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Original Article

Cytotoxic and Immunomodulatory Activity of Curcumin and Chitosan on Experimental Toxoplasmosis

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Abstract

Background: *Toxoplasma gondii* is a pathogenic parasite with worldwide distribution. We investigated curcumin and chitosan in combination on the viability of *T. gondii* tachyzoites in silico, in vitro and in vivo.

Methods: A 3D model was employed in Urmia University of Medical Sciences, Urmia, Iran in 2021 to study the interaction between curcumin and dihydrofolate reductase (DHFR). Ramachandran root-mean-square deviation and VERIFY3D validated the model. Cytotoxicity of curcumin and chitosan was evaluated by MTT viability assay. BALB/c mice infected with 10^4 *Toxoplasma* organisms were treated with curcumin, chitosan, and the combination of curcumin+chitosan. Serum levels of inducible NO synthetase (iNOs), interferon gamma (IFN-γ), interleukin (IL)-5, glutamate oxaloacetic transaminases(SGOT), and glutamic pyruvate transaminase (SGPT) were determined.

Result: Curcumin-DHFR and curcumin-DHPS (dihydropteroate synthase) interactions and calculated enzyme energy indicated an excellent affinity for curcumin with DHFR, but not DHPS. MTT results of concurrent treatments demonstrated IC_{50} rates of 0.1, 0.05, and 0.01 mg/ml at 24, 48, and 72h, respectively. IFN-γ, IL-5 and iNOs levels in curcumin+chitosan treated mice were 1.71, 0.51, and 1.51 IU/L, while those of SGOT and SGPT were 76 and 84 IU/L, respectively.

Conclusion: The combination of curcumin and chitosan increased survival time of infected mice by seven days. Curcumin and chitosan in combination regulated the immune system and reduced liver damage, potentially forming the basis of a new treatment for toxoplasmosis.

Introduction

Toxoplasma gondii is an obligatory protozoan parasite infecting up to a third of the world population (1). This parasite infects the nucleated cells of warm-blooded animals and humans (2). Human infection is caused by ingesting cysts in living tissue and raw meat, as well as by eating mature oocysts in contaminated vegetables and water (3-5). Infections in pregnant women can give rise to miscarriage and infant encephalitis, and may lead to death in humans with a compromised immune system (5). Individuals with a healthy immune system may be asymptomatic or show mild symptoms against chronic infection, but in immunocompromised patients, *Toxoplasma* infection may cause severe complications such as encephalitis, pneumonia, myocarditis and chorioretinitis (6-7).

Currently, available treatments for toxoplasmosis, such as the combination of pyrimethamine with sulfadiazine, have some limitations and side effects that include nausea, headache, folic acid deficiency, aplastic anemia, skin rash and hematologic toxicity (8-10). Investigations into the utility of medicinal plants as potential treatments for toxoplasmosis are currently underway. Computational or *in silico* methods have become a dominant tool for addressing drug discovery as a consequence of their capability to minimize the time required for identification and design of drug compounds, and also for optimization of their structure (11).

Curcumin, the main component of the plant *Curcuma longa*, has been revealed as an antiparasitic agent capable of killing cells infected with parasites such as *Schistosoma japonicum*, *Leishmania*, *Giardia lamblia* and *Plasmodium falciparum* (12). Chitosan, a deacetylated derivative of chitin, is naturally synthesized in the cell wall of fungal cells. Chitosan increases the secretion of cytokines and stimulates cytotoxic T cells (13). It also has an inhibitory effect on many bacteria, filamentous fungi, yeasts, pathogenic viruses, helminthic parasites and protozoa (14).

To date, anti-*Toxoplasma* activity of curcumin in combination with chitosan has not been investigated. Utilizing in vivo, in vitroandin silico methods, this study assessed the potential anti-*Toxoplasma* activity of curcumin and chitosan in combination. It was hypothesized that the combination of curcumin and chitosan could provide a novel therapeutic synergy for the treatment of toxoplasmosis.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of theUrmia University of Medical Sciences, Code: IR.UMSU.REC.1399.092, Ethics approval date: 2020-06-24.

Data preparation and model generation for in silico study

The study was conducted in Urmia University of Medical Sciences, Urmia, Iran in 2021. To investigate the mode of interaction, between curcumin and *T. gondii* dihydrofolate reductase (DHFR), together with the interaction between curcumin and dihydropteroate synthase (DHPS), the crystal structures of DHFR and DHPS were retrieved from the protein data bank (www.rcsb.org). Therefore, the crystal structure of DHFR was modeled based on sequences retrieved from the UniProt database (https://www.uniprot.org), followed by submission to I-TASSER (Iterative Threading ASSEmbly Refinement) webserver (15). Hyperchem software (version 8.0.8) was utilized to generate the 3D structure of curcumin.

Docking and molecular dynamic (MD) simulation studies

For the docking study, the binding sites (grid centers) were set to coordinate the crystalized ligands in the protein complexes. To investigate the dynamics of curcumin with target proteins, the topologies of selected poses in each docking analysis were generated using PRODRG (16), for MD simulation. The particle mesh Ewald method was applied for electrostatic interactions. In addition, the actual frame was recorded for each 1.0 ps.

Molecular Mechanics/Poisson-Boltzmann surface area (MMPBSA) binding free energy calculation

The binding energy, ΔGbind, was calculated based on the equation as follows: ΔGbind = Grlc- (Grec + Glig), where Grlc is the free energy of the receptor-ligand complex, and Grec and Glig are the unbound receptor and ligand, respectively. The g_mmpbsa tool was used to calculate the binding energy components of the protein-ligand complexes using the MM-PBSA method, apart from the entropic term and energetic contribution of each residue to the binding by use of the energy decomposition scheme (17). A total of 250 snapshots was considered for the MMPBSA calculation of protein-ligand complexes.

Preparation of curcumin and chitosan

Curcumin with the purity of 94% and chitosan with medium molecular weight (190–310 kDa) were purchased from Sigma Aldrich, USA (Cat nos. 7727 and 48877, respectively). For the preparation of desired concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, and 0.0005 mg/ml), curcumin (20 mg) was dissolved in 1% DMSO as solvent, while chitosan (20 mg) was prepared at the same concentrations by its dilution in 10 ml of glacial acetic acid solvent (2%) and mixed using a shaker until the mixture was completely diluted.

Parasite and mouse treatment groups

T. gondii RH strain was obtained from the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran), diluted 10^4 with PBS (pH 7.4) for intraperitoneal injection into female BALB/c mice (6–8 weeks of age and weighing 20 g). After 5–6 days, active tachyzoites were harvested from the peritoneum and 28 parasitically infected mice were assigned to four equal groups (7 in each group), including curcumin (at 1,5,10, and 25 mg/kg/day), chitosan (at 0.5,1,5, and 10 mg/kg/day), mixed (curcumin 10 + chitosan 5, curcumin 1+ chitosan 0.5, curcumin 5 + chitosan 1, curcumin 25+ chitosan 10mg/kg/day), and PBS as negative control. For the survival test, 52 mice were allocated to the same four groups, but the number of mice in each of the first three groups was 16, and in the PBS group, was 4.

MTT assay

T.gondii tachyzoites of RH strain were harvested from the peritoneum of infected BALB/c mice after four days. Parasites were counted and diluted to 10^4 tachyzoites in 1 ml of RPMI-1640 and then treated with the previously described concentrations of curcumin, chitosan, and their combination in 96-well microplates. The cytotoxic activity of components was evaluated by MTT assay after 24, 48, and 72 h, respectively (18-20). All values are means of triplicate wells. Results were expressed as the percentage of IC_{50} (half maximal inhibitory concentration).

Hepatic cytotoxicity and measurement of IFN-γ, IL-5 and iNOs

Four days after infection with *Toxoplasma* parasites, infected mice were treated with different concentrations of curcumin, chitosan and their combination. Three days after treatment, blood samples were collected and serum was separated by centrifugation at 2,000 ×g at 37°C for 10 min. Liver enzymes, SGOT and SGPT, and also IFN-γ, IL-5, and iNOs levels were measured by a spectrophotometer (Spectronic20D) using biomedical kits, including MABTECH Product code: 3321_1HP_20 (Iran), Mouse iNOs ELISA Kit (ab253219, USA), and Mouse IL-5 ELISA Kit (ab204523,

USA), as per instructions provided by the manufacturer.

Survival rate

The mouse challenge test is highly specific and ideally suited to show the effectiveness of a test compound. Four days after infection with *Toxoplasma* parasite, all mice except the control group were treated with different concentrations of curcumin, chitosan and their combination. The survival days were evaluated daily, and results were compared.

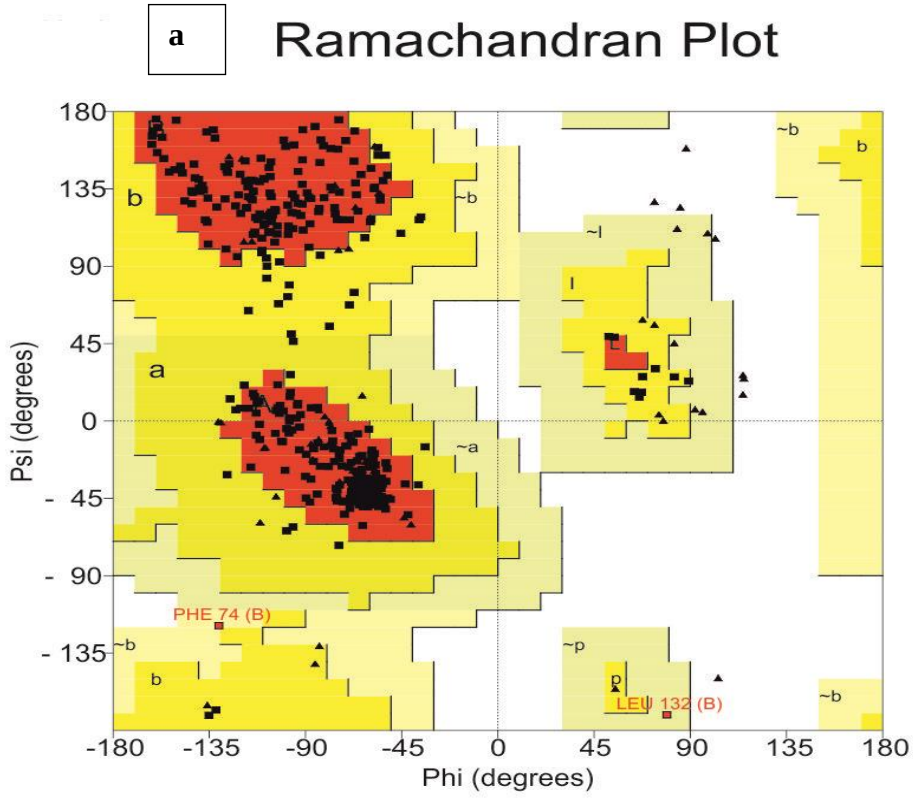
Statistical analysis

One-way ANOVA was utilizedto compare the MTT test results and mean values of cytokines. The survival test required Kaplan-Meier analysis, and SPSS16 (Chicago, IL, USA) software was applied for the statistical analyses.

Results

Generated model validation

A 3D model was generated for DHFR by the I-TASSER web server, to study curcumin-DHFR interaction. Also, PROCHECK and VERIFY3D web servers were employed to evaluate the quality of the model. Fig. 1(a) shows the Ramachandran plot of the model in which the majority (91.5%) of the residues are in the most favored regions, indicative of the favorable quality of the model. The VERIFY3D results in Fig. 1(b) indicated that 86.24% of the residues averaged 3D-1D score ≥ 0.2. For a reliable model, at least 80% of the amino acids should have a score greater than 0.2.



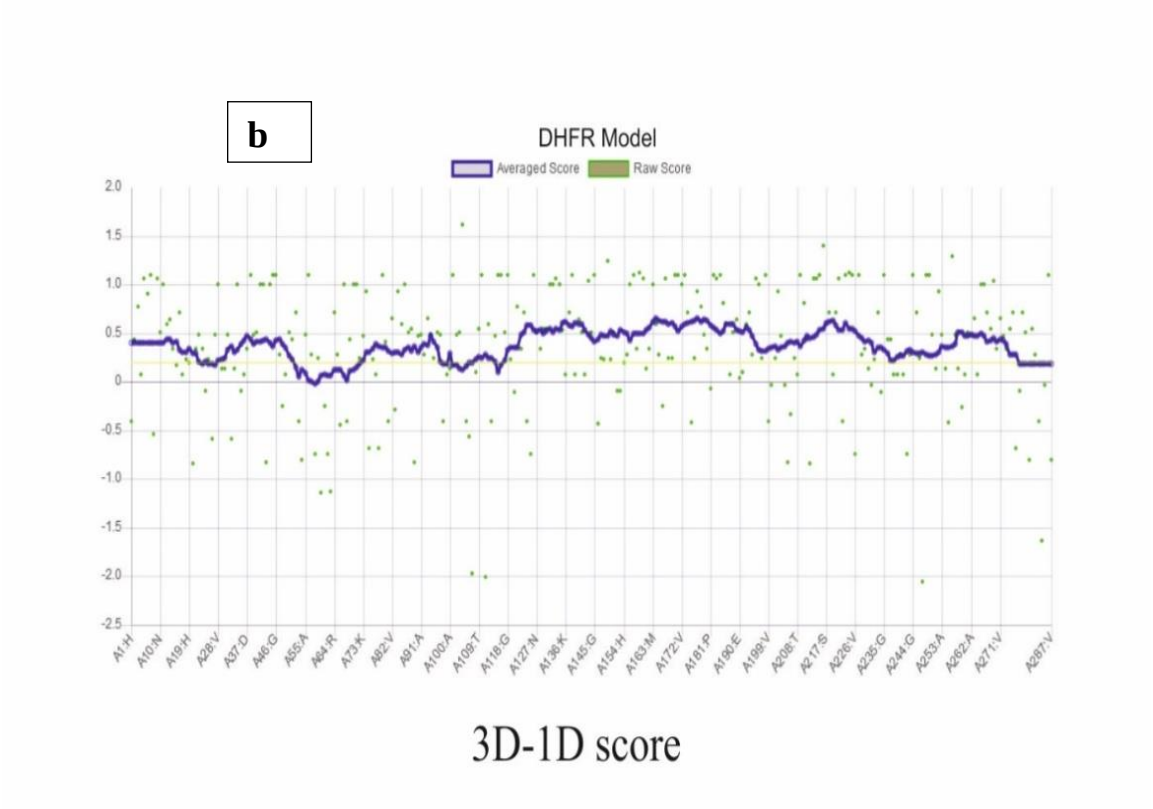


Fig. 1: Generated model validations of DHFR protein. (a) Ramachandran plot of the model and (b) VERIFY3D results

Interaction of DHFR and DHPS with curcumin-

The results of the docking study are represented in Table 1. The results predicted that unlike DHPS, DHFR had an excellent affinity of curcumin for the binding site. The 2D and 3D interaction diagrams of curcumin complexes are illustrated in Figure 2(a). Fig. 2(b) shows that curcumin could reach the hydrophobic regions of DHFR, giving a high binding score, while the same behavior did not occur in the DHPS protein. Curcumin could not reach the inner sites of the DHPS protein, providing only shallow hydrophilic interactions.

Table 1: The contribution of each residue in the binding energy of the curcumin-protein

	DHFR			***DHPS***		
Model No.	Affinity	RMSD u.b	RMSD l.b.	Affinity	RMSD u.b	RMSD l.b.
1	-9.1	0	0	-5.5	0	0
2	-8.9	1.615	2.899	-5.2	13.004	16.715
3	-8.9	1.965	3.098	-5.1	25.105	27.455
4	-7.6	1.533	2.832	-5.1	30.138	33.1
5	-7.1	20845	23.889	-4.8	14.661	17.3

RMSD: root-mean square deviation, **u.b**.: upper bound, **l.b**.: lower bound

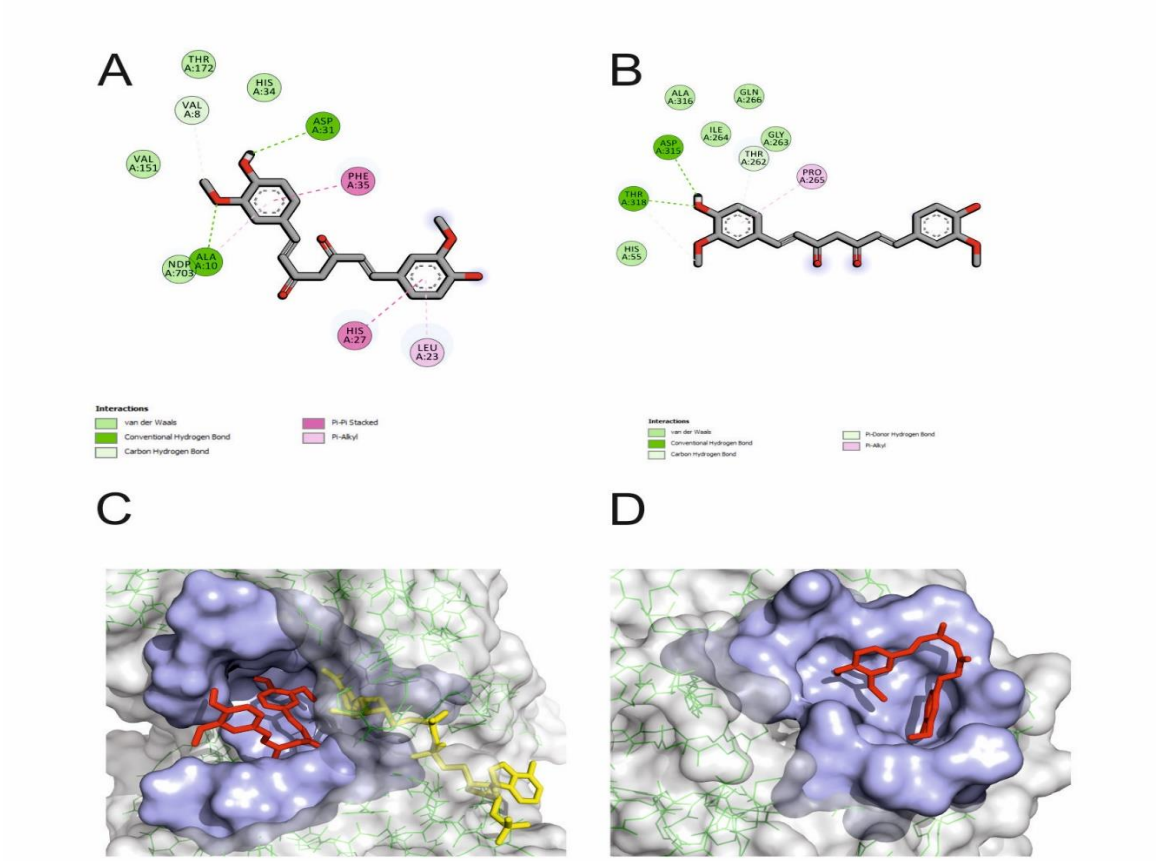


Fig. 2(a): 2D and 3D interaction diagrams of curcumin complexes

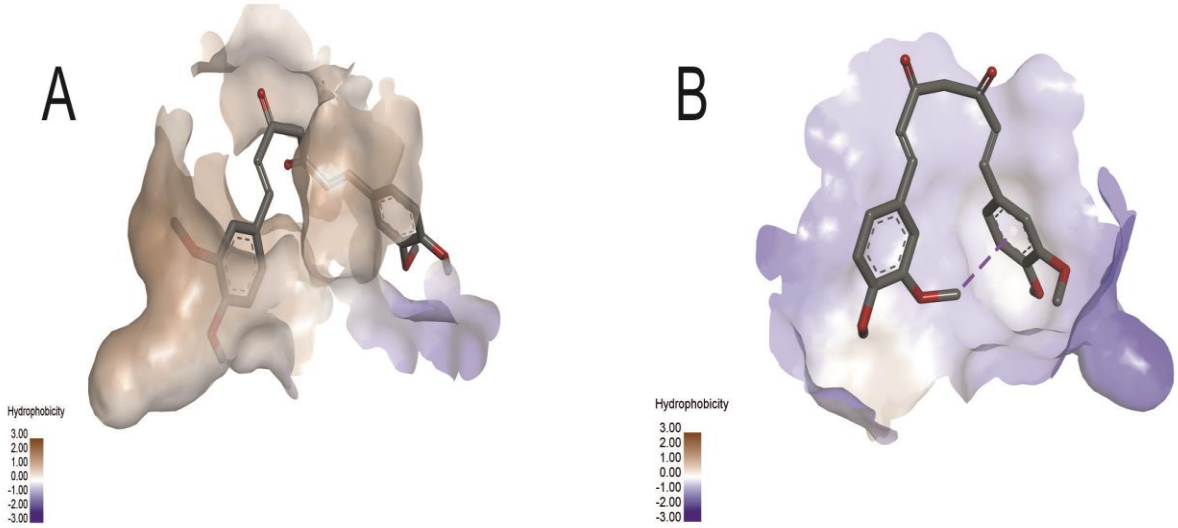


Fig. 2 (b): hydrophobic and hydrogen bond interactions of curcumin

MD simulation studies

The systems were run for 50ns, and then RMSD values were calculated to assess the stability of the proteins in the simulations. Fig. 3(a) displays the RMSD plot for DHFR and DHPS proteins in complex with curcumin. The plots indicate that the conformational changes in proteins reach a steady-state (stable condition) after 20ns of simulation, demonstrating the stability of the macromolecules in the simulation. The contribution of each residue in the binding energy of the curcumin protein is illustrated in Fig. 3(b) where negative values (kJ/mol) indicate favored interactions, while positive energies show unfavored interactions. In the curcumin-DHFR complex, amino acids at positions 40, 100, and 150 had a positive contribution (negative values)in binding energy. Moreover, the energy summaries for DHFR and DHPS complexes with curcumin were calculated by g_mmpbsa package in kJ/mol, as depicted in Table 2.

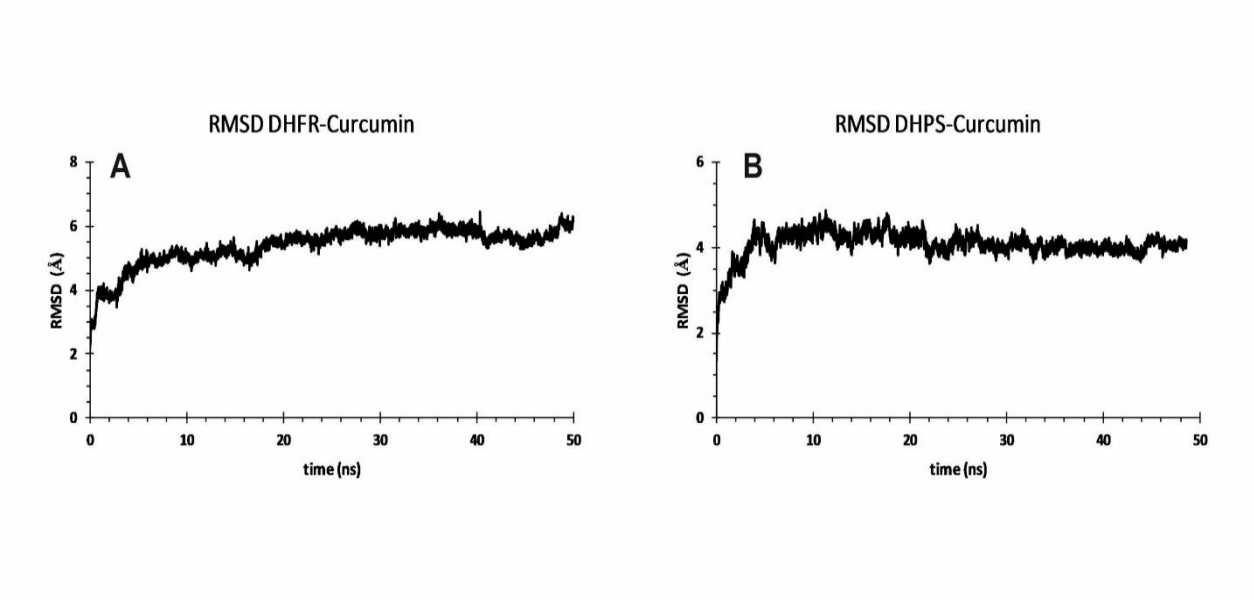


Fig. 3: RMSD plot for DHFR and DHPS proteins in complex with curcumin

Table 2.: Energy summaries for DHFR and DHPS complexed with curcumin calculated by g_mmpbsa package. Energies were measured in KJ/mol.

	DHFR	***DHPS***
Van der Waals energy	-205.394	149.727
Electrostatic energy	-34.815	-40-12
Polar Solvation energy	112.483	126.091
SASA energy	-21.455	-17.842
SAV energy	0	0
WCA energy	0	0
Binding energy	149.182	-81.598

SASA: solvent-accessible surface area, **SAV**: solvent accessible volume, **WCA**: Weeks–Chandler–Andersen, **DHFR**: dihydrofolate reductase, **DHPS**: dihydropteroate synthase

Cytotoxic effect

The cytotoxic effects of different curcumin and chitosan concentrations on *Toxoplasma* viability are presented in (Fig. 4). Both curcumin and chitosan demonstrated an anti-*T. gondii* effect revealing that the IC_{50} levels of curcumin and chitosan were 0.1, 0.05, and 0.01 mg/ml after 24, 48, and 72 h, , respectively. Thus, combination therapy was more effective than single therapy and had a significant destructive effect on *T.gondii* tachyzoites.

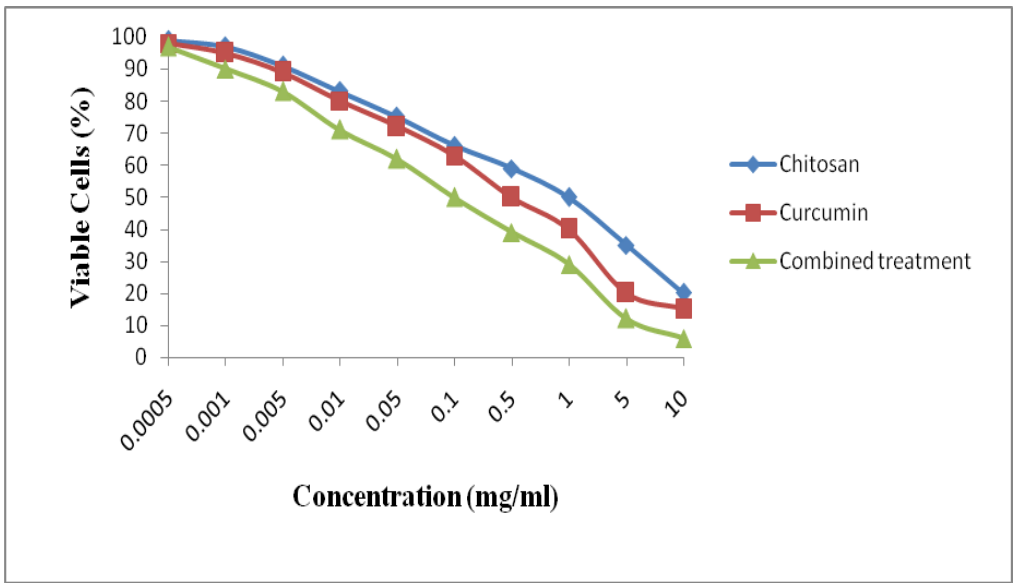


Fig. 4(a): Comparison of mean light absorption (OD) of live tachyzoites after 24 hours of incubation determined by the MTT assay

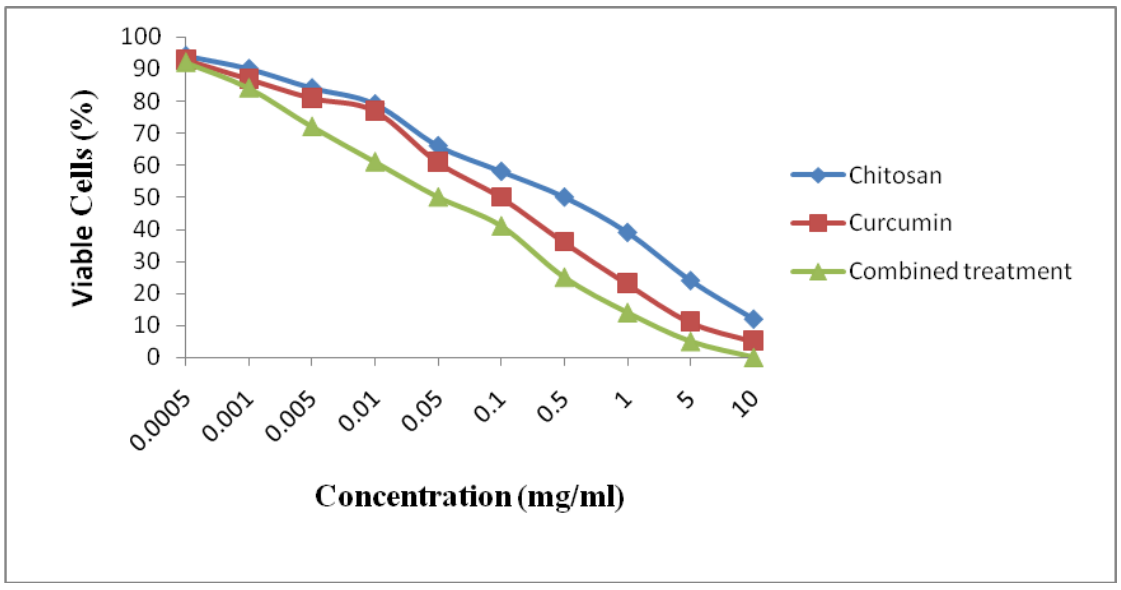


Fig. 4(b): Comparison of mean light absorption (OD) of live tachyzoites after 48 hours of incubation determined by the MTT assay

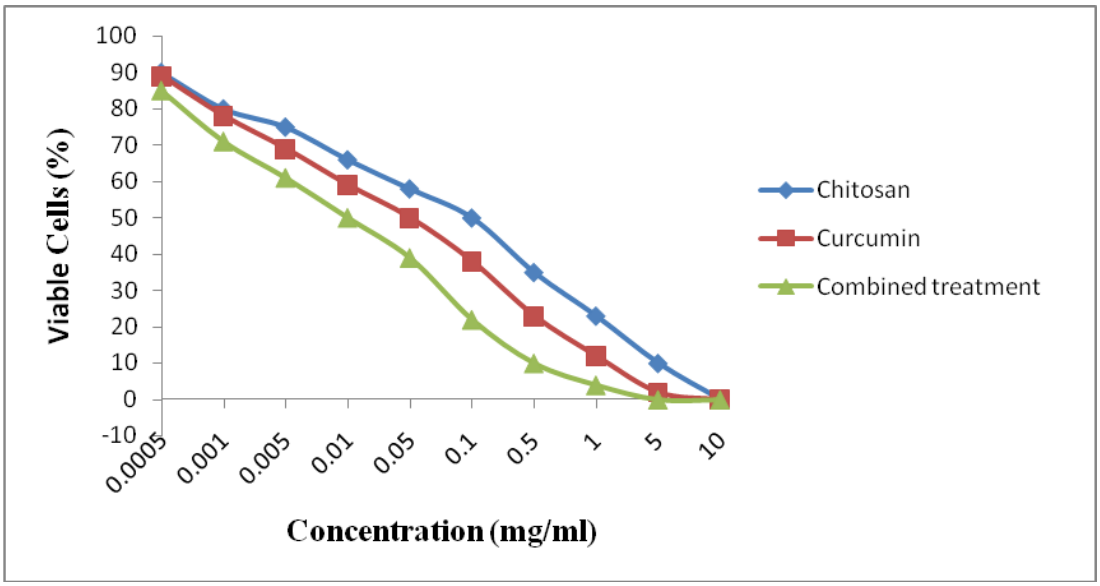


Fig. 4(c): Comparison of mean light absorption (OD) of live tachyzoites after 72 hours of incubation determined by the MTT assay

Liver enzyme assessment

The assessment of liver function and the role of oxidative damage, inflammation and infection with *Toxoplasma* was accomplished by evaluating levels of the liver enzymes SGPT (ALT) and SGOT (AST), which increased in cytotoxic liver injuries. In curcumin-treated, and curcumin and chitosan-treated mice, both ALT and AST serum levels were decreased compared to the negative control group (Untreated/ infected mice) (P<0.05) (Table 3).

Table 3: Serum SGPT and SGOT levels of mice infected with T. gondii

Mice	*SGPT (u/l)*	*SGOT (u/l)*
Control	68.16±2.4	72.5±2.1
Curcumin-treated	59.5±2.0#	62.66±2.1#
Chitosan-treated	66.16±2.7	70.66±1.9
Curcumin and Chitosan-treated	59.83±1.4#	64.16±1.2#

Values are means ±SEM (n=7). #: significant change at P<0.05

IFN-γ, IL-5, and iNOs production

Compared to controls, serumlevels of IFN-γ and iNOs were increased, whilst IL-5 levels were decreased in mice treated with the combination of curcumin and chitosan (P<0.05)(Fig. 5). Serum levels of IL-5 were not significant in mice receiving curcumin and chitosan alone.

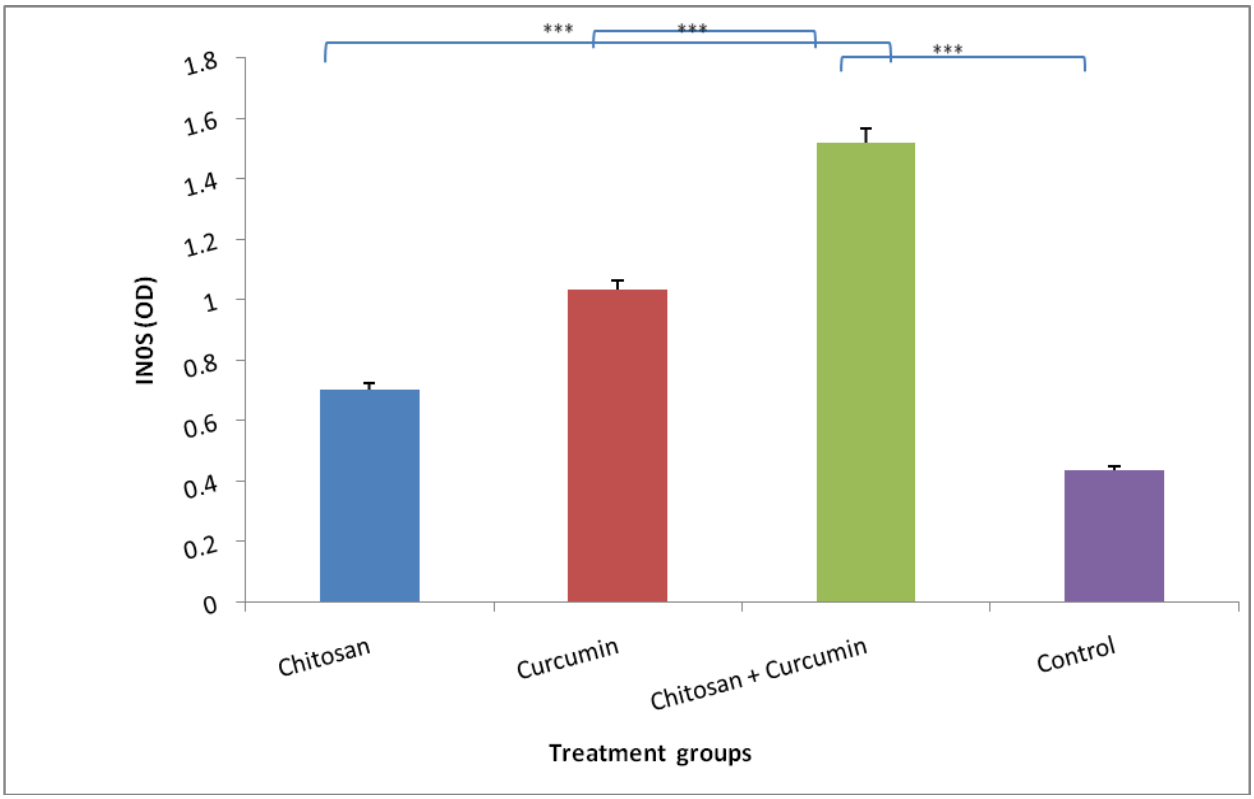


Fig. 5:(a) Effect of curcumin and chitosan on iNOs production (P< 0.05). A statistically significant increase in the level of iNOs was observed in mice receiving curcumin and chitosan (significant = *** P< 0.05)

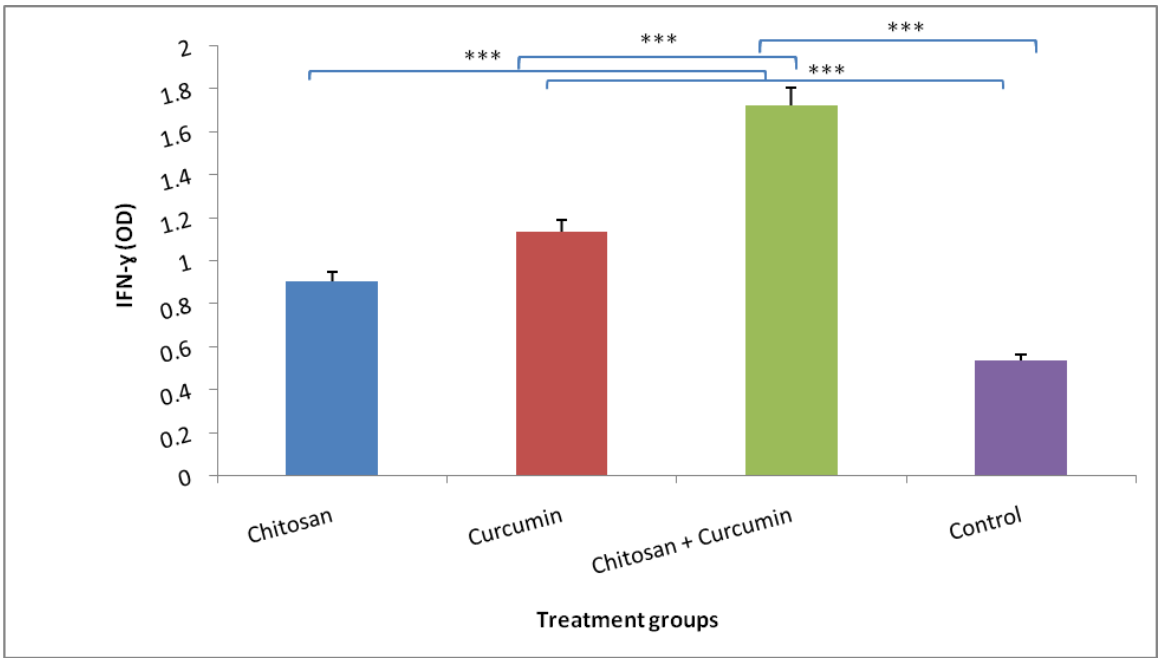


Fig. 5: (b)A statistically significant increase in the level of IFN-γ was observed in mice receiving chitosan and curcumin (significant = *** P< 0.05)

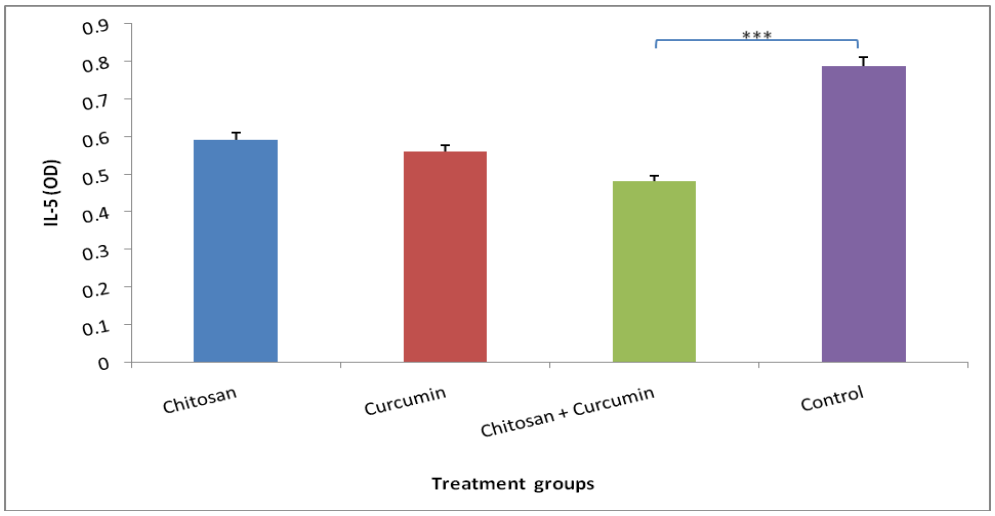


Fig. 5: **(c)**A statistically significant increase in the level of IL-5 was observed in mice receiving chitosan and curcumin (significant = *** P< 0.05)

Survival function

The greatest survival time (12 days) was observed in mice treated with the combination of curcumin and chitosan (25 and 10 mg/kg/day, respectively) after the challenge with live parasites, whereas the shortest survival time (7 days) was detected in the negative control group (Fig. 6). The *in vivo* study affirmed that combined and separate treatment of infected mice could increase survival rate compared to uninfected mice. Additionally, mice treated with the combination of curcumin and chitosan had a higher survival rate than those treated with curcumin and chitosan alone (Fig. 6).

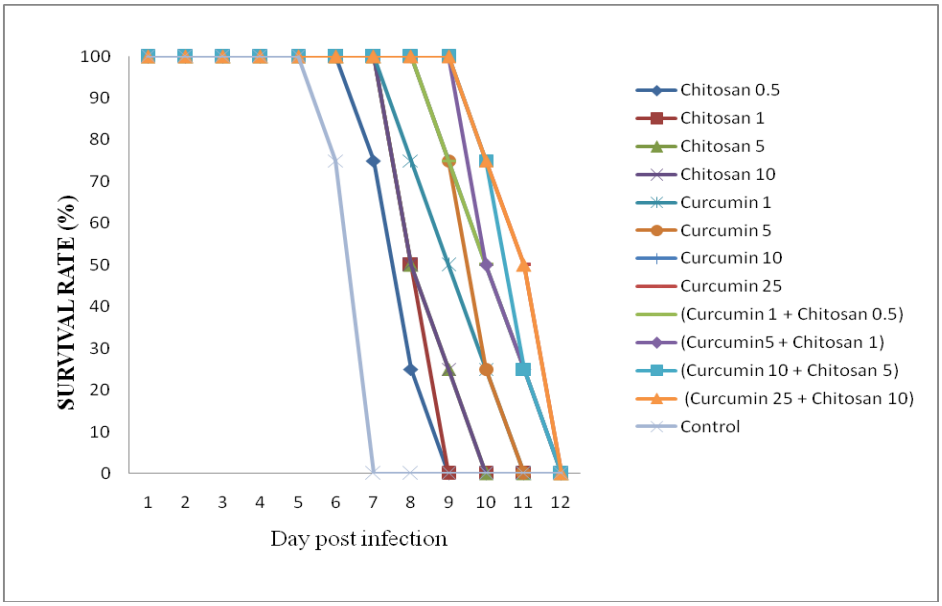


Fig. 6: The administration of curcumin, chitosan and their combination (mg/kg/day) on the survival rate of experimental mice. The survival time for the control group was seven days

Discussion

The current study revealedCurcumin was binded strongly to the active site of the DHFR protein. Therefore, it could be a potential candidate for inhibiting *Toxoplasma gondii* viability. In a similar study (10), using a molecular docking method on FDA approved drugs, which included Cefpiramide, Cefotiam and Ponatinib, attempted to develop a selective treatment by inhibiting Protein Kinase 1 from *T. gondii*. The authors found that all compounds could bind strongly to the active site of the protein.

Our results revealed greater favorable affinity of curcumin for DHFR than DHPS binding. Likewise, owing to the static representation of the interaction between two molecules in the docking studies, it was necessary to conduct a dynamic analysis of the interactions by MD simulation. In this regard, the RMSD values were calculated to evaluate the stability of the proteins in the whole simulation time. The results showed the stability of curcumin complexes to target proteins, DHFR and DHPS, after 20 ns of simulation. Ashwinder et al. (21) investigated Targeting Heat Shock Proteins 60 and 70 of *T. gondii* as a potential drug target. Their finding implied virtual screening of certain compounds expedited the development of inhibitors for Hsp60 and Hsp70 in *T. gondii* (21).

In the current study, evaluation of the cytotoxicity of both curcumin and chitosan using the MTT assay exhibited antiparasitic effects of these compounds. The cytotoxicity of curcumin and chitosan on various parasites and bacteria (12, 22) divulged that these compounds potentially affect metabolic activity and parasite survival. The MTT assay is highly applicable to identify plant compounds affecting viability of *Toxoplasma* (23). The rationale for using the combination of curcumin and chitosan in the current study was to stimulate the immune system toward cellular immunity. Combined use in the treatment of toxoplasmosis has not been studied previously. However, chitosan and curcumin have shown their independent immunomodulatory effects in previous studies (13, 24). Curcumin is a beneficial regulator of the immune system and increases mitogenesis and antigen-induced T cell proliferation (24).

Our findings revealed that IFN-γ serum levels were significantly higher in mice treated with curcumin. However, this finding is in contrast to a previous report in which there was no considerable variation in IFN-γ levels between control and curcumin treated animals (24). One of the possible reasons for the inconsistency could relate to the role of NK cells in secretion of IFN-γ. Curcumin modulates the activation ofNK cells and increases its cytotoxicity (25). NK cells also produce IFN-γ and IL-22, leading to an increase in phagolysosome fusion that has a role in inhibiting intracellular pathogen growth. In addition, studies by Gertsch et al. revealed that high concentrations of curcumin upregulated IFN-*γ* mRNAs (26).

In the current study, serum IFN-γ levels increased in mice treated with the combination of curcumin and chitosan. IFN-γ is considered as a key factor in both immune and inflammatory responses via multiple mechanisms including increased expression of iNOs, NO and reactive oxygen species (ROS). IFN-γ is responsible for changes in host metabolism that restrict *T. gondii* replication (27-28). Toxoplasmosisis known to cause hepatic injury and hepatotoxicity, evidenced by increased serum SGOT and SGPT levels (29-30), whereas the current study revealed that treatment with curcumin and chitosan yielded a significant decrease in the levels of both liver function enzymes (Table 3).

Computational methods have been considered a new approach for drug design and development due to their low cost and high speed. Furthermore, using in silico methods, it

is possible to better understand the underlying mechanism of ligand-receptor interactions before performing more intensive experimental studies. Based on our findings, curcumin and chitosan can be used in combination for stimulation and elevation of IFN-γ and iNOs levels, reduction of toxicity and liver damage, and potentially, treatment of toxoplasmosis.

Acknowledgments

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Conflict of Interest

The authors declare that there is no conflict of interests.

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