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Research Article

Mechanism of anti-toxoplasmosis ethanol extract of mangosteen peel (*Garcinia mangostana* Linn) on the expression of IFNγ, interleukin 12, matrix metallopeptidase 9, and histopathological of mice kidney

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ABSTRACT

Aim: Toxoplasmosis is a dangerous infectious disease caused by *Toxoplasma gondii* in the blood or tissue. This disease is zoonotic which is source of transmission from animals to humans. Therapy herbal medicine for toxoplasmosis can be determined by looking at IFNγ, interleukin 12 (IL-12), and matrix metallopeptidase 9 (MMP-9) expression, and seeing the level of organ damage. This study is to prove the mechanism of anti-toxoplasmosis of mangosteen peel extract against *T. gondii*. **Materials and Methods:** The study was divided into two stages: Stage I: Determination of the effective dose 50% of mangosteen peel extract against *T. gondii* and Stage II: Mechanism of anti-toxoplasmosis of mangosteen peel extract against *T. gondii*. This study used 50 mice divided into five groups: Healthy control group (P0) given 0.5% CMC-Na, P1 group without treatment, P2 group given Cotrimoxazole 60 mg/kgB, and P3 and P4 groups given mangosteen peel extract 200 and 400 mg/kgBW (orally twice a day). **Results:** After injection of *T. gondii* 10^2 intraperitoneally and given treatment for 5 days then blood samples taken for measurement of blood urea nitrogen (BUN) and creatinine; kidney organ samples to determine organ damage, IFNγ, IL-12, and MMP-9 expression. The effective dose 50% (ED 50%) of Mangosteen peel extract was at a dose of 60 mg/kgBW. Mangosteen peel extract 200 mg/kgBW (P3) and 400 mg/kgBB (P4) with significance ($P > 0.05$) can reduce the number of parasites, levels of BUN and creatinine, reduce kidney damage, increases IFNγ and IL-12, and decreases MMP-9 expression. Mangosteen peel extract 200 mg/kgBW (P3) was proven as anti-toxoplasmosis in mice. **Conclusion:** This research is: ED 50% at a dose of 60 mg/kgBW. Mangosteen peel extract 200 mg/kgBW (P3) and 400 mg/kgBB (P4) with significance ($P > 0.05$) can reduce the number of parasites, levels of BUN and creatinine, reduce kidney damage, increases IFNγ and IL-12, and decreases MMP-9 expression.

KEY WORDS: Anti-toxoplasmosis, Ethanol extract of mangosteen peel, *Toxoplasma gondii*

INTRODUCTION

Toxoplasmosis is a dangerous infectious disease caused by *Toxoplasma gondii* which is in the blood or tissue. This zoonotic disease transmitted to humans. Toxoplasmosis cases in Indonesia were reported in range of 50–88% for all provinces except in NTB which reported only 28.95% in 2003.[1] The prevalence of toxoplasmosis in animals showed domestic poultry 30%, cats 35–73%, dogs 75%, pigs 11–36%, goats 11–61%, sheep 66%, and horses 44%.[2,3]

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Mangosteen fruit peel is one of the herbal remedies that contain xanthone, flavonoids, and phenols. Xanthone in mangosteen peel containing α-mangosteen and α-mangosteen is known to have aromatic group complexes with several clusters to inhibit the process of reduction and oxidation reactions in microbial or parasitic metabolism.[4] Previous research has shown that the mangosteen peel extract (*Garcinia mangostana* Linn) has the potential as an antioxidant, anti-inflammatory, anticancer, anti-infectious microorganism (as an antibacterial and antiparasitic), and enhances the cellular and humoral immune system. Xanthone, phenol, and flavonoids in mangosteen peel extract have the ability to work as an antioxidant oxidants, which capable of reducing damage in the

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host's body due to free radicals or radicals reactive.[5] This research strategy of the *T. gondii* parasite murder plan with the herbal medicine compound of the ethanol extract of mangosteen peel regulated dosage and the time interval of such giving has not been done by any researcher.

MATERIALS AND METHODS

Materials

Drugs used are mangosteen peel take from Boyolali district in the Central Java. Mangosteen plant is identified in detail at LIPI Biology Research Center Bogor-Indonesia. The standardized extract using α-mangosteen standard from Sigma-Aldrich Co. The virulence isolate parasite of T. gondii is RH strain. The research was divided in two stages: Stage I, determination of the effective dose 50% of mangosteen peel extract against *T. gondii* was used 60 male mice and Stage II mechanism of anti-toxoplasmosis of mangosteen peel extract against *T. gondii* used was 60 male mice. This study used adult male mice which has body weight 20–30 g and had obtained approval from Animal Clearance Committee from Faculty of Veterinary Medicine, Airlangga University with a certificate of ethical eligibility with number: 2.KE.005.01.2018. The other materials of examination were the sample of blood from cardiac vein and the peritoneal fluid from peritoneal cavity mice which was collected after the administration of mangosteen peel extract in day 5, cotton, alcohol, ether solution, 10% formalin buffer solution, ethanol and xylol solution, Immunohistochemical staining and HE staining. 1 ml and 3 ml syringe, venoject tube, thoma board, evaporator, electron microscope, thin-layer chromatography (TLC), densitometer, cover and object glass, surgical equipment for necropsy and organ harvesting, equipment for white rat enclosures that have been filled with sawdust for bedding, feeding, eating, and drinking mice.

Methods

Preparation and standardization of mangosteen peel extract

A total of 8000 g of mangosteen leather powder, then put into extraction flask and soaked with absolute ethanol solvent with a high ratio of mangosteen peel powder and 1:3 ethanol solvent. This solution should be shaken as often as possible, and after 48 h the solution is filtered. The ethanol extract of mangosteen peel was then evaporated until the extract was obtained in black, dry, and thick with a vacuum rotary evaporator at 40°C until.[5] The roots, stems, leaves, and the flesh of mangosteen peel sent to LIPI Bogor to determine exactly which species of mangosteen peel will be used in the study. However, standardization of mangosteen peel extract is done qualitatively and quantitatively to identify chemical compounds: Alkaloids, flavonoids, phenol (Tannin) triterpenoids, and others with TLC using the stationary phase: Kiesel gel GF 254, mobile phase: Chloroform:ethyl acetate:format acid (0.5; 9:0.5) and the appearance of stains or reagent of $FeCl_2$.[6,7]

Treatment of laboratory animals

Research on mechanism of antitoxoplasmosis drug is used 50 male mice which are divided into five treatment groups with each treatment group as much as 10 mice consisted of: The first group (P0): As a healthy control, group P1: As a negative control, a group of mice infected *T. gondii* and only given CMC-Na 0.5%. P2: As a positive control, a group of mice infected *T. gondii* dose 10^2 and given Cotrimoxazole 60 mg/kgBW twice a day orally. P3: Groups of mice infected with *T. gondii* dose 10^2 and given a mangosteen peel extract 200 mg/kgBW given twice a day orally. P4: Group of mice infected with *T. gondii* dose 10^2 was then given a mangosteen peel extract 400 mg/kgBW given twice a day orally. Male mice in this treatment group were treated on day 1 after infection. Treatment was done for 5 days then performed some working procedure that is: Taking the sample of mice blood from the venous cardiac of 1–2 ml and then inserted into the venoject tube without ethylenediaminetetraacetic acid anticoagulants performed to determine levels of blood urea nitrogen (BUN) and creatinine; furthermore, to determine the extent of kidney damage due to parasite infection of *T. gondii*.

Histopathological analysis

The kidney for the histopathologic preparations HE staining on the 6th-day post infection. Preparation of histopathology by indirect immunohistochemical technique begins by cutting the kidney organ preparations in a glass object, which is then immersed in xylol twice and stratified alcohols. To remove residual xylol and alcohols containing the remaining tissues of kidney organs then the object glass is washed with Aquadest. The object glass containing kidney tissue was then washed with phosphate-buffered saline (PBS) pH 7.4 3 times, each for 5 min, then immersed with 3% hydrogen peroxidase (H_2O_2) for 5–10 min. The next process is to soak a glass object containing kidney tissue with 1% bovine serum albumin in PBS for 10–30 min and left at room temperature. Then enter the next stage of the coating with the primary antibodies IFNγ, Interleukin 12 (IL 12), and matrix metallopeptidase 9 (MMP-9) by soaking glass objects that contain kidney tissue with primary antibodies IFNγ, IL 12, and MMP-9 for 1 h at room temperature. Furthermore, to remove the remaining primary antibodies is done by adding PBS pH 7.4 and silencing for 3 × 5 min. The next step adds antibody Goat anti-mouse immunoglobulin G Biotin labeled Strep Avidin-Horseradish Peroxidase for 30 min at

room temperature and PBS pH 7.4 for 3 × 5 min. For the antigen and primary antibodies to be perfectly bonded, Cromogen DAB (3,3-diaminobenzidine tetrahydrochloride) is added for 10–20 min. The rest of the cromogene was cleaned by immersing in Aquadest for 3 × 5 min, then added with the commuted blue counterstain to renal cortex cells expressing IFNγ, IL 12, and MMP-9 and those not expressing IFNγ, IL 12, and MMP-9 can be seen contrast.

Interpretation of immunohistochemistry

The Examination expression of IFNγ, IL 12, and MMP-9 was conducted with Remmele Immunoreactive Score (IRS) scale index. The Remmele immunoreactive score (IRS) scale index is the result of multiplication of immunoreactive cell percentage score with color intensity score on the immunoreactive cell. The data of each sample are the average IRS value observed at five fields of view at ×100 and ×400 magnification. All examinations of histopathologic preparations of kidney organ with this immunohistochemical staining using ordinary light microscope Nikon 500L brand equipped with digital camera brand DS Fi2 300 megapixel and image processing software Nikkon Image System.[8]

Statistical Analysis

The comparison test was carried out using the unpaired *t*-test formula if the distribution was normal, and the non-parametric test using the Mann–Whitney U-test if the data distribution was not normal ($P < 0.05$).

RESULTS

The results showed that the mangosteen peel was derived from the species *G. mangostana Linn* from the *Clusiaceae* tribe with a chemical compound consisting of xanthan = 3.86%, polyphenols = 28.11%, tannins=11.32%,saponins=4.91%,α-mangostin=2.94%, β-mangostin = 0.35%, and γ-mangostin = 0.31%. The effectiveness of the dose (ED 50%) of the mangosteen peel ethanol extract was obtained at a dose of 60 mg/kgBB. Mangosteen peel extract dose of 200 mg/kgBB (P3) and a dose of 400 mg/kgBB (P4) was proven to reduce the number of *T. gondii* parasites in mice peritoneal fluid, reduce levels of BUN, and creatinine in the blood of mice, and reduce renal organ damage (tubular necrosis, interstitial inflammation, and hemorrhage), also proven to increase IFNγ and IL-12 and decrease MMP-9 expression. Different test results of the number of parasites in the peritoneal fluid of mice can be shown in Tables 1 and 2.

Table 1: The number of parasites in peritoneal fluid mice (takizoit/ml)×10⁴

Group	Sample size (*n*)	The number of parasites (X±SD)	Median	Minimum-maximum	Kruskal–Wallis (*P*)
P0	5	0.00±0.00[a]	0	0–0	
P1	5	350040±81894.62[d]	332.400	232800–432000	
P2	5	165000±24295.68[cd]	163.200	134400–192000	0.008*
P3	5	128660±17109.88[c]	123.100	102200–144000	
P4	5	50320±12919.44[b]	56.400	40800–72000	

Description: Different Superscript shows significant differences (with Mann–Whitney U-test and Independence *t* test)

Table 2: The result of average BUN and creatinine

Group treatment	Average±standard deviation (X±SD) BUN	Average±standard deviation (X±SD) creatinine
P0	29.40±1.673[a]	0.076±0.023[a]
P1	49.20±5.891[c]	0.238±0.030[d]
P2	33.20±3.271[ab]	0.144±0.034[c]
P3	32.80±2.168[ab]	0.080±0.012[ab]
P4	35.40±4.159[b]	0.110±0.010[b]

Description: Different Superscript shows significant differences, BUN: Blood urea nitrogen

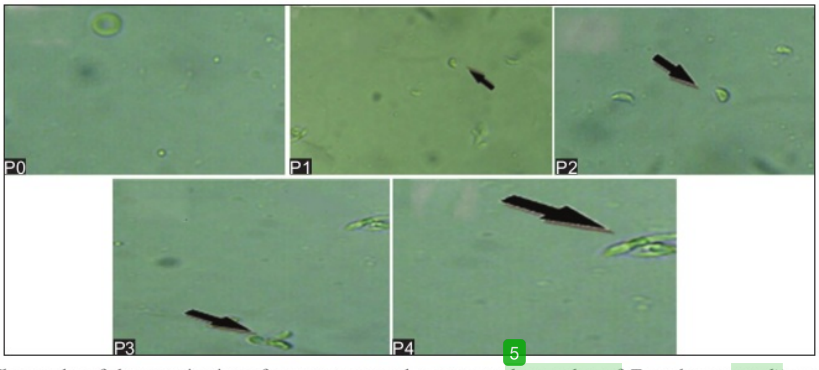

Figure 1: The results of the examination of mangosteen peel extract on the number of *Toxoplasma gondii* parasites in the peritoneal fluid of mice. The *T. gondii* parasite is indicated by a black arrow (×400 magnification)

Examination of *T. gondii* parasite in peritoneal fluid of mice with ×400 magnification microscope can be shown in Figures 1 and 2.

Different tubular necrosis test results, interstitial inflammation, and hemorrhage between groups can be shown in Table 3.

Microscopic observation results of the expression of sitokin IFNγ and IL-12, MMP-9 in the cell korteks in the kidney using immunohistochemical staining can be seen in Table 4 and Figures 3-6.

DISCUSSION

Examination of urea or BUN and creatinine levels was performed to determine the effect of giving mangosteen peel extract to the treatment group, whether there was an increase in urea and creatinine levels. Elevated levels of BUN and creatinine are among the best indicators for identifying kidney damage.[9] The BUN examination performed using this method is based on a complex colored reaction, due to the presence of urease enzymes that hydrolyze urea into ammonium and CO_2 ion. Then, the ammonium ion reacts with chloride and salicylate to form a blue-green complex. The color complex formed is proportional to the urea content in the sample read at 578 nm wavelength. Measurement of BUN can provide an idea of the state of kidney function, but BUN can also be affected by a condition not related to the kidney, one of which is due to an increase or decrease in protein intake in food. This happens because the liver turns ammonia into urea, so the more protein metabolized, the more urea is formed. Thus, an increase in BUN levels in each group does not imply any damage to kidney function. This is evidenced by creatinine levels that are still within the normal range. This increase occurs only due to the high protein catabolism, resulting in high levels of BUN as well.[9,10]

The method of the examination of creatinine levels is based on the reaction of the formation of orange-red complexes between the alkaline solution with picric

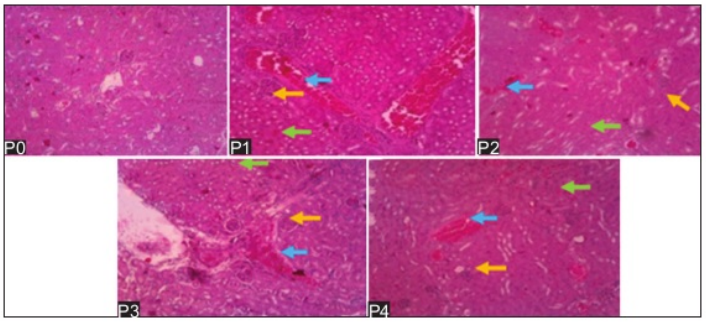

Figure 2: The results of renal histopathology examination with HE staining in the healthy control group (P0) are still normal, and no changes have been found in the cells around the kidney glomerulus. In those infected without drug (P1), Cotrimoxazole (P2) infected and treated groups or groups that were infected and treated with mangosteen peel extract (P3 and P4) in the glomerulus of the kidney were seen necrosis (yellow arrows), hemorraghe (green arrows), and congesti (blue arrows)

Table 3: Different tubular necrosis, interstitial inflammatory, and hemorrhagic test between group

Group treatment	Average±standard deviation (X±SD) tubular necrosis	Average±standard deviation (X±SD) interstitial inflammatory	Average±standard deviation (X±SD) haemorhagi
P0	0.04±0.089^{a}	0.00±0.00^{a}	0.00±0.00^{a}
P1	2.48±0.179^{b}	1.04±0.385^{b}	1.04±0.385^{b}
P2	1.52±0.593^{bc}	0.92±0.415^{b}	0.92±0.415^{b}
P3	1.12±0.438^{c}	0.72±0.228^{b}	0.72±0.228^{b}
P4	0.52±0.179^{c}	0.48±0.228^{b}	0.48±0.228^{b}

Description: Different superscript shows significant

Table 4: Different test of IFNγ, IL-12, and MMP-9 between groups in the kidney organs

Group treatment	Average±standard deviation (X±SD) IFNγ	Average±standard deviation (X±SD) IL-12	Average±standard deviation (X±SD) MMP-9
P0	0.76±0.555^{a}	0.80±0.469^{a}	1.40±1.068^{a}
P1	3.88±0.944^{b}	5.52±1.792^{b}	6.20±1.643^{c}
P2	4.36±0.740^{b}	7.52±0.986^{c}	4.96±0.537^{bc}
P3	7.04±0.727^{c}	7.44±1.664^{c}	4.80±1.530^{bc}
P4	7.28±1.591^{c}	8.24±1.367^{c}	3.88±0.268^{b}

Description: Different superscript shows significant differences, IL-12: Interleukin 12, MMP-9: Matrix metallopeptidase 9

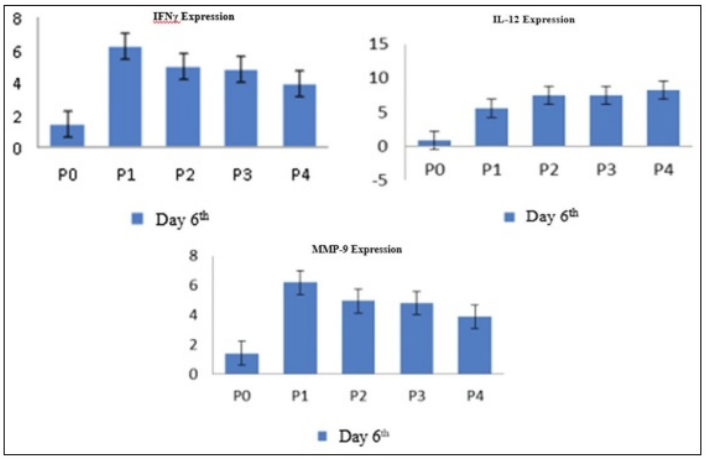

Figure 3: The mangosteen peel extract proven to increase IFNγ and interleukin 12 and decrease matrix metallopeptidase 9 expression

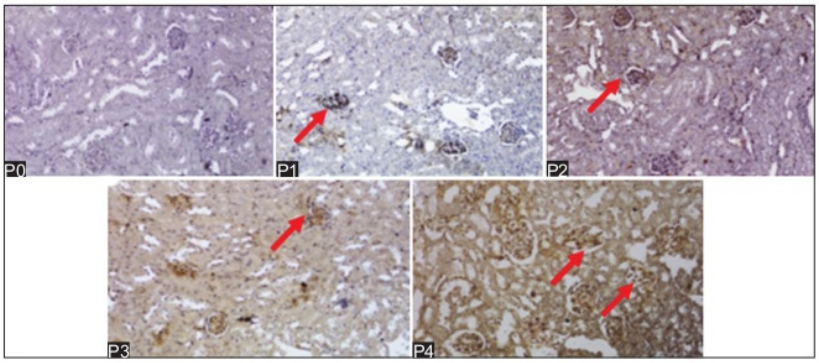

Figure 4: Examination results of mangosteen peel extract on IFNγ. There is no healthy control group (P0) found in dark brown spots on the kidney glomerulus cortex cells, the untreated (P1), Cotrimoxazole infected group (P2) or mangosteen peel extract (P3 and P4), in kidney cortex cells are found in brown spots. Brown spots around the renal glomerular cortex cells show IFNγ cytokine expression (red arrows) (100x magnification microscope)

acid. The absorbance of this complex is proportional to the creatinine concentration in the sample. Creatinine is a muscle degradation product that indicates a renal dysfunction if the levels exceed the normal limits. Serum creatinine is a strong indicator of kidney function and its concentration is relatively constant from day to day.[9]

As a result of *T. gondii* infection through the parasitic circulation of *T. gondii* spreads throughout the kidney, which causes damage to various types of nucleated cells in the kidney organs. Acute kidney damage is often characterized by the discovery of polymorphonuclear cell (eosinophil) infiltration and mononuclear cell infiltration (leukocytes).[11] In acute *T. gondii* infection is often marked by very fast takizoit replication and can be found almost in all the tissues of kidney organs.[10] However, the infection of acute *T. gondii* stadium takizoit rarely or not found in the epithelium tubules or renal glomerulus, only infiltration of polymorphonuclear cells (eosinophil) and mononuclear cell infiltration (leukocytes), which later developed into necrosis and congestion in the kidney.[3,11,12]

Cytokines TNFa, IL-12, IL-10, and IFNg are cytokines that result from acute or chronic inflammatory inflammation of kidney tissue due to parasite infection *T. gondii* phase Takizoit with a dose of infection 10^2 that enter into the body of mice.[13] If the expression activity of IFNγ and IL-12 cytokines is increased then the multiplication and replication of *T. gondii* parasites can also be inhibited. Thus, the expression activity of IFNγ and IL-12 cytokines is the main target of drugs derived from the herbaceous plant of mangosteen peel extract used as anti-toxoplasmosis.[14] Active chemical compounds in mangosteen peel extract can reduce the negative impact of free radicals that cause inflammatory processes and tissue damage of kidney organ tissues due to the parasite infection of *T. gondii*.[15] Xanthone

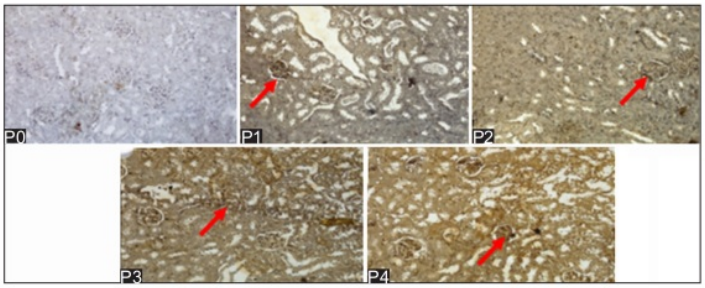

Figure 5: Results of the examination of mangosteen peel extract on Interleukin 12 (IL-12) cytokine expression in kidney organs. There is no healthy control group (P0) found in dark brown spots on the kidney glomerulus cortex cells, the untreated (P1), Cotrimoxazole infected group (P2) or mangosteen peel extract (P3 and P4), in kidney cortex cells are found in brown spots. Brown spots around the renal glomerular cortex cells show cytokine IL-12 expression (red arrows) (×100 magnification microscope)

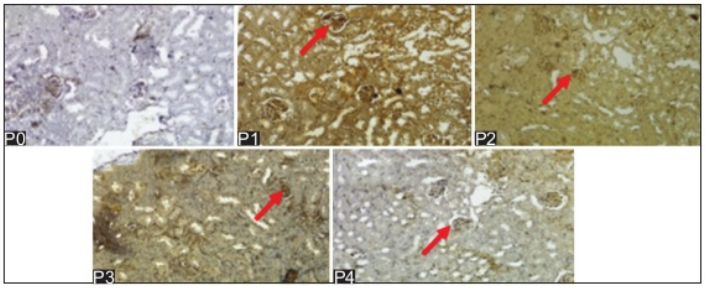

Figure 6: Results of the examination of mangosteen peel extract on matrix metallopeptidase 9 (MMP-9) cytokine expressions in kidney organs. There is no healthy control group (P0) found in dark brown spots on the kidney glomerulus cortex cells, the untreated (P1), Cotrimoxazole infected group (P2) or mangosteen peel extract (P3 and P4), in kidney cortex cells are found in brown spots. Brown spots around the renal glomerular cortex cells show MMP-9 cytokine expression (red arrows) (×100 magnification microscope)

contained within the mangosteen peel is known to have a complex of aromatic groups with several groups to assist the process of reduction and oxidation reactions in microbial or parasitic metabolism so that it can be utilized as antitoxoplasm.[16]

Xanthone contains many α-mangosteen and b-mangosteen activate protein kinase enzymes, which can inhibit free radical, apoptotic (cell death) process and inhibits the formation of bacterial, parasitic, fungal, and viral cell structure, and function.[16] Enzyme the activated metalloproteinase matrix due to flavonoids especially flavonol has anti-inflammatory drug receptor cyclooxygenase-2. The activated protein kinase enzyme due to xanthone will activate Toll-like receptors (TLRs) which are drug receptors that act as immunomodulators to parasites. TLRs that are activated by the *T. gondii* parasite infection are TLRs2, TLRs4, TLRs9, and TLRs11. TLRs2, TLRs4, and TLRs9 can only recognize lipopolysaccharide of infectious bacterial PAMPs. However, if TLRs11 is activated, it will be very easy to recognize PAMPs on the part of flagelin protein and PAMPs profilin from an infectious agent of *T. gondii* parasite. After the part of the protein flagellin and profilin of the parasite is recognized and there will be a strong bond between TLRs11 and PAMPs from the infectious agent of *T. gondii* parasite. TLRs11 is activated, it will activate macrophage cells and NK cells. Increased macrophage activity may be stimulated by increased activity of cytokines TNFα, IL-12, IL-10, and IFNγ.[16-19] Increased macrophage activity will stimulate increased activity of cytokines TNFα, IL-12, IL-10, and IFNγ will accelerate the phagocytocytes process against parasitic infectious agents, resulting in an increase in the number of deaths of infectious agents of parasites. IFNγ also has a role in inhibiting the multiplication of parasites that may inhibit growth and result in the death of *T. gondii* parasites. IL-12 in collaboration with IFNγ will stimulate the activation of T cells (Thelper1) especially CD4+ and CD8+ cells. Activation of CD4+ and CD8+ cells play an important role in acute and chronic toxoplasmosis infection that stimulates a cellular immune response (activates phagocyte cell activity) and humoral immune response (G Ig) so that the infected host body is able to fight the infectious agent of *T. gondii* parasite. IL-12 heterodimer consisting of IL-12p35, IL-12p40, and IL-12p70 is able to control T cell response to infection and parasitic replication of *T. gondii* with genotypic variation so

that the *T. gondii* parasite with genotypic variation will be more easily recognizable and phagocytized by phagocytes or NK cells. IL-12 and IL-18 cytokine in collaboration with IFNγ in plays an important role in inhibiting parasitic replication and decreasing the number of parasites of *T. gondii* in perite host fluids (mice). While flavonoids, especially flavonol groups in mangosteen ethanol extract of mangosteen peel, have mechanism of action inhibiting the enzyme of MMP-9 indirectly but through MMP-2 barrier first, then Tissue Inhibitor of Matrix Metalo Proteinase (TIMP) occurs. After TIMP is formed then the enzyme MMP-9 is inhibited which will eventually inhibit the membrane of *T. gondii* parasitic cell tissue causing membranes of parasite lysed cell tissue then parasites die. If the lysis cells are parasitic and successfully removed from the host's body, the decreased prisma levels are characterized by decreased parasites in the peritoneal fluid, increased expression of IFNγ and IL-12 cytokines, and decreased MMP-9 expression. In addition, there is a reduction in tissue damage of kidney organs. If the damage of kidney tissue has been reduced, there will be a decrease in BUN and creatinine levels in the blood serum of the host.[20-23]

CONCLUSIONS

This research is: ED 50% at dose of 60 mg/kgBW. Mangosteen peel extract 200 mg/kgBW (P3) and 400 mg/kgBB (P4) with significance ($P > 0.05$) can reduce the number of parasites, levels of BUN and creatinine, reduce kidney damage, increases IFNγ and IL-12, and decreases MMP-9 expression.

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