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Exploring Endophytic *Aspergillus flavus* Isolated from *Callistemon citrinus:* Characterization, Kojic Acid Isolation, and Bioactivity Studies

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ABSTRACT

Objectives: Plant-associated fungi are a biodiversity-rich group of microorganisms that are normally found within plant tissues or in the intercellular spaces. Endophytic fungi are capable of producing important "phytochemicals" that displayed variable therapeutic activities. The current study aimed to isolate the endophytic fungi associated with *Callistemon citrinus* (L.) stem tissues and evaluate their potential therapeutic activity. *C. citrinus* is a well-known plant of family Myrtaceae, known as the 'red bottlebrush. **Methods**: The isolated endophytic fungus was characterized phenotypically and genotypically, subjected to large-scale fermentation and extracted with ethyl acetate to obtain a crude extract. The antimicrobial activity of the pure isolated compound was evaluated against *Propionibacterium acne* ATCC 6919 using agar well-diffusion assay. The MTT assay was utilized to evaluate the cytotoxicity of the crude extract against MCF-7 (human breast adenocarcinoma) cells. Apoptotic and necrotic investigation were applied. **Results:** *Aspergillus flavus* AUMC 16367 was isolated, characterized phenotypically and genotypically, and subjected to large-scale fermentation. Kojic acid, a well-known bioactive compound, was the major metabolite isolated and identified from *A. flavus* extract. *A. flavus* crude extract displayed strong cytotoxic activity against MCF-7 cell line showing IC_{50} value of 15.89±0.33 µg/ml and apoptotic and necrotic effects. Kojic acid exhibited no antimicrobial activity against *Propionibacterium acnes* ATCC 6919. **Conclusion:** Kojic acid was isolated from the fungal extract in significant amount constituting nearly 67% of the total weight of the crude extract. *A. flavus* crude extract displayed significant cytotoxic effects against MCF-7 and highlighting the potential therapeutic applications of this natural extract.

***Keywords**: Callistemon citrinus*, *Aspergillus flavus*, kojic, antimicrobial, cytotoxic.

INTRODUCTION

Current pharmaceutical industries rely heavily on the discovery of secondary metabolites found in natural resources to aid in the development of novel drugs with potential biological activity. Among the challenges, the exploration of endophytic microorganisms has emerged as a promising avenue for drug discovery, Endophytes, residing within the internal tissues of plants without causing disease symptoms, have

garnered attention for their ability to produce bioactive secondary metabolites with diverse pharmacological properties [1].

In this context, our study focuses on *Callistemon citrinus* (L.), commonly known as the 'red bottlebrush,' as a potential source of endophytic microorganisms and associated bioactive compounds [2]. *C. citrinus* (L.), a member of the Myrtaceae family, is recognized for its distinctive cylindrical, brush-like flowers and adaptability to various environmental conditions. *C. citrinus* is native to Australia; this plant is not only appreciated for its ornamental value but also for its traditional uses, including antimicrobial and antifungal properties and treatment of respiratory conditions such as coughs and bronchitis. Its essential oils have demonstrated significant biological activities, including antimicrobial, antifungal, and antioxidant effects, making it a valuable resource for further research on bioactive compounds and therapeutic applications [3,4]. Of particular interest are endophytic fungi, which have demonstrated remarkable bioactivity and chemical diversity, notably the genus *Aspergillus*, renowned for its diverse bioactivity and chemical richness [5]. *Aspergillus* species, abundant in various environments, have demonstrated potential in producing bioactive metabolites with therapeutic applications [6,7].

While previous studies have explored endophytic fungi from various plant species, the endophytic fungi of *C. citrinus* remains relatively unexplored. By filling this knowledge gap, our study contributes to the growing body of research on plant-associated fungi and their pharmaceutical relevance. Through the isolation and characterization of endophytic strains from *C. citrinus*, we aim to elucidate their potential in producing bioactive metabolites for pharmaceutical applications. Furthermore, our investigation extends to the isolation and characterization of the secondary metabolites from the crude extract of the identified endophytic strain.

Kojic acid, widely produced by *Aspergillus* species, possesses diverse applications in cosmetics, medicine, and other industries [8]. Despite its extensive use, comprehensive biological assays are lacking, necessitating further investigation into its antimicrobial. Considering this, our study aims to address this gap by conducting thorough biological assays to assess the therapeutic potential of both the crude extract derived from the fungi strain and kojic acid. The crude extract will undergo comprehensive evaluation to determine its anticancer activity, whereas kojic acid will be examined to ascertain its antimicrobial activity. Through the elucidation of their bioactivity profiles, our goal is to enhance our understanding of their pharmacological attributes, thus facilitating their potential application across various domains.

MATERIAL AND METHODS

Microbial Host material

The fungal strain was isolated from the stem tissues of (L.), *Callistemon citrinus*, which was collected from Helwan University Garden, Ain Helwan, Cairo, Egypt, in August 2022. Voucher specimen was labeled as 16Cci5/2024 and deposited in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Ain Helwan, Cairo, Egypt. The plant was identified by Dr. Therese Labib, Consultant of Botanical Gardens and Plant Taxonomy, Ministry of Agriculture.

Isolation of Endophytic Fungi

Endophytic fungi were isolated from the stem of *C. citrinus* (L.) following the method described by Tolulope *et al*., 2015 [9]. The plant samples (10 gm of fresh stem) underwent a series of surface sterilization steps, including washing with tap water, rinsing with distilled water, and sequential immersion in 70% ethanol for 30 seconds, 0.5% sodium hypochlorite (NaOCl) for 2–3 minutes, and another rinse with 70% ethanol for 2 minutes. Finally, they were washed multiple times with sterile distilled water. The sterilized plant materials were dried between sterile filter papers and then aseptically dissected. Three segments were placed onto petri dishes containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (500 mg/L). The petri dishes were sealed with Para film and incubated at 27°C for 3-6 days; the isolated fungus was purified by transferring hyphal tips to fresh PDA plates without antibiotics. Non-sterilized plant tissues were cultured as a positive control. The purified endophytic isolate was then transferred to PDA slants and maintained at 4°C until further use.

Phenotypic Characterization

Phenotypic characterization of the fungal isolate was preliminarily identified using cultural and morphological features such as colony growth pattern and conidial morphology [10].

Genotypic Identification of Fungal Strain

The cultures were grown on Czapek Yeast Autolysate agar (CYA) medium and incubated at 25°C for 7 days [11]. A small amount of mycelium was scraped, suspended in 100 µl of distilled water, boiled at 100°C for 15 minutes and finally preserved at -70°C to facilitate DNA extraction and sequencing. DNA extraction and sequencing were conducted by SolGent Company (Daejeon, South Korea). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using primers ITS5 (5' - GGAAGTAAAAGTCGTAACAAGG - 3') and ITS4 (5'

- TCC TCC GCT TAT TGA TAT GC - 3'), following the protocol by (Bruns et al., 1990).[12].

The amplification conditions comprised an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 40 seconds, and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 5 minutes. Purification of the PCR product was achieved using the SolGent PCR Purification Kit-Ultra, and confirmation of the product's purity was ascertained through electrophoresis on a 1% agarose gel.

Large-Scale Fermentation

The cultivation process of the isolated fungus involved using potato dextrose broth, consisting of 20% potato and 2% glucose [13]. Initially, the strain was cultured on potato dextrose agar (PDA) medium and then incubated at 28°C for 5 days. Following this, mycelial agar plugs (each measuring 0.5 × 0.5 cm^2) were inoculated into 500 ml Erlenmeyer flasks containing 250 ml of potato dextrose broth. After 4 days of incubation at 28°C on a rotary shaker set to 120 r/m, 25 ml seed cultures were aseptically transferred into larger 1000 ml flasks containing 500 ml of potato dextrose broth. This liquid cultivation was maintained for 14 days at 28°C and 120 r/m on a rotary shaker, as described by Liu, *et al*., 2019 [14]. To remove the spores and mycelia, the strain was subjected to filtration, and the resulting filtrate was concentrated to approximately 10% of its original volume using a vacuum rotary evaporator at 40-45°C. The concentrated filtrate underwent extraction three times using liquid-liquid partition with ethyl acetate (EtOAc) solvent in a 1:1 ratio (v/v). The crude extracts were then collected and concentrated to dryness using a vacuum rotary evaporator at 40-45°C, following the method outlined by Jantarach *et al*., 2010 and Pan *et al*., 2016 [15,16] .

Isolation and Purification of the fermentation Broth

During concentration of the extract (6 g) using a rotary evaporator, white crystals precipitated out in significant amounts, (4 g) constituting nearly 67% of the total weight of the crude extract. Subsequently, the precipitate underwent washes to remove impurities. The resulting precipitate was then dissolved in dichloromethane and subjected to recrystallization using acetone.

Instruments and materials for chromatographic techniques

1D and 2D NMR spectra (^{1}H NMR, ^{13}C NMR, APT, HMBC) were recorded at (Nuclear magnetic resonance unit - Central Laboratory-Helwan university, Ain Helwan, Cairo, Egypt.) using Bruker a 500 MHz for ^{1}H NMR and 125.40 MHz for ^{13}C NMR. The spectra were run in DMSO, and chemical shifts were given as δ ppm relative to tetramethyl silane (TMS) as an internal standard.

Materials used for biological activity studies

Antimicrobial activity

Tested microorganisms: *Propionibacterium acnes* ATCC 6919 obtained from the Regional Center for Mycology and Biotechnology at Al-Azhar University, Cairo, Egypt. Gentamycin (Garamycin, MUP for Schering-Plough, Egypt).

Susceptibility Test

Agar well-diffusion assay was applied [9]. The procedure commenced by inoculating *Propionibacterium acne* onto Mueller Hinton agar plates using sterile cotton swabs. Wells, approximately 6 mm in diameter, were then created in the agar using a sterile cork borer. The initial solution of kojic acid was prepared by dissolving 10 mg in 1 mL dimethyl sulfoxide to obtain concentration of 10 mg/mL. Subsequently, 100 µl of kojic acid solution was introduced into each well. Following an incubation period at 37°C for 24 hours, the plates were examined for the presence of inhibition zones. The sensitivity of the bacterium to kojic acid was determined by measuring the diameter of any inhibition zones observed. These results were then compared with those obtained using the standard antibiotic gentamicin, which served as the control in the experiment.

Evaluation of cytotoxic activity

Materials for cytotoxicity assay

Cancer cell line

MCF-7 (Human breast adenocarcinoma) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA).

Culture media

RPMI -1640, (Rockville, MD, USA). MCF-7 Cells were maintained in Roswell Park Memorial Institute (RPMI -1640) media supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells inoculated in a humidified atmosphere with 5% CO_2 at 37 °C [17,18].

Sub-culturing: monolayer cells of the third or fourth generation when confluence had reached 90 % were taken for the experiment after examination under inverted microscope were harvested after trypsin/EDTA treatment at 37°C [19].

96-well culture plates with a clear bottom (Rockville, MD, USA), Trypan blue dye (Sigma-Aldrich, St. Louis, MO, USA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA), prepared as solution, 5mg/ml of MTT in 0.9%NaCl, Acidified Isopropanol (Thermo Fisher Scientific, Waltham, MA, USA)

prepared as 0.04 N HCl in absolute isopropanol, Doxorubicin (standard reference drug as a positive control) (Sigma-Aldrich, St. Louis, MO, USA), Dimethyl sulfoxide (DMSO) as a negative control in MTT assay (Sigma-Aldrich, St. Louis, MO, USA), Phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA).

Method of Metabolic Cytotoxicity by using MTT assay

The metabolic cytotoxicity of the *Aspergillus flavus* AUMC 16367 crude extract was evaluated against the MCF-7 cell line using the MTT assay. This assay exploits the ability of mitochondrial dehydrogenase enzymes in living cells to convert the yellow tetrazolium salt (MTT) into dark blue formazan crystals, which accumulate in viable cells[17]. The level of formazan formation, detected by measuring absorbance at 570 nm, directly correlates with cell viability[20].

The cancer cells were cultured until reaching 90% confluence, harvested using 0.025% trypsin, and their viability was assessed using trypan blue dye with an inverted microscope. Viable cells were then seeded into 96-well microtiter plates at a concentration of $5x10^4$ cells per well in fresh medium and allowed to attach for 24 hours. The crude extract of AUMC 16367, along with the standard drug doxorubicin, was prepared at an initial concentration of 1 mg/ml in DMSO. These solutions were further diluted to working concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, and 12.5 µg/ml before being added to the cell cultures.

Each concentration was added to three wells, while control cancer cells were treated with DMSO. Following 48-hour incubation at 37°C in 5% CO_2, the cells were treated with 40 µl MTT solution (5 mg/ml in 0.9% NaCl) for 4 hours. After the incubation, unbound dye was removed by washing with phosphate-buffered saline (PBS). The formazan crystals were then solubilized by adding 180 µl of acidified isopropanol to each well. The absorbance was measured, and the results were normalized to control values. The IC50 value, representing the concentration required to inhibit 50% of cell viability, was determined and compared to the cytotoxic effect of doxorubicin[17,21]

Materials for Apoptotic and necrotic investigation

Double nucleic acid-binding dyes, acridine orange (AO), and ethidium bromide (EB) (Sigma-Aldrich, St. Louis, MO, USA).

Apoptotic and necrotic investigation

MCF-7 cells were cultured and treated with a 10 µg/ml concentration of crude extract. To evaluate apoptosis and necrosis, the cells were stained with a mixture of acridine orange (AO) and ethidium bromide (EB). AO, which permeates all cells, causes live cells to exhibit a green nucleus. In contrast, EB is taken up only by cells with compromised membranes, resulting in a red nucleus. Apoptotic cells showed condensed and fragmented chromatin, which appeared green to yellow-green, reflecting various stages of apoptosis. Cells undergoing necrosis displayed a structurally intact but orange to red nucleus. The percentages of apoptotic and necrotic cells were calculated by counting the stained cells, providing insight into the cytotoxic effects of the crude extract [22].

Statistical analysis

Data was analysed using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test. The experimental results were expressed as a mean, ± Standard deviation (SD). The difference between groups were considered significant when $p<0.001$. All analyses were performed using Graphpad Prism software (San Diego, CA. USA) software.

RESULTS

Phenotypic Characterization of Isolate

Colonies appeared greenish yellow on PDA, (**Figure 1**). Microscopically, the specimen displayed a conidiophore with an elongated, rough, and thick -walled structure accompanied by a larger conidial head, additionally, it featured a globose shape with thin, rough-walled conidia (**Figure 2**).

Figure 1. Fungal strain AUMC 16367 culture (7-days age) on potato-dextrose agar (PDA).

Figure. 2. Light microscopy of sporulating hyphae (1000×) of AUMC 16367 showing conidia and conidiophore morphology.

Genotypic Identification Results

The fungal sequence data has been officially submitted and cataloged in the GenBank database. Specifically, the AUMC 16367 strain, isolated from *C. citrinus* (L.), has been assigned accession number OR984863. Further analysis revealed that the AUMC 16367 strain exhibits a high degree of similarity, ranging from 99.18% to 99.50%, with various strains of the same species. Notably, this includes a significant match with *Aspergillus flavus* strain ATCC 16883, with a GenBank accession number of KU729026.

Characterization and identification of isolated compounds

The purified crystalline metabolite was subjected to NMR analysis and results compiled in **Table 1**. The ^{1}H NMR data together with ^{13}C NMR data and HMBC spectrum found in good agreement with data published before; thus the compound was confirmed to be kojic acid (**Figure 3**) [23,24].

Table 1: ^{1}H NMR (400 MHz), ^{13}C NMR and APT (100 MHz), H-H COSY and HMBC spectroscopic data for kojic acid in DMSO.

position	δ_H (mult, *J* in HZ)	δ_C (APT)	HMBC
1			
2		168.56 (C)	
3	6.39 (1H, S)	110.28 (CH)	C-2, C-4, C-5, C-6, C-7
4		174.40 (C)	
5	8.06 (1H, S)	146.15 (C)	
6	4.34 (2H, S)	139.70 (CH)	C-2, C-3, C-4, C-5, C-7
7		59.93 (CH_2)	C-2, C-3

^{1}H NMR (500 MHz); ^{13}C NMR (125 MHz); J-value is expresses in Hz between parentheses.

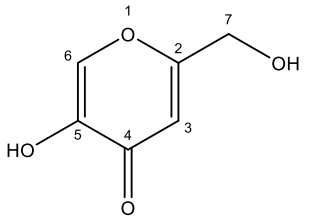

Figure 3. Structure of kojic acid

Antimicrobial Activity:

Results are compiled in **Table 2**. Results showed that kojic acid exhibited no antimicrobial activity against *Propionibacterium acnes* ATCC 6919, as indicated by the absence of an inhibition zone. In contrast, the positive control, gentamicin, produced a substantial zone of inhibition measuring 25 mm.

Table 2. Antimicrobial Activity of kojic Acid and Gentamicin against *Propionibacterium acnes* ATCC 6919

Tested microorganisms	**Kojic acid**	**Control (Gentamicin)**
Propionibacterium acnes ATCC 6919	NA	25 mm

Cytotoxic Activity

The cytotoxic activity of *Aspergillus flavus* AUMC 16367 crude extract against the MCF-7 cell line was evaluated using the MTT assay. The IC_{50} value of the crude extract was determined to be 15.89±0.33 µg/ml. In comparison, the standard drug doxorubicin demonstrated IC_{50} value of 3.50±0.45 µg/ml (**Figure 4**).

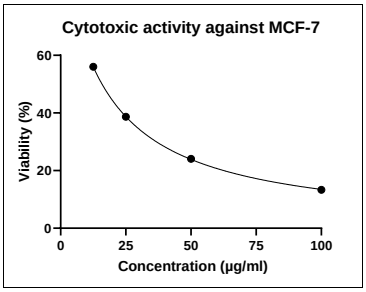

Figure 4. Dose-response curve of *Aspergillus flavus* AUMC 16367 extract against MCF-7 cells.

Staining of Apoptotic and Necrotic Cells

The staining assay employing acridine orange/ethidium bromide (AO/EB) was utilized to assess the cytotoxic effects of the *Aspergillus flavus* AUMC 16367 crude extract on MCF-7 cells, focusing on the percentage of apoptotic and necrotic cells. This assay provides valuable insights into the cellular morphology and viability alterations induced by the fungal extract.
The results revealed that treatment with the *Aspergillus flavus* AUMC 16367 extract led to a notable decrease in the number of normal cells, accompanied by a moderate increase in apoptotic cells and a significant elevation in necrotic cells **Fig. 5**. Conversely, doxorubicin, the standard cytotoxic agent, exhibited a predominant necrotic effect with minimal observable normal or apoptotic cells **Fig. 6**. These findings underscore the potent cytotoxicity of doxorubicin against MCF-7 cells, consistent with its established efficacy.

The AO/EB staining results suggest that the cytotoxic mechanism of the fungal extract primarily involves necrosis, with a concurrent induction of apoptosis. This dual mode of cell death induction may be attributed to the presence of bioactive compounds within the extract, which could disrupt cellular integrity and induce programmed cell death pathways.

Further elucidation of the specific bioactive constituents responsible for the observed cytotoxic effects, as well as their underlying mechanisms of action, is warranted. Additionally, exploring the synergistic interactions between different compounds present in the fungal extract may provide valuable insights into optimizing its cytotoxic potential against breast cancer cells.

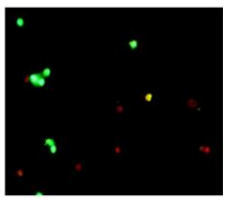

Figure 5. Apoptosis staining of MCF-7 cells Treated with *Aspergillus flavus* AUMC 16367 extract.

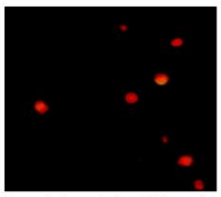

Figure 6. Apoptosis staining of MCF-7 cells treated with doxorubicin.

DISCUSSION

Endophytes, microorganisms residing within plant tissues, are recognized for their ability to produce bioactive compounds. A prominent example is kojic acid, which is produced in large quantities by fermenting *Aspergillus* species [25,26]. Kojic acid, or 5-hydroxymethyl-2-pyrone, has a wide range of applications: it is used in cosmetics for skin-whitening and UV protection, in medicine for its anti-inflammatory and analgesic properties, and in the food industry as an anti-browning agent [8]. Despite these diverse uses, its effectiveness against *Propionibacterium acnes*, a common acne bacterium, has not been thoroughly investigated, presenting an opportunity for further research. So, we deemed it of interest to evaluate its antimicrobial activity. Results showed the lack of antibacterial activity against *Propionibacterium acnes*, which indicates that it may not be effective as a treatment for acne through antibacterial mechanisms. These findings highlight the specificity of kojic acid's biological activities and suggest that its primary applications should remain focused on pigmentation and cosmetic uses rather than antibacterial treatments. In addition to their antimicrobial potential, endophytes are significant sources of novel anticancer compounds due to their ability to produce bioactive substances with notable cytotoxicity against cancer cells[27]. This capacity underscores their value in developing cancer therapies and drug discovery. For instance, the diterpenoid paclitaxel (taxol), derived from endophytes, demonstrates a unique mechanism of action compared to other anticancer agents, showcasing the potential of these microorganisms to produce effective and distinctive anticancer agents[28]. In our study, we extended this exploration by evaluating the cytotoxic effects of *Aspergillus flavus* AUMC 16367 crude extract against the MCF-7 cell line. The results indicated a significant cytotoxic effect with an IC_{50} value of 15.89±0.33 µg/ml, in comparison; the standard drug doxorubicin demonstrated an IC50 value of 3.50±0.45 µg/ml. suggesting potential therapeutic value in anticancer applications. Further investigation using the AO/EB staining assay indicated that treatment with *A. flavus* AUMC 16367 crude extract led to a notable decrease in normal cells, with a moderate increase in apoptotic cells and a significant elevation in necrotic cells. This dual mode of cell death suggests that the fungal extract's cytotoxic mechanism involves both necrosis and apoptosis. These findings align with the broader understanding of endophytes as producers of effective and distinctive anticancer agents, reinforcing the importance of exploring these microorganisms for innovative therapeutic options.

CONCLUSION

Aspergillus flavus AUMC 16367 was isolated from *Callistemon citrinus* (L.) stem tissues. Kojic acid was isolated from the fungal extract in significant amount constituting nearly 67% of the total weight of the crude extract. It didn't display activity against *Propionibacterium acnes*. *A. flavus* crude extract displayed significant cytotoxic effects against MCF-7 showing apoptotic and necrotic activity.

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Conflict of interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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