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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIFUNGAL ACTIVITY OF METHYL GALLATE AND SYRINGIC ACID ISOLATED FROM *ASTERISCUS GRAVEOLENS* AGAINST *FUSARIUM OXYSPORUM* F. SP. *ALBEDINIS*

ASTERISCUS GRAVEOLENS'TEN İZOLE EDİLEN METİL GALAT VE SİRİNGİK ASİDİN FUSARIUM OXYSPORUM F. SP. ALBEDINIS'E KARŞI ANTİFUNGAL AKTİVİTESİ

Zeyneb BELHI[1]*, Zahira ROUCHAM[1], Noureddine BOULENOUAR[1,2], Abdelkrim CHERITI[1]

[1]Tahri Mohamed University, Phytochemistry and Organic Synthesis Laboratory, 08000, Bechar, Algeria
[2]Nour Bachir University Center, Biological Sciences Department, 32000, El-Bayadh, Algeria

ABSTRACT

Objective: *The objective of this study was to isolate and identify the compounds responsible for the antifungal activity against Fusarium oxysporum f. sp. albedinis (Foa) from Asteriscus graveolens aerial parts extract, and to evaluate the effects in vitro of selected compounds for control of Fusarium wilt.*
Material and Method: *We reveal the presence of the phenolic compounds in Asteriscus graveolens, from which the antifungal activities of aerial parts extracts were investigated for effects on the growth of mycelia against Fusarium oxysporum f. sp. albedinis (Foa) by direct bioautography. The antifungal compounds were isolated from A. graveolens extract using silica gel column chromatography and thin-layer chromatography. Structural identification of the antifungal compounds was conducted using NMR (^{1}H and ^{13}C) spectrophotometry and LC-MS.*
Result and Discussion: *The isolated compounds were identified as methyl gallate (MG) and syringic acid (SA) based on comparing their spectral and physical data with the literature.*
Keywords: *Asteriscus graveolents, Fusarium oxysporum f. sp. Albedinis, methyl gallate, syringic acid*

ÖZ

Amaç: *Bu çalışmanın amacı, Asteriscus graveolens toprak üstü kısımları ekstresinden Fusarium oxysporum f. sp. albedinis'e (Foa) karşı antifungal aktiviteden sorumlu bileşikleri izole etmek ve tanımlamak ve Fusarium solgunluğunun kontrolü için seçilen bileşiklerin in vitro etkilerini değerlendirmektir.*
Gereç ve Yöntem: *Asteriscus graveolens'te fenolik bileşiklerin varlığını ortaya koyduk ve bu bileşiklerden elde edilen toprak üstü kısım ekstrelerinin antifungal aktiviteleri doğrudan biyootografi ile Fusarium oxysporum f. sp. albedinis'e (Foa) karşı misel büyümesi üzerindeki etkileri açısından araştırıldı. Antifungal bileşikler silika jel kolon kromatografisi ve ince tabaka kromatografisi kullanılarak A. graveolens ekstresinden izole edilmiştir. Antifungal bileşiklerin*

* **Corresponding Author / Sorumlu Yazar:** Zeyneb Belhi
e-mail / e-posta: belhi.zeyneb@univ-bechar.dz, **Phone / Tel.:** +213664043808

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yapısal tanımlaması NMR (^{1}H ve ^{13}C) spektrofotometrisi[A1] ve LC-MS/MS[A2] kullanılarak yapılmıştır.
Sonuç ve Tartışma: *İzole edilen bileşikler, spektral ve fiziksel verilerinin literatürle karşılaştırılmasına dayanarak metil gallat (MG) ve siringik asit (SA) olarak tanımlanmıştır.*
Anahtar Kelimeler: *Asteriscus graveolents, Fusarium oxysporum f. sp. Albedinis, metil gallat, siringik asit*

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) constitutes an important in the social and economic life of the Algerian Sahara. It represents the food, shade, garden, and refuge for the Saharan people [1]. This crop belongs to the Arecaceae family and is used in diet and traditional medicine due to its nutritive and pharmacological importance [2]. Besides, they provide a suitable microclimate for other crops (fruit, cereals, etc.) and they also protect them against the wind. For this, palm trees represent food and ecological security measures[3]. However, its culture is threatened by several pests and diseases such as *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *albedinis* [4].

Fusarium oxysporum is well-known as a plant pathogen causing severe damage in many crops, both in the field and during postharvest storage. Strains of *F. oxysporum* can grow under very low oxygen tensions and often have been detected as contaminants in ultrahigh-temperature processed fruit juices. Some strains are known to produce fumonisin mycotoxins [5]. The plant pathogenic strains are divided into special forms or *formae speciales* according to the plant species on which they cause disease [6,7]

Diseases caused by *F. oxysporum* are widespread in the world. They are harmful to many vegetables (tomato, cucurbit ...) and ornamental (carnation) plants, as well as to field crops such as cotton [8], chili [9], wheat [10], banana (Panama disease) [7,11], and date palm (Bayoud disease) [1,3].

Asteriscus graveolens, a member of the Asteraceae family, is the subject of research in numerous pharmacological and chemical studies. This plant mainly contains alkaloids, flavonoids, and terpenoids. These molecules exhibit various pharmacological benefits, such as anti-inflammatory, anticancer, and anti-viral effects, on the cardiovascular system.

The objective of this study was to isolate and identify the compounds responsible for the antifungal activity against *Fusarium oxysporum* f. sp. *albedinis* (Foa) from *Asteriscus graveolens* aerial parts extract, and to evaluate the effects *in vitro* of selected compounds for control of *Fusarium* wilt.

MATERIAL AND METHOD

Plant Materials

The aerial parts of *Asteriscus graveolens* were collected from Bechar (road of Lahmer, Bechar, Algeria). The collected plants were identified, and voucher specimens were conserved at the herbarium of the Phytochemistry and Organic Synthesis Laboratory under accession No CA00/14. The aerial parts were air-dried at room temperature in a shady place and then ground in the blender. After grinding, the material was stored at room temperature.

Figure 1. General view of *Asteriscus graveolens*

Extraction and Bioguided Fractionation

The dried aerial part plants were extracted with 80% ethanol for 18 h using Soxhlet apparatus and then evaporated to dryness by a rotary evaporator (Büchi Rotavapor R-210) at 55°C under reduced pressure. This extract was suspended in distilled water and partitioned sequentially with n-hexane, dichloromethane, ethyl acetate, and n-butanol, respectively. This extract was suspended in distilled water and portioned sequentially with hexane, dichloromethane, ethyl acetate, and n-Butanol. The organic phase was evaporated to dryness under reduced pressure.

Thin-Layer Chromatography (TLC)

The extracts of each solvent were subjected to TLC. TLC was carried out on silica gel 60 F_{254} plates (Merck, Germany The used solvent system was ethyl acetate: heptane (75:25). Spots were detected on TLC under UV light. R_f values of evaluated spots were recorded.

Determination of the Total Phenolic Contents (TPC)

The total phenolic content (TPC) of the extracts was determined by the Folin–Ciocalteu method using a modified procedure of Sengul et al., 2009 [12] and [13].

Gallic acid was used as the standard phenolic compound. The calibration was plotted by mixing aliquots of 1000; 500; 250; 125; 62.5 and 31.25 ppm of gallic acid solutions with 5 ml of Folin Ciocalteu reagent and 5 ml of crude extract. After 3 min, a solution of sodium carbonate 10 % Na_2CO_3 was added and the mixture was allowed to stand for 1 h with intermittent shaking. The color was developed and absorbance was measured at 760 nm in a Shimadzu UV 1800 Spectrophotometer after 30 min using Gallic acid as a standard. The total phenolic content (TPC) was calculated from the calibration curve, and the results were expressed as µg of gallic acid equivalent per mg dry weight (mg GA/g).

Determination of the Total Flavonoid Contents (TFC)

The aluminum chloride colorimetric method was used for the determination of the total flavonoid content of the samples; quercetin was used to make the standard calibration curve [14].

Antifungal Screening by Direct Bioautography

To screen for and identify compounds with antifungal activity present in the plant extracts, direct bioautography was used as described by Boulenouar et al. [15]. This approach involves directly immersing and cultivating a suspension of fungal spores on a developed TLC chromatogram.

Fungal Strain

The phytopathogenic filamentous fungus (Foa) used in this work was obtained from The Technical Institute for Saharian Agronomy (TISA), Adrar, Algeria. The strain was identified, and a voucher specimen was stored at the Phytochemistry and Organic Synthesis Laboratory under N° POSL/2011/01.

Spore suspensions of plant pathogens (Foa) were used. The concentration of Foa spores was adjusted to approximately 10^7 spores/ ml by dilution and counting.

Antifungal Activity of the Plant Extracts

The antifungal potential of the plant extracts was assessed by applying 80 µg/µl of each extract onto silica gel 60 F_{254} TLC plates (7 × 1.5 cm). These chromatograms were then immediately transferred into Petri dishes containing 20 ml of a spore solution with a concentration of 2×10^7 spores/ml, and left for 10 seconds. The development of fungal growth was monitored periodically until the TLC plates were completely covered with mycelial growth. Control plates, spotted with the respective organic solvent, were concurrently processed [15]. For visualization of microbial growth, tetrazolium salts, particularly p-iodonitrotetrazolium violet (INT) solution at a concentration of 2 mg/ml, were sprayed onto the Bioautograms [16]. Following overnight reincubation at 21°C, clear white zones against a purple background on the TLC plate indicated the presence of antimicrobial activity in the sample [17]. To identify the active compound, the R_f values on the plates were compared with those of reference plates.

Fractions

Among all extracts and fractions, ethyl acetate fractions exhibited a great antifungal effect on Foa and have been further characterized by chemical methods (TPC, TFC, NMR, and LC-MS/MS analysis).

The ethyl acetate fractions were chromatographed over silica gel open Column chromatography (30 g) using a mobile phase: (ethyl acetate: heptane) with the report in the following volume: (75: 25). Column chromatography was performed over silica gel 60 (Merck, particle size 290-320 mesh).

The recovered fractions were analyzed again by TLC, and fractions with identical spots and R_f values were pooled together for the antifungal evaluation using the antimicrobial assays described below.

Characterization of Isolated Compounds

The extracted and purified bioactive compounds from *Asteriscus graveolens* were characterized by nuclear magnetic resonance (NMR) techniques: Routine ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Model Avance AMX spectrometer (^{1}H 400 MHz and ^{13}C 100 MHz respectively) in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal reference. Mass spectrometry detection was conducted using a Shimadzu LC-MS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. Data were acquired by Lab Solutions software (Appendix C). Ethyl acetate fractions of *Asteriscus graveolens* were analyzed by the LCMS-8040 system (Shimadzu, Kyoto, Japan). The mobile phase consisted of 100% methanol (solvent A) and acetonitrile (solvent B) (1:1 v/v). The mobile phase flow rate was 0.3 ml/min. The column temperature was fixed at 40°C. Plant compounds were detected by a full scan mode ranging from m/z 100 - 1000 amu.

The LC-MS/MS, ^{13}C NMR, and ^{1}H NMR analyses were carried out in the laboratory of the "Catalysis Research and Application Center" of the University of İnönü, Malatya, Turkey.

Preliminary Evaluation of the Antifungal Activity

Preliminary analysis of the antifungal activity was performed using the agar-disc diffusion bioassay [19] and the agar-well diffusion bioassay [20] for the evaluation of ethyl acetate fractions.

For the disc diffusion bioassay, sterile discs (6 mm in diameter) of Whatman filter paper No.10 were impregnated with (20, 50, 80, and 100 µl) of each extract. The solvent was left to evaporate at room temperature, and the discs were placed on the surface of the plates previously seeded. Paper discs impregnated with ethyl acetate were used as controls.

For the well-diffusion bioassay, wells were made in the agar using an inverted sterile Pasteur pipette (6 mm in diameter), and (20, 50, 80, and 100 µl) of ethyl acetate extracts were deposited in the wells. Ethyl acetate was used as a control (all manipulations were done in sterile conditions). Plates were incubated at 21°C for 5 days.

Antimicrobial activity was detected by the presence of a growth inhibition zone surrounding the disc or well. The diameter of this zone was measured and recorded. The tests were realized in triplicate (the standard errors were less than 10%).

RESULT AND DISCUSSION

Phytochemical Study of the Bioactive Extracts/Fractions

Total Phenolic Contents

The crude extracts and ethyl acetate fractions of the investigated plant underwent phytochemical screening, revealing the presence of phenolics. Total phenolic contents were quantified utilizing the Folin-Ciocalteu method and expressed as Gallic acid equivalents (GAE) in µg GA/mg of the extract. The determination of total phenolic content was facilitated by reference to the graph depicted in Figure 1, and the standard curve equation was $y = 0.00146x + 0.02028$, where $R^2 = 0.99913$. The total phenolic contents (Gallic acid equivalents, µg GA/mg) in the samples were calculated to be 1144,879 and 366,052 µg GA/mg in *A. graveolens*,respectively (Table 1).

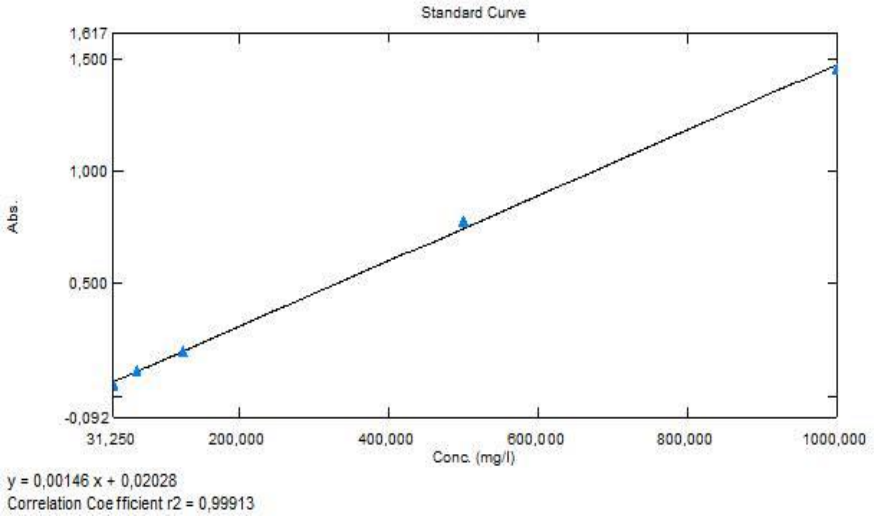

Figure 2. Standard curve of gallic acid

Figure 2 shows the total phenolic content in the samples of aerial parts of *A. graveolens* spontaneously grown in the southwest of Algeria.

The high amount of phenolic compounds from *A. graveolens* was reported by Ramdane et al. (2017). The variance in total phenolic content could be due to the chemical composition of the extract but also to the extreme conditions of growth and an arid ecosystem.

Total Flavonoid Contents

The concentration of total flavonoid contents in the test samples was calculated from the calibration plot (Y=0.00535 – 0.00381; R^2=0.99917) and expressed as µg quercetin equivalents per mg of dry extract (µg QE/mg). The total flavonoid contents in different extracts are shown in Figure 3.

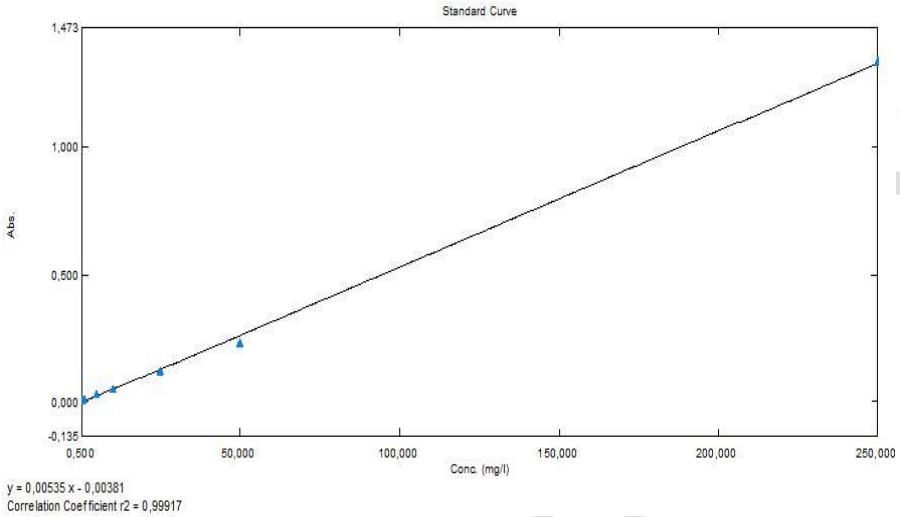

Figure 3. The total flavonoid content in the samples of aerial parts of *Asteriscus graveolens*

Many studies on the phytochemical composition of *A. graveolens* showed that this plant produced phenolic compounds including flavonoids:

Ahmed et al. (1991) have identified kaempferol 3-*O*-β-glucoside, kaempferol 3-*O*-β-galactoside, kaempferol 7-*O*-β-galactoside, quercetin 7-*O*-β-glucoside, luteolin 7-*O*-β-glucoside, and quercetin as major compounds in *A. graveolens* in Egypt.

Table 1. Total phenolic and flavonoid contents of crude extracts and ethyl acetate fractions of *A. graveolens*

Sample Code	TPC (mg GAE/gDry extract wt)	TFC (mg QE/g dry extract wt)
A graveolens Crude extract	1144.879	13.824
A graveolens EtOAc fraction	366.052	5.573

TPC: total phenol content; TFC: total flavonoid content; GAE: gallic acid equivalents; QE: quercetin equivalents; wt: weight; EtOH: ethanol; EtOAc: ethyl acetate [18]

The total phenolic content of the ethyl acetate fractions, calculated from the calibration curve (R^2 = 0.99913), was 366.052 µg GA/mg in *A. graveolens* and the total flavonoid content (R^2 = 0.99917) was 5.573 µg QE/mg in*A. graveolens* (Table 1).

A recent study on the phytochemical composition of *A. graveolens* [19] showed that ethyl acetate is the most suitable solvent for the extraction of bioactive compounds from this plant.

Direct Bioautography

Frequently, TLC-Direct Bioautography is used as a bio-guiding method to destine substances with biological activity that can be further analyzed by spectroscopic methods to obtain information on their structure [20].

The richness of natural substances reported by Cheriti *et al.* (2007) can explain the antifungal activity in certain extracts of *A. graveolens* (Table 2).

Table 2. Direct bioautography results of the extracts of *Asteriscus graveolens*

Specie	Eluent	Extraction Solvent	Antifungal Effect
Asteriscus graveolens	**EtOAc: Hep 75:25**	EtOH	++
		Hex	-
		DCM	++
		EtOAc	++
		n-But	+

Hep: heptane; EtOH: ethanol; Hex: hexane; DCM: dichloromethane; EtOAc: ethyl acetate; *n*-But: butanol

The absence of observed effects from testing an extract on a specific biological target does not necessarily negate the presence of active substances, as synergy between components may occur. Additionally, in some instances, the concentration of these substances may be sufficiently low that their activity can only be detected on TLC plates.

Characterization of Bioactive Compounds by NMR and LC-MS

LC-MS/MS analyses showed that plant extracts were decomposed topreviously known ones. The structures of compounds were elucidated by NMR techniques and mass spectroscopy. The compounds isolated from the ethyl acetate fractions of the species *Asteriscus graveolens* display a powerful antifungal effect.

The known compounds were identified as methyl gallate and syringic acid based on comparing their spectral and physical data with the literature (Figure 4). Effectively, the *m*/*z* values of 185 and 198 corresponded to their molecular weight of 184.15 and 198.17 g/mol respectively, thus validating the output of the mass spectrometer. Figure 4 shows the structure of the compounds isolated.

Identification of molecules by NMR (^{1}H and ^{13}C) spectrophotometry and LC-MS/MS showed the presence of methyl gallate and syringic acid in aerial parts of *A. graveolens*.

NMR spectra of methyl gallate ($C_8H_8O_5$): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.95 (s, 3H, CH_3), 6.91 (s, 2H, C_6H_2), 8.73 (s, 3H, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 52.08 (CH_3O), 110.8, 123.82, 137.57, and 146.65 (C_6H_2), 166.51 (CO). The molecular mass of isolated methyl gallate was determined as 185 using LC-MS/MSanalysis. Results obtained with ^{1}H-NMR; ^{13}C-NMR and LC-mass spectroscopy were identical to published data [21].

NMR spectra of syringic acid ($C_8H_8O_5$): ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 3.82 (s, 6H, CH_3), 7.07 (s, 2H, C_6H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 56.78 (CH_3O), 106.09, 121.39, 141.78 and 149.19 (C_6H_2), 167.49 (CO). The molecular mass of isolated syringic acid was determined as 198 using LC-MS/MSanalysis.

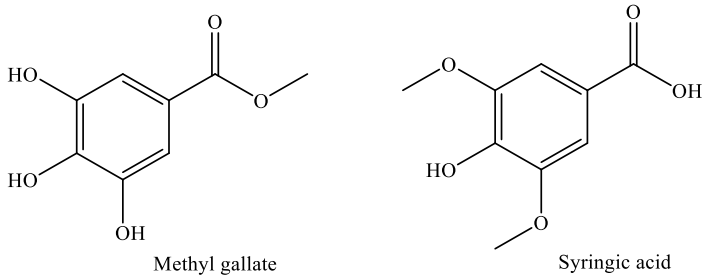

Figure 4. Compounds isolated and identified in aerial parts of *Asteriscus graveolens*

In this research, the assessment of *A. graveolens* extracts against the pathogen responsible for Bayoud disease, *Fusarium oxysporum* f. sp. *albedinis* (Foa), incorporated innovative principles into direct bioautography. Previous research conducted at the Phytochemistry and Organic Synthesis Laboratory (POSL, Bechar University, Algeria) has established that this plant contains secondary metabolites possessing various biological activities.

This medicinal plant has previously been investigated by our research group (POSL team) for its antibacterial and antifungal properties. They were chosen for initial testing based on a systematic review conducted on promising bioactive plants which highlighted the above species [1,22-24].

Plant extracts were selected for inclusion in this study because their ability to inhibit the respective enzymes and biological activities has already been established in studies published by others and in previous studies carried out by our research group [1,22-24].

Bioautography is notably significant to avoid the time-consuming isolation of inactive compounds [25]. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture [26]. The bioautography technique is inexpensive, so beneficial for screening large numbers of samples (particularly crude extracts). Although results are not completely quantitative, they can give information about how many and which substances in a mixture showed antifungal activity [27].

The phytochemical analysis aimed to identify the specific metabolite accountable for the observed antifungal activity. Based on TLC profiling results, it is conjectured that the inhibition may be attributed to flavonoids found in the ethyl acetate extracts derived from the aerial parts of *A. graveolens*, with R_f values of 0.24 and 0.88.

The number of active compounds in the plant extracts was determined using the bioautography method, those compounds were separated with CC and had similar R_f values of 0.24, and 0.88 in *A. graveolens* ethyl acetate fractions.

Apart from the advantages of rapidly detecting active compounds in mixtures and high sensitivity, the depicted bioautography also points to a potential disadvantage of this diffusion assay. Its applicability is limited to microorganisms that easily grow on TLC plates [28].

Recently, the fungicidal activities of plant extracts have been extensively reported [27]. Research investigating the fungicidal effects of *A. graveolens* extracts on the pathogen *F. oxysporum*, responsible for *Fusarium* wilt in date palms, remains limited. This disease presents a significant threat to date palm cultivation. However, the medicinal potential of *A. graveolens* is bolstered by the presence of phenolics and flavonoids, indicating promising therapeutic applications.

The effects of the extracts utilized were demonstrated by Boulenouar et al. (2014) using the disc diffusion technique. Results indicated detectable effects against Foa in at least two tests, thus confirming the presence of antifungal substances despite the variance in the techniques employed. The notable impact observed across different parts of the plant may be attributed to variations in the components present. This discrepancy could stem from differences in chemical composition or mechanism of action. It's noteworthy that certain substances exhibit antifungal activity against Foa but not against its toxins, highlighting the intricate nature of the pathogenic mechanism.

Indeed, Foa is known to produce multiple toxins, which play a crucial role in its pathogenicity. Consequently, previous research conducted against Foa has identified active substances that can influence one or more of these mycotoxins. This influence may occur through the modification of their metabolism or their effects, thereby affecting the pathogenic behavior of the fungus [24,29].

Flavonoids represent a class of compounds known for their ability to inhibit various enzymes. Through phytochemical screening, our study identified a diverse array of phytoconstituents, with phenolic compounds being particularly abundant [30].While numerous investigations have explored the structure-activity relationship of various polyphenols and their antifungal properties, the precise relationship remains unclear despite the vast number of these compoundsclear [31]. Plants synthesize a wide range of metabolites to ensure their survival, growth, development, and defense against a broad spectrum of pathogens, including bacteria, fungi, and viruses. In our study, we isolated methyl gallate (MG) and syringic acid (SA) as major metabolites exhibiting antifungal activity from the aerial parts of *A. graveolens*.

The LC-MS/MSchromatogram data of the EtOAc extract revealed a group of peaks that were fractionated from one to seven by open silica column chromatography. The active compounds of fractions 5 and 6 were purified and identified as MG using NMR and LC-MS/MSanalysis. MG and SA are natural constituents isolated from different plants [21,32]. *In vitro* studies on the antifungal activity of SA were done by Chong et al. using concentrations ranging from 50 to 110 mL μg^{-1}, those typically recorded in oil palm roots. SA was found to be antifungal against *G. boninense* [33,34].

Phenylpropanoid metabolism produces an enormous array of secondary metabolites. The biosynthesis of GA and its derivative MG takes place via phenylpropanoid metabolism [35].

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AUTHOR CONTRIBUTIONS

Concept: Z.B., Z.R., N.B., A.C.; Design: Z.B., Z.R., N.B., A.C.; Control: Z.B., A.C.; Sources: Z.B., Z.R., N.B., A.C.; Materials: A.C.; Data Collection and/or Processing: Z.B., Z.R., N.B., A.C.; Analysis and/or Interpretation: Z.B., Z.R., N.B., A.C.; Literature Review: N.B., A.C; Manuscript Writing: Z.B.; Critical Review: Z.B., Z.R., N.B., A.C.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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