasis, inducing loss of viability, cytopathogenic and hemolytic activity of *T. vaginalis* isolates with a mechanism of action that overcomes MTZ resistance. Further studies are recommended to evaluate the effect of netropsin analogous on *T. vaginalis*.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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