International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 12, Issue 3, 2020

Original Article

CYTOTOXIC METABOLITES OF *ALTERNARIA ALTERNATA,* AN ENDOPHYTE OF THE MEDICINAL PLANT *BIDENS BIPINNATA*

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Received: 09 Nov 2019, Revised and Accepted: 22 Jan 2020

ABSTRACT

Objective: Endophytes are widely spread in the plant kingdom and represent a very promising source of biologically active natural products. The medicinal plant *Bidens bipinnata* Lin. (Asteraceae) which is known for its anti-inflammatory, antifungal and antitumor effects has been chosen for the investigation of its endophyte to search for bioactive metabolites.

Methods: An endophytic *Alternaria alternata* species was isolated from the leaves of the plant *B. bipinnata* Lin. To investigate the metabolic profile of this endophytic fungus it was cultivated in several culture media as static and shaken culture. The antimicrobial and cytotoxic activities of the ethyl acetate extracts of the fungus were examined. Extracts exhibiting highest antimicrobial activities in agar diffusion assay and cytotoxicity against HeLa cancer cell line were subjected to activity-guided chromatographic fractionation for the identification of bioactive metabolites. A cytotoxic assay was performed on the isolated compounds against HeLa cancer cell lines as well as cytostatic activity tests against HUVEC and K-562 cell lines.

Results: Chromatographic fractionation resulted in the isolation and identification of alternariol and tentoxin from the extract of the fungus cultivated in medium M5 while sterigmatocystin was isolated in addition to alternariol and tentoxin from the extract of the fungus grown in medium M25. Both alternariol and sterigmatocystin proved to be of moderate cytotoxicity and weak cytostatic activity with alternariol showing higher cytotoxic activity than sterigmatocystin. Highest cytotoxicity against HeLa cell lines was observed for tentoxin with a CC_{50} of 22.5 µg/ml.

Conclusion: This study presents the isolation and identification of the bioactive metabolites alternariol, sterigmatocystin and tentoxin from the endophyte *A. alternata* in addition to the antifungal activity of the strain extract as well as the cytotoxic and cytostatic activities of the isolated metabolites against HeLa, HUVEC and K-562 cell lines, respectively.

Keywords: Alternariol, Sterigmatocystin, Tentoxin, Endophytes, *Alternaria* alternata, *Bidens bipinnata*

DOI: http://dx.doi.org/10.22159/ijpps.2020v12i3.36317. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps

INTRODUCTION

Endophytes are considered as a promising source for bioactive metabolites and only few plant species of the entire plant kingdom on earth have been examined so far [1-3].

Plants having special uses and biological activities are considered as a promising source for study [4]. Accordingly, the medicinal plant *Bidens bipinnata* has been chosen for the investigation of its endophytic content. *B. bipinnata* L. is a widely distributed herbaceous plant that was used in traditional medicine for the treatment of inflammation accompanying several diseases such as hepatitis and diabetes [5].

A study has been conducted to investigate the role of total flavonoids of *B. bipinnata* in suppressing tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) release in HUVECs cultured with sera from active Henoch-Schonlein purpura (HSP) patients. Results showed that total flavonoids of the plant may inhibit the inflammatory mediators in HUVECs induced by sera from active HSP patients [5]. Furthermore, polyacetylenes from *B. bipinnata* were reported to exert anti-inflammatory activities on lipopolysaccharide (LPS)-induced interleukin 1 and tumor necrosis factor (TNF-α) production in macrophage (RAW264.7) cells [6]. In a recent study, the plant extract exerted inhibitory effects on human HepG2 cell lines and Hela cell lines with IC_{50} values of 14.80 µg/ml and 13.50 µg/ml respectively [7].

Since mainly the plant but not its endophytes has been studied so far and as it is a biologically active plant that could represent a promising source of bioactive endophytes according to the plant selection strategies identified by Strobel [4], it has been chosen for the investigation of its endpophytes. In a previous study an endophytic *Khuskia oryzae* species have been isolated from the stem parts of *B. bipinnata* and two bioactive oxylipins, as well as the mycotoxin sterigmatocystin, have been isolated from it [8].

This study presents the isolation and identification of biologically active metabolites of another endophytic fungus, *Alternaria alternata* from the leaves of *B. bipinnata.*

Alternaria fungi are widely spread in nature and have been reported to act as phytopathogens, plant pathogens, parasites, saprophytes and endophytes [9]. Accordingly, a wide variety of biological activities have been reported for *Alternaria* metabolites such as phytotoxic, cytotoxic, and antimicrobial properties [10-14]. Porritoxin isolated from an endophytic *Alternaria* species has been studied as a potential cancer chemopreventive agent [15]. Depudecin, an inhibitor of histone deacetylase (HDAC) from *A. brassicicola*, also showed antitumor potency [16, 17]. Some *Alternaria* metabolites such as tenuazonic acid and tentoxin have been studied as herbicidal agents [18-20].

Due to the importance of culture medium composition for microbial production of secondary metabolites [21-23], it was necessary to use different culture media for the cultivation of this endophytic fungus. Thus, it was cultivated in three different culture media (M4, M5 and M25) under shaking and stationary conditions to investigate the effect of varying culture media and growth conditions on the production of secondary metabolites. HPLC chromatograms of the ethyl acetate (EtOAc) extracts of the obtained cultures showed different chemical patterns and different antimicrobial and cytotoxic activities in preliminary biological screening tests.

MATERIALS AND METHODS

The wildly growing plant material was collected near Cairo/Egypt and identified by Dr. Abdel Megid (Head of Department of Botany at the Museum of Agriculture, Cairo/Egypt). Leaves of the plant were cut into small pieces, washed with sterilized water, followed by treatment with 70% ethanol for 1-2 min and air-drying under a

laminar flow hood. This was performed in order to eliminate contaminants. Outer tissues were removed from the plant samples and the inner tissues were carefully dissected under sterile conditions and placed onto malt agar plates containing antibiotics. After four weeks of incubation, hyphal tips of the fungus were transferred to fresh malt agar medium. The fungus was cultivated on malt agar medium at room temperature for several days. When fungal hyphae almost covered the surface of the MA plate, cultures were stored at 4 °C for a maximum period of 6 mo, and then re-inoculated onto fresh MA media.

Identification of the fungal strain was carried out at the Centraalbureau voor Schimmelcultures in the Netherlands using a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region.

Large-scale fermentation (40 L) of the endophytic fungus was carried out under static conditions in two different culture media (M5 and M25). The first medium used for cultivation of the investigated fungus was M5, which was composed of glycerin (20 g/l), glucose (2 g/l), peptone (10 g/l) and sodium chloride (0.5 g/l). In addition static cultures were also prepared in medium M25 which consisted of glucose (10 g/l), malt extract (20 g/l), soybean flour (2 g/l), yeast extract (1 g/l), potassium dihydrogen phosphate (1 g/l) and magnesium sulfate heptahydrate (0.5 g/l). Extraction with ethyl acetate resulted in a dry weight of 15 g crude extract for the cultures in M5 and a yield of 17 g dry extract for the culture in M25. After defatting with n-hexane final extracts of 10 g and 12 g were obtained for both cultures respectively. Both extracts were subjected to bioactivity guided chromatographic fractionation using hexane; ethyl acetate (9:1) followed by a gradual increase of polarity till elution with 100% ethyl acetate. Bioactive fractions were furthermore purified using Sephadex LH-20 followed by preparative HPLC using reversed-phase silica as a stationary phase and a mixture of acetonitrile and water as a mobile phase. These purification steps resulted in the isolation of 1.7 mg of alternariol, 1.5 mg of tentoxin and 1.4 mg of sterigmatocystin.

Alternariol: reddish white needles, UV (MeOH) λ_{max} 206.1, 255.8, 299.8 and 339.7 nm; HRESIMS *m/z* 259.2106 $[M+H]^+$, calcd 259.2109 for $C_{14}H_{11}O_5$.

Tentoxin: colourless needles, $[a]_D^{20}$ -117 (*c* 0.3, MeOH). UV (MeOH) λ_{max} 220, 280, 300 nm. IR (film) vmax: 3350, 2950, 1450, 1670, 1630, 1520, 760, 700 cm^{-1}. HRESIMS *m/z*: 415.2254 $[M+H]^+$, calcd 415.2259, for $C_{22}H_{30}N_4O_4$.

Sterigmatocystin: pale yellow powder, $[\alpha]_D^{20}$ -363 (*c* 1.0, $CHCl_3$), UV (MeOH) λ_{max} (log ϵ) 233 (27200), 248 (34000), 275sh (7700), 330 (19200) nm; IR (film): 3125, 1655, 1635, 1610, 1595 cm^{-1}; HRESIMS *m/z* 325.2506 $[M+H]^+$calcd 325.2510 $[M+H]^+$for $C_{18}H_{13}0_6$.

Antimicrobial screening

Antimicrobial activities were examined by agar diffusion tests according to the literature [24, 25] and minimal inhibitory concentration (MIC) was determined using the broth microdilution method according to the NCCLS guidelines [26].

Antiproliferative and cytotoxic assays

The cells used in this assay were HUVEC (ATCC CRL-1730), K-562 (DSM ACC 10) and HeLa (DSM ACC 57) which were cultured in DMEM (CAMBREX 12-614F), RPMI 1640 (CAMBREX 12-167F) and RPMI 1640 (CAMBREX 12-167F) respectively. The culture medium of these cells was supplemented with 10 ml l^{-1} ultraglutamine 1 (Cambrex 17-605E/U1), 500 µl l^{-1} gentamicin sulfate (CAMBREX 17-518Z), and 10 % heat-inactivated fetal bovine serum (PAA A15-144) at 37 °C in high-density polyethylene flasks (NUNC 156340).

Antiproliferative assay

Before dilution in DMEM, the test substances were dissolved in DMSO. After soft trypsinization the adherent cells were harvested at the logarithmic growth phase using 0.25 % trypsin in PBS containing 0.02 % EDTA (Biochrom KG L 2163). Approximately 10.000 cells were seeded with 0.1 ml culture medium per well of the 96-well microplates for each experiment (NUNC 167008).

Cytotoxic assay

HeLa cells were pre-incubated for 48 h without the test substances for the cytotoxic assay. After the pre-incubation time dilutions of the compounds were carried out carefully on the subconfluent monolayers of HeLa cells. In a humidified atmosphere and 5 % CO_2 cells were incubated with dilutions of the test substances for 72 h at 37 °C.

Method of evaluation

To estimate the influence of chemical compounds on cell proliferation of K-562, determination of the numbers of viable cells present in multiwell plates was carried out via CellTiter-Blue® assay. To measure the metabolic capacity of the cells, resazurin was used as an indicator of cell viability. It was observed that viable cells of untreated control retain the ability to reduce resazurin into resorufin, which is highly fluorescent, while nonviable cells do not generate a fluorescent signal since they rapidly lose metabolic capacity and do not reduce the indicator dye. Under these experimental conditions, the signal obtained from the CellTiter-Blue® reagent is proportional to the number of viable cells. Glutaraldehyde was used to fix adherent HUVEC and HeLa cells and a 0.05 % solution of methylene blue was used for for 15 min to stain them. The stain was eluted with 0.2 ml of 0.33 N HCl in the wells after gentle washing. SUNRISE microplate reader (TECAN) was used to measure optical densities at 660 nm. GI_{50} and CC_{50} values were determined as being where the dose-response curve intersected the 50% line, compared to untreated control. Magellan (TECAN) software was used to perform the comparisons of the different values.

RESULTS AND DISCUSSION

The pure fungal strain was cultivated in three different culture media (M4, M5 and M25) both as shaken and as stationary culture, in order to examine the effect of different growth conditions on the secondary metabolite production of this endophytic strain. The extracts obtained for the fungal strain after cultivation in each medium were tested for antimicrobial activity against several microbial strains (*S. salmonicolor, K. marxianus, C. albicans, A. niger, A. fumigatus, P. avellaneum* and *A. terreus*) (table 1). Highest antimicrobial activity was observed for the strain cultivated in medium M25 as a static culture. Extracts of shaken cultures were less active than static culture extracts. The obtained fungal extracts after cultivation in different media were subjected to a cytotoxic assay against HeLa cancer line. Highest cytotoxicity was observed for the extract of the fungus cultivated in medium M25 (fig. 1).

Table 1: Antimicrobial activity[a] of extracts of different cultures of *Alternaria alternata*

Strain	*S. salmonicolor*	*K. marxianus*	*C. albicans*	*A. niger*	*A. fumigatus*	*P. avellaneum*	*A. terreus*
Extract of stationary culture in M25	17	28	0	22	16	18	23
Extract of stationary culture in M5	13	17	0	0	12	17	18
Extract of stationary culture in M4	10	13	0	0	0	15	13
Extract of shaken culture in M25	13	18	24	20	14	13	20
Extract of shaken culture in M4	12	12	0	0	0	12	10
Extract of shaken culture in M5	12	16	18	0	12	10	14
Nystatin	25	28	24	20	20	20	20

a = measured in terms of the diameter of the inhibition zone in millimeters

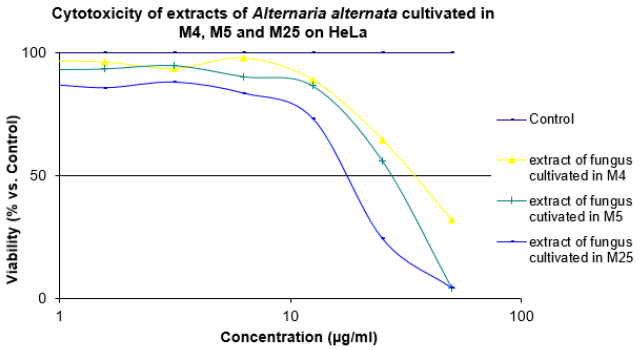

Fig. 1: Cytotoxicity of *Alternaria* extracts on Hela cell line

Activity guided chromatographic fractionation resulted in the isolation of the bioactive secondary metabolites of this fungal strain. The fungus grown in media M5 and M25 produced two common major secondary metabolites.

For the first compound a molecular formula of $C_{14}H_{11}O_5$ (*m/z* 259.2106 $[M+H]^+$) was determined by HRESIMS thus indicating 10 degrees of unsaturation. The number of carbons and hydrogens suggested by HRESIMS was in agreement with the number of signals detected in the ^{13}C NMR and ^1H NMR data (table 2). The ^1H NMR spectrum exhibited signals for four aromatic hydrogens at δ 6.4, 6.6, 6.7 and 7.2 ppm; one methyl group at δ 2.7 ppm and three phenolic hydroxyl groups at δ 10.3, 10.8 and 11.7 ppm. Each two of the four aromatic protons were *meta* coupled with each other thus indicating the presence of two different aromatic rings. The ^{13}C NMR spectrum exhibited signals for a methyl group (δ 25.23 ppm), eight olefinic carbons (δ 97.40, 100.86, 101.59, 104.3, 108.95, 117.52, 138.12, 138.32 ppm), four olefinic carbon atoms bearing oxygen atoms (δ 152.61, 158.41, 164.06, 164.69 ppm) and a carbonyl group (δ 165.39 ppm). From the HMBC correlations, it was observed that one phenyl ring contained the two *meta* positioned protons at δ 7.2 and 6.4 ppm together with the two hydroxyls at δ 10.8 and 11.7 ppm. This was concluded from the correlations observed between H-4 (δ 6.4 ppm) and C-6, 2, 3 and those of H-6 (δ 7.2 ppm) with C-2, 4, 7 and 1'. Furthermore HMBC correlations were observed for the second phenyl group suggesting its substitution by one hydroxyl and one methyl group due to the correlations observed between H-3` (δ 6.6 ppm) and C-1', 4', 5', 6' and for H-5` (δ 6.7 ppm) with C-1', 3', 4', 7'. The upfield shift of C-2 (δ 97.40 ppm) suggests its connection to a carbonyl group while the downfield shift observed for C-2' (δ 138.32 ppm) indicates its connection to an ether group. According to literature data the deduced structure identified the isolated compound as alternariol (fig. 2) [27].

Alternariol is considered as a mycotoxin that has been previously isolated from several *Alternaria* species reported of infecting various fruits, such as tomatoes, olives, mandarins, melons, peppers and apples [28].

In addition, alternariol was previously isolated from a mangrove endophytic fungus from the South China Sea Coast and was reported to have strong cytotoxic activity against KB cell lines with an IC_{50} value of 4.82 µg ml^{-1}. Antifungal activity and choline esterase inhibitory activity have also been reported for it [29]. Further investigation showed that alternariol [26] has been identified as a topoisomerase I and II poison which might contribute to the impairment of DNA integrity in human colon carcinoma cells [30]. It induced cell death by activation of the mitochondrial pathway of apoptosis in human colon carcinoma cells [31]. A more recent study investigated the mechanism of action of immune suppression of alternariol. It was found to suppress lipopolysaccharide (LPS)-induced NF-κB pathway activation in THO-1 derived macrophages, decrease secretion of proinflammatory cytokines IL-8, IL-6 and to induce secretion of anti-inflammatory IL-10. In absence of LPS stimulus alternariol was found to increase IL-10 transcription only. Consequently it was concluded that alternariol is capable of repressing inflammation in an inflamed environment by targeting the NF-κB signaling pathway [32]. Furthermore, alternariol belongs to the toxins of *Alternaria*, which has phytotoxic properties and is important in the development of some plant disease processes such as black spot and seedling chlorosis [29].

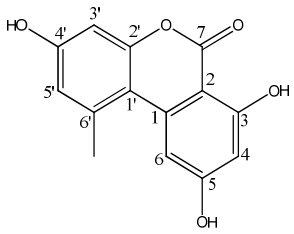

Fig. 2: Chemical structure of alternariol

Table 2: NMR spectroscopic data (125 MHz, DMSO-d_6) of alternariol

Position	δ ^{13}C	δ ^1H (*J in Hz*)	HMBC
1	138.1, qC		
2	97.4, qC		
3	164.7, qC		
4	100.9, CH	6.4, *d* (1.95)	2, 3, 6
5	164.1, qC		
6	104.3, CH	7.2, *d* (1.95)	2, 4, 7, 1`
7	165.4, qC		
1`	109.0, qC		
2`	138.3, qC		
3`	101.6, CH	6.6, *d* (2.60)	1`, 4`, 5`, 6`
4`	158.4, qC		
5`	117.5, CH	6.7, *d* (2.45)	1`, 3`, 4`, 7`
6`	152.6, qC		
7`	25,2, CH_3	2.7, *s*	6, 8, 9
OH		10.3, *s*	3`, 4`, 5`
OH		10.8, *s*	4, 6, 7
OH		11.7, *s*	2, 3, 4

Another major metabolite was produced by the fungus cultivated in M25 but was not observed in HPLC chromatograms of extracts of the fungus grown in M4 or M5. This compound appeared as a yellow solution in methanol and a molecular formula of $C_{18}H_{13}O_6$ (*m/z* 325.2506 $[M+H]^+$) was determined for it by HRESIMS. The ^{1}H-NMR spectrum showed eight proton signals, a hydroxyl proton appearing at δ 13.28 ppm indicating the presence of a chelated phenolic hydroxyl group and a methoxy group at δ 56.88 ppm. The ^{13}C-NMR data (table 3) revealed the presence of 18 carbon signals and thus together with the ^{1}H-NMR confirmed the suggested molecular formula. The HMBC correlations (table 3) clearly revealed the first part of the structure as being a benzo-x-pyrone. The downfield shift of C-7 and C-2 indicates their connection to oxygen atoms and thus reveals this part of the structure as being a tetrahydrodifurano ring system. By comparing the obtained structure with literature data it was found to be the mycotoxin sterigmatocystin (fig. 3), which has been previously isolated from several *Aspergillus* species like *Aspergillus versicolor* and *Aspergillus multicolor* [33]. Through the xanthone nucleus attached to a bifuran structure, it closely resembles the aflatoxins and has similarly been shown to be toxic to mice [34], rats [35] monkeys [36], ducklings [34] and is carcinogenic and mutagenic when injected or fed to rats [35]. Sterigmatocystin and has been detected in wheat [36] and coffee beans [37]. It belongs to the main 20 mycotoxins that are known to occur in foodstuffs at significant levels and frequency to be of food safety concern [33]. These mycotoxins have been reported to be produced by five fungal genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* [33].

A cytotoxic assay against HeLa cancer cell line was performed on the isolated compounds and observed results showed that both alternariol and sterigmatocystin exerted moderate cytotoxicity against HeLa cell lines and weak cytostatic activity against both HUVEC and K-562 cell lines with alternariol exerting higher cytotoxic and cytostatic activities (fig. 4). A CC_{50} value of more than 50 µg ml^{-1} and GI_{50} values of more than 50 and 44.7 µg ml^{-1} were observed for alternariol in the cytotoxic and cytostatic assay against HUVEC and K-562 cell lines respectively (fig. 4).

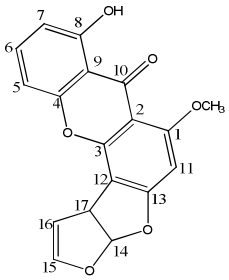

Fig. 3: Chemical structure of sterigmatocystin

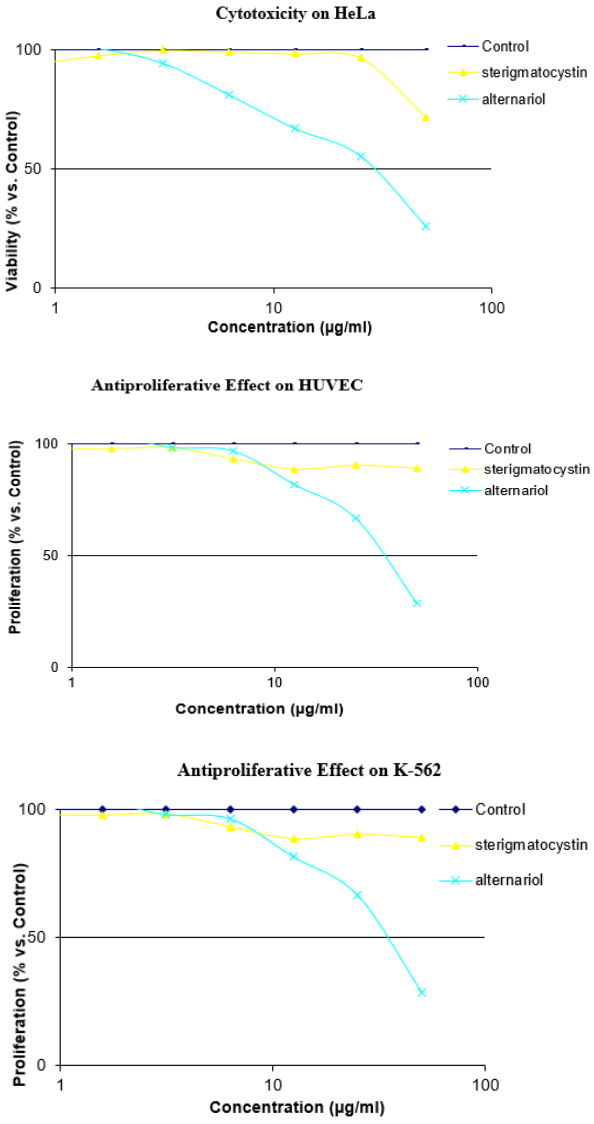

Fig. 4: Antiproliferative and cytotoxic activities of sterigmatocystin and alternariol

Table 3: NMR spectroscopic data (150 MHz, DMSO-d_6) of sterigmatocystin

Position	δ ^{13}C	δ ^{1}H (*J* in Hz)	HMBC
1	110.8, CH	6.72, *d*	8, 17, 9
2	106.6, qC		
3	180.5, qC		
4	136.3, CH	7.62, *t*	8, 4, 9
5	161.4, qC		
6	162.9, qC		
7	102.7, CH	5.53, *t*	3, 15
8	153.4, qC		
9	105.0, qC		
10	145.7, CH	6.74, *d*	9, 17
11	113.4, CH	6.90, *d*	15, 13, 16, 5, 17
12	154.5, qC		
13	106.6, CH	7.00, *d*	4, 9, 7, 14, 17
14	164.5, the		
15	91.1, CH	6.71, *s*	8, 1, 13, 12
16	108.3, qC		
17	47.3, CH	4.86, *d*	16, 5, 11
18	56.9, OCH_3	3.89, *s*	1

The second main active constituent of the strain extracts obtained after cultivated in both M25 and M5 was found to be tentoxin (fig. 5). A molecular weight of 414 g/mol (base peak at 415.23 $[M+H]^+$ in the HPLC-MS) was suggested for the compound. By dereplication with authentic samples, it has been found to have an identical UV chromatogram to that of tentoxin (fig. 5). Also the IR spectrum and chromatographic properties were identical to those of tentoxin. This metabolite is a phytotoxin, which causes chlorosis in the seedlings of many plants [38]. Chemically, tentoxin is cyclo-[L-leucyl-*N*-methyl-(Z)-dehydrophenylalanylglycyl-*N*-methyl-L-alanyl]. Since dihydro-tentoxin has almost no chlorotic effect, the presence of the styrene structure in the dehydrophenylalanyl residue is essential for the chlorotic activity exerted by tentoxin [38].

A cytotoxic assay of tentoxin against HeLa cancer cell line was carried out and results revealed strong cytotoxicity for tentoxin with a CC_{50} of 22.5 µg/ml (fig. 6). This result is in agreement with previously conducted studies on tentoxin which stated that it exerted cytotoxic activity against both lung cancer cell line A549 and breast cancer cell line MDA-MB-231[39]. Previous studies also reported cytotoxic activities of *Alternaria alternata* against cultured tobacco BY-2 cells and detected the production of tentoxin by the fungus. The phytotoxin was reported to exert potent inhibition of the chloroplastic ATP synthase of certain plant species [40].

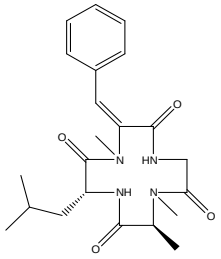

Fig. 5: Chemical structure of tentoxin

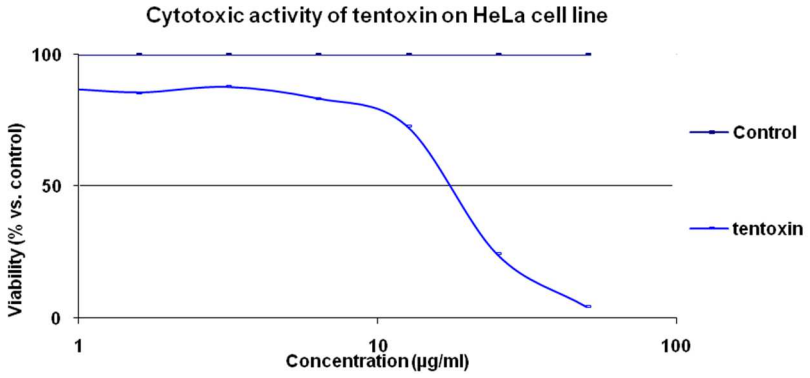

Fig. 6: Cytotoxic activity of tentoxin on HeLa cell line

Now the question arises about the role of these bioactive metabolites in the plant-endophyte interaction. Several hypotheses have been made to find an answer to this question. It has been suggested that endophytes may contribute to their host plant by producing many bioactive substances to provide protection that helps the host plant to survive stressful conditions. Some endophytes were found to be capable of increasing the hosts' effects on other plant species co-growing with them which compete with them for nutritional sources and habitat [4]. This could be the reason why dominant plant species were found to harbor special endophytes that support them in their competition with other species [4]. Indeed, this might be true for the plant *B. bipinnata*, as it is known to be a dominant, widely spreading species distributed in many regions of the world [41], which could be referred to the bioactive metabolites produced by its endophyte. Therefore, the detected production of bioactive natural products, especially cytotoxic and antifungal compounds by the fungal endophyte of *B. bipinnata* suggests their possible role in protecting themselves from competitors and the host plant from invaders.

At the time the endophyte was isolated from the plant, it was free from any symptoms of diseases, which could be due to the absence of pathogenic endophytes or due to the presence of latent pathogens. This assumption is based on previous hypotheses

suggesting that it is possible to isolate a latent pathogen from an asymptomatic plant proposing that some pathogens may develop from endophytes [42].

This could explain the detection of phytotoxins such as tentoxin, sterigmatocystin, and alternariol known to cause chlorosis in plants, as secondary metabolites of the endophytic strain *Alternaria alternata,* from healthy samples of the plant *Bidens bipinnata*. It could be assumed that these toxins are produced by latent pathogens which are waiting for a suitable time of reduced immune response of the plant or senescence to exert their pathogenic effects, since being isolated as an endophyte, does not exclude the possibility that a fungus may become pathogenic when the host is stressed or senescent [42].

CONCLUSION

In conclusion, the endophyte *Alternaria alternata* has been isolated from the leaves of the medicinal plant *Bidens bipinnata* and its extract was found to exert antifungal activity against several fungal species, cytotoxicity against HeLa cell line as well as weak cytostatic activities against HUVEC and K-562 cell lines. Bioactivity-guided chromatographic fractionation resulted in the isolation and identification of alternariol, sterigmatocystin and tentoxin as bioactive metabolites of this fungal endophyte. Results of this study support previously made assumption of possible protective effects of endophytes on their host.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

Randa Abdou performed the study steps, evaluated the research results and wrote the manuscript. Mohamed Dawoud analyzed data.

CONFLICTS OF INTERESTS

The authors report no conflicts of interest in this work.

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