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First report of *Alternaria alternata* associated with seed of Asafetida (*Ferula assa-foetida*)

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ABSTRACT

Kojic acid is a fungal metabolic product produced by a few species of Aspergillus, especially by A. oryzae, which has the Japanese common name koji. This compound is an inhibitor of growth of bacteria and multiplication of viruses. In this study, kojic acid derivative, Kojic acid glucoside, was evaluated as DNA gyrase activity inhibitors. DNA gyrase has long been known as an attractive target for antibacterial drugs. In order to investigate the mode of interaction of the compound with DNA gyrase active site, the chemical structures of kojic acid glucoside wase designed using ChemDraw program, then transferred into Hyperchem software for energy minimization. Docking study was performed by AutoDock 4.2 program and the resulting docking poses were analyzed in AutoDockTools, DS Visualizer 3.5 and Ligplot software. Binding model and the best docked pose of this compound showed Kojic acid glucoside formed a hydrogen bond with Asp73, Asn46, Glu50, Thr165, Val71, Arg136 of DNA gyrase in active site. The insilico molecular docking study results showed that, Kojic acid glucoside have minimum binding energy and good affinity toward the active pocket, thus, this may be considered as inhibitor of DNA gyrase.

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Introduction

Asafetida (Ferula assa-foetida belonging Apiaceae family), is an important spice crop with medicinal properties and native to central Asia, eastern Iran to Afghanistan. In previous studies, antioxidant (Kavoosi and Rowshan 2013; Dehpour et al., 2009), antimicrobial (Bhatnager et al., 2015; Divya et al., 2014), antifungal (Rani et al., 2009; Kamble and Patil, 2008) and antiviral (Mahendra and Bisht 2012) effects of this plant have been demonstrated. To date, no fungal pathogens have been reported on F. assa-foetida.

Materials and Methods

In May 2020, samples of 400 seeds from each of Asafetida seed crops in Shiraz, Mashhad, Isfahan and Yasuj, Iran were tested for germination on moistened paper in petri dishes. The germination test was conducted in four replications of 100 seeds each sample by using paper (between papers) medium and was incubated at 25 ± 1 ˚C temperature and 85 ± 5 % relative humidity for 14 d. Different seed health testing methods viz standard blotter and agar plate tests were used to investigate the fungal pathogens present in these seeds and their effect on germination (ISTA 2013). The seeds were surface-sterilized in 1% sodium hypochlorite solution for one minute and rinsed with sterile distilled water. The collected seeds samples were plated in petri dishes containing sterile wet filter paper or potato dextrose

agar (PDA) medium and incubated for 7 days at 25±1 ˚C under alternating cycles of 12 hours light and darkness. Among different Asafetida samples in the investigated regions, fungi-infected seeds was detected only in sample Shiraz.

The emerged fungi were picked up, purified and identified based on morphological and molecular characteristics. Pure cultures were obtained by using single spore and hyphal tip methods at 23 ± 1 ˚C on 2% water agar (2% WA) and 0.05% potato dextrose agar (0.05% PDA) media. For morphological identification, the isolates were cultured on Potato Carrot Agar medium (PCA) and incubated at 25 ± 1 ˚C under cool-white fluorescent illumination with 16 h dark, 8 h light photoperiod for 14 d (Simmons, 2007). Preliminary identification of fungal isolates was carried out by using standard keys as described by Barnett and Hunter (1998), and Simmons (2007).

Further confirmation of the identification was obtained by molecular characterization in which genomic DNA was extracted using DNA extraction kit (Genomic DNA isolation kit IV; DENA Zist Asia, Iran) according to the manufacturer's instructions. To verify the identity, the internal transcribed spacer (ITS) of rDNA was amplified and sequenced using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), specifically designed for *A. alternata* (Zheng et al., 2015) and sequenced by

Macrogen (Korea). Polymerase chain reaction (PCR) products were sequenced and the identity of fungal isolates confirmed by a BLAST search on the GenBank database. PCR identification for the isolate was confirmed using AaltFor (5′-GTGCCTTCCCCCAAGGTC TCCG-3′) and AaltRev (5′-CGGAAACGAGGTGGTTCA GGTC-3′) primer specific as the *A. alternate* which produced fragment size of approximately 184 base pair (bp) (Kordalewska et al., 2015). In order to determinate if *A. alternata* was the possible cause of discolored seeds and abnormal seedlings and also to confirm Koch's postulates, the fungal isolate was subjected to pathogenicity tests on seeds and seedlings. Asafetida seeds were inoculated with a conidial suspension (1×10^5 conidia mL^{-1} amended with 0.05% Tween 20) and incubated for 10 d at 25±2 °C in a growth chamber under 12 h photoperiod. For pathogenicity test on seedlings, 1000 µl of spore suspension (1×10^5 conidia mL^{-1} amended with 0.05% Tween 20) was sprayed onto the leaves of seedlings at the two- to three leaf stage. Plants were covered by polyethylene bags for 48 hours, maintained in a greenhouse at 25 ± 2 °C and 90-100% humidity. After 72 hours of incubation, the appeared chlorosis and blighting developed on the leaves of inoculated plants, whereas control plants remained asymptomatic. The pathogenicity tests were

repeated twice under the same conditions. After germinating the seeds, 19% of the resulting seedlings were abnormal (deformed and diseased), discolored, and most of them (average of 17% of all seedlings) died shortly after emergence. The mean frequency of fungi recorded was found to be significantly highest in agar plate test (19%) when compared to standard blotter test (15%). All the 19 fungal isolates isolated were similar in morphological features.

Results and Discussion

The fungi isolated were initially light grey in color and changed to dark green, and then the whole upper surface of the colony turned black after 3 days of incubation on PDA medium. The color of the colony was gray-olive green on PCA medium. Conidiophores that developed singly or in clusters, were light brown to brown, were long or short being either ramified or simple and measured 33-94 µm in length and 4-10 µm in width. Conidia were produced in acropetal chains on conidiophores, light olivaceous to dark brown, varied in shape from ovoid to ellipsoidal with 4-7 transverse septa and 0-3 longitudinal septa, and measured 18-30 µm in length and 6-11 µm in width (Fig. 1). Based on morphological characterization, the fungi isolated from seed were tentatively identified as *A. alternata* (Simmons, 2007).

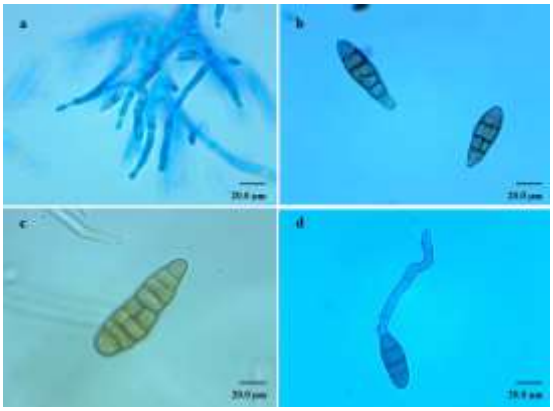

Fig. 1. *Alternaria alternata* isolate AANKH 568 from 10-day-old colony on PCA (a) conidiophores with conidia developing at the tips, (b, c) mature conidia, (d) germinating conidium. Scale bars 20 µm.

Ten days after inoculation, the seedlings inoculated with fungal isolate showed chlorosis and blight symptoms. The symptoms on the tissues resembled those observed in the field. No symptoms were detected on the controls. *A. alternata* was recovered from the diseased tissues but not from the control. Fungal isolate recovered from seedling was identified based on morphological characteristics including cultural morphology, size and shape of conidia, and sporulation patterns and compared with the initial isolate.

This combination of data confirmed that the pathogen was *A. alternata*.

A. alternata was re-isolated from the seeds and seedling exhibited the similar morphology and molecular of the earlier described one and also proving pathogenicity and confirming Koch's postulates. The results showed that the *A. alternata* causes seed discoloration, shrivelling, reduction in seed germination and increase in the

number of abnormal (deformed and diseased) seedlings. The isolate of *A. alternata* has been lodged in the culture collection of the Seed Health Laboratory, the Seed and Plant Certification and Registration Institute (SPCRI) in Iran with the accession number AANKH 568.

The fungus *A. alternata* is one of the most common pathogens and usually reduce the quality of seed that experience stress during the ripening period, particularly from excessive rain and delayed harvest. This Alternaria-infected seeds is may be attributed to cultures of the sensitive native population, varying climate and environmental conditions, crop rotation with vegetables and also sensitive crops in the Shiraz region of Iran.

Further studies should be considered to determine potential yield loss caused by *A. alternata*, as well as control strategies to limit the spread of this pathogen. However, some management strategies, such as crop rotation with non-host crops, field sanitation, seed treatment with fungicides and use of healthy seeds may be helpful reducing disease severity. *A. alternata* is seed-borne and seed-transmitted pathogen in Asafetida, and it is introduced into new regions through the movement of *A. alternata*-infected seed. To our knowledge, this is the first report of *A. alternata* has been isolated from Asafetida seeds produced in world.

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