

## Research Article



## Isolation, Identification and Evaluation of Antimicrobial and Cytotoxic Activities of the Marine Fungus *Aspergillus unguis* RSPG\_204

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### ABSTRACT

Natural products derived from marine organisms have been the focus of many investigations. Marine derived biomolecules such as peptides, enzymes, enzyme inhibitors and lipids have the potential for the prevention and treatment for many medical conditions including microbial infection and cancer. Therefore, these biomolecules might be useful as molecular models in drug research. In the current study, secondary metabolites were extracted from the marine sponge associated fungus *Aspergillus unguis* RSPG\_204 following the isolation and identification of the fungus. The extracted metabolites were investigated for their antimicrobial, antioxidant and cytotoxic activities. The fungal extracts exhibited a potent antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* while they were not effective against *Aspergillus niger*. The static culture of the fungus showed the highest antioxidant activity as measured by DPPH radical scavenging activity. Meanwhile, Mycelial extract showed the highest cytotoxic activity against MCF7 (breast) cell line with IC<sub>50</sub> of 9.98µg/mL. While, the cytotoxic activity of both static culture and mycelial extracts against HEPG2 (liver) cell line were non significant. DNA fragmentation assay and quantitative determination of interleukin-2 (IL-2) levels by ELISA were used as suggested methods for the detection of apoptosis and evaluation of the immune response. It could be concluded that, the results in general indicated the possibility of using marine fungi derived metabolites as potential antimicrobial and anticancer agents.

**Keywords:** *Aspergillus unguis* RSPG\_204, *Agelas* sp., Marine fungi, Antimicrobial, Antioxidant, Cytotoxic activity.

### INTRODUCTION

The screening for medically important metabolites from marine organisms has been initiated in the late forties by the isolation of the antibiotics cephalosporins and other metabolites from the marine fungi *Cephalosporium* sp.<sup>1</sup> In a relatively short time more than 10,000 new compounds have been isolated from fungi, sponges, soft corals and other marine organisms.<sup>2</sup> A broad spectrum of biological activities has been detected for metabolites extracted from marine fungi including antibacterial, antifungal, anti-inflammatory and antineoplastic.<sup>3</sup> Emerging evidence suggests that marine natural products, especially the secondary metabolites from marine organisms, are far more likely to yield anticancer drugs than terrestrial sources.<sup>4,5</sup> In the last decade, there has been a dramatic increase in the number of preclinical anticancer lead compounds extracted from metabolites of marine-derived fungi.<sup>6-8</sup> Cancer is not only a human disease but also it threatens the survival of veterinary species. Although the majority of both the scientific and clinical effort has thus far focused on the dog, a number of veterinary species, including the cat, horse and ferret, develop cancers that are of comparative interest.<sup>9,10</sup>

The use of cancer cell line as an *in vitro* model is a very important approach to provide novel opportunities for cancer diagnosis and treatment for both veterinary and

human cancer patient. Cancer cell lines proved to be an excellent model for the study of the biological mechanisms underlying cancer.<sup>11</sup>

Oxidative stress describes a set of intracellular or extracellular conditions that lead to the chemical or metabolic generation of reactive oxygen species (ROS), including hydroxyl radicals, singlet oxygen, lipid hydroperoxides and related species. (ROS), can cause oxidative damage to essential cellular constituents, such as membrane lipids, proteins and DNA, which may ultimately result in cell death.<sup>12</sup> Aerobic organisms attempt to protect themselves against oxidative damage with exogenous antioxidants obtained through the diet as well as endogenous antioxidants. Furthermore, oxidative damage mediated by (ROS) is known to contribute to the aging process and the pathogenesis of cancer.<sup>13,14</sup> Free radicals, generated as by-products of normal cellular metabolism, have been implicated in the etiology of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes. The compounds that can scavenge free radicals have tremendous potential in ameliorating these disease conditions.

As far as known till now, there are no reports on marine compounds isolated from microorganisms of the Red Sea area of Egypt that have antioxidant and cytotoxic activities. Hence, we tried to study the sponge associated fungus *Aspergillus unguis* RSPG\_204 and some of its



biological activities. The present investigation is an outcome of such a study on the fungus *Aspergillus unguis* RSPG\_204 isolated from the Sponge *Agelas* sp. and screen for its antimicrobial, antioxidant, and cytotoxic activities, using *in vitro* techniques to screen these potentials.

## MATERIALS AND METHODS

### Sponge materials

Sponge sample; *Agelas* sp., from which *Aspergillus unguis* RSPG\_204 was isolated. It was collected from Hurghada coast, Red Sea, Egypt. The site is located north Hurghada at El- Gouna , latitude N 27° 24' 7.5", E 33° 41' 16.8" the samples were collected at depth of 5m-8m in January 2013 and kept frozen until the work-up. The morphological taxonomy of the sponges was identified by Mohamed A. Ghani–environmental researcher -Red Sea Marine parks, Hurghada, Red Sea, Egypt.

### Preparation of animal material

Small pieces of inner tissue of fresh Sponge materials were rinsed three times with sterile sea water (SW); then aseptically cut into small cubes, approx. (0.5 cm<sup>3</sup>). A total of 50-75 cubes of each sample were placed on different isolation media. During the initial investigations, cubes from Sponge sample were placed in EtOH (70 %) for various times between 5 and 30s and subsequently squeezed three times in sterile sea water (SW) before inoculation.

### Isolation of the fungus from sponge sample

A measured area of Sponge tissue (about 1cm<sup>3</sup>) was excised from the middle internal mesohyl area of the Sponge using a sterile scalpel. These Sponge cubes were placed directly on the surface of the agar plates<sup>15</sup> or the excised tissue was then homogenized with sterile aged sea water, using a sterile mortar and pestle. The resultant homogenization was serially diluted until 10<sup>-6</sup> and preincubated at room temperature for 1hr for the activation of dormant cells. From dilution 10<sup>-3</sup> to 10<sup>-6</sup> 0.1ml of each dilution was used to inoculate suitable solid medium containing antibacterial antibiotics. The plates were then incubated at 30°C for 7-14 days.<sup>16</sup> The appeared single fungal colonies were picked up and inoculated on PDA (sea water) slants. The medium used in isolation exhibited the following composition (g/L): yeast extract (1), glucose (1), Ammonium nitrate (1), peptone (0.25), agar (20) and sea water (1000). The pH was adjusted to 7.4.<sup>17</sup> The medium<sup>18</sup> was supplemented with Streptomycin sulphate (0.1 g/L) and Penicillin G (0.1 g/L).

### Screening medium (Wickerham Medium for Liquid Culture)

For both shake and static cultures this broth medium with the following ingredients (g/L): Yeast extract (3), Malt extract (3), peptone (5), glucose (10) and sea water to make 1000 ml.

### Small and large scale cultivation for screening

One fungal slant (7-10 days old) was used to inoculate two Erlenmeyer flasks; (1L) and each containing 300ml of Wickerham medium for liquid cultures; by making spore suspension using 10ml sterile marine water. The cultures were then incubated at room temperature (shaking and static) for 8 days. Large scale cultivation was carried out using twenty 1L Erlenmeyer flasks for liquid cultures.

### Extraction of secondary metabolites

The fungus was harvested at the end of incubation period, centrifuged at 8,000 rpm and subjected to extraction. The culture supernatant was extracted with ethyl acetate (3x or till exhaustion) and then evaporated under vacuum. On the other hand the fungal mycelia were first extracted using acetone and evaporated till dryness. The residual part was re-extracted using small volume of ethyl acetate.

### Identification of fungal cultures

Fungal culture was identified by DNA isolation, amplification (PCR) and sequencing of the Internal Transcribed Spacer (ITS) region. The primers ITS2 (GCTGCGTTCTTCATCGATGC) and ITS3 (GCATCGATGAAGAACGCAGC) were used for PCR amplification while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used for sequencing. The purification of the PCR products was carried out to remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). *Candida* sp. was used as control.

### Antimicrobial activity Test

Disc agar plate method was done to evaluate the antimicrobial activity of fungal extracts.<sup>19</sup> Investigated samples were solubilized in methanol. The antimicrobial activities of 0.5-cm-diameter filter paper disc saturated with about 1mg sample were tested against four different microbial strains including *Staphylococcus aureus* (Gram-positive), *Pseudomonas aeruginosa* (Gram-negative), *Candida albicans* (yeast) and *Aspergillus niger* (fungus). The two bacterial strains and yeast were grown on nutrient agar (DSNZ 1) medium (g/L): beef extract (3), peptone (10), and agar (20). Whereas, the tested fungus was grown on Szapek-Dox (DSMZ130) medium (g/L): sucrose (30), NaNO<sub>3</sub> (3), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.5), KCl (0.5), FeSO<sub>4</sub>.7H<sub>2</sub>O (0.001), K<sub>2</sub>HPO<sub>4</sub> (1) and agar (20).

The culture of each microorganism was diluted by sterile distilled water to 10<sup>7</sup> to 10<sup>8</sup> CFU/ml to be used as inoculum. A total of 0.1 ml of the previous inoculum was used to inoculate 1L of agar medium (just before solidification) then poured in Petri-dishes (10 cm diameter containing 25 ml). Discs (5 mm diameter) were



placed on the surface of the agar plates previously inoculated with the test microbes and incubated at 37 °C for 24 hrs for the bacteria and yeast and at 30 °C for 48 hrs for the fungus.

### DPPH radical scavenging activity

DPPH radical scavenging activity of all extracts was analyzed according to a modified procedure of Matsushige and his group.<sup>20</sup> 1 ml of methanol solution for each extract (100 µg/ml) was added to 1 ml of methanol solution of DPPH (60 µM). The prepared solutions were mixed and left for 30 min at room temperature. The optical density was measured at 520 nm. Mean of three measurements for each compound was calculated.

### Evaluation of cytotoxic activity

The extracts showed high antioxidant activity were selected for further investigation of their cytotoxic activity. The cytotoxic activity of the selected fungal extracts were tested against four cancer cell lines including; HELA (cervical), MCF-7 (breast), HCT116 (colon) and HEPG2 (liver). All cell lines were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). All cell lines were cultured in RPMI-1640 medium (Sigma Aldrich Chemical Co., St. Louis. Mo. U.S.A) supplemented with 10% FBS (Fetal bovine serum), penicillin (100 U/mL) and streptomycin (2 mg/mL) at 5% CO<sub>2</sub> in a 37 °C incubator.

The cells were plated in 96-well plate at a density of 3.0x10<sup>3</sup> in 150 µL of medium per well. Tested extracts dissolved in DMSO were added to the wells in triplicates with concentrations of 0, 5, 12.5, 25 and 50 µg/mL for 48 hrs. The cytotoxic activity was determined using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara (2006).<sup>21</sup> The IC<sub>50</sub> values (the concentrations of extract required to produce 50% inhibition of cell growth) were also calculated.

### Scaling up of cultured cells

The volume of cell culture was scaled-up to provide more cells for DNA extraction and to provide more media for the quantitative measurement of IL-2. Extracts were further selected based on their low IC<sub>50</sub> against specific cell lines. The selected extracts were added separately to a tissue culture flask containing the corresponding cell line in RPMI-1640 medium at a density of 5x10<sup>6</sup>/15 mL. Normal fibroblasts cell line (HFB-4) was used as control. The cells were grown under the same conditions as mentioned earlier. The cells were subjected to centrifugation for 5 minutes at 3,000 rpm to collect the cells. The pellet was washed twice with PBS (pH 7.4). The collected cells were used for DNA extraction and the media were used to measure IL-2.

### Measurement of apoptosis by DNA fragmentation assay

DNA was extracted from cultured cell line using DNA extraction kit (One-4-all genomic DNA mini-preps kit, Bio

Basic Canada Inc.) according to the manufacturer protocol. This was followed by agarose gel electrophoresis to detect DNA laddering.

### Quantitative measurement of Interleukin-2 (IL-2) levels

The level of IL-2 was measured in cell culture media using IL-2 ELISA kit (Koma Biotech Inc.) according to the manufacturer protocol. The assay diluent was used as a standard blank. All samples were measured in duplicates for statistical relevance and IL-2 levels were measured and represented in optical density (OD) at wavelength of 450 nm.

## RESULTS AND DISCUSSION

### Identification of fungal cultures

BLAST search for the fungus isolate revealed 99% similarity to *Aspergillus unguis* RSPG\_204. The phylogentic tree of this fungal isolate was also constructed (Figure 1).

### Antimicrobial activity

The supernatant and mycelial extracts from the shake and static cultures; respectively, of the identified fungus (*Aspergillus unguis* RSPG\_204) isolated from the sponge *Agelas* sp. were highly effective against *P. aeruginosa*, *S. aureus* and *C. albicans* (Figure 2).

The supernatant extract from shake culture exhibited the highest activity followed by mycelial extract of static culture against both *S. aureus* and *C. albicans* and this was followed by the supernatant extract from static culture. All extracts did not show any activity against *A. niger*. Meanwhile, mycelial extract from shake culture did not show any activity against all tested microbes. The supernatant extract from static culture also did not show any activity against *P. aeruginosa*.

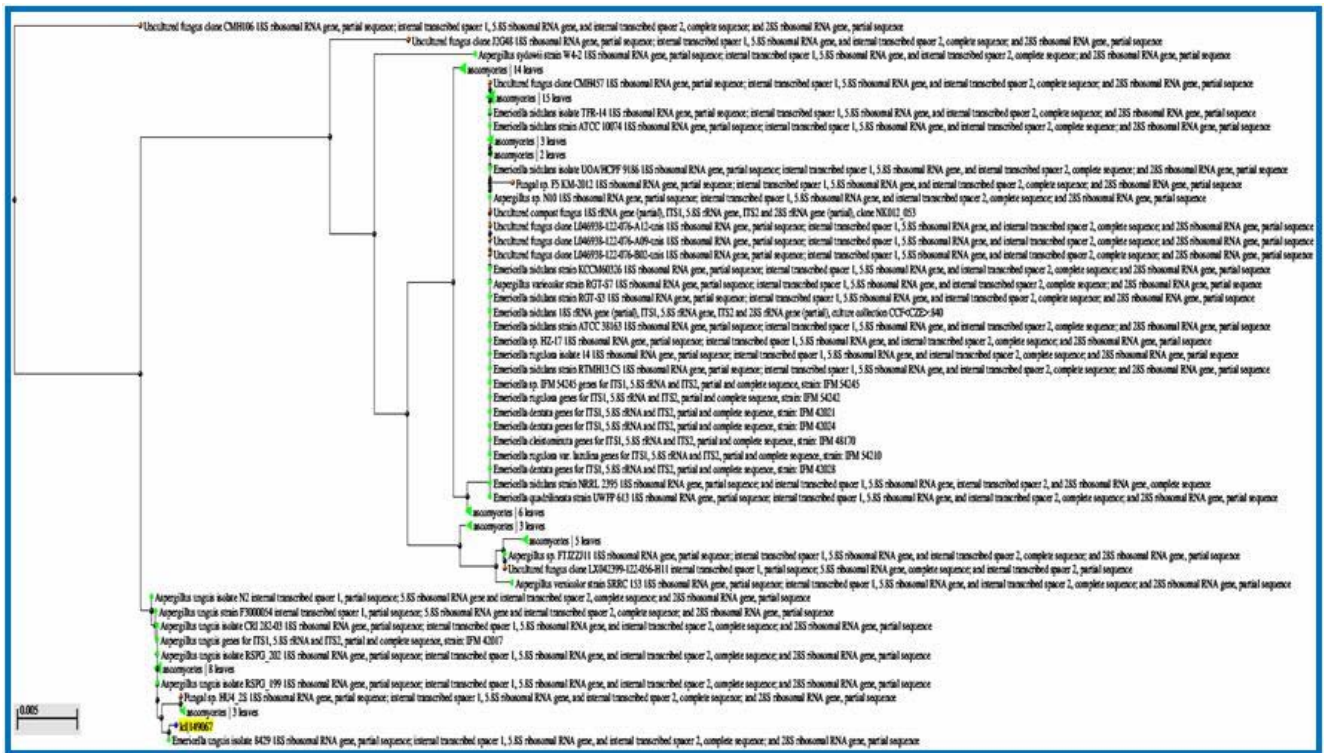
Marine invertebrates-associated microorganisms gained a great attention as important sources of new bioactive secondary metabolites. Genus *Aspergillus* was investigated previously as a producer of considerable numbers of cytotoxic and biologically active compounds.<sup>22</sup>

### DPPH-free radical scavenging activity

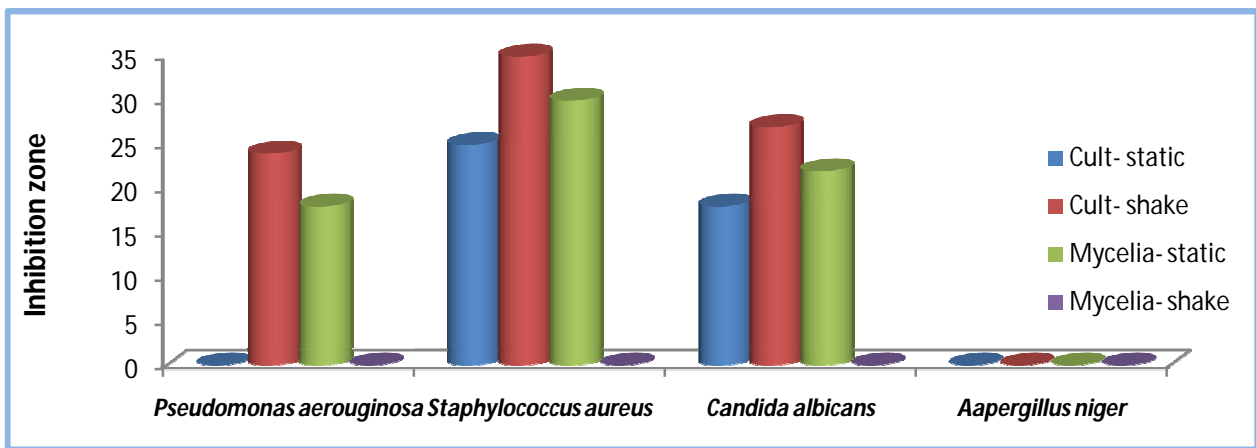
The supernatant extract from the static culture of the identified fungus (*Aspergillus unguis* RSPG\_204) showed the highest free radical scavenging activity (FRSA) against DPPH radical, while its mycelial extract showed moderate activity (Figure 3).

The DPPH assay is based on the principle that a hydrogen donor is an antioxidant. It measures the activity of an antioxidant to directly scavenge DPPH radical and determining its absorbance spectrophotometrically at 520 nm. The DPPH is stable organic nitrogen centered free radical with a dark purple color that becomes colorless when it reacts with antioxidant to form non-radicals.<sup>23</sup> Our data are in agreement with previous studies.<sup>24,25</sup>

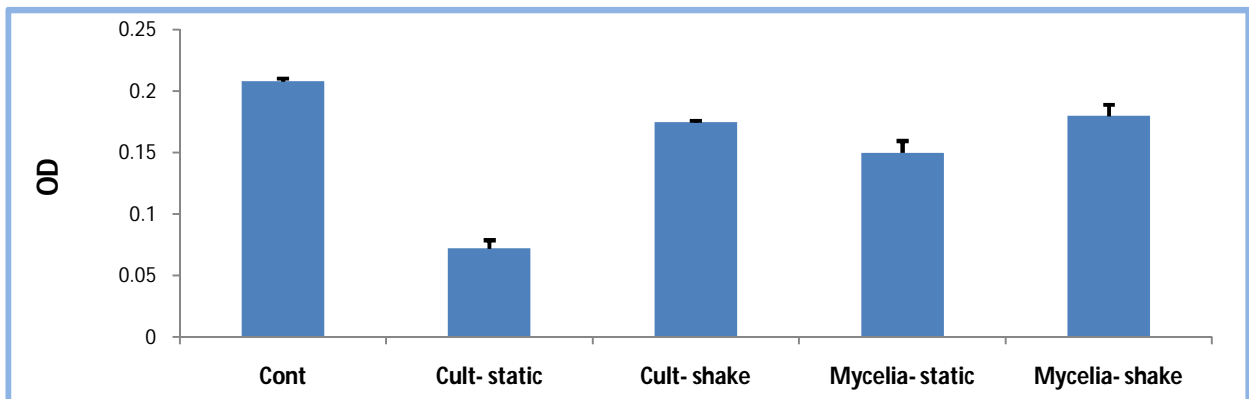




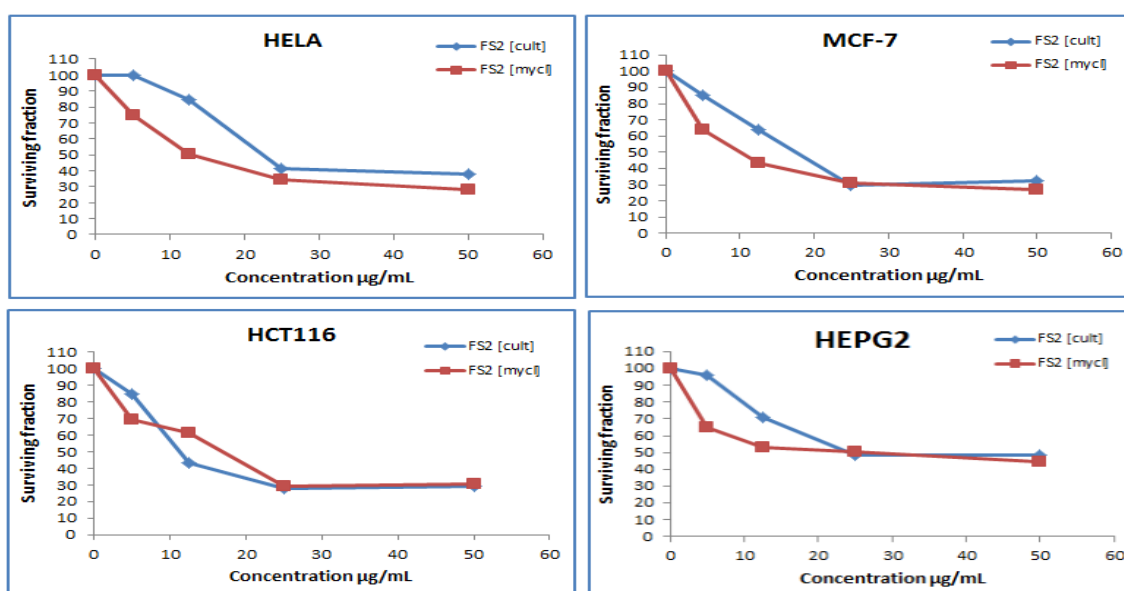
**Figure 1:** The phylogenetic tree of the fungus *Aspergillus unguis* RSPG\_204 (99% similarity) isolated from the Sponge (*Agelas* sp.).



**Figure 2:** Antimicrobial activity of 2ry metabolites extracted from culture and mycelia (static and shake) of the marine fungus *Aspergillus unguis* (RSPG 204).



**Figure 3 :** Free radical scavenging activity [DPPH] of 2ry metabolites extracts from culture and mycelia (static and shake) of the fungus *Aspergillus unguis* (RSPG 204). Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml).



**Figure 4:** Effects of 2ry metabolites extracted from culture static (blue) and mycelia static (red) of the fungus *Aspergillus unguis* (RSPG 204) on HELA (Cervical), MCF-7 (Breast), HCT116 (Colon), HEPG2 (Liver carcinoma) cell lines viability.

### Cytotoxic activity

Secondary metabolites extracts from both culture and mycelia exhibited *in vitro* cytotoxic activity against all investigated cell lines, in a dose dependent manner (Figure 4 and Table 1).

**Table 1:** Evaluation of IC<sub>50</sub> (µg/mL) for the selected extracts

Cancer cell line	<i>Aspergillus unguis</i> (RSPG 204)	
	Culture (static)	Mycelia (static)
HELA	22.4	12.6
MCF7	17.6	9.98
HCT116	11	17
HEPG2	24	26

The solvent used did not affect the viability of cells, proving that the cytotoxic effects were exclusively due to the extracts. Mycelial extract (static culture) showed the highest cytotoxic activity against MCF7 with IC<sub>50</sub> of 9.98 µg/mL compared with 17.6 µg/mL for culture extract. This was followed by culture extract against HCT116 with IC<sub>50</sub> of 11 µg/mL compared with 17 µg/mL for mycelial extract. The values of IC<sub>50</sub> for culture and mycelial extracts against HELA were 22.4 and 12.6 µg/mL respectively. Meanwhile, the cytotoxic activities of both culture and mycelial extracts against HEPG2 were non significant and were almost the same with IC<sub>50</sub> values of 24 and 26 µg/mL respectively.

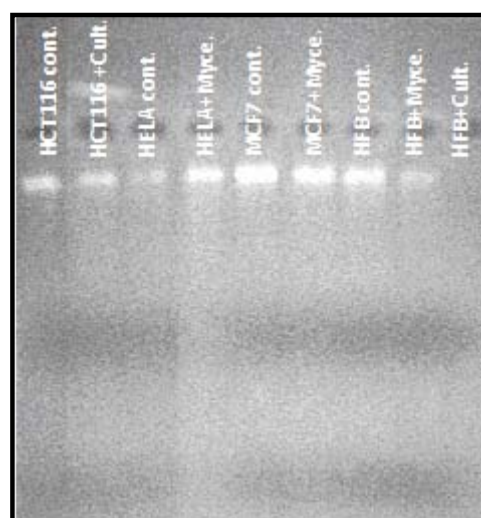
Marine-derived fungi are a source of significant chemical diversity with various biological activities including cytotoxic and anticancer activities. Gymnastatins were the first group of cytotoxic metabolites to be reported as novel cytotoxic metabolites from a sponge derived fungus, *Gymnascella dankaliensis*.<sup>26</sup> Gymnastatins

exhibited growth inhibition against human cancer cell lines including BSY-1 (breast) and MKN7 (stomach).

Meanwhile, dankastatins extracted from the same fungus exhibited growth inhibition against P388 (murine lymphocytic leukemia) cell line.<sup>27</sup>

### Measurement of apoptosis by DNA fragmentation assay

Extracts showed very low IC<sub>50</sub> with different cell lines (Table 1) were subjected to measurement of apoptosis by DNA fragmentation assay. Although, photographed gel did not show a clear laddering pattern for genomic DNA in any of the tested cell lines (Figure 5), some lanes looked like they caught early apoptotic event as indicated by the tailing pattern of the DNA band down the gel. Genomic DNA was undetectable in HFB-4 cell line treated with culture extract.



**Figure 5:** Gel picture showing electrophoretic mobility of genomic DNA extracted from cell lines subjected to culture extract (Cult.) and mycelial extract (Myce.) of the fungus *Aspergillus unguis* (RSPG 204) as compared to cell lines control.

DNA fragmentation is a hallmark of apoptosis.<sup>28,29</sup> Apoptosis leads to the cleavage of genomic DNA into multiple oligonucleosomal fragments characterizing an apoptotic event.<sup>30</sup> Analysis of DNA fragmentation using agarose gel electrophoresis provides a qualitative method for assessing apoptosis or cell death. DNA fragmentation is characterized by the presence of typical ladder DNA fragments of 180-200 base pairs on an agarose gel. In contrast, random cleavage of DNA in necrotic cells will produce a diffuse smear upon electrophoresis of DNA.<sup>31</sup>

The absence of characteristic DNA fragmentation as shown in photographed gel does not necessarily mean that apoptosis did not occur since DNA fragmentation occurs in late phase of apoptosis. It has been found that ladder formation in lymphocytes occurs very late and probably as terminal event in the cascade of events that lead to DNA degradation and cell death.<sup>32</sup> Additional evidence suggests that apoptosis in cells of epithelial or mesenchymal origin may not involve DNA degradation into oligonucleosomal multimers and that different forms of the endonuclease appear to be active in different cell types.<sup>32,33</sup> On the other hand we used only one concentration of the extract (IC<sub>50</sub> concentration) and we need to try different concentrations especially the higher ones to check if we can get clear laddering pattern as an indication of more advanced apoptotic event. Undetectable DNA in case of treating HFB-4 cells with culture extract may indicate that these cells experienced a late phase of apoptosis and their DNA was diminished. The employment of different assays for the detection of apoptosis including electron microscopy, flow cytometry and detection of active caspases can be used as more accurate methods for the detection of apoptosis.<sup>34</sup>

#### Quantitative measurement of Interleukin-2 (IL-2) levels

The measured ODs for IL-2 in different media are listed in Table 2. The results indicated that all ODs were comparable with control (assay diluent) with no significant changes.

**Table 2:** Optical densities indicating the level of IL-2 in culture media of different cell lines.

S.No.	Cell line or Cell line+ extract	Mean±SD
1	HCT116	0.046±0.010
2	HCT116+Culture extract	0.031±0.002
3	HELA	0.049±0.021
4	HELA+Mycelia extract	0.030±0.000
5	MCF7	0.035±0.002
6	MCF7+Mycelia extract	0.063±0.026
7	HFB	0.046±0.004
8	HFB+Mycelia extract	0.036±0.000
9	HFB Culture extract	0.041±0.003
10	Control	0.037±0.001

Interleukin 2 (IL-2) has been approved by the FDA for the treatment of some types of cancer. The importance of IL-

2 emerges from its role in regulating the activities of lymphocytes. Fearon et al.<sup>35</sup> demonstrated that the antitumor activity of IL-2 is mainly due to its ability to stimulate the cell mediated killing activity of cytotoxic T lymphocytes (CTLs). IL-2 has also the ability to induce lymphokine-activated killer cells,<sup>36,37</sup> and to activate tumor-infiltrating lymphocytes (TILs).<sup>38,39</sup>

#### CONCLUSION

Based on our results it can be concluded that, the isolated marine fungus *Aspergillus unguis* RSPG\_204 showed different biological activities including antioxidant, antimicrobial and cytotoxic. This is primary evidence provided by our study for the first time for this fungus. Further *in vitro* and *in vivo* studies are needed to confirm our results and to investigate the underlying mechanisms controlling these activities.

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