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Research Report

TNF-α expression on rats after *Candida albicans* inoculation and neem (*Azadirachta indica*) extract feeding

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ABSTRACT

Background: *Neem is a known traditional medicine from trees which function as immunomodulator. Candidiasis found in mouth is 80% caused by Candida albicans (C. albicans). Immunity is important to limit C. albicans since medicine price is relatively traditional medicine may become a good choice. In the other side the medicine price may not be reached by the citizen, cause citizen choose the traditional medicine.* ***Purpose:*** *The research is aimed to explain of TNF-α expression on rats after inoculated by C. albicans and fed with neem extract (Azadirachta Indica).* ***Methods:*** *There were 5 groups, the first group which was called as control group (KO) hadn't been fed aqueous extract from neem leaves and was not inoculated by C. albicans, the other group (treatment) was classified into 4 groups. The first group was inoculated by C. albicans only (KP1), second group was fed with 50 mg/day/kg body weight aqueous extracts from Neem leaves, then inoculated with C. albicans starting from day 8 until day 21 (KP2), third group was fed with 100 mg/day/kg body weight aqueous extract from Neem leaves, then inoculated with C. albicans start from day 8 until day 21 (KP3), fourth group was fed with 200 mg/day/kg body weight aqueous extract from Neem leaves, then inoculated by C. albicans start from day 8 until day 21 (KP4). The data was collected from by swabbing the rat's tongue to calculate C. albicans colonies. The rats were acclimated and collected for immunohistochemistry measurement.* ***Results:*** *The study showed that there were different result on ANOVA, HSD test, and linier regression. ANOVA showed significant difference ($p < 0.01$) between groups. The HSD test showed significant difference ($p < 0.05$) between each groups. TNF-α was the stimuli sensor from environment, and used as parameter to see the effect from the change of innate immunity component to C. albicans.* ***Conclusion:*** *Aqueous extract from neem leaves increased the macrophage TNF-α expression on in rat in oculated with C. albicans.*

Key words: *Neem leaves aqueous extract, Azadirachta indica, macrophage, phagocytosis, Candida albicans*

ABSTRAK

Latar belakang: *Mimba merupakan salah satu tanaman obat tradisional yang telah dikenal masyarakat dan berfungsi sebagai imunomodulator. Penyakit infeksi yang paling banyak dijumpai di rongga mulut (80%) adalah kandidiasis dengan penyebab utama Candida albicans (C. albicans). Di mana peran imunitas sangat penting pada C. albicans. Di sisi lain harga obat yang semakin mahal semakin tidak terjangkau masyarakat, menyebabkan masyarakat memilih obat tradisional.* ***Tujuan:*** *Riset ini untuk menjelaskan tentang ekspresi TNF-α makrofag pada tikus wistar yang diinokulasi C. albicans dan diberi konsumsi ekstrak cair daun mimba.* ***Metode:*** *Penelitian ini terbagi menjadi 5 kelompok, kelompok kontrol (KO) tidak diberi perlakuan, kelompok yang diinokulasi C. albicans (KP1), kelompok yang diberi konsumsi 50 mg/hari/kg dan diinokulasi C. albicans mulai dari hari 8 sampai hari 21 (KP2), kelompok yang diberi konsumsi 100 mg/hari/kg dan diinokulasi C. albicans mulai dari hari 8 sampai hari 21 (KP3), kelompok yang diberi konsumsi 200 mg/hari/kg dan diinokulasi C. albicans mulai dari hari 8 sampai hari 21 (KP4). Data dikumpulkan dari swabbing lidah untuk dihitung koloni C. albicans dan jaringan lidah dengan metode immunohistochemistry untuk penghitungan sel makrofag yang mengekspresikanTNF-α.* ***Hasil:*** *Studi menunjukkan terdapat perbedaan yang signifikan dari hasil ANOVA, uji HSD, regresi linier. ANOVA menunjukkan perbedaan ($p < 0,01$) antar kelompok. Uji HSD menunjukkan perbedaan ($p < 0,05$) antar kelompok. Hal ini dapat dikatakan bahwa TNF-α adalah sensor stimuli dari lingkungan, yang digunakan sebagai parameter untuk melihat pengaruh dari*

perubahan dari komponen respons innate terhadap C. albicans. ***Kesimpulan:*** *Ekstrak cair daun mimba dapa meningkatkan ekspresi makrofag TNF-α dari tikus yang diinokulasi C. albicans.*

Kata kunci: *Ekstrak cair daun mimba, Azadirachta Indica, makrofag, fagositosis, Candida albicans*

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INTRODUCTION

Neem (*Azadirachta indica*) contains bioactive component such as azadirachtin, salanine, meliantriole, nimbin, nimbolide, gedunine, mahmodine, gallic acid, catechin, epicatechin, margolone, margolonone, isomargolonone, cyclictrisulphide, cyclictetrasulphide and polysaccharide. Neem has been widely used by the community to treat diseases including worm infection, scabies, malaria, fungal infection, tumor, and allergy.[1,2] Researches had proven that neem modulates innate and adaptive immunity,[3–6] while innate immunity (phagocytosis) especially macrophage, plays important role in fighting *Candida albicans* (*C. albicans*) which was the main etiology for oral candidiasis.[7,8] Oral candidiasis is one of the most common infectious diseases found in oral cavity (80%).[7] Previous researches from the author had proved that aqueous extract from Neem leaves could inhibit the growth of *C. albicans* in vitro.[9] Other than having antifungal effect, Neem leaves could also function as immunomodulator. Many antifungal drugs have no immunomodulator properties, while infection of *C. albicans* is highly depending on the state of immune system. Destruction and elimination by phagocytic cells, could occur by both oxidative and non oxidative pathways. Oxidative pathway including the production of superoxide and NO by iNOS system, where both activities could be induced by TNF-α, while phagocytic activity and fungicidal uptake functions and intracellular fungal destruction. Non oxidative measures including production of cytokines, such as TNF-α that may modulate the activity of phagocytosis.[10-12] Other researches had proved that Neem leaves could increase macrophage activity in vitro, so it was assumed that Neem leaves might affect TNF-α which was a cytokine that play role in activating phagocytosis, but until today, the mechanism of the increasing activity of TNF-α to *C. albicans* had not yet fully explained.[13] The aim of the research was to know TNF-α expression on rats were inoculated by *C. albicans* and fed with neem extract.

MATERIALS AND METHODS

This research was an experimental laboratory research with sample of 25 male wistar rats that have fulfilled "*Declaration of Helsinki*". Each rat was 100-200 grams in weight, age 2–3 months that received one week adaptation. There were five groups which were control group (KO) which were not provided with aqueous extract of Neem leaves and not inoculated with *C. albicans*, treatment groups which consisted of the group that were inoculated with *C. albicans* only (KP1), a group which consumed aqueous extract of neem leaves with a dose of 50 mg/day/kg bodyweight, then were inoculated with *C. albicans* started from day 8 to day 21 (KP2), a group that were provided with aqueous extract of Neem extract with a dose of 100 mg/day/kg body weight, and were inoculated with *C. albicans* from day 8 to day 21 (KP3), and a group that were provided with aqueous extract of Neem leaves with a dose of 200 mg/day/kg body weight and were inoculated with *C. albicans* (KP4). All groups were observed in day 22 by conducting light swab with cotton bud on the rats' dorsal tongue with one swab to count the number of *C. albicans colony*. The rats were collected and lingual tissues were obtained and prepared, then TNF-α was analyzed with immunohistochemistry methods, through: deparanization with ethanol started from absolute to 70%, water, phosphate buffer saline (PBS) pH 7.4 and were provided with tripsin. Preparation was flooded within the solution of 3% H_2O_2, washed with PBS twice and were undergoing blocking process with 3% BSA. Anti rat TNF-α was then reacted, and was incubated for 24 hours in 4° C temperature in a humidity chamber. The substances were then reacted with biotiyilized secondary anti rabbit (Ab). Washed three times with PBS, and were provided with peroxidase-labeled streptavidin and were incubated for 1 hour. Substances were then re-washed three times with PBS, reacted with diamine Benzidine (DAB) substrate, and were added with "Meyer-HE". Data obtained were analyzed with ANOVA and continued with HSD test.

RESULTS

The result showed less TNF-α in macrophages on groups inoculated with *C. albicans* compared to control group. The higher d the dose of neem extract, the higher the TNF-α expression.

ANOVA test show of TNF-α expression showed that there was a significant difference ($p<0.01$), this is continued with HSD test which also gave significant difference. Thus indicates that aqueous extract from neem leaves can increase TNF-α expression with dose 50, 100, 200 mg/weight/day, on the other side *C. albicans* reduces TNF-α expression. Linear regression showed a strong positive correlation (0.985), meaning the higher the dose of neem leaves aqueous extract, the higher the number of TNF-

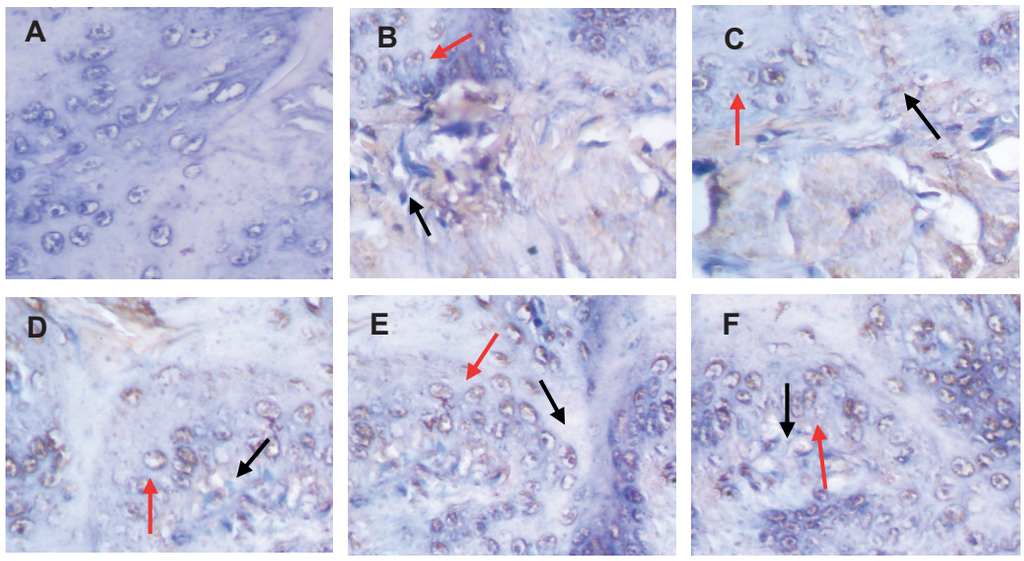

Figure 1. Expression of TNF-α in macrophage with immunohistochemistry technique (400× magnification). Brown color shows presence of TNF-α expression (↗) Blue color shows presence of TNF-α expression (↗) A) Staining control (macrophage appeared in blue staining); B) Expression of TNF-α in macrophage of control group (K0); C) Expression of TNF-α in macrophage of treatment group 1 (KP1); D) Expression of TNF-α in macrophage of treatment group 2 (KP2); E) Expression of TNF-α in macrophage of treatment group 3 (KP3); F) Expression of TNF-α in macrophage of treatment group 4 (KP4)

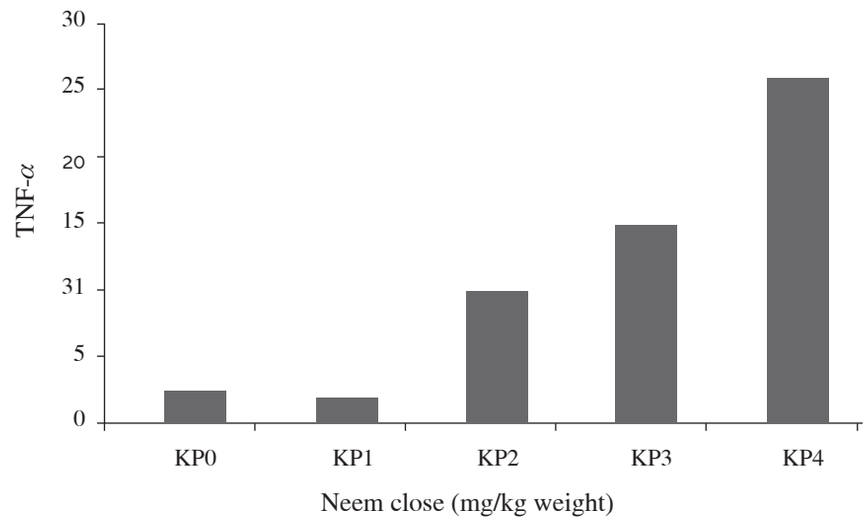

Figure 2. Macrophage expression of TNF-α.

α expression macrophage (Figure 3). Expression of TNF-α (circles) were scattered around the rightward straight line with upward position, which indicated that the higher the dose of neem given, the higher the expression of TNF-α (Figure 3). *C. albicans* colony was counted by colony counter after grown on Saburound's agar (Figure 4).

There are no C. *albicans* at control group. The highest number of colony was found at KPI, and the smallest at KP4 (Figure 4). ANOVA test showed there are a significant difference between groups, and using HSD test indicated that aqueous extract from Neem leaves with dose 200mg/weight/day can reduce *C. albicans* colony.

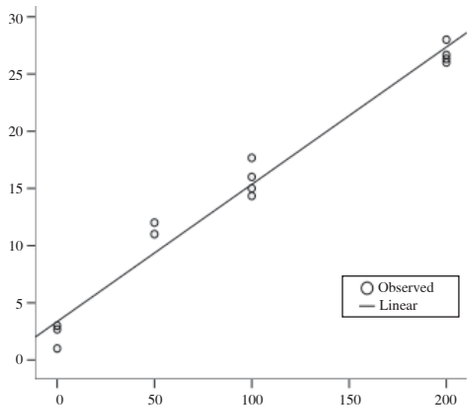

Figure 3. Linear graphic of TNF-α expression.

Tabel 1. HSD test of TNF-α expression to macrophage

Groups	N	Mean	SD
KPo	5	2.6666	.47146a
KP1	5	1.7334	1.01113b
KP2	5	11.2000	.44721c
KP3	5	15.8000	1.26073d
KP4	5	26.6666	.78181e
Total	25	11.6133	9.40825f

Description: different letter shows existence of difference significant TNF-α expression

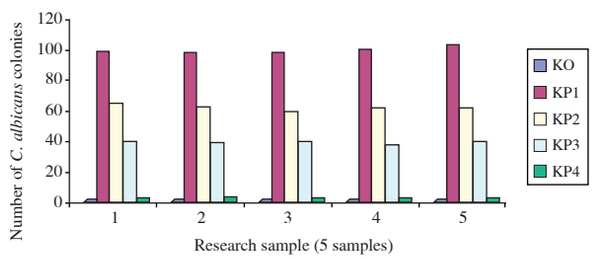

Figure 4. Number of *C. albicans* colony between groups.

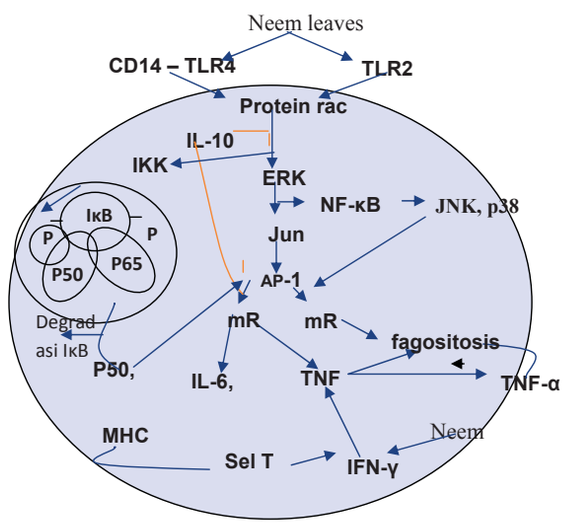

Figure 5. The activity of neem leaves to immune response.

DISCUSSION

Stimuli response was evaluated through oral mucosa immunity mucous membrane, oral lymphoid tissue, extraoral lymphoid, intraoral lymphoid tissue, lymphoid gland saliva, gingiva lymphoid tissue. In this research, the sampling was done on mouse tongue because candidiasis is most commonly found at tongue. Oral mucosa immune system will influence systematical immune system through vascular immune system.

Aqueous extract of neem leaves containing galic acid, catechin, epicatechin which can influence macrophage response through two ways, one was directly influences *C. albicans* and indirectly by influencing macrophage. Effect of *C. albicans* was anticipated to causes change at *C. albicans* cell membrane, so it is easier to be recognized by macrophage to do phagocytosis. Indirect effect of aqueous extract from Neem leaves by the way of changing macrophage activity to *C. albicans*, presentation by the way of immunemodulation.

This research was conducted to solve the problem about aqueous extract from Neem leaves to TNF-α activity related to macrophage phagocytosis activity to *C. albicans.* CD14, TLR2, TLR4, TNF-α, phagocytosis activity and number of *C. albicans* colonies, because CD14, TLR2, TLR4, TNF-α, phagocytosis activity were component at commissioned innate immune system as censor stimuli. While colony amounts of *C. albicans* was applied as parameter existence of effect from change of innate immune component to *C. albicans*. Stimuli can be recognized by censor system CD14, TLR2, TLR4 and will be followed up with selection, organizational and interpretation so that response to stimuli can be adapted for requirement of host to survive candidiasis to cause CD14 suppression which is to functioning increase TLR activity, pursues TLR2 and TLR4 resulting degradation protein of transcription activity residing in macrophage cell. Function of phagocytosis and TNF-α is playing important role at macrophage level which also influence the degradation of adaptive immune response on *C. albicans* on the other side with existence of aqueous extract from neem leaves situation of imunosuppresion resulted from *C. albicans* will be improve and repaired.

Macrophage as professional phagocyte function to break immunogen as antigen presenting cells (APC) which recognizes microbe through some receptors its to stimulate migration of cell to the place of infection and stimulates the production of substance. Activation of macrophage at innate immunity through CD14 expressed to surface of cell and activates toll-like receptors (TLRs) showed that neem leaves with their immunomodulatory components (assumed as galic acid, catechin, epicatechin) built a new balance through immune system regulation in which we could recognize the outcomes of products from macrophage cells in facing stressors. Improvement mechanism of TNF-α by Neem to *C. albicans* started by the existence of improvement of CD14, TLR2 and TLR4 (*C. albicans* reduces all immune activities). TLR2, TLR4 affected

phospatidilinositol in macrophage cell membrane and would then activate Rac protein, which then activated NF-κB and AP-1 through jun kinase via the mitogen-activated protein kinase (MAPK) pathway. Included within this pathways was extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38. ERK affected jun activity, while p38 affected the production of IL-6, IL-8 and IL-12. Activity of p38 and ERK could activate AP-1. These three MAPK pathways could be activated simultaneously at the same time. NF-κB was a regulator from early response to pathogen and as an activator of immune system. NF-κB was p50–p65 from a family of heterodimer protein that transcribed many genes. Activation of *NF-*κB require I-κB protein phosporilation, which was continued by degradation of p50–p65 within the nucleus and it would activate the gene. After the release of I-κB, an increase would occur in the activity of transcription factor NF-κB which stimulated gene expression that affected the production of TNF-α and phagocytic activity. Stimulation of gene expression among others affected the production of TNF-α. In immune response to *C. albicans,* TNF-α played the role as primary immunity in immune system regulation. Specifically to macrophage, this cytokine increased the activity in killing pathogens, in which this action became an important mediator in inflammation. Activity of innate immune response may affected MHC, so it would give effect on the activity of adaptive immunity (T cell and B cell).[14-16] Briefly, the mechanism of the increased expression of TNF-α induced by aqueous extract of Neem was explained in figure 5. aqueous extract of Neem with component galic acid, epicatechin, catechin, can reduce number of colonies *C. albicans* through improvement of TNF-α activity, though the number is not absolute to show the existence of infection, but applicable to show the existence of infection. Amount of which more than control group serve the purpose of parameter the happening of infection. This research shows number of *C. albicans* which increasingly declines with aqueous extract from neem leaves dose excelsior mimba, where dose 200 mg/kg/weight seen number of least colonies. Degradation of *C. albicans* colonies number is anticipated by immunomodulator content (galic acid, epicatechin, catechin) and nimbidin content, azadirachtin, gedunin, cyclic trisulphide, cyclic tetrasulphide which can function as antifungal. Immunomodulator content can increase immune response to *C. albicans*, while antifungal content can kill candida directly and destructive to its cell membrane.

It concluded that aqueous extract from neem leaves could increase the expression of TNF-α in rats inoculated with *C. albicans.*

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