Original Article

Parasitological, histopathological and immunological assessment of *Eugenia caryophyllata* aqueous extract alone and combined with Spiramycin© against chronic toxoplasmosis in murine model

Dalia A Shafey[1], Sahar M Selim[1], Soraya A Sharaf[1], Marwa A Gouda[1], Hebatallah M Sallam[1], Dina M Sweed[2], Dalia F Slima[3], Sawsan S Shendi[1]

Departments of Clinical and Molecular Parasitology[1], Pathology[2], and Plant Ecology[3], National Liver Institute[1,3], Faculty of Medicine[2], Menoufia University[1-3], Shebin El-Kom, Menoufia, Egypt.

ABSTRACT

Background: Nowadays, interest in the use of herbs for medical purposes is increasing. The most frequently used chemotherapeutics for treating chronic toxoplasmosis showed a variety of side effects. Therefore, it is crucial to discover new alternative therapy from natural products.

Objective: To evaluate the *in vivo* effect of *Eugenia caryophyllata* aqueous extract alone or combined with Spiramycin© against chronic toxoplasmosis.

Material and Methods: The study utilized 50 mice, equally divided into five groups. They included the normal control group (GI), infected control group (GII), Spiramycin©-treated group (GIII), *E. caryophyllata*-treated group (GIV), and Spiramycin© combined with *E. caryophyllata* treated group (GV). Treatment started six weeks after the infection and continued for 15 d. Parameters used for evaluation included parasitological, and histopathological assessments. As well, an immunohistochemical study was conducted to measure expression levels of tumor growth factor-β (TGFβ), inducible nitric oxide synthase (iNOS), and phosphorylated signal transducer and activator of transcription 1 (pSTAT-1). Interleukins 10 and 12 (IL-10, IL-12), and liver enzymes (ALT and AST) were also measured.

Results: Brain cyst counts significantly decreased in all treated groups. Combined treatment was better than Spiramycin© alone. Mice receiving *E. caryophyllata* alone or combined with Spiramycin© showed improvement in the histopathological changes in all examined tissues, with decreased expression levels of TGFβ, iNOS, and pSTAT-1. Additionally, there was an increase in IL-12 level in the combined treatment group, and a decrease in the IL-10 level. Moreover, combined treatment reduced the high levels of liver enzymes (ALT and AST).

Conclusion: *E. caryophyllata* extract exhibited promising potential *in vivo* activity against chronic toxoplasmosis.

Keywords: chronic toxoplasmosis; clove; cytokines; iNOS, mice, natural products; pSTAT-1; TGFβ; treatment.

Received: 27 August, 2024; **Accepted:** 24 September, 2024.

Corresponding Author: Hebatallah M. Sallam; **Tel.:** +20 1061083107; **Email:** heba.mohamed750@liver.menofia.edu.eg

Print ISSN: 1687-7942, **Online ISSN:** 2090-2646, **Vol. 17, No. 3, December, 2024.**

INTRODUCTION

Toxoplasmosis is a worldwide infection caused by *T. gondii*, which is an obligate intracellular protozoan[1]. Although most cases of toxoplasmosis are asymptomatic or mild in immunocompetent individuals, infection could be life-threatening in immunocompromised conditions, such as HIV, cancer, and organ transplantation, *i.e.*, receiving immunosuppressive drugs[2]. Reactivation of latent infection could progress to lethal diseases, *e.g.*, encephalitis, pneumonitis, and myocarditis[3]. Besides, toxoplasmosis during pregnancy can cause abortion, stillbirths, and dangerous neonatal and fetal complications. Among them, encephalitis, pneumonitis, chorioretinitis, epilepsy, psychomotor or mental retardation were the most reported[1].

Toxoplasmosis is treated with commercial medications; however, these have serious side effects if administered for long durations, are poorly tolerated, and cannot go beyond biological barriers[3]. Th reported mechanism of action is inhibition of folate metabolism, with subsequent arrest of DNA synthesis[4]. Unfortunately, available therapeutics have limited activity against encysted bradyzoites[5]. The first-line treatment is the combination of pyrimethamine-sulfadiazine and folinic acid to avoid hematological damage. In patients allergic to sulfa medications, or intolerance to pyrimethamine, trimethoprim-sulfamethoxazole offered a satisfactory alternative[6]. On the other hand, atovaquone or azithromycin are also therapeutic alternatives; however, their use accounted for some clinical

 DOI: 10.21608/puj.2024.316097.1266

conditions[7]. Spiramycin©, a macrolide antibiotic, is the first choice for treating acute toxoplasmosis. It is less risky and can reach high levels in the placenta. Despite its great tissue diffusion, it has low blood-brain barrier penetration and does not achieve effective concentrations in brain tissue[5].

As a result, there is an urgent need for the development of novel, effective, and safe medications with less toxicity[4]. Since herbal extracts contain a variety of bioactive components, they are now potential resources for developing novel drugs, *i.e.*, possible alternatives for many synthetic drugs[8]. Notably, herbs utilize flavonoids, alkaloids, saponins, tannins, quinones and phenolic compounds to defend against microbial activities[9]. Multiple natural products were assessed for their efficacy against toxoplasmosis. *Cuminum cyminum* (L.) seed oil[10], and *Azadirachta indica* (Neem) extract[11] proved to be useful as a natural safe therapy for acute toxoplasmosis. Coconut and ginger oils also exhibited promising therapeutic efficacy against chronic toxoplasmosis[12].

For ages, *E. caryophyllata*, or cloves were used in folk medicine due to their antioxidant properties[13], anesthetic, antiseptic, antiviral, antibacterial, and antifungal potential activities[14]. Besides, its extract displayed anti-parasitic properties against *Trypanosoma* spp.[15,16], and *T. spiralis*[17]. Moreover, it was reported to decrease hepatic fibrosis potentiating the role of eugenol compound constituent in cloves, as a promising anti-*S. mansoni* agent[18]. Cloves supplementation also decreased gastrointestinal egg shedding of several nematodes in sheep such as *H. contortus* as well as species of *Trichostrongylus, Ostertagia*, and *Cooperia*[19].

Various cytokines were associated with host immune response against toxoplasmosis. In fact, TGF-β promotes immune reactions against *T. gondii* by activating Th17 and eliminating toxoplasmosis. However, its beneficial effects are limited to *T. gondii* pre-infection. Research suggests that TGF-β could inhibit the immune system's response to *T. gondii* after infection, worsening the pathogenesis of toxoplasmosis[20]. Nitric oxide (NO), a pro-inflammatory agent produced by the enzyme iNOS, is released by macrophage cells. It increases the permeability of vessels, and triggers macrophages to produce different pro-inflammatory cytokines and mediators[21]. Besides, IFN-γ-STAT-1 signaling is required to activate anti-*T. gondii* programs by monocyte-derived cells, such as NO and antigen presentation molecules for T cell activation and IFN-γ production[22]. Finally, IL-12 is a key cytokine for the generation of IFN-γ-producing natural killer (NK) cells as well as the CD4 and CD8 T cells, which are critical for adaptive immunity against parasites[23].

Accordingly, the current investigation was designed to assess the therapeutic efficacy of *E. caryophyllata* aqueous extract alone and in combination with Spiramycin© against chronic toxoplasmosis in an animal model.

MATERIAL AND METHODS

This experimental case-control study was conducted at the Medical Parasitology Department, National Liver Institute (NLI), Menoufia University, Shebin El-Kom, and Theodor Bilharz Research Institute (TBRI), Giza, Egypt during the period from September 2021 to January 2022.

Study design: Mice were divided into five groups to evaluate the potential efficacy of *E. caryophyllata* aqueous extract in comparison to Spiramycin©. Treatment started 6 w post-infection (pi), and continued for 15 d. Mice were sacrificed at 60 dpi, and blood samples were collected from each mouse. Parameters used for evaluation included parasitological, histopathological studies, and measurement of ILs 10 and 12 levels. Drug toxicity was also evaluated by measuring liver function enzymes.

Sample size calculation: Using G power, a confidence interval of 95% and a power of 80%[24], the sample size was calculated = nine rats in each group to be increased to ten rats in each group.

Experimental animals: Fifty laboratory-bred pathogen-free Swiss female albino mice 6–8 w weighing ~20±2 g, were obtained from TBRI, Giza, Egypt. Mice were housed under standard conditions with a controlled room temperature of 25°C, a humidity of 70%, a 12-hour light and 12-hour dark cycle, free access to water, and a rodent diet in accordance.

***Toxoplasma* strain and maintenance:** The ME49 *T. gondii* strain was kindly obtained from TBRI. To sustain the infection, mice were administered every 8 w 100 µl of brain suspension containing ~100 cysts obtained from previously infected animals *via* an esophageal tube[25].

Drugs, extract preparation and doses: Spiramycin© tablets were purchased from Pharo Pharma in Egypt. After six weeks of infection, Spiramycin© pills were fragmented and diluted in distilled water to form a suspension, which was then delivered orally at a dosage of 200 mg/kg for 15 d[26]. Clove buds were purchased from a nearby herbal store in Shebin El-Kom, Egypt. They were separately reduced to coarse forms using a mortar and pestle and slowly evaporated to dryness in a hot water bath (100°C) to prepare an aqueous extract with a concentration of 50 mg/ml. The extract was given at a dose of 200 mg/kg orally for 14 d[15]. Fresh drug solutions were prepared every day.

Determination of the active ingredients for *E. caryophyllata* extract: A spectrophotometer

(LaboMed Metertek sP850) was used to measure the concentrations of alkaloids[27], tannins[27,28], phenols[29], and falvonoids[30]. All concentrations were expressed as mg/100 g extract.

Experimental groups: The experimental mice were divided into five groups as follows: normal control (GI), infected control (GII), infected and treated with Spiramycin© (GIII), infected and treated with *E. caryophyllata* aqueous extract (GIV), infected and treated with combined therapy (GV).

Mice infection: Each mouse was inoculated by oral gavage with 100 µl of brain suspension containing ~100 cysts[25].

Mice sacrifice and samples collection: The experiment was terminated at 60 dpi. All mice surviving the infection period and treatment were anaesthetized with chloroform. Mice were sacrificed by cervical dislocation, and the brain was dissected to count *Toxoplasma* cysts, and for immunocytochemical staining. Besides, brain, liver, lungs, and spleen were used for histopathological examination. Blood samples were collected, and sera were separated and kept at -20°C to measure IL-10, and IL-12, and evaluate drug toxicity by measurement of liver function enzymes.

Mortality rate: Mice were checked twice daily, and the mortality rate was recorded for each group.

Brain cysts count: At necropsy of each mouse, brains cysts were counted by a light microscope[31].

Histopathological study: Parts of the brains and different organs from all studied groups were fixed in neutral buffered formalin (10%) and processed into blocks. Serial sections of five µm thickness were cut, then stained with H&E[32]. Representative images were taken using an Olympus microscope CX41 and an Olympus camera DP26 in Shinjuku, Tokyo, Japan.

Immunohistochemical (IHC) study: Immunostaining was performed using the streptavidin-biotin-amplified technique[33]. Sections of 5 µm thickness from mouse brain tissue in all groups were cut and processed. Slides were stained for TGF-β (Santa Cruz, Texas, USA), the inducible form of iNOS (Santa Cruz, Texas, USA), and pSTAT-1 (ABclonal, Woburn, USA). Each run contained a negative control that did not contain the main antibody. A positive control (as preferred per the antibody's datasheet) was included in each run. The data of IHC were evaluated using a semi-quantitative visual technique. Coding the slides helped to reduce unintentional bias. The expression of TGF-β was regarded as positive when cytoplasmic staining was observed in any number of cells[34]. The positive expression of iNOS was evaluated when cytoplasmic staining was observed in any number of cells[35]. The positive expression of pSTAT-1 was regarded when nuclear or cytoplasmic staining was observed in any set of cells[36]. The histoscore (H-score) system was used as a common method of assessment of all the studied markers. The H-score was assessed by calculating the percentage of cells at each staining intensity level. A final score was assigned using the following formula: [(1 X (% of cells with mild staining) + 2 X (% of cells with moderate staining) + 3 X (% of cells with strong staining)]. Then a final score of 0 to 300 was assigned[35,36].

Cytokines' analysis: According to Engvall[37], IL-10 (Abcam ab100697-Mouse ELISA Kits) and IL-12 (Abcam ab171179-IL-12 p40 Mouse ELISA Kits) were measured in serum samples from mice by ELISA. The values of IL-10 and IL-12 in serum samples were measured and expressed as pg/ml using the BioTeK ELx808 ELISA reader at the Clinical and Molecular Parasitology laboratory, National Liver Institute.

Biochemical study: For the assessment of drug toxicity, hepatic enzymes [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] activities in the serum were determined using an automated Cobas C 502 (Roche, Switzerland) at the Clinical Pathology laboratory, National Liver Institute. The enzyme activity was represented as IU/l.

Statistical analysis: The acquired data was tabulated and analyzed using the statistical software IBM SPSS Statistics for Windows, Version 23 (Armonk, NY: IBM Crop). Descriptive statistics were reported using percentage (%), mean, and standard deviation (SD). An analysis of variance (ANOVA) test was performed to compare the means of continuous quantitative parametric parameters. Results with a $P<0.05$ were regarded as statistically significant.

Ethical considerations: The scientific research ethics committee authorized the procedures, National Liver Institute (NLI), Menoufia University, with IRB NO. 0003413. The animal experiment was conducted according to the criteria and principles of the Institute of Laboratory Animal Resources Commission (ILARC) for treating and utilizing laboratory animals.

RESULTS

Active ingredients in *E. caryophyllata* extract: The concentrations (mg/100 g) of the active ingredients of *E. caryophyllata* extract were 581.8, 339.3, 10.7, and 49.6 for alkaloids, tannins, phenols and flavonoids, respectively.

Mortality rate: The present study recorded a 10% mortality rate in in the infected non-treated group (GII) and 0% in all treated groups.

Cyst count: Brain cyst count was significantly reduced in all treated groups (P<0.001). The highest reduction in brain cyst count was found in GV, followed by GIII (Table 1).

Table 1. Comparison between cysts counts in brain tissues among the different studied groups.

Study groups	Brain cyst count (Mean±SD)	ANOVA text	*P* value	POST HOC test
GII (n=9)	34.6 ± 3.17	525.036	<0.001*	*P*5<0.001*
GIII (n=10)	9.8 ± 1.54			*P*6<0.001*
GIV (n=10)	17.6 ± 1.71			*P*7<0.001*
GV (n=10)	7.4 ± 1.07			*P*8<0.001*, *P*9=0.005*, *P*10<0.001*

GII: Positive control; **GIII:** Spiramycin© therapy; **GIV:** *E. caryophyllata* extract therapy; **GV:** Combined therapy; ***P*5:** GII vs GIII; ***P*6:** GII vs GIV; ***P*7:** GII vs GV; ***P*8:** GIII vs GIV; ***P*9:** GIII vs GV; ***P*10:** GIV vs GV. ***:** Significant (P<0.05).

Histopathological results

- **Brain:** The present study recorded marked meningeal inflammation, perivascular hypertrophy, and gliosis in the brain tissue of GII with lymphocyte infiltration. The degree of inflammation decreased in GV, GIII, and GIV (Fig. 1).

- **Liver:** Stained sections showed marked portal inflammation, central venous necrosis, and vascular congestion with infiltration of lymphocytes and eosinophils in the liver tissues of GII. Compared to GII, GV and GIII demonstrated marked improvement in liver tissue pathology, followed by GIV (Fig. 2).

- **Spleen:** There was marked white pulp atrophy and congestion of red pulp with megakaryocyte infiltration in the spleen of GII. The degree of white pulp atrophy and red pulp congestion improved in all treated groups, especially GV (Fig. 3).

- **Lung:** There was marked peri bronchial inflammation with lymphocytes, macrophages, and eosinophils infiltration and alveolar occlusion by inflammatory cells and hemorrhage in lung tissues of GII. GV and GIII significantly ameliorated inflammation and hemorrhage, followed by GIV (Fig. 4).

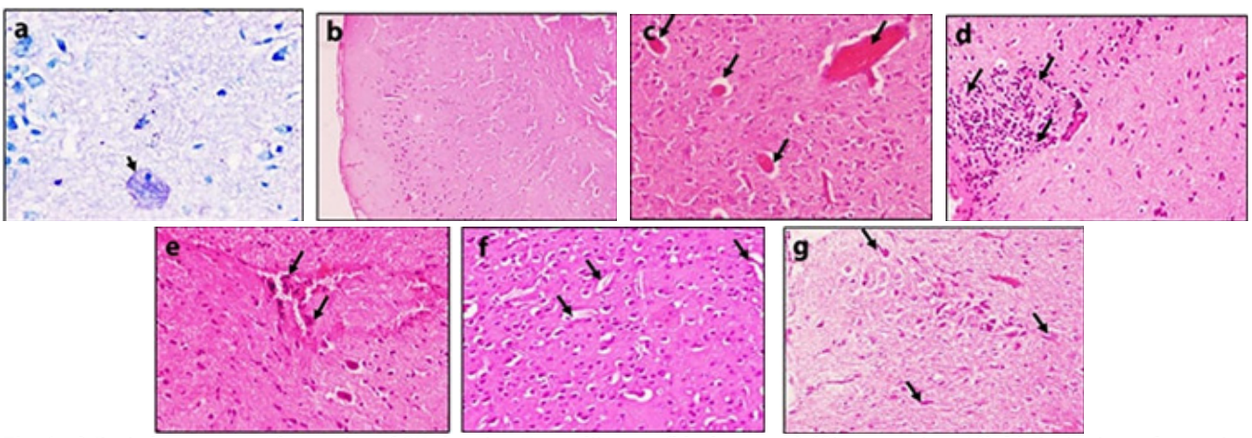

Fig. 1. a) Brain homogenate from mice of GII showing *T. gondii* cysts (black arrows) (Giemsa stain, ×40). **b)** Brain tissue from mice of GI with normal histology (100x). **c-g)** Brain tissue from infected mice stained with H&E showing **c)** GII: abnormal prominent vasculature and congestion (black arrows); **d)** GII: perivascular inflammation with infiltration of inflammatory cells (black arrows) around blood vessels; **e)** GIII: mild perivascular inflammation (arrows), and mild gliosis with abnormal glial cells; **f)** GIV: moderate meningeal irritation, inflammation (arrows) and gliosis; **g)** GV: mild vascular hypertrophy, and mild gliosis (arrows). [c, d (200x), e, f, g (100x)].

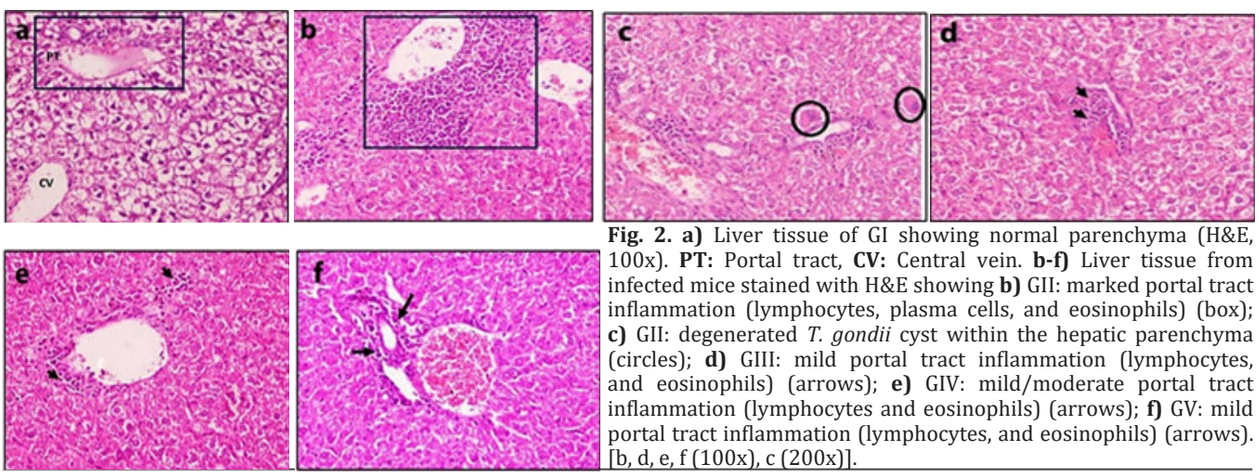

Fig. 2. a) Liver tissue of GI showing normal parenchyma (H&E, 100x). **PT:** Portal tract, **CV:** Central vein. **b-f)** Liver tissue from infected mice stained with H&E showing **b)** GII: marked portal tract inflammation (lymphocytes, plasma cells, and eosinophils) (box); **c)** GII: degenerated *T. gondii* cyst within the hepatic parenchyma (circles); **d)** GIII: mild portal tract inflammation (lymphocytes, and eosinophils) (arrows); **e)** GIV: mild/moderate portal tract inflammation (lymphocytes and eosinophils) (arrows); **f)** GV: mild portal tract inflammation (lymphocytes, and eosinophils) (arrows). [b, d, e, f (100x), c (200x)].

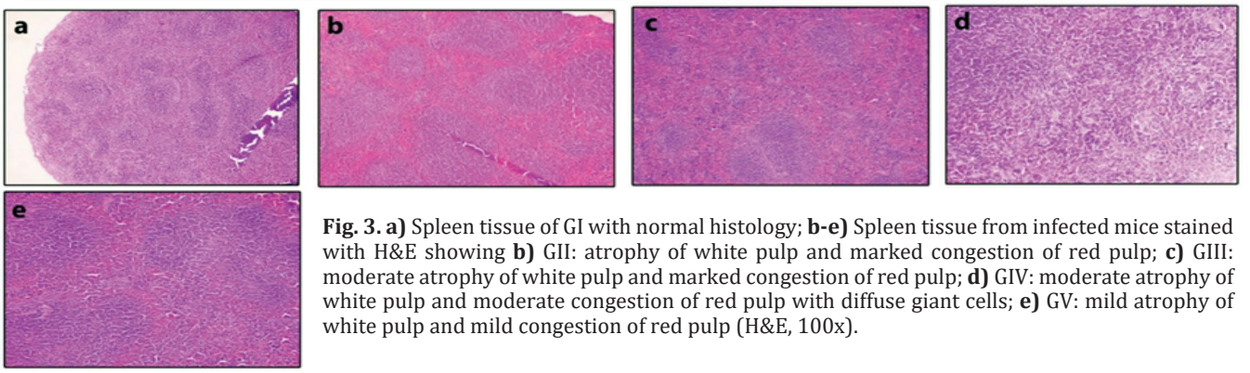

Fig. 3. a) Spleen tissue of GI with normal histology; **b-e)** Spleen tissue from infected mice stained with H&E showing **b)** GII: atrophy of white pulp and marked congestion of red pulp; **c)** GIII: moderate atrophy of white pulp and marked congestion of red pulp; **d)** GIV: moderate atrophy of white pulp and moderate congestion of red pulp with diffuse giant cells; **e)** GV: mild atrophy of white pulp and mild congestion of red pulp (H&E, 100x).

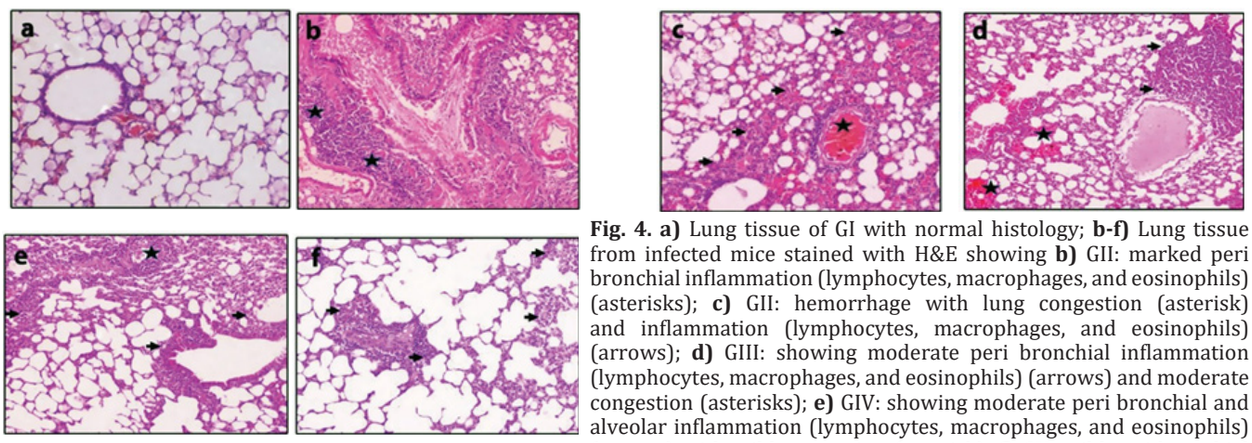

Fig. 4. a) Lung tissue of GI with normal histology; **b-f)** Lung tissue from infected mice stained with H&E showing **b)** GII: marked peri bronchial inflammation (lymphocytes, macrophages, and eosinophils) (asterisks); **c)** GII: hemorrhage with lung congestion (asterisk) and inflammation (lymphocytes, macrophages, and eosinophils) (arrows); **d)** GIII: showing moderate peri bronchial inflammation (lymphocytes, macrophages, and eosinophils) (arrows) and moderate congestion (asterisks); **e)** GIV: showing moderate peri bronchial and alveolar inflammation (lymphocytes, macrophages, and eosinophils) (arrows) with mild congestion (asterisk); **f)** GV: mild peri bronchial inflammation (lymphocytes, macrophages, and eosinophils) and alveolar inflammation (arrows) (H&E, 100x).

Immunohistochemistry: The present study demonstrated that brain tissues of GII exhibited cytoplasmic staining with a high TGF-β H-score. Low to moderate TGF-β, H-score cytoplasmic staining, was seen in GV, GIII and GIV (Fig. 5). The brain tissue of GII had a high iNOS H-score and cytoplasmic staining. In every treatment group, the iNOS H-score cytoplasmic staining ranged from low to moderate (Fig. 6). Brain tissue of GII showed high pSTAT-1 H-score cytoplasmic and nuclear staining. GV, GIII, and GIV groups showed low to moderate pSTAT-1 H-score cytoplasmic and nuclear staining (Fig. 7).

Cytokines: A significant difference regarding serum IL-10 among the infected untreated group and treated groups (P<0.001) was noted. All the treated groups

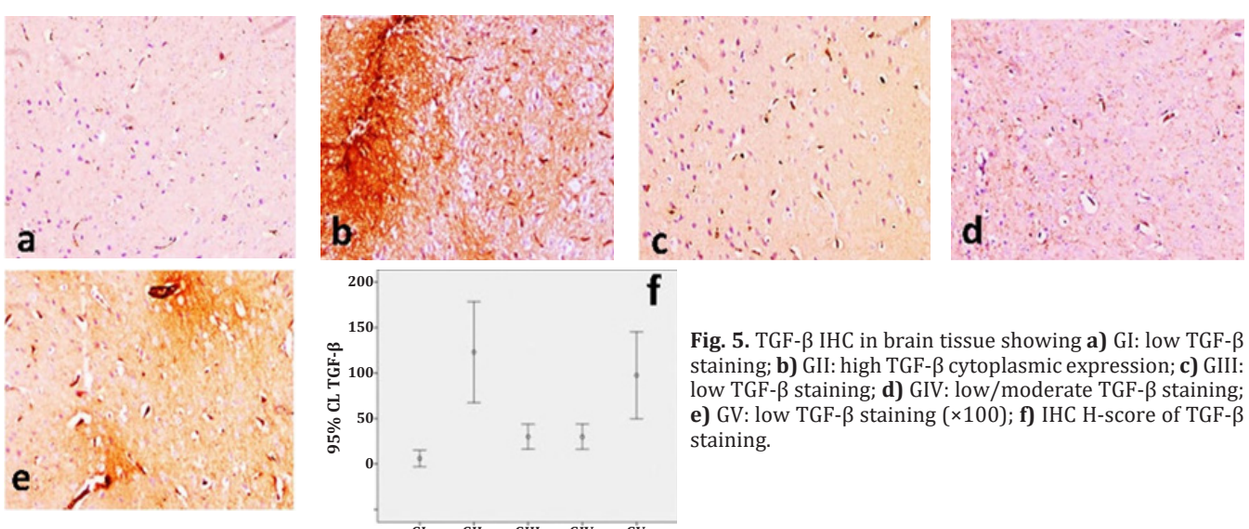

Fig. 5. TGF-β IHC in brain tissue showing **a)** GI: low TGF-β staining; **b)** GII: high TGF-β cytoplasmic expression; **c)** GIII: low TGF-β staining; **d)** GIV: low/moderate TGF-β staining; **e)** GV: low TGF-β staining (×100); **f)** IHC H-score of TGF-β staining.

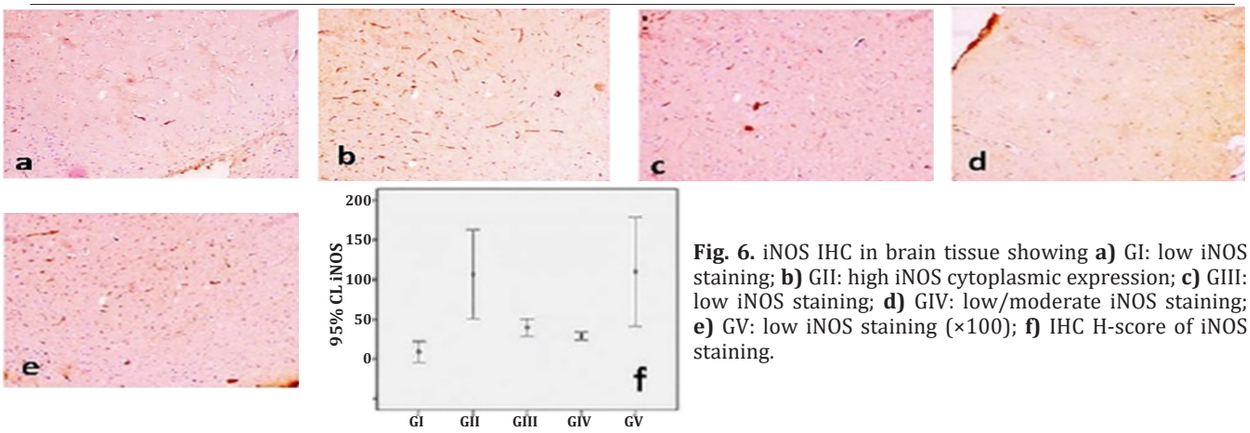

Fig. 6. iNOS IHC in brain tissue showing **a)** GI: low iNOS staining; **b)** GII: high iNOS cytoplasmic expression; **c)** GIII: low iNOS staining; **d)** GIV: low/moderate iNOS staining; **e)** GV: low iNOS staining (×100); **f)** IHC H-score of iNOS staining.

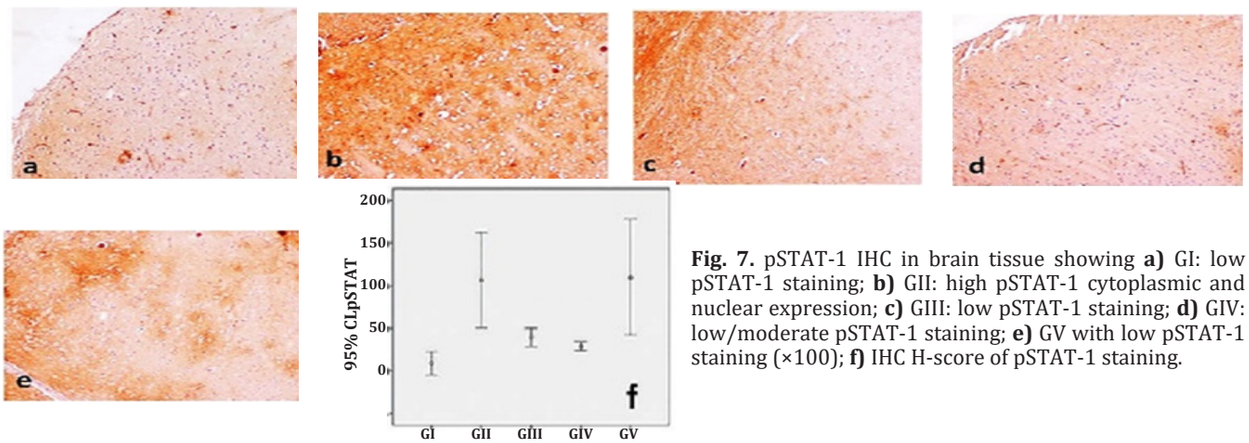

Fig. 7. pSTAT-1 IHC in brain tissue showing **a)** GI: low pSTAT-1 staining; **b)** GII: high pSTAT-1 cytoplasmic and nuclear expression; **c)** GIII: low pSTAT-1 staining; **d)** GIV: low/moderate pSTAT-1 staining; **e)** GV with low pSTAT-1 staining (×100); **f)** IHC H-score of pSTAT-1 staining.

showed significant reduction. The highest IL-10 was in GII, while the lowest was observed in GIV (Fig. 8). Regarding serum IL-12, there was a significant difference among the infected untreated group and treated groups (P<0.001). The highest IL-12 was found in GV (Fig. 8).

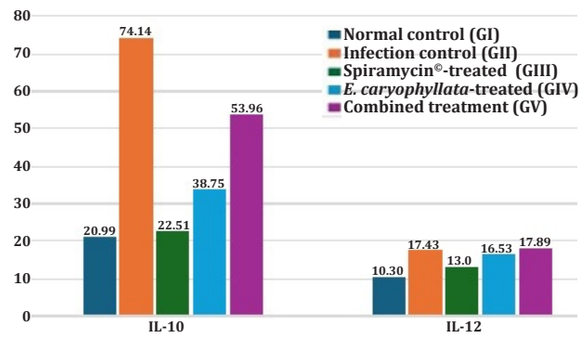

Fig. 8. Comparison between serum IL-10 and IL-12 among the study groups (P<0.001).

Biochemical results: A statistically significant difference (P<0.05) was detected in ALT and AST levels between the infected control and treatment groups. A distinct improvement in ALT levels was observed in all treated groups compared to GII. Levels of AST were slightly improved in GIII and GV (Fig. 9).

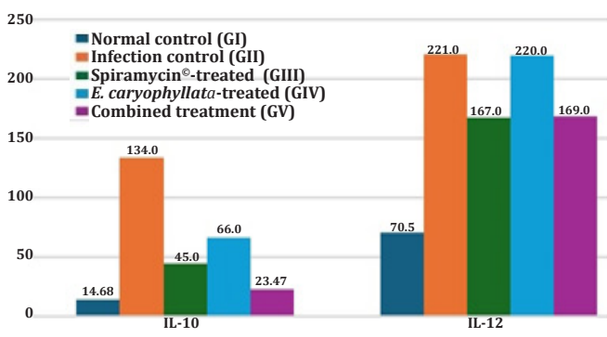

Fig. 9. Comparison of the measured serum ALT and AST among the study groups (P<0.05).

DISCUSSION

As far as we know, no prior research has been conducted to assess the effects of *E. caryophyllata* on experimental toxoplasmosis. Our study evaluated its isolated impact and its combined effect with Spiramycin© *in vivo*. Regarding mortality rates in *T. gondii*-infected mice, the findings are in harmony with Castaño Barrios *et al.*[38] who demonstrated that the

survival rate of the infected mice was 100% up to 60 dpi, and 83% survival at 90 dpi when compared to 100% survival in the non-infected control group.

For the mean brain cyst count, Castaño Barrios *et al*.[38] estimation agreed with our results as they found that the number of cysts decreased at the early 30 dpi chronic infection course up to the long-term chronic infection at 60 dpi and at 90 dpi, suggesting gradual control of infection. A similar significant reduction was noticed by Peyvandi *et al*.[39], when *Urtica dioica* L. aqueous extract decreased cyst burden to 88.2% and 83.4% for intraperitoneal and intragastric treatments, respectively. For treatment of other parasites, eugenol caused massive destruction of *T. spiralis* larvae[17]. El-Kady *et al*.[18] recorded significant amelioration of liver enzymes and hepatic fibrosis, which potentiated eugenol's role as a promising antifibrotic and complementary anti-Schistosoma agent. These data indicate the potential use of *E. caryophyllata* as a herbal and safe antiparasitic therapy. This impact is attributed to the presence of bioactive phytochemicals like alkaloids, phenols, flavonoids, and tannins with their antimicrobial activity.

The brain meningeal inflammation, perivascular hypertrophy, and gliosis changes were consistent with Castaño Barrios *et al*.[38] who reported that infected mice had meningoencephalitis and perivascular inflammatory cuffs composed of mononuclear inflammatory cells in all examined parts of the CNS. Furthermore, Nurinasari *et al*.[40] demonstrated that administering Spiramycin© and an ethanol extract from Moringa leaves effectively repaired gliosis in neurons and neuroglia of toxoplasmosis rat fetuses. Inflammatory changes detected in the liver, lung, and spleen agreed with El-Kady *et al*.[18] who discovered that PZQ or eugenol therapy in *S. mansoni*-infected mice reduced collagen accumulation in the portal tract and inside granulomas, resulting in considerable reduction of granuloma volume, particularly in the eugenol-treated group. This could be explained by the anti-inflammatory and antioxidant properties of the bioactive phytochemicals found in *E. caryophyllata* extract.

The IHC expression of TGF-β in brain tissues was consistent with Zare-Bidaki *et al*.[20] who found that it inhibited the immune system's response in post *T. gondii* infection, worsening the pathogenesis of toxoplasmosis. On the contrary, Xu *et al*.[41] demonstrated that Inonotus obliquus polysaccharide enhanced the production of the anti-inflammatory cytokine TGF-β in pregnant *T. gondii* infected mice. The iNOS expression in brain tissues was consistent with Kang *et al*.[21], who discovered that Spiramycin© had a strong inhibitory effect on iNOS expression. Their findings showed that Spiramycin© effectively reduces the expression of pro-inflammatory mediators, preventing the onset of inflammatory illness or treating aggravated inflammation[21]. On the contrary, Omar *et al*.[42] studied IHC labelling of iNOS in brain, liver, and heart tissues from various experimental groups. The enzyme was strongly expressed in both the infected untreated and Spiramycin©-treated groups. Our results of the pSTAT-1 expression in infected animal brain tissues contrasted with Cowan *et al*.[43] who stated that pSTAT-1 reduction in microglia causes higher brain parasite load and mortality in mice infected with *T. gondii*, despite immune cell activation and migration from bloodstream to brain tissues during infection. The IFN-γ-STAT1 signaling in monocyte-derived cells has a role in upregulating anti- *T. gondii* programs, *e.g.*, NO and antigen presentation substances needed for stimulation of T cells and IFN-γ secretion from T cells. The reduction of pSTAT-1 IHC expression upon treatment in our study can be explained by the parasite burden's reduction following therapy, and by the inflammatory reaction.

Regarding serum IL-10 levels, Meira *et al*.[44] previous report agreed with our results which revealed that an alteration to the Th2 immune response, including the release of anti-inflammatory cytokines such as IL-10, could increase parasite persistence, resulting in tissue immunological damage. Production of IL-10 in *T. gondii*-infected brains may enhance parasite survival by suppressing the intracerebral immune response. Xu *et al*.[41] disagreed with our current results and showed that I. obliquus polysaccharide increased the production of the anti-inflammatory cytokine IL-10 in *T. gondii*-infected pregnant mice. Anti-IL-10 treatment of infected severe-combined-immune -deficiency (SCID) mice delayed death and increased IFN-γ production in splenocyte cultures. For serum IL-12, the observed results were consistent with Jiang *et al*.[45] who observed that IL-12 production decreased for the first 24 h following infection with *T. gondii* but then reactivated. Other investigations revealed that the proliferation of $CD8^+$ T cells, double-negative T cells, and NK cells by S4B6 IL-2 complex pre-treatment enhanced the longevity rates of infected mice relying on IL-12, IL-18, and IFN-γ[46]. Mahmoudzadeh *et al*.[23] identified IL-12 as a significant activator of IFN-γ release in NK cells. Directory factors are required to regulate and inhibit the immune system from being excessively stimulated. Excess stimulation of the immune system, and production of IFN-γ and IL-12 may cause harmful effects Therefore, regulating and controlling the immune response by IL-10 is essential due to its inhibition of macrophages to produce IL-12 and other cytokines[47].

In the present study, results of ALT and AST agreed with Niazi *et al*.[48] who reported that eugenol protected the liver from damage in a rat model of fructose-induced metabolic syndrome by enhancing antioxidant status and lowering oxidative stress and lipoperoxidation. It also decreased hepatic inflammation, fat accumulation, and liver cell fibrosis.

Similarly, Tiwari *et al*.[49] discovered that *N. sativa* supplementation can considerably lower liver enzymes while also improving mitochondrial activity and ATP generation. Furthermore, Zhang *et al*.[50] discovered that toxoplasmosis resulted in elevated blood levels of ALT and AST activity, indicating liver injury. In comparison to the levels of ALT and AST in the control group, Hederagenin-treated mice and infected animals treated with Spiramycin© had considerably lower levels of these enzymes.

In conclusion, our investigation revealed that the aqueous extract of *E. caryophyllata* possesses anti-inflammatory and immunomodulatory activities rendering it efficacious in combating chronic toxoplasmosis. Furthermore, a combination of *E. caryophyllata* and Spiramycin© demonstrated a significant efficacy in reducing *T. gondii* cysts in the brain and promoting significant histopathological improvement in various tissues. The accuracy of our findings was corroborated using IHC stain and cytokine measurements. The current findings offer novel insights into the creation of new therapeutic targets to combat chronic toxoplasmosis, potentially mitigating the adverse effects associated with existing chemical medications.

Acknowledgments: We acknowledge TRBI for providing the ME49 strain. The authors express their gratitude to colleagues in the Clinical Pathology laboratory, National Liver Institute, Menoufia University for measurement of AST and ALT.

Author contributions: Shafey DA, Selim SS, Sharaf SA, Gouda MA, Sallam HM and Shendi SS contributed to the study design, assessment of the parasitological parameters and data analysis. Slima DF and Sallam HM prepared the herbal aqueous extract. Sweed DM performed the histopathological and IHC studies. Sallam HM and Shendi SS supervised the animal accommodation conditions, and their scheduled sacrifice, as well as maintenance of *T. gondii* ME49 strain. All authors revised and accepted the manuscript before publication and asserted the authorship order.

Conflict of interest: The authors do not have any relevant competing interests to disclose regarding the content of this work.

Funding statement: No financial support was obtained to aid in the development of this manuscript.

REFERENCES

1. El-Zawawy LA, El-Said D, Hegazy IH, Shalaby TI, Arán VJ, Hezema NN. Effect of VAM2-2 and VAM2-2 loaded chitosan nanoparticles in treatment of experimental toxoplasmosis: An experimental study. Senses Sci 2020; 7(3):1068-1104.
2. Manuel L, Santos-Gomes G, Noormahomed EV. Human toxoplasmosis in Mozambique: Gaps in knowledge and research opportunities. Parasit Vectors 2020; 13(1):571.
3. Abd Elgawad H, Alhusseiny SM, Taman A, Youssef MY, Mansour B, Massoud M, *et al*. Biological evaluation of newly synthesized quinoline–based compound PPQ-8 in acute and chronic toxoplasmosis: An experimental study. Exp Parasitol 2019; 206:107756.
4. 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.
5. 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.
6. Shammaa AM, Powell TG, Benmerzouga I. Adverse outcomes associated with the treatment of *Toxoplasma* infections. Sci Rep 2021; 11(1):1035.
7. Ibrahim NI, Nada SM, Salama MA, Abd El-Rahman EM. Treatment modalities of toxoplasmosis. Tob Regul Sci 2023; 9:995-1010.
8. Saboon, Chaudhari SK, Arshad S, Amjad MS, Akhtar MS. Natural compounds extracted from medicinal plants and their applications. In: Natural Bioactive Compounds; 2019; pp: 193-207; available online at: https://doi.org/10.1007/978-981-13-7154-7_7
9. Jubair N, Rajagopal M, Chinnappan S, Abdullah NB, Fatima A. Review on the antibacterial mechanism of plant-derived compounds against multidrug-resistant bacteria (MDR). Evid Based Complement Alternat Med 2021; 2021:3663315.
10. Gomaa M, Sheta E. Efficacy of *Cuminum cyminum* (L.) seed oil on acute toxoplasmosis: An experimental study on albino mice. PUJ 2022; 15(1):98-109.
11. Rashed HA, Younis S, Abdel Bary A, A Elmorsy E. *Azadirachta indica* (Neem) extract as natural agent against experimental toxoplasmosis. PUJ 2023; 16(3):253-260.
12. El Naggar H, Shehata M, Abdelmaksoud H, Barakat A, Abdelhameed R, Mohammad O, *et al*. Therapeutic potential of coconut and ginger oils in chronic murine toxoplasmosis. PUJ 2024; 17(1):37-45.
13. Batiha GE, Alkazmi LM, Wasef LG, Beshbishy AM, Nadwa EH, Rashwan EK. *Syzygium aromaticum* L. (Myrtaceae): Traditional uses, bioactive chemical constituents, pharmacological and toxicological activities. Biomolecules 2020; 10(2):202.
14. Muñoz Castellanos L, Amaya Olivas N, Ayala-Soto J, De La O Contreras CM, Zermeño Ortega M, Sandoval Salas F, *et al*. *In vitro* and *in vivo* antifungal activity of clove (*Eugenia caryophyllata*) and pepper (*Piper nigrum* L.) essential oils and functional extracts against *Fusarium oxysporum* and *Aspergillus niger* in tomato (*Solanum lycopersicum* L.). Int J Microbiol 2020; 2020:1702037.
15. Sani AA, Maigari AK, Malami AI, Omotosho K, Hamisu HS, Abubakar Y *et al*. Preliminary phytochemical screening and *in vivo* anti-trypanosomal activities of *Eugenia caryophyllata* (clove flower bud) in Wister rats (*Rattus norvegicus*) infected with *Trypanosoma evansi* (Evans, 1880). IJAAR-STE 2020:130-138.
16. Sarto MPM, Lucas da Silva HF, de Souza Fernandes N, de Abreu AP, Zanusso Junior G, de Ornelas Toledo MJ. Essential oils from *Syzygium aromaticum* and *Zingiber officinale*, administered alone or in combination with

benznidazole, reduce the parasite load in mice orally inoculated with *Trypanosoma cruzi* II. BMC Complement Med Ther 2021; 21(1):77.
17. ElGhannam M, Dar Y, ElMehlawy MH, Mokhtar FA, Bakr L. Eugenol: Effective anthelmintic compound against foodborne parasite *Trichinella spiralis* muscle larvae and adult. Pathogens 2023; 12(1):127.
18. El-Kady AM, Ahmad AA, Hassan TM, El-Deek HEM, Fouad SS, Althagfan SS. Eugenol: A potential schistosomicidal agent with anti-inflammatory and anti-fibrotic effects against *Schistosoma mansoni*-induced liver pathology. Infect Drug Resist 2019; 12:709-719.
19. Ježek J, Mirtič K, Rešetič N, Hodnik JJ, Vergles Rataj A. The effect of pumpkin seed cake and ground cloves (*Syzygium aromaticum*) supplementation on gastrointestinal nematode egg shedding in sheep. Parasite 2021; 28:78.
20. Zare-Bidaki M, Assar S, Hakimi H, Abdollahi SH, Nosratabadi R, Kennedy D, *et al*. TGF-β in toxoplasmosis: Friend or foe? Cytokine 2016; 86:29-35.
21. Kang JK, Kang HK, Hyun CG. Anti-inflammatory effects of spiramycin in LPS-activated RAW 264.7 macrophages. Molecules 2022; 27(10):3202.
22. Kak G, Raza M, Tiwari BK. Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases. Biomol Concepts 2018; 9(1):64-79.
23. Mahmoudzadeh S, Nozad Charoudeh H, Marques CS, Bahadory S, Ahmadpour E. The role of IL-12 in stimulating NK cells against *Toxoplasma gondii* infection: A mini-review. Parasitol Res 2021; 120(7):2303-2309.
24. Faul F, Erdfelder E, Lang AG, Buchner A. G-power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 2007; 39(2):175-191.
25. Wang Q, Sibley LD. Assays for monitoring *Toxoplasma gondii* infectivity in the laboratory mouse. Methods Mol Biol 2020; 2071:99-116.
26. Grujić J, Djurković-Djaković O, Nikolić A, Klun I, Bobić B. Effectiveness of spiramycin in murine models of acute and chronic toxoplasmosis. Int J Antimicrob Agents 2005; 25(3):226-230.
27. Slima DF, Alhobishi HA, Turki ZA. Phytochemical screening on *Deverra tortuosa* (Desf.) DC. collected from different habitats in Egypt. Egypt J Bot 2021; 61(3):849-866.
28. Ali AM, Saeed AA, Fdhel TA. Phytochemical analysis and antimicrobial screening of selected Yemeni folk medicinal plants. JMPS 2019; 7(5):108-114.
29. Jindal KK, Singh RN. Phenolic content in male and female *Carica papaya*: A possible physiological marker for sex identification of vegetative seedlings. Physiol Plant 1975; 33(1):104-107.
30. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. JFDA 2002; 10(3):3.
31. Araujo FG, Huskinson J, Remington JS. Remarkable *in vitro* and *in vivo* activities of the hydroxynaphthoquinone 566C80 against tachyzoites and tissue cysts of *Toxoplasma gondii*. Antimicrob Agents Chemother 1991; 35(2):293-299.
32. Carleton HM, Drury RAB, Wallington EA. Carleton's Histological Techniques. Wallington EA and Drury RAB. Oxford Univ Press New York 1980: 140-222.
33. Hsu SM, Raine L, Fanger H. A comparative study of the peroxidase-anti-peroxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am J Clin Pathol 1981; 75(5):734-738.
34. Kimawaha P, Jusakul A, Junsawang P, Loilome W, Khuntikeo N, Techasen A. Circulating TGF-β1 as the potential epithelial mesenchymal transition-biomarker for diagnosis of cholangiocarcinoma. J Gastrointest Oncol 2020; 11(2):304-318.
35. Aydemir I, Özkut MM, Fırat F, Gümürüdü A, Sal DH, Erdoğan K, *et al*. Effects of stem cells applications on oxidative stress and apoptosis during implantation. Asian Pac J Reprod 2018; 7(5):229-235.
36. Farag AGA, Samaka R, Elshafey EN, Shehata WA, El Sherbiny EG, Hammam MA. Immunohistochemical study of Janus kinase 1/signal transducer and activator of transcription 3 in psoriasis vulgaris. Clin Cosmet Investig Dermatol 2019; 12:497-508.
37. Engvall E. The ELISA, enzyme-linked immunosorbent assay. Clin Chem 2010; 56(2):319-320.
38. Castaño Barrios L, Da Silva Pinheiro AP, Gibaldi D, Silva AA, Rodrigues-Silva PM, Roffê E, *et al*. Behavioral alterations in long-term *Toxoplasma gondii* infection of C57BL/6 mice are associated with neuroinflammation and disruption of the blood brain barrier. PloS One 2021; 16(10):e0258199.
39. Peyvandi A, Gorgani-Firouzjaee T, Najafzadehvarzi H, Jafarzadeh J. *Urtica dioica* extract leads to cyst reduction, enhanced cell-mediated immune response, and antioxidant activity in experimental toxoplasmosis. Acta Parasitol 2023; 68(4):880-890.
40. Nurinasari H, Purwanto B, Indarto D, Subekti DT, Apriningsih H, Syiva'a A. Effect of Spiramycin and Moringa leaf feeding in improving placenta, heart, histopathological neurons and glia in fetal models with toxoplasmosis. Bali Med J 2023; 12(1):996-1000.
41. Xu L, Yu Y, Sang R, Ge B, Wang M, Zhou H, *et al*. *Inonotus obliquus* polysaccharide protects against adverse pregnancy caused by *Toxoplasma gondii* infection through regulating Th17/Treg balance *via* TLR4/NF-κB pathway. Int J Biol Macromol 2020; 146:832-840.
42. Omar M, Abaza BE, Mousa E, Ibrahim SM, Rashed HE, Farag TI. Effect of Spiramycin versus aminoguanidine and their combined use in experimental toxoplasmosis. J Parasit Dis 2021; 45(4):1014-1025.
43. Cowan MN, Kovacs MA, Sethi I, Babcock IW, Still K, Batista SJ, *et al*. Microglial STAT1-sufficiency is required for resistance to toxoplasmic encephalitis. PLoS Pathog 2022; 18(9): e1010637.
44. Meira CS, Pereira-Chioccola VL, Vidal JE, de Mattos CC, Motoie G, Costa-Silva TA, *et al*. *Toxoplasma* groups: Cerebral and ocular toxoplasmosis related with IFN-γ, TNF-α, and IL-10 levels. Front Microbiol 2014; 5:492.
45. Jiang H, Zhai T, Yu Y, Li X, Gong P, Zhang X, *et al*. Delayed IL-12 production by macrophages during *Toxoplasma*

gondii infection is regulated by miR-187. Parasitol Res 2020; 119:1023-1033.

46. Kupz A, Pai S, Giacomin PR, Whan JA, Walker RA, Hammoudi PM, *et al*. Treatment of mice with S4B6 IL-2 complex prevents lethal toxoplasmosis via IL-12- and IL-18-dependent interferon-gamma production by non-CD4 immune cells. Sci Rep 2020; 10(1):13115.
47. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, *et al*. Macrophage IL-10 blocks $CD8^+$ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intra-tumoral dendritic cells. Cancer Cell 2014; 26(5):623-637.
48. Niazi AA, Gharaei KF, Saebinasab Z, Maleki M, Maghool F, Fereidooni F, *et al*. Eugenol administration improves liver damage induced by a fructose-rich diet. Adv Biomed Res 2021; 10:42.
49. Tiwari A, Surendra G, Meka S, Varghese B, Vishwakarma G, Adela R. The effect of *Nigella sativa* on non-alcoholic fatty liver disease: A systematic review and meta-analysis. Hum Nutr Metab 2022; 28:200146.
50. Zhang RH, Jin R, Deng H, Shen QK, Quan ZS, Jin CM. Evaluation of the anti-*Toxoplasma gondii* activity of hederagenin *In vitro* and *in vivo*. Korean J Parasitol 2021; 59(3):297-301.