Molecular and Biochemical Assessment of Certain Antioxidants as Potential Anti-Toxoplasmic Agents in Murine Toxoplasmosis

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Receive date:28/6/2024
Revise date:28/6/2024
Accept date:26/8/2024
Publish date:16 /9/2024
Keywords: Toxoplasma gondii, Resveratrol, Lepidium sativum, Olive, and MDA.

Background and study aim: *Toxoplasma gondii* (*T. gondii*) is a worldwide distributed obligate intracellular protozoan. Oxidative stress is the main mechanism of the host's defense against protozoan infection. It has an essential role throughout *Toxoplasma* infection in the host and the parasite and is proposed to participate in the mechanism of neuropathology and neurodegeneration. At present, treatment of toxoplasmosis is not optimal. The current work aimed to assess the therapeutic and antioxidant indices of Lepidium sativum seeds extract (LSSE), eucalyptus leaf extract, olive leaf extract (OLE), and Resveratrol (RSV)against experimental toxoplasmosis.

Patients and Methods: 176 male Swiss albino mice were divided into 11 groups. Animals were sacrificed 8 weeks post-infection. The efficacy of tested drugs was evaluated using parasitological, biochemical, oxidants/ antioxidants parameters, total antioxidant capacity, and cytokines gene expression.

Results: Natural plants (OLE, LSSE, Eucalyptus) and RSV showed anti-toxoplasma effect via reducing the brain cysts count, hepato-renal protective activity through reducing ALT, AST, urea, and creatinine as well as a significant antioxidant potential through increasing levels of TAC, GSH, catalase, and decreasing levels of MDA and NO either when used as a monotherapy or combined with pyrimethamine and sulfadiazine. Additionally, olive leaf extract showed an anti-inflammatory effect through decreasing the pro-inflammatory cytokine IL1β besides increasing the anti-inflammatory cytokine IL-10.

Conclusion: In conclusion, natural plants mainly olive leaf extract and Lepidium sativum can be promising sources for anti-*Toxoplasma* medications.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan that infects an extensive variety of hosts, comprising humans and other warm-blooded animals [1]. The discrepancy between the reactive oxygen species (ROS) production and the antioxidant system of the organism is known as oxidative stress [2]. The ROS distinctive action is the polyunsaturated fatty acids degradation in the process called lipid peroxidation, which results in harmful molecule production, comprising malondialdehyde (MDA) [3]. Oxidative stress has an important role in the host and the parasite throughout *Toxoplasma* infection [4]. Zhuang et al. [5] mentioned that the oxidative stress produced by the host response is lethal to parasites. Furthermore, it is fundamental in toxoplasmosis establishment in both humans and animals [6]. At present, a combination of sulfadiazine and pyrimethamine (PRY) is being used in toxoplasmosis treatment [7]. Regrettably, they have considerable toxicity comprising bone marrow suppression plus teratogenic eff ects in the first trimester of pregnancy [8]. Also, these treatments are eff icient only against tachyzoites in the acute phase of the disease with no eff ect on the bradyzoites [9]. So, Ebrahimzadeh

et al. [10] showed that medical plants plus natural herb extracts are extensively used as substitutive therapy for many parasitic diseases and are well-thought-out to be safe with low toxicity when compared to synthetic drugs. Resveratrol (RSV, 3, 4′, 5-trihydroxy-trans-stilbene) is a non-flavonoid polyphenol, obviously existing at great concentrations in red wine in addition to grape seeds. Pharmacologically, it has been turned into therapeutic neuromodulation because of its antioxidant and anti-inflammatory properties [11]. Meng et al. [12] stated that RSV can reduce immune cell activation and the consequent production and release of pro-inflammatory mediators. Former studies show that RSV allows neuroprotection via inhibiting activation of microglia plus decreasing the pro-inflammatory factors production through cellular cascade signaling [13]. Lepidium sativum is a widespread herb, grown in numerous areas in Saudi Arabia for instance Al-Qaseem, Hijaz, and the Eastern Province. It is usually recognized as (Hab El Rashaad or Thufa) [14]. Phytochemically, Lepidium Sativum seeds have shown the existence of tannins, flavonoids, benzyl isothiocyanate, alkaloids, sterols, and triterpenes that are identified to have analgesic, anti-inflammatory, antioxidant, and anti-parasitic effects [15].

Myrtaceae family has a big genus named Eucalyptus which includes 900 species and subspecies. Eucalyptus is native to Australia where native Australians used its leaves for fungal infection therapy in addition to wound healing. It exists everywhere in the world [16]. It had *in vivo* and *in vitro* activities against *Toxoplasma*. Moreover, it exhibited greater survival rates and anti-oxidant effects [17].

The Olive (Olea europaea L.) is a minor tree related to the family Oleaceae. It is found in tropical and warm temperate districts of the world, also it is one of the oldest recognized cultivated plants [18]. Briante et al. [19] reported that olive leaves are identified as a low-cost raw material that can be used as a basis for great-value products like phenolic compounds. Oleuropein, verbascoside, hydroxytyrosol, luteolin-7-glucoside, and apigenin-7-glucoside are the chief phenolic compounds in olive leaf extracts [20]. In numerous studies in humans and animals, olive leaf extract revealed a great antioxidant ability [21] besides anti-inflammatory efficacy [22]. This work aimed to assess the therapeutic effects of resveratrol and other natural herbs (Lepidium sativum, eucalyptus leaves extract and olive leaves extract) against *Toxoplasma gondii* in comparison to traditional therapy pyrimethamine and sulfadiazine.

PATIENTS/MATERIALS AND METHODS

Parasites: Non-virulent *T. gondii* ME49 strain was utilized to induce chronic infection in mice. This strain was obtained from the Department of Parasitology, Faculty of Medicine, Zagazig University, Egypt. It was maintained in the Animal House Center, Faculty of Medicine, Zagazig University. Infected mice were sacrificed, and under sterile conditions, brains were removed and then homogenized using 1ml of normal saline, the tissue cysts number was detected by putting 2 drops of each 20µl brain homogenate on slides then counted using light microscopy with magnifying (lens ×40) and the count was multiplied by 20 to get the number of tissue cyst per brain [23].

Mice: 176 male Swiss albino mice, apparently healthy laboratory-bred, aged 5 weeks, weighing about 20-25gm each were carefully chosen from the Animal House Center, Faculty of Medicine, Zagazig University, and conducted in this study.

Drugs: Mice received pyrimethamine (12.5mg/kg) and sulfadiazine (200mg/kg) (Sigma Aldrich). Their active constituents were calculated for every mouse for each dose, then dissolved in 0.5ml of Tween-80 solution 0.5% and given as a combination according to (Köksal et al. 2015). Resveratrol (100mg/kg) was purchased as a powder from (Sigma Aldrich). Its active constituents were calculated for every mouse for each dose, then dissolved in 0.5ml of saline 0.9% [24].

Plant material: The Olive (*Olea europ*aea) & Eucalyptus (*Eucalyptus camaldulensis*) fresh leaves were gathered from the Pharmacognosy department experimental farm, Faculty of Pharmacy, Zagazig University, Egypt. Dried seeds of cress (*Lepidium sativum*) were purchased from the local Egyptian market.

Plant extraction: Extract was prepared in the Pharmacognosy department, Faculty of

Pharmacy, Zagazig University, Egypt. 500 g of fresh olive and *Eucalyptus* leaves and 300 g of dried cress seeds were cut into small pieces then, separately macerated into 80% methanol till complete exhaustion. The extracts were filtrated over filter paper and then the methanol was detached at 50 ^{0} C under decreased pressure to get semi-solid residues of crude plant extracts of 50 and 70 grams, respectively [25].

Preparation of plants` extracts for oral administration: The dried extracts suspensions were primed for oral route administration using 0.5% Tween-80 (ADWIC, Egypt) as a suspending agent in normal saline. Adjustment of each preparation concentration that each 0.1 ml of the prepared suspension contains 1 mg of the plant extract, to achieve a dose of 200mg/kg for eucalyptus & olives leaves & Lepidium sativum seeds extract [26].

Experimental design: Mice were divided into eleven experimental groups as follows: Group (1) Control non-infected Group (2) Control infected non-treated Group (3) infected and treated with pyrimethamine-sulfadiazine. Group(4): infected and treated with RSV Group(5): infected and treated with *Eucalyptus* leaves extract Group(6) infected and treated with *Lepidium sativum* seeds extract Group(7) infected and treated with olives leaves extract Group (8) infected and treated with both pyrimethamine-sulfadiazine and RSV Group(9) infected and treated with both pyrimethamine-sulfadiazine and *Eucalyptus* leaves extract Group(10) infected and treated with both pyrimethamine-sulfadiazine and *Lepidium sativum* seeds Group(11) infected and treated with both pyrimethamine-sulfadiazine and olives leaves extract. All drugs and plant extracts were given once daily for 2 weeks, started 24 hrs. post-infection, orally as a liquid suspension by gavage.

Mice inoculation: Orally, mice were infected with 10 cysts/ mouse by a 19-gauge gavage needle. They were sacrificed six weeks post-infection; their brain was cut into two portions. One brain segment was used for counting tissue cysts. The second one was well-preserved for gene expression in RNA later at ─ 80 c.

Blood and tissue sampling: Preparation of blood samples was conducted as described by Vdoviaková et al. [27], at 6 weeks post-infection, mice were anesthetized through injection of sodium thiopental (50mg/kg) intraperitoneally (i.p) before scarification. The blood was collected into two types of tubes from the heart as well as the abdominal veins. (a) Tubes containing heparin (El-Gomhorya Co. Egypt): some of the heparin tubes were centrifuged immediately at 3000rpm for 15 minutes by Hittech® centrifuge with separation of plasma free of hemolysis, the rest of these tubes were not centrifuged and used as a whole blood sample (b) Tubes devoid of anticoagulant: were centrifuged after 1hr and hemolysis free serum was separated. Blood, serum, in addition to plasma were used to assess the biochemical parameters and oxidants/antioxidant levels.

1.1. Parasitological study: Counting of *T. gondii cysts* in the brain (parasitic burden) [28], after mice were sacrificed; their brains were removed, one hemisphere of the brain from the infected mice was homogenized in a tissue homogenizer with 1ml saline for each brain. 0.1ml of brain suspension was put on the microscopic slide and then counted by high power lens (×40). Calculation of mean cyst count was done using the following equation: Mean cyst number = cyst count in 100µl×10×2.

1.2. Biochemical study: Serum liver enzymes (alanine aminotransferase, ALT plus aspartate aminotransferase, AST) levels were detected by commercial kits (Egy. Co. for biotechnology, Cairo, Egypt) and kidney parameters urea and creatinine using commercial kits (Genesis Co. Egypt) as described in the manufacturer's instructions.

1.3. Oxidant / anti-oxidant study: Plasma total antioxidant capacity concentration (TAC), both Malondialdehyde (MDA) concentration and nitric oxide (NO)concentration in liver homogenate, blood glutathione (GSH) concentration and plasma catalase concentration were assessed via commercial kits (Egy. Co. for biotechnology, Cairo, Egypt) according to the manufacturer's instructions.

1.4. Molecular (gene expression study): The molecular assay was carried out at

the Biotechnology unit, reference laboratory for veterinary quality control on poultry production, and animal health research institute. Quantitative real-time PCR was used to evaluate gene expression alteration of inflammatory cytokines (IL1β and IL10) in brain samples.

RNA extraction (RNeasy mini kit was supplied by Qiagen, Germany): Extraction of RNA (according to manufacturer's protocol), brain sample (30 mg) was weighed then placed in 2ml screw covered tubes then 600µl of Buffer RLT (with 10 µl ß-Mercaptoethanol/ ml Buffer RLT) was added into the tubes which in turn were positioned into the adaptor sets, that are fixed into Tissue Lyser clamps to be homogenized. Disruption was completed in 2 minutes high-speed shaking step (30 Hz). The lysate was centrifugated at 14000 rpm for 3 min. One volume of ethanol 70% was added to the cleared lysate and then directly mixed via pipetting. Up to 700µl of the sample, comprising any precipitate which may have been produced, was transmitted to the RNeasy spin column located in a 2ml collection tube, centrifugation was done at 14000 rpm for 1 min. The flow-through was thrown and the excess volume was processed in the same way. Buffer RW1(700 µl) was added, and centrifugation at 10000 rpm for 1 min. Was performed. The flow-through was thrown. Buffer RPE (500 µl) was added. Centrifugation was performed twice at 10000 rpm for 1 min then 2 min. Respectively. The flow-through was discarded.

Oligonucleotide primers and probes used in SYBR Green real-time PCR were purchased from Metabion (Germany): IL10 primer sequences involved forward (5'GCGGCTGAGGCGCTGTCAT 3') plus reverse (5' CGCCTTGTAGACACCTTGGTCTTGG 3') [29], for IL1β, included forward (5' CTCTGTGACTCGTGGGATGATGAC3') and reverse (5' TCTTCTTCTTTGGGTATTGTTTGG 3') [30] and for *T. gondii* reference control, included forward (5' CAGCCTTCCTTCTTG GGTAT3') and reverse (5' TGGCATAGAGGTCTTTACGG3') [31]. In the SYBR green rt-PCR results study, amplification curves besides Ct values were specified via the Stratagene MX3005P software. To evaluate the gene expression modification on the various samples of RNA, each sample CT was compared with that of the control group according to the "ΔΔ Ct" process identified by Yuan et al. [32] via the subsequent relation: ($2^{-\square\square ct}$). Whereas ΔΔ Ct = Δ Ct reference – Δ Ct target = Ct control – Ct treatment and Δ Ct reference = Ct control- Ct treatment.

Statistical analysis:

Statistical analyses were performed using the SPSS version 23. Continuous variables were analyzed as means and standard deviations. Categorical variables were expressed as medians. The chi-square or Fisher exact test was used for categorical parameters and the Student t test for continuous parameters. The different outcomes were assessed using the Odds ratio. All tests were two-sided. P-value < 0.05 was considered statistically significant.

RESULTS

Our results showed that olive leaf extract (OLE) in combination with PYR and SDZ(G11) gave the greatest reduction in brain cyst count with a reduction percentage of 81% followed by G10(treated with LSSE combined with PYR and SDZ), G9(treated with eucalyptus combined with PYR and SDZ) and G8(treated with RSV combined with PYR and SDZ) with reduction percentage 79%,78% and 77% respectively. The lowest reduction percentage was 55% which was found in G3(treated with PYR and SDZ), p<0.001.

As regards mean values of ALT enzyme. We found that the lowest value was observed in G7 and G11 with mean values of 21.2 and 21 respectively. Regarding mean values of AST enzyme, the lowest value was detected in G 10 with a mean value of 24.8. As regards mean values of urea, the lowest value was observed in G11 with a mean value of 13.6. Moreover, the lowest value of creatinine was detected in G11 with a mean value of 0.6. On the other hand, the highest value of ALT, AST, urea, and creatinine was observed in G3 with mean values 31.40, 37,29,1.25 respectively, p<0.001.

Our data displayed that the highest value of TAC was observed in G7, and G11 with mean 1068.6 and 1068.8 respectively, while the least mean

value was seen in G3 (703.8). Further, the greatest mean value of catalase and GSH was seen in G7 (9.42 and 29.2 respectively). On the other hand, the smallest mean value was recorded in G3 (3.08 and 19.2 respectively). Moreover, the lowest mean value of MD and NO was observed in G7 (3.96 and 0.83 respectively) and the highest mean value was observed in G3 (10.64 and 2.44 respectively), with p<0.001.

We reported that the mean value of cytokine expression; IL-1B; the lowest value was detected in G7 with a mean value of 3.41 however the highest value was found in G6 with a mean value of 9.50. On the other side, IL-10 expression the highest value was detected in G7 with a mean value of 6.00, while the lowest values were observed in G3 with mean values 1.02, P<0.001.

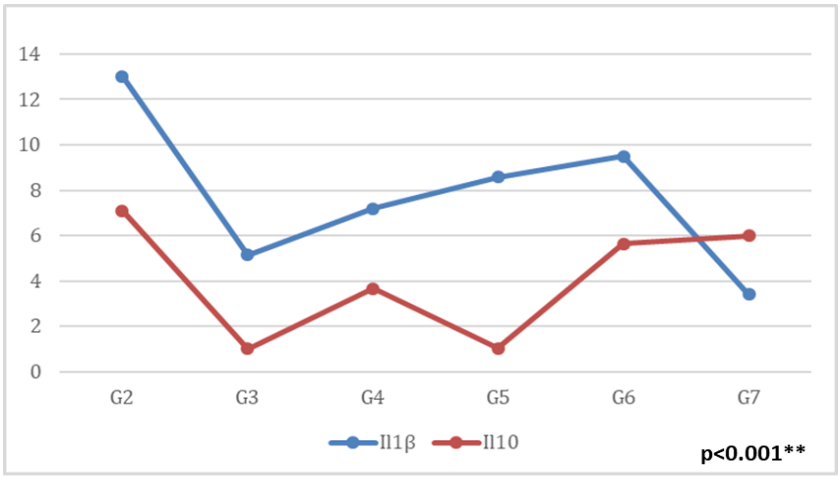

Figure 1: The difference of il1β & il10 gene expression in studied groups

Table 1: Therapeutic effect of individual & combined RSV, LSSE, Eucalyptus, and OLE on brain cyst number in infected mice

Group		brain cyst count			
		Mean	±	SD	R%
G2		880	±	130.38 [a]	
G3		392	±	123.77 [b]	**55%**
G4		288	±	43.82 [c]	67%
G5		260	±	40.00[c]	70%
G6		264	±	45.61 [c]	70%
G7		248	±	30.33[c,d]	**72%**
G8		200	±	37.42 [d]	77%
G9		196	±	49.80[d]	78%
G10		188	±	22.80 [d]	79%
G11		168	±	22.80[d]	**81%**
	F-test	50.869			
	P-value	<0.001**			

a, b, c, d: No significant difference between any two groups, within the same column have the same superscript letter, **Mean±SD** : mean ± standard deviation, **P:** Probability, ****:** Highly significant difference, **F**: ANOVA test**.**

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DOI: 10.21608/AEJI.2024.299873.1393

Table 2: Liver enzymes (ALT& AST) plus kidney function tests (urea & creatinine) in studied groups

Groups		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	F-test
ALT	Mean ± SD	17.2 ±1.30^{g}	36.4 ±2.30^{a}	31.4 ±1.14^{b}	27.6 ±1.14^{c}	25.8 ±0.84^{d}	21.2 ±1.30^{f}	21.2 ±1.87^{f}	29.8 ±1.30^{b}	26.2 ±1.10^{c,d}	23.8 ±0.84^{e}	21 ±1.30^{f}	80.913
AST		21.2 ±1.30^{d}	39 ±1.22^{a}	37 ±3.32^{a}	29.6 ±1.52^{b}	27.0 0±2.00^{c}	25 ±2.45^{c}	26.6 ±0.89^{c}	30.8 ±1.64^{b}	26.8 ±1.48^{c}	24.8 ±0.84^{c}	25.4 ±1.14^{c}	46.088
Urea		12.6 ±1.82^{e}	31.8 ±2.17^{a}	29 ±2.12^{b}	22.8 ±2.17^{c}	23.8 ±2.68^{c}	22.2 ±3.70^{c}	18.8 ±1.30^{d}	18.6 ±1.14^{d}	19 ±1.00^{d}	16.8 ±2.59^{d}	13.6 ±1.34^{e}	39.873
Creatinine		0.57 ±0.06^{d}	1.26 ±0.08^{a}	1.25 ±0.05^{a}	0.96 ±0.04^{b}	0.94 ±0.04^{b}	0.95 ±0.08^{b}	0.82 ±0.02^{c}	0.81 ±0.01^{c}	0.8 0±0.01^{c}	0.79 ±0.05^{c}	0.6 ±0.04^{d}	96.886
P-value		<0.001**											

a, b, c, d, e, f & g: There is no significant difference between any two groups, within the same column have the same superscript letter, **Mean±SD**: mean ± standard deviation, **P:** Probability, ****:** Highly significant difference, **F**: ANOVA test

Table 3: anti-oxidant parameters (TAC & catalase & GSH) and oxidant parameters (MDA & NO) in studied groups

Groups		G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	F-test
TAC	Mean ± SD	1084 ±15.3^{a}	807.8 ±19.1^{d}	703.8 ±10.9e	924.6 ±58.1c	971.6 ±30.8b	975. ±11.00b	1086.6 ±22.73a	898.4 ±46.7c	974.4 ±4.39b	978.8 ±11.9b	1086.8 ±28.13a	97.743
Catalase		4.26 ±0.26g	2.30 ±0.19i	3.08 ±0.08h	8.78 ±0.08b	8.46 ±0.29b	9.12 ±0.19a	9.42 ±0.48a	6.42 ±0.13d	5.86 ±0.27e	5.38 ±0.08f	7.12 ±0.40c	461.65
GSH		28.40 ±0.55a	17.8 ±0.84d	19.2 ±0.84d	22.8 ±0.84c	24 ±2.55c	26.2 ±1.79b	29.2 ±0.84a	23.4 ±1.52c	24.4 ±0.55c	24.2 ±1.10c	28 ±0.71a	41.665
MDA		2.88 ±0.19f	11.82 ±0.63a	10.64 ±0.88b	8.14 ±0.63c	6.2 ±0.16d	4.36 ±0.26f	3.96 ±0.21f	8.18 ±0.27c	6.74 ±0.25d	5.00 ±0.16e	4.3 ±0.19f	231.953
NO		0.95 ±0.1e,f	2.32 ±0.09a	2.44 ±0.22b	1.12 ±0.1cd	1.14 ±0.1cd	0.94 ±0.09e	0.83 ±0.07f	1.2 ±0.07c	1.05 ±0.11d, e	0.95 ±0.04e	0.88 ±0.04f	124.478
P-value		<0.001**											

a, b, c, d, e, f & g: There is no significant difference between any two groups, within the same column have the same superscript letter, **Mean±SD**: mean ± standard deviation, **P:** Probability, ****:** Highly significant difference, **F**: ANOVA test.

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DOI: 10.21608/AEJI.2024.299873.1393

DISCUSSION

Toxoplasma gondii shows a distinctive pathogenesis, making treatment challenging **[26].** The blood-brain barrier prevents adequate drug concentration passage **[33].** The present standard treatments against toxoplasmosis are restricted. Therefore, the exploration of substitutive compounds with new modes of action is mandatory **[9].** Natural compounds and conventional herbal medicine have great accessibility and fewer side effects in comparison to the existing anti-*toxoplasma* therapies **[10].**

In the current work, we aimed to evaluate the antiparasitic and antioxidant effects of resveratrol, *Lepidium sativum, Eucalyptus, and olive leaf extract* against chronic experimental *T. gondii* infection in mice. Drugs were used both individually and in combination with PYR and SDZ. The assessment was conducted by parasitological, biochemical, and molecular analysis.

Concerning parasitological assessment, our results showed that all groups treated with natural compounds revealed a significant decrease in the number of brain cysts compared to the PYR and SDZ-treated group (G3) either when used as a monotherapy or combined with PYR and SDZ. Moreover, we observed that olive leaf extract (OLE) gave the highest reduction in brain cyst count when used alone (G7) or in combination with PYR and SDZ (G11) with reduction percentages of 72% and 81% respectively (table 1).

Regarding groups treated with OLE (G7 & G11), the obtained results can be attributed to the effect of maslinic acid which is the active ingredient of OLE. These results are agreed with **Martín-Navarro *et al.* [34]** who stated that maslinic acid (MA) has been reported to be a potent inhibitor of glycogen phosphorylase and other proteases of *T. gondii*. It is well-known that *T. gondii* secret different types of proteolytic enzymes such as metalloproteases, serine protease, cysteine protease, and threonine protease which cause the organism pathogenicity **[35].** Additionally, the existence of amylopectin granules is a feature of *T. gondii* bradyzoites in tissue cysts. *T. gondii* genome encodes a presumed glycogen phosphorylase (TgGP) which is required for harmonizing amylopectin storage in addition to efficient brain cyst production **[36].**

Additionally, **De Pablos *et al.* [37]** found that MA caused alterations in the ultrastructure and gliding motility of *Toxoplasma* tachyzoites, blocking the parasite entry into Vero culture cells (through its inhibitory effect on proteases). Interestingly, this inhibition is dose-dependent. This can add another explanation for the obtained reduction in brain cyst numbers in both G7 and G11.

Analysis of results obtained in groups treated with *Lepidium sativum* seeds extract (LSSE) in (G6 & G10), revealed a significant reduction in brain cysts count with reduction percentage 70% and 79% respectively (table 1).

The anti-parasitic activity of LSSE may be attributed to the seeds covering that comprises numerous quantities of diverse bioactive compounds that are qualified to prevent the parasite's development and division or induce immunity within the host **[38]**.

Previous studies discussed the antiparasitic effect of *Lepidium* species. **Montazeri *et al.* [39]** reported the anti-*Toxoplasma* activity of hydroalcoholic extract of LSSD via inhibition of *T. gondii* tachyzoites growth in infected Vero cells. *Lepidium* species are rich in aromatic glucosinolate (GSLs) which are converted into isothiocyanates like benzyl isothiocyanates (BITCs) **[40]**. The anti-*Toxoplasma* activity of LSSE is supposed to be attributed to GSLs and BITCs contents **[39].**

Regarding the groups treated with *Eucalyptus* leaf extract (G5 & G9), there was a significant reduction in brain cyst count with reduction percentages of 70% and 78% respectively (table 1). *Eucalyptus* extract can exert its action through its antioxidant activity. Likewise, the *in vivo* anti-*Toxoplasma* activity of *Eucalyptus* extract has been previously observed by **Mirzaalizadeh *et al.* [17].** They noticed better survival rates in mice infected with tachyzoites of *T. gondii* RH strain followed by injection with the extract of various concentrations intra-peritoneally. At the same time, *Eucalyptus* treatment minimized the enlarged bulk of the liver and spleen which induced by infection, they proposed that the mechanism of action of *Eucalyptus* was due to its anti-oxidant effect during *T. gondii* infection.

Regarding resveratrol (RSV) treated groups (G4 & G8), we reported a significant reduction in brain cyst count with reduction percentages of 67% and 77% respectively (table 1). **Tonin *et al.***

[41] demonstrated the increased acetylcholinesterase enzyme (AchE) activity in the brains of *T. gondii*-infected animals. Consequently, increases the hydrolysis of acetylcholine (Ach) which has anti-inflammatory activity so, this leads to an increase in the inflammatory response.

These results are close to that obtained by **Bottari *et al.* [42]** who reported a significant decrease in brain cysts number in mice infected with *T. gondii* following the administration of sulfamethoxazole-trimethoprim (ST) combined with RSV. However, they observed no significant difference in brain cyst count when RSV was used as monotherapy compared to the infected untreated group and attributed the effect of RSV in lowering the number of brain cysts to its anti-inflammatory protection activity through modulation of the activity of AChE.

On the other hand, **Chen *et al.* [43]** demonstrated that RSV in different concentrations possesses an inhibitory effect on extracellular and intracellular *T. gondii* RH strain tachyzoites. RSV showed a direct action against *Toxoplasma* through decreasing the extracellularly developed tachyzoites numbers. Former researchers attributed RSV anti-*Toxoplasma* effect to the disruption of redox hemostasis of parasites, furthermore, RSV was qualified to discharge the cellular stress load, encourage apoptosis, and preserve the autophagic condition of macrophages, which turned out to be controlled via intracellular parasites. Furthermore, **Contreras *et al.* [44]** showed that RSV prevented intracellular *T. gondii* tachyzoite development at concentrations under the host cell's toxic threshold. They suggested a potential connection between RSV and DNA injury or repair procedures which is probably related to DNA replication stress.

T. gondii infection causes oxidative stress associated with an increase in liver enzymes (AST &ALT) and renal function tests (urea and creatinine) due to peroxidation of unsaturated fatty acids in biological membranes of cells leading to a decrease in membrane fluidity and disruption of the membrane integrity causing leakage of enzymes from the cytosol to the extracellular compartment **[45].**

Regarding the biochemical study, an improvement in liver functions (AST &ALT) and kidney functions (urea and creatinine) was noticed in all treated groups. The maximum decrease in serum levels of ALT, AST, urea, and creatinine was noted in G 11 &7 (OLE-treated groups), however, the least reduction was in group 3 (treated with pyrimethamine + sulfadiazine) (Table 2).

The hepatoprotective activity of OLE may be attributed to its biologically active compounds like MA which work as free radical scavengers **[45].** Also, the renal protective activity was attributed to the anti-inflammatory and antioxidant effects of MA **[46].** The same results were obtained by **Al-Attar and Alsalmi [47]** who proved that OLE played a vital role against the hepatorenal injury induced by diabetes with a remarkable reduction of liver and renal parameters through its antioxidant activities.

Concerning oxidant-antioxidant assessment in our research, a strong antioxidant activity was clear in the targeted treated groups demonstrated by an increase in total antioxidant capacity (TAC) and antioxidant parameters (GSH and catalase) levels and a decrease in oxidant parameters (MDA and NO) levels (tables 3).

In the present study, treatment with OLE induced the highest antioxidant activity. This antioxidant efficiency was attributed to its MA content **[48]** and oleuropein content **[49].** The anti-oxidant activity of oleuropein may be attributed to the presence of an ortho-diphenolic group in its structure which is capable of scavenging ROS via hydrogen contribution and steadying oxygen radicals by an intramolecular hydrogen bond **[50].**

Oxidative stress is frequently accompanied by nitrosative stress produced through NO and reactive nitrogen species production, which are the main motivators of inflammation and pro-inflammatory signaling. So, antioxidants can be considered as anti-inflammatory molecules.

In the existing research, we observed that the targeted drugs had an anti-inflammatory activity proved by a reduction in the pro-inflammatory cytokine (IL-1β) and a rise in the anti-inflammatory cytokine (IL-10) (fig.1). Both OLE and LSSE treated groups showed a strong anti-inflammatory activity compared by PYR-SDZ treated group.

Referring to OLE-treated groups, the observed anti-inflammatory effect may be due to its MA and oleuropein content. Also, **[51]** proved the anti-inflammatory effect of MA, the anti-

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DOI: 10.21608/AEJI.2024.299873.1393

inflammatory effect of oleuropein was attributed to inhibition of lipoxygenase activity and the production of leukotriene B4 as demonstrated by **Omer [49].**

Some studies have demonstrated the antioxidant activity of LSSE in various dissolvent, that can prohibit inflammation **[52].** The chief constituent of *L. sativum* seeds is α-Linolenic acid which stops NO formation and blocks inducible NO synthase gene expression. α-Linolenic acid possibly will do this role by obstructing NF-κB efficacy and the mitogen-activated protein kinase (MAPK) phosphorylation in macrophages **[53].** The ethanolic extract of *L. sativum* prominently lowered iNOS-2 expression and nitrate content. The decline in nitrosative stress considerably downregulated the expression of nuclear factor kappa-B (NF-κB) and NF-κB DNA binding action and decreased cytokines (TNF-α & IL-6) in a dose-reliant manner **[15].** Finally, we concluded that natural plants (OLE, LSSE, *Eucalyptus*) and RSV showed an anti-*Toxoplasma* effect via significant reduction of the rain cysts count when used either as monotherapy or combined with PYR &SDZ. Moreover, natural plants and RSV have hepato-renal protective activity by reducing ALT, AST, urea, and creatinine. Furthermore, natural plants and RSV showed strong anti-oxidant potential mainly OLE and LSSE through increasing levels of TAC, GSH, and catalase and decreasing levels of MDA and NO. OLE showed an anti-inflammatory effect through decreasing levels of pro-inflammatory cytokine IL1β besides rising anti-inflammatory cytokine IL-10. Further studies including characterization and nanoparticle formula in herbs should be performed to afford novel mechanisms of action to clarify the effects previously described or extend the range of its biological actions.

CONCLUSION:

We concluded that in this order; OLE, LSSE, Eucalyptus, and RSV showed anti-*Toxoplasma* effect via reducing the brain cysts count, hepato-renal protective activity as well as a significant antioxidant potential. Additionally, OLE showed an anti-inflammatory effect through decreasing the pro-inflammatory cytokine IL1β besides OLE and LSSE showed increasing the anti-inflammatory cytokine IL10. So, OLE and to a lesser extent LSSE can be promising anti-*toxoplasma* medications.

Conflict of interest: None.

Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Authors 'contributions: All authors contributed to the study's conception and design. Marwa A Salama, Eman M Abd El Rahman, and Soad M Nada contributed to performing the laboratory work, interpreting the results, and writing the manuscript. Rasha A Attia, Nagwa Ibrahim, and Samira M Mohammad participated in the laboratory work, collecting references, and writing the manuscript. All authors read and approved the final manuscript.

Ethical consideration: Mice were reared and sacrificed as stated by the rules of The Institutional Animal Care and Use Committee according to Zagazig University (ZU-IACUC) for Animal Use in Research and Teaching. All surgeries were conducted under anesthesia and all exertions were done to confirm negligible animal suffering. Suitable precautions were followed while handling *the T. gondii* parasite because it is a bio-safety level 2 (Bl-2) pathogen. During the parasite-animal passage, caution was taken to prevent assisting personnel infection. The study protocol was permitted by the Parasitology Department Review Board (Approval No, ZU-IACUC/3/F/95/2020).

HIGHLIGHTS:

- Oxidative stress is the main mechanism of the host's defense against *Toxoplasma* infection
- At present, treatment of toxoplasmosis is not optimal
- OLE, LSSE, Eucalyptus, and RSV showed anti-*Toxoplasma* effect via reducing the brain cysts count, hepato-renal protective activity as well as a significant antioxidant potential. Additionally, OLE showed anti-inflammatory effects.

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Site as: Salama, M., Nada, S., Mohammad, S., Attia, R., Ibrahim, N., Abd El Rahman, E. Molecular and Biochemical Assessment of Certain Antioxidants as Potential Anti-Toxoplasmic Agents in Murine Toxoplasmosis. *Afro-Egyptian Journal of Infectious and Endemic Diseases*, 2024;14(4):420-431. doi: 10.21608/aeji.2024.299873.1393