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Screening of kojic acid antimicrobial activity against skin pathogens

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ABSTRACT

The antibacterial and antifungal activity of kojic acid was evaluated against medically important skin pathogens. Tube Dilution Assay was used for analyzing of kojic acid minimum inhibitory concentrations (MIC), it was varied from 64 µg/ml to 128 µg/ml. There was no bacterial growth was appeared at MIC 64 µg/ml. Kojic acid inhibited the Trychophyton rubrum MTCC296 and Candidus albicans MTCC3018 growth at MIC 128 µg/ml. Disc diffusion method was chosen to elucidate the antimicrobial activity of the kojic acid. Kojic acid inhibitory zone observed was 17 mm, 14 mm, 12 mm and 9 mm, respectively against Pseudomonas aeruginosa MTCC1688, Trychophyton rubrum MTCC296, Streptococcus pyogenes MTCC1924, Candidus albicans MTCC3018, respectively.

Key words: Kojic acid, Antimicrobial activity, *Trichophyton rubrum, Candida albicans, Pseudomonas aeruginosa* and *Streptococcus pyogenes*

INTRODUCTION

The name "Kojic acid" was derived from the word "Koji", a fungus used as a starter inoculums in oriental fermented food products in japan. This crystalline substance was firstly isolated by Saito in 1907, from the mycelia of *Aspergillus oryzae* grown on steamed rice. The chemical structure was determined as 5-hydroxy-2-hydroxymethyl-δ-pyrone by Yabuta in 1924. Kojic acid crystallizes in form colorless and prismatic needles (Lin *et al.,* 1976). It is a biologically important natural antibiotic produced by various fungal strains such as *Aspergillus or Penicillium spp*. in an aerobic process from a wide range of carbon sources (Bentley, 2006). It plays an important role in iron-overload diseases such as β-thalassemia or anemia, since it possesses iron chelating activity (Brtko *et al.,* 2004). The primary application of kojic acid in market is in the cosmetic industry in which it plays key role in skin care treatments (Brtko *et al.,* 2004). Non cosmetic uses reported for

kojic acid include therapeutic uses for melasma, antioxidant and preservative in foods, antibiotic, chemical intermediate, metal chelate, pesticide, and antimicrobial agents (Chaudhary *et al.,* 2014).

The skin is the primary defense against invasion by bacteria, viruses, fungi and other toxic elements and is the largest organ. The superficial skin infections commonly encountered are of fungal and bacterial origin and the examples include *Trichophyton rubrum, Candida albicans, Pseudomonas aeruginosa* and *Streptococcus pyogenes. 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 (Nir-Paz *et al.,* 2003).

Hot tub folliculitis is a common type of folliculitis, a condition which causes inflammation of hair follicles. This condition is caused by an infection of hair follicles due to the bacterium *Pseudomonas aeruginosa*. Hot tub folliculitis appears on the skin in the form of a rash, roughly resembling chicken pox and then develops further to appear as a pimple, if the rash is aggravated, it can stay, worsen, and spread, lasting for months. By that time, it is much more difficult to treat (Kerr and Snelling, 2009). *Streptococcus pyogenes* is cause of many important human diseases, such as impetigo, cellulites, pharyngitis, cellulities and erysipelas, is an acute infection typically with a skin rash, usually on any of the face, fingers, legs, arms and toes. It is an infection of the superficial lymphatics and dermis, usually caused on infected areas throat infections associated with release of certain toxins lead to scarlet fever (Carapetis *et al.,* 2005).

It is crucial to monitor the changing trends in skin infections and their antimicrobial susceptibility pattern to provide suitable antimicrobial therapy for curbing infection, reducing morbidity and ameliorate the quality of life.

METHODOLOGY

Skin pathogens

Totally four important skin pathogens were procured from microbial typical culture collection (MTCC), Chandigarh and those are subcultured every month and maintained at 4°C in refrigerator. Four variant microbes were selected for analysing the antimicrobial activity of kojic acid.

1. *Pseudomonas aeruginosa* MTCC1688 (G^-).
2. *Streptococcus pyogenes* MTCC 1924 (G^+)
3. *Trichophyton rubrum* MTCC 296 (Fungus)
4. *Candida albicans* MTCC 3018 (Yeast)

Evaluation of in vitro susceptibility testing had been hampered due to lack of reliable in vitro techniques for testing of antifungal agents against dermatophytes. Various methods such as agar dilution, broth micro & macro dilution, E test, colorimetric micro dilution and disk diffusion have been available (Karaca and Koç, 2004, Niewerth *et al.,* 1998). A disk diffusion method to test yeasts has recently been standardised (NCCLS, 2004). The agar based disc diffusion (ABDD) susceptibility method for dermatophytes is quick, easy and a good option (Matar *et al., 2003)*.

Preparation of inculum

About 16 - 18hrs old culture of selected bacteria was mixed with physiological saline and turbidity was corrected by adding sterile physiological saline until a MacFarland turbidity standard of 0.5 (10^8 Colony Forming Units CFU/ml) reached. Respectively Inoculum suspensions of fungi and yeast were prepared from the seven days and two days cultures grown on sabouraud dextrose agar at 28°C. The fungal and yeast colonies were covered with approximately 10 mL of distilled water, and the suspensions were made by scraping the surface with the tip of a sterile loop. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to sterile tubes and left for 15 to 20 min at room temperature to sediment the heavy particles. The optical density of the suspensions containing conidia and hyphal fragments was read at 530 nm, adjusted to transmittance of 65 to 70% (10^6 cells/mL) (Santos *et al., 2006)*.

Minimum inhibitory concentration (MIC)

Kojic acid was isolated from *Aspergillus oryzae* RMS2 (GenBank accession number: KX756390) culture through submerged fermentation (Ranjit kumar and Jayalakshmi, 2017). Minimum inhibitory concentration of the kojic acid was tested by the Two-Fold serial Tube Dilution Assay (Karaca and Koç, 2004). The test kojic acid was dissolved in sterilized water to obtain 256 µg/ml stock solution. Then 1 ml of stock solution was incorporated in 1 ml of Muller Hinton broth for bacteria (5% ship blood was added for *Streptococcus pyogenes* MTCC 1924) and saboraud dextrose broth for fungi and yeast to get concentration of 256 µg/ml and serially diluted by double technique to achieve 128, 64, 32, 16, 8, 4, 2 and 1 µg/ml, respectively. Next 50 µl of

standardized suspension of the test organism was transferred to each tube. To the control tube kojic acid was not added and it contained only organism. The culture tubes were incubated at 37°C for 24 hrs (bacteria), for 48 hrs (yeast) and at 28°C for 5 days (fungi). Tube Dilution Assay was performed by constantly diluting the percent concentration of antimicrobial agent to microbial rich broth in a series of tubes. It is used to measure the Minimum Inhibitory Concentration [MIC] of an antimicrobial agent, which is the lowest concentration of antimicrobial agent that will inhibit the growth of microbes. The turbidity of the tubes indicates the amount of microbe growth, with the least turbid, or clear, tubes correlating with the absence of microbes. The tube with no antimicrobial agent presents as opaque and most turbid because the microbes are able to flourish. As antimicrobial concentration increases, the turbidity decreases until the MIC was reached and microbes can no longer survive. The lowest concentration which did not show any macroscopic growth of tested organism was determined as MIC.

Disc diffusion test

Disc diffusion test was tested for measuring the zone of inhibition (Matar *et al., 2003)*. Inoculum was drawn from the four isolated strains agar plate culture, and the inculum was transferred into a tube containing 4 to 5 ml of a respective broth medium. The broth culture is incubated at 35°C until it achieved the turbidity of the 0.5 McFarland standard. This results in a suspension containing approximately $1x10^8$ CFU/ml for bacteria and 1×10^6 for fungi. Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This was removed excess inoculum from the swab. The dried surfaces of a Müeller-Hinton agar plate (5% ship blood was added for *Streptococcus pyogenes* MTCC 1924) and saboraud dextrose agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. The lid was left aside for 3 min, to allow for any excess surface moisture to be absorbed before applying the kojic acid impregnated discs. The MIC of kojic acid concentrations (kojic acid at MIC 64 µg/ml for bacteria, kojic acid at MIC 128 µg/ml for yeast and fungi was impregnated on antibiotic free discs purchased from Himedia, kojic acid was not impregnated on control discs). The impregnated discs were dispensed onto the surface of the inoculated agar plates. Each disc was pressed down to ensure complete contact with the agar surface. The plates were inverted and placed in an incubator at 35°C after the discs were applied. After completion of incubation (24hrs for bacteria, 48hrs for yeast and 5days for fungi) each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the discs. Zones were measured to the nearest whole millimeter, using a ruler, which wass held on the back of the inverted petri plate. The petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light.

RESULTS AND DISCUSSION

After completion of incubation period ((24h for bacteria, 48h for yeast and 5days for fungi), that all of the microorganisms growth was checked on the antibiotic-free control plate. The MIC was defined as the lowest concentration of inhibitory compound at which there is no visible growth of the organism. Kojic acid minimum inhibitory concentrations was varied from 64 µg/ml to 128 µg/ml. Kojic acid inhibited the bacterial growth at MIC 64 µg/ml, the *Trychophyton rubrum* MTCC296 growth at MIC 128 µg/ml and *Candida albicans* MTCC3018 growth at MIC 128 µg/ml [Table-1].

Table-1: Kojic acid antimicrobial activity

Pathogen	Kojic acid MIC (µg/ml)	Zone of Inhibition (mm)
Pseudomonas aeruginosa MTCC1688	64	17
Streptococcus pyogenes MTCC1924	64	12
Trychophyton rubrum MTCC296	124	14
Candida albicans MTCC3018	124	9

Kojic acid zone of inhibition varied from 9 to 17 mm, kojic acid shown highest antimicrobial activity against *Pseudomonas aeruginosa* MTCC1688 [Figure 2(A)], with a zone of inhibition 17 mm, followed by *Trychophyton rubrum* MTCC296, with a zone of inhibition 14 mm [Figure 3(A)]. kojic acid shown moderate antimicrobial activity against *Streptococcus pyogenes* MTCC1924 with

a zone of inhibition 12 mm [Figure 2(B)]. It showed the lowest inhibitory activity against *Candidus albicans* MTCC3018, with a zone of inhibition just 9 mm [Figure 3(B)].

Figure 1: Pure crystals of kojic acid

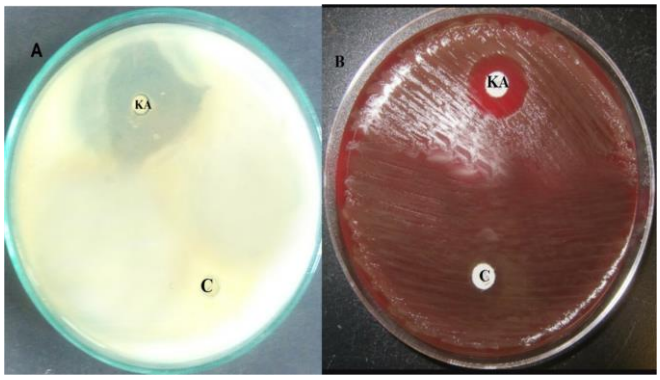

Figure 2: Disc diffusion testing; A) *Pseudomonas aeruginosa* MTCC1688, B) *Streptococcus pyogenes* MTCC1924,

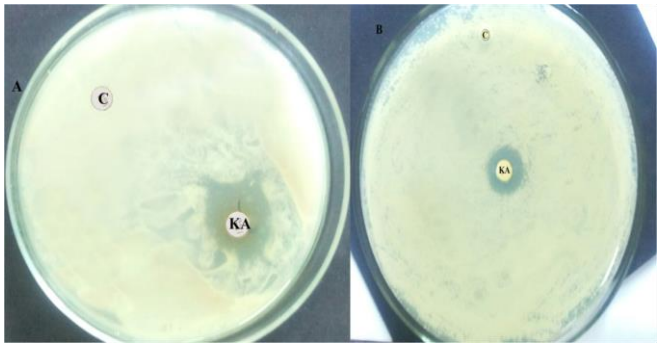

Figure 3: Disc diffusion testing; A)*Trichophyton rubrum* MTCC296, B) *Candida albicans* MTCC3018

The antibacterial and antifungal activity of kojic acid against medically important skin pathogens was evaluated in the present work, Among kojic acid was showing antimicrobial activity against all skin pathogens showing zone of inhibition more than 9 mm, with highest zone of inhibition 17 mm against *Pseudomonas aeruginosa* MTCC1688, followed by *Trychophyton rubrum* MTCC296 with zone of inhibition 14 mm, *Streptococcus pyogenes* MTCC1924 with zone of inhibition 12 mm and *Candida albicans* MTCC3018 with zone of inhibition 9 mm. Previous antimicrobial activity studies showed that kojic acid was more active against gram negative bacteria than gram positive ones, the present study also endorsed the same having strong antibacterial activity against gram negative bacteriaum (Bentley, 2006). *Pseudomonas aeruginosa* MTCC1688 and *Trychophyton rubrum* MTCC 296 shown high sensitivity with kojic acid.

Kojic acid possess antibacterial properties against gram-negative as well as gram-positive bacteria and also acted an antifungal agent (Hassan *et al.,* 2014). Kojic acid can potentially inhibit pathogen infection since: (1) it enhances host immunity by stimulating phagocytosis, generating reactive oxygen species (ROS) in macrophages, and potentiating phytohemagglutinin-based proliferation of lymphocytes; (2) Kojic acid directly exert antimicrobial activity against fungal/bacterial pathogens. For instance, kojic acid functions as an antifungal agent against Cryptococcus neoformans (cryptococcosis), where kojic acid also inhibits melanin synthesis necessary for fungal infectivity (Chee and Lee, 2003, Rodrigues *et al.,* 2011).

Kojic acid had been inhibited several genera of bacteria including *Escherichia, Salmonella, Pseudomonas, Bacillus, Staphylococcus, Klebsiella, Vibrio, Neisseria, Chromobacterium, Clostridium, Aerobacter, Corynebacterium, Diplococcus, Micrococcus, Pasteurella* and *Proteus (*Morton *et al.,*1945). Lee *et al.,* 1950 reported antituberculosis activity against of kojic acid grown under a variety of cultural conditions. Aytemir *et al.,* 2003 reported the MIC of kojic acid for *S. aureus* (MIC 256 µg/ml), *E. coli* (128 µg/ml) and *Candida albicans* (128 µg/ml). Durgadevi *et al.,* 2015 reported the zone of inhibition produced by kojic acid against cultures of *Staphylococcus aureus* and *Escherichia coli* (12 mm) and *Bacillus subtilis* (11 mm). The present study along with other studies showed that kojic acid can be used as an antibiotic and antifungal agent which in turn may have many commercial applications.

Kojic acid and its peptide derivatives has also been reported as potential antibacterial agents. Emami *et al.,* 2013 found novel mannich bases of 7-piperazinylquinolones with kojic acid and chlorokojic

acid showed significant effect as antibacterial agents. Particularly chlorokojic acid derivative was found to be most potent compound against *Staphylococcus aureus* and *Pseudomonas aeruginosa,* it showed activity about 4-8 times higher than standard drug norfloxacin. The factor that enhances the anti-microbial activity is attributable to the high hydrophobicity of the substituent at the end 7 position. Kojic acid greatly lowers minimum inhibitory concentrations (MIC) of commercial antifungal agents used in medicinal and agricultural field (i.e) activity of amphotericin B and strobilurin respectively against pathogenic yeasts and fungi. *A. fumigatus* causative agent of human invasive aspergillosis, which is being treated with H_2O_2 or amphotericin B indicated a chemo-sensitizing activity of kojic acid. Hence kojic acid can be applied as chemo-sensitizer to improve the efficiency conventional fungal drugs and it influence complex III inhibitors disrupting the mitochondrial respiratory chain in fungi (Kim *et al.,* 2013).

The antifungal and antibacterial property of kojic acid against common human skin pathogens is important for cosmetic industry. The incidence of dermatophytosis is increasing in recent times especially in paediatric population and in immuno compromised persons, although *Trichophyton rubrum* among other dermatophytes is a major causative agent for superficial dermatophytosis, it is known to cause deep infections in immuno compromised patients. Since kojic acid has been widely used as a depigmenting component in cosmetic products, the discovery of antimicrobial activity against skin pathogens deserves attention.

CONCLUSION

The antibacterial and antifungal activity of kojic acid was evaluated against medically important skin pathogens. Kojic acid minimum inhibitory concentrations was varied from 64 μg/ml to 128 μg/ml. There was no bacterial growth was appeared at MIC 64 μg/ml. Kojic acid inhibited the *Trychophyton rubrum* MTCC296 and *Candidus albicans* MTCC3018 growth at MIC 128 μg/ml.

Kojic acid inhibitory zone observed was 17 mm, 14 mm, 12 mm and 9 mm, respectively against *Pseudomonas aeruginosa* MTCC1688, *Trychophyton rubrum* MTCC296, *Streptococcus pyogenes* MTCC1924, *Candidus albicans* MTCC3018, respectively.

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