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Research Report

# The effects of *Acanthus ilicifolius* chloroform extract on TLR-2 expression of macrophages in oral candidiasis

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***ABSTRACT***

***Background:*** *Immunosuppressed conditions due to long-term corticosteroid and tetracycline consumption are susceptible to fungal invasion, especially by Candida albicans (C. albicans), that requires treatment of oral candidiasis. Toll like receptor-2 (TLR-2) plays a role in candida recognition. Nystatin is regularly employed for oral candidiasis, but produces certain side-effects. Chloroform extract of Acanthus ilicifolius (A. ilicifolius) leaves represents both a potent inhibitor of C. albicans growth and an antioxidant.* ***Purpose:*** *This study aimed to compare the effect of A. ilicifolius leaf chloroform extract and nystatin treatment on TLR-2 expression in oral candidiasis immunosupressed models.* ***Methods:*** *This study constitutes a true experimental investigation incorporating a post test-only control group design. 20 healthy male Rattus novergicus (Wistar), aged 12 weeks and with an average weight of 250g, were immunosuppressed through oral administration of dexamethasoneand tetracycline for a period of 21 days before being induced with C. albicans (ATCC-10231) 6 x $10^8$ for two weeks. The subjects were divided into five groups (n=4/group): healthy (H), no-treatment(P), nystatin treatment(N), A. Ilicifollius (8%) treatment (AI-1) and A. ilicifollius (16%) treatment (AI-2). The subjects were treated for 14 days, with their tongue being subsequently biopsied. TLR-2 expression was subjected to immunohistochemical examination, observed under a microscope (400x magnification) and statistically analyzed (one-way Anova, LSD-test, $p<0.05$).* ***Results:*** *TLR-2 expression of P (6.25 ± 2.5), N (11.25 ± 0.96), AI-1 (13.00 ± 1.15), AI-2 (12.75 ± 1.7) was higher than H (1.75 ± 0.5). Significant differences existed between N to P, N, AI-1, AI-2; P to N, AI-1 and AI-2 ($p<0.05$). No significant differences were present between N, AI-1 and AI-2 ($p < 0.05$).* ***Conclusion:*** *A. ilicifolius extract can increase expression of TLR-2 in oral Candidiasis-immunosuppressed models. A. ilicifolius extract produces the same effect in increasing TLR-2 expression when compared to nystatin.*

***Keywords:*** *achanthus ilicifolius; Candida albicans; immunosupressed; oral candidiasis; TLR-2*

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## INTRODUCTION

Infection in humans can be either bacterial or fungal in nature. Although often a commensal organism in humans, several fungi can prove to be pathogenic under certain conditions. Fungal infections can occur in immunocompromised patients suffering from trauma, HIV infection, immunosuppression and neutropenia and in whom the normally protective bacterial microflora is disrupted.[1] Prolonged use of systemic drugs, for example broad-spectrum antibiotics, immune-suppressants and drugs with xerostomic side-effects, alter the local oral flora or disrupt the mucosal surface or reduce salivary flow, thereby creating a favorable environment for candida to grow and cause oral infection.[2] Drug induced oral candidiasis has been reported in asthma patients treated with steroid inhalers for approximately three months.[3]

The most common fungal infection affecting the oral cavity is oral candidiasis approximately 60% of the cases of which are caused by *Candida albicans* (*C. albicans*) infection.[4] According to research conducted in Surabaya during 2007, following candida isolation in AIDS patients suffering from oral candidiasis with clinical features of acute pseudomembrane candidiasis, acute erythematosus

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candidiasis, chronic hyperplastic candidiasis, acute erythematosus candidiasis and perleche, *C. albicans* species was found in approximately 35.29% of cases, while *non-C. albicans* species were detected in the other 64.71%.[5]

Neutrophils and macrophages constitute the immune cell types involved in immune mechanism defense against fungal infections. They recognize *candida* through pattern recognition receptors (PRRs) which interact with specific molecules called pathogen-associated molecular patterns (PAMPs) exposed on the surface of the *candida*.[6] Recognition of fungal cells occurs by means of PRR, dectin-1 (-1,3 glucan) and several other PRRs involved in identifying different cell wall polysaccharides of this pathogen. These include toll like receptors including: (TLR) 2 which recognizes phospholipomannan of *C. albicans*, TLR4 which recognizes O-mannan of *C. albicans* and mannose receptor (MR) which recognizes N-mannan of *C. albicans* [7]. These cells release cytokines and chemokines to further modulate the immune response for mucosal protection.[8,9] Activation of TLRs, includingTLR-2, not only induces inflammatory responses, but also the development of antigen-specific adaptive immunity against fungal infection.

Nystatin is a drug which, in addition to amphotericin B, miconazole, itraconazole and fluconazole, is commonly used in oral candidiasis therapy. It produces several side-effects including: unpleasant tastes in the mouth, vomiting, diarrhea, nausea, anorexia, abdominal pain, thrush and headaches.[10] *Acanthus ilicifolius* (*A. ilicifolius*) is a mangrove plant of the Acanthaceae family native to tropical regions in Asia and Africa employed by communities in Malaysia, India and Thailand to treat rheumatism, neuralgia, wounds resulting from poisoned arrows, coughs, asthma, influenza and dermatitis.[11,12] *A. ilicifolius* leaf extract contains several active phytochemical components such as proteins, resins, steroids, tannins, glycosides, reducing sugars, saponins, sterols, terpenoids, phenols, cardioglycosides and catachols.[11] This plant also has the ability to act as an antimicrobial.[13]

Chloroform and n-hexane extracts from *A. ilicifolius* leaves have a powerful inhibitive effect on *Bacillus subtilis*, *Staphylococcus aureus*, *C. albicans*, *Aspergilus fumigatus* and *Aspergilus niger*.[12] The chloroform extract from *A. ilicifolius* leaves at a dose of 400 mg/kgBW has been reported to have an anti-ulcerative effect in mice, while one of 500 mg/kgBW has been demonstrated to be a practical anti-carcinogenic.[14,15] The chloroform extract of *A. ilicifolius* leaves has an anti-candida effect and produces the highest inhibitory zone compared with other extraction methods.[16] Chloroform extract of *A. ilicifolius* leaf 1% is effective in inhibiting *C. albicans* through thermoplastic nylon soaking. The administration of chloroform extract *A. ilicifolius* 8 mg/ml may increase IL-17 in immunosuppressed subjects suffering from oral candidiasis.[17,18]

The purpose of this study was to compare the effect of *A. ilicifolius* chloroform extract and nystatin on TLR-2 expression immunosuppressed models as an alternative medicine derived from natural materials for oral candidiasis.

## MATERIALS AND METHODS

This research represented a true experiment incorporating post test-only control group design. It employed 20 healthy, 12-week old, male, *Rattus novergicus* (Wistar), each 250 grams in weight, which were divided into five groups (n=4/group). Group-1(H): healthy/normal subject group, Group-2 (P): subjects induced with *C. albicans* and 0.1% CMCNa, Group-3 (N): subjects induced with *C. albicans* and treated with nystatin topical, Group *A. ilicifollius 8%* treatment (AI-1): subjects induced with *C. albicans* and treated with 8% *A. ilicifolius* chloroform extract topically, Group- *A. ilicifollius 16%* treatment (AI-2): subjects induced with *C. albicans* and treated topically with 16% *A. ilicifolius* chloroform extract. The method of animal subject induction in this study was based on Chami's *et al.* research, with several modifications. Immunosuppression was achieved by orally administering dexamethasone 0.5 mg/day and 1% tetracycline /day for 21 days. Between the 7$^{th}$ and 21$^{st}$ days the subjects were induced with 0.1 cc of *C. albicans* (ATCC-10231) 6 x 10$^{8}$, applied to their tongues with a sterile cotton bud three times a week for two weeks.[19]

*A. ilicifolius* chloroform extract was produced by dehydrating leaves in the open air for two days. 10 grams of dried *A. ilicifolius* leaves were subsequently mixed with 200 ml of chloroform using a mortal and pastle, covered and allowed to stand for five hours. The solvent was removed and filtered with Whatman's no. 1 filter paper before being evaporated at low pressure by using a Buchi Rotavapor R-200 at 45°C. The chloroform extract was then stored in a refrigerator for future use.[20]

Treatment using *A. ilicifolius* chloroform extract 8% (Group AI-1), 16% (Group AI-2) and Nystatin as control groups was performed by applying 0.5 cc of the material to the surface of the tongue at the same time on 14 consecutive days, after which the subjects in each group were sacrificed and biopsed. TLR-2 expression was examined using a immunohistochemical staining method and then observed by means of a light microscope at 400x magnification.

The statistical analysis used both one-way ANOVA and LSD statistical tests to determine the significance of differences between groups.

## RESULTS

The expression of TLR-2 in an oral candidiasis immunosuppressed model derived from the tongue of a subject is shown in Figure 1. Figure 2 indicates that the healthy/ normal group (H) had the lowest expression of TLR-2 (1.75 ± 0.5) compared to P (6.25 ± 2.5), N (11.25 ± 0.96), AI-1 (13.00 ± 1.15) and AI-2 (12.75 ± 1.7), while

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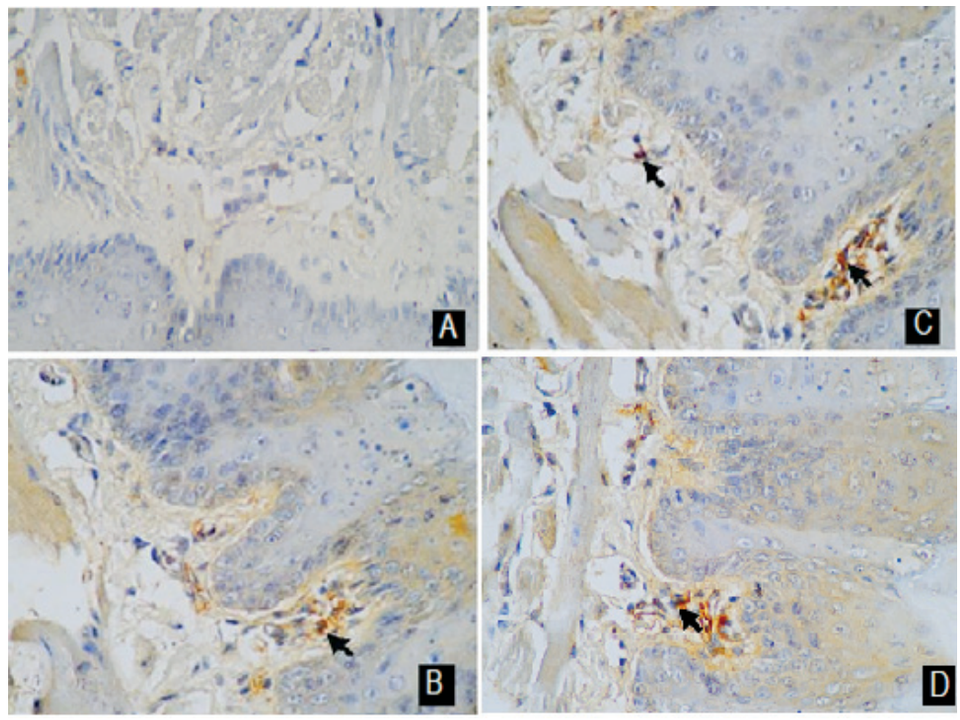


**Figure 1.** TLR-2 expression of macrophage in the tongue of *Rattus novergicus* (Wistar) (black arrow). A: Healthy/ normal group (H), B: Oral candidiasis group (P), C: group of subjects induced by *C. albicans* and treated with nystatin topical (N), D: group of subjects induced by *C. albicans* and treated with *A. ilicifolius* chloroform extract topically (AI) (400x Magnification).

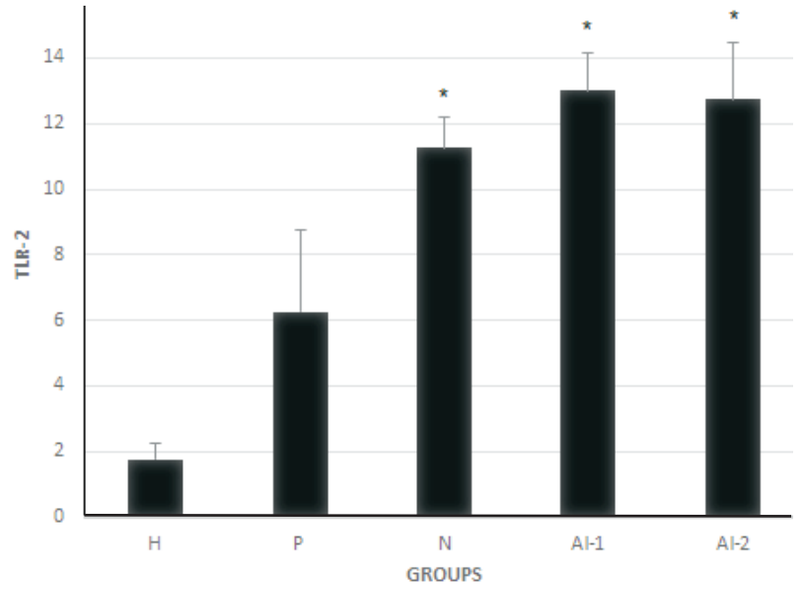


**Figure 2.** Means of TLR-2 expression of macrophage**.**
This graphic represents the mean ± standard deviation in each group of TLR-2 expression of macrophage after treatment with nystatin (N) and *A. ilicifolius* 8% (AI-1) and *A. ilicifolius* 16% (AI-2) compared with healthy subjects (H) and those receiving no treatment (P). There were significant differences between the healthy/normal group (H) and P, N, AI-1 and AI-2; P compared to N, AI-1 and AI-2 ($p < 0.05$). while there were no significant differences between N compared to AI-1 and AI-2, and AI-1 compared to AI-2 ($p > 0.05$). (*$p < 0.05$ vs H and P).

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the highest expression of TLR-2 appeared in the group of subjects which had been induced with *C. albicans* and treated topically with 8% *A. ilicifolius* chloroform extract (AI-1) compared to other groups.

A one-way ANOVA statistical test was employed which indicated the significant differences between groups ($p<0.05$). A subsequent LSD statistical test confirmed significant differences between the healthy/normal group (H) compared to groups P (0.018), N (0.017), AI-1 (0.017) and AI-2 (0.018) ($p<0.05$). Significant differences also existed in P compared to N (0.020), AI-1 (0.019) and AI-2 (0.021) ($p<0.05$), while there was no significant differences between N and AI-1 (0.063) or AI-2 (0.18) and AI-1 compared to AI-2 (0.766) ($p>0.05$).

## DISCUSSION

Immunosuppressive conditions and long-term antibiotic consumption represent predisposing factors that can lead to oral candidiasis. In this study, subjects were immunosuppressed through the administering of dexamethasone and combined with tetracycline, the latter of which is intended to cleanse the oral cavity of bacteria, thereby enabling *C. albicans* to proliferate without contamination by other bacteria. The tongues of subjects were used as a site of inoculation, treatment and sampling.

In this study, the level of TLR-2 expression, the nystatin treatment, 8% *A. ilicifolius* treatment, and 16% *A. ilicifolius* treatment, was found to be lower in the healthy group when compared to the candidiasis group. The untreated candidiasis also possessed lower TLR-2 expression compared to that of the treatment groups. Immunosuppressive factors (catecholamines and steroids) induced by stress may also down-regulate the expression of TLRs. A number of studies have reported that TLRs are up-regulated in inflammatory conditions and down-regulated in immunosuppressive conditions.[21] Therefore, it is speculated that immunosuppressed conditions may have down-regulated the expression of TLR-2 in this study.

There was no significant difference between the treatment group of nystatin, the treatment of 8% *A. ilicifolius* and the treatment of 16% *A. ilicifolius* ($p < 0.05$). This indicates that nystatin therapy involving both 8% *A. ilicifolius*, and 16% *A. ilicifolius* administered to subjects with immunosuppressed candidiasis demonstrated the same ability to increase TLR-2 expression which was possibly due to the phytochemical content of the extract. Chloroform leaf extract of *A. ilicifolius* contains phenol, flavanoid and tannin, which promote both antimicrobial and antioxidant activity.[21] One study reported that the pentacyclic triterpenoid (isolated chloroform extract) present has the potential to restore vascular disorders associated with hypertension, obesity, diabetes, atherosclerosis. It could also be used in cancer therapy, as anti-ulcer drugs, as well as for the prevention and treatment of metabolic diseases.[12,22]

Previous studies of both nystatin and *A. ilicifollius* extract (8% and 16%) found that the latter were similar to nystatin in increasing the amount of IL-17 expression in oral candidiasis immunosuppressed conditions models.[18] This indicates that the capture of TLR-2 leads to a decrease in the number of candida and increased pro-inflammatory cytokines. In this study, the concentration of 8% *A. ilicifolius* extract produced the effect on the increase of proinflammatory cytokines via TLR-2 pathway. Up-regulation of TLRs may lead to an inflammatory response and protective function against infection, while down-regulation of TLRs may suppress inflammation and facilitate subsequent infection.[23]

Another study reported an increase in the capture capability of TLR-2 in mice afflicted with candidiasis which was associated with decreased TNF and chemokine production.[24] TLR-2 can enhance the ability of macrophages in *C. albicans*-induced mice[25], while its absence can reduce neutrophil chemotaxis and antifungal mechanisms.[26] TLR-2 binds phospholipomannans from *C. albicans* in the presence of galectin-3 and $\beta$-mannosides to induce a macrophage pro-inflammatory response.[27] TLR-2 in combination with dectin-1 can also bind the β-glucan fungus. Inhibition of TLR-2 in human mononuclear cells can cause a 40-50% reduction in the production of proinflammatory cytokines in vitro. A decrease in the amount of TLR-2 may escalate susceptibility to infection by *C. albicans*.[28] TLR activation, in particular that of TLR-2, can suppress defense immunity against *C. albicans* through IL-10 induction and T-regulatory cells.[29] TLR signals through the MyD88 pathway leading to activation of MAPK and induces the translocation of nuclear factor kappa B to the nucleus which promotes the transcription and synthesis of proinflammatory cytokines.[30]

In conclusion, *A. ilicifolius* chloroform extract can increase expression of TLR-2 in oral candidiasis-immunosupressed models. *A. ilicifolius* chloroform extract produces the same effect by increasing TLR-2 expression compared with nystatin. Further research is required regarding the function of acanthus in this model and its role in the mechanism of oral candidiasis.

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