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Efficacy of Newly Introduced Synthetic Compounds for Treatment of Experimental Intestinal Giardiasis

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Absract: This study was conducted to evaluate the anti-protozoal effect of the two new compounds, (4-Hydroxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)-2-oxoacetic acid (M1) and Ethyl 2-(4-Hydroxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)-2-oxoacetate (M2) on *Giardia lamblia* infection and to study the pathological impact of these drugs on the duodenal mucosa of infected hamsters. Forty hamsters were used and divided into four groups. Group (1): *G. lamblia* infected-untreated hamsters served as control. Group(2): infected with *G. lamblia* and treated with metronidazole. Group (3): *G. lamblia* infected and orally-treated with M1 compound (100 mg/kg/5 successive days). Group (4): *G. lamblia* infected and orally-treated with M2 compound (100 mg/kg/5 successive days). Treatment of Giardiasis was given 2 weeks post-infection. Orally administered compounds M1 and M2 gave a highly statistical significant reduction ($p< 0.001$) in the number of cysts/gm stool (76.38% &82.71%, respectively). Number of vegetative forms (trophozoite) in the small intestine of sacrificed hamsters was reduced significantly ($p< 0.001$) (71.99% after M1 treatment, & 80.12% after M2 treatment) when compared to infected-untreated control group. Pathological examination of the upper third of the duodenum revealed complete villous shortening and atrophy, polymorphonuclear and eosinophilic cells with diffuse loss of brush border microvillus surface area on group of hamster treated with compound M2. In conclusion, compound M2, had a promising effect on the *Giardia lambila* infection and was superior than treated with Metronidazole.

Key words: Infected hamsters · *Giardia Lamblia* cysts · Intestinal giardiasis · Newly synthetic compound · Histopathological Studies

INTRODUCTION

Giardia duodenalis is a protozoan of public health interest that causes gastroenteritis in humans and other animals [1].

The flagellate protozoan *Giardia lamblia* (*G. lamblia*), its causative agent, is the most common protozoal intestinal parasite isolated worldwide [2-5]. Infection is more common in children than in adults [6, 7]. Diarrhea is the most common symptom of acute *Giardia* infection, occurring in 90% of symptomatic subjects. Abdominal cramping, bloating and flatulence occur in 70-75% of symptomatic patients. Weight loss, disaccharidase deficiency, malabsorption and growth retardation are possible complications [8]. Transmission takes place through ingestion of viable cysts. Infection can be acquired from food or water and directly person-to-person by the oro-fecal route, often there are outbreaks due to poor sanitation facilities [9-11]. Pharmaceutical agents of traditional use are Metroinidazole, Quancrine, Furazolidone and Paromomcine [12]. Other drugs of more recent introduction, such as Albendazole and Nitazoxanide, have also been applied in clinical practice [13, 14]. Of these, Metronidazole and Albendazole may be considered the most representative anti-*Giardia* agents of traditional and recent use, respectively. However, evidence points to an increasing frequency of cases refractory to treatment with these drugs [15, 16]. Therapeutic failure is occurring more and more frequently, due to reinfection or parasite resistance to drug related compounds [17]. Recently, a series of novel synthesized, derived from 3-Acylquinolin-2-ones, were assayed

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activities against *Giardia lamblia*. From this series, (4-Hydroxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)-2-oxoacetic acid (M1) and Ethyl 2-(4-Hydroxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)-2-oxoacetate (M2), showed considerable parasitological activity of these compounds against *Schistosomiasis mansoni* [18, 19] *Schistosomiasis haematobium* [20] and *Giardia lamblia* [21]. In addition, this derivative effects on both trophozoite and cysts reduced, M1&M2 can thus be considered are well-known antiparasites activity. The aim of this work is to study the therapeutic effects of newly introduced synthetic compounds on *G. lamblia*.

MATERIALS AND METHODS

Experimental Animals: Forty laboratory bred male golden hamsters (100-110 grams) were used in the study. The animals were supplied and housed in Schistosome Biological Supply Center (SBSC) at Theodor Bilharz Research Institute (TBRI). The animals' care was in accordance with institutional guidelines. They were kept for three weeks in air conditioned room at 21°C, receiving food containing 24% protein.

Parasites and Infection: G.lamblia cysts were obtained from diarrheic patients attending Parasitology laboratory in outpatient clinic of TBRI. Stool containing *G. lamblia* cysts were repeatedly washed with saline and sieved in order to get rid of debris and to concentrate the cysts. Faecal samples were collected in clean, wide-mouthed covered containers and examined by direct smear [22].

MIF Merthiolate-iodine-formaldhyde Concentration Technique (MIFC): one gm of faecal specimen was added to 5ml MIF solution, mixed well and filtrated in other cup. This was followed by the addition of 7ml ether. The prepared specimen was centrifuge for 5 min at 3000 g. A drop of mixed sediment was placed on a slide, covered and examined under light microscope [23] (MIF solution is a mixture of 2 solutions with ratio 4:1. Solution A compos iodide, iodine and dist. H2O). Each hamster was infected orally by10000 cysts of *G. Lamblia*.

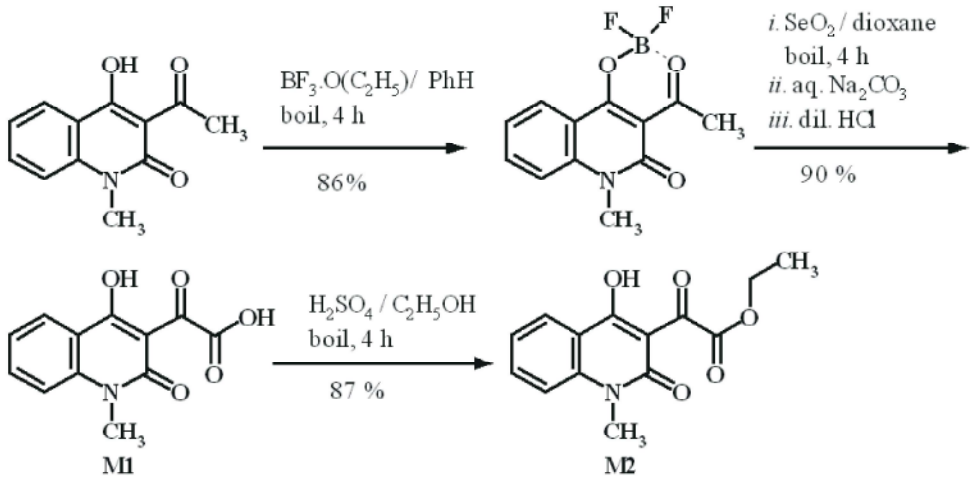
Drugs: Metronidazole (Flagyle) was supplied by Rhone Opulence Rorer Company, as suspension. The dose given to each hamster was 120 µg/kg twice daily for 5 successive days. The dose for hamsters was calculated according to the chart for drug doses in experimental animals [24].

Newly derivatives synthetic compound (M1&M2): supplied by manufactured for ecological formulas research program devoted to synthesis of new 3-substituted 4-hydroxyquinolin-2(1H)-one. The dose was 100mg/kg given for 5 days, post infection [25].

Preparation of Synthetic Compound (M1 &M2): In continuation to our current research program devoted to synthesis of new 3-substituted 4-hydroxyquinolin-2(1H)-one derivatives which are associated with important biological activity [18, 26] we have recently described the preparation of two 3-quinolinyl-keto carboxylic acid derivatives (M1) [27]. As depicted in *Scheme 1*, keto acid (M1) was selectively obtained *via* oxidation of the corresponding difluoroboryl complex of 3-acetylquinolinones with selenium dioxide. Esterification of keto acid (M1), using boiling absolute ethanol in concentrated sulfuric acid, led to the respective ethyl 3-quinolinyl keto carboxylates (M2), in high yields (*Scheme 1*). The structures of the new compounds were elucidated on basis of their elemental microanalyses, as well as IR, ^{1}H NMR, ^{13}C NMR and mass spectral data.

Chemistry: Melting points are uncorrected and were determined in open capillary tubes on a digital Stuart-SMP3 melting point apparatus. IR spectra were recorded on a Perkin-Elmer FT-IR 1650 spectrophotometer, using samples in KBr disks. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on Mercury–300BB or Gemini–300BB spectrometers (δ), using DMSO-d6 or CDCl3 as solvents and TMS as an internal reference. Mass spectra (70 eV) were obtained using a Shimadzu GC-2010 Gas–Chromatography instrument mass spectrometer. Elemental microanalyses were performed on a Perkin-Elmer CHN-2400 analyzer.

A- *2-(4-Hydroxy-1-methyl-2-oxo-1, 2-dihydroquinolin-3-yl)-2-oxoacetic acid (M1)*: It was prepared as previously described in literature [2] in 90 % yield. M.p. 203–205°C (Decomp.). IR (KBr, cm^{-1}), *v*: 3447–2800 (H-bonded O–H), 3030 (C–H_{arom}), 2986 (C–H_{aliph}), 1751 (C=$O_{carboxylic}$), 1690 (C=$O_{\alpha\text{-keto}}$) and 1632 (C=$O_{quinolone}$). ^{1}H NMR (300 MHz, DMSO-d_6), δ: 3.50 (s, 3H, N–CH_3), 5.75 (s, 1H, tautomeric 4-OH?3-CH, disappeared on addition of D_2O), 7.35 (t, J = 7.8 Hz, 1H, 6-CH), 7.55 (d, J = 8.4 Hz, 1H, 8-CH), 7.83 (t, J = 7.8 Hz, 1H, 7-CH) and 8.08 (d, J = 8.1 Hz, 1H, 5-CH). ^{13}C NMR (75 MHz, DMSO-d_6), δ: 192.5, 171.6, 165.0, 159.7, 141.9, 136.0, 125.0, 122.6, 115.4, 113.6, 102.4 and 28.6. MS (*m/z, I_r* %): 248 (2.01) (M+1), 247 (12.58) ($M^{+\cdot}$), 230 (0.54), 219 (4.72), 203 (22.89), 175 (100), 161 (0.65), 133 (11) and 115 (1.64). Anal. Calcd. for $C_{12}H_9NO_5$ (247.21): C, 58.30; H, 3.67; N, 5.67 %. Found C, 58.00; H, 3.50; N, 5.40 %.



Scheme 1: Preparation of keto acids (M1) and keto esters (M2)

***Ethyl 2-(4-Hydroxy-1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)-2-oxoacetate (M2)*:** A solution of the 10 mmol of α-keto acid M1 (2.47 g) in absolute ethanol (50 mL) and drops of conc. sulfuric acid was heated under reflux for 4 h. The reaction mixture was left to cool and the crystalline precipitate so obtained was filtered and recrystallized from ethanol to give the ester M2, crystallized from ethanol, yield: 2.39 g (87 %), m.p. 140–142°C. IR (KBr, cm^{-1}),: 3400-2900 (H-bonded O–H), 3080 (C–H_{arom}), 2950 (C–H_{aliph}), 1739 (C=O_{ester}), 1646 (C=$O_{\alpha\text{-}keto}$), 1630 (C=$O_{quinolone}$), 1559, 765. ^{1}H NMR (300 MHz, DMSO-d_6),: 1.30 (t, J = 6.6 Hz, 3H, O–CH_2CH_3), 3.53 (s, 3H, N–CH_3), 4.31 (q, J = 7.8 Hz, 2H, O–CH_2CH_3), 7.36 (t, J = 7.2 Hz, 1H, 6-CH), 7.58 (d, J = 8.4 Hz, 1H, 8-CH), 7.84 (t, J = 7.5 Hz, 1H, 7-CH), 8.11 (d, J = 7.8 Hz, 1H, 5-CH). ^{13}C NMR (75 MHz, DMSO-d_6),: 190.5, 171.8, 163.6, 160.1, 142.0, 136.2, 125.2, 122.8, 115.5, 113.9, 102.6, 61.8, 28.8, 13.6. MS (*m/z*, I_r %): 276 (3.65) (M+1), 275 (17.45) (M+), 247 (5.08), 230 (1.32), 229 (6.16), 219 (6.63), 203 (13.80), 202 (100), 188(3.59), 175 (22.89), 161 (7.89), 134 (39.39), 118 (6.22), 106 (24.51), 91 (15.18), 77 (23.25). Anal. Calcd. for $C_{14}H_{13}NO_5$ (275.26): C, 61.09; H, 4.76; N, 5.09 %. Found C, 61.07; H, 4.75; N, 5.06 %.

Experimental Design: A group of forty Golden Syrian hamsters were used, which were further subdivided into four small groups: each hamster was infected by orally with 10,000 *G. lamblia* cysts using stainless steel esophageal tube, the divided into four groups. Group (1): Infected animals acted as infected control group (10 animals). Group (2): Infected with *Giardia* cysts (10 animals) and treated with Metronidazole in a dose of 120 µg/ml received twice daily for 5 successive days after infection. Group (3): Infected with *Giardia* cysts (10 animals) received a dose of 100 mg/ml M1 for 5 consecutive days post infection. Group (4): Infected with *Giardia* cysts (10 animals) received 100 mg/kg M2 for 5 consecutive days post infection. The numbers of trophozoites were investigated in the first duodenal part of duodenum of the infected hamsters.

Histopathological Examination: After the mice were killed, the small bowel was removed. A 1 cm segment was excised 5, 15 and 25 cm from gastro duodenal junction. The excised segment was opened longitudinal, oriented on filter paper and fixed in formaldehyde 4% for histopathological examination. After fixation, pieces of tissues were processed for paraffin embedding. Histopathological paraffin sections (3-5um) were stained with Hematoxylin and Eosin (H&E) and the *Giardia* were identified by Giemsa stain [28].

Statistics: Comparison was done between each treated group and its respective untreated control. The percentage change between each two groups to be compared was assessed using the formula:

$$\frac{\text{(Mean value of the infected untreated group - mean value of infected treated group)}}{\text{Mean value of infected untreated group}} \times 100$$

Mean Value of Infected Untreated Group: Difference between the mean scores of any of the two groups to be compared, were tested for significance using an unpaired 2 tailed studentis t-test. The data were considered significant if P values were less 0.05.

Ethical Cosiderations: The experimental animal studies were conducted in accordance with international valid guidelines and they were maintained under convenient conditions at the Schistosoma Biological Supply Program (SBSP) animal house of Theodor Bilharz Research Institute (TBRI).

RESEULTS

Parasitological Results: In *G. lamblia* infected group, results showed significant reduction in both vegetative and cystic forms which treated with M1 after infection (62.85% and 76.38%, respectively) compared to infected control group (Tables 1 & 2).

On the other hand, the M2 treated hamsters showed a highly significant reduction in trophozoite and cystic forms in intestinal contents (71.99% and 82.71, respectively) (Tables 1 & 2).

Pathological Results: Intestinal sections of infected non treated control hamsters revealed focal villous blunting, moderate exudation of mononuclear inflammatory cells and plenty of *Giardia* organisms (As teardrop (Pear) shaped organisms with paired nuclei and flagella) seen on the intestinal surface (Figs. 1,2&3).

Intestinal sections of infected group treated with M1 revealed persistent foci of moderate exudation of mononuclear inflammatory cells and focal villous blunting (Figs. 4&5). In the same context, Metronidazole treated group showed focal similar findings to M1 treated group. On the contrary, intestinal sections of infected group treated with M2 revealed the best results where the villi were near normal with no villous blunting. However, there were persistent foci of moderate exudation of mononuclear inflammatory cells (Figs. 6&7).

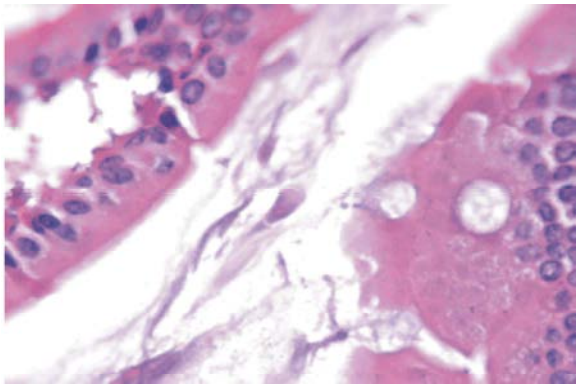


Fig. 1: Infected control group. Giardia trophozoit form. H&E x1000

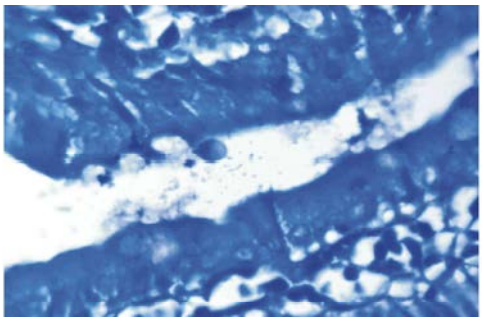


Fig. 2: Infected control group. Giardia. Giemsa x1000

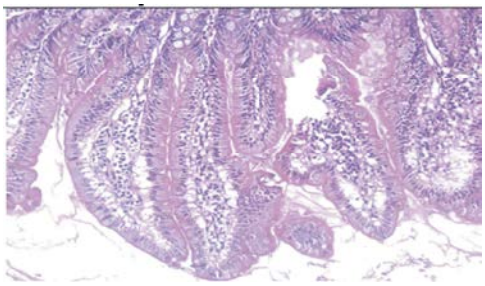


Fig. 3: Infected control group. Villi with moderate mononuclear inflammatory exudation and focal villous blunting (Arrow). H&E x100

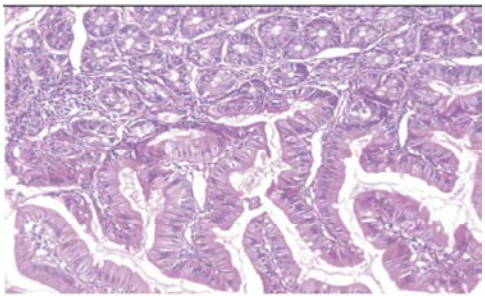


Fig. 4: M1 treated group. Foci of persistent moderate mononuclear inflammatory exudation (arrow) (less effective treatment). H&E x100

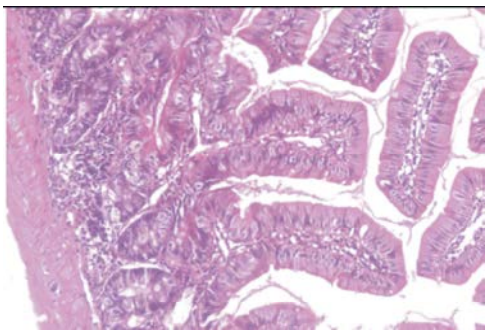


Fig. 5: M1 treated group. Foci of persistent moderate mononuclear inflammatory exudation and focal villous blunting (arrows) (less effective treatment). H&E x100

Table 1: Effect of compound M1 &M2 on vegetative forms (Trophozoite) in the small intestine infected with *Giardia lamblia*

Groups	Vegetative forms in small intestine (Trophozoite) Mean± SE	% reduction
1-Control infected groups	35.7±2.1	0
2-Infected treated with Metronidazole	3.1±0.95*	92.15%
3-Treated with M1-after infection.	13.0±0.20**	62.85%
4-Treated with M2 after-infection.	10.0±1.2***	71.99%

Date were as mean ± SE

* Significant difference compared to the infected control group (P >0.001)

Table 2. Effect of compound M1 &M2 on the number of cysts excreted in stool infected with *Giardia lamblia*

Group	Cysts/gm stool Mean± SE	% reduction
1-Control groups	7957.6± 35.35	
2-Infected treated with Metronidazole	463.2± 24.22*	94.18%
3-Treated with M1-after infection	1879.5±21.24**	76.38%
4-Treated with M2-post infection	1371.1± 30.22***	82.71%

Date were as mean ± SE

* Significant difference compared to the infected control group (P >0.001)

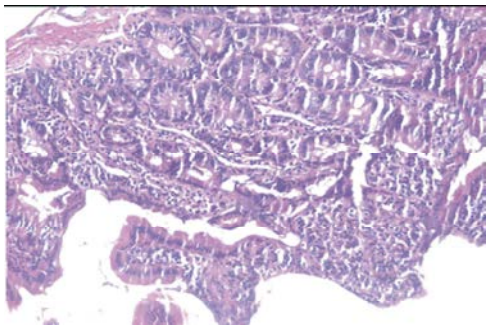


Fig. 6: M2 treated group. No Giardia butthere is still foci of persistent moderate mononuclear inflammatory exudation (more effective treatment). H&E x200

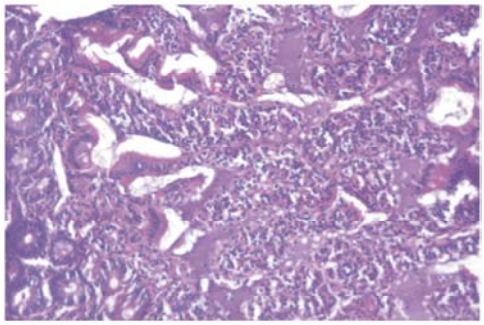


Fig. 7: M2 treated group. No Giardia but there are still foci of persistent moderate mononuclear inflammatory exudation (More effective treatment). H&E x200

DISCUSSION

Giardia lamblia is a binucleate flagellated protozoan parasite that infects the upper intestinal tract of human and many animals' species [29]. It was one of the first protozoan's which discovered by Leeuwenhoek in 1681 in his own diarrheal stool [30]. Nowadays *Giardia* is recognized as the most common parasitological cause of diarrhea in human patients, with an estimated 280 million infections per year and is a major concern to drinking water authorities, as it is a frequently diagnosed waterborne infection [31].

Therapeutic strategy recommended for treatment of giardiasis includes the nitroheterocyclic drugs eg., Tinidazole, Metronidazole, Furazolidine, Benzaimidazole and Albendazole [31].

Metronidazole is the most studied drug for treatment of giardiasis and is available worldwide at low costs. Useful reviews of pharmacology and mechanism have been published. Treatment courses ranging from single dose to 10 days have been used. The results with single dose therapy have been poor with parasitological cure of 36-76%. In contrast, cure rates have been much better with 5 (75-100%), 7 (89-100%) and 10 days (83-100%). These 5-10 day courses have generally used doses of 250 mg to 750 mg tid for adults, or the equivalent dose for children. It is not clear whether the higher dose results in significantly improved outcomes [33, 34].

Parasitic infections caused by *G. lamblia* are still major threats against public health, especially in developing countries. A paucity of new therapeutics has been tested for treatment of Giardiasis in recent years, despite the discovery of Metronidazole resistant *Giardia* isolates. As drug resistance becomes a larger problem, an increased need for new therapeutics is apparent and will have to be met, facilitated by high throughout drug screening [35].

In this study, treatment of giardiasis 2 weeks post-infection with compound M1 and M2, orally administered gave a highly significant reduction was

found in the mean number of *G.lamblia* trophozoite forms in small intestinal contents in the group treated with compound M1 (71.99%), M2 (80.12%) compared to infected control group. These results go in harmony with the histopathological changes that occurred at this time period. There was focal mucosal erosions, villous blunting and moderate inflamatory exudation. This result goes with the previous findings of El-Shennawy *et al*. [21] who recorded the effect of compounds 3-{(2E)-3-(dimethylamino) Prop-2-enoyl}-5, 6-diphenyl-1, 2, 4-triazin-3 (2H)-one (T1), 3-{(2E)-3-(dimethylamino) Prop-2-enoyl}- (2H)-chromen-2-one (C1), 3-(Hexa-2, 4 dienoyl)- 4 hydroxy-1-methyl quinolin-2 (1H)-one (Q1) and N, N' –Bis{2,4-dioxo-1-methyl-1, 2, 3, 4-tetrhydroquinol-3-yl) methyl} benzidine (Q2) on the vegetative (trophozoites) forms in the small intestine of sacrificed hamsters. Compound T1 orally administered in a dose (100mg/kg and 60mg/kg) gave a highly significant reduction in the number of trophozoites (96.7% and 91.7%). However compound C1 resulted to a significant reduction in the number of trophozoites (75.2% and 60.2%). They was found that in the treatment with compounds Q1 and Q2 (80mg/kg and 60mg/kg) the former was much more efficient than the later. The variation between reduction percent in the two studies may due to difference in the function group in compounds. On the other hand, certain aminoglycosides have activity against *Giardia;* most notably Paromomycin and Neomycin. Paromomycin has been evaluated in a few earlier studies, demonstrating an efficacy of about 55-90% [36].

In our study, a high significant difference in the number of cysts/gm s tool between control and treated *G. lamblia* infected group. The reduction rate reported for compound M1 and M2 given in a resulted 76.38%, 82.71%, respectively. These results are in accordance with El-Shennawy *et al*. [21] who reported that a significant reduction in the number of cysts/gm stool in treatment of giardiasis with compounds T1, C1, Q1, Q2 orally administered. The reduction rate reported for compound T1 given in a dose of (100mg/kg and 60mg/kg) was 94.3% and 83.8%, respectively. However, groups treated with compound C1 given in a dose of (100mg/kg and 60mg/kg) resulted 96.2% and 89.1%. Percent reduction reported for compound Q1 (80mg/kg and 60mg/kg 89.6% and 72.6% respectively). Where in compound Q2, the reduction percents were 80.35% and 68.9%.

Bahadur *et al.* [37] studied the effect of Chalcones, 1,3-diphenyl-2-propen-1-one derivatives belonging to the Flavonoid family, are open-chain unsaturated carbonyl systems in which two aromatic rings are joined by a three-carbon α, β-unsaturated carbonyl framework on *Giardia intestinalis*. He synthesized, purified, characterized and tested toxicity against *G. intestinalis* of two libraries of novel chalcone derivatives, for a total of 46 compounds. Active compounds were comparatively assayed under both anaerobic and more physiological microaerobic conditions. This novel approach allowed us to identify two chalcone derivatives that under microaerobic conditions are able to kill *Giardia* trophozoites selectively and more efficiently than Metronidazole.

The results of the present study showed that Metronidazole treatment when compared to the infected control group led to 92.15% reduction in the number of trophozoite form and 94.18% in reduction of the number of *Giardia* cysts/ gm in stool. These results agreed with Amer *et al.* [12] who reported a percentage of reduction of 93.91% in the number of cysts and 92.22% in the number of trophozoites following Metronidazole treatment in hamsters. Similarly, Gardner and Hill [38] reported a cure rate of more than 90% using Metronidazole for treatment of *Giardia* infection.

In conclusion, M1& M2 is as efficient as Metronidazole on *Giardia lamblia* in vivo with added advantage of being a newly derivatives synthetic compound. The present results hold the perspective for the finding of new therapeutic alternative to *Giardia lamblia* treatment. New and efficient synthetic compound inhibiting the growth of M1& M2 trophozoites without side effects may be very useful in the treatment of the infection.

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