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Original Article

A Promising Natural Anticancer Compound Derived from *Gymnoascus dankaliensis*

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Abstract

Cancer is the uncontrolled growth of malignant cells and is universally estimated to be a common cause of death. No decisive treatment has been identified to cure cancer; therefore, scientists have focused on developing safe and effective treatments. The activity of natural compounds isolated from living organisms, such as fungi, has been investigated in cancer cells. This study aimed to isolate and analyze natural products, as secondary metabolites (SM), of the fungus *Gymnoascus dankaliensis (G. dankaliensis)* and identify their activity against SR and HCT-18 (HRT-18) cell lines. *G. dankaliensis* was isolated from dung samples and identified using a molecular method. The internal transcribed spacer region was amplified from the isolated genomic DNA and sequenced afterward. The isolate was grown on a rice medium as a solid-state fermentation medium to extract natural metabolite products using the ethyl acetate extraction method. The GC-MS analyzed the compound of the natural extract, and the activity of the natural extract was identified against SR and HCT-18 cell lines. The results revealed the ability of *G. dankaliensis* to produce a natural product as an SM composed of five compounds. The growth of the treated SR and HCT-8 cell lines with the natural extract was inhibited after incubation for 27 h, with the IC50 being 3.57 and 8.61 µg/mL on the HCT-18 and SR cell lines, respectively. In conclusion, the natural extract isolated from the SM of *G. dankaliensis* showed activity against cancer cells, affecting the SR and HCT-18 cell lines, compared to the control. These results revealed that the product is a promising anticancer treatment.

Keywords: Anticancer, Cancer, GC-MS analysis, *Gymnoascus dankaliensis,* Internal Transcribed Spacer, Natural Products, Solid-State Fermentation

1. Introduction

The uncontrolled growth of cells produces a malignant or benign tumor which is defined as cancer (1). Since cancer is a common cause of death in modern life, scientists have intensified their efforts to develop new strategies to address this problem (1). The use of natural products to control the growth of lesions is a valuable and safe strategy for cancer therapy (2). Great efforts have been made to solve the issues occurring during cancer treatment, such as resistance to chemotherapy (3). The expectations for the application of natural products have increased recently. Fungi are widespread organisms that inhabit marine and terrestrial environments (4). They synthesize organic and natural products called secondary metabolites (SMs), such as lovastatin, which have improved their activities (5). Based on their chemical structure, SMs are divided into four classes: polyketides, terpenoids, shikimic acid-derived compounds, and non-ribosomal peptides (6).

These natural compounds are considered a common source of pharmaceutical drugs, such as

products that act against carcinogenic tumors (anticancer), antibiotics, and cholesterol-reducing drugs. For example, *Leptosphaeria* species (spp.) produce a natural compound called leptons which induce an apoptosis pathway (7). Several natural compounds have been identified, including myriocin, triornicin, and apicidin, produced by *Melanconis flavovirens*, *Epicoccum purpurascens*, and *Fusarium semitectum*, respectively (3).

Gymnoascus dankaliensis (G. dankaliensis) is a soil-borne fungus isolated from desert soil samples from the Giza pyramids in Egypt (8). *G. dankaliensis* is classified into the kingdom fungi, phylum *Ascomycota*, order *Onygenales*, family *Gymnoascaceae*, and genus *Gymnoascu*. Hammerschmidt, Aly (8) observed that *G. dankaliensis* produced bioactive SMs after cultivation in a solid rice medium supplemented with NaCl or KBr. Fungal SMs have been poorly investigated in Iraq, and only a few studies have examined SMs in other species. No studies were identified that investigated the SMs of *G. dankaliensis* in Iraq.

This study aimed to investigate fungal isolates from sheep dung and identify the bioactive compound produced as an SM by the *G. dankaliensis* isolate. The biological activities of the bioactive compound and its toxicity against cancer cell lines were also investigated.

2. Materials and Methods

2.1. Isolation of *G. daneklinsis* from Sheep Dung

Sheep dung samples were collected in March 2020 using sterile forceps, transferred to sterile paper bags, and dried at room temperature. They were cultured following the moist chamber method (9), in which samples were separated into moist sterile filter paper and placed on a Petri dish. Dishes were incubated at 27°C for three weeks with the regular addition of distilled water **(**DW) (Figure 1).

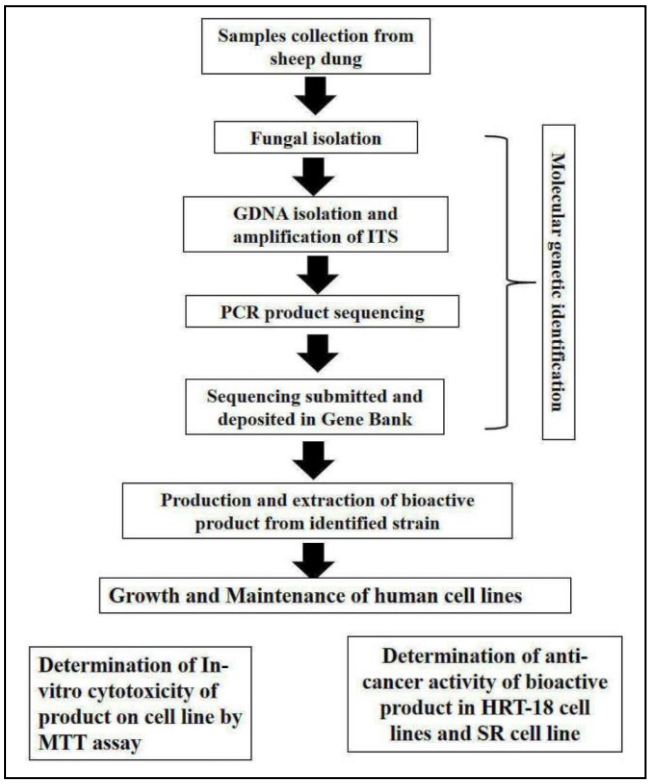

Figure 1. A schematic diagram highlighting the principle steps of the workflow

2.2. Fungal Growth and Maintenance

Fungal isolates were sub-cultured on potato dextrose agar (PDA) and incubated at 27°C for 14 days. The purified isolates were maintained in slant culture for later study steps.

2.3. Fungal Identification

2.3.1. Molecular Genetic Characterization

The genomic DNA of the fungi was isolated using the Presto™ Mini gDNA Yeast Kit (Geneaid, Taiwan). The protocol was performed according to the manufacturer's instructions (https://www.geneaid.com/data/files/160566422130805 5331.pdf). The genomic DNA was kept at -80°C for further analysis. The universal primer pair and the protocol by Mirhendi, Makimura (10) were used to amplify the conservative internal transcribed spacer (ITS) region. The quantity and quality of the genomic DNA and polymerase chain reaction (PCR) products were analyzed using NanoDrop and electrophoresis.

The PCR products were sent for sequencing (Macrogen, Korea) (http://dna.macrogen.com). The sequence was aligned with the public database to compare the homology in the GenBank using the Basic Local Alignment Search Tool (BLAST) (Figure 1).

2.4. Preparation of Fungal Extracts

The *G. daneklinsis* strain was grown on (rice medium) as a solid-state fermentation (SSF) medium for 30 days at 27°C. The SSF medium was prepared from 30 g of rice, 0.5 g of NaCl, and 35 mL of DW. The extraction was carried out using ethyl acetate, and the supernatant was filtered using Whatman No. 1 filter paper (11). The extract was dried at room temperature and stored at 4°C until use.

2.5. Characterization of the Bioactive Compound Using GC-MAS

The crude extract of the *G. daneklinsis* SM was analyzed by the GC-MS at the Basrah Oil Company, Nahr Bin Omar Laboratory. The analysis was performed using an Agilent Technologies 7890 B GC system coupled to an Agilent Technologies 5977A MSD with an EI ion source using HP-5MS 5% phenyl methyl siloxane (30 m×250 Um×0.25 mm). Helium gas was used as the carrier gas at a constant flow mode of 1 mL/min and a purge flow of 3 mL/min. The oven temperature was set at 40°C, held for 5 min, raised 1°C/min to 280°C for 1 min, and then held at 280°C for the remaining 20 min. The injection mode was pulsed-split less with an injection temperature of 290°C, and the injection sample volume was 1 µL. The mass spectrometer ion source temperature was set at 230°C, with a scan speed of 1562 (N2), and the electron ionization was obtained over a mass range of 35-650 m/z. The data were analyzed using the NIST 2014 library database.

2.6. Maintenance of Human Cell Lines

HCT-18 and SR cell lines were obtained from the cell bank unit of the Iraqi Center for Cancer and Medical Genetics Research, Baghdad, Iraq. The cells were stored in liquid nitrogen until further use. Cell lines were maintained and cultured in a minimum essential medium containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. A Sanyo CO_2 incubator (MCO-17A) was used to incubate the flask at 37°C with 5% CO_2. The routine sub-culture for maintenance was carried out every two days.

2.7. Determination of *in vitro* Cytotoxicity by MTT Assay

The cytotoxic effect of the bioactive extract was determined using the MTT assay by evaluating the viability of cells (Figure 1). Cell lines were seeded at 1×40 cells/well and incubated for 24 h in a Sanyo CO_2 incubator (MCO-17A) at 37°C with 5% CO_2. Afterward, the cells were washed with phosphate-buffered saline, treated with crude extracts (3.125, 6.25, 12.5, 25, 50, and 100 µg\mL), and incubated for 27 h at 37°C. Subsequently, 28 µL of MTT (2 mg\mL) was added and incubated for 90 min at 37°C. The cells were washed to remove the MTT solution, and 130 µL of dimethyl sulfoxide was added to solubilize the crystals. The plate was incubated at 37°C for 15 min with shaking. Optical density (OD) was measured using a spectrophotometer at 492 nm. The experiment was repeated three times. The inhibition rate of cell growth was calculated using the following equation:

Inhibition rate=A-B/A*100

(A) represents the OD of control, and **(B)** is the OD of the sample.

3. Results

3.1. Identification of *G. dankaliensis* Isolate

The *G. dankaliensis* isolate was identified using the advanced molecular characterization method. The ITS region was amplified as a conserved region and sequenced afterward. The ITS region sequences with a size of about 500-600 bp were compared to the public database sequences, which were deposited in the GeneBank (Figure 2). The homology was investigated using the BLAST. The identified *G. dankaliensis* isolate was deposited at GenBank with the accession number LC647058.

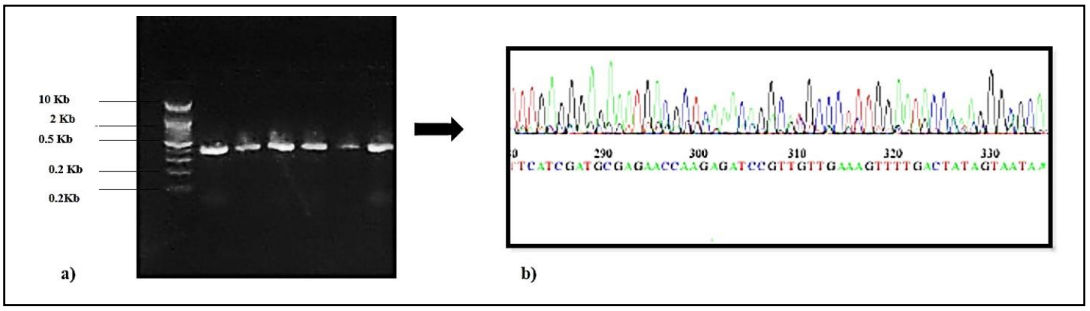

Figure 2. a) Gel electrophoresis of the PCR products of the ITS region on an agarose gel stained with ethidium bromide. The size of the products was about 500 bp. **b)** The sequences of the PCR products were aligned with the deposited public database

3.2. Growth of *G. dankaliensis* Isolate

The isolate was grown on PDA for routine maintenance. To induce the isolate to produce an SM, the fungi were grown on SSF media (Figure 3a) for 30 days at 27°C.

3.3. Production and Extraction of Bioactive Metabolites from *G. dankaliensis*

The organic solvent ethyl acetate was used to extract the crude extract of *G. dankaliensis* (Figure 3).

3.4. Fungal Extract Analysis by GC-MAS

The extract of *G. dankaliensis* was analyzed using GC-MS, and the results showed that the extract provided five peaks with retention times of 3.507, 13.144, 18.658, 19.230, and 24.531 min (Figure 4I).

The results of GC-MAS revealed that the crude fungal extract was composed of five compounds: dimethylsulfoxonium formylmethylide (12), 2-butenoic acid (13), formamide (14), 2-methyl propyl ester (15), and oxalic acid (16). The chemical structure of each identified compound is shown in Figure 4II.

3.5. Examination of the Cytotoxicity of the Crude Extracts of the *G. dankaliensis* Isolate on Cell Lines

Two cell lines, including HCT-18 and SR, were used to investigate the cytotoxicity of the dried crude fungal extract. There were six different concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg\mL) of the crude extract. After 27 h of incubation, an MTT assay was conducted to investigate the cytotoxicity of the crude fungal extract on the SR and HCT-18 cell lines. The results showed that the crude extract had cytotoxic activity on the HCT-18 and SR cell lines, with the IC50 being 3.57 and 8.61 µg/mL, respectively. The inhibitory effect of the crude extract increased with increasing extract concentrations (Figure 5).

The growth inhibition (GI) and IC50 were distinguished based on the percentage of viable cells. The percentage of viable cells was compared between the cells treated with the extract and the controls (untreated cells). The determination of GI and IC50 was compared to the cells unexposed to the crude fungal extract as the control. Figure 6 shows morphological differences between the treated and control cell lines with bioactive compounds. The growth of the treated and control (untreated) cell lines was inhibited, as shown in figure 6.

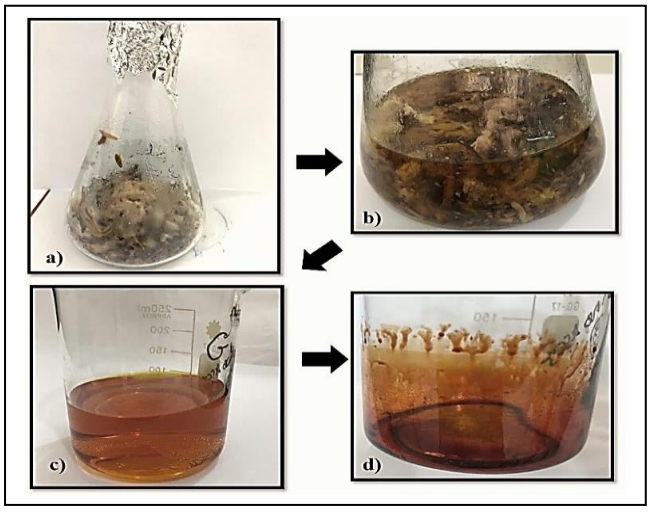

Figure 3. Growth of *the G. dankaliensis* strain and preparation of bioactive metabolites extract. **a)** Growth of *G. dankaliensis* strain on SSF medium (rice medium) for 30 days at 27 °C. **b)** Extraction of bioactive metabolite by ethyl acetate. **c)** The extracted bioactive compound after filtration. **d)** Dried extract of organic solvent at room temperature

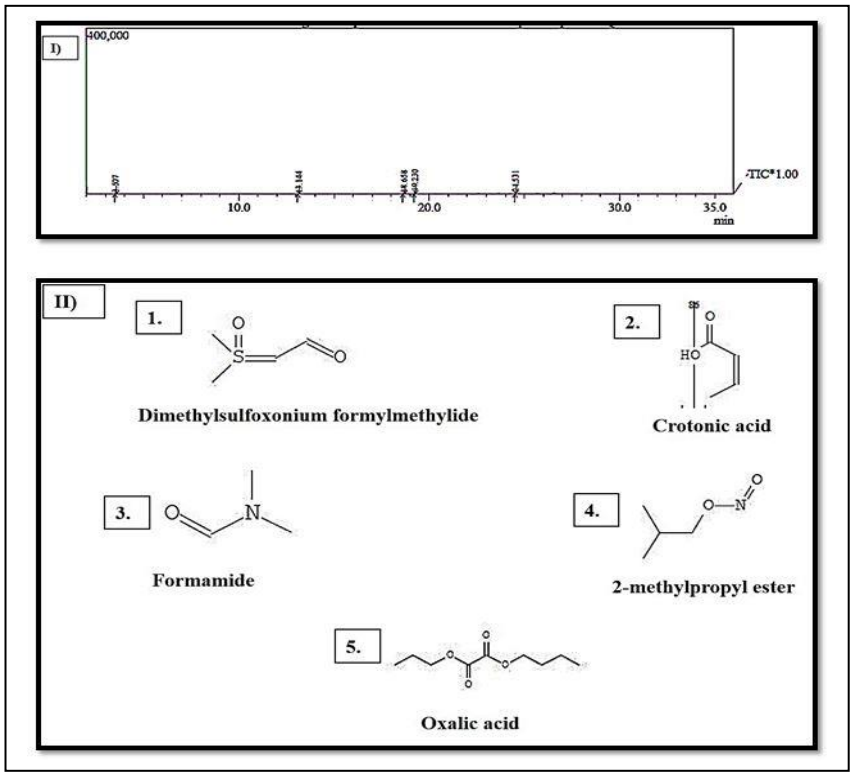

Figure 4. GC-MS analysis of the fungal extract. **I)** Analysis of the chromatogram with the retention time. **II)** Chemical structures of the five identified compounds of the fungal extract

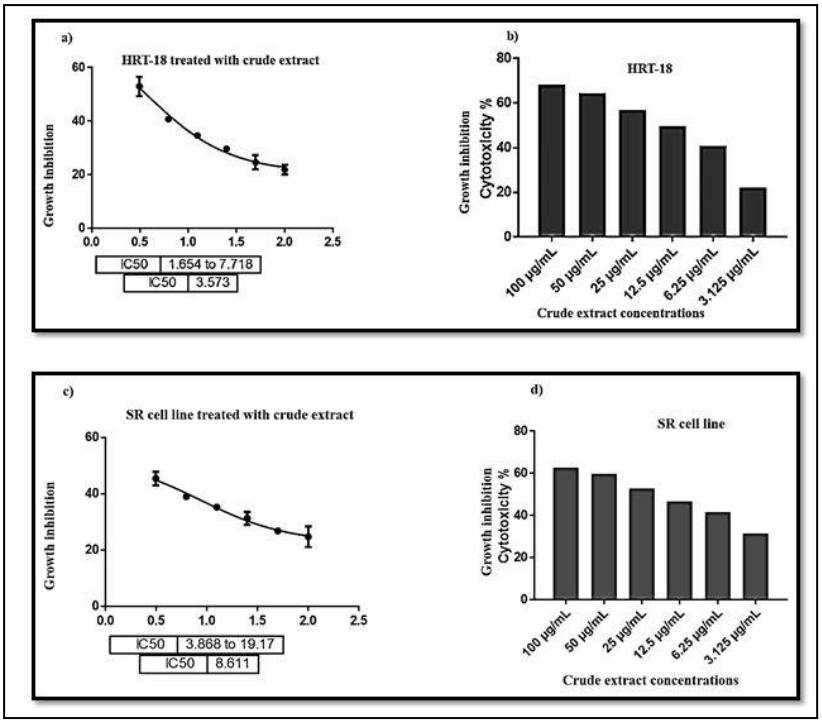

Figure 5. *In vitro* cytotoxicity effect of the crude extract of the *Gymnoascus dankaliensis* on the cell line. **a)** and **c)** The IC50 of the crude extract on the HRT-18(HCT-18) and SR cell lines, respectively. **b)** and **d)** The GI of the HRT-18 and SR cell lines at different crude extract concentrations, respectively

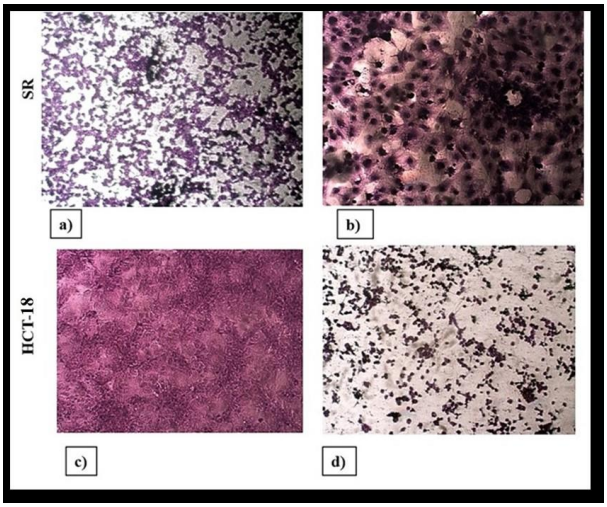

Figure 6. The morphological appearance of treated and untreated (control) cell lines. The cell line was incubated with extract for 27h at 37 °C. **a)** Growth of the SR cell line was inhibited after the treatment with the extracted bioactive product while **b)** shows the control. **c)** and **d)** represent the treated and control HCT-18 cell lines, respectively. The growth of the treated cell lines was inhibited in contrast with the control. An inverted microscope is used for scrutinizing the photos with 10X magnification power

4. Discussion

This study aimed to isolate and identify *G. daneklinsis* spp. from sheep dung and investigate the activity of the natural metabolite product of *G. daneklinsis.* In a previous study, *G. daneklinsis* was recovered from a soil sample collected from an area close to the Giza pyramids in Egypt, while Surjushe (17) isolated it as a clinical sample. In addition, *G. dankaliensis* was obtained from the sponge *Halichondria japonica* (8). Doveri (18) used dung samples to recover different fungal isolates.

The activity of the extract was investigated, and the data showed that the product has activity against two types of cancer cell lines (HCT-18 and SR). Amagata, Minoura (19) observed that the growth of the P388 cancer cell line was inhibited by *G. dankaliensis* extract. Similarly, the activity of the extract was also investigated against the murine lymphoma cell line, L5178Y (8).

The identification of the extract components was conducted by GC-MS analysis. The results revealed that the extract was composed of five elements based on the five peaks and the retention time. The five components are displayed in figure 3.3II. Dimethylsulfoxonium formylmethylide was identified as an antioxidant isolated from the leaves of *Mundulea sericea* in a previous study by Khyade and Waman (20). Furthermore, it was reported in the extract of *Dendrobium crepidatum* (21).

Secondly, a 2-methyl propyl ester compound was identified, with two biological activities, including the inhibition of α-Glucosidase and an *in vivo* hypoglycaemic effect (15). It was also found that it has antimicrobial and antioxidant activity (15).

Oxalic acid is produced by *Penicillium canescens* and *P. jenseii* (16). Previous studies have identified oxalic acid as a pathogenic factor for *Sclerotinia sclerotiorum* (22).

The antioxidant activities of the two compounds (2-methyl propyl ester *and* dimethylsulfoxonium formylmethylide) may have a role in the growth reduction of the two types of cancer cell lines.

The role of antioxidants in scavenging reactive oxygen species and inhibiting tumor growth has been previously reported (23).

This study had several limitations. Firstly, the bioactivity of a natural product against cancerous cell lines was investigated in vitro. We recommend that further studies be conducted to scrutinize this natural bio-products' activity and toxicity in animal models. The mechanism of cancer cell line growth reduction was also investigated.

The production of bioactive compounds, such as SMs, has been previously reported although limited concentrations have been obtained. Many methods have been reported to enhance the production of bioactive compounds, such as modifying growth conditions (24). This encouraged us to extend our study to analyze the genetic production pathway and genetically modify the fungus to enhance its production.

This study isolated *G. dankaliensis* and investigated the biological activities of its natural product extract

against two types of cancer cell lines (HCT-18 and SR). This activity may relate to antioxidants in the analyzed extract compound. These results highlight that the product may be a promising anticancer treatment.

Authors' Contribution

Study concept and design: F. N. J.
Acquisition of data: A. A. L.
Analysis and interpretation of data: F. N. J.
Drafting of the manuscript: F. N. J.
Critical revision of the manuscript for important intellectual content: A. A. L.
Statistical analysis: A. A. L.
Administrative, technical, and material support: F. N. J.

Ethics

The study protocol was approved by the thics committee of the University of Basrah, Basrah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

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