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Original Article

***Moringa oleifera* Leaf Ethanol Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication**

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ABSTRACT

The various infection routes of *Toxoplasma gondii* that are close to daily life strongly support the incidence of toxoplasmosis. The emergence of drug-resistant *Toxoplasma gondii* strains raises future concerns. Moringa leaf ethanol extract has been shown to have several anti-pathogen activities, which could have an anti-*Toxoplasma* effect. This research was conducted to analyze the anti-*Toxoplasma* effect of moringa leaf ethanol extract against tachyzoites replication in *Toxoplasma gondii* and the correlation between extract doses with the number of tachyzoites. Mice were divided into five groups. The negative control group (Group I) received CMC-Na solution. The positive control group (Group II) received spiramycin 100 mg/kg BW. The treatment groups received moringa leaf ethanol extract 250 mg/kg BW (group III), 500 mg/kg BW (group IV), and 1000 mg/kg BW (group V), respectively. Mice were injected with 1×10^5 tachyzoites/0.1 mL/mice intraperitoneally on the first day. Moringa leaf ethanol extract and spiramycin were given orally once daily for three days. The number of tachyzoites in the intraperitoneal fluid was calculated on the fifth day. The results have shown that there were significantly lower differences ($P < 0.05$) in group IV ($P = 0.021$) and group V ($P = 0.022$) compared to group I. There was also a significant negative correlation between the extract doses and the number of tachyzoites ($P = 0.000$; $r = -0.781$). *Moringa oleifera* leaf ethanol extract has an anti-*Toxoplasma* effect by inhibiting the tachyzoite replication at 500 mg/kg BW and 1000 mg/kg BW.

Keywords: *Moringa oleifera*; tachyzoites; *Toxoplasma gondii*

Highlights: This research provides the first study that proved the effectiveness of *Moringa oleifera* leaf ethanol extract in inhibiting *Toxoplasma gondii* tachyzoites replication.

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INTRODUCTION

Toxoplasma gondii is an obligate apicomplexan intracellular protozoan that causes toxoplasmosis in warm-blooded animals, including humans. It has been reported that approximately 30-50% of the world's human population is infected by this parasite.¹ The prevalence of toxoplasmosis seropositivity in humans has also reached 32,6% among 9 of 47 primary health centers in Makassar, Indonesia.² The high incidence of toxoplasmosis makes this disease a global health problem that needs attention because it can cause severe clinical manifestations in immunocompromised patients and permanent fetal disability.³

The various infection routes of *T. gondii* that are close to daily life could strongly support the incidence of toxoplasmosis. This zoonotic infection can occur in several ways: accidentally ingesting cat faeces that contain oocysts; eating undercooked meat that contains tissue cysts; transplacental transmission from an infected mother to a fetus; and other possibilities, such as receiving a blood transfusion or an organ transplant from an infected donor.⁴

Recent studies in clinical cases of toxoplasmosis have shown that drug resistance in *T. gondii* is ongoing. The emergence of drug-resistant *T. gondii* strains raises future concerns, not only in terms of treatment failure but also of increasing clinical severity in immunocompromised patients.⁵

Using natural ingredients such as plants or fruits as herbal medicine can be an alternative. This alternative is also considered less toxic than synthetic drugs and is better in terms of economy, practicality, and accessibility. Research on natural resources in Indonesia should always be carried out due to the vast and abundant biodiversity in Indonesia, which has excellent potential to bring benefits to the health sector.

Moringa (Indonesian: kelor) or *Moringa oleifera* is a plant often found in Indonesia. Parts of the plants that can be utilized are

roots, stems, fruits, flowers, seeds, and leaves. *In vitro* study of *M. oleifera* seeds has been shown to inhibit the replication of tachyzoites.⁶ The leaves are part of the plant that is often consumed by Indonesian people and have been shown to have anti-inflammatory, antifungal, and antibacterial effects.⁷⁻⁹ It also acts as a larvicidal.¹⁰

A phytochemical analysis of *M. oleifera* leaf ethanol extract revealed alkaloids, phenolics, flavonoids, tannins, saponins, and terpenes as their bioactive compounds.^{8,11-13} It has rutin as its major flavonoid, gallic acid as its major phenolic acid, and lutein as its major carotenoid. Several alkaloid compounds were also detected, such as pyrazoline alkaloids, piperidine alkaloids, and quinoline alkaloids.^{14,15}

Quinoline alkaloids are one of the typical deoxyribonucleic acid (DNA) intercalating alkaloids that have cytotoxic and antiparasitic effects from their intercalating actions between the nucleotide pairs of the parasite.¹⁶ *M. oleifera* leaf ethanol extract could have an anti-*Toxoplasma* effect through its DNA-damaging compounds. Therefore, this research was conducted to prove the potential of *M. oleifera* leaf ethanol extract as the new anti-*Toxoplasma* drug against tachyzoites replication in *T. gondii*.

MATERIALS AND METHODS

Experimental Materials and Tools

M. oleifera leaves were purchased from and identified by the Technical Implementation Unit of the Herbal Laboratory, Materia Medica Batu, East Java, Indonesia (reference number 074/656/102.20-A/2022). The RH strains of *T. gondii* tachyzoites were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. *Deutschland-Denken-Yoken* (DDY) mice (male, 20-30 grams, 2-3 months old) were purchased from a certified local experimental animal breeder. Other materials used in this research were spiramycin (500 mg,

Spirasin®, SANBE, Bandung, Indonesia) and sodium chloride 0.9% (Otsu®, PT Otsuka Indonesia, East Java, Indonesia).

Tools used in this research were Neubauer counting chamber (0.1 mm depth, Assistant®, Germany), cover glass (22 x 22 mm, OneLab®, Indonesia), light microscope (Nikon®, Nikon Corporation, Japan), disposable syringe 3 cc (Terumo®, Terumo Company, Tokyo, Japan), sterilized falcon tubes (NEST®, NEST Biotech, China), micropipette (DLAB®, DLAB Scientific Co., Ltd., Beijing, China), and hand tally counter (MARAS®, Togoshi Seiki, Taiwan).

Plant Extraction

M. oleifera leaves were washed, dried at 40°C temperature using an oven, and ground into powder. The powder was macerated with 96% ethanol for 24 hours while being stirred occasionally. The first maceration results were filtered, and the residue was re-macerated with the same stage until the second maceration results were obtained. Both maceration results were mixed and evaporated using a rotary evaporator at 40°C until they became a dense mass.¹⁷

Phytochemical Analysis

Table 1. Chemical Reaction Tests for Some Bioactive Compounds from *M. Oleifera* Leaf Ethanol Extract^{7,17}

Constituent	Method
Alkaloids	Mayer test
Flavonoids	Ammonium test
Phenolics	Ferric chloride test
Steroids	Lieberman-Burchad test
Saponins	Froth test
Tannins	Ferric chloride test

The phytochemical analysis was performed to determine secondary metabolites (alkaloids, flavonoids, phenols, steroids, saponins, and tannins) present in the *M. oleifera* leaf ethanol

extract using the color and precipitate reaction methods (Table 1).

Thin-Layer Chromatography Analysis

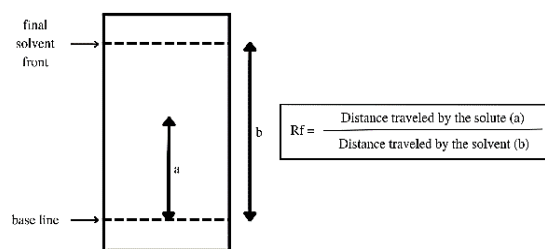


Figure 1. Retention factor (Rf) Values Calculation Formula in Thin-Layer Chromatography Analysis.

The thin-layer chromatography (TLC) analysis was performed to isolate the specific compounds present in the *M. oleifera* leaf ethanol extract. The extract was applied on silica gel 60 F₂₅₄ plates as the stationary phase. The mobile phases were (chloroform: methanol: water) (50:65:10) for alkaloids and steroids, (n-butanol: acetic acid: water) (4:1:5) for flavonoids, phenols, and tannins. After leaving the developed plates to dry, they were observed under Ultra-Violet (UV) light at both 254 nm and 366 nm, then sprayed with iodine reagents to detect the bands.¹⁸ The movement of the separated compounds was expressed by retention factor (Rf) values, which were calculated by the formula (Figure 1).

Animals

All the mice have been declared healthy by the veterinarian. The mice were acclimated for one week under laboratory conditions in wire-covered cages with paddy husk as bedding at a temperature of 24±4°C, relative humidity of 44-56%, and 12 hours of light and dark cycle. Four mice per cage were given free access to distilled water and standard mouse food.

Parasite Culture

The tachyzoite culture was performed *in vivo* on male DDY mice. They were maintained by routine intraperitoneal passage every 72 hours. The number of tachyzoites was determined by counting them in a counting chamber, then diluted to sodium chloride 0.9% solution before being inoculated into the experimental mice.¹⁹ The toxoplasmosis induction used in this research was 1×10^5 tachyzoites/0.1 mL/mice.

Toxoplasmosis Drug Reference

This research used spiramycin as the toxoplasmosis drug reference.²⁰ It was administered orally at a dose of 100 mg/kg BW. The tablets were crushed into powder and dissolved with sodium carboxymethyl cellulose (CMC-Na) solution until they became a homogenous suspension.

Experimental Design and Protocol

Mice were divided into five *T. gondii*-infected groups (n = four mice for each group). The infected group were divided as follows: negative control group (Group I) received CMC-Na solution 0.5 mL/mice orally as a placebo, positive control group (Group II) received spiramycin 100 mg/kg W, group III received *M. oleifera* leaf ethanol extract 250 mg/kg BW, group IV received *M. oleifera* leaf ethanol extract 500 mg/kg BW, and group V received *M. oleifera* leaf ethanol extract 1000 mg/kg BW.

Mice in the infected group were injected with 1×10^5 tachyzoites/0.1 mL/mice intraperitoneally on the first day. *M. oleifera* leaf ethanol extract and spiramycin were diluted into 0.5 mL of CMC-Na solution and

given orally once daily for three days from the second to the fourth day. On the fifth day, all mice were sacrificed with cervical dislocation, and the intraperitoneal fluid was collected to count the tachyzoites.

Intraperitoneal Fluid Collection

The outer skin of the peritoneum was cut using scissors and tissue forceps, then gently pulled back to expose the inner skin lining the peritoneal cavity. The peritoneal cavity was washed with 3 mL of normal saline. The abdomen was shaken slowly to dislodge the tachyzoites into the saline solution. Aspiration of the intraperitoneal fluid was carried out using a syringe.²¹

Count of Parasites

The number of parasites was carried out by blind-direct examination using a counting chamber at 400x magnification of a light microscope.¹⁹ Blind-direct examination means the counter does not know from which group the sample was taken. The mean of tachyzoites was expressed in a multiplication factor of 10^4 .

Statistical Analysis

The research results were analyzed using Statistical Product and Service Solutions software (IBM Corp., Armonk, NY) version 25. The significant differences were statistically determined using the Kruskal-Wallis test, followed by the Mann-Whitney test. Values at $P < 0.05$ are considered significant. The Pearson correlation coefficient (r) was used to determine the correlation between extract doses and the number of tachyzoites.

RESULTS AND DISCUSSION

Phytochemical Analysis

Table 2. Phytochemical Analysis Results of *M. oleifera* Leaf Ethanol Extract

Constituent	Result	Interpretation
Alkaloids	Development of cream-yellow precipitate	Positive
Flavonoids	Development of red or pink color	Positive
Phenols	Development of dark green color	Positive
Steroids	Development of blue color	Positive
Tannins	Development of brownish-green color	Positive
Saponins	No formation of stable foam	Negative

The phytochemical analysis of *M. oleifera* leaf ethanol extract revealed the absence of saponins and the presence of alkaloids, flavonoids, phenols, steroids, and tannins (Table 2). These results were not aligned with the previous research, which revealed that the *M. oleifera* leaf ethanol extract contained saponins as its secondary metabolite compound.^{7,10,12} The absence of saponins in this research could have occurred due to several factors that affected the extraction process. Factors influencing the maceration process results are temperature, solvent types and concentration, duration, and other factors.²²

The extraction of *M. oleifera* leaves using methanol as a solvent with 72 hours of maceration showed positive saponin results on the phytochemical screening.⁷ Positive saponin results were also obtained in the extraction using ethanol as the solvent with a maceration time of 72 hours.¹² Another study using ethanol as a solvent with 48 hours of maceration time showed negative screening results for saponins, which were the same as the results of the phytochemical analysis in this research using the same type of solvent but with 24 hours of maceration time.¹¹

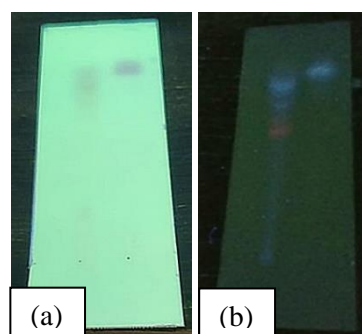


Figure 2. TLC results of alkaloids under UV light (a) 254 nm (b) 366 nm

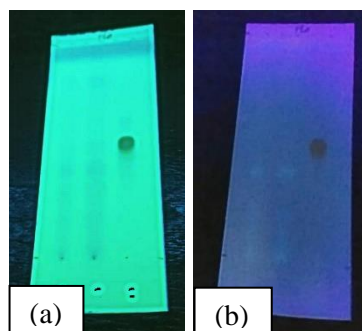


Figure 3. TLC results of flavonoids under UV light (a) 254 nm and (b) 366 nm

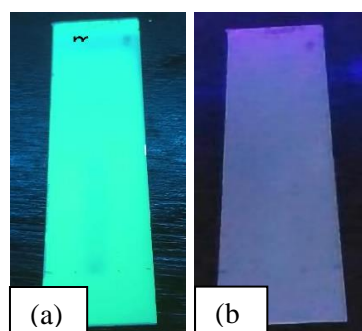


Figure 4. TLC results of phenols and tannins under UV light (a) 254 nm and (b) 366 nm

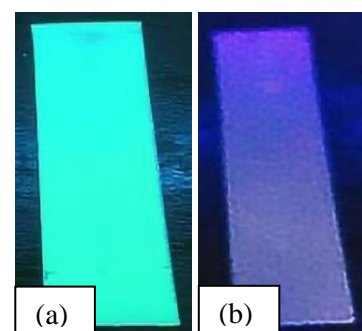


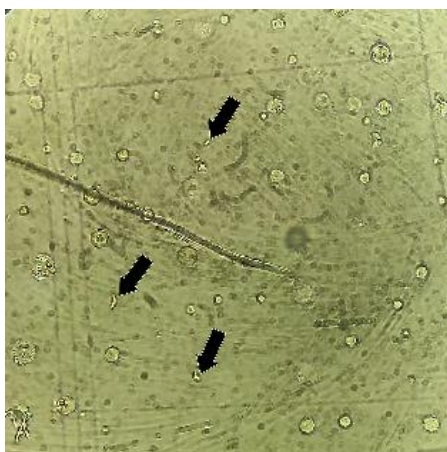
Figure 5. TLC results of steroids under UV light (a) 254 nm and (b) 366 nm

Table 3. Rf values and colors of peaks of each compound of *M. oleifera* leaf ethanol extract

Constituent	Rf values	Colors of peaks
Alkaloids	0.63	Red
	0.73	Blue
	0.81	Blue
Flavonoids	0.35	Yellow
	0.43	Yellow
	0.48	Yellow
	0.80	Yellow
	0.88	Yellow
Phenols	0.78	Blackish green
Steroids	0.75	Yellowish blue
	0.88	Yellowish blue
	0.93	Yellowish blue
Tannins	0.78	Blackish green

The positive bioactive compound results were also confirmed with TLC analysis as shown in Figure 2–5. Alkaloids were detected with Rf values of 0.63, 0.73 and 0.81. Flavonoids were detected with Rf values of 0.35, 0.43, 0.48, 0.80, and 0.88. Phenols were detected with Rf values of 0.78. Steroids were detected with Rf values of 0.75, 0.88, and 0.93. Tannins were detected with Rf values of 0.78 (Table 3).

Number of Tachyzoites

**Figure 6.** *T. gondii*-infected group. Tachyzoites (black arrows) on intraperitoneal fluid from the infected group under a light microscope with 400x magnification.

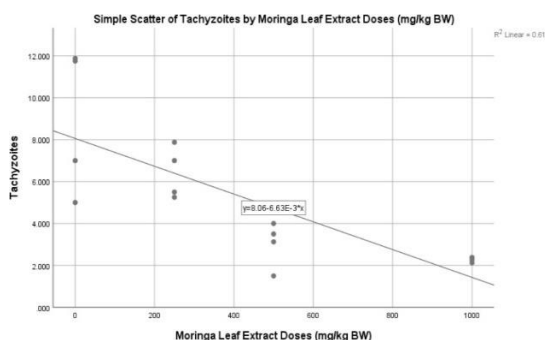
Identification of tachyzoites was carried out based on its distinctive morphology, which is a crescent-like shape, sharp at the anterior and blunt at the posterior, with a length of about 4–8 μm and a width of about

2–3 μm . The color of tachyzoites in the intraperitoneal fluid preparation was clear and transparent, accompanied by movement, indicating that the protozoa are still alive and have motility (Figure 6).

Table 4. Tachyzoites count results in the intraperitoneal fluid of the *T. gondii*-infected group (n = four mice per group)

Group	Infection	Mean \pm SD ($\times 10^4$)
I	+	8.91 \pm 3.45
II	+	2.88 \pm 2.14*
III	+	6.41 \pm 1.25
IV	+	3.03 \pm 1.08*
V	+	2.28 \pm 0.12*

Group I: Negative Control Group; Group II: Positive Control Group; Group III: Treatment Group 1; Group IV: Treatment Group 2; Group V: Treatment Group 3; +: Infected; SD: Standard Deviation; *: $p < 0.05$ compared to Group I

**Figure 7.** Simple scatter plot of tachyzoites count results (y-axis) by the *M. oleifera* leaf ethanol extract doses (x-axis). The number of tachyzoites tends to increase as the extract doses decrease, with $P = 0.000$ and $r = -0.781$

The highest tachyzoites were in group I with a total of $8.91 \pm 3.45 \times 10^4$. Group II was significantly lower than group I, with a total of $2.88 \pm 2.14 \times 10^4$. There were two extract treatment groups with a significantly lower number of tachyzoites compared to group I: group IV with a total of $3.03 \pm 1.08 \times 10^4$ and group V with a total of $2.28 \pm 0.12 \times 10^4$. The number of tachyzoites in group III, with a total of $6.41 \pm 1.25 \times 10^4$, was not significantly different compared to group I as shown in Table 4.

The toxoplasmosis intraperitoneally induction used in this research was 1×10^5 tachyzoites/0.1 mL/mice. Tachyzoites can invade almost all the host nucleated cells

and replicate rapidly.³ The cell will eventually suffer damage and rupture due to this state, and the released tachyzoites will continue to look for other cells, perpetuating the cycle. The severity of the clinical symptoms depends on the degree of tachyzoite replication, which means that an individual's immune system is crucial in defining the clinical manifestations.²³

Administration of spiramycin at 100 mg/kg BW orally for three days effectively inhibited tachyzoite replication, as proved by the significantly lower difference in the mean number of tachyzoites present in the peritoneal fluid between group II and group I (Table 4). Spiramycin is an antibiotic and an antiparasitic macrolide agent that is considered the drug of choice against *T. gondii* in pregnancy. The mechanism of action of the drug was to inhibit the synthesis of proteins and the growth of protozoan cells.²⁴ Its effectiveness as a toxoplasmosis drug has also been proven through previous research, which could reduce the number of *T. gondii* cysts in brain tissues.²⁰

The doses of *M. oleifera* leaf ethanol extract used in this research were 250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW. The tachyzoite count differences were significantly lower in groups IV and V compared to group I. There was no significant difference in the mean number of tachyzoites between group III and group I (Table 4).

The results revealed that *M. oleifera* leaf ethanol extract at doses of 500 mg/kg BW and 1000 mg/kg BW could inhibit tachyzoite replication, but the dose of 250 mg/kg BW could not. These results occurred because the concentration of chemical properties in *M. oleifera* leaf ethanol extract increased with increasing doses (Figure 7). Our results are also align with other research on *Plasmodium yoelii*, in which the higher the dose of *M. oleifera* leaf ethanol extract, the greater the inhibitory activity against the parasites.²⁵

Quinoline alkaloids (*3-methylquinoline*) are typical DNA intercalating compounds found in the *M. oleifera* leaf ethanol extract.¹⁵

Their antiparasitic effect occurred through their hydrophobic, aromatic, and planar properties, which allow them to intercalate between the nucleotide pairs of the parasite. These cause mutations, such as deletions or frame-shift mutations, which will disrupt the replication of the parasite.¹⁶ If the mutation occurs in an essential protein-coding gene, it causes the death of the parasite.¹³ This theory also aligns with previous research, which proved that the moringa seeds extract promotes apoptosis-like death in *T. gondii* tachyzoites *in vitro*.⁶

A simple scatter plot showed a negative correlation between the extract doses and the number of tachyzoites (Figure 7). The decrease in tachyzoite count results, along with increasing doses of *M. oleifera* leaf ethanol extract, occurred because the concentration of alkaloids in the extract is directly proportional to the dose. The higher concentration of alkaloids causes more intercalated DNA in the parasites, resulting in more disruption in the replication of the tachyzoites.

This research proved the effectiveness of *M. oleifera* leaf ethanol extract in inhibiting tachyzoites replication. Future research needs to conduct more specific studies on the effect of the extract as an anti-*Toxoplasma*, whether isolating the specific antiparasitic bioactive compound and examining the histopathological variables on *T. gondii* target organs or other variables of its pathway of antioxidant properties.

STRENGTH AND LIMITATION

The blind-direct examination method in this research provided minimal occurrence of bias due to the subjective perspective of the researcher. This research also verified and explained the antiparasitic mechanism of *M. oleifera* leaf ethanol extract through the combination of phytochemical screening, TLC analysis, and the count of parasites.

Although we adopt the blind-direct examination for the count of parasites method, it does not provide full objective

results in this research. More objective parameters, such as histopathological or hematological variables, in order to examine the impact of the *T. gondii* tachyzoites replication, should be carried out in future study to support the results of this research.

CONCLUSIONS

The *M. oleifera* leaf ethanol extract has an anti-*Toxoplasma* effect by inhibiting the tachyzoite replication at 500 mg/kg BW and 1000 mg/kg BW.

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ETHICAL CLEARANCE

The research protocol was approved by the Health Research Ethics Committee (HREC) of the Medical Faculty of Widya Mandala Catholic University, Surabaya, Indonesia (reference number 0326/WM12/KEPK/DSN/T/2022).

This research was carried out following the ethical principles outlined in the Council for International Organizations of Medical Sciences (CIOMS) and World Health Organization (WHO) International Ethical Guidelines for Health-Related Research Involving Humans.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

AUTHOR CONTRIBUTION

Experimental design: LW, GLW, CPT. Extract preparation: GLW, CPT. Materials preparation: LW, CPT. Research implementation: LW, CPT, HP. Research supervision: LW, HP. Data analysis: LW, GLW. Manuscript writing: LW, CPT. Manuscript editing: LW, GLW, CPT, HP.

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