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Original Article

Anti-*Toxoplasma gondii* activity of 5-oxo-hexahydroquinoline derivatives: synthesis, *in vitro* and *in vivo* evaluations, and molecular docking analysis

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Abstract

Background and purpose: The aim of this study was to evaluate the *in vitro* and *in vivo* anti-*Toxoplasma gondii* (*T. gondii)* effect of 5-oxo-hexahydroquinoline compounds. Moreover, molecular docking study of the compounds into the active site of enoyl-acyl carrier protein reductase (ENR) as a necessary enzyme for the vitality of apicoplast was carried out.

Experimental approach: A number of 5-oxo-hexahydoquinoline derivatives (Z1-Z4) were synthesized. The *T. gondii* tachyzoites of RH strain were treated by different concentrations (1-64 μg/mL) of the compounds. The viability of the encountered parasites with compounds was assessed using flow cytometry and propidium iodide (PI) staining. Due to the high mortality effect of Z_3 and Z_4 *in vitro*, their chemotherapy effect was assessed by inoculation of tachyzoites to four BALB/c mice groups (n = 5), followed by the gavage of various concentrations of the compounds to the mice. Molecular docking was done to study the binding affinity of the synthesized 5-oxo-hexahydroquinolines into ENR enzyme active site byusing AutoDock Vina® software. Docking was performed by a Lamarckian Genetic Algorithm with 100 runs.

Findings / Results: Flow cytometry assay results indicated compounds Z_3 and Z_4 had relevant mortality effect on parasite tachyzoites. Besides, *in vivo* experiments were also performed and a partial increase of mice longevity between control and experiment groups was recorded. Molecular docking of Z_3 and Z_4 in the binding site of ENR enzyme indicated that the compounds were well accommodated within the binding site. Therefore, it could be suggested that these compounds may exert their anti-*T. gondii* activity through the inhibition of the ENR enzyme.

Conclusion and implications: Compounds Z_3 and Z_4 are good leads in order to develop better anti-*T. gondii* agents as they demonstrated both *in vitro* and *in vivo* inhibitory effects on tachyzoites viability and infection. Further studies on altering the route of administration along with additional pharmacokinetics evaluations are needed to improve the anti-*T. gondii* impacts of 5-oxo-hexahydroquinoline compounds.

Keywords: Flow cytometry; Molecular docking; 5-Oxo-hexahydroquinoline; Propidium iodide; *Toxoplasma.*

INTRODUCTION

Toxoplasma gondii (T. gondii) is an intracellular protozoan parasite which causes toxoplasmosis, a common zoonotic infection in humans and animals (1). The estimation indicates that disease prevalence rate in humans is one-third of the world population people who have been chronically infected. The parasite engenders severe medical complications in the fetus and also immunocompromised people (2, 3).

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Moreover, despite of the relatively low rate of toxoplasmosis outbreak in immunocompetent ones, some studies report severe toxoplasmosis in healthy patients (4, 5). *T. gondii* is transmitted by felids, whereas their coccidian lifestyle through sexual reproduction leads to pouring oocysts in outside of their bodies (6-8). Rodents and other intermediate hosts may ingest oocysts, bringing about the initial expansion of growing tachyzoites and thus disseminating to all over the body which leads to acute infection of the parasite. In response to tachyzoites attack throughout the body, the immune reaction of the intermediate host's body occurs, and then parasite converts into the recumbent and slow-growing form of the parasite called the bradyzoite (9). Finally, the ingestion of raw meats or water involving oocysts causes infection in humans.

The conventional treatment of toxoplasmosis in humans comprises of a combination of drugs of sulfadiazine (blocks dihydropteroate synthetase) and pyrimethamine (or trimethoprime which inhibits dihydrofolate reductase) (10). *T. gondii* first disseminates as tachyzoites then resides in tissue cysts causing a prolonged latent infection. In immunocompromised patients, bradyzoites can turn into tachyzoites that may cause pneumonia and encephalitis. Despite all advancements in drug discovery, encysted bradyzoites have not been yet destroyed by suitable drugs (11).

Quinolines are a group of heterocyclic aromatic organic compounds with a multitude of medicinal properties (12-15). Quinoline ring and one of its derivatives, 2-methyl-3-carbethoxyquinoline, were eligible to inhibit the *T. gondii* growth, 52-57% loss of a unique non-photosynthetic organelle of *T. gondii* tachyzoites called apicoplast (16). The synthesis of heme, isoprenoid precursors and fatty-acid synthesis (FAS) II pathway is done within this particular organelle(17,18). The enoyl-acyl carrier protein reductase (ENR) is among the specific enzymes FAS II pathway, the final and integral stage in the fatty acid chain elongation (17). Attempts have far revealed the vulnerability of a host of *T. gondii* to inhibition of fatty acids pathway and ENR subsequently by triclosan (19,20). As to compare with previous studies, quinoline derived drugs such as 4-aminoquinolines and 8-amino quinolines were more efficacious in treating malaria disease than revealing inhibition effect on *T. gondii* growth. On the other hand, benzylquine and bisquinoline were among the promising compounds against *T. gondii* with EC_{50} values less than 1 μM, in comparison with other candidates that have been used in the study which killed less *T. gondii* (21). Another research showed that endochin-like quinolone can reduce brain *T. gondii* tissue cyst numbers in mice due to inhibition of *T. gondii* cytochrome bc1 function (22). A review study reveals that the quinoline ring has anti-*T. gondii* activity through mechanisms of heme polymerization and degradation in the food vacuole, membrane transport interruption in the parasite (19), can disrupt apicoplast DNA and finally the whole apicoplast in the parasite (16,23).

Regarding quinoline derivatives biological effects, we synthesized 5-oxo-hexahydroquinoline analogs bearing different aromatic substitutions at C_4 while, having different pyridyl alkyl carboxylates at C_3 (Fig. 1) and evaluated them for their *in vitro* and *in vivo* inhibitory effects on the viability and infectivity of *Toxoplasma* tachyzoites.

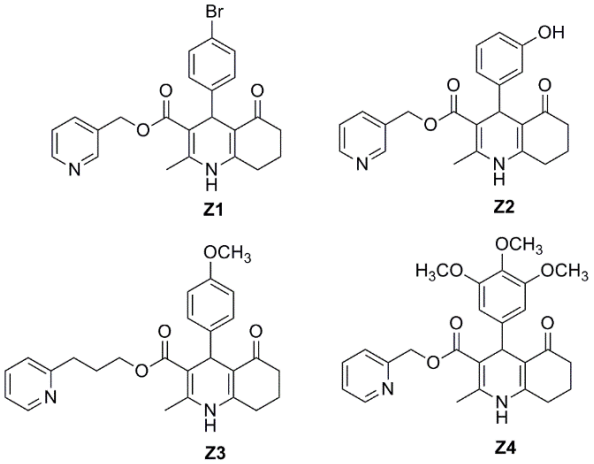


Fig. 1. Structural formula of 5-oxo-hexahydroquinoline compounds (pyridin-3-yl methyl 4-(4-bromophenyl)-2-methyl- 5- oxo-1,4,5,6,7,8- hexahydroquinoline-3-carboxylate (Z1), pyridin-3-yl methyl 4-(3-hydroxyhenyl) -2- methyl- 5- oxo -1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z2), 3-(pyridin-3-yl)propyl 4-(4-methoxyphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z3), and pyridin- 2-ylmethyl 2-methyl -5-oxo-4-(3,4,5-trimethoxyphenyl) -1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z4).

MATERIALS AND METHODS

Chemicals

Analytical degree chemicals and solvents were used in this experiment. Pyridine-2-yl-methanol, pyridine-3-yl-methanol, pyridine-3-yl- propanol, aldehydes, and 1,3-cyclohexanedione were provided from Merck, Darmstadt, Germany. Propidium iodide (PI) was obtained from Sigma-Aldrich, USA.

Apparatuses

Carbon-13 nuclear magnetic resonance (^{13}C NMR) and proton (^{1}H) NMR spectra were detected with a Brucker 300 spectrometer. Infrared (IR) spectra were acquired on a Perkin-Elmer spectrometer (KBr disk) (Perkin-Elmer, Waltham, MA). Chemical shifts were reported as ppm and tetramethylsilane was used as an internal standard. Mass spectra (MS) were recorded with an Agilent spectrometer (Agilent technologies 9575c inert MSD, USA). A Costech elemental analysis instrument (Costech ESC 4010, Italy) was achieved as the elemental assessment and was within 0.4 % of the calculated value. A hot stage apparatus (Electrothermal, Essex, UK) was used to attain melting points.

Synthesis of 5-oxo-hexahydroquinoline derivatives (Z_1-Z_4)

Oxobutanoates (2a, 2b, and 2c) were synthesized by reacting pyridin-3-ylmethanol (1a), pyridin-2-ylmethanol (1b) or pyridin-3-ylpropanol (1c) with 2, 2, 6-trimethyl-4H-1, 3-dioxin-4-one in high yields as shown in Fig. 2. Alternatively, 5-oxo-hexahydroquinoline derivatives were obtained by the reaction among oxobutanoate (2a, 2b, or 2c), corresponding aromatic aldehyde (2 mmol) and 1,3-cyclohexanedione (2 mmol) in the presence of ammonium acetate (10 mmol) in ethanol (7 mL) for 8 h, while protected from light (Fig. 2) (15). The reaction was monitored by thin-layer chromatography (TLC). Ethanol was removed under reduced pressure and the residue was dissolved in chloroform. Then the reaction mixture was purified by TLC with chloroform-ethanol as the mobile phase. Finally, the product was recrystallized from the proper solvent to yield pure compounds (Fig. 2). All the molecular structures of final products were classified and confirmed by IR, ^{1}H NMR, mass spectrometry (MS), and elemental analysis. Chemical structures and physical properties of the synthesized compounds are shown in Table 1.

Biological activity procedures

Animals

Inbred BALB/c mice aging 6-8 weeks, and weighing 25-27 g were purchased from Pasteur Institute, Tehran, I.R. Iran. The whole procedures of trials and sacrifices were identical for all animals. In the course of the experiments, starting from November 2017 and ending up to February 2018, animals were maintained at 22 °C with about 40-50% relative humidity, and also standard food and water *ad libitum* were available. The *in vivo* study was conducted based on the laboratory animal's guidelines in literary and research disciplines (24).

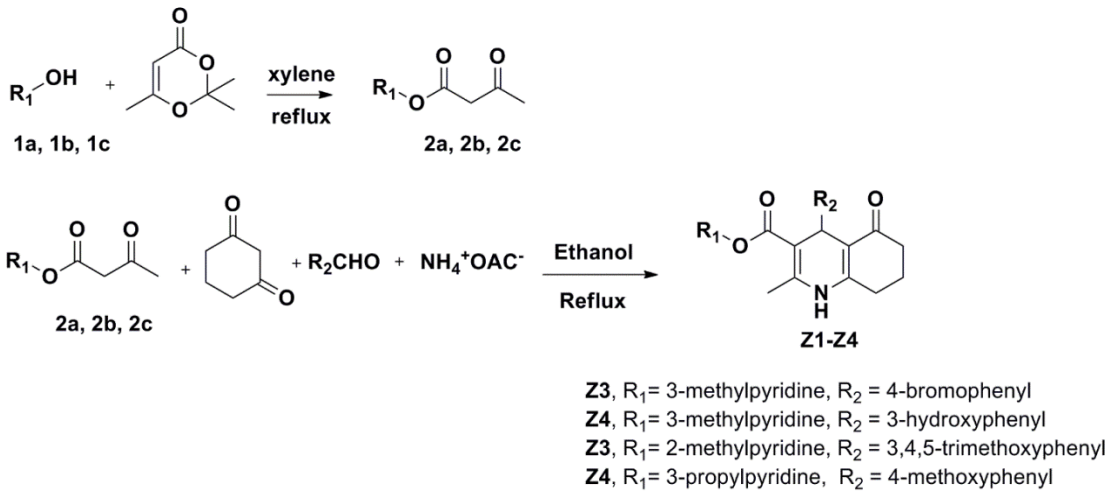
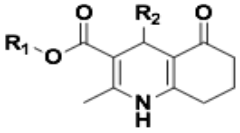
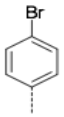
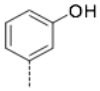
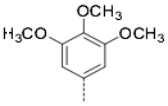
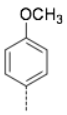


Fig. 2. Synthetic approaches of compounds Z1-Z4.

Table 1. Structures and drug likeness scores of synthesized compounds.



Code	R1	R2	Yield (%)	Log P	HBA	HBD	TPSA	nRB	MW
Z_1	3-methylpyridine		43	2.048	5	1	67.76	6	453
Z_2	3-methylpyridine		24	1.214	6	2	87.99	5	390
Z_3	2-methylpyridine		68	2.173	8	1	95.45	8	464
Z_4	3-propylpyridine		48	2.051	6	1	64.98	6	432

LogP, Logarithm of partition coefficient between n-octanol and water; HBA, number of hydrogen bond acceptors; HBD, number of hydrogen bond donors; TPSA, topological polar surface area; nRB, number of rotatable bonds; MW, molecular weight.

Parasites

The virulent *T. gondii* RH strain was provided from Tehran University of Medical Sciences, Tehran, I.R. Iran. *T. gondii* Tachyzoites were taken by a serial intraperitoneal passage in BALB/c inbred mice and then they were collected 72 h after the inoculation of 106 parasites in the mice, by repeated flushing in the peritoneal cavity using phosphate-buffered saline (PBS) at a pH of 7.2. Next, tachyzoites were harvested and centrifuged for 10 min at 200 g at 25 °C to eliminate the peritoneal cells and cellular debris. The supernatant was then gathered and centrifuged for 10 min at 1200 rpm (800 g) (25).

Extracellular viability assay (flow cytometry)

Compounds Z_1-Z_4 were dissolved in dimethyl sulfoxide (DMSO) to attain a 10 mM stock solution. The tachyzoites (2×10^5/mL of PBS) were treated with DMSO (0.1% v/v) as the control and various concentrations (1-64 µg/mL) of 5-oxo-hexahydroquinolines for 2 h at 4 °C. Then, the tachyzoites were gathered in Eppendorf tubes staining with 50 µg/mL concentration of PI for 30 min at 4 °C. Finally, the parasites were preserved on ice and in dark condition until analysis. Positive controls were obtained by incubation of the parasites in the vicinity of 0.2% saponin. The cell suspension was transferred into polystyrene flow cytometry tubes (BD Falcon, USA). Data achievement and evaluation were performed with a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, USA) and Cell Quest Pro software (25).

Chemotherapy effect of the compounds

A number of 2×10^5 tachyzoites were subcutaneously inoculated into 5 groups, 5 mices in each group, including three concentrations (16, 32, 64 µg/mL) of compounds Z_3, Z_4.The other groups were negative and positive control (Sufadiazine 4 µg/mL). Based on IC_{50} determination study of compounds Z_3, Z_4, these compounds were

selected for chemotherapy in mice. Toxicity assessment of compounds Z_3 and Z_4 was performed in the 6th group of mice; the compounds were orally gavaged into mice that have not been inoculated by the intact parasites. For the negative control, the mice only have been inoculated with parasites. Sulfadiazine (15 mg/kg) as an active substance was orally administered against *T. gondii* in the positive control group.

Molecular docking analysis

The 3D structures of the synthesized compounds were sketched using ChemOffice software (26) Then, the achieved structures were optimized in their conformation by the energy minimization process, in which the results were saved in pdb formats. After energy optimization and ligand preparation, the Gastiger's partial charges and the number of torque's angles of molecules were calculated. The ligands were also saved in pdbqt formats. In this study, we applied AutoDock Vina software to inquire about the affinity and binding modes of the synthesized derivatives to the binding pocket of P-gp. X-ray crystal structure of Enoyl-acyl Carrier Protein Reductase (Enoyl ACP Reductase) in complex with triclosan and NAD (PDB ID: 2O2S) was got from RCSB Protein Data Bank (http://www.rcsb.org). To prepare the enzyme for docking, we removed the innate ligand and water molecules, added hydrogen atoms, merged non-polar hydrogens, and added Gasteiger charges. All rotatable bonds of ligands, defined by default of the program, were allowed to rotate during the automated docking process, and then prepared protein and ligand structures were saved in the PDBQT format suitable for calculating energy grid maps. Auto Dock Tools were used to prepare the molecules and parameters before submitting it for docking analysis with Auto Dock (27). The grid dimensions were 30 × 30 × 30 Å along with a grid space value of 1 Å. The grid was centered at 3.177, 67.997, and 67.535 Å (X, Y, and Z) which involved the active site of triclosan. It is notable to say that macromolecule sequences were considered rigid and inflexible. Besides, the exhaustiveness factor was considered "8", and then each of the conformational structures resulting from docking was ranked due to the free energy of the interactions of ligand-receptors. Validation of molecular docking was done by extracting the structure of the cocrystallized ligand and re-docking it into the receptor (self-docking). The root mean square deviation (RMSD) was used to evaluate, optimize, and validate the docking calculations. We investigated the binding affinities of the synthesized 5-oxo-hexahydroquinolines into ENR enzyme active site. The docking process used a Lamarckian genetic algorithm with 100 runs for each ligand, leaving other parameters at their default measures (28). Conformations were clustered and ranked by the energy. The conformation with the best-scored pose and the lowest binding energy was selected. The docking results were depicted using ChemOffice® software.

Statistical analysis

Data analysis was done by FACS Calibur flow, Cell Quest Pro software and SPSS software (version 22, Armonk, NY, USA) using the one way ANOVA test as appropriate. Intergroup comparisons were undertaken using Tukey's posthoc test, after log transformation for non-parametrically distributed variables. $P \leq 0.05$ was contemplated statistically significant.

RESULTS

Synthesis of 5-oxo-hexahydroquinoline derivatives

The designed compounds were synthesized through conventional synthetic procedures and characterized by different methods.

Pyridin-3-yl methyl 4-(4-bromophenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z_1)

Recrystallized from ethanol; pale yellow crystals; M.P: 233 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ_H (ppm): 1.72-1.91 (m, 2H, cyclohexenone), 2.14-2.21 (m, 2H, cyclohexenone), 2.30 (s, 3H, dihexadecylphosphate (DHP)-CH_3), 2.44-2.50 (m, 2H, cyclohexenone overlapped with DMSO), 4.87 (s, 1H, DHP-C_4-H), 5.02 and 5.10 (AB system, 2H, J_{AB} = 12.6 Hz, $COOCH_2$), 7.05 (d, 2H, J = 8.4 Hz, phenyl-H-2, 6), 7.29-

7.35 (m, 3H, pyridine-H-5 and phenyl-H-3, 5), 7.53 (dt, 1H, *J* = 7.8, 1.8 Hz, pyridine-H-4), 8.45 (d, 1H, *J* = 1.8 Hz, pyridine-H-2), 8.50 (dd, 1H, *J* = 4.8, 1.5 Hz, pyridine-H-6), 9.28 (brs, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ_H (ppm): 18.78, 21.16, 26.45, 35.88, 37.08, 63.02, 102.62, 111.37, 119.26, 123.87, 130.17, 131.17, 132.69, 136.10, 146.88, 147.39, 149.48, 149.56, 151.73, 166.82, 195.19. MS (EI), m/z (%): 452 (M^+, 3), 362 (18), 361 (3), 360 (20), 297 (100), 237 (3), 205 (5), 180, 161 (7), 133 (4), 92 (11), 65 (5). IR (KBr): υ 3207 (NH), 3079 (CH-aromatic), 2952 (CH-aliphatic), 1667, 1634 cm^{-1} (CO). Found C, 70.21; H, 4.68; N, 6.16%. Anal. ($C_{23}H_{21}BrN_2O_3$) requires C, 60.94; H, 4.67; N, 6.18%.

Pyridin-3-yl methyl 4-(3-hydroxyhenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z_2)

Recrystallized from ethyl acetate and ethanol; pale yellow crystals; M.P: 214 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ_H (ppm): 1.66-1.93 (m, 2H, cyclohexenone), 2.18-2.20 (m, 2H, cyclohexenone) 2.30 (s, 3H, DHP-CH_3), 2.44-2.50 (m, 2H, cyclohexenone, overlapped with DMSO), 4.87 (s, 1H, DHP-C_4-H), 5.04 and 5.12 (AB system, 2H, J_{AB} = 12.9 Hz, $COOCH_2$), 6.46-6.49 (m, 1H, phenyl-H-4), 6.54-6.57 (m, 2H, phenyl-H-2,6), 6.93 (t, 1H, *J* = 7.5 Hz, phenyl-H-5), 7.29-7.34 (m, 1H, pyridine-H-5), 7.53 (dt, 1H, *J* = 7.8, 1.5 Hz, pyridine-H-4), 8.46 (d, 1H, *J* = 1.5 Hz, pyridine-H-2), 8.49 (dd, 1H, *J* = 4.8, 1.5 Hz, pyridine-H-6), 9.13 (brs, 1H, NH), 9.21 (brs, 1H, OH). MS (EI), m/z (%): 390 (M^+, 11), 312 (14), 297 (100), 254 (4), 216 (4), 161 (7), 133 (4), 93 (18). IR (KBr): υ 3276 (NH), 3072 (CH-aromatic), 2938 (CH-aliphatic), 1699, 1645 cm^{-1} (CO). Found C, 70.57; H, 5.64; N, 7.19%. Anal. ($C_{23}H_{22}N_2O_4$) requires C, 70.75; H, 5.68; N, 7.17%.

3-(pyridin-3-yl)propyl 4-(4-methoxyphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z_3)

Recrystallized from ethanol; pale yellow crystals; M.P: 165 °C. ^{1}H NMR (acetone, 300 MHz) δ_H (ppm): 1.62-1.77 (m, 4H, cyclohexenone and $COOCH_2CH_2CH_2$), 2.07-2.10 (m, 2H, cyclohexanone), 2.27 (s, 3H, DHP-CH_3), 2.36-2.43 (m, 4H, cyclohexenone, and $COOCH_2CH_2CH_2$), 3.55 (s, 3H, phenyl-OCH_3), 3.78-3.98 (2m, 2H, $COOCH_2CH_2CH_2$), 4.94 (s, 1H, DHP-C_4-H), 6.60 (d, 2H, *J* = 9 Hz, phenyl-H-3,5), 7.10 (d, 3H, *J* = 9 Hz, phenyl-H-2,6 and pyridine-H-5), 7.34 (d, 1H, *J* = 7.8 Hz, pyridine-H-4), 8.06 (brs, 1H, NH), 8.21 (s, 1H, pyridine-H-2), 8.25 (d, 1H, *J* = 0.6 Hz, pyridine-H-6). MS (EI) m/z (%): 432 (M^+, 45), 414 (59), 325 (100), 312 (27), 294 (9), 268 (18), 206 (27), 161 (7), 120 (21), 92 (36). IR (KBr): υ 3279 (NH), 3074 (CH-aromatic), 2952 (CH-aliphatic), 1694, 1646 cm^{-1} (COC). Anal. Calcd for $C_{25}H_{26}N_2O_4$: C 72.35, H 6.50, N 6.49%, found: C 72.20, H 6.53, N 6.48%.

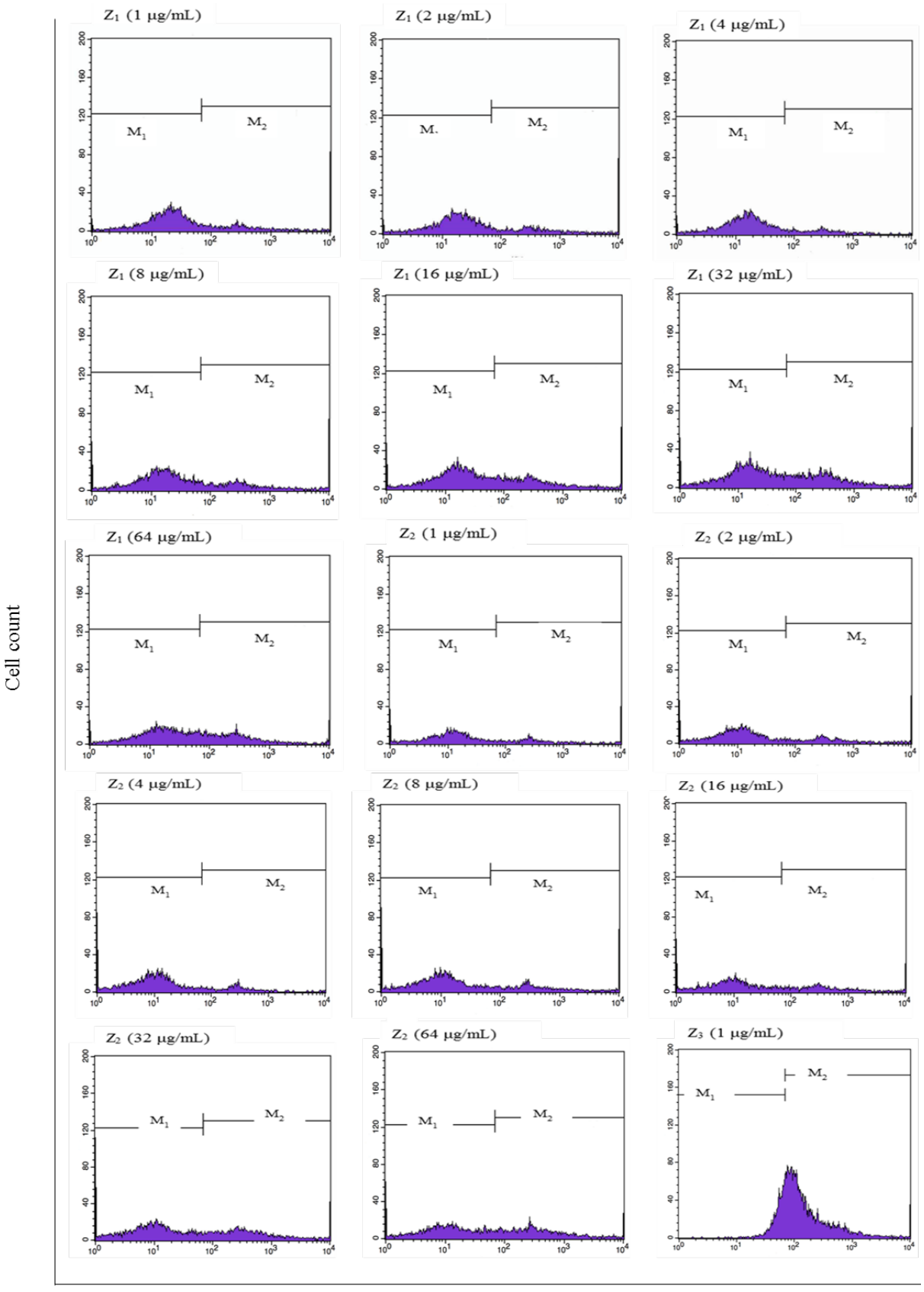
Pyridin-2-ylmethyl 2-methyl-5-oxo-4-(3,4,5-trimethoxyphenyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (Z_4)

Recrystallized from ethyl acetate; pale yellow crystals, M.P: 174 °C. ^{1}H NMR (DMSO-d_6, 300 MHz) δ_H (ppm): 1.77-194 (m, 2H, cyclohexenone), 2.21-2.25 (m, 2H, cyclohexenone), 2.32 (s, 3H, DHP-CH_3), 2.46-2.50 (m, 2H, cyclohexenone, overlapped with DMSO), 3.59 (s, 3H, phenyl-4-OCH_3), 3.61 (s, 6H, phenyl-3,5-OCH_3), 4.95 (s, 1H, DHP-C_4-H), 5.10 and 5.18 (AB system, 2H, J_{AB} = 13.8 Hz, $COOCH_2$), 6.42 (s, 2H, phenyl-H-2,6), 7.07 (d, 1H, *J* = 8.1 Hz, pyridine-H-3), 7.28-7.32 (m, 1H, pyridine-H-5), 7.67-7.72 (m, 1H, pyridine-H-4), 8.51-8.53 (d, 1H, *J* = 4.5 Hz, pyridine-H-6), 9.12 (brs, 1H, NH). MS (EI), m/z (%): 464 (M^+, 2), 433 (4), 372 (100), 328 (11), 297 (36), 188 (35), 160 (13), 133 (4), 93 (14), 65 (5). IR (KBr): υ 3276 (NH), 3073 (CH-aromatic), 2935 (CH-aliphatic), 1702, 1647 (CO), 1070, 1230 cm^{-1} (COC). Found C, 67.42; H, 6.05; N, 6.00%. Anal. ($C_{26}H_{28}N_2O_6$) requires C, 67.23; H, 6.08; N, 6.03%.

Biological section

Flow cytometric determination of T. gondii tachyzoite mortality evaluation in the presense of 5-oxo-hexahydroquinolines

The synthesized and purified 5-oxo-hexahydroquinolines (Z_1-Z_4) were evaluated for their effect on *T. gondii* tachyzoite mortality using flow cytometric determination of PI. Flow cytometry analysis of different concentrations (1-64 μg/mL) of the compounds against *T. gondii* tachyzoites viability is demonstrated in Fig. 3.


Z1 (1 µg/mL)
Z1 (2 µg/mL)
Z1 (4 µg/mL)
Z1 (8 µg/mL)
Z1 (16 µg/mL)
Z1 (32 µg/mL)
Z1 (64 µg/mL)
Z2 (1 µg/mL)
Z2 (2 µg/mL)
Z2 (4 µg/mL)
Z2 (8 µg/mL)
Z2 (16 µg/mL)
Z2 (32 µg/mL)
Z2 (64 µg/mL)
Z3 (1 µg/mL)
M1
M2
Cell count
FL 1

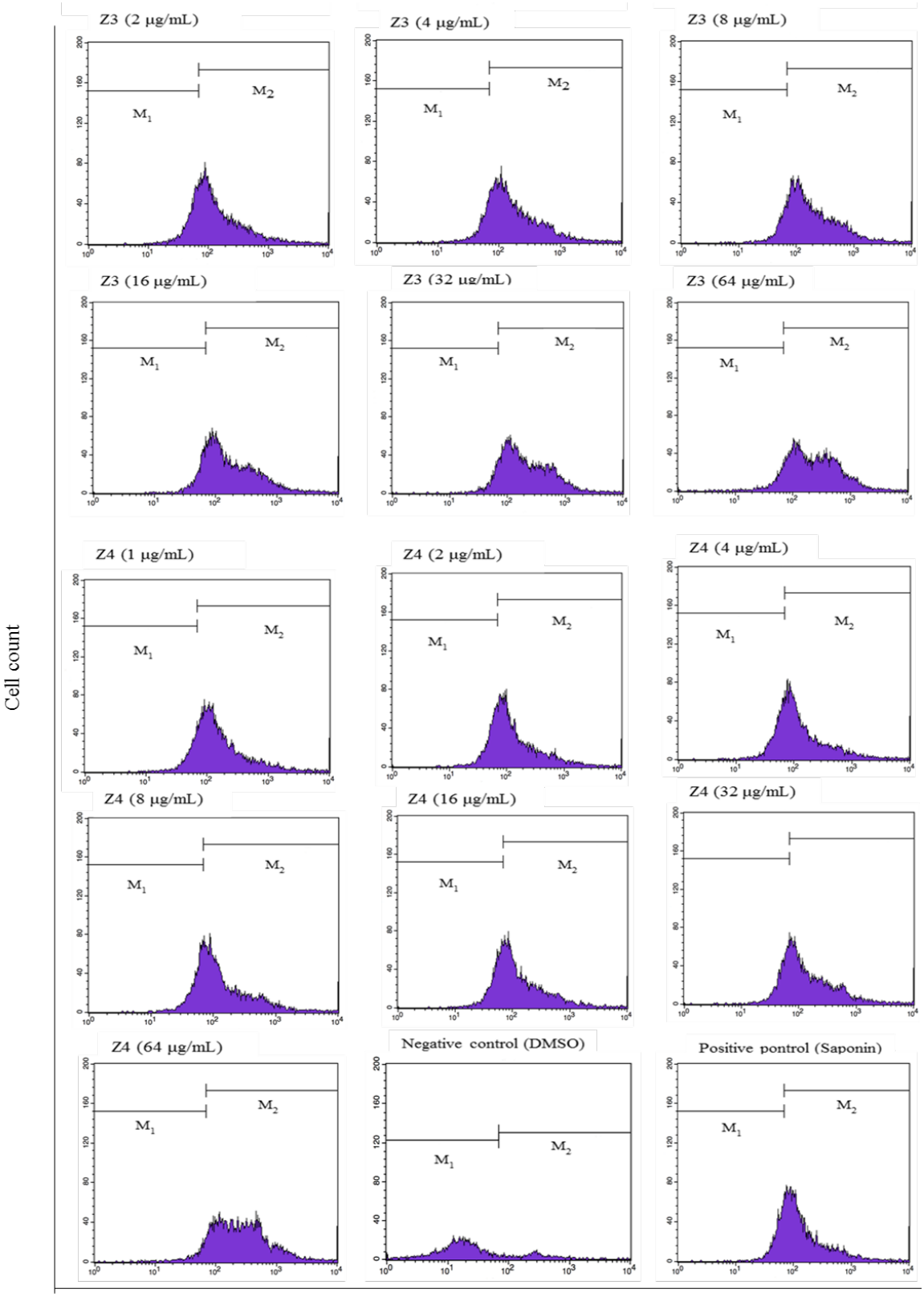


Fig. 3. Flow cytometry analysis of *Toxoplasma gondii* tachyzoites exposed to different concentrations of Z1, Z2, Z3, Z4, negative and positive controls.

Compounds Z_3 and Z_4 caused over 50% mortality of tachyzoites at high concentrations (8-64 µg/mL). The test results on tachyzoite of *T. gondii* exposed to DMSO, saponin as a positive control, and various doses of Z_1-Z_4 are shown in Table 2. The percentages of mortality

caused by the compounds improved in a concentration-dependent manner. Saponin (0.2%) was used to trigger mortality or apoptosis which was seen about 96.69% of the exposing tachyzoites.

in vivo anti-T. gondii activity of 5-oxo-hexahydroquinolines

The most active compounds, Z3 and Z4, were evaluated for their *in vivo* anti-*T. gondii* activity in the mice exposed to the tachyzoites in three different concentrations (16, 32, 64 μg/mL). Results revealed that after about 10 days all the mice died and the mean longevity of the mice treated with 16, 32 and 64 μg/mL of Z3 was 7, 8.5 and 8.6 days and for Z4 were 7.8, 9.1 and 9.6, respectively, while, the mean survival of the mice in the negative control group was 5.4 days (Fig. 4). The results showed a correlation between Z3 and Z4 administration and the mice resistance against *T. gondii* tachyzoites. It seems that increasing the administered doses of Z3 and Z4, leads to higher mice longevity.

Table 2. The mortality (%) of *Toxoplasma gondii* tachyzoites after 1.5 h exposure to different concentrations of 5-oxo-hexahydroquinoline derivatives. 0.2% of saponin was used as the positive control, in the meantime, DMSO (0.1% v/v) was considered as the negative control.

Concentrations (μg/mL)	Compounds				Controls	
	Z_1	Z_2	Z_3	Z_4	DMSO	Saponin
0	0.41	0.41	0.41	0.41	-	-
1	20.06	20.30	77.94	76.68	-	-
2	21.48	21.49	73.03	75.59	-	-
4	13.27	14.22	72.14	85.47	-	-
8	26.46	24.51	63.70	85.77	-	-
16	33.52	26.92	64.94	83.93	-	-
32	38.86	38.20	74.56	90.28	-	-
64	35.21	42.43	78.03	90.57	-	-
	-	-	-	-	16.32	96.69

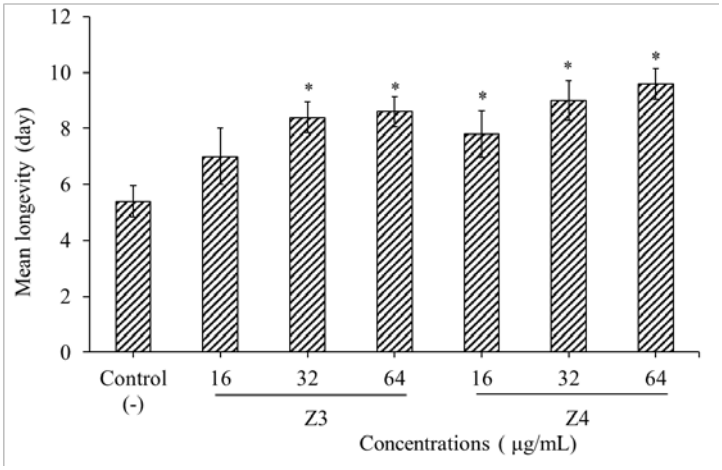


Fig. 4. The mean of longevity (days) of mice groups inoculated by *Toxoplasma gondii* tachyzoites exposed to three different concentrations of compounds Z3 and Z4 (μg/mL). *$P \leq 0.05$ indicates significant differences compared to the negative control group (DMSO). The mice treated with sulfadiazine as positive control but due to a longevity more than 30 days, therefore the positive control is not shown on the graph. Data represent mean ± SD, n = 5.

Molecular docking analysis

We investigated the binding affinities of the synthesized 5-oxo-hexahydroquinolines into ENR enzyme active site. Docking results are listed in Table 3. Molecular docking process was validated by extracting the structure of the co-crystallized ligand and redocking it into the receptor (self-docking).

Table 3. Docking results of 5-oxo-hexahydroquinoline derivatives into the *Toxoplasma gondii* Enoyl- acyl carrier protein Reductase enzyme binding site.

Ligands	ΔG (Kcal/mol)	Interaction	Atom of ligand	Amino acid
Z_1	-8.4	H-bonding	Oxygen (carbonyl)	Gly131
Z_2	-7.8	H-bonding	Oxygen (carbonyl)	Gly131
Z_3	-8.5	H-bonding H-bonding	NH Oxygen (methoxy)	Ala129 Lys237
Z_4	-9.2	H-bonding H-bonding H-bonding	NH Nitrogen (pyridine ring) Oxygen (carbonyl)	Ala129 Arg230 Ala231
Triclosan	-9.1	H-bonding	OH	Tyr189

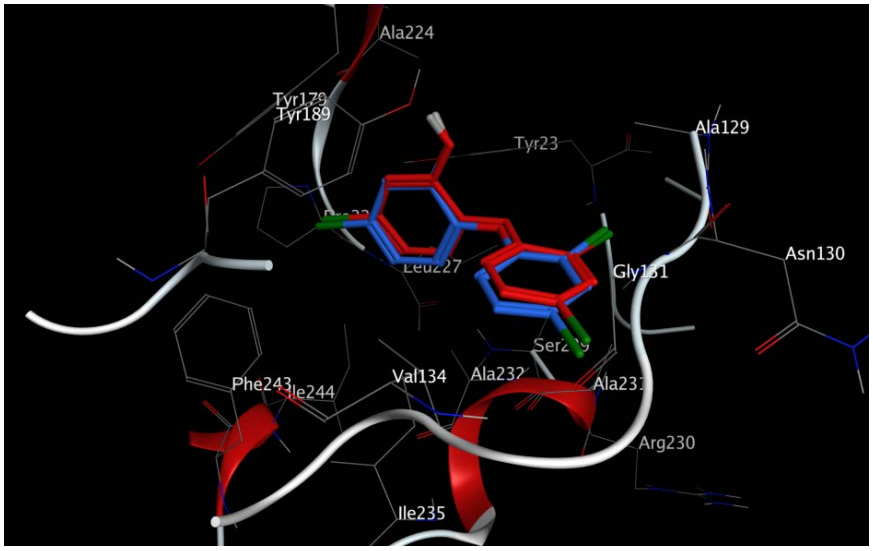


Fig 5. Representation of the co-crystallized inhibitor (blue) docked into the binding site and superimposed on co-crystallized inhibitor (red) in the crystal structure of *T. gondii* Enoyl-ACP Reductase (PDB ID: 2O2S).

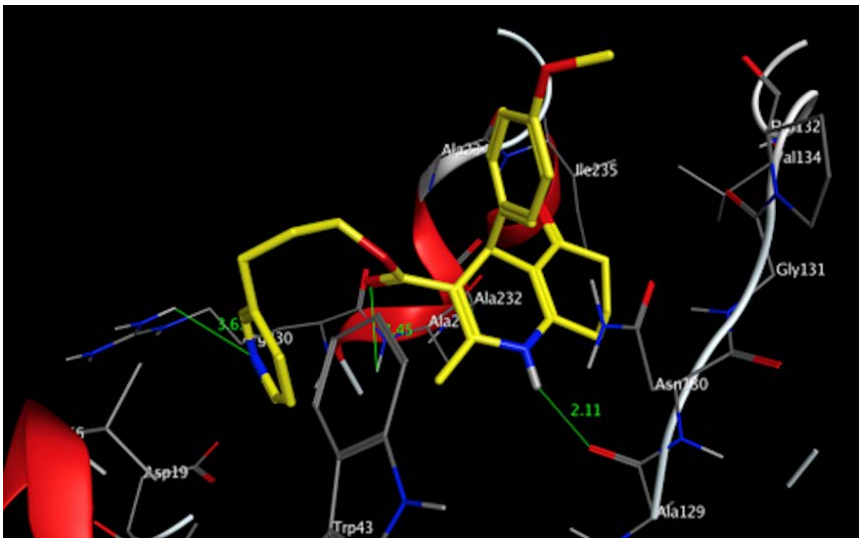


Fig. 6. Docking model of compound Z4 in the *Toxoplasma gondii* enoyl-acyl carrier protein Reductase enzyme active site (PDB ID: 2O2S).

RMSD between the best conformation of the native ligand docked into the binding site of *T. gondii* ENR and the experimental one was 0.51 Å (Fig. 5). Binding interactions of the synthesized derivatives established H-bonding and H-Pi interactions with different amino acids including Gly131, Ala231, Asn130, Ala129, Lys237, and Arg230 (Table 3). Compounds Z_3 and Z_4 possessed the most negative binding free energies (-8.5 and -9.2 kcal/mol) which were comparable to that of the innate ligand (-9.1 kcal/mol), were well accumulated in the binding pocket of ENR by making hydrogen bonds. The binding model of Z_3 and Z_4 is illustrated in Fig. 6. In the two active compounds (Z_3 and Z_4) a key hydrogen bond interaction could be seen between NH of the hexahydroquinolinone ring and Ala129. It seems that the residue Ala129 played a crucial role in making stable ligand-protein complexes.

DISCUSSION

5-Oxo-hexahydroquinoline compounds synthesis and evaluation were done to assess their anti-*T. gondii* effects. Flow cytometry results using PI staining on *T. gondii* extracellular tachyzoites indicated that compound Z_3 (baering 2-pyridyl methyl carboxylate and 3,4,5-trimethoxyphenyl at C_3 and C_4) and Z_4 (containing 23-pyridyl propyl carboxylate and 4-methoxyphenyl moiety at C_3 and C_4) are potential substances to exert anti-*T. gondii* effects. Treating toxoplasmosis has some difficulties, such as adverse effects of currently available drugs, which could be resolved by looking for novel compounds with new effective mechanisms. One other major problem to address is the treatment of immunocompromised patients, which can be complicated due to their latent period. Furthermore, rising microbial resistance is also very challenging; therefore, developing new effective drugs that are devoid of previous problems are of great importance. A number of studies were conducted on quinolone derivatives evaluation. By way of example, an study showed that 1-hydroxyquinolones inhibited the growth of *T. gondii* by means of pyrimidine starvation associated with the ATP depletion in dihydroorotate dehydrogenase pathway (29). Endochin-like quinolone structure was also previously surveyed for its potential use in clinics. The relevant results revealed that two of endochin-like derivatives were remarkably successful in the inhibition of acute and latent murine toxoplasmosis. Inhibition of the cytochrome bc1 complex in the parasites was suggested as a reason for this outstanding effect (22). Moreover, the other quinolone that reported to be effective against toxoplasmosis was enrofloxacin, which was better than sulfadiazine (as a strong anti-*T. gondii* compound) to protect human foreskin fibroblasts infected cells against *T. gondii*. Enrofloxacin proved to be an active compound for cleaning up the tissue parasites as well as it could alleviate the brain inflammations of *T. gondii*'s infected *Calomys callosus*. In addition, the reduction percentage of intracellular replication during the usage of enrofloxacin was more than sulfadiazine (30). Another research that was based on the combination of enrofloxacin and toltrazuril, resulted killing a significant number of parasites and entirely broking down the tachyzoite structure (31). Gatifloxacin, as a fluoroquinolone, has ultimately inhibited human foreskin fibroblasts *T. gondii* intracellular replication, therefore leads to increase the *T. gondii*'s infected mice survival, and it has protected the mice against acute toxoplasmosis. As like as enrofloxacin, a synergistic effect, *in vitro* and *in vivo*, was observed by using gatifloxacin along with pyrimethamine. Moreover, juxtaposing infected mice associated with concurrent use of gatifloxacin and interferon gamma demonstrates appropriate effect of the drugs which was much higher than using them separated (32). *In vivo* study of several quinolone derivatives against acute and latent toxoplasmosis showed that two of the compounds had ED_{50} values of 0.14 and 0.08 mg/kg in comparison with atovaquone having an ED_{50} value of 0.85 mg/kg (22). In our study, 5-oxo-hexahydroquinoline derivatives were evaluated for their anti-*T. gondii* effects. Reviewing the literature of quinoline derivatives in the previous studies, two compounds were analyzed and proved to be active agents against *T. gondii* with EC_{50} value of 0.03 mg/kg. Furthermore, they had an inhibitory effect on *Plasmodium falciparum* regarding the mitochondrial electron transport mechanism, a necessary phase of pyrimidines synthesis (33).

4-Aminoquinoline and 8-aminoquinoline derivatives such as tafenoquine were safer than primaquine, they are utilizing to prevent the relapse of *Plasmodium vivax* as well, but these compounds were ineffective against *T. gondii* eventually (34). Another study that was conducted on the effects of quinolone-like compounds on *T. gondii* indicated that quinolone has less EC_{50} than atovaquone, at the same time, the quinolone-like compounds were among the ones with higher EC_{50} amounts than other common drugs in toxoplasmosis treatment (21). A survey on the effect of 8-hydroxyquinoline compounds as anti-*T. gondii* agents showed that 58 derivatives of these compounds containing one or two quinoline rings had very high effects (16). In the present study, the most active compounds (Z_3 and Z_4) were evaluated for their anti-*T. gondii* activity in mice at three different concentrations. *in vivo* results showed significant statistical differences between all doses of both compounds and the negative control group ($P \leq 0.05$) except the compound Z3 at 16 μg/mL, the mean longevity was increased compared to the negative control group. Besides, toxicity effects were also analyzed, and the mice which were administered the highest dose (64 μg/mL) of compound Z_3 and Z_4 showed no signs of a certain amount of toxicity (weight loss, ruffled fur, inactivity and so forth). Biologically, *T. gondii* and the other Apicomplexa family parasites have an essential organelle for parasite living which is called apicoplast. FAS pathway is of utmost importance mechanism in the apicoplast. The FAS process in mammalians is type I, but in Apicomplexa parasites is type II. The fatty acid production in microorganisms is well studied, it helped scientists to discover relevant enzymes and substrates. Review studies show the key role of FAS II pathway inhibition in pathogen mortality (35,36). FAS comprised of 4 stages of compression, reduction, hydration, and elongation, respectively. Elongation is the most important stage of the process that is catalyzed by ENR (37). ENR reduces the double bond between acetyl CoA and malonyl CoA in associated with NADH cofactor. Studies demonstrated that ENR is of great necessity for FAS (38,39). Drugs such as isoniazid, triclosan, diaborazine derivatives, and aminopyridine inhibitors are able to inhibit this enzyme in bacteria, triclosan certainly inhibits FAS in both *T. gondii* and *Plasmodium falciparum* (18,39). As previously mentioned, the quinolone derivatives were among the anti-*T. gondii* apicoplast agents. Thus, the ENR enzyme within the apicoplast of the parasite provided us a chance to assess the hexahydroquinoline based compounds inhibition effect on the FAS process (23). These results propose using hexahydroquinoline derivatives with a similar structure to quinolone might exert anti-*T. gondii* effects. Back to our study, docking results of the 5-oxo-hexahydroquinolines showed that compound Z_3 and Z_4 was well located within the active site of *T. gondii* ENR enzyme and possessed the least estimated free energy of binding compared to the innate ligand. In addition, the correlation between biological activities and the binding affinities anticipated by AutoDock Vina® modeling was appropriate for compound Z_3 and Z_4. Therefore, it is suggested that this compound may exert its anti-*T. gondii* activity through the inhibition of the ENR enzyme. Several studies surveyed ENR, for instance, in one study isoxazole-3-carboxamides, 4-hydroxypyridine-2(1H)-ones, and 3-aminopyrazoles were considered the best inhibitors of ENR. However, this is not the only enzyme that should be inhibited and other mechanisms of fatty acid synthesis should be blocked as well to kill tachyzoites (19). The result of infectivity test on tachyzoites indicated that compound Z_3 and Z_4 prolonged the life duration of mice groups inoculated by *Toxoplasma* tachyzoites more effectively than negative control group; moreover, considrable statistical differences were observed between the compounds of intrest and the control group. Docking results of the 5-oxo-hexahydroquinolines exposed that compounds Z_4 and Z_3 well located within the active site of *T. gondii* ENR enzyme and possessed the most negative estimated free energies of binding comparable to the innate ligand. In addition, the correlation between biological activities and the binding affinities predicted by AutoDock modeling was highly good for some compounds. Therefore, it could be suggested that these compounds may exert their anti-*T. gondii* activity through the inhibition of the ENR enzyme.

CONCLUSION

5-Oxo-hexahydroquinoline derivatives have been synthesized and evaluated for their anti-*T. gondii* activities. In conclusion, 5-oxo-hexahydroquinoline seems to be an attractive scaffold in this field and provides the context of further research for lead optimization and drug discovery. Compounds Z_3 (baering 2-pyridyl methyl carboxylate and 3,4,5-trimethoxyphenyl at C_3 and C_4) and Z_4 (containing 23-pyridyl propyl carboxylate and 4-methoxyphenyl moiety at C_3 and C_4) are good leads in order to develop better anti-*T. gondii* agents as they demonstrated both *in vitro* and *in vivo* inhibitory effects on tachyzoites viability and infection.

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CONFLICTS OF INTEREST STATMENT

All authors declare no conflict of interest in this study.

AUTHORS' CONTRIBUTION

All authors contributed equally to this study.

REFERENCES

1. Dunay IR, Gajurel K, Dhakal R, Liesenfeld O, Montoya JG. Treatment of toxoplasmosis: historical perspective, animal models, and current clinical practice. Clin Microbiol Rev. 2018;31(4):e00057-17,1-33.
DOI: 10.1128/CMR.00057-17.
2. Hoffmann S, Batz MB, Morris JG. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J Food Prot. 2012;75(7):1292-1302.
DOI: 10.4315/0362-028X.JFP-11-417.
3. Weiss LM, Dubey JP. Toxoplasmosis: A history of clinical observations. . Int J Parasitol. 2009;39(8):895-901.
DOI: 10.1016/j.ijpara.2009.02.004.
4. Carme B, Demar M, Ajzenberg D, Dardé ML. Severe acquired toxoplasmosis caused by wild cycle of Toxoplasma gondii, French Guiana. Emerg Infect Dis. 2009;15(4):656-658.
DOI: 10.3201/eid1504.081306.
5. Khan A, Ajzenberg D, Mercier A, Demar M, Simon S, Darde ML, *et al*. Geographic separation of domestic and wild strains of Toxoplasma gondii in French Guiana correlates with a monomorphic version of chromosome1a. PLOS Neglect Trop Dis. 2014;8(9):e3182,1-12.
DOI: 10.1371/journal.pntd.0003182.
6. Freppel W, Ferguson DJ, Shapiro K, Dubey JP, Puech P-H, Dumètre A. Structure, composition, and roles of the Toxoplasma gondii oocyst and sporocyst walls. Cell Surf. 2019;5:100016-100026.
DOI: 10.1016/j.tcsw.2018.100016.
7. Dubey JP. The history and life cycle of Toxoplasma gondii. In: Weiss LM, Kim K, editors. The model Apicomplexan-perspectives and methods. 3th ed. New York: Academic Press; 2020. pp. 1-19.
DOI: 10.1016/C2011-0-07157-0.
8. Ramakrishnan C, Maier S, Walker RA, Rehrauer H, Joekel DE, Winiger RR, *et al*. An experimental genetically attenuated live vaccine to prevent transmission of Toxoplasma gondii by cats. Sci Rep. 2019;9(1):1474-1487.
DOI: 10.1038/s41598-018-37671-8.
9. Saraf P, Shwab EK, Dubey JP, Su C. On the determination of Toxoplasma gondii virulence in mice. Exp Parasitol. 2017;174:25-30.
DOI: 10.1016/j.exppara.2017.01.009.
10. Konstantinovic N, Guegan H, Stäjner T, Belaz S, Robert-Gangneux F. Treatment of toxoplasmosis: current options and future perspectives. Food Waterborne Parasitol. 2019;15:e00036,1-15.
DOI: 10.1016/j.fawpar.2019.e00036.
11. Murata Y, Sugi T, Weiss LM, Kato K. Identification of compounds that suppress Toxoplasma gondii tachyzoites and bradyzoites. PloS One. 2017;12(6):e0178203,1-14.
DOI: 10.1371/journal.pone.0178203
12. Kumari L, Mazumder A, Pandey D, Yar MS, Kumar R, Mazumder R, *et al*. Synthesis and biological potentials of quinoline analogues: A review of literature. Mini-Rev Org Chem. 2019;16:653-88.
DOI: 10.2174/1570193X16666190213105146
13. Ranjbar S, Edraki N, Firuzi O, Khoshneviszadeh M, Miri R. 5-Oxo-hexahydroquinoline: An attractive scaffold with diverse biological activities. Mol. Divers. 2019;23:471-508.
DOI: 10.1007/s11030-018-9886-4
14. Ranjbar S, Khonkarn R, Moreno A, Baubichon-Cortay H, Miri R, Khoshneviszadeh M, *et al*. 5-Oxo-hexahydroquinoline derivatives as modulators of P-gp, MRP1 and BCRP transporters to overcome multidrug resistance in cancer cells. Toxicol Appl Pharm. 2019;362:136-49.
DOI: 10.1016/j.taap.2018.10.025
15. Ranjbar S, Firuzi O, Edraki N, Shahraki O, Saso L, Khoshneviszadeh M, *et al*. Tetrahydroquinolinone derivatives as potent P-glycoprotein inhibitors: design, synthesis, biological evaluation and molecular docking analysis. MedChemComm. 2017;8:1919-33.
DOI: 10.1039/C7MD00178A
16. Kadri D, Crater AK, Lee H, Solomon VR, Ananvoranich S. The potential of quinoline derivatives

for the treatment of Toxoplasma gondii infection. Exp Parasitol. 2014;145:135-144.
DOI: 10.1016/j.exppara.2014.08.008.
17. Brinster S, Lamberet G, Staels B, Trieu-Cuot P, Gruss A, Poyart C. Type II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens. Nature. 2009;458(7234):83-86.
DOI: 10.1038/nature07772.
18. Salas-Navarrete C, Hernández-Chávez G, Flores N, Martínez LM, Martinez A, Bolívar F, *et al*. Increasing pinosylvin production in Escherichia coli by reducing the expression level of the gene fabI-encoded enoyl-acyl carrier protein reductase. Electron J Biotechnol. 2018;33:11-16.
DOI: 10.1016/j.ejbt.2018.03.001.
19. Anquetin G, Greiner J, Vierling P. Quinolone-based drugs against Toxoplasma gondii and Plasmodium spp. Curr Drug Targets Infect Disord. 2005;5(3):227-245.
DOI: 10.2174/1568005054880172.
20. McLeod R, Muench SP, Rafferty JB, Kyle DE, Mui EJ, Kirisits MJ, *et al*. Triclosan inhibits the growth of Plasmodium falciparum and Toxoplasma gondii by inhibition of apicomplexan Fab I. Int J Parasitol. 2001;31(2):109-113.
DOI: 10.1016/s0020-7519(01)00111-4.
21. Radke JB, Burrows JN, Goldberg DE, Sibley LD. Evaluation of current and emerging antimalarial medicines for inhibition of Toxoplasma gondii growth in vitro. Int J Parasitol. 2001;31(2):109-113.
DOI: 10.1016/s0020-7519(01)00111-4.
22. Doggett JS, Nilsen A, Forquer I, Wegmann KW, Jones-Brando L, Yolken RH, *et al*. Endochin-like quinolones are highly efficacious against acute and latent experimental toxoplasmosis. Proc Natl Acad Sci U S A. 2012;109(39):15936-15941.
DOI: 10.1073/pnas.1208069109.
23. Smith AT, Livingston MR, Mai A, Filetici P, Queener SF, Sullivan WJ. Quinoline derivative MC1626, a putative GCN5 histone acetyltransferase (HAT) inhibitor, exhibits HAT-independent activity against Toxoplasma gondii. Antimicrob Agents Chemother. 2007;51(3):1109-1111.
DOI: 10.1128/AAC.01256-06.
24. Akins CK, Panicker SE, Cunningham CL. Laboratory animals in research and teaching: Ethics, care, and methods: Am Psychol Assoc. 2005.
25. Asgari Q, Keshavarz H, Rezaeian M, Motazedian MH, Shojaee S, Mohebali M, *et al*. Direct effect of two naphthalene-sulfonyl-indole compounds on Toxoplasma gondii tachyzoite. J Parasitol Res. 2013;2013,1-8.
DOI: 10.1155/2013/716976.
26. Buntrock RE. ChemOffice Ultra 7.0. J Chem Inf Model Comput Sci. 2002;42(6):1505-1506.
DOI: 10.1021/ci025575p.
27. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 2010;31(2):455-461.
DOI: 10.1002/jcc.21334.
28. Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, *et al*. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem. 1998;19(14):1639-1662.
DOI: 10.1002/(SICI)1096.
29. Hegewald J, Gross U, Bohne W. Identification of dihydroorotate dehydrogenase as a relevant drug target for 1-hydroxyquinolones in Toxoplasma gondii. Mol Biochem Parasitol. 2013;190(1):6-15.
DOI: 10.1016/j.molbiopara.2013.05.008.
30. Barbosa BF, Gomes AO, Ferro EAV, Napolitano DR, Mineo JR, Silva NM. Enrofloxacin is able to control Toxoplasma gondii infection in both in vitro and in vivo experimental models. Vet Parasitol. 2012;187(1-2):44-52.
DOI: 10.1016/j.vetpar.2011.12.039.
31. da Silva RJ, Gomes AO, Franco PS, Pereira AS, Milian IC, Ribeiro M, *et al*. Enrofloxacin and toltrazuril are able to reduce Toxoplasma gondii growth in human BeWo trophoblastic cells and villous explants from human third trimester pregnancy. Front Cell Infect Microbiol. 2017;7:340-360.
DOI: 10.3389/fcimb.2017.00340.
32. Khan AA, Slifer TR, Araujo FG, Remington JS. Activity of gatifloxacin alone or in combination with pyrimethamine or gamma interferon against Toxoplasma gondii. Antimicrob Agents Chemother. 2001;45(1):48-51.
DOI: 10.1128/AAC.45.1.48-51.2001.
33. McPhillie M, Zhou Y, El Bissati K, Dubey J, Lorenzi H, Capper M, *et al*. New paradigms for understanding and step changes in treating active and chronic, persistent apicomplexan infections. S Sci Rep. 2016;6:29179-29202.
DOI: 10.1038/srep29179.
34. Burrows JN, Burlot E, Campo B, Cherbuin S, Jeanneret S, Leroy D, *et al*. Antimalarial drug discovery–the path towards eradication. Parasitol. 2014;141(1):128-139.
DOI: 10.1017/S0031182013000826.
35. Parsons JB, Frank MW, Subramanian C, Saenkham P, Rock CO. Metabolic basis for the differential susceptibility of Gram-positive pathogens to fatty acid synthesis inhibitors. Proc Natl Acad Sci U S A. 2011;108(37):15378-15383.
DOI: 10.1073/pnas.1109208108.
36. Yao J, Rock CO. How bacterial pathogens eat host lipids: implications for the development of fatty acid synthesis therapeutics. J J Biol Chem. 2015;290(10):5940-5946.
DOI: 10.1074/jbc.R114.636241.
37. Vaughan AM, O'Neill MT, Tarun AS, Camargo N, Phuong TM, Aly AS, *et al*. Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. Cell Microbiol. 2009;11(3):506-520.
DOI: 10.1111/j.1462-5822.2008.01270.x.
38. Goodman C, McFadden G. Fatty acid biosynthesis as a drug target in apicomplexan parasites. Current drug targets. 2007;8:15-30.
39. Surolia N, Surolia A. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of Plasmodium falciparum. Nat Med. 2001;7(2):167-173.
DOI: 10.1038/84612.