Mycobiology 31(4): 248-250 (2003)

Fungistatic Activity of Kojic Acid Against Human Pathogenic Fungi and Inhibition of Melanin-production in *Cryptococcus neoformans*

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(Received August 12, 2003)

Kojic acid was investigated for its antifungal activity against the human pathogenic fungi including *Candida albicans, Cryptococcus neoformans* and *Trichophyton rubrum.* For *C. albicans, C. neoformans* and *T. rubrum*, the MIC (minimum inhibitory concentration) of kojic acid was 640, 80 and 160 μg/ml, respectively. In *C. neoformans*, melanin-producing yeast, kojic acid-treated nonmelanized cell was more susceptible to magainin than melanized cell, suggesting melanin give a protective function against microbial peptide.

KEYWORDS: Antifungal activity, Kojic acid, Magainin, Melanin

Kojic acid [5-hydroxy-2-(hydroxymethyl-pyrone)] is a fungal metabolic product produced by fungi such as *Aspergillus* or *Penicillium* (Rosfarizan *et al.*, 1998). In animal, kojic acid inhibits the activity of catechol oxidation of phenol oxidase in the biosynthesis of skin pigment melanin (Cabanes *et al.*, 1994). Thus kojic acid has been widely used as a major skin-depigmenting agent in skin care cosmetic products. In Japan kojic acid is also consummed in diet with the belief that it is of benefit to health. Indeed it has been shown to enhance neutrophil phagocytosis and lymphocyte proliferation (Niwa and Akamatsu, 1991). However, studies in the antimicrobial activity of kojic aicd have been limited. In this paper, we evaluated the antifungal activity of kojic acid against medically important fungi. *Trichophyton rubrum* and *Candida albicans* are among the important human pathogenic fungi which cause several human fungal diseases such as athelet's foot, ringworm of the nail and cutaneous candidadiasis. *Cryptococcus neoformans* is a yeast-like fungal pathogen that cause life-threatening meningitidis in immunocompromised individuals, especially AIDS patients (Zuger *et al.*, 1986).

In *C. neoformans*, melanin-producing fungus, melanin pigment has been known to be associated with its virulence (Rhodes *et al.*, 1982). It was also reported that melanin formation was involved in decreased susceptibility of *C. neoforman* to UV light and enzymatic degradation as well as protection against heat and cold (Wang and Casadevall, 1994; Rosas and Casadevall, 1997, 2000). In this study we evaluated the effect of kojic acid on the melanin production of *C. neoformans* as well as its susceptibility against antifungal agent.

C. neoformans strain 7129, *T. rubrum* strain 6345 and *C. albicans* 7965 were obtained from KCTC (Korean Collection for Type Culture), and maintained on Sabouraud dextrose agar (SDA) at 26°C until the study was performed. Kojic acid and magainin were purchased from Sigma Chemical Co. Stock solution of the kojic acid (25 mg/ml) was prepared with distilled water and sterilized through a milipore filter with the pore size of 0.22 μm.

In disc diffusion test, sterilized Whatman filter paper disc of 6 mm diameter was impregnated with 400 μg/ml of kojic acid and placed over the center of surface of SDA plate seeded with fungal cells. The culture was incubated for 72 h at 26 and 37°C to obtain maximum growth for yeast cells in the culture media. The culture of *T. rubrum* was incubated for 6 days at 26°C. The diameter of inhibition was measured to estimate the degree of antifungal activity of kojic acid. In order to investigate the fungicidal activity of kojic acid, SDA was inoculated with yeast cell suspension using cotton swap. Plates were incubated at 26°C untill SDA was fully covered with growth of yeast cells. Paper disk impregnated with kojic acid (400 μg/ml) was placed on the surface of SDA plate. Plate was incubated for 48 hr at 26°C and observed for the appearance of clear zone around disk.

In order to estimate MIC (minimum inhibitory concentration), inoculum of *C. neoformans* and *C. albicans* was prepared. One loop of 3 day-old culture was suspended in malt extract broth. For *T. rubrum*, 10-day-old culture was flooded with sterile distilled water and were gently agitated to remove spores. Spore suspension was adjusted with distilled water to 1×10^6 spore per milliliter using haematocytometer. For the antifungal activity test, 800 μl of malt extract broth was dispensed into each well of 24 well plate. Then each well was inoculated with 100 μl of inoculum suspension prepared as above method. Final concentrations of kojic acid at a 20, 40, 80, 160, 320, 640, 1,280 μg/ml were added to each well. Plates was incubated at

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26°C for 24 h and 72 h for *C. albicans* and *T. rubrum*, respectively. In controls, sterile water was added to each well instead of kojic acid. For each set of experiment, four replicates were carried out. Inhibition of growth was determined by counting the number of yeast cells in case of *C. albicans* and by observing hyphal growth for *T. rubrum*. The minimum inhibition concentration (MIC) value was determined as the lowest concentration at which no growth of fungal cell was observed.

Isolates of *C. neoformans* were grown in defined minimal media composed of 15 mM glucose, 30 mM KH_2PO_4, 10 mM $MgSO_4$, 15 mM glycine, 5 uM vitamin B, pH 5.5 with or without 1.0 mM L-3,4 dihydroxyphenylalanine(L-dopa) at 26°C in a rotary shaker at 150 rpm. In order to investigate the effect of kojic acid on the melanization of the cells, culture was prepared in defined minimal media supplemented with L-dopa and kojic acid (40 *μg/ml*) and incubated under the same condition. Control culture was incubated in defined minimal media without L-dopa. After 8 days of incubaton, melanization of fungal cell was observed visually.

Isolate was grown in defined minimal media with L-dopa or without L-dopa as well as in defined minimal media supplemented with both L-dopa and kojic acid (40 *μg/ml*) at 26°C for 8 days. Equal concentration of inoculumn of each culture was prepared spectrophotometrically and placed into 24 well plate containing 1 *ml* of malt extract broth supplememted with magainin. Plate was incubated at 26°C for 24 h. Antifungal activity of magainin was determined by measuring optical density of culture at 540 nm.

Our results showed that kojic acid possess antifungal activity against human pathogenic fungi such as *C. albicans*, *C. neoformans*, and *T. rubrum*. Kojic acid significantly inhibited the growth of fungi on SDA. In disc diffusion assay, *C. neoformans* and *T. rubrum* were very susceptible to the kojic acid whereas *C. albicans* was less susceptible (Fig. 1). For *C. neoformans*, *T. rubrum*, and *C. albicans*, the MIC of kojic acid was 80, 160, and 640 *μg/ml*, respectively at 26°C (Table 1). For *C. albicans*, no significant difference of antifungal activity of kojic acid was observed between 37°C and 26°C. However, *C. neoformans* was more susceptible to kojic acid at 37°C than 26°C. We suspect that this might be related with the physiological state of *C. neoformans* at a higher tempaertature. In disc plate method, no clear zone around paper disc impregnated with kojic acid was formed. This result showed that kojic acid does not possess killing activity against fungal cell. The antifungal property of kojic acid against *T. rubrum* and *C. albicans*, common human fungal skin pathogen is important fact for cosmetic industry. Since kojic acid has been widely used as a depigmenting components in cosmetic products, the discovery of antifungal activity of kojic acid may provide additional usefulness of kojic acid-containing cosmetic products in that it possess antifungal effect against dermatophytes in addition to depigmenting effect.

Table 1. MIC (minimum inhibitory concentration) of kojic acid (*μg/ml*) (determined by microbroth culture assay)

Fugus / Temp.	*C. neoformans*	*C. albicans*	*T. rubrum*
26°C	80	640	160
37°C	40	640	N.D

N.D : Not done.

C. neoformans grown in minimal media with L-dopa became lightly melanized after 3 days incubation and heavily melanized by 8 days whereas isolates grown without L-dopa was not melanized during the course of incubation. Isolates grown in minimal media supplemented with both L-dopa and kojic acid was not heavily melanized after 8 days (Fig. 2). This result showed that kojic acid significantly reduced the melanization of *C. neoformans* at sub-MIC value. In *C. neoformans*, melanin production is thought to be a major virulence factor in that melanin-deficient mutants lose virulence and in that conversion of the melanin phenotype restore virulence

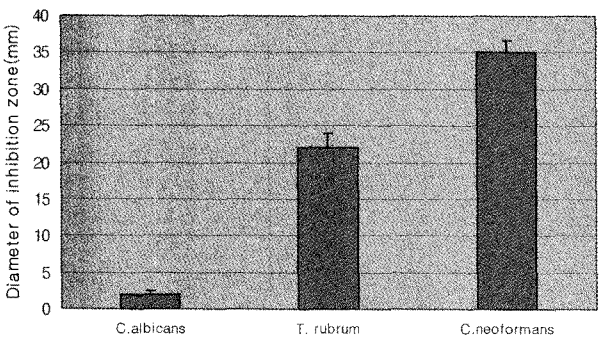

Fig. 1. *In vitro* antifungal activity of kojic acid (determined by diameter of inhibition zone).

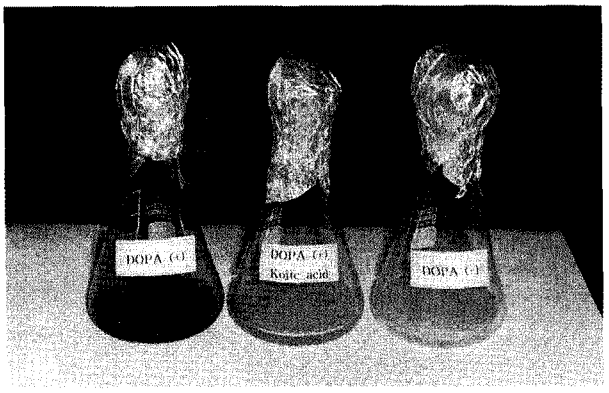

Fig. 2. Effet of kojic acid on melanization of *C. neoformans*. (left): DOPA supplemented. (middle): DOPA + kojic acid supplemented. (right): without DOPA.

(Rhodes *et al.*, 1982). In *Wangiella dermatitis,* melanin-deficient strain of fungus have reduced pathogenic effect in mice (Wheeler and Bell, 1988). It has been suggested that the melanin may aid in protecting *C. neoformans* against host immune system by scavenging leukocytic antimicrobial oxidant (Jacobson and Emery, 1991). Thus, these facts suggested that inhibitor for melanin synthesis in melanin-producing fungus could be potential target for antifungal agent., similar to the melanin inhibitors such as tricyclazole and pyroquilon which used to prevent rice blast disease caused by the plant pathogen, *Pyricularia oryzae*. In this study, our result may provide a possibility that the treatment of kojic acid inhibit melanin production and give a chance to reduce the virulence of *C. neoformans*.

Doering *et al.* (1999) revealed the ability of melanin in *C. neoformans* to protect against microbicidal peptides such as magainin and defensin. They suggested that protection of melanin against microbicidal peptide might be due to the binding ability of melanin to peptide. In this study, melanized cell was less susceptible to magainin than non-melanized cell. Kojic acid-treated cell grown in media with L-dopa was more susceptible to magainin than melanized cell. MIC of magainin against kojic acid-treated cell was 50 ng/*ml* whereas MIC of magainin against melanized cell was 200 ng/*ml*. Thus, our results are in accordance with the previous reports, suggesting that melanin give a protective function against microbicidal peptide.

The present study revealed that kojic acid diminish melanin formation of *C. neoformans* and melanin play a role in protecting against microbicidal peptide. Further studies for *in vivo* activity of kojic acid as a antifungal agent will be needed.

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