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 endophytes. In a previous study an alternative strategy identified by Strobel [4], it has been chosen for the investigation of its endophytes to search for bioactive metabolites.

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-Schönlein 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 IC50 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 endophytes. 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 tenamoxaxic acid and tenoxin 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 is 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
Antimicrobial screening

Antiproliferative and cytotoxic assays

Antimicrobial activities were examined by agar diffusion tests according to DSM ACC 10 and HeLa (DSM ACC 57) which were cultured in NCCLS guidelines [26].

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 purification steps resulting in the isolation of 1.7 mg of tentoxin and 1.4 mg of sterigmatocystin. Further purification was carried out 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) \(\lambda_{max} = 206.1, 255.8, 299.8\) and 339.7 nm; HRESIMS \(m/z = 259.2106 [M+H]^+\), calcld \(259.2109\) for C14H13O5.

**Tentoxin:** colourless needles, \([\alpha]_D^{20} = -117\) [c 0.3, MeOH]. UV (MeOH) \(\lambda_{max} = 220, 280, 300\) nm. IR (film) \(\nu_{max} = 3350, 2950, 1450, 1670, 1630, 1520, 760, 700\) cm\(^{-1}\). HRESIMS \(m/z = 415.2254 [M+H]^+\), calcld \(415.2259\) for C24H19O11.

**Sterigmatocystin:** pale yellow powder, \([\alpha]_D^{20} = -363\) [c 1.0, CHCl3]. UV (MeOH) \(\lambda_{max} = 233 (27200), 248 (34000), 275sh (77000), 330 (19200)\) nm; IR (film): 325, 1655, 1635, 1610, 1595 cm\(^{-1}\). HRESIMS \(m/z = 325.2506 [M+H]^+\), calcld 325.2510 [M+H]^+ for C17H13O6.

**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 extracts of the fungal strains were subjected to a cytotoxic assay against HeLa cancer line. Highest cytotoxicity 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 extracts of the fungus cultivated in medium M25 (fig. 1).

| Table 1: Antimicrobial activity 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           | 0       | 0           | 12            | 17        |
| Extract of stationary culture in M5  | 13              | 17           | 0           | 0       | 0           | 12            | 17        |
| Extract of stationary culture in M4  | 10              | 13           | 0           | 0       | 0           | 12            | 17        |
| 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        |

\(\text{a = measured in terms of the diameter of the inhibition zone in millimeters}\)
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 $^1$H NMR data (table 2). The $^1$H NMR spectrum exhibited signals for four aromatic hydrogens at $\delta$ 6.4, 6.6, 6.7 and 7.2 ppm; one methyl group at $\delta$ 2.7 ppm and three phenolic hydroxyl groups at $\delta$ 10.8, 11.0 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 a methyl group at $\delta$ 25.23 ppm, eight olefinic carbons ($\delta$ 97.40, 100.86, 101.59, 104.3, 108.95, 117.52, 130.12, 138.52 ppm), four olefinic carbon atoms bearing oxygen atoms ($\delta$ 152.61, 158.41, 164.06, 164.69 ppm) and a carbonyl group ($\delta$ 165.39 ppm). From the HMBC correlations, it was observed that one phenyl ring contained the two meta positioned protons at $\delta$ 7.2 and 6.4 ppm together with the two hydroxyls at $\delta$ 10.8 and 11.7 ppm. This was concluded from the correlations observed between H-4 ($\delta$ 6.4 ppm) and C-6, 2, 3 and those of H-6 ($\delta$ 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' ($\delta$ 6.6 ppm) and C-1', 4', 5', 6' and for H-5' ($\delta$ 6.7 ppm) with C-1', 3', 4', 7'. The upfield shift of C-2' ($\delta$ 97.40 ppm) suggests its connection to a carbonyl group while the downfield shift observed for C-2' ($\delta$ 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 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].

| Position | $\delta$ $^{13}$C | $\delta$ $^1$H ($J$ in Hz) | HMBC |
|----------|-----------------|--------------------------|------|
| 1        | 138.1, qC       | $6.4, d(1.95)$          | 2, 3, 6 |
| 2        | 97.4, qC        |                          |      |
| 3        | 164.7, qC       |                          |      |
| 4        | 100.9, CH       |                          |      |
| 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       | 6.7, $d(2.45)$          | 1', 3', 4', 7' |
| 5        | 117.5, CH       | 6.7, $d(2.45)$          | 1', 3', 4', 7' |
| 6'       | 152.6, qC       | 2.7, s                  | 6, 8, 9 |
| 7'       | 25.2, CH$_3$    | 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 $\delta$ 13.28 ppm indicating the presence of a chelated phenolic hydroxyl group and a methoxy group at $\delta$ 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-$\alpha$-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 tetrahydrofuran 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 $\mu$g ml$^{-1}$ and GI$_{50}$ values of more than 50 and 44.7 $\mu$g 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](image)

![Fig. 4: Antiproliferative and cytotoxic activities of sterigmatocystin and alternariol](image)
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 CC₅₀ 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].

![Chemical structure of tentoxin](image)

**Table 3: NMR spectroscopic data (150 MHz, DMSO-d₆) of sterigmatocystin**

| Position | δ¹³C     | δ¹H (J/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, OCH₃| 3.89, s    | 1    |

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.

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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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