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Bioguided isolation of alternariol derivatives from *Ficus*-derived endophyte *Alternaria alternata*

Randa Abdou [1, 2*] Gouda H Attia [3] Mariam Mojally [4] Mohamed Dawoud [5] and Mostafa E. Rateb [6]

[1] Pharmacognosy Department, Faculty of Pharmacy, Umm Al-Qura University, Makkah, Saudi Arabia

[2] Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt

[3] Pharmacognosy Department, Faculty of Pharmacy, Najran University, Najran, Saudi Arabia

[4] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Umm Al-Qura University, Makkah, Saudi Arabia

[5] Department of Pharmaceutics, Faculty of Pharmacy, Umm Al-Qura University, Makkah, Saudi Arabia

[6] School of Computing, Engineering & Physical Sciences, University of the West of Scotland, Paisley PA1 2BE, U.K.

*** Corresponding author:** Randa Abdou, Department of Pharmacognosy, Faculty of Pharmacy, Umm Al-Qura University, 24231 Makkah, KSA. Tel. 00966546132281. E-mail address: rfabdou@uqu.edu.sa

Research Article

Running title: Bioactive metabolites of *Alternaria alternata* endophyte

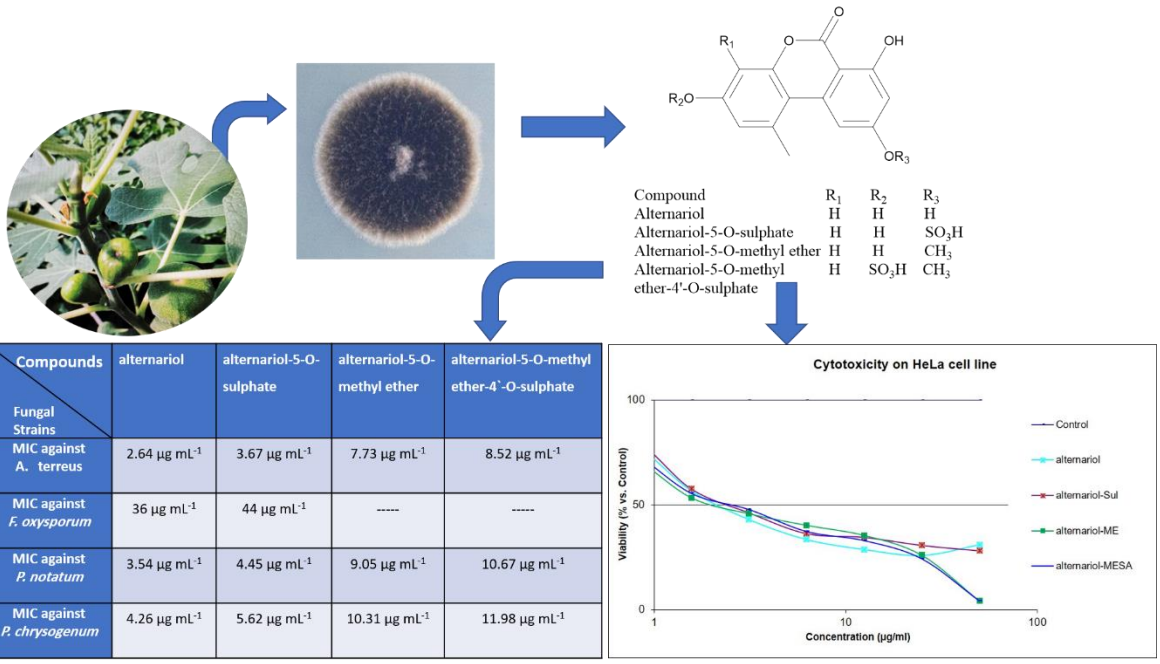
Bioguided Isolation of Alternariol Derivatives from *Ficus*-derived Endophyte *Alternaria alternata*

ABSTRACT

Background: Endophytes are a rich source of bioactive natural products and suggested to contribute to the biological or defense activities of their host plants. Following our research on the discovery of bioactive metabolites from endophytes, *Alternaria alternata* was isolated from the leaves of *Ficus carica* L. fam *Moraceae*. Materials and methods: Large scale cultivation of the endophytic strain was carried out and the obtained extract was subjected to preliminary screening of antifungal and cytotoxic activities. Results: Promising antifungal and cytotoxic activities were obtained for the extract. Bio-guided fractionation resulted in the isolation and identification of four alternariol derivatives (alternariol, alternariol-5-O-sulphate, alternariol-5-O-methyl ether, alternariol-5-O-methyl ether-4`-O-sulphate). The isolated compounds were tested for antifungal and cytotoxic effects. Results revealed highest antifungal activity for alternariol against *A. terreus* (MIC= 2.64 µg mL^{-1}) and *F. oxysporum* (MIC= 36 µg mL^{-1}) while alternariol-5-O-methyl ether exhibited the highest cytotoxicity against K-562 (CC_{50}= 3.72 µg mL^{-1}) and HUVEC (CC_{50}= 2.06 µg mL^{-1}) cell lines. Conclusion: All alternariol derivatives showed potent cytotoxic and antifungal activities against *A. terreus* suggesting the contribution of this endophyte in the known antimicrobial and anticancer activities of the host plant.

Key words: Endophyte, Alternariol, *Alternaria alternata*, Anticancer, *Ficus carica*.

Pictorial Abstract



Compounds / Fungal Strains	alternariol	alternariol-5-O-sulphate	alternariol-5-O-methyl ether	alternariol-5-O-methyl ether-4`-O-sulphate
MIC against *A. terreus*	2.64 µg mL^{-1}	3.67 µg mL^{-1}	7.73 µg mL^{-1}	8.52 µg mL^{-1}
MIC against *F. oxysporum*	36 µg mL^{-1}	44 µg mL^{-1}	-----	-----
MIC against *P. notatum*	3.54 µg mL^{-1}	4.45 µg mL^{-1}	9.05 µg mL^{-1}	10.67 µg mL^{-1}
MIC against *P. chrysogenum*	4.26 µg mL^{-1}	5.62 µg mL^{-1}	10.31 µg mL^{-1}	11.98 µg mL^{-1}

INTRODUCTION

Plants and plant-derived microbial endophytes represent rich sources of natural products with different chemical classes and diverse biological activities [1]. Biological activity and growth conditions are important criteria in the adequate selection of a host plant for endophyte investigation [2]. *Ficus* species were employed for the treatment of many diseases such as gastrointestinal, cardiovascular, respiratory disorders and cancers [3]. Studies performed on *Ficus carica* L. extract revealed its antioxidant, cancer suppressive and antiviral effects [4, 5]. Antimicrobial activity of *F. carica* extract was reported against several bacterial strains with MIC values ranging from 0.3-5mg/mL [6]. Additionally, antifungal activity of *F. carica* against both *Microsporum canis* (MIC 75 μg/mL) and *C. albicans* (MIC 500 μg/mL) was also proven [7]. The anticancer activity of the plant leaves extract was reported against Huh7it liver cancer cells with an IC_{50} of 653 μg/mL [8]. Taking the reported biological activities [5] and the hot and dry growth conditions in Makkah, Saudi Arabia into consideration, *F. carica* L. was chosen as a host plant for endophyte study [9]. Previous studies suggested endophytes' contribution in the biological effects of host plants [10]. Additionally, investigation of *Ficus* spp. mainly focused on the plant itself. These two facts encouraged us to investigate the chemical profile of the endophyte *Alternaria alternata* recovered from *F. carica* leaves in addition to its anticancer and antimicrobial effects.

MATERIALS AND METHODS

Plant collection and endophyte isolation

The medicinal plant *Ficus carica* L. fam. *Moraceae* was collected from Makkah (Wadi Fatima), KSA. Plant identification was carried out by Dr. Hany (Pharmacognosy Department, college of Pharmacy, Najran University). A voucher specimen of the plant (UQU-2019-1) is available at the herbarium of the college of Pharmacy (Department of Pharmacognosy), UQU, Makkah, KSA. Collected plant material was decreased in size, washed and its surface sterilized followed by drying under laminar flow. By the aid of a sterile scalpel outer plant tissues were removed, and internal tissues were cut under aseptic conditions. Endophyte isolation and cultivation was performed as previously published [11, 12].

Endophyte identification

Identification of the fungal endophyte was carried out as previously described in our study on all endophytes isolated from *F. carica* [13]. The standard protocol based on the cultural and microscopic properties of the endophyte [14] was first employed for identification and afterwards it was confirmed using molecular biological techniques through DNA extraction followed by amplification using Polymerase chain reaction (PCR), and finally sequencing was performed using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers as previously published [13, 14].

Fermentation and fractionation of the endophytic extract

Cultivation of the isolated fungal endophyte was carried out in potato dextrose agar for two weeks at 23ºC. Formed mycelia were employed for inoculation of Erlenmeyer flasks each containing 250 mL of the MPG-medium that consisted of malt extract (20 g/L), soybean flour (2 g/L), glucose (10 g/L), KH_2PO_4 (1g/L), $MgSO_4.7H_2O$ (0.5 g/L) and yeast extract (1g/L). A stationary culture (40 L) was incubated at 23 ºC for three weeks. After the incubation period, culture filtrate and mycelium of each flask were mixed homogenously followed by maceration in 200 mL ethyl acetate (EtOAc) for 24 h and afterwards decantation and collection of the supernatant. The collected EtOAc extract was evaporated and defatted with *n*-hexane. Using the agar diffusion assay, the antimicrobial activity of the fungal extract was tested and found to be effective against several bacterial and fungal strains which encouraged us to subject it to bioactivity guided chromatographic fractionation for determination of the active metabolites. Accordingly, Silica gel was used as a stationary phase and a mixture of methanol and chloroform (1:9) as a mobile phase in the first bioguided chromatographic fractionation step of the extract. Polarity of the mobile phase was gradually increased till 100% methanol was used as the last eluent. Further purification was performed on Sephadex LH-20 using methanol as an eluent. Isolation of the bioactive metabolite from the active fraction was finally achieved using preparative HPLC using a gradient mobile phase composed of 25% acetonitrile in H_2O till 100 % acetonitrile over 45 min and a flow rate of 10 mL min^{-1}. This resulted in the isolation of four metabolites; alternariol (5 mg), alternariol-5-O-

sulphate (5.5 mg), Alternariol-5-O-methyl ether (5.8 mg) and alternariol-5-O-methyl ether-4`-O-sulphate (4.8 mg) (Fig 1) which were identified by different spectroscopic analyses.

Antimicrobial screening

Antimicrobial effects of the extract and isolated compounds were examined by agar diffusion and minimum inhibitory concentration (MIC) was calculated by the aid of the broth microdilution method as in literature [15-17].

Statistical Analysis

Student's t-test was used to evaluate the significant difference and compare results of the antimicrobial activities of the different tested samples. A statistically significant difference was considered when the p value was smaller than 0.05.

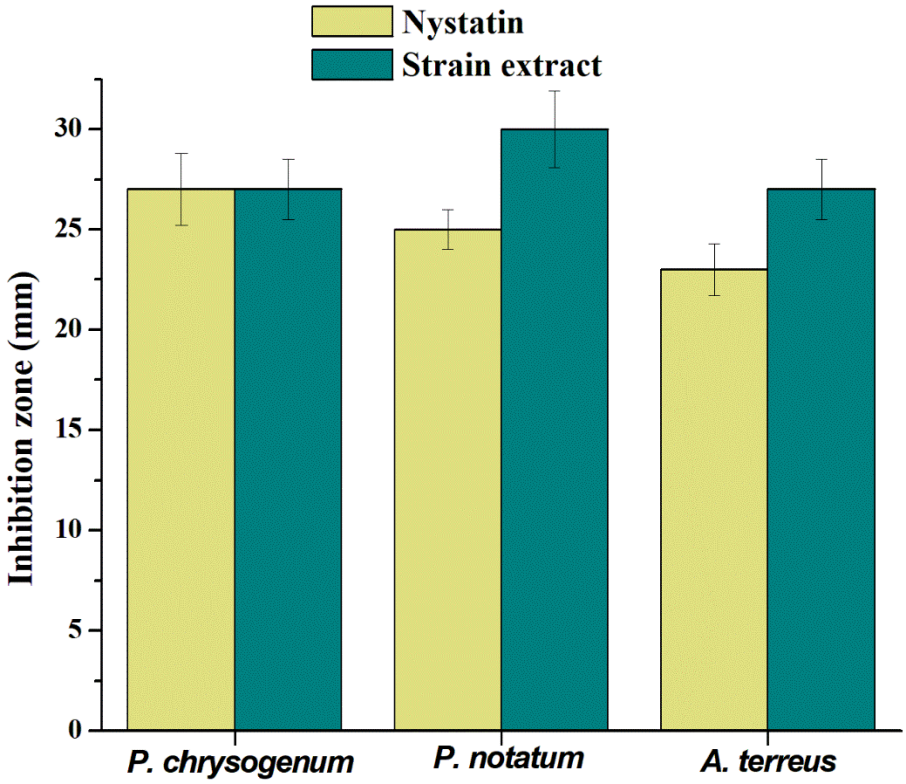


Figure 1: Antifungal activity of *A. alternata* extract measured in terms of the diameter of the inhibition zone in millimeters using nystatin as a positive control

Cytotoxic assay

The cancer cell lines K-562, HUVEC and HeLa were cultured in Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's Modified Eagle's Medium (DMEM), and RPMI 1640, respectively. 10 mL l^{-1} ultraglutamine 1, 500 µl l^{-1} gentamicin sulfate, and 10 % heat inactivated fetal bovine serum were added at 37°C in high density polyethylene flasks for supplementation of the cell culture medium and the cytotoxic assay was conducted as previously published [11, 18].

RESULTS AND DISCUSSION

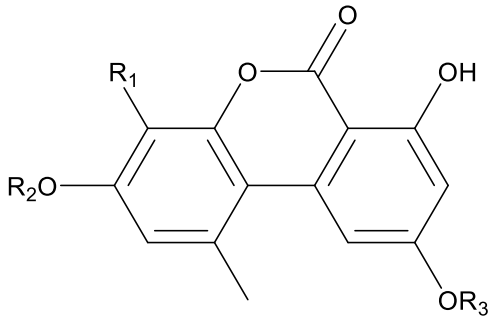
Isolation of secondary metabolites

The endophytic extract revealed significant cytotoxicity (CC_{50}= 3.71 µg mL^{-1}) against human immortal cervical cancer (HeLa), human immortalized myelogenous leukemia (K-562) and human umbilical vein endothelial (HUVEC) cell lines (CC_{50} = 3.65 µg mL^{-1} and 3.86 µg mL^{-1}, respectively). Moreover, the extract exerted antifungal activity against several fungal strains (Fig. 1) in agar diffusion assay. Accordingly, bio-guided fractionation using different chromatographic approaches was conducted on the bioactive fractions to explore the active metabolites which resulted in the isolation of four fungal secondary metabolites (Fig. 2).

Structure elucidation of bioactive metabolites

The molecular weight of 258 g/mol was deduced for the first metabolite by the obtained negative and positive ESI-MS at *m/z* 257.4 $[M-H]^-$ and *m/z* 259.2 $[M+H]^+$. The NMR data of the compound led to the deduction of a molecular formula of $C_{15}H_{12}O_5$. The ^{13}C and ^{1}H NMR spectra revealed four aromatic protons and an aromatic methyl group for the compound. All spectral data obtained for this metabolite were identical to previously published data for alternariol [19] (Fig. 2). The second metabolite was obtained with similar UV absorbances to alternariol derivatives. Its HRESI-MS indicated a molecular formula of $C_{14}H_{10}O_8S$ which was corroborated with the equimolecular ion peak at *m/z* 339.0170 $[M+H]^+$. ^{1}H NMR indicated the presence of an aromatic methyl group in addition to two pairs of meta-coupled aromatic protons. The ^{13}C NMR data of alternariol were comparable to those obtained for this metabolite except for the up-field shift of C-5 and downfield shifts of C-4 and C-6 suggesting the presence of substitution by a sulphate group at C-5 [20], which was

confirmed by literature [21] and resulted in the identification of this metabolite as alternariol-5-O-sulphate (alternariol-Sul). Further, alternariol-5-O-methyl ether (alternariol-ME) (Fig. 2) showed typical UV absorbances for alternariol derivatives. A molecular weight of 272 g/mol and a molecular formula of $C_{15}H_{12}O_5$ were deduced through negative and positive ESI-MS which showed molecular ion peaks at *m/z* 271.3 $[M-H]^-$ and *m/z* 273.2 $[M+H]^+$. From the ^1H and ^{13}C NMR spectra, it was concluded that the compound contained a methoxy group, an aromatic methyl group and four aromatic protons. Comparison of the obtained spectral data for this compound with previously published data confirmed its identity as alternariol-ME [22]. The molecular formula $C_{15}H_{12}O_8S$ of the fourth endophytic metabolite was revealed for its HRESIMS with the equimolecular ion peak at *m/z* 353.0320 $[M+H]^+$ which showed 14 mass units higher compared to alternariol-Sul. A close resemblance of the structure of this secondary metabolite with alternariol-Sul and alternariol-ME was concluded from the ^1H and ^{13}C NMR spectra. The main difference observed in this compound was the up-field shift of C-4`and downfield shifts of C-3` and C-5`, indicating the attachment of a sulphate group to C-4` [20]. The obtained spectral data were identical with literature data and led to its identification as alternariol-5-O-methyl ether-4`-O-sulphate (alternariol-MESA) [21].



Compound	R_1	R_2	R_3
Alternariol	H	H	H
Alternariol-5-O-sulphate	H	H	SO_3H
Alternariol-5-O-methyl ether	H	H	CH_3
Alternariol-5-O-methyl ether-4'-O-sulphate	H	SO_3H	CH_3

Figure 2: Chemical structures of alternariol, alternariol-Sul, alternariol-ME, alternariol-MESA

Bioactivity of isolated metabolites

The isolated fungal metabolites were tested for their antifungal activity in agar diffusion assay against several fungal strains (*Aspergillus terreus* ATCC 74135, *Penicillium notatum* ATCC 9478, *Penicillium chrysogenum* ATCC 10106) using nystatin (1 µg mL^{-1}) and as a positive control. Highest antifungal activity was observed for all metabolites against *A. terreus* with a MIC of 2.64 µg mL^{-1} for alternariol, 3.67 µg mL^{-1} for alternariol-Sul, 7.73 µg mL^{-1} for alternariol-ME and 8.52 µg mL^{-1} for alternariol-MESA (Table 1). Alternariol and alternariol-Sul also exhibited antifungal effect against the plant pathogen *Fusarium oxysporum* with MIC values of 36 and 44 µg mL^{-1}, respectively compared to the positive standard amphotericin B (MIC= 2.9 µg mL^{-1}). Alternariol exerted higher antifungal activity against *P. notatum* and *P. chrysogenum* followed by alternariol-Sul (Table 1).

Compounds / **Fungal strain**	**alternariol**	**alternariol-5-O-sulphate**	**alternariol-5-O-methyl ether**	**alternariol-5-O-methyl ether-4`-O-sulphate**
MIC against *A. terreus*	2.64 µg mL^{-1}	3.67 µg mL^{-1}	7.73 µg mL^{-1}	8.52 µg mL^{-1}
MIC against *F. oxysporum*	36 µg mL^{-1}	44 µg mL^{-1}	-----	-----
MIC against *P. notatum*	3.54 µg mL^{-1}	4.45 µg mL^{-1}	9.05 µg mL^{-1}	10.67 µg mL^{-1}
MIC against *P. chrysogenum*	4.26 µg mL^{-1}	5.62 µg mL^{-1}	10.31 µg mL^{-1}	11.98 µg mL^{-1}

Table 1: Antifungal activities of the isolated compounds against *A. terreus* and *F. oxysporum*

Furthermore, the isolated molecules were subjected to a cytotoxic assay against the cancer cell lines K-562, HUVEC and HeLa. All metabolites exerted significant cytotoxic activities against HeLa cell line (Fig. 3-5) with highest cytotoxicity observed for alternariol-ME (CC_{50} = 2.06 µg mL^{-1}) followed by alternariol-MESA (CC_{50} = 2.16 µg mL^{-1}). Strong cytotoxic activity was observed for all compounds against HUVEC cell line with the highest activity detected for alternariol-ME (CC_{50}=3.72 µg mL^{-1}). All isolated metabolites exerted similar cytotoxicity against K-562 cells with CC_{50} values ranging from 4.31 to 4.75 µg mL^{-1} (Fig 3-5). These results highlight the importance of *Alternaria alternata* as a rich source of bioactive metabolites which has been supported by the detected cytotoxicity of a recently discovered natural product, alternate C against the cancer cell lines MDA-MB-231 and MCF-7 [23].

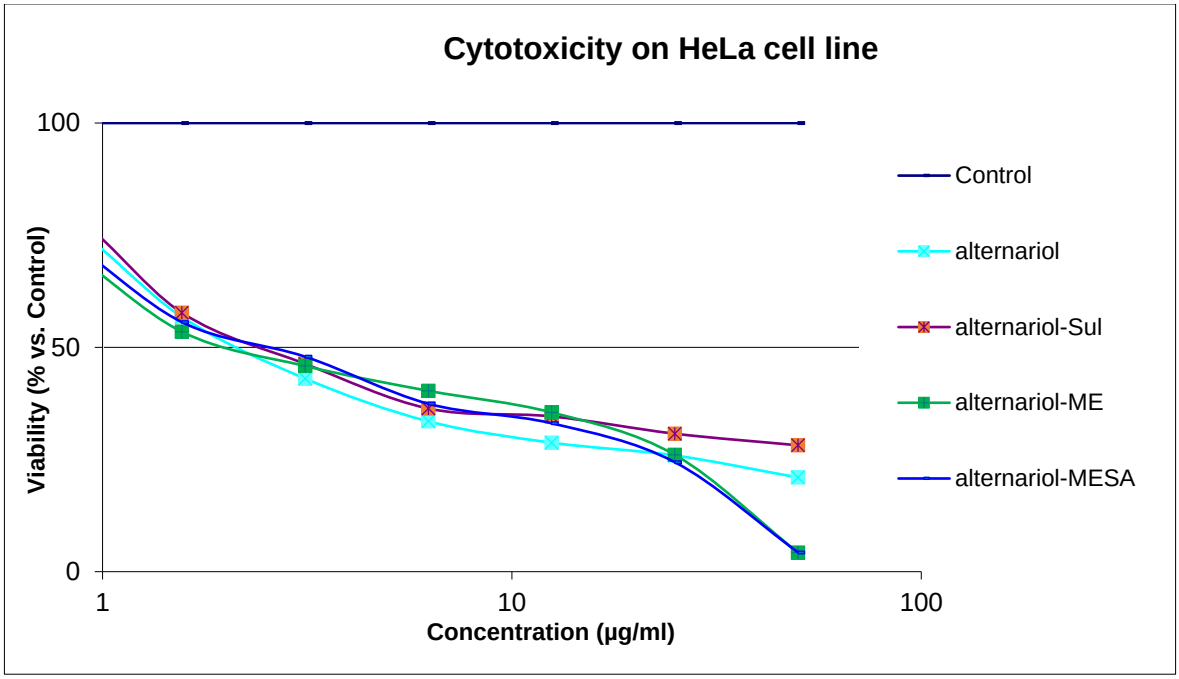


Figure 3: Cytotoxic (CC_{50}) activities of the isolated metabolites on HeLa

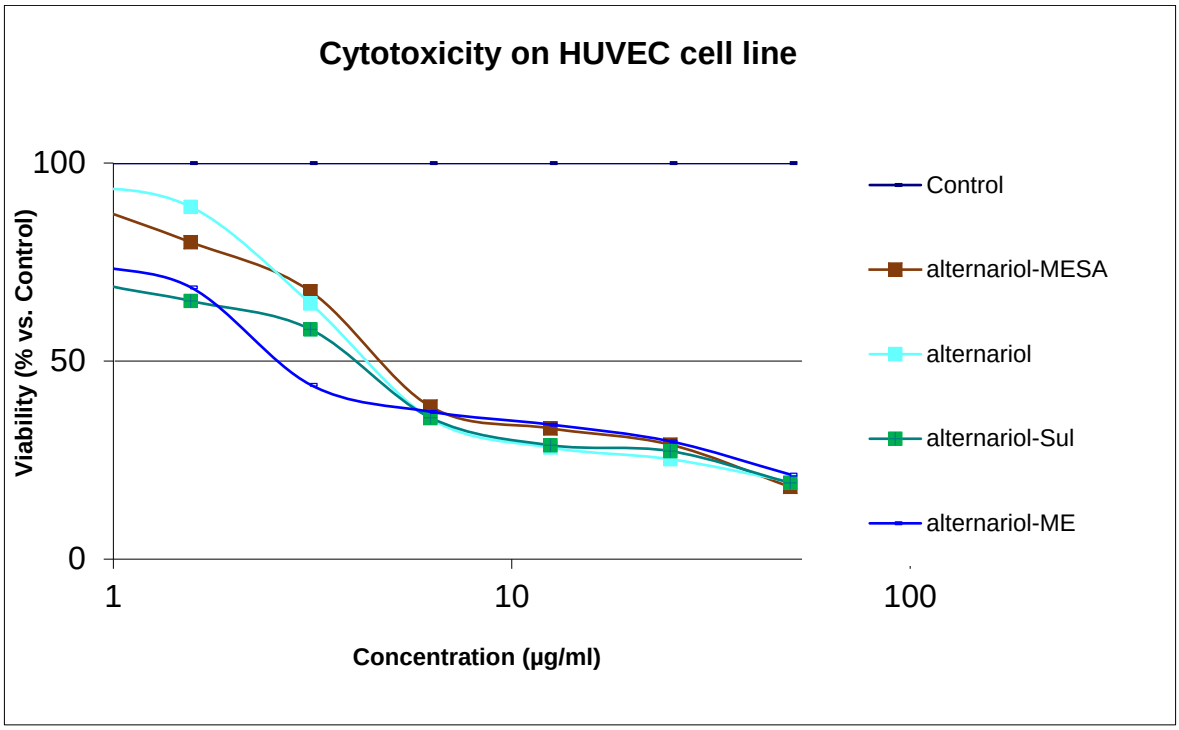


Figure 4: Cytotoxic (CC_{50}) activities of the isolated metabolites on HUVEC

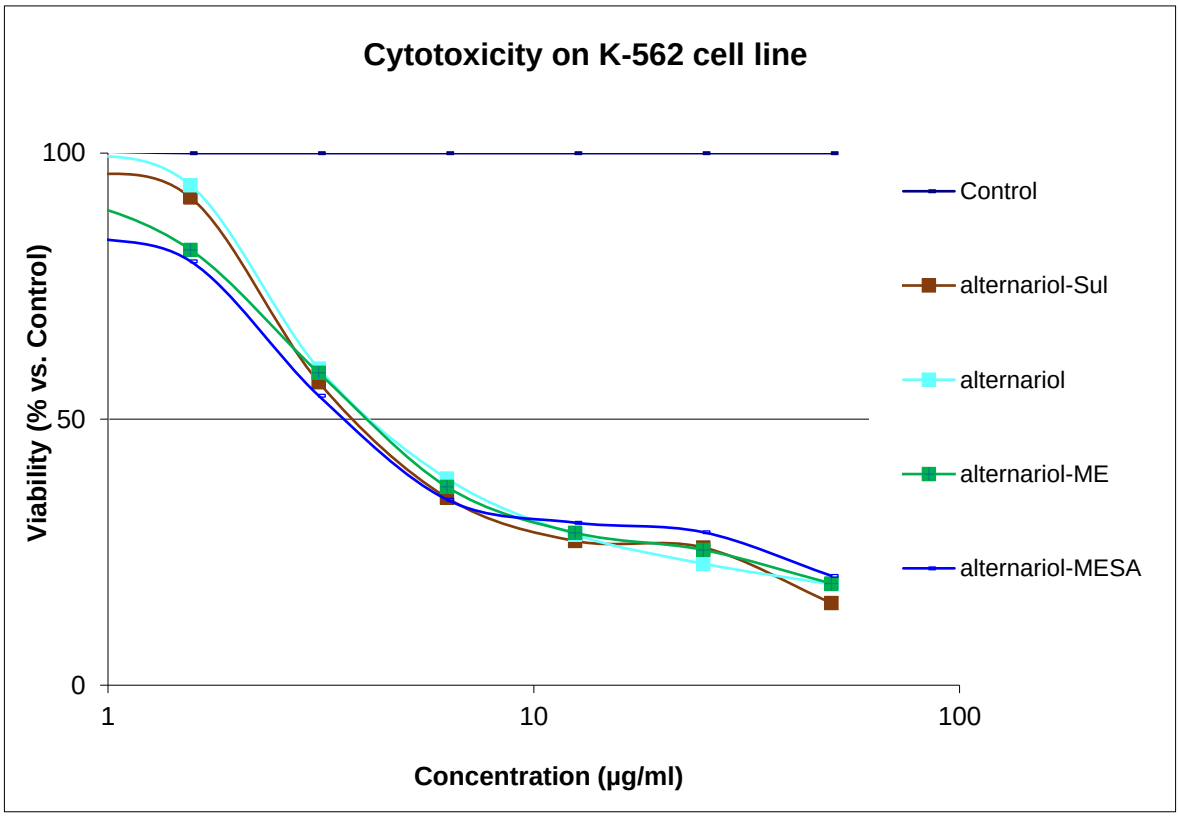


Figure 5: Cytotoxic (CC_{50}) activities of the isolated metabolites on K-562

CONCLUSION:

In conclusion, from the medicinal plant *Ficus carica* L. fam *Moraceae* growing in the tropical weather of Makkah, KSA the endophyte *Alternaria alternata* was isolated and studied for its bioactive metabolites. Bioguided fractionation led to the isolation of four alternariol derivatives from their bioactive fraction and identified by different spectroscopic analyses. The highest cytotoxicity against HeLa and HUVEC cell lines was observed for alternariol-5-O-methyl ether. Interestingly, all alternariol derivatives showed potent cytotoxic and antifungal activities suggesting contribution of this endophyte at least in part in the antimicrobial and anticancer activities reported for the host plant *F. carica*. Furthermore, the detected antifungal effects of these compounds suggest a possible protection of the host plant by this endophyte which supports previous assumptions on the protective relationship between endophytes and host plants.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

ABBREVIATIONS

CC_{50}: cytotoxic concentration 50; **MIC:** minimum inhibitory concentration; **GI_{50}:** growth inhibition 50%; **NMR:** nuclear magnetic resonance; **HRESIMS:** high-resolution electrospray ionization mass spectrometry; **HMBC:** heteronuclear multiple bond correlations; **HUVEC:** human umbilical vein endothelial cell; **K-562:** human immortalized myelogenous leukemia; **HeLa:** human immortal cervical cancer.

SUMMARY

Endophytes are a rich source of bioactive natural products and suggested to contribute to the biological or defense activities of their host plants. Following our research on the discovery

of bioactive metabolites from endophytes, the fungal strain *Alternaria alternata* was isolated from the leaves of *Ficus carica* L. fam *Moraceae*. In preliminary screening, this endophytic extract exerted promising antifungal and cytotoxic activities. Bio-guided fractionation resulted in the isolation and identification of four alternariol derivatives. The isolated compounds were tested for their cytotoxicity against HeLa, K-562 and HUVEC cancer cells. Alternariol-5-O-methyl ether exhibited the highest cytotoxicity against HeLa and HUVEC cell lines. All alternariol derivatives showed potent cytotoxicity and antifungal activity suggesting contribution of this endophyte at least in part in the biological activities reported for the host plant *F. carica*. Results revealed highest antifungal activity for alternariol against *A. terreus* (MIC= 2.64 µg mL^{-1}) and *F. oxysporum* (MIC= 36 µg mL^{-1}). The detected antifungal effects of these compounds suggest a possible protection of the host plant by this endophyte which supports previous assumptions on the protective relationship between endophytes and host plants [24].

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