1. Submitted to the journal " Indonesian Journal of Tropical and Infectious Disease" (18-1-2023)
2. First revision: Accepted with major revision (07-02-2023)
3. Resubmit for review from another reviewer (13-02-2023)
4. First revision submitted (26-02-2023)

   -Revised version with highlights

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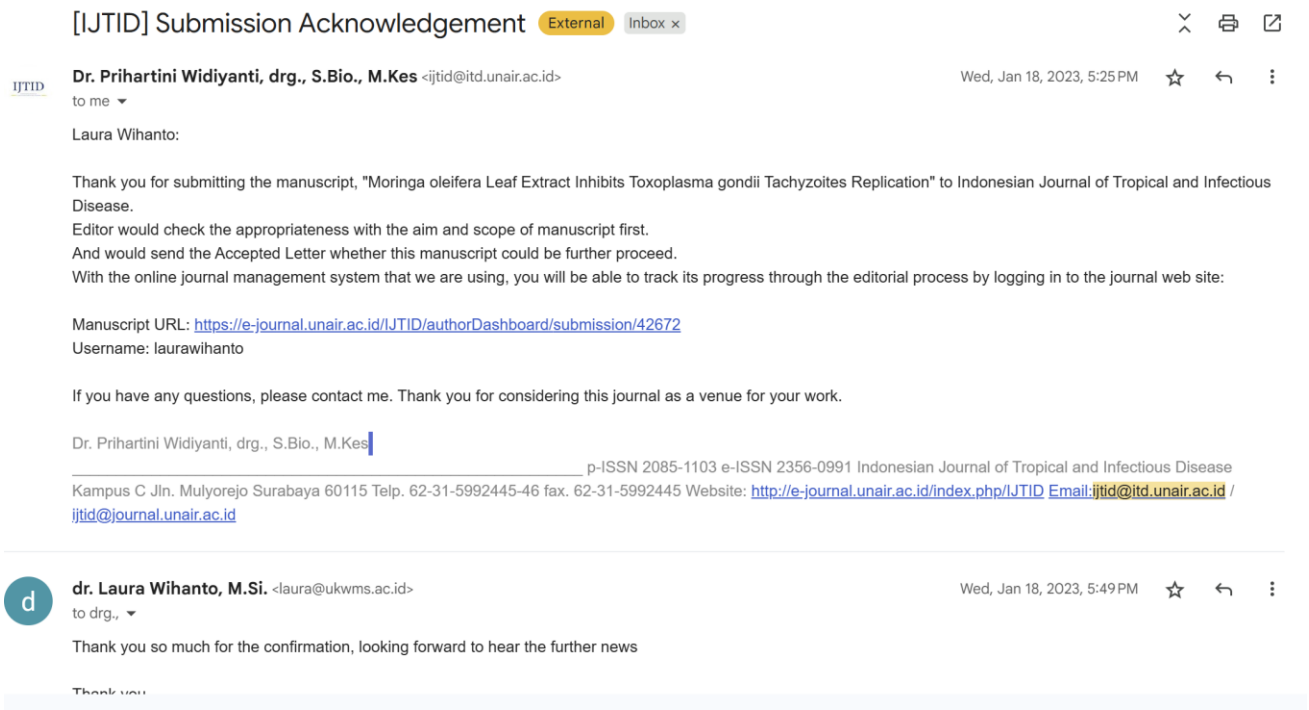
Submitted to the journal " Indonesian Journal of Tropical and Infectious Disease" (18-1-2023)



[IJTID] Submission Acknowledgement External Inbox ×

IJTID **Dr. Prihartini Widiyanti, drg., S.Bio., M.Kes** <ijtid@itd.unair.ac.id> Wed, Jan 18, 2023, 5:25 PM

to me

Laura Wihanto:

Thank you for submitting the manuscript, "Moringa oleifera Leaf Extract Inhibits Toxoplasma gondii Tachyzoites Replication" to Indonesian Journal of Tropical and Infectious Disease.
Editor would check the appropriateness with the aim and scope of manuscript first.
And would send the Accepted Letter whether this manuscript could be further proceed.
With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

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Username: laurawihanto

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Dr. Prihartini Widiyanti, drg., S.Bio., M.Kes

p-ISSN 2085-1103 e-ISSN 2356-0991 Indonesian Journal of Tropical and Infectious Disease
Kampus C Jln. Mulyorejo Surabaya 60115 Telp. 62-31-5992445-46 fax. 62-31-5992445 Website: http://e-journal.unair.ac.id/index.php/IJTID Email:ijtid@itd.unair.ac.id / ijtid@journal.unair.ac.id

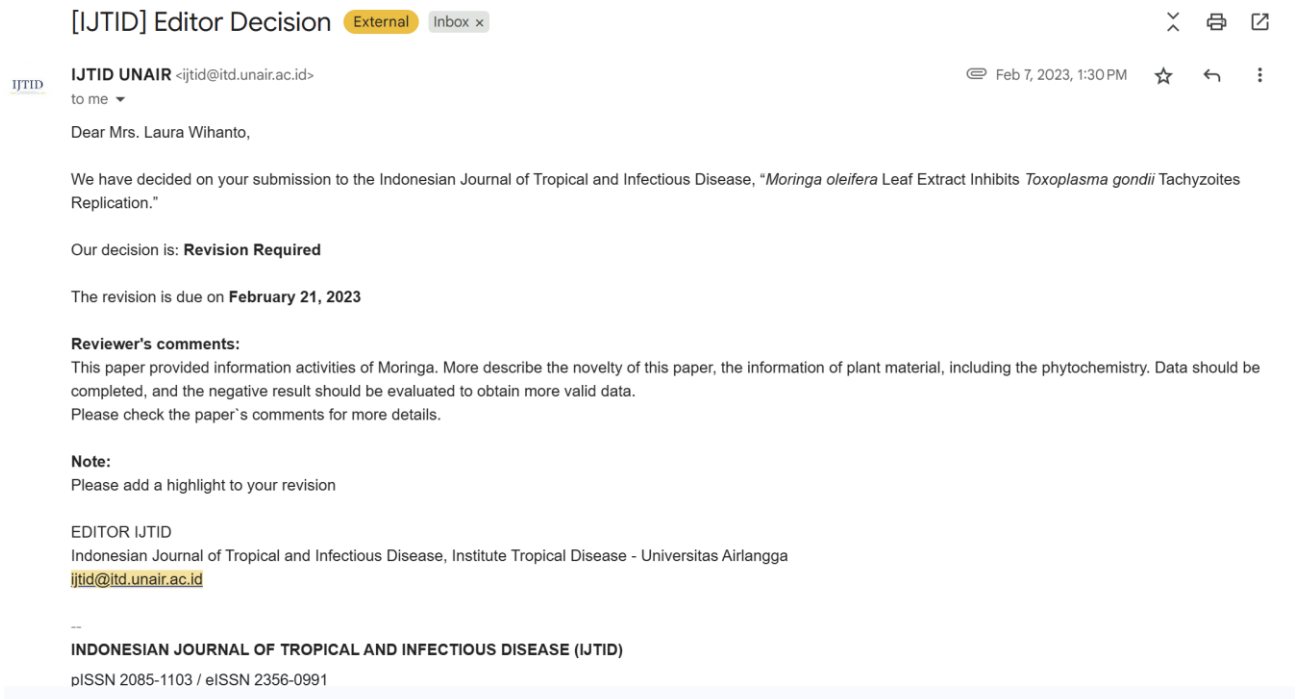
d **dr. Laura Wihanto, M.Si.** <laura@ukwms.ac.id> Wed, Jan 18, 2023, 5:49 PM

to drg.,

Thank you so much for the confirmation, looking forward to hear the further news

Thank you

First revision: Accepted with major revision (07-02-2023)



[IJTID] Editor Decision External Inbox ×

IJTID **IJTID UNAIR** <ijtid@itd.unair.ac.id> Feb 7, 2023, 1:30 PM

to me

Dear Mrs. Laura Wihanto,

We have decided on your submission to the Indonesian Journal of Tropical and Infectious Disease, "*Moringa oleifera* Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication."

Our decision is: **Revision Required**

The revision is due on **February 21, 2023**

Reviewer's comments:
This paper provided information activities of Moringa. More describe the novelty of this paper, the information of plant material, including the phytochemistry. Data should be completed, and the negative result should be evaluated to obtain more valid data.
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EDITOR IJTID
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Original Article

Moringa oleifera Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication

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 extract has been shown to have several anti-parasite activities, which could have an anti-Toxoplasma effect. To analyze the anti-Toxoplasma effect of moringa leaf 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 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 tachyzoites intraperitoneally on the first day. Moringa leaf 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. 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 a significant correlation between the extract doses and the number of tachyzoites (P = 0.000; r = -0.781). Moringa leaf extract has an anti-Toxoplasma effect at 500 mg/kg BW and 1000 mg/kg BW, and there was a negative correlation between the extract doses and the number of tachyzoites.

Keywords: *Toxoplasma gondii; tachyzoites; toxoplasmosis; Moringa oleifera; moringa leaf extract*

ABSTRAK

Berbagai jalan masuknya infeksi Toxoplasma gondii yang dekat dengan kehidupan sehari-hari sangat mendukung tingginya angka kejadian toksoplasmosis. Munculnya strain Toxoplasma gondii yang resisten terhadap pengobatan menimbulkan kekhawatiran di masa mendatang. Ekstrak daun kelor telah dibuktikan memiliki beberapa aktivitas anti parasit, salah satunya dapat berupa efek anti-Toxoplasma. Menganalisis efek anti-Toxoplasma pada ekstrak daun kelor dalam menghambat replikasi Toxoplasma gondii stadium takizoit dan korelasi antara dosis ekstrak daun kelor dengan jumlah takizoit. Mencit dibagi menjadi 5 kelompok. Kelompok kontrol negatif (grup I) diberi larutan CMC-Na. Kelompok kontrol positif (grup II) diberi spiramycin 100 mg/kgBB. Kelompok perlakuan masing-masing diberi ekstrak daun kelor 250 mg/kgBB (grup III), 500 mg/kgBB (grup IV), dan 1000 mg/kgBB (grup V). Induksi toksoplasmosis pada mencit dilakukan secara intraperitoneal pada hari ke-1. Ekstrak daun kelor dan spiramycin diberikan secara peroral selama 3 hari. Perhitungan takizoit pada sampel cairan intraperitoneal dilakukan pada hari ke-5. Terdapat perbedaan yang signifikan lebih rendah (P < 0.05) pada grup IV (P = 0.021) dan grup V (P = 0.022) dibandingkan dengan grup I. Terdapat korelasi yang signifikan antara dosis ekstrak daun kelor dengan jumlah takizoit (P = 0.000; r = -0.781) Ekstrak daun kelor memiliki efek anti-Toxoplasma pada dosis 500 mg/kgBB dan 1000 mg/kgBB, serta terdapat korelasi negatif antara dosis ekstrak daun kelor dengan jumlah takizoit

Kata kunci: *Toxoplasma gondii; takizoit; toksoplasmosis; Moringa oleifera; ekstrak daun kelor*

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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 been reported to reach 43-88% of the total population in various regions of 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 *Toxoplasma 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 *Toxoplasma gondii* is ongoing. The emergence of drug-resistant *Toxoplasma 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. 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.[6-8] It also acts as a larvicidal.[9] A phytochemical screening test of moringa leaf extract revealed alkaloids, phenolics, flavonoids, tannins, saponins, and triterpenoids as their bioactive compounds.[7,10-12]

Alkaloids are secondary metabolites that have antiparasitic effects.[13] Other natural ingredients that contain alkaloids, such as pulai leaves, cinnamon, chili pepper, and turmeric, have been shown to have an anti-Toxoplasma effect.[14-16] In vitro study of moringa seeds has also been carried out and has been shown to inhibit the replication of tachyzoites.[17] Therefore, this research was conducted to prove the potential of moringa leaf extract as the new anti-Toxoplasma drug against tachyzoites replication in *Toxoplasma gondii*.

MATERIALS AND METHODS

Ethics Statement

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.

Plant Materials and Extraction

Moringa leaves were purchased from and identified by the Technical Implementation Unit of the Herbal Laboratory, Materia Medica Batu, East Java, Indonesia

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Moringa leaves were washed, dried at 40ºC 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 40ºC until they became a dense mass.[18]

Phytochemical Analyses

The phytochemical analysis was performed to determine secondary metabolites (alkaloids, flavonoids, phenols, steroids, saponins, and tannins) present in the moringa leaf extract using the color and precipitate reaction methods. Alkaloids were determined using the Mayer test, and a cream-yellow precipitate confirmed the positive result. Flavonoids were determined by adding the sample with hydrochloric acid and magnesium powder, and red or pink color reaction changes indicated the test was positive. The extract was regarded as positive for phenols if a dark green color developed after adding a few drops of ferric chloride solution. Saponins were determined by shaking the sample with hydrochloric acid, and the positive result was confirmed by a foam forming on the sample. Tannin was determined by adding ferric chloride solution to the boiled extract suspension, and brownish-green color changes indicated the test was positive. Steroids were determined using the Lieberman-Burchad method, and blue color changes in the sample indicated the test as positive.[18]

Animals

Male Deutschland-Denken-Yoken (DDY) mice (20-30 grams, 2-3 months old) were purchased from a certified local experimental animal breeder. 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±4ºC, 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 RH strains of *Toxoplasma gondii* tachyzoites were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. The tachyzoite culture was performed in vivo on mice. They were maintained by routine intraperitoneal passage every 72 hours. The concentration of tachyzoites used for toxoplasmosis induction in this research was 10 x 10^4 tachyzoites/mL/mice. The number of tachyzoites was determined by counting the means with a counting chamber before being inoculated into the experimental mice.[19]

Toxoplasmosis Drug Reference

This research used spiramycin (500 mg, Spirasin®, SANBE, Bandung, Indonesia) 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 *Toxoplasma 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 orally as a placebo, positive control group (Group II) received spiramycin 100 mg/kg BW, treatment group 1 (Group III) received moringa leaf extract 250 mg/kg BW, treatment group 2 (Group IV) received moringa leaf extract 500 mg/kg BW, and treatment group 3 (Group V) received moringa leaf extract 1000 mg/kg BW. Mice in the infected group were injected with tachyzoites intraperitoneally on the first day. Moringa leaf extract and spiramycin were given orally once daily for three days on the second until fourth day. On the fifth day, all mice were sacrificed with cervical

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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 Neubauer counting chamber (0.1 mm depth, Assistent®, Germany) at 40x objective lenses 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 Analyses

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 1. Phytochemical analysis results of moringa leaf extract

Constituent	Result
Alkaloids	+
Flavonoids	+
Phenols	+
Steroids	+
Tannins	+
Saponins	-

+: Positive; -: Negative

The phytochemical analysis of moringa leaf extract revealed the absence of saponins and the presence of alkaloids, flavonoids, phenols, steroids, and tannins (Table 1). These results were not aligned with the previous research, which revealed that the moringa leaf extract contained saponins as its secondary metabolite compound.[7,10-12] ~~The first process to separate the bioactive compounds from the raw materials of natural products is called extraction. The extraction techniques play an essential role in the extraction output, two of which are the quantity and quality of bioactive compounds extracted.~~ 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]

Number of Tachyzoites

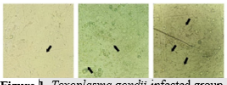

Figure 1. *Toxoplasma gondii*-infected group. Tachyzoites (black arrows) on intraperitoneal fluid from the infected group under a 10x40 light microscope.

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 1).

Table 2. Tachyzoites count results

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

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

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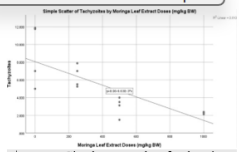

Figure 2. Simple scatter plot of tachyzoites count results (y-axis) by the moringa leaf extract doses (x-axis) ($P = 0.000$; $r = -0.781$).

The highest tachyzoites were in group I with a total of 8.91±3.45 x 10^4. There were three groups with a significantly lower number of tachyzoites compared to group I: group II with a total of 2.88±2.14 x 10^4, group IV with a total of 3.03±1.08 x 10^4, and group V with a total of 2.28±0.12 x 10^4. The number of tachyzoites in group III, with a total of 6.41±1.25 x 10^4, was not significantly different compared to group I (Table 2). Simple scatter plot showed a negative correlation between the extract doses and the number of tachyzoites (Figure 2).

The concentration of tachyzoites for toxoplasmosis peritoneally induction in this research was 10 x 10^4 tachyzoites/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 2). Spiramycin is an antibiotic and an antiparasitic macrolide agent that is considered the drug of choice against *Toxoplasma 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 *Toxoplasma gondii* cysts in brain tissues.[20]

The doses of moringa leaf extract used in this research were 250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW. The results shown in Table 2 revealed that moringa leaf 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 moringa leaf extract increased with increasing doses (Figure 2). These results are also align with other research on *Plasmodium yoelii*, in which the higher the dose of moringa leaf extract, the greater the inhibitory activity against the parasites.[25]

Alkaloids are one of the antiparasitic bioactive compounds in the moringa leaf extract. Their antiparasitic effect occurred through their covalent bonds with the deoxyribonucleic acid (DNA) of the parasite. These covalent bonds cause mutations, such as deletions or frame-shift mutations, which will disrupt the next round of parasite replication if the DNA repair enzymes are unable to restore these covalent bonds. 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 *Toxoplasma gondii* tachyzoites in vitro.[17]

This research proved the effectiveness of moringa leaf 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 *Toxoplasma gondii* target organs or

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CONCLUSIONS

The moringa leaf extract has an anti-Toxoplasma effect by inhibiting the tachyzoite stadium replication at 500 mg/kg BW and 1000 mg/kg BW. There was a negative correlation between the doses of moringa leaf extract and the number of tachyzoites.

ACKNOWLEDGEMENT

This research was supported by grants from the Institution of Research and Community Service of Widya Mandala Catholic University, Surabaya, Indonesia.

CONFLICT OF INTEREST

The authors conaffirm no conflict of interest in this research.

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Resubmit for review from another reviewer (13-02-2023)

IJTID UNAIR <ijtid@itd.unair.ac.id> Mon, Feb 13, 2023, 9:45 AM

to me

Dear Mrs. Laura Wihanto,

We have decided on your submission to the Indonesian Journal of Tropical and Infectious Disease, "*Moringa oleifera* Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication."

Our decision is: **Resubmit for review**

The revision is due on **February 21, 2023**

Reviewer's comments:
In general, the manuscript is good, but it needs some improvement in writing and content.
1. The writing of the species is repeated, only at the beginning it is written completely and then it is quite abbreviated. Examples of Toxoplasma gondii: T. gondii, Moringa oleifera: M. oleifera. Writing of genus and species, including foreign words (not B. English) in italics.
2. In the abstract, the infectious dose of Toxoplasma should be stated. Do not use difficult words repeatedly.
3. In Methods, it is necessary to include the multiplication of isolates in animals before infecting animals. It is necessary to convey the volume of Toxoplasma infection and administration of the extract in mL (not dose)
4. In the results and discussion, the picture is not clear. It is necessary to provide an enlarged image with good resolution. It needs discussion, in group IV there was an increase in the number of tachyzoites. This is very important to convey.
5. In the bibliography, writing can be adjusted according to the guidelines of the journal, which includes writing species and abbreviating journal names.
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Original Article

Moringa oleifera Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication

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 extract has been shown to have several anti-parasite activities, which could have an anti-Toxoplasma effect. To analyze the anti-Toxoplasma effect of moringa leaf 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 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 tachyzoites intraperitoneally on the first day. Moringa leaf 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. 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 a significant correlation between the extract doses and the number of tachyzoites ($P = 0.000$; $r = -0.781$). Moringa leaf extract has an anti-Toxoplasma effect at 500 mg/kg BW and 1000 mg/kg BW, and there was a negative correlation between the extract doses and the number of tachyzoites.

Keywords: *Toxoplasma gondii; tachyzoites; toxoplasmosis; Moringa oleifera; moringa leaf extract*

ABSTRAK

Berbagai jalan masuknya infeksi Toxoplasma gondii yang dekat dengan kehidupan sehari-hari sangat mendukung tingginya angka kejadian toksoplasmosis. Munculnya strain Toxoplasma gondii yang resisten terhadap pengobatan menimbulkan kekhawatiran di masa mendatang. Ekstrak daun kelor telah dibuktikan memiliki beberapa aktivitas anti parasit, salah satunya dapat berupa efek anti-Toxoplasma. Menganalisis efek anti-Toxoplasma pada ekstrak daun kelor dalam menghambat replikasi Toxoplasma gondii stadium takizoit dan korelasi antara dosis ekstrak daun kelor dengan jumlah takizoit. Mencit dibagi menjadi 5 kelompok. Kelompok kontrol negatif (grup I) diberi larutan CMC-Na. Kelompok kontrol positif (grup II) diberi spiramycin 100 mg/kgBB. Kelompok perlakuan masing-masing diberi ekstrak daun kelor 250 mg/kgBB (grup III), 500 mg/kgBB (grup IV), dan 1000 mg/kgBB (grup V). Induksi toksoplasmosis pada mencit dilakukan secara intraperitoneal pada hari ke-1. Ekstrak daun kelor dan spiramycin diberikan secara peroral selama 3 hari. Perhitungan takizoit pada sampel cairan intraperitoneal dilakukan pada hari ke-5. Terdapat perbedaan yang signifikan lebih rendah ($P < 0.05$) pada grup IV ($P = 0.021$) dan grup V ($P = 0.022$) dibandingkan dengan grup I. Terdapat korelasi yang signifikan antara dosis ekstrak daun kelor dengan jumlah takizoit ($P = 0.000$; $r = -0.781$) Ekstrak daun kelor memiliki efek anti-Toxoplasma pada dosis 500 mg/kgBB dan 1000 mg/kgBB, serta terdapat korelasi negatif antara dosis ekstrak daun kelor dengan jumlah takizoit.

Kata kunci: *Toxoplasma gondii; takizoit; toksoplasmosis; Moringa oleifera; ekstrak daun kelor*

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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 been reported to reach 43-88% of the total population in various regions of 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 *Toxoplasma 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 *Toxoplasma gondii* is ongoing. The emergence of drug-resistant *Toxoplasma 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. 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.[6-8] It also acts as a larvicidal.[9] A phytochemical screening test of moringa leaf extract revealed alkaloids, phenolics, flavonoids, tannins, saponins, and triterpenoids as their bioactive compounds.[7,10-12]

Alkaloids are secondary metabolites that have antiparasitic effects.[13] Other natural ingredients that contain alkaloids, such as pulai leaves, cinnamon, chili pepper, and turmeric, have been shown to have an anti-Toxoplasma effect.[14-16] In vitro study of moringa seeds has also been carried out and has been shown to inhibit the replication of tachyzoites.[17] Therefore, this research was conducted to prove the potential of moringa leaf extract as the new anti-Toxoplasma drug against tachyzoites replication in *Toxoplasma gondii*.

MATERIALS AND METHODS

Ethics Statement

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.

Plant Materials and Extraction

Moringa leaves were purchased from and identified by the Technical Implementation Unit of the Herbal Laboratory, Materia Medica Batu, East Java, Indonesia.

Moringa 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.[18]

Phytochemical Analyses

The phytochemical analysis was performed to determine secondary metabolites (alkaloids, flavonoids, phenols, steroids, saponins, and tannins) present in the moringa leaf extract using the color and precipitate reaction methods. Alkaloids were determined using the Mayer test, and a cream-yellow precipitate confirmed the positive result. Flavonoids were determined by adding the sample with hydrochloric acid and magnesium powder, and red or pink color reaction changes indicated the test was positive. The extract was regarded as positive for phenols if a dark green color developed after adding a few drops of ferric chloride solution. Saponins were determined by shaking the sample with hydrochloric acid, and the positive result was confirmed by a foam forming on the sample. Tannin was determined by adding ferric chloride solution to the boiled extract suspension, and brownish-green color changes indicated the test was positive. Steroids were determined using the Lieberman-Burchad method, and blue color changes in the sample indicated the test as positive.[18]

Animals

Male Deutschland-Denken-Yoken (DDY) mice (20-30 grams, 2-3 months old) were purchased from a certified local experimental animal breeder. 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 RH strains of *Toxoplasma gondii* tachyzoites were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. The tachyzoite culture was performed in vivo on mice. They were maintained by routine intraperitoneal passage every 72 hours. The concentration of tachyzoites used for toxoplasmosis induction in this research was 10 x 10^4 tachyzoites/mL/mice. The number of tachyzoites was determined by counting the means with a counting chamber before being inoculated into the experimental mice.[19]

Toxoplasmosis Drug Reference

This research used spiramycin (500 mg, Spirasin®, SANBE, Bandung, Indonesia) 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 *Toxoplasma 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 orally as a placebo, positive control group (Group II) received spiramycin 100 mg/kg BW, treatment group 1 (Group III) received moringa leaf extract 250 mg/kg BW, treatment group 2 (Group IV) received moringa leaf extract 500 mg/kg BW, and treatment group 3 (Group V) received moringa leaf extract 1000 mg/kg BW. Mice in the infected group were injected with tachyzoites intraperitoneally on the first day. Moringa leaf extract and spiramycin were given orally once daily for three days on the second until 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 Neubauer counting chamber (0.1 mm depth, Assistent®, Germany) at 40x objective lenses 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 Analyses

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 1. Phytochemical analysis results of moringa leaf extract

Constituent	Result
Alkaloids	+
Flavonoids	+
Phenols	+
Steroids	+
Tannins	+
Saponins	-

+: Positive; -: Negative

The phytochemical analysis of moringa leaf extract revealed the absence of saponins and the presence of alkaloids, flavonoids, phenols, steroids, and tannins (Table 1). These results were not aligned with the previous research, which revealed that the moringa leaf extract contained saponins as its secondary metabolite compound.[7,10-12] The first process to separate the bioactive compounds from the raw materials of natural products is called extraction. The extraction techniques play an essential role in the extraction output, two of which are the quantity and quality of bioactive compounds extracted. 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]

Number of Tachyzoites

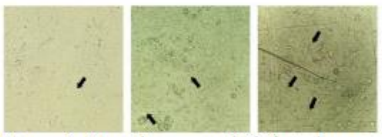

Figure 1. *Toxoplasma gondii*-infected group. Tachyzoites (black arrows) on intraperitoneal fluid from the infected group under a 10x40 light microscope.

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 1).

Table 2. Tachyzoites count results

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

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

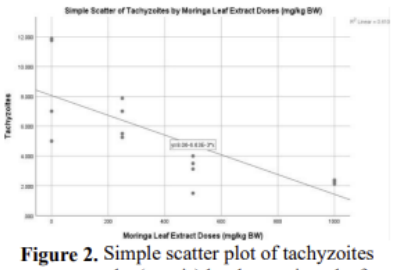

Figure 2. Simple scatter plot of tachyzoites count results (y-axis) by the moringa leaf extract doses (x-axis) ($P = 0.000$; $r = -0.781$).

The highest tachyzoites were in group I with a total of $8.91 \pm 3.45 \times 10^4$. There were three groups with a significantly lower number of tachyzoites compared to group I: group II with a total of $2.88 \pm 2.14 \times 10^4$, 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 (Table 2). Simple scatter plot showed a negative correlation between the extract doses and the number of tachyzoites (Figure 2).

The concentration of tachyzoites for toxoplasmosis peritoneally induction in this research was 10×10^4 tachyzoites/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 2). Spiramycin is an antibiotic and an antiparasitic macrolide agent that is considered the drug of choice against *Toxoplasma 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 *Toxoplasma gondii* cysts in brain tissues.[20] The doses of moringa leaf extract used in this research were 250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW. The results shown in Table 2 revealed that moringa leaf 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 moringa leaf extract increased with increasing doses (Figure 2). These results are also align with other research on *Plasmodium yoelii*, in which the higher the dose of moringa leaf extract, the greater the inhibitory activity against the parasites.[25]

Alkaloids are one of the antiparasitic bioactive compounds in the moringa leaf extract. Their antiparasitic effect occurred through their covalent bonds with the deoxyribonucleic acid (DNA) of the parasite. These covalent bonds cause mutations, such as deletions or frame-shift mutations, which will disrupt the next round of parasite replication if the DNA repair enzymes are unable to restore these covalent bonds. 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 *Toxoplasma gondii* tachyzoites in vitro.[17] This research proved the effectiveness of moringa leaf 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 *Toxoplasma gondii* target organs or

other variables of its pathway of antioxidant properties.

CONCLUSIONS

The moringa leaf extract has an anti-Toxoplasma effect by inhibiting the tachyzoite stadium replication at 500 mg/kg BW and 1000 mg/kg BW. There was a negative correlation between the doses of moringa leaf extract and the number of tachyzoites.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors affirm no conflict of interest in this research.

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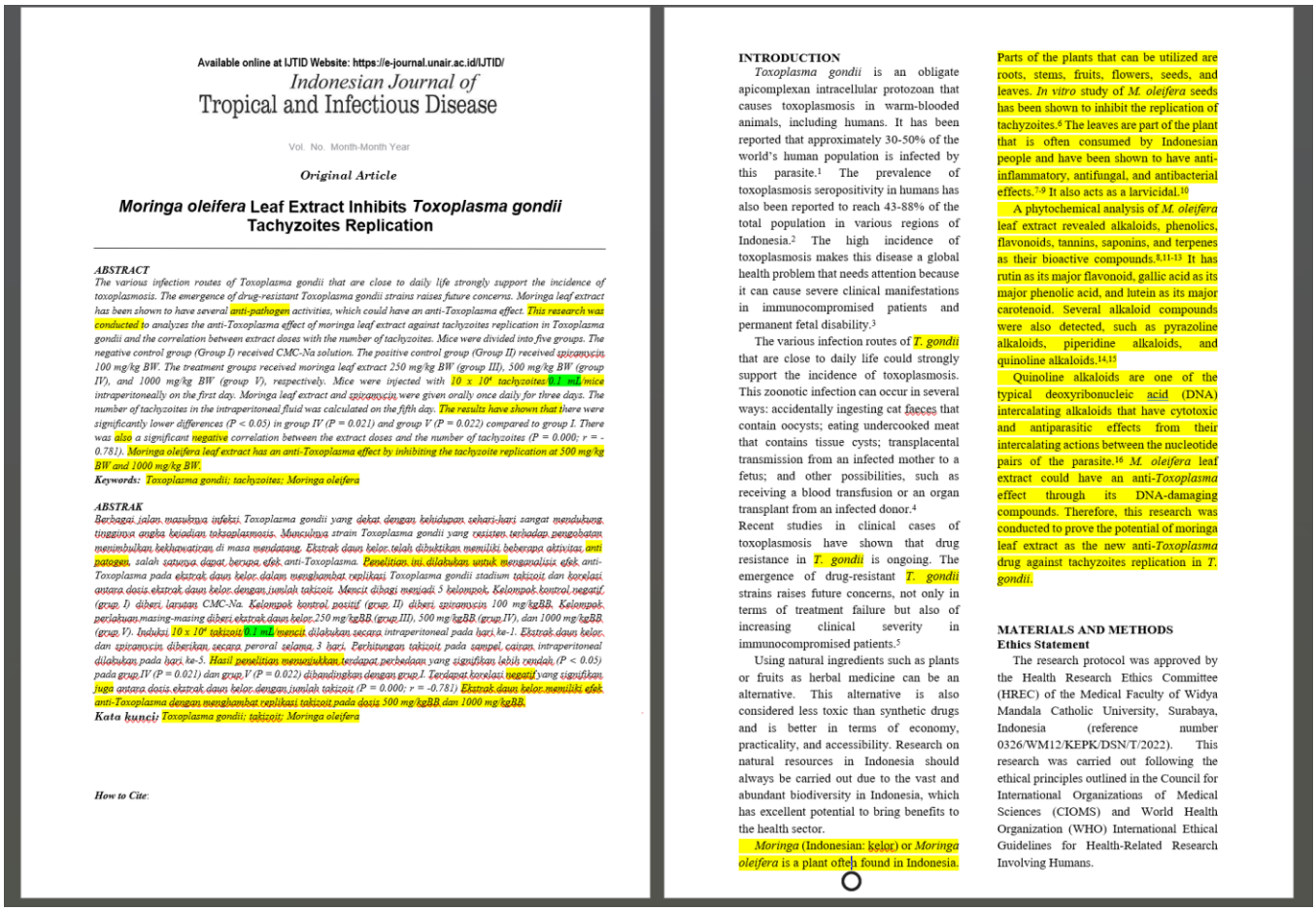

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Original Article

Moringa oleifera Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication

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 extract has been shown to have several anti-pathogen activities, which could have an anti-Toxoplasma effect. This research was conducted to analyzes the anti-Toxoplasma effect of moringa leaf 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 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 10 x 10⁴ tachyzoites 0.1 mL/mice intraperitoneally on the first day. Moringa leaf 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 extract has an anti-Toxoplasma effect by inhibiting the tachyzoite replication at 500 mg/kg BW and 1000 mg/kg BW.

Keywords: *Toxoplasma gondii; tachyzoites; Moringa oleifera*

ABSTRAK

Berbagai jalan masuknya infeksi Toxoplasma gondii yang dekat dengan kehidupan sehari-hari sangat mendukung tingginya angka kejadian toksoplasmosis. Munculnya strain Toxoplasma gondii yang resisten terhadap pengobatan menimbulkan kekhawatiran di masa mendatang. Ekstrak daun kelor telah dibuktikan memiliki beberapa aktivitas anti patogen, salah satunya dapat berupa efek anti-Toxoplasma. Penelitian ini dilakukan untuk menganalisis efek anti-Toxoplasma pada ekstrak daun kelor dalam menghambat replikasi Toxoplasma gondii stadium takizoit dan korelasi antara dosis ekstrak daun kelor dengan jumlah takizoit. Mencit dibagi menjadi 5 kelompok. Kelompok kontrol negatif (grup I) diberi larutan CMC-Na. Kelompok kontrol positif (grup II) diberi spiramycin 100 mg/kgBB. Kelompok perlakuan masing-masing diberi ekstrak daun kelor 250 mg/kgBB (grup III), 500 mg/kgBB (grup IV), dan 1000 mg/kgBB (grup V). Induksi 10 x 10⁴ takizoit 0.1 mL/mencit dilakukan secara intraperitoneal pada hari ke-1. Ekstrak daun kelor dan spiramycin diberikan secara peroral selama 3 hari. Perhitungan takizoit pada sampel cairan intraperitoneal dilakukan pada hari ke-5. Hasil penelitian menunjukkan terdapat perbedaan yang signifikan lebih rendah (P < 0.05) pada grup IV (P = 0.021) dan grup V (P = 0.022) dibandingkan dengan grup I. Terdapat korelasi negatif yang signifikan juga antara dosis ekstrak daun kelor dengan jumlah takizoit (P = 0.000; r = -0.781) Ekstrak daun kelor memiliki efek anti-Toxoplasma dengan menghambat replikasi takizoit pada dosis 500 mg/kgBB dan 1000 mg/kgBB.

Kata kunci: *Toxoplasma gondii; takizoit; Moringa oleifera*

How to Cite:

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 been reported to reach 43-88% of the total population in various regions of 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 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 extract could have an anti-*Toxoplasma* effect through its DNA-damaging compounds. Therefore, this research was conducted to prove the potential of moringa leaf extract as the new anti-*Toxoplasma* drug against tachyzoites replication in *T. gondii*.

MATERIALS AND METHODS

Ethics Statement

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.

Plant Materials and Extraction

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

Thin-Layer Chromatography Analysis

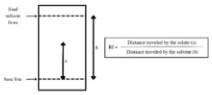


Figure 1. Retention factor (Rf) values calculation formula in thin-layer chromatography analysis.

The thin-layer chromatography (TLC) analysis was performed to isolate the specific compounds present in the *M. oleifera* leaf extract. The extract was applied on silica gel 60 F_{254} 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

Deutschland-Denken-Yoken (DDY) mice (male, 20-30 grams, 2-3 months old) were purchased from a certified local experimental animal breeder. 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 RH strains of *T. gondii* tachyzoites were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. 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 solution before being inoculated into the experimental mice.[19] The toxoplasmosis induction used in this research was 10 x 10^4 tachyzoites/0.1 mL/mice.

Toxoplasmosis Drug Reference

This research used spiramycin (500 mg, Spirasin®, SANBE, Bandung, Indonesia) 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 BW, group III received *M. oleifera* leaf extract 250 mg/kg BW, group IV received M. oleifera leaf extract 500 mg/kg BW, and group V received *M. oleifera* leaf extract 1000 mg/kg BW.

Mice in the infected group were injected with 10 x 10^4 tachyzoites/0.1 mL/mice intraperitoneally on the first day. *M. oleifera* leaf 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 Neubauer counting chamber (0.1 mm depth, Assistent®, Germany) at 10 x 40 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 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 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 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]

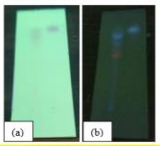


Figure 2. TLC results of alkaloids under UV light (a) 254 nm (b) 366 nm

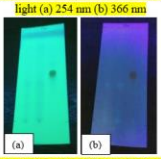


Figure 3. TLC results of flavonoids under UV light (a) 254 nm and (b) 366 nm

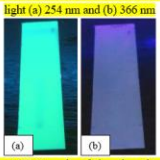


Figure 4. TLC results of phenols and tannins under UV light (a) 254 nm and (b) 366 nm

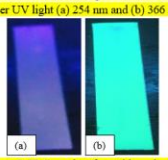


Figure 5. TLC results of steroids under UV light (a) 254 nm and (b) 366 nm

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

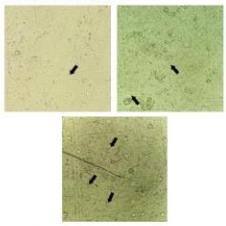


Figure 6. *T. gondii*-infected group. Tachyzoites (black arrows) on intraperitoneal fluid from the infected group under a light microscope with 10 x 40 magnification.

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±SD (x 10^4)
I	+	8.91±3.45
II	+	2.88±2.14*
III	+	6.41±1.25
IV	+	3.03±1.08*
V	+	2.28±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

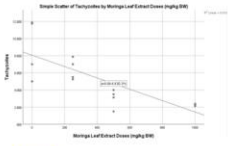


Figure 7. Simple scatter plot of tachyzoites count results (y-axis) by the *M. oleifera* leaf extract doses (x-axis). The number of tachyzoites tends to increase as the extract doses decrease, with $P = 0.000$ and $r = -0.781$.

The highest tachyzoites were in group I with a total of 8.91±3.45 x 10^4. Group II was significantly lower than group I, with a total of 2.88±2.14 x 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±1.08 x 10^4 and group V with a total of 2.28±0.12 x 10^4. The number of tachyzoites in group III, with a total of 6.41±1.25 x 10^4, was not significantly different compared to group I (Table 4).

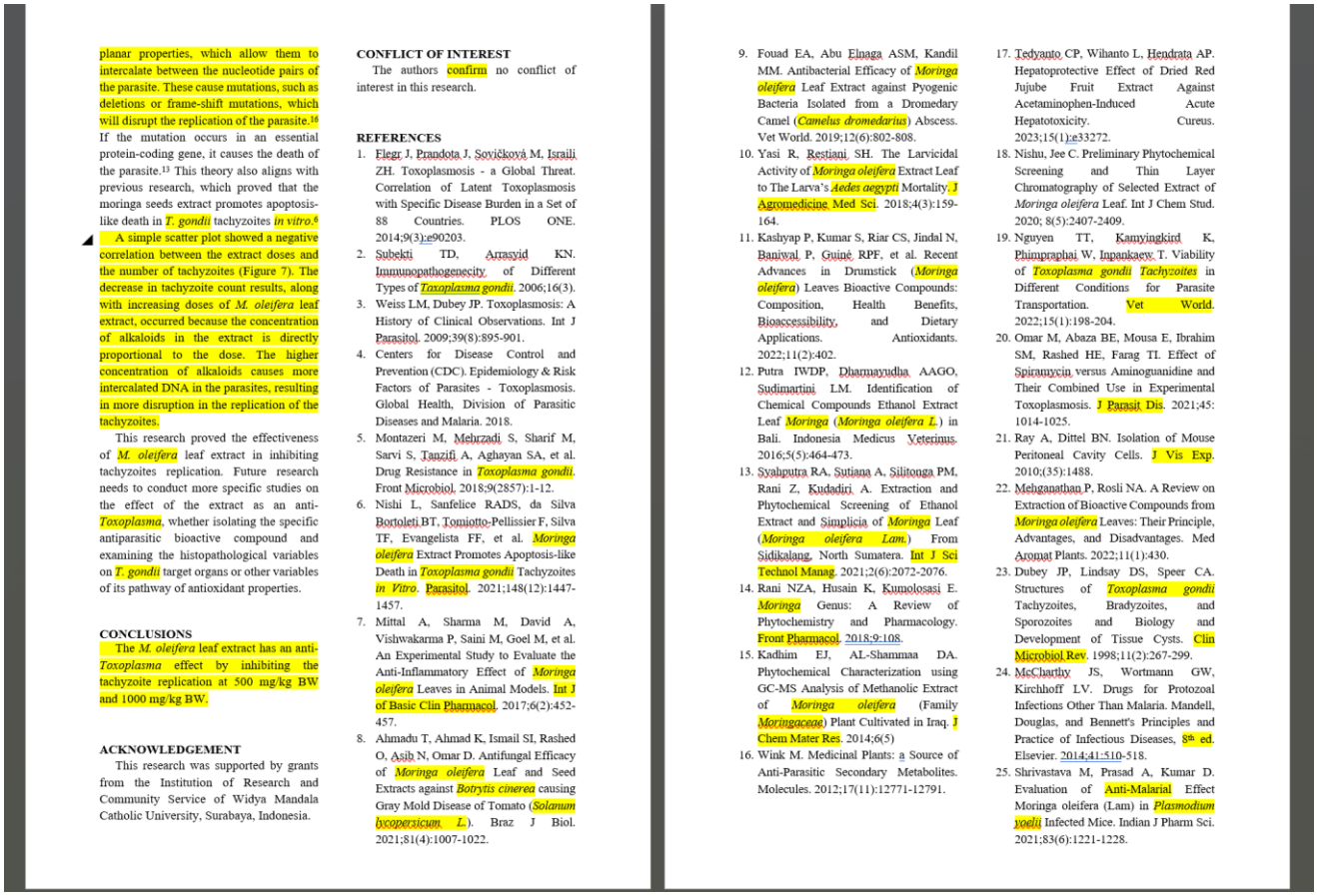
The toxoplasmosis intraperitoneally induction used in this research was 10 x 10^4 tachyzoites/0.1 mL/mice. Tachyzoites can invade almost all the host nucleated cells and replicate rapidly.[3] The cell will eventually suffer damage and rupture due to this state, and the released tachyzoites will continue 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 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 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 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 extract, the greater the inhibitory activity against the parasites.[25]

Quinoline alkaloids (Quinoline, 3-methyl) are typical DNA intercalating compounds found in the *M. oleifera* leaf 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 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 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.

CONCLUSIONS

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

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors confirm no conflict of interest in this research.

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Second revision: Minor revisions (16-03-2023)

[IJTID] Editor Decision External Inbox ×

IJTID **IJTID UNAIR** <ijtid@itd.unair.ac.id> Thu, Mar 16, 2023, 3:27 PM

to me

Dear Mrs. Laura Wihanto,

We have decided on your submission to the Indonesian Journal of Tropical and Infectious Disease, "*Moringa oleifera* Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication."

Our decision is: **Revision required**

The revision is due on **March 23, 2023**

Reviewer's comments (Round 2):

1. There are a few small notes about writing doses and image magnification.
2. Correction of concern on the image of tachyzoites in the intraperitoneal fluid.
3. What do you mean by three pictures? Is it from some treatment? If it represents the treatment, provide a description. However, if you only want to show the presence of tachyzoites, just one image is sufficient, with a size large enough for the reader to see.

Note:

Please add a highlight to your revision

EDITOR IJTID

Indonesian Journal of Tropical and Infectious Disease, Institute Tropical Disease - Universitas Airlangga

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Indonesian Journal of
Tropical and Infectious Disease

Vol. No. Month-Month Year

Original Article

Moringa oleifera Leaf Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication

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 extract has been shown to have several anti-pathogen activities, which could have an anti-Toxoplasma effect. This research was conducted to analyzes the anti-Toxoplasma effect of moringa leaf 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 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 10 x 10^4 tachyzoites/0.1 mL/mice intraperitoneally on the first day. Moringa leaf 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 extract has an anti-Toxoplasma effect by inhibiting the tachyzoite replication at 500 mg/kg BW and 1000 mg/kg BW.

Keywords: *Toxoplasma gondii; tachyzoites; Moringa oleifera*

ABSTRAK

Berbagai jalan masuknya infeksi Toxoplasma gondii yang dekat dengan kehidupan sehari-hari sangat mendukung tingginya angka kejadian toksoplasmosis. Munculnya strain Toxoplasma gondii yang resisten terhadap pengobatan menimbulkan kekhawatiran di masa mendatang. Ekstrak daun kelor telah dibuktikan memiliki beberapa aktivitas anti patogen, salah satunya dapat berupa efek anti-Toxoplasma. Penelitian ini dilakukan untuk menganalisis efek anti-Toxoplasma pada ekstrak daun kelor dalam menghambat replikasi Toxoplasma gondii stadium takizoit dan korelasi antara dosis ekstrak daun kelor dengan jumlah takizoit. Mencit dibagi menjadi 5 kelompok. Kelompok kontrol negatif (grup I) diberi larutan CMC-Na. Kelompok kontrol positif (grup II) diberi spiramycin 100 mg/kgBB. Kelompok perlakuan masing-masing diberi ekstrak daun kelor 250 mg/kgBB (grup III), 500 mg/kgBB (grup IV), dan 1000 mg/kgBB (grup V). Induksi 10 x 10^4 takizoit/0.1 mL/mencit dilakukan secara intraperitoneal pada hari ke-1. Ekstrak daun kelor dan spiramycin diberikan secara peroral selama 3 hari. Perhitungan takizoit pada sampel cairan intraperitoneal dilakukan pada hari ke-5. Hasil penelitian menunjukkan terdapat perbedaan yang signifikan lebih rendah ($P < 0.05$) pada grup IV ($P = 0.021$) dan grup V ($P = 0.022$) dibandingkan dengan grup I. Terdapat korelasi negatif yang signifikan juga antara dosis ekstrak daun kelor dengan jumlah takizoit ($P = 0.000$; $r = -0.781$) Ekstrak daun kelor memiliki efek anti-Toxoplasma dengan menghambat replikasi takizoit pada dosis 500 mg/kgBB dan 1000 mg/kgBB.

Kata kunci: *Toxoplasma gondii; takizoit; Moringa oleifera*

How to Cite:

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 been reported to reach 43-88% of the total population in various regions of 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 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 extract could have an anti-*Toxoplasma* effect through its DNA-damaging compounds. Therefore, this research was conducted to prove the potential of moringa leaf extract as the new anti-*Toxoplasma* drug against tachyzoites replication in *T. gondii*.

MATERIALS AND METHODS

Ethics Statement

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.

Plant Materials and Extraction

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

Thin-Layer Chromatography Analysis

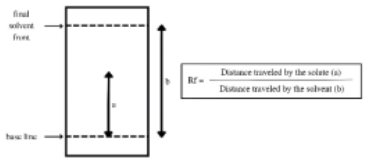

Figure 1. Retention factor (Rf) values calculation formula in thin-layer chromatography analysis.

The thin-layer chromatography (TLC) analysis was performed to isolate the specific compounds present in the *M. oleifera* leaf extract. The extract was applied on silica gel 60 F_{254} 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

Deutschland-Denken-Yoken (DDY) mice (male, 20-30 grams, 2-3 months old) were purchased from a certified local experimental animal breeder. 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 RH strains of *T. gondii* tachyzoites were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. 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 solution before being inoculated into the experimental mice.[19] The toxoplasmosis induction used in this research was 10 x 10^4 tachyzoites/0.1 mL/mice. 1 x 10^5

Toxoplasmosis Drug Reference

This research used spiramycin (500 mg, Spirasin®, SANBE, Bandung, Indonesia) 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 BW, group III received *M. oleifera* leaf extract 250 mg/kg BW, group IV received M. oleifera leaf extract 500 mg/kg BW, and group V received *M. oleifera* leaf extract 1000 mg/kg BW.

Mice in the infected group were injected with 10 x 10^4 tachyzoites/0.1 mL/mice intraperitoneally on the first day. *M. oleifera* leaf 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 Neubauer counting chamber (0.1 mm depth, Assistent®, Germany) at 10 x 40 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.

400x

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 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 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 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]

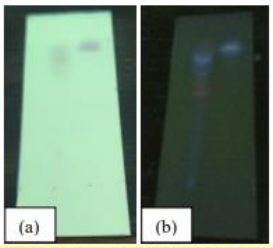

Figure 2. TLC results of alkaloids under UV light (a) 254 nm (b) 366 nm

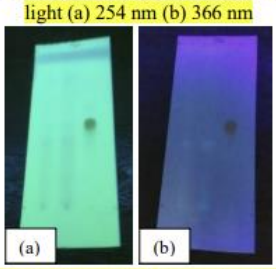

Figure 3. TLC results of flavonoids under UV light (a) 254 nm and (b) 366 nm

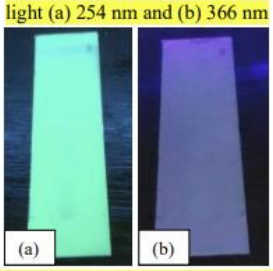

Figure 4. TLC results of phenols and tannins under UV light (a) 254 nm and (b) 366 nm

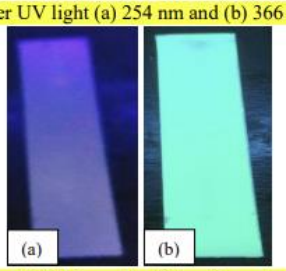

Figure 5. TLC results of steroids under UV light (a) 254 nm and (b) 366 nm

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

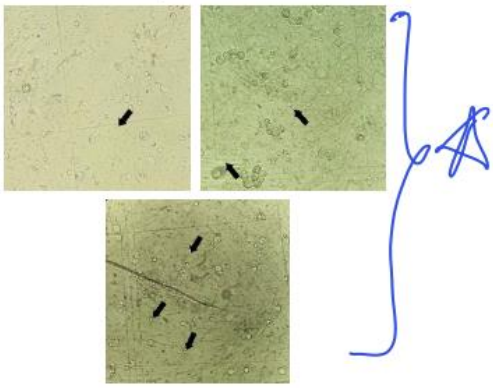

Figure 6. *T. gondii*-infected group. Tachyzoites (black arrows) on intraperitoneal fluid from the infected group under a light microscope with 10 x 40 magnification.

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±SD (x 10^4)
I	+	8.91±3.45
II	+	2.88±2.14*
III	+	6.41±1.25
IV	+	3.03±1.08*
V	+	2.28±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

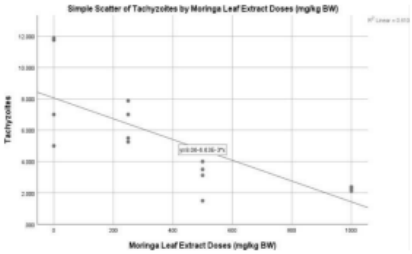

Figure 7. Simple scatter plot of tachyzoites count results (y-axis) by the *M. oleifera* leaf extract doses (x-axis). The number of tachyzoites tends to increase as the extract doses decrease, with $P = 0.000$ and $r = -0.781$.

The highest tachyzoites were in group I with a total of 8.91±3.45 x 10^4. Group II was significantly lower than group I, with a total of 2.88±2.14 x 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±1.08 x 10^4 and group V with a total of 2.28±0.12 x 10^4. The number of tachyzoites in group III, with a total of 6.41±1.25 x 10^4, was not significantly different compared to group I (Table 4).

The toxoplasmosis intraperitoneally induction used in this research was 10 x 10^4 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 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 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 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 extract, the greater the inhibitory activity against the parasites.[25]

Quinoline alkaloids (Quinoline, 3-methyl) are typical DNA intercalating compounds found in the *M. oleifera* leaf 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 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 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.

CONCLUSIONS

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

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors confirm no conflict of interest in this research.

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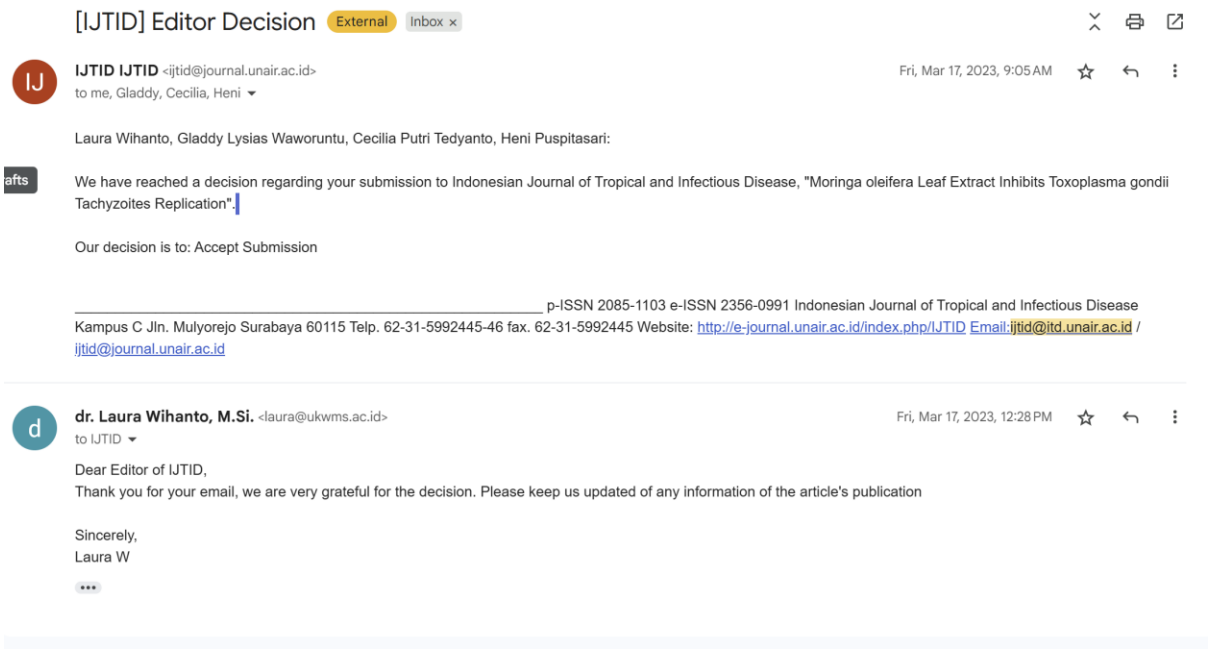

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We have reached a decision regarding your submission to Indonesian Journal of Tropical and Infectious Disease, "Moringa oleifera Leaf Extract Inhibits Toxoplasma gondii Tachyzoites Replication".

Our decision is to: Accept Submission

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No : 28/IJTID-3/III/2023 March 17th, 2023
Regard : Acceptance Letter

Dear
Laura Wihanto

We are pleased to inform you that the following paper has been officially accepted for publication:

Manuscript ID	:	IJTID #42672
Type of manuscript	:	Original Article
Authors	:	Laura Wihanto, Gladdy Lysias Waworuntu, Cecilia Putri Tedyanto, Heni Puspitasari
Received	:	January 18th, 2023
E-mail	:	laura@ukwms.ac.id

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Your manuscript titled ***Moringa oleifera*** **Leaf Ethanol Extract Inhibits** ***Toxoplasma gondii*** **Tachyzoites Replication** has been accepted and will be finalized for the coming issue Vol. 11 No. 1 2023. However, before sending the manuscript to the press, I will send you the press-ready copy (galley format) for your final checking.

We will now make the final preparations for publication, then return the manuscript to you for your approval.

Sincerely,
Editor in Chief

Dr. Prihartini Widiyanti, drg., M.Kes., S.Bio

Paper published (04-09-2023)

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Dr. Prihartini Widiyanti, drg., S.Bio., M.Kes

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