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IN VITRO CYTOTOXIC PROPERTIES OF *CLADOSPORIUM CLADOSPORIOIDES* EXTRACT AGAINST HUMAN OVARIAN CANCER CELLS A2780 AND MOUSE LYMPHOMA CELLS L5178Y

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ABSTRACT

Fungi have been a major source of new bioactive molecules, including anticancer agents. In this study, three soil fungal isolates were fermented in agar media and extracted with ethyl acetate after 21 days. The extracts were tested for cytotoxicity properties against murine lymphoma cell line (L5178Y) and epithelial ovarian cancer cell line (A2780). Identification of the fungal metabolites was carried out using HPLC analysis. Results showed potent growth inhibition against both A2780 and L5178Y by *Cladosporium cladosporioides* crude extract. HPLC analysis of *C. cladosporioides'* extract revealed the compound ergosterol as the major component, which may be responsible for the cytotoxic activity of the extract. The results of this study support further investigation of this fungus for anticancer metabolites.

KEYWORDS: ***Cladosporium cladosporioides, cytotoxicity, fungal metabolites***

INTRODUCTION

Cancer remains one of the leading causes of death worldwide second to only cardiovascular diseases [1,2]. Effective treatment for cancer is still an unmet healthcare need. While most of the available treatments are often very toxic [3], resistance against more selective ones often develops rapidly.[4] Also, most of the available treatments are not curative such that mortality rate is still high contributing about 13% of all deaths according to World Health organization. There is therefore an urgent need for new, safer and more effective anticancer agents. Generally, natural products have been the source of new drugs for the treatment of various diseases including cancer [5]. Many of the available antineoplastic agents are also of natural origin [6, 7]. Some of them such as the vinca alkaloids, paclitaxel and camptothecin were derived from plants [8]. A few of them such as bleomycin [9], boningmycin [10], epoxomicin [11], doxorubicin [12], were derived from soil bacteria. Although many fungal metabolites with potent cytotoxic properties have been reported [13] and a few such as simvastatin are undergoing clinical trials [14,15], fungal metabolites are largely unexplored [6] and none has been approved for clinical use as an anticancer agent. Soil microbiota has been the source of many drugs in clinical use especially the antibiotics such as the tetracycline, streptomycin and chloramphenicol [16]. Cyclosporin A, an immunosuppressant, was isolated from

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Trichoderma polysporum a soil fungi [17]. *Aspergillus terreus*, another soil fungus also produces lovastatin which is an antilipidemic agent [18]. In this study, the *in vitro* cytotoxic property of the extract of *Cladosporium cladosporioides* against two cancer cell lines - mouse lymphoma and human epithelial ovarian cancer cell lines is reported.

MATERIALS AND METHODS

Isolation and purification of soil fungi

The soil fungi were isolated using the soil-plate method described by Warcup [19]. One gram of the soil sample was suspended in 10 mL of double-distilled water and was serially diluted to make microbial suspensions (10^{-1} to 10^{-5}). Dilutions of 10^{-2} to 10^{-4} were taken and introduced into differently labelled sterile Petri-dishes (triplicate of each dilution). Unto the Petri dishes, sterile on Malt Extract Agar (MEA) (Oxoid, UK) was added and swirled evenly to ensure homogeneity of the mixture and production of discrete colonies. Chloramphenicol 50 mg/L was added to the MEA medium to inhibit bacterial growth. The Petri dishes were left to set for 30 min on the bench before incubation at room temperature for 7 days in the dark. At the end of the incubation period, the culture plates were inspected. Based on differences and similarities in the colony morphology of the mixed cultures, purification was carried out by subculturing onto freshly prepared MEA. This process was repeated until pure cultures were obtained. A total of three pure isolates labelled FG2, FG4 and FG8 were successfully obtained.

Identification of purified fungal cultures

The soil fungal isolates were identified according to a molecular biology protocol by DNA amplification and sequencing of the ITS region, which was described previously by Kjer *et al*. [20]. The sequence data have been submitted to GenBank, with accession numbers KY034289 for *Trichoderma longibrachiatum,* KY034291 for *Cladosporium cladosporioides* and KY034290 for *Trichoderma erinaceum*. Voucher fungal specimens (Reference Numbers FG2, FG4 and FG8 respectively) were deposited at the Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Düsseldorf, Germany.

Small scale fermentation and extraction

This was carried out using the multiple-plate method in which multiple cultures of each fungal isolate was grown in 30 sterile Petri dishes containing MEA for a total of 21 days at 28°C in the dark. At the end of the fermentation period, the agar was cut into small pieces using sterile spatulas. Exactly 500 mL of ethyl acetate (Sigma-Aldrich, Germany) was poured into appropriately labelled conical flasks and the fungi transferred into their respective flasks and agitated for two days. Thereafter filtration was carried out and the filtrates concentrated using the vacuum rotary evaporated at 45°C.

Cytotoxicity assay (MTT colorimetric assay) using epithelial ovarian cancer cells (A2780)

The extracts were tested for cytotoxic activity against human ovarian cancer cells (A2780). The cells were obtained from ECACC (Salisbury, Wiltshire/UK) and cultivated in RPMI-1640 medium supplemented with 10% FBS, 120 µg/ml streptomycin and 120 U/ml penicillin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The cells were seeded into 96-well plates (Greiner, Frickenhausen, Germany) at a density of 20% per well in a fixed volume of 90 µL and kept under 5% CO_2 at 37 °C for 6 h. The seeding density depended on the growth characteristics of the cells and was chosen to avoid a 100% confluency of untreated cells. After 6 h, cells were attached as controlled by microscopy and treated with the fungal extracts, resulting in a final volume of 100 µL per well. After 72 h incubation, 20 µL of MTT solution (5 mg/mL) were added to each well. Incubation with MTT was terminated after 50-70 min (before cell-damaging formazan needles were formed) by injecting 150 µL of a mixture of 2-propanol:1 M HCl (50 mL:165 µL) to each well with an injector by using a FLUOstar microplate reader (BMG Labtechnologies, Offenburg, Germany). Then, the 96-well plates were kept at 5 °C for 1-2 h. Finally, the absorption was measured at 595 nm (test wavelength) and 690 nm (reference wavelength) using the BMG FLUOstar. Absorption at the reference wavelength was subtracted from the absorption at the test wavelength.

Cytotoxicity assay (MTT colorimetric assay) using mouse lymphoma cells (L5178Y)

The extracts were tested for cytotoxic activity against mouse lymphoma cells (L5178Y). The murine cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were maintained in a humified

atmosphere at 37° C with 5% CO_2. Stock solutions in ethanol 96% (v/v) of each of the fungal extracts were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µL containing 3750 cells were pipetted into 96-well microtiter plates. Subsequently, 50 µL of a solution of the test samples (10 µg/mL) containing the appropriate concentration was added to each well. The small amount of ethanol present in the wells did not affect the experiments. The test plates were incubated at 37° C with 5% CO2 for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH2PO4, 6.5 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3 h 45 min at 37° C in a humidified incubator with 5% CO2, the medium was centrifuged (15 min, 20 °C, 210 x g) with 200 µL DMSO, the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microliter-well spectrophotometer. The color intensity is correlated with the number of healthy living cells.

Cell survival was calculated using the formula:
Survival % = 100 x (TC_{abs} - CM_{abs})/(UTC_{abs} -CM_{abs})
Where TC_{abs} = absorbance of treated cells; UTC_{abs} = absorbance of untreated cells; CM_{abs} = absorbance of culture medium
All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

High Performance Liquid Chromatography (HPLC)

The extracts of the four isolates were subjected to HPLC for preliminary identification of their components. Exactly 1 mg/mL of each extract was dissolved in HPLC grade methanol, sonicated for 10 min and centrifuged at 3,000 rpm for 5 min. A 1:5 serial dilution was carried out to get 0.2 mg/mL solution and 20 µL of those solutions was analysed in Dionex HPLC system equipped with photodiode array detector (UVD340s, Dionex Softron GmbH, Germany) using 125 mm Eurospher-10 C18 prefilled column (Knauer, Germany) with 4 mm internal diameter and 5 µm particle size. The mobile phase comprised of nano-pure water adjusted to pH 2 with formic acid and methanol. Separation was monitored at 254 nm and peaks were identified by dereplication and MS result.

Purification of precipitate using drowning-out crystallization methods

During the sample preparation of *C. cladosporioides'* extract for HPLC analysis, copious milky white precipitate was formed when the extract was dissolved in methanol. This was separated and re-dissolved in dichloromethane. Using methanol as anti-solvent, the precipitate was further precipitated and purified from solution. Purity was confirmed analytical HPLC and was identified using proton nuclear magnetic resonance and mass spectrometry.

RESULTS

Three fungal strains were successfully isolated and purified from the soil. The pure fungi strains were fermented and extracted. The dried extract of *C. cladosporioides* was 200 mg, 2 g for *T. erinaceum* and 2.7 g for *T. longibrachiatum*. The fungal extracts were subjected to cytotoxicity testing against two cell lines. In the test against mouse lymphoma cell line, *C. cladosporioides* showed up to 53% inhibition at 10 µg/mL concentration. An inhibition of 20% was recorded for *T. longibrachiatum*, and *T. erinaceum* extract showed no cytotoxic activity (Table 1). At 100 µg/mL, an inhibition of 83% for *C. cladosporioides'* extract quite comparable to the positive control cisplatin (cDDP), which showed an inhibition of 91% at 10^{-4}M. *T. longibrachiatum* and *T. erinaceum* extracts showed poor cytotoxic activity, with % inhibition not different from what was recorded for the negative controls DMSO and sodium chloride.

Table 1: Cytotoxic activities of fungal extracts against mouse lymphoma cell line (L5178Y)

Fungal Extracts	% Inhibition
T. longibrachiatum	20.2
C. cladosporioides	53.4
T. erinaceum	0.0
Control	0.0

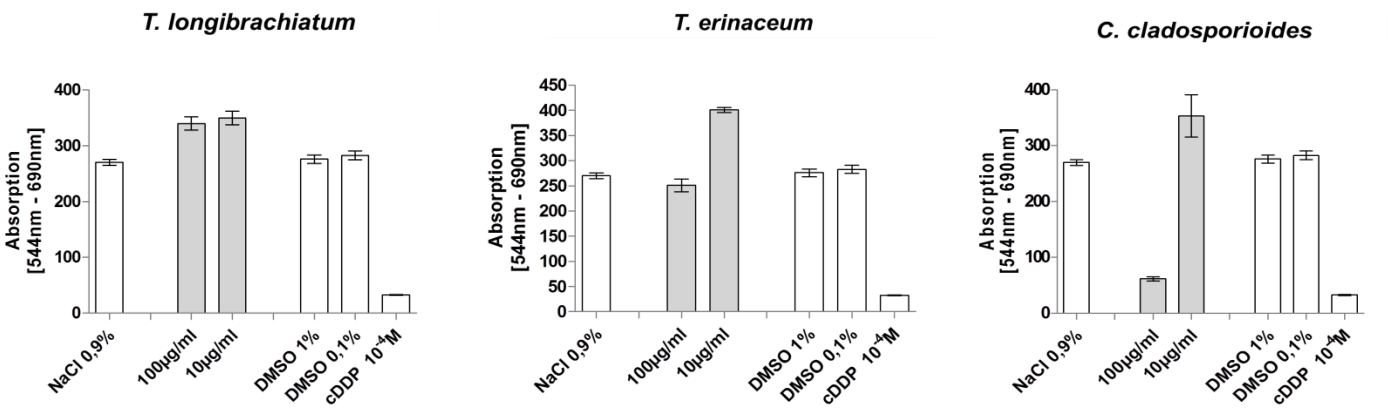
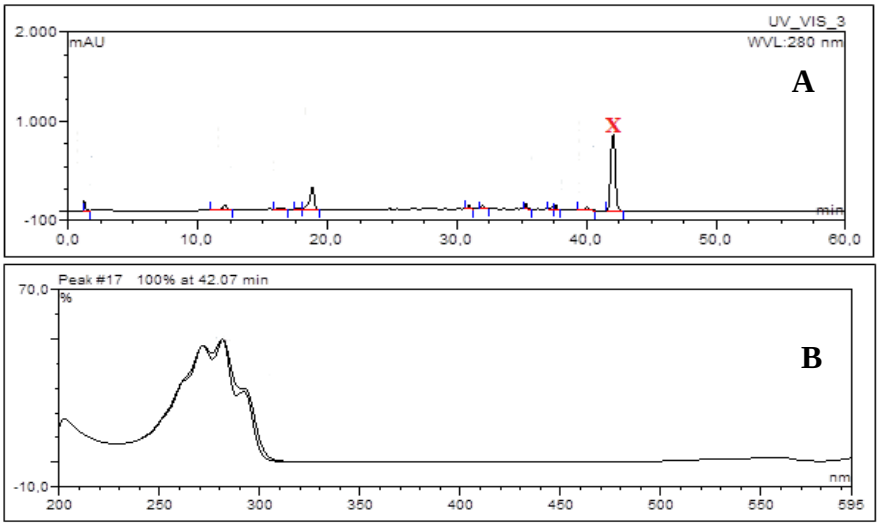
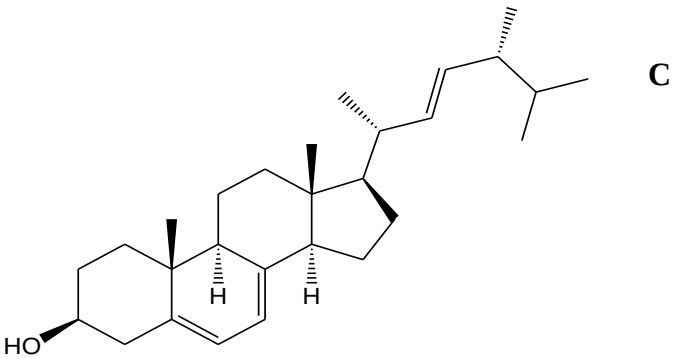


Figure 1: Cytotoxic activities of fungal extracts against the ovarian cancer cells (A2780)





$C_{28}H_{44}O$; 396.65 $gmol^{-1}$

Figure 2: HPLC chromatogram of *C. cladopsorium* extract showing detection of Ergosterol [A]; the UV spectrum [B]; and chemical structure of Ergosterol [C].

DISCUSSION

The *C. cladosporioides* extract showed about 83 % growth inhibition against the ovarian cancer cells (A2780); at 100 µg/mL, while cisplatin which is an approved antitumor agent had 91 % inhibition at 10^{-4}M. It should be noted that while cisplatin is a pure compound, the extract of *C. cladosporioides* is a crude unrefined extract with many components. Therefore, an 83% inhibition at 100 µg/mL is an interesting result. Similarly, its inhibitory effect against the mouse lymphoma cells (L5178Y) can also be considered significant. An inhibition of 53% at 10 µg/mL for a crude extract is a very significant result with an IC_{50} below 10 µg/mL. Coming from

the same extract that had 83% inhibition against the human ovarian cancer cells (A2780), it could be expected that it could have 100% inhibition against the mouse lymphoma cells (L5178Y) at far below 100 µg/mL if the results are to be compared and extrapolated. An IC_{50} of 20 µg/mL has been referred to as highly promising [21], therefore, a 53% inhibition at 10 µg/mL is obviously highly promising using the same MTT assay method. The result shows that the murine lymphoma cell line was more sensitive to the extract than the epithelial ovarian cancer cell line.

Two naphthoquinone compounds, anhydrofusarubin and methyl ether of fusarubin, isolated from *Cladosporium sp*. have been shown to have inhibitory activity against human leukemia cells [22]. Another compound, a viriditoxin derivative, cladosporinone with potent cytotoxic activity against mouse lymphoma cell line L5178Y was reported from a rice culture of *C. cladosporioides* [23]. HPLC-DAD analysis of the *C. cladosporioides* extract did not indicate the presence of any of these compounds, however, the HPLC chromatogram and UV spectrum showed the presence of the compound ergosterol as the major compound in the fungal extract (Figure 2). The UV spectrum of the detected ergosterol is confirmed by literature [24, 25]. The cytotoxic property of ergosterol has been previously reported [26, 27]. This property of ergosterol could have contributed to the observed cytotoxic property of the extract. The effectiveness of this extract against two different cancer cell lines at considerably low dose is an indication that the extract may have broad spectrum anticancer properties.

The potentials of fungal metabolites as the source of anticancer agents are high. Even though no fungal metabolite has been approved for clinical use in cancer therapy [13], a whole lot of them are at different phases of clinical trial [28]. Although paclitaxel was first isolated from a plant, *Taxia brevifolia* [29], it has since been isolated from a fungal endophyte, *Taxomyces adreanae* associated with the plant [30]. From recent reports, it is possible that paclitaxel which was isolated from *T. brevifolia* was actually produced by the endophytic fungi that inhabits the plants or produced as a result of metabolic partnership. Similarities between the metabolites isolated from plants and their associated endophytes have been reported, suggesting that some of the plant compounds could actually be fungal metabolites [31, 32]. The results of this study support the claim that fungal metabolites are potential sources of potent anticancer agents.

CONCLUSION

Three fungi *C. cladosporioides, T. longibrachiatum* and *T. erinaceum* were isolated from a soil sample. *C. cladosporioides* showed significant cytotoxic properties property against two cancer cell lines. The results strongly support further investigation of this fungus for anticancer metabolites.

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CONFLICT OF INTEREST

authors declared no conflict of interests in te conduct and reporting of this study

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