Isolation, Characterization and Biological evaluation of secondary metabolite from *Aspergillus funiculosus*

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Abstract Screening of *Aspergillus funiculosus* for bioactive secondary metabolites produced kojic acid, which is known to have wide range of biological properties. It is very active against Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli*, but moderately active against yeasts and Gram-positive bacteria except *Staphylococcus epidermidis*. Filamentous Fungi are more sensitive to kojic acid. When it exposed to larvicidal activity on *Aedes aegypti* third instar larvae are more sensitive than early fourth instar larvae.

Keywords *Aspergillus funiculosus* · Secondary metabolites · Kojic acid · Antibacterial · *Aedes aegypti* · Larvicidal

Introduction

Microbial natural products remain the most promising source of novel antibiotics. The impact of microbial biodiversity favors the chance of isolating new antibiotics [1–3]. Fungi are the most promising group of bioactive compounds producer [4]. The most well known examples of natural product are antibiotics [5]. Microbial natural products have also been developed as anti-diabetic drugs, hormone (ion-channel or receptor) antagonists, anti-cancer drugs, and agricultural and pharmaceutical agents [6].

Aspergillus funiculosus (NCIM 1029) was procured from National Collection of Industrial Microorganisms (NCIM), NCL, Pune. *Aspergillus funiculosus* grows rapidly on potato dextrose agar medium at a temperature of 27°C. After three days of incubation it produced colonies ranging 1–2 cm. The colonies were powdery in texture; an early stage of the colony was pale yellow in colour and turned to yellow after the formation of conidia. The microscopic features of the fungi include long hyphae bearing conidiophore and chains of conidia on it (Fig. 2). Cultivation of the fungus was carried out in 2000 ml of Erlenmeyer flask containing 1000 ml of potato dextrose medium. Culture flasks were incubated at optimized culture conditions (medium pH 7.0, temperature 27°C) for 3 days. Mycelial mat was removed and ethyl acetate was added to the medium (1:1 v/v). After thorough mixing, immiscible portion of the ethyl acetate (pale yellow colored) was separated from the medium. The mycelial mat was also washed twice with ethyl acetate. The separated ethyl acetate portion was rotaevaporated at 45°C.

The crude obtained was subjected to silica gel column chromatography and the eluted pure compound was analyzed on TLC plates. Kojic acid was tentatively identified

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by spraying the thin-layer plates with a 1% solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a 0.12 N HCl [7]. Further its structure was determined by NMR spectrum (Table 2 and Fig. 1).

The antibacterial and antifungal activities of the extract were evaluated by agar well diffusion method [8–9] against *Bacillus subtilis* (MTCC 619), *Bacillus sphaericus* (MTCC 511), *Staphylococcus aureus* (MTCC 737), *Staphylococcus epidermidis* (MTCC 435), *Escherichia coli* (MTCC 1687), *Pseudomonas aeruginosa* (MTCC 1688), *Pseudomonas oleovorans* (MTCC 617), *Klebsiella pneumoniae* (MTCC 109), *Candida albicans* (MTCC 227), *Saccharomyces cerevisiae* (MTCC 170), *Aspergillus niger* (MTCC 282) and

Rhizopus oryzae (MTCC 262), procured from IMTECH, Chandigarh. The inhibition zone diameter (IZD) was measured to the nearest millimeter. The minimum inhibitory concentration (MIC) was tested as per the NCCLS standards [10] against the bacteria.

Larvae of laboratory-reared strains of *Aedes aegypti* the late 3rd instar and early 4th instar stages were exposed to sub lethal concentrations of 150, 300 and 450 ppm of the crude extracts in distilled water for 24 h at room temperature ($32^\circ\text{C} \pm 2^\circ\text{C}$) according to standard WHO procedure [11] by dissolving the compound in acetone (99.8 %). For comparison commercial Malathion was used as positive

Fig. 2 Unstained wet mount preparation of *Aspergillus funiculosus* under magnification of $80 \times$ (A) and $1300 \times$ (B, C, D) using a Polyvar Compound Microscope attached to a CCD camera (Sony) with an aid of software (Easy-Grab; Noldus Information Technology).

Table 1 Antibacterial, antifungal and larvicidal activity of kojic acid

Microorganism	Zone of inhibition			MIC $\mu\text{g} / \text{ml}$	
	Kojic acid (50 $\mu\text{g} / \text{ml}$)	Kojic acid (100 $\mu\text{g} / \text{ml}$)	Control (30 $\mu\text{g} / \text{ml}$)	Kojic acid	Control- Nitrofurantoin
Gram +ve Bacteria			Penicillin-G		
<i>Bacillus sphericus</i>	12	15	20	200 μg	100 μg
<i>Bacillus subtilis</i>	12	15	20	200 μg	100 μg
<i>Staphylococcus aureus</i>	11	13	18	100 μg	50 μg
<i>Staphylococcus epidermidis</i>	16	19	18	100 μg	50 μg
Gram –ve Bacteria			Streptomycin		
<i>Pseudomonas aeruginosa</i>	19	23	34	50 μg	75 μg
<i>Pseudomonas oleovorans</i>	13	15	30	100 μg	75 μg
<i>Escherichia coli</i>	16	18	29	100 μg	50 μg
<i>Klebsiella aerogenes</i>	13	15	30	100 μg	50 μg
Fungi			Clotrimazole	Larvicidal activity LC_{50}	
<i>Candida albicans</i>	13	15	18	(Aedes aegypti)	
<i>Saccharomyces serviseae</i>	16	19	19	3rd instar	Early 4th instar
<i>Aspergillus niger</i>	17	20	22		
<i>Rhizopus oryzae</i>	17	21	23	204.51	271.64

Antifungal and antibacterial activity Inhibitory zone diameters are in mm.

All the concentrations are in ppm for larvicidal activity.

LC_{50} = Lethal concentration (ppm) at which 50 % of the larvae showed mortality.

Table 2 ^1H and ^{13}C NMR spectral data for Kojic acid

S.NO	^1H NMR	^{13}C NMR
1	6.35	139.5
2.	—	145.8
3.	—	174.2
4.	8.02	110.04
5.	—	168.30
6.	4.30(–CH ₂)	59.6
7.	–OH 9.05 (PHENOLIC)	
8.	–OH 5.72(–CH ₂)	

MASS: 142.0

control. 150 ppm of Malathion was prepared in 250 ml of water. The larvae were fed with dry yeast powder by sprinkling on the water surface. The dead larvae were counted after 24 h and percentage mortality was reported from the average for the two replicates taken together. A probit analysis using a computer program [12] was employed on the results to determine LC_{50} values.

The *invitro* antimicrobial (antibacterial, antifungal) and larvicidal results were summarized in the Table 1. The secondary metabolite isolated from *Aspergillus funiculosus*, kojic acid showed prominent inhibition against *Pseudomonas aeruginosa* and moderate activity against all other bacteria and fungi. The LC_{50} values for 3rd and 4th instar larvae of *Aedes aegypti* was 204.51 and 271.64 ppm respectively.

From the LC_{50} values the extract was found relatively more toxic to the 3rd instar larvae than 4th instar larvae.

The present study of screening bioactive secondary metabolites revealed that *Aspergillus funiculosus* as a new source for the production of kojic acid. Because of various commercial applications, by using strain-improvement techniques the rate of production of kojic acid can be improved from *Aspergillus funiculosus*.

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