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Identification of secondary metabolites from *Alternaria* spp. In Afghanistan

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Abstract
Secondary metabolites of fungi have low molecular weight which play a significant role in agriculture during pre- and post-harvest stages. *Alternaria* is one of the fungi that produce toxins and have a major impact on plant pathogenesis. The aim of this study was to find secondary metabolites from *Alternaria* fungi on tomato plants. In this study, *Alternaria* spp. was isolated from tomato fields in the Jalriz district of Maidan Wardak province, Afghanistan. After isolation and purification, the fungus was cultured on wheat seeds to stimulate secondary metabolite production. Following 21 days of cultivation, ethyl acetate was employed to extract the secondary metabolites, which were subsequently dissolved in N-hexane and analyzed using gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis revealed the presence of thirteen chemical compounds, including Tetradecane 2,6,10-trimethyl, Heptadecane 2,6,10,15 tetramethyl, Heptacosane, Docosane, Heneicosane, Bis(2-ethylhexyl) phthalate, Phenol 2,4-bis(1,1 dimethylethyl) phosphite (3:1), Cardamonin bis(tert-butyldimethylsilyl) ether, N-Hexadecanoic acid, 1-Hexadecanol 2-methyl, Squalene, Eicosane, and 3-(Benzylthio) acrylic acid, methyl ester. All the identified compounds from *Alternaria* exhibit varying effects on plants at different stages of their life cycle.
Keywords: *Alternari,* fungus, Secondary metabolites

شناسایی میتابولایت های ثانویه از جنس قارچ لترناریا در افغانستان

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میتابولایت‌های ثانویه قارچ‌ها وزن مالیکولی پایینی دارند که در مراحل قبل و بعد از ذخیره سازی محصولات نقش مهمی دارند. الترناریا یکی از قارچ‌هایی است که زهر تولید می‌کند و تأثیر زیادی بر مرض‌زایی نباتات دارد. هدف از این مطالعه، یافتن میتابولایت‌های ثانویه قارچ الترناریا در نبات بادنجان رومی بود. در این مطالعه، Alternaria spp. از مزارع بادنجان رومی در ولسوالی جلریز ولایت میدان وردک افغانستان جدا شده است. پس از جداسازی و خالص سازی قارچ برای تحریک تولید میتابولایت ثانویه بر روی دانه گندم کشت شد. پس از ۲۱ روز از کشت، ایتایل اسیتیت برای استخراج میتابولایت‌های ثانویه استفاده شد، که متعاقباً درنایتروجن-هگزان حل شده و با استفاده از کروماتوگرافی گازی - طیف سنجی کتلوی (GC-MS) تجزیه و تحلیل شدند. تجزیه و تحلیل با استفاده از GC-MS موجودیتی سیزده ترکیب کیمیاوی از جمله تترادیکان ٦،١٠،٢ ترای میتایل، هپتادیکان ١٥،٦،١٠،٢-تترا میتایل، هپتاکوزان، دوکوزان، هنیکوزان، بایس(٢-ایتایل هگزایل) فتالیت، فینول ۴،٢-بایس(١،١-دای میتایل ایتایل) فاسفیت (٣:١)، کاردامونین بایس(ترت-بوتایل میتایل سیلیل) ایتر، نایتروجن‌هگزادیکانوئیک اسید، ١-هگزادیکانول ٢-میتایل، اسکوالن، ایکوزان و ٣- (بنزایل تیو) اکریلیک اسید، میتایل استر را نشان داد. تمام ترکیبات شناسایی شده از *Alternaria* اثرات متفاوتی بر روی نباتات در مراحل مختلف دوران حیات خود نشان می دهند.
واژه‌های کلیدی: الترناریا، قارچ، میتابولایت‌های ثانویه

Introduction

Alternaria is recognized as saprophytic and pathogenic fungus that has significant implications for plant growth throughout various stages of its life cycle. The several species of *Alternaria* such as *Alternaria radicina*, *A. brassicola, A. brassicae*, *A. alternata*, *A. solani*, *A. aborescens*, and *A. infectoria* are very important because these species are responsible for causing diseases in various vegetables, including tomatoes, potatoes, peppers and some fruits (10).

The identification of the *Alternaria* genus dates back to the 19th century, when it was initially recognized as *A. tenuis* by Nees in 1816. Subsequently, Von Keissler designated *A. alternata* as the type isolate in 1912. *Alternaria* has been reported worldwide, with its sexual form found in a limited number of species related to Ascomycetes, while its asexual form is related to Deuteromycetes (18).

Mycotoxins, which are low molecular weight of secondary metabolites, play a significant role in agriculture during pre- and post-harvest stages. *Alternaria* is one of the fungi that produce such Mycotoxins and therefore have a major impact on plant growth (17). Over the past few decades, approximately 260 chemical compounds have been identified as secondary metabolites in *Alternaria*. These compounds include terpenoids, quinones, steroids, pyranones, and nitrogen-containing compounds. While some metabolites are unique to specific *Alternaria* species, others have been isolated from other genera of fungi. For instance, AAl toxin was isolated from *Fusarium* spp (8).

The *Alternaria* fungus causes important diseases in plants, leading to reduced crop production and economic losses. The secondary metabolites produced by pathogenic fungi are typically toxic to plants and are known as phytotoxins. These toxins can be categorized into two groups: host-specific toxins and non-host-specific toxins (8). Fungal metabolites can inhibit of plants seed germination. For example, secondary metabolites of *Cladosporium cladosporioides* LWL5 prevented the lettuce seed germination at different concentrations (19).

Previous studies have shown various effects of *Alternaria's* secondary metabolites on different plants. For instance, *A. alternata* metabolites caused necrotic symptoms on Datura leaves within 24 hours of spraying, leading to the death of treated weeds after one week (1). Another study demonstrated that Tenuazonic acid (TeA) had a perforating effect on *Ageratina adenophora* leaves. Spraying relatively high concentrations of TeA on seedlings of the same plants at the 4-6 leaf stage resulted in necrosis within 12 hours and eventually led to plant death (21). Research has shown that the spores suspension of *A. alternata* can damage 4-leaf seedlings of Amaranths, although its effect on older plants is less pronounced (5).

Isotentoxin, an isomer of tentoxin has a lesser impact on leaf chlorosis but causes rapid wilting in *Galium apaine*. Additionally, seven secondary metabolites of *Fusarium* (Fusarnon-X, Nivalenol, Diacetoxoxysirphenol, Nisolanol, Dioxinovalenol, HT-2, and T-2) inhibited seed germination of *Orobanche ramosa* seeds by 90-100% (22).

Considering the significance of extracting and identifying the secondary metabolites of *Alternaria* pathogenic fungus, this study aims to characterize and utilize these metabolites within Afghanistan.

Material and methods

The fungus was initially isolated from tomato fields in the Jalriz district of Maidan Wardak province and cultured on potato dextrose agar (PDA) medium. Subsequently, the fungus was transferred to water agar medium and the purification was done through monosporing technique.

To prepare the water agar, 16-20 grams of agar was added to 1 liter of water and sterilized in an autoclave at 120 °C for 20 minutes. The *Alternaria* isolate was then transferred to the water agar, and after 24 hours, a single spore (monospore) was transferred to PDA for further cultivation.

For the production of secondary metabolites, the *Alternaria* fungus was cultured on wheat media. Wheat soaked in water at a ratio of 1 liter per kilogram of grains for 2 hours.

The excess water was removed by air-drying the grains for a few minutes. The grains were then transferred to flasks and sterilized in an autoclave at 120°C for 20 minutes. Subsequently, the fungus was added to the wheat in a laminar hood and incubated at 27±1°C and dark condition for 21-28 days in an incubator. After 21 days of incubation, the fully grown fungus was mixed with a wheat media and transferred to a 1-liter flask. 100 ml Water and 40 ml ethyl acetate were added to the flask in a ratio of 5:2. The solution separated into two phases, with ethyl acetate forming the upper phase and water the bottom phase (Fig.1).

Figure 1: two phases of the solution; the upper phase, which contains ethyl acetate and *Alternaria* secondary metabolites and lower phase consisting of water.

Figure 2: *Alternaraia* culture after 21 days on wheat media.

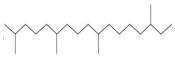
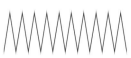
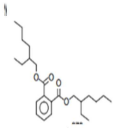
The ethyl acetate phase was extracted using a pipette and transferred to a beaker. The ethyl acetate was then evaporated in an open-air environment, and 1 cc of n-hexane was added to the remaining ingredients. Gas chromatography is a vital technique used for the identification of compounds, especially hydrocarbons with long chains such as alcohols, acids, esters, ethers, etc. Gas chromatography has revolutionized the analysis of active chemical compounds and has greatly contributed to the identification of secondary metabolites produced by fungi. In this study, the secondary metabolites of *Alternaria* dissolved in n-hexane were directly injected into the gas chromatography device.

The gas chromatography device used for this study was the Agilent 690 GC (Gas Chromatograph) with 5973 MSD (Mass Spectrometer) version. The sample containing the secondary metabolites was injected directly into the device, and the data analysis of the chromatography was performed using the NIST version 2.0f library available on the website. Thirteen chemical compounds were identified through gas chromatography, most of which have an impact on the germination and development of weeds, leading to their degeneration.

Results

The fungus required a 21-days incubation period in the media to generate a sufficient quantity of potent secondary metabolites. During the research, the media exhibited a darkening in color, ranging from black to brown due to the dense growth of fungal mycelium and fungal spores. Given the superior performance of wheat as growth media for *Alternaria* Figure 2, it is feasible to utilize this medium for the production of *Alternaria* toxins. In gas chromatography graph, the X-axis represents the molecular mass and charge change ratio (m/z) of the chemical compounds, while the Y-axis denotes the relative abundance. The m/z value of 57, corresponding to the molecular mass and charge change ratio in hexane, is considered as a Base pick for scaling the graph, with other picks drawn based on this base scale Figure 3. Through this chromatographic analysis, a total of 13 chemical compounds were identified, considerable of them including phthalates, alcohols, ethers, esters, and phenols (Table 1).

Table 1: Thirteen chemical compounds in secondary metabolites of Alternaria identified by Gas Chromatography

No	Chemical name	Molecular formula	Chemical group	Characteristics	Constriction formula
1	Tetradecane, 2, 6, 10- trimehyle-	$C_{17}H_{36}$	Alkane		
2	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	Alkane		
3	Heptacosane	$C_{27}H_{56}$	Alkane	Antifungal (4)	
4	Docosane	$C_{22}H_{46}$	Alkane		
5	Heneicosane	$C_{21}H_{44}$	Alkane	Acts as pheromones (15)	
6	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	Phthalate	Herbicidal properties (20)	
7	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	Phenol	Herbicidal properties (3)	
8	Cardamonin, bis(tert-butyldimethylsilyl) ether	$C_{28}H_{42}O_4Si_2$	Ether	Herbicidal properties (7)	
9	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Acid		
10	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$		Cytotoxicity (1)	
11	Squalene	$C_{30}H_{50}$		Considered as a cancer treatment (16)	
12	Eicosane	$C_{20}H_{42}$			
13	3-(Benzylthio)acrylic acid, methyl ester	$C_{11}H_{12}O_{2S}$		Herbicidal properties (11)	

Discussion

In the current investigation, the cultivation of fungi in wheat grain was employed to facilitate the synthesis of the secondary metabolites associated with *Alternaria*. Subsequently, the obtained metabolites were dissolved in n-hexane for identification. Notably, the production of these metabolites exhibited variations when compared to the findings of Kamal et al. (20). In their study, the secondary metabolites of *A. alternata* were cultivated in Potato Dextrose Broth (PDB) and subsequently extracted using methanol for dissolution.

On the other hand, Morgavi et al. (13) conducted a study focusing on the production of fungal metabolites from *Monascus* spp. in rice grain culture medium. It is noteworthy that the present research aligns with the aforementioned study conducted by Morgavi et al. (13).

In the investigation conducted by Al Mousa et al. (2) *A. tenuissima* was subjected to solid medium cultivation using rice as the substrate to facilitate the production of secondary metabolites. Subsequently, the secondary metabolites were extracted using ethyl acetate and subjected to gas chromatography for the identification of thirteen chemical compounds. Notably, several secondary metabolites such as Squalene, 1-Hexadecanol, 2-methyl-, and Tetradecane, 2,6,10-trimethyl were found to be common in both studies. However, there were divergences in the identification of Hexadecanoic acid between (2), and the present study. In Al- Mousa et al. (2) hexadecanoic acid, methyl ester with the molecular formula C_{17} H_{34} O_2, but in the present study, Hexadecanoic acid has been identified with $C_{16}H_{32}O_2$ molecular formula. Despite this difference, the present study exhibits a considerable degree of consistency with (2) since both studies utilized the same culture medium for the fungi and employed ethyl acetate for secondary metabolite extraction in both studies. It is important to note that dissimilarities in the identified chemical compounds could be attributed to the species of *Alternaria* under investigation, as Al-Mousa et al. (2), focused on *A. tenuissima* metabolites while the present study involved secondary metabolites extracted from *Alternaria* spp.

In the review conducted by Kausar et al. (7) regarding the secondary metabolites of *A. brassicicola* and *A. gaisen*, their impact on the growth of *Parthenium hysterophorus* was elucidated, demonstrating a significant inhibitory effect on seed germination, with an approximate inhibition rate of 88%. Gas chromatography analysis of the secondary metabolites from these two *Alternaria* species revealed the presence of N-hexadecanoic acid. In this investigation, the secondary metabolites of two types of *Alternaria* were extracted using ethyl acetate as the solvent. Subsequently, the obtained metabolites were dissolved n-hexane, and gas chromatography was employed to analyze these chemical compounds. Similarly, in the present study, the identification of n-hexadecanoic acid was also observed among the secondary metabolites produced by various *Alternaria* species (Table 1).

The researches findings indicate the presence of compounds in the secondary metabolites of *Alternaria* fungus that exhibit biological activities (1). These chemical compounds have effects on photosynthesis, stomatal activity, nutrient and water transfer and regulate germination and growth of several plants. Additionally, these metabolites detrimentally affect various cell organelles such as mitochondria, ribosomes, cell membranes, chloroplasts, Golgi bodies, and nuclei, ultimately leading to plants destruction (9).

The other biological activities encompass anti-cancer, antimicrobial, phytotoxic, and cytotoxic activities. The findings of this study have led to the identification of 13 secondary metabolites derived from *Alternaria* species, which have also been compatible to previously identifications of the compounds in other research studies.

Considering the aforementioned studies and the compounds identified in the secondary metabolites of *Alternaria*, this research serves as a fundamental principle for detection and

identification. Therefore, researchers and organizations involved in this field should dedicate more attention to bioactivities of the secondary metabolites of *Alternaria* (14).

Conclusion

After detailed studies using a chromatography device, a total of 13 chemical compounds were identified, namely tetradecane, 2,6,10-trimethyl; heptadecane, 2,6,10,15-tetramethyl; heptacosane; docosane; henicosane; bis(2-ethylhexyl) phthalate; phenol; 2,4-bis(1,1-dimethylethyl)-phosphite (3:1); cardamonin; bis(tert-butyldimethylsilyl) ether; n-hexadecanoic acid; 1-hexadecanol, 2-methyl; squalene; eicosane; and 3-(benzylthio) acrylic acid, methyl ester.

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