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Acanthus ilicifolius L. Treatment for Oral Candidiasis with Immunosuppressive Conditions Subjected to p38 MAPK Enhancement

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ABSTRACT

Methanolic extract from the leaves of *Acanthus ilicifolius* L. (*A. ilicifolius* L.) is a potent inhibitor of *Candida albicans* (*C. albicans*) growth and anti-inflammatory. *C. albicans* causes oral candidiasis in immunosuppressive condition. Mitogen-activated protein kinase (MAPK) signalling via p38 appears to discriminate between yeast and hyphal cells of *C. albicans*. Activation of p38 MAPK by hyphae results in the upregulation of proinflammatory cytokines. The p38 MAPK activation is known to impair corticosteroid action. The research was conducted to investigate the effect of methanolic extract *A. ilicifolius* L. treatment of oral candidiasis with the immunosuppressive condition through enhancement of p38 MAPK expression in the epithelial cells. Immunosuppressed conditions were obtained when 16 healthy male *Rattus norvergicus* (Wistar) was given oral administration of dexamethasone and tetracycline for 14 days and induced with *C. albicans* (ATCC-10231) 1 McFarland. The subjects were divided into four groups (n = 4/group): immunosuppression (IS), immunosuppression with oral candidiasis without treatment (ISC), immunosuppression with oral candidiasis and nystatin treatment (ISC+N), and immunosuppression with oral candidiasis and *A. ilicifolius* L. treatment (ISC+AI), and were treated for 14 days. Later, the rats were euthanised, and their tongue were biopsied. The p38 MAPK expression was subjected to immunohistochemical examination, observed under a microscope (400× magnification) and statistically analysed (one-way ANOVA, LSD-test, $p < 0.05$). The p38 MAPK expression of ISC+AI (36.05 ± 1.54) was higher than IS (26 ± 2.32), ISC (26.4 ± 3.71), IS+N (34.2 ± 0.99). Significant differences existed between ISC+AI and ISC+N to IS and ISC ($p < 0.05$). No significant differences were present between IS and ISC; ISC+AI and ISC+N ($p > 0.05$). Therefore, this treatment could enhance p38 MAPK expression in oral candidiasis with the immunosuppressed condition.

Keywords: *Corticosteroid; immunosuppressed condition; nystatin; oral candidiasis; p38 MAPK*

INTRODUCTION

Immunosuppressive drugs are the drugs that suppress the immune system used to prevent the production of antibodies. However, due to their non-selective action, they have various side effects (Kant *et al.*, 2009). Increased risk of opportunistic infections such as fungal infection are associated with these drugs. Oral candidiasis is associated with predisposing factors, which includes the disruption of the oral bacterial community by

the lengthy use of broad-spectrum antibiotics and any disorder that leads to an impaired immune response (Hebecker *et al.*, 2014). The *Candida* species is one of the common human fungal pathogens, which are benign commensals of the normal skin and mucosal flora in approximately 80% of the population (Tang *et al.*, 2016). Among the *Candida* spp., *Candida albicans* (*C. albicans*) ranks first in isolation frequency and morphological flexibility that plays crucial roles in several aspects of infection and host recognition (Brunke & Hube, 2013; Noble *et al.*, 2017).

The epithelial cells are the crucial first-line barrier as they are constantly exposed to the external environment (Tang *et al.*, 2016). The initial stage of *C. albicans* before colonisation and growth is adhesion to the epithelial cells, which are prerequisites for mucosal candidiasis (Zakikhany *et al.*, 2007; Wächtler *et al.*, 2011). Epithelial cells are capable of discriminating between the *C. albicans* yeast and hyphal form via Mitogen-activated protein kinase (MAPK) signalling pathways and activating immune responses (Moyes *et al.*, 2010). Recognition via specific MAPK of the filamentous form appears to initiate a danger response mechanism constituting MAPK phosphatase 1 (MKP-1) and c-Fos activation which may inform the host of when this normally commensal fungus has become invasive (pathogenic) (Tang *et al.*, 2016). p38 MAPK or c-Fos signalling is only activated by *Candida* species that form hyphae such as *C. albicans* and *C. dubliniensis*, but not by pseudo hypha-forming *Candida* species such as *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* (Moyes *et al.*, 2012). Activation of p38 MAPK results c-Fos transcription factor recruitment which play essential roles in the transcription of cytokines secreted by the epithelial cells. This mechanism then leads to the upregulation of cytokines and inflammatory mediators (Naglik & Moyes, 2011; Moyes & Naglik, 2011). Anti-inflammation mechanism of corticosteroid works by increasing the synthesis of anti-inflammatory proteins, such as MKP-1 which functions contrary to p38 MAPK (Adcock & Lane, 2003). The p38 MAPK activation impairs corticosteroid action.

The previous study reported that *A. ilicifolius* L. leaves extract showed promising effect against *C. albicans*. *A. ilicifolius* L. leaves extract could increase TLR2 and IL-22 expression in oral candidiasis with the immunosuppressive condition (Andriani & Pargaputri, 2018; Andriani & Pargaputri, 2019). Methanolic *A. ilicifolius* L. leaves extract 20% has the ability to increase macrophages oral candidiasis under the immunosuppressive condition. Macrophages are recognised as the innate immune cells involved in *C. albicans* infection (Setyawan *et al.*, 2019). A methanol extract of *A. ilicifolius* L. exhibit the highest antioxidant activity than the other solvent extracts and also has the ability in inhibiting *C. albicans* (Sofia & Merlee, 2017).

Based on the background above, we show that the activation of p38 MAPK pathways is playing an important role in the inflammation mechanism in oral candidiasis. Immunosuppressive conditions result in a weakening of the immune system. Therefore, this research aim was to determine the effect of methanolic extract *A. ilicifolius* L. treatment for oral candidiasis with the immunosuppressive condition through enhancement of p38 MAPK in epithelial cells.

MATERIALS AND METHODS

Animal Model

This was a true experiment with post-test only control group design. Sixteen healthy male *Rattus novergicus* strain Wistar aged 12 weeks, 200 g to 250 g in weight were taken as the animal model for immunosuppressed oral candidiasis, divided into four groups (n = 4/group) of immunosuppression

(IS), immunosuppression with oral candidiasis without treatment (ISC), immunosuppression with oral candidiasis and nystatin treatment (ISC+N), and immunosuppression with oral candidiasis and *A. ilicifolius* L. treatment (ISC+AI). Immunosuppression was performed by giving dexamethasone 0.5 mg/day and tetracycline 1%/day orally for seven days. On 8th day, we reduced the dose until 10% for dexamethasone and tetracycline, 1%. On 8th day, up to 20 rats were induced with 0.1 cc *C. albicans* ATCC-10231 1 McFarland, applied on the rats' tongues using sterile cotton bud three times a week for two weeks (Chami *et al.*, 2004).

Extract Preparation

Fresh leaves of the *A. ilicifolius* L. are taken from Wonorejo Mangrove Forest, on the east coast of Surabaya, Indonesia and were identified by the staff from Bioscience and Plant Technology Laboratory, Department of Biology, Institute Technology Sepuluh Nopember, Surabaya. Preparation for *A. ilicifolius* L. methanolic extraction methods based on Andriani *et al.* (2020). *A. ilicifolius* L. methanolic was made of the dried leaves that were ground to a coarse powder. About 1,000 g of *A. ilicifolius* L. powder was soaked with methanol 98% in Erlenmeyer flask covered with aluminium foil for 48 hours with occasional shaking and stirring. After that, it was filtered with Whatman's no.1 filter paper. The solvent was then evaporated at low pressure by using a rotary evaporator to get a viscous mass. Then it was evaporated with a water bath to separate the extract from the solvent. The methanolic extract was dissolved in 0.2% CMCNa to get a concentration of 20% (Andriani *et al.*, 2020).

Treatment and Examination

Nystatin as control group was applied on tongue surface, 0.5 cc at the same hours twice a day for 14 days as *A. ilicifolius* L. methanolic extract 20%. After treated for 14 days, the rats in each group were sacrificed and biopsied. The p38 MAPK expression was examined using the immunohistochemical staining method (p38 MAPK polyclonal antibody, Stressgen) and then observed under a light microscope with 400× magnification. The statistical analysis was performed by using one-way ANOVA to verify the significance of differences between groups, then least significant different (LSD) statistical test was used to verify the significance of differences between each group.

RESULTS

The expression of p38 MAPK on epithelial cells derived from the tongue of a subject is shown in Fig. 1. The immunosuppressed group (IS) had the lowest expression of p38 MAPK (26.00 ± 2.32) compared to ISC (26.40 ± 3.71), ISC+N (34.20 ± 0.99), and ISC+AI (36.05 ± 1.54), while the highest expression of p38 MAPK appeared in the group of subjects which had been induced with *C. albicans* and treated topically with 20% *A. ilicifolius* L. methanolic extract (ISC+AI) compared to other groups (Fig. 2). The data was normally distributed and homogeneous. A one-way ANOVA statistical test was utilised which indicated the significant differences between groups ($p < 0.05$). LSD statistical test confirmed significant differences existed between ISC+AI and ISC+N to IS and ISC ($p < 0.05$). No significant differences were present between IS and ISC; ISC+AI and ISC+N ($p > 0.05$).

DISCUSSION

The cellular signalling mechanisms against microorganism, in particular *C. albicans*, involve MAPK, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and phosphatidylinositide 3-kinase (PI3K) pathways (Naglik & Moyes, 2011). MAPK signalling via p38 appears to discriminate between yeast and hyphal cells of *C. albicans* (Mercado *et al.*, 2012). Recognition

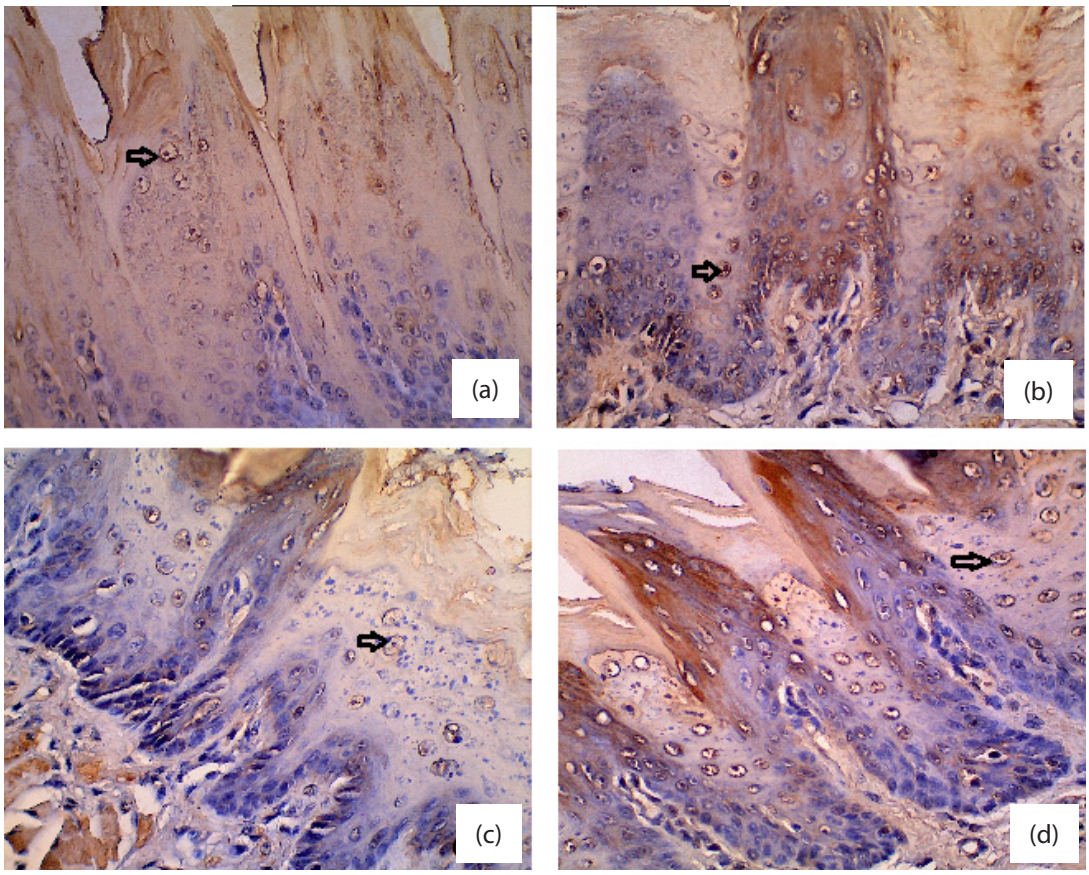

Fig. 1 The p38 MAPK expression on the epithelial cell of the tongue (black arrow). (a) Immunosuppression (IS) group; (b) Immunosuppression with oral candidiasis without treatment (ISC) group; (c) Immunosuppression with oral candidiasis and nystatin treatment (ISC+N) group; and (d) Immunosuppression with oral candidiasis and *A. ilicifolius* L. methanolic extract 20% treatment (ISC+AI) group (400× magnification).

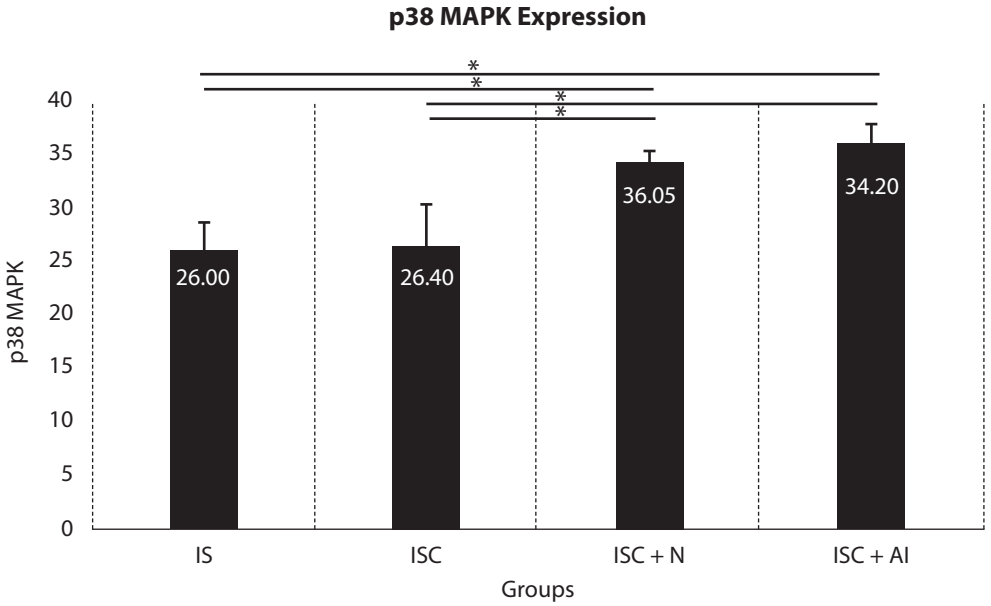

Fig. 2 The means of p38 MAPK expression on the epithelial cell of the tongue in each group.

mechanism of *C. albicans* of yeast and hyphal cells can occur via pathogen-associated molecular patterns (PAMPs), e.g., mannans or β-glucans, and pattern recognition receptors (PRRs), e.g., toll-like receptors (TLR) (Moyes *et al.*, 2010). Recognition via PRRs results to the activation of NF-κB, MAPK and PI3K signalling pathways. Signalling MAPK via p38 induced c-Fos transcription factor activation and together with NF-κB and PI3K signalling leads to cytokines and inflammatory mediators' productions, consequently activating the immune cells (Naglik & Moyes, 2011; Tang *et al.*, 2016). The IL-8 recruits neutrophils which is activated by members of the GM-CSF, G-CSF, and IL-1 family. Neutrophils protects directly through phagocytosis, and indirectly activates Th, including Th-17 which produces IL-17 and IL-22, that play a role in the protection mechanism against *C. albicans* (Moyes *et al.*, 2012).

Activation of p38 MAPK by *C. albicans* hyphae results in the upregulation of proinflammatory cytokines (Tang *et al.*, 2016). The p38 MAPK activation is also known to induce corticosteroid insensitivity. Activation of p38 MAPK can stabilise pro-inflammatory cytokines and chemokines transcripts (Mercado *et al.*, 2012). In this research, subjects were immunosuppressed through the administering of dexamethasone and combined with tetracycline orally to acquire an environment that supports the occurrence of oral candidiasis. Dexamethasone is one of corticosteroid drug that may affect the p38 MAPK expression.

The level of p38 MAPK expression immunosuppression group with or without oral candidiasis from this research was found similar and tend to be low when compared to the treatment group. Immunosuppression condition by corticosteroid may cause inactivation of the p38 via MAPK phosphatase 1. Glucocorticoid-induced MAPK phosphatase 1 dephosphorylates and inactivates all members of the MAPK family of proteins, including Jun N-terminal kinase, extracellular-signal-related kinase 1 and 2, and p38 kinase (De Bosscher *et al.*, 2003; Rhen & Cidlowski, 2005). This pathway is one of corticosteroid antiinflammation mechanisms and possibly increase the opportunistic pathogen, *C. albicans* to develop and invade the host.

The treated oral candidiasis group either nystatin or *A. ilicifolius* L. methanolic extract, the level of p38 MAPK expression was increased compared with the immunosuppression group with or without oral candidiasis. This result indicates that either nystatin or *A. ilicifolius* L. methanolic extract was involved in p38 MAPK activation. Baek *et al.* (2013) reported that treatment with nystatin resulted in elevated phosphorylation of ERK, p38 MAPK, and JNK, indicating activation of the three MAPKs by nystatin. The presence of nystatin possibly helps to dismiss infection via recruitment of inflammatory cells (Baek *et al.*, 2013). Previous studies have reported that *A. ilicifolius* L. leaves extract could increase the amount of receptor (TLR-2), cytokine proinflammatory (IL-22) and macrophage in oral candidiasis with immunosuppressive condition (Andriani & Pargaputri, 2018; Andriani & Pargaputri, 2019; Setyawan *et al.*, 2019). These properties are involved in the defence mechanism against *C. albicans*. The present study also reported a similar effect between the treatment group of nystatin and *A. ilicifolius* L. methanolic extract 20%. Previous studies of both nystatin and *A. ilicifolius* L. extract have similar effect in increasing the amount of TLR2 and IL22 expression in oral candidiasis immunosuppressed conditions models (Andriani & Pargaputri, 2018; Andriani & Pargaputri, 2019).

Methanol leaves *A. ilicifolius* L. at a concentration of 20% has the best antifungal activity (Andriani *et al.*, 2020). This extract contains flavonoid, tannin, steroid, saponin, terpenoid, glycoside and polyphenol (Gayathri & Gayathri, 2014; Andriani *et al.*, 2020). The 2-Benzoxazolinone (BOA) and benzoxazinoids which presents in methanol

leaves extract of *A. ilicifolius* L., promote antifungal and antioxidant activity (Saranya *et al.*, 2015). Methanol leaves extract of *A. ilicifolius* L. have strong antioxidant activity (Avijit *et al.*, 2012; Andriani *et al.*, 2020). Flavonoids and phenolic compounds that were isolated from methanolic leaves extract *A. ilicifolius* L. showed high antioxidant activity in rats. This extract also possessed considerable significant antinociceptive activity on experimental laboratory animals (Saranya *et al.*, 2015).

CONCLUSION

Methanolic extract *A. ilicifolius* L. treatment could enhance p38 MAPK expression in oral candidiasis with the immunosuppressed condition. Within the limitation of this study, it can be concluded that this extract might work as an antifungal and involved in the MAPK signalling pathway. However, a more in-depth study of this extract is needed, so that it can be used as potential antifungal drugs.

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