Original Article

The therapeutic efficacy of curcumin nanoemulsion versus Spiramycin in *Toxoplasma gondii* (ME49 strain) chronically infected mice

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ABSTRACT

Background: Previously, we showed that curcumin (CUR) nanoemulsion exhibited a promising prophylactic effect on acute toxoplasmosis, and decreased parasite burden. Scanning electron microscopy (SEM) of the peritoneal exudates showed deformed tachyzoites in both prophylactic and treated subgroups.
Objective: The present study is designed to evaluate the therapeutic effect of CUR nanoemulsion compared to that of Spiramycin on *T. gondii* type II, ME49 strain causing chronic toxoplasmosis in experimentally infected mice.
Material and Methods: This case-control experimental study included 30 Swiss albino mice, divided into three equal groups. All mice were infected with avirulent ME49 strain to induce chronic toxoplasmosis. The study included group I (infected non-treated), II (infected treated with CUR nanoemulsion), and III (infected and treated with Spiramycin). The assessment parameters included estimation of the mortality rate, and parasite burden (cyst number and size) in livers and spleens impression smears, and in brains homogenates.
Results: The mortality rate was 40% in the infected non-treated group with no mortality in all treated mice. There was a significant decrease of cyst number and size in livers, spleens, and brains of both treated groups as compared to the infected non-treated mice.
Conclusion: It was concluded that CUR nanoemulsion had a promising therapeutic effect on chronic toxoplasmosis.

Keywords: chronic toxoplasmosis; curcumin; experimental study; nanoemulsion; Spiramycin.

Received: 7 July, 2022; **Accepted:** 10 August, 2022.

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Print ISSN: 1687-7942, **Online ISSN:** 2090-2646, **Vol. 15, No. 2, August, 2022.**

INTRODUCTION

Toxoplasmosis is a global zoonotic disease, and one-third of the human population is reportedly chronically infected by *T. gondii*[1]. From the United States, an estimated 11% of the population, 6 years and older, were at risk for acquiring toxoplasmosis[2]. Recently, in a case-control study conducted at Zagazig University Pediatric Hospital, Egypt for estimating *Toxoplasma* IgG and IgM seroprevalence in 67 children on regular hemodialysis and 50 healthy controls, the total seropositivity was 23%. While anti-*Toxoplasma* IgM antibodies were not detected, IgG seroprevalence was 16% and 28% in control and hemodialysis groups, respectively with insignificant statistical differences. Significant risk factors included contact with cats or soil, ingestion of semi-cooked meat and blood transfusion[3].

A significant fraction of toxoplasmosis cases is caused by reactivation of existing chronic infections, and *T. gondii* can persist and encyst chronically in the brain leading to a broad spectrum of neurological complications[4]. In reactivated toxoplasmosis, excysted bradyzoites transform to tachyzoites and proliferate uncontrollably in the brain causing widespread inflammation and critical morbidity. Reactivated toxoplasmosis develops in immunosuppressed patients, such as those afflicted by human immunodeficiency virus (HIV)/AIDS, and solid organ transplant recipients. Reactivation of *T. gondii* brain cysts often manifests as toxoplasmic encephalitis[5].

Since all currently available therapeutic drugs are only effective against tachyzoites with poor efficacy against tissue cysts, there is much controversy in treatment of chronic toxoplasmosis. As previously reviewed[6], drug resistance developed against several anti-*Toxoplasma* drugs due to gene mutations. Recent evolutionary technology enabled investigators to understand *T. gondii* biology system and uncover new

DOI: 10.21608/puj.2022.149674.1175

drug targets. At least 50 promising anti-cyst candidates were identified through screening of compound libraries, target-based drug design, or repurposing of Food and Drug Administration (FDA)-approved drugs[7]. Deficiency of suitable effective drugs that safely eliminate brain cysts encouraged researchers to investigate natural products. Singaporean reviewers tabulated all nutritional products extracted from food plants, phytochemicals, vitamins, and minerals that were evaluated in the last two decades. The reviewers observed that the majority of these nutritional products are promising toxoplasmacidal agents without identification of a specific drug target. They also discussed different nanotechnology strategies to increase natural products bioavailability and biosolubility. Due to lack of evidence supporting successful human clinical trials, the reviewers recommended further large-scale clinical trials to confirm the importance of nutritional therapy in prevention and/or treatment of chronic toxoplasmosis by determining the mechanism of action against bradyzoite biology and brain cyst development[8].

Due to its porous structure with high surface area, metal-organic frameworks (MOFs) were utilized as a drug delivery system. An Egyptian study investigated the therapeutic efficacy of CUR@MOFs nanocomposite in treatment of chronic toxoplasmosis in experimentally infected mice with ME49 strain. Parameters evaluated were brain cyst count and histopathological examination of tissues obtained from liver, spleen, and lung. It was concluded that the new nanocomposite significantly decreased the number of brains cysts, and ameliorated the histopathological changes with preserved parenchyma, and stromal tissues in the examined organs[9]. Therefore, the present study aimed to assess the therapeutic effects of CUR nanoemulsion against experimental chronic toxoplasmosis as compared to specific therapeutic medication with Spiramycin.

MATERIAL AND METHODS

This case-control study was conducted at Medical Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt, during the period from August, 2018 to January, 2019.

Study design: The study included three groups of mice, infected non-treated (GI), infected treated with CUR nanoemulsion (GII), and infected treated with Spiramycin (GIII). All mice were sacrificed at the end of the 9th w post infection (PI) for assessment of mortality rate, parasite burden in livers and spleens impression smears as well as in brains homogenates.

Experimental animals: The study included thirty female Swiss albino pathogen-free mice (*Mus musculus domesticus*), 6-8 weeks of age and weighing 20-25 g. Mice were purchased from animal house, Faculty of Medicine, Suez Canal University. They were housed under controlled temperature and light conditions, and provided with water and commercial chow *ad libitum*.

Parasites: *T. gondii* avirulent strain (ME49) was obtained from the Zoonotic Disease Department, National Research Center, Giza, Egypt. It was maintained by serial oral inoculation of mice every 8 weeks with 0.1 ml of brain suspension containing 20 cysts of previously infected mice[9].

Drugs: Spiramycin and CUR were purchased from Sigma-Aldrich, Germany. Preparation, dose and methods of administration were previously described[10].

Preparation and characterization of CUR nanoemulsion: Previously described[10].

Mice infection: A brain of previously infected mouse was homogenized in one ml saline (0.85% NaCl). Each mouse received orally 0.1 ml that contained 20 cysts[11].

Pilot study: As previously described[10], a pilot study was conducted to determine the least effective dose of CUR nanoemulsion, and a dose of 20 mg/kg/d was established as the lowest effective dose.

Mice grouping: Table (1) describes the study groups.

Mortality rate (MR): This was calculated according to the following equation; MR= (number of dead mice at the sacrifice time)/(number of mice at the beginning of the experiment) X 100[12].

Cyst counts and sizes in livers and spleens: Impression smears from the cut surface of livers and spleens were performed. Liver and spleen of each mouse was cut into two halves exposing a fresh surface that was lightly blotted using a paper towel and then pressed to a clean glass slide once. The impression smears were stained with Giemsa stain and examined under oil immersion lens[15]. Cyst number was counted

Table 1. Study groups.

	No.	Name	Characteristics
Group I	10	Control	Infected, non-treated.
Group II	10	CUR nanoemulsion	Infected and treated with CUR nanoemulsion 20 mg\kg\d orally once daily for 10 d after 6 w PI[13].
Group III	10	Spiramycin	Infected and treated with Spiramycin 100 mg/kg once daily for 10 d after 6 w PI[14].

in 10 high power fields (HPF) for each mouse, and the mean number of cysts in each group was determined[16]. Cyst size was measured by calibrated ocular micrometer, and the mean cyst size was calculated. Reduction percentage (R%) in cyst count and size was calculated according to the following equation: R% = [(C-E)/C] X 100; where C = mean cyst count or size in the control group (GI), and E = mean cyst count or size in each treated group[16].

Cyst counts and sizes in brains homogenates: Smears were prepared from a half ml saline homogenate of one half of the brain of each mouse[17]. Air dried smears were stained with Giemsa stain and examined under oil immersion lens[15]. Parasite burden was evaluated microscopically by counting the cysts in 10 HPFs of the brain homogenate smear of each mouse. Similarly, the mean cyst counts and sizes and R% were calculated as previously conducted in livers and spleens impression smears[16].

Statistical analysis: Results were recorded, tabulated, statistically analyzed by statistical package SPSS. Descriptive statistics including mean±standard deviation (SD) and analytical statistics using ANOVA (F test). Statistical significance was considered at *P*<0.05.

Ethical consideration: The study was approved by the ethical committee of Suez Canal University. Animal experiments were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

RESULTS

Mortality rate: The highest rate was observed in infected non-treated group (I), that had 40% mortality rate at the end of the 9th week PI whereas zero mortality rates were observed in CUR nanoemulsion and Spiramycin treated groups (II and III, respectively) with statistical significance (*P*<0.05).

Cyst count and size in liver impression smears: The mean cyst count in infected non-treated group (GI) was 20.33±1.53. Comparatively, mean cyst counts of 6.67±1.53 and 8.0±1.0 were reduced due to treatment with CUR nanoemulsion (GII) and Spiramycin (GIII) groups, respectively. Percentage of reduction in both treated groups was consequently calculated as 60.67%, and 60.21%, respectively (Table 2). Mean size of cysts in infected non-treated GI was 26.67±8.51 μ, while mean cyst sizes in GII and GIII were reduced to 8.0±4.58 μ and 8.0±3.61 μ, respectively. Both treated groups had the same percentage of reduction (70%). There were statistically significant (*P*>0.01) decreases in cyst counts and sizes in both treated groups compared to infected non-treated mice (Table 2).

Table 2. The effect of CUR nanoemulsion and Spiramycin treatments on mean cysts count and sizes in livers impression smears (/10HPFs), and R% in *T. gondii* ME49 strain infected groups.

Groups	Cyst counts		Statistical analysis	Cyst sizes		Statistical analysis
	Mean ± SD	Reduction%	*F* test (*P* value)	Mean ± SD	Reduction%	*F* test (*P* value)
Infected non-treated (I)	20.33±1.53	--	**90.17 (<0.01)**	26.67±8.51	--	**9.83 (<0.01)**
CUR treated (II)	6.67±1.53	60.67		8.0±4.58	70	
Spiramycin treated (III)	8.0±1.0	60.21		8.0±3.61	70	

***:** Significant (*P* < 0.05).

Cyst counts and sizes in spleens: The mean cyst count in infected non-treated GI was 24.33±2.08. The highest reduction of 75.34% occurred in CUR nanoemulsion treated GII with a mean cyst count of 6.0±1.0. The 73.93% reduction in Spiramycin treated GIII was equivalent to a mean cyst count of 6.33±1.53 (Table 3). Mean size of cysts in infected non-treated GI was 21.2±8.1 μ, while in CUR nanoemulsion (GII) and Spiramycin (GIII) treated groups the sizes were reduced to 8.38±1.4 μ and 8.17±2.0 μ, respectively. The percentage reduction of 60.47%, and 61.46% were respectively similar in both treated groups. Significant differences (*P*>0.05) were recorded for both treated groups as compared to the non-treated GI regarding cyst counts and sizes (Table 3).

Table 3. The effect of CUR nanoemulsion and Spiramycin treatments on mean cyst counts and sizes in spleens impression smears (/10HPFs), and R% in *T. gondii* ME49 strain infected groups.

Groups	Cyst counts		Statistical analysis	Cyst sizes		Statistical analysis
	Mean ± SD	Reduction%	*F* test (*P* value)	Mean ± SD	Reduction%	*F* test (*P* value)
Infected non-treated (I)	24.33±2.08	--	**129.17 (<0.01)**	21.2±8.1	--	**15.97 (<0.01)**
CUR treated (II)	6.0±1.0	75.34		8.38± 1.4	60.47	
Spiramycin treated (III)	6.33±1.53	73.93		8.17±2.0	61.46	

***:** Significant (*P* < 0.05).

Cyst counts and sizes in brains homogenates: The mean cyst count in infected non-treated GI was 17.33±2.52. The percentage reduction in CUR nanoemulsion (GII) treated was apparently higher at 73.08% with a mean cyst count of 4.67±0.58, as compared to Spiramycin treated GIII in which percentage reduction was 61.54% and mean parasite count 6.67±1.53 (Table 4). Mean size of cysts in infected non-treated GI was 26.0±8.54 μ while that in CUR nanoemulsion and Spiramycin treated groups were 7.0±2.0 μ and 8.67±2.89 μ, respectively. A higher percentage of reduction (73.08%) was recorded for nanoemulsion treated GII, while that of Spiramycin treated GIII was 66.67%. There was a statistically significant (P>0.05) decrease of cyst counts and sizes in both treated groups as compared to the non-treated group (Table 4).

Table 4. The effect of CUR nanoemulsion and Spiramycin treatments on mean cysts count and sizes in brains homogenates (/10HPFs), and R% in *T. gondii* ME49 strain infected groups.

Groups	Cyst counts		Statistical analysis	Cyst sizes		Statistical analysis
	Mean ± SD	Reduction%	*F* test (*P* value)	Mean ± SD	Reduction%	*F* test (*P* value)
Infected non-treated (I)	17.33±2.52	--		26.0±8.54	--	
CUR treated (II)	4.67±0.58	73.08	**46.37 (<0.01)**	7.0±2.0	73.08	**11.68 (<0.01)**
Spiramycin treated (III)	6.67±1.53	61.54		8.67±2.89	66.67	

***:** Significant (P < 0.05).

DISCUSSION

Replicating tachyzoites, after invading intestinal epithelial cells, disseminate *via* the blood stream and lymphatics to all organs including the brain. Inside neurons and astrocytes, the preferred brain cells[18], tachyzoites differentiate into slow-dividing bradyzoites within a cyst containing thousands of bradyzoites. The cyst wall protects bradyzoites against host immune system and therapeutic drugs. Since *T. gondii* is an opportunistic parasite, brain cysts establish a symptomless chronic infection and become activated by a decrease in host immune response. Excysted bradyzoites transform into tachyzoites that proliferate causing widespread inflammation and critical morbidity. In fact, brain cyst reactivation with toxoplasmic encephalitis (TE) is not commonly reported in immunocompetent individuals with latent toxoplasmosis[8]. In their review[8], the researchers observed that brain cysts were commonly reported in the cerebral cortex, basal ganglia, and cerebellum based on studies conducted on autopsies of AIDS patients with TE[18]. Moreover, the reviewers noticed that latent toxoplasmosis negatively affects neurocognitive functions associated with behavioral changes, e.g. anxiety, depression, epilepsy, and schizophrenia. The strong tropism and persistence of brain cysts was attributed to impairment of a complex of brain functions leading to clinical manifestations that range from long-term behavioral and cognitive changes to lethal morbidity[19]. However, clinical studies attributed the behavioral changes and neurological manifestations to the influence of brain cysts on either neurotransmitter levels[20,21]; or neuroinflammation[22]; or neuroendocrine alteration[23]; or specific brain regions for cognition, mood, and emotion processing[24]. Notably, the long duration of treatment and inability to eliminate brain cysts, combined with frequency of side effects, make current chemotherapeutic options less than ideal and highlight the need for new anti-*Toxoplasma* chemotherapeutics[25].

In the present study, CUR nanoemulsion exhibited a statistically significant difference in mortality rate in comparison to infected non-treated group, and equivalent to that obtained in Spiramycin-treated group. This lowering of mortality rate was in agreement with two other studies that utilized nanotechnology for treatment[14,26]. In contrast, a study conducted in Alexandria, Egypt investigating the preventive effect of triclosan-liposomal nanoparticles on *T. gondii* ME49 strain, observed a high mortality rate. It was attributed to the daily stress caused by the twice-oral administration of the drug[27].

In our study, the recorded cyst counts, and sizes showed significant decrease in numbers and size in impression smears of livers and spleens, and emulsion smears of brains in both CUR and Spiramycin treated groups in comparison to the infected non-treated group. Similar results were obtained from two studies that utilized CUR as nanoemulsion[17] and CUR@ MOFs nanocomposite[9]. Furthermore, similar results were obtained in several studies that utilized natural products in treatment of chronic toxoplasmosis. *Artemisia annua* (wormwood) was the first herb investigated in treatment of toxoplasmosis, and artemisinin derivatives exhibited better potency than artemisinin in controlling acute toxoplasmosis and protecting chronically infected mice from reactivated toxoplasmosis[28]. An Egyptian study investigated seed oil of *Nigella sativa* (black cumin) and showed promising prophylactic and therapeutic effects in chronic murine infection induced by ME49 strain. Results revealed significant reduction in the mortality rate and brain cyst burden that was explained as due to increased level of inducible nitric oxide synthase protecting mice against immunopathology[29]. Another Egyptian study evaluated the ethanolic extract from *Thymus vulgaris* leaves (mint) in comparison to combined treatment by sulfadiazine and pyrimethamine. Results revealed significant reduction of the severity of chronic infection as demonstrated by decreased both count and size of brain cysts[30]. Besides, the results obtained from a third Egyptian study revealed significant reduction in the mean number of brain cysts (47.5%) in mice treated with *T. vulgaris* extract[31].

Similarly, *Salvia miltiorrhiza* (red sage) plant proved to be a promising therapeutic candidate against tachyzoites and bradyzoites *in vitro*. The investigators recommended further studies to assess its effects against brain cysts *in vivo*[32]. An Egyptian study was conducted to investigate the therapeutic effects of *Araucaria heterophylla* (conifer) resin extract and its major component (13-epi-cupressic acid) in chronic toxoplasmosis compared to cotrimoxazole. Results revealed that both agents exhibited higher toxoplasmicidal activity *in vitro*, and significant reduction in brain cyst burden (96% and 98%, respectively) *in vivo*[33]. Two studies investigated the ethanolic extract of *Myristica fragrans* (nutmeg) as a prophylactic agent for protection against brain cyst development[31], and acute cerebral toxoplasmosis[34] in experimentally infected mice with ME49 and RH strains, respectively. Although the first study showed that *M. fragrans* ethanolic extract exhibited mild reduction (0.8%) in brain cyst count, the second study demonstrated that myrislignan, isolated from *M. fragrans* peels, had potent anti-*T. gondii* activities both *in vitro* and *in vivo*. These activities were attributed to the interruption of mitochondrial function[34].

Lately in 2020, the ethanol extract of *Anogeissus leiocarpa* bark (African birch) was evaluated against acute and chronic toxoplasmosis. It showed significant inhibitory activity on RH strain tachyzoites, with significant reduction on host cell invasion *in vitro*. Besides, it was able to increase mice survival rate in chronic infection with ME49 strain[35]. Pomegranate is a rich source of Urolithin-A, a powerful protecting agent against neurodegeneration. Its administration significantly reduced brain cysts number and size in chronically infected mice than those in untreated animals[36]. One year later, three studies were conducted also investigating natural products[1,37,38]. Essential oils of *Pelargonium X. asperum* showed significant reduction in tachyzoites burden as demonstrated by their inability for growth and invasion[1]. Those of *Myrtus communis* (flowering myrtie) exhibited significant prophylactic results and improvement of innate immunity[37]. Leaf oil extracts of *Rosmarinus officinalis* (rosemary) showed a therapeutic rather than prophylactic potential, with reduction rates of cyst burden (81%) and cyst viability (23%). Ultrastructurally, tachyzoites with multiple depressions, protrusions, obtained from brain cysts with irregularities on cyst wall surface were unable to produce successful secondary infection after inoculation into naive mice[38].

Other natural products targeting *Toxoplasma* were suggested. In one *in vitro* study, CUR was utilized to promote a detoxification pathway producing inhibition of *T. gondii* glyoxalase 1 (*Tg*Glo1). The investigators recommended further studies utilizing drug delivery nanotechnology system to overcome CUR bioavailability and biosolubility aiming to explore CUR potential inhibitory effect on *Tg*Glo1 enzymatic activity[39]. Other studies hypothesized that constituents of some natural products inhibited heat shock proteins 70[40] and 90[41]; bradyzoites biosynthesis pathways for unsaturated fatty acid[42]; calcium homeostasis and mitochondrial physiology[43]; and cyst wall integrity[44]. However, inhibition of tachyzoites differentiation into encysted bradyzoites remains the most commonly reported mechanism of action of natural products in chronic toxoplasmosis[45].

In conclusion, CUR nanoemulsion proved to be a safe novel therapeutic agent against chronic toxoplasmosis, and we recommend further studies to evaluate its prophylactic effects for administration to immunocompromised patients either alone or in combination with the currently available drugs targeting tachyzoites growth and viability. On the other hand, due to the variable virulence among *T. gondii* strains and different incubation periods for chronic infection among host species, no single animal model can reflect the development and progression of chronic toxoplasmosis in humans. Since natural products are safe without side effects even for prolonged periods of administration time, extensive optimization, standardization as well as assessment of the pharmacokinetics are required to transfer experimental studies to clinical human trials.

Author contribution: Rageh EA performed mice infection, all parasitological parameters and analyzed the results. El-Gayar EK and Alabbassy MM proposed the study topic, suggested the study design, interpreted the results, and wrote the draft. Barakat AM maintained the strain and shared Rageh EA in mice infection and practical assessment of the parasitological parameters. Abaza SM supervised and finalized the article for publication. All authors agreed on the authorship and final version of publication.
Conflict of interest: No conflict of interest.
Funding statement: No grants received.

REFERENCES

1. Huangs S-Y, Yao N, He J-K, Pan M, Hou Z-F, Fan Y-M, *et al.* *In vitro* anti-parasitic activity of *Pelargonium X. asperum* essential oil against *Toxoplasma gondii*. Front Cell Dev Biol 2021; 9:616340.

2. Centers for Disease Control (CDC). Toxoplasmosis: Epidemiology and risk factors. Available online at https://www.cdc.gov/parasites/toxoplasmosis/epi.html, 2017. Last reviewed: September 4, 2018.
3. Moawad HSF, Etewa SE, Mohammad SM, Neemat-Allah MA, Degheili JA, Sarhan MH. Seropositivity of toxoplasmosis among hemodialysis children patients at Zagazig University Pediatrics Hospital, Egypt. PUJ 2022; 15(1):53-59.
4. Lyu C, Yang X, Yang J, Hou L, Zhou Y, Zhao J, *et al*. Role of amylopectin synthesis in *Toxoplasma gondii* and its implication in vaccine development against toxoplasmosis. Open Biol 2021; 11(6):200384.
5. Vidal JE. HIV-related cerebral toxoplasmosis revisited: Current concepts and controversies of an old disease. J Int Assoc Provid AIDS Care 2019; 18: DOI: 1177/2325958219867315.
6. Abaza SM. Recent advances in identification of potential drug targets and development of novel drugs in parasitic diseases. I. Drug resistance. PUJ 2021; 14(3):244-260.
7. Montazeri M, Mehrzadi S, Sharif M, Sarvi S, Tanzifi A, Aghayan SA, *et al*. Drug resistance in *Toxoplasma gondii*. Front Microbiol 2018; 9:2587.
8. Tan S, Tong WH, Vyas A. Impact of plant-based foods and nutraceuticals on *Toxoplasma gondii* cysts: Nutritional therapy as a viable approach for managing chronic brain toxoplasmosis. Front Nutr 2022; 9:827286.
9. El-Shafey AAM, Hegab MHA, Seliem MME, Barakat AMA, Mostafa NE, Abdel-Maksoud HA, *et al*. Curcumin@metal organic frameworks nanocomposite for treatment of chronic toxoplasmosis. J Mater Sci Mater Med 2020; 31(11):90.
10. Rageh EA, El-Gayar EK, Abaza SM, Alabbassy MM. Assessment of the prophylactic and therapeutic effects of curcumin nanoemulsion in comparison with Spiramycin in mice experimentally infected with *T. gondii* (RH strain). PUJ 2022; 15(2):154-161.
11. Djurkovi-Djakovi O, Milenkovi V. Murine model of drug-induced reactivation of *Toxoplasma gondii*. Acta Protozool 2001; 40:99-106.
12. Eissa MM, El-Azzouni MZ, Mady RF, Fathy FM Baddour NM. Initial characterization of an autoclaved *Toxoplasma* vaccine in mice. Exp Parasitol 2012; 131:310–316,
13. Teimouri A, Azami S, Keshavarz H, Esmaeili F, Alimi R, Mavi SA *et al*. Anti-*Toxoplasma* activity of various molecular weights and concentrations of chitosan nanoparticles on tachyzoites of RH strain. Int J Nanomedicine 2018; 13:1341-1351.
14. Etewa SE, El-Maaty DAA, Hamza RS, Metwaly AS, Sarhan MH, Abdel-Rahman SA, *et al*. Assessment of Spiramycin-loaded chitosan nanoparticles treatment on acute and chronic toxoplasmosis in mice. J Parasit Dis 2018; 42(1):102-113.
15. Ayele L, Mohammed C, Yimer L. Review on diagnostic cytology: Techniques and applications in veterinary medicine. J Vet Sci Technol 2017; 8:1.
16. Barakat A. Some diagnostic studies on male New Zealand rabbit experimentally infected with *Toxoplasma gondii* strain. Glob Vet 2007; 1(1):17-23.
17. Azami SJ, Teimouri A, Keshavarz H, Amani A, Esmaeili F, Hasanpour H, *et al*. Curcumin nanoemulsion as a novel chemical for the treatment of acute and chronic toxoplasmosis in mice. Int J Nanomedicine 2018; 13:7363-7374.
18. Alvarado-Esquivel C, Sánchez-Anguiano LF, Mendoza-Larios A, Hernández-Tinoco J, Pérez-Ochoa JF, Antuna-Salcido EI, *et al*. Prevalence of *Toxoplasma gondii* infection in brain and heart by immunohistochemistry in a hospital-based autopsy series in Durango, Mexico. Eur J Microbiol Immunol 2015; 5:143-149.
19. Daher D, Shaghlil A, Sobh E, Hamie M, Hassan ME, Moumneh MB, *et al*. Comprehensive overview of *Toxoplasma gondii*-induced and associated diseases. Pathogens 2021; 10:1351.
20. David CN, Frias ES, Szu JI, Vieira PA, Hubbard JA, Lovelace J, *et al*. GLT-1-dependent disruption of CNS glutamate homeostasis and neuronal function by the protozoan parasite *Toxoplasma gondii*. PLoS Pathog 2016; 12:e1005643.
21. Li Y, Viscidi RP, Kannan G, McFarland R, Pletnikov MV, Severance EG, *et al*. Chronic *Toxoplasma gondii* infection induces anti-N-methyl-d-aspartate receptor autoantibodies and associated behavioral changes and neuropathology. Infect Immunity 2018; 86(10):e00398-18.
22. Boillat M, Hammoudi P-M, Dogga SK, Pagès S, Goubran M, Rodriguez I, *et al*. Neuroinflammation-associated a specific manipulation of mouse predator fear by *Toxoplasma gondii*. Cell Rep 2020; 30:320-334.e6.
23. Singh DK, Hari Dass SA, Abdulai-Saiku S, Vyas A. Testosterone acts within the medial amygdala of rats to reduce innate fear to predator odor akin to the effects of *Toxoplasma gondii* infection. Front Psychiatry 2020; 11:630.
24. Abdulai-Saiku S, Tong WH, Vyas A. Behavioral manipulation by *Toxoplasma gondii*: Does brain residence matter? Trends Parasitol 2021; 37:381-390.
25. Smith NC, Goulart C, Hayward JA, Kupz A, Miller CM, van Dooren GG. Control of human toxoplasmosis. Int J Parasitol 2021; 51(2-3):95-121.
26. El-Temsahy MM, El Kerdany ED, Eissa MM, Shalaby TI, Talaat IM, Mogahed NM. The effect of chitosan nanospheres on the immunogenicity of *Toxoplasma* lysate vaccine in mice. J Parasit Dis 2016; 40(3):611-626.
27. El-Zawawy L, El-Said D, Mossallam S, Ramadan H, Younis S. Preventive prospective of triclosan and triclosan-liposomal nanoparticles against experimental infection with a cystogenic ME49 strain of *Toxoplasma gondii*. Acta Tropic 2015; 141:103-111.
28. Dunay IR, Chan WC, Haynes RK, Sibley LD. Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. Antimicrob Agents Chemother 2009; 53:4450-4456.
29. Rayan HZ, Wagih H, Atwa M. Efficacy of black seed oil from *Nigella sativa* against murine infection with cysts of ME49 strain of *Toxoplasma gondii*. PUJ 2011; 4:165-176.
30. Eraky MA, El-Fakahany AF, El-Sayed NM, Abou-Ouf EA, Yaseen DI. Effects of *Thymus vulgaris* ethanolic extract on

chronic toxoplasmosis in a mouse model. Parasitol Res 2016; 115(7):2863-2871.

31. Farag TI, Salama MA, Yahia SH, Elfeqy RA. Therapeutic efficacy of *Thymus vulgaris* and *Myristica fragrance* (nutmeg) ethanolic extract against toxoplasmosis in murine model. J Egypt Soc Parasitol 2019; 49:73-79.
32. Murata Y, Sugi T, Weiss LM, Kato K. Identification of compounds that suppress *Toxoplasma gondii* tachyzoites and bradyzoites. PLoS ONE 2017; 12:e0178203.
33. El-Tantawy NL, Soliman AF, Abdel-Magied A, Ghorab D, Khalil AT, Naeem ZM, *et al*. Could *Araucaria heterophylla* resin extract be used as a new treatment for toxoplasmosis? Exp Parasitol 2018; 195:44-53.
34. Zhang J, Si H, Li B, Zhou X, Zhang J. Myrislignan exhibits activities against *Toxoplasma gondii* RH strain by triggering mitochondrial dysfunction. Front Microbiol 2019; 10:2152.
35. Spalenka J, Hubert J, Voutquenne-Nazabadioko L, Escotte-Binet S, Borie N, Velard F, *et al*. *In vitro* and *in vivo* activity of *Anogeissus leiocarpa* bark extract and isolated metabolites against *Toxoplasma gondii*. Planta Med 2020; 86(4):294-302.
36. Tan S, Tong WH, Vyas A. Urolithin-A attenuates neurotoxoplasmosis and alters innate response towards predator odor. Brain Behav Immun Health 2020; 8:100128.
37. Shaapan RF, Al-Abodi HR, Alanazi AD, Abdel-Shafy S, Rashidipour M, Shater AF, *et al*. *Myrtus communis* essential oil; anti-parasitic effects and induction of the innate immune system in mice with *Toxoplasma gondii* infection. Molecules 2021; 26(4):819.
38. Abdel-Hamed EF, Mostafa NE, Fawzy EM, Ibrahim MN, Attia R, Salama MA. The delayed death-causing nature of *Rosmarinus officinalis* leaf extracts and their mixture within experimental chronic toxoplasmosis: Therapeutic and prophylactic implications. Acta Trop 2021; 221:105992.
39. Goo Y-K, Yamagishi J, Ueno A, Terkawi MA, Aboge GO, Kwak D, *et al*. Characterization of *Toxoplasma gondii* glyoxalase 1 and evaluation of inhibitory effects of curcumin on the enzyme and parasite cultures. Parasit Vectors 2015; 8:654.
40. Barenco PVC, Lourenco EV, Cunha-Júnior JP, Almeida KC, Roque-Barreira MC, Silva DAO, *et al*. *Toxoplasma gondii* 70-kDa heat shock protein: Systemic detection is associated with the death of the parasites by the immune response and its increased expression in the brain is associated with parasite replication. PLoS ONE 2014; 9:e96527.
41. Sun H, Zhuo X, Zhao X, Yang Y, Chen X, Yao C, *et al*. The heat shock protein 90 of *Toxoplasma gondii* is essential for invasion of host cells and tachyzoite growth. Parasite 2017; 24:22.
42. Ma J, He J-J, Hou J-L, Zhou C-X, Zhang F-K, Elsheikha HM, *et al*. Metabolomic signature of mouse cerebral cortex following *Toxoplasma gondii* infection. Parasit Vectors 2019; 12:1-11.
43. Rosenberg A, Luth MR, Winzeler EA, Behnke M, Sibley LD. Evolution of resistance *in vitro* reveals mechanisms of artemisinin activity in *Toxoplasma gondii*. Proc Natl Acad Sci (USA) 2019; 116:26881-26891.
44. Guevara RB, Fox BA, Bzik DJ. *Toxoplasma gondii* parasitophorous vacuole membrane-associated dense granule proteins regulate maturation of the cyst wall. mSphere 2020; 5:e00851–19.
45. Waldman BS, Schwarz D, Wadsworth II MH, Saeij JP, Shalek AK, Lourido S. Identification of a master regulator of differentiation in *Toxoplasma*. Cell 2020; 180(2):359-373.