Aspergillus fumigatus and Cylindrocarpon candidum fungi induced apoptosis in HepG2 cell line through activation of caspases enzymes


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Abstract

Introduction: Many investigations are now interested to discover naturally occurring compounds, which can be used for the prevention and treatment of cancer. Most natural products which may be used as adjuvant therapy or to reduce the side effect of chemotherapy and radiotherapy. More than 300 products obtained from microorganisms have antitumor activities.

Results: In the study we isolated N-(3-4-Dichlorophenyl) 2-Methyl, 2,3Dihydroxypropio amide from Aspergillus fumigatus and 2.4.6. Triphenyl pyridine from Cylindrocarpon candidum and investigate the cytotoxic effect and apoptotic effect on HepG2 cell line. The results revealed high cytotoxic effect at the concentration of 400µg/ml for both N-(3-4-Dichlorophenyl) 2-Methyl, 2,3Dihydroxypropio amide and 2.4.6. Triphenyl pyridine and effect is increase with time of incubation. The apoptotic effect of both products were investigated by measurement the caspase enzymes, the results showed highest activity of caspase 3 and caspase 9. Also at concentration 400µg/ml in both products.

Conclusion: From this data we observe that two isolated product have antitumor effect and this effect is related to the concentration of the products and incubation period. Also, the two products induce apoptosis through increase activation of caspase 3 and caspase 9 which lead to programmed of cell death. This study need to furthermore study on experimental animal to confirm our results.

Introduction

The first line of cancer treatment is surgery which has significantly reduced the cancer mortality. The use of additional treatment such as radiotherapy and chemotherapy has resulted in no more than 5% reduction of death (Samantha et al., 2003). The development of tumor drug resistance during treatment is the major factor limiting the success of chemotherapeutic management of tumors. Such resistance may occur during primary therapy or be acquired during subsequent treatment (Curt et al., 1984 and Cairo et al., 1989).

Many investigations are now interested to discover naturally occurring compounds, which can be used for the prevention and treatment of cancer despite little understanding about their molecular and cellular basis of action Hala et al. (2004). Wainwright (1992) reported that 300 antitumor agents have been isolated from microorganisms, 13% have been obtained from fungi. Of 43 antitumor agents obtained from fungi, 23 come from the imperfect fungi, 15 from the Basidiomycetes and 5 from the Ascomycetes. Zhang et al. (1994) isolated sixteen polysaccharides from the mycelium of G. tsngac three of them showed antitumor activity against the solid cancer sarcoma 180/mice. Polysaccharides extracted from Sargassum thunbergii have strong antitumor effects against transplanted tumors such as sarcoma 180 and Ehrlich solid carcinoma (Zhuang et al., 1995).

Acebal et al. (1999) isolated agrochelin as a new alkaloid cytotoxic substance by the fermentation of Agrobacterium sp. The compound was obtained from the bacterial cell by solvent extraction and purified
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by silica gell chromatography. Rimpler et al. (1996) described the ability of fungi in the families Tricholomataceae and Polyporaceae to produce antitumor activity. The effects of a polysaccharide-protein complex (PSPC) isolated from the culture filtrate of T. lobayense were investigated in mice injected with S-180 cells. PSPC restored the phagocytic function of peritoneal exudate cells (PEC) and T-cell mitogenic activity in tumor-bearing mice. (Liu 1996).

Gary et al. (1997) purified a series of peptide antifungal – anticancer agents known as leucinostatin A from Acremonium sp. Leucinostatin A possess activity against certain human cancer cell lines. Raha et al. (1990) found that L-Asparaginase from Cylindrocarpon obtusiporum MB-10 inhibits the growth of ascites fibro sarcoma and Dalton’s Lymphoma tumor cells in vivo and significantly increases the survi-val rate of tumor bearing mice. The enzymetr-eated normal mice become healthier and survive longer than their usual life span.

In this work we continue the previous study on the Aspergillus fumigatus and Cylindrocarpon candidum to emphasis has been imposed for isolation, purification and identification active principles in the both extracts of the two mentioned organisms. Furthermore we study the cytotoxic effect of the two organisms on HepG2 cells and trying to identify their mechanism of action.

Material and methods

Microorganisms.

Experimental fungal species

Two fungal species were used in the study:

1- Aspergillus fumigatus was obtained during the studies curried by Mahmod (2004)

2- Cylindrocarpon candidum was obtained during the studies curried by Abo EL- Nasser (2000).

Culture media.

-Liquid Malt extract for Production of antitumor agents.

This medium was described by Fang, et al. (1997). It has the following composition (g/L): malt extract, 20; peptone, 1; glucose, 20 and distilled water, 1000 ml.

Production and Extraction of antitumor agents.

Aliquots of 1L liquid malt extract medium placed in conical flask (2L) and autoclaved at 121 °C for 20 min. Each flask was inoculated by 5ml spore suspension of the experimental organism and incubated at 25 °C for 5 days. The broth media was separated into a supernatant and mycelial cake. The mycelial cake was extracted with acetone and concentrated under vacuum and the supernatant was extracted with ethyl acetate and also concentrated under vacuum. The resulting residues were combined and extracted with ethyl acetate to obtain a crude material. This residue was tested for its toxicity against tumor cells.

Purification and analysis of the extracts obtained from Aspergillus fumigatus and Cylindrocarpon candidum.

100 liters of the broth media was separated into a supernatant and mycelial cake. The mycelial cake was extracted with acetone and concentrated under vacuum condition and the supernatant was extracted with ethyl acetate and concentrated under vacuum condition. The resulting residues were combined and extracted with ethyl acetate to obtain a crude material. The residue was fractionated on column chromatography packed with silical gell 60 G F254 for column chromatography and eluted with ethyl acetate flowed by an increasing proportion of methanol. Fractions were collected at 15 °C.
Fig (1) Purification of antitumor agent of *Aspergillus fumigates* crud.

100L Culture broth

**separation**

**Mycelium (225g)**

- Extraction with acetone
  - Resinous residue (3.5g)

- Extraction with EtOAc
  - Residue (7g)
    - Fractionation on silical gell column by EtOAc: methanol (1:1)
      - F1  F2  F3  F4  F5  F6  F7

**Aqueous filtrate (97L)**

- Extraction with EtOAc
  - Resinous residue (5g)

All fractions were tested using HepG2 cell line

- Active fraction
  - F5 (0.83g)
Aspergillus fumigatus and Cylindrocarpon candidum……..

Fig (2) Purification of antitumor agent of Cylindrocarpon candidum crud.

100L Culture broth

Mycelium (150g)

Extraction with acetone

Aqueous filtrate (98.4L)

Extraction with EtOAc

Resinous residue (1.5g)

Resinous residue (3.7g)

Extraction with EtOAc

Residue (4.2g)

Fractionation on silical gell column by EtOAc: methanol (1:1)

F1  F2  F3  F4  F5

All fractions were tested using HepG2 cell line

Active fraction

F3 (0.5g)
Standard Protein Assay

The protein concentration in the investigated samples was determined according to the methods of (Bradford 1976).

Cell line

Human liver carcinoma cell line HepG2 obtained from American type culture collection.

Cell culture

HepG2 cell line was maintained and subculture in 75 cm² cell culture flask (Fisher Scientific Pittsburgh, PA). Using 10 ml of RPMI – 1640 [Roswell park Memorial Institute medium]. (Supplemented with 1 % (2 mM) glutamic acid, 10 % unheated fetal bovine serum (FBS), 100 µ/ ml penicillin and 100 µg/ ml streptomycin). All cells were grown at 37 °C in atmosphere 5% CO2 – 95 % air in a high humidity atmosphere in water – Jacteted incubator.

Cell viability assays.

1- Trypan blue exclusion test.

0.05 % trypan blue solution was added to cell suspension (v/v) from each control and treated cells. Cells were examined under the light microscope. Total cell counts and viable cell number (survival) were determined by a standard Heamocytometer procedure. Live viable cells were seen colorless (impermeable to the dye due to intact cell membrane) and dead cells were seen as blue (permeable to dye due to disruption of cell membrane):

% live (Recovery rate) =

\[
\frac{\text{Count of living cells}}{\text{Count of total cells}} \times 100
\]

2- MTT assay

Cytotoxicity was measured using the yellow MTT and form a dark blue in solution formazan crystals which is largely impermeable to cell membranes, resulting in it accumulation within healthy cells. Solubilization of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the optical absorbance (OD) at 570nm (Mosmann, 1998).

Procedure

HepG2 cells (0.5 X 10⁵ cells/ well) in serum. Free media were plated in fetal bottom 96.well microtiter plate, and treated with compound for 24h at 37 °C in humidified 5% CO2 atmosphere. After incubation media were removed and 40 µl MTT solution /well was added and incubated for an 4hrs. MTT crystals were solublized by adding 180 µl of acidified isopropanal /well and plate was shacked at room temperature. The absorbance was read at 570 nm using ELISA reader (meter tech 960, USA). Triplicate wells were prepared for control and each individual dose and the average was calculated. Data were expressed as the percentage of relative viability comparison, the untreated cells compared with control. The cytotoxicity indicated by < 100 % relative viability. Percentage of relative viability was calculated using the following equation:

Absorbance of treated cells / Absorbance of control X 100.

Then the half maximum inhibitory concentration IC 50 was calculated from the equation of the dose response curve.

3- Colorimetric Protease assays.

Caspase 3 and Caspase 9 (the ApoTarget)

The ApoTarget Colorimetric Protease assays Catalog # KHZ0101 and KHZ0102. Caspase 3 and caspase 9 protease assays are to be used for in vitro determination of proteolytic activity in lysates of mammalian cells (biosource International, Inc.542 Flynn Road Camarillo, California 93012, USA). Assay procedure briefly. Induce apoptosis in cells by different
concentration of each compound. Cells were counted and pellet 3–5 million cells per sample. Resuspend cells in 50 µl of chilled cell lysis buffer and cells were incubated on ice for 10 min. then centrifuged and the protein concentration was assayed by the Bradford methods (FISI) in cytosol extract. Each cytosol was a diluted to a concentration of 50–200µg protein per 50 µl. cell lysis buffer. Reaction buffer [containing DDT (dithiothreitol)] was added to each sample. 5µl of the 4 mµ DEVD – pNA [amino acid sequence composed of the chromophore, p-nitroanilide (pNA)] substrate (200µm final concentration) was added and incubated at 37 °C for 2 hrs in dark. Then the absorbance was recorded at 405 nm using spectrophotometer.

Results

Fractionation, purification and Identification of the anticancer agents from Aspergillus fumigates and Cylindrocarpon candidum.

The culture broth of both organisms were extracted to obtain crude antitumor active agents (figs1,2). These crude fractions were fractionated by silica gel column chromatography using ethyl acetate: methanol 1:1 as eluting agents. Seven fractions and five fractions were obtained from Aspergillus fumigates and Cylindrocarpon candidum respectively (table 1). These different fractions were examined for their anticancer activity against HepG2 cell line. It was found that F5 from Aspergillus fumigates and F3 from Cylindrocarpon candidum showed the highest activity (fig 3).

Each product was tested by Infrared absorption spectra (IR) were measured with FLIR spectrophotometer. NEXUS-460 (USA) using KB6 as standard material, Ultra violet-visible spectra (UV spectra) were measured with Microlet Evolution 300. UV and IR were determined in Desert Research Center (DRC). Micro analytical unit and G.C Mass spectra were measured with Fimmigan mat. SSQ 7000 (Thermo. Inst. Sys. Inc. U.S.A. mass spectrometer at an-ionization Voltage 870 ev and El mode. These measured in Ain Shams University, Faculty of Science, Central Laboratory, to know the structure of each product.

The first products was the (N-(3,4-Dichloropheny)l2-Methyl 2, 3 Dihydroxypropio amide) produced from Aspergillus fumigates it shows λmax in the UV regin at 266nm indication of substituted was being (NH,AC) (Fig 4). In IR spectrum showed absorption bands at 3621cm⁻¹ OH, 3421 cm⁻¹ NH and 1693 CO cm⁻¹. Mass spectra showed M/Z 229, 149, 73 and 41 the spectra data confirm assigned structure I.

The second product (Fig 5) was the (2,4,6. Triphenyl pyridine) produced from Cylindrocarpon candidum. In IR spectrum showed absorption band at 3405 cm⁻¹ NH. Mass spectra showed M/Z 307, 242,206,154 and 125 the spectra data confirm assigned structure II.

Measurement of anticancer activity of the two active fractions (DMD and TPP) on HepG2 cell line (liver cancer).

HepG2 cells were treated with graded concentrations (25-400 µg/ml) of DMD and TPP for 12-24 h. and then the cell proliferation and cell viability were monitored. The results showed that treatment of HepG2 cells with two compounds of DMD and TPP resulted in significant decrease viability of the cells in a dose and time- dependent manner (12, 24 h) as compared with control (Figs 6,7). The effect of DMD and TPP on cell proliferation was found to remarkably inhibit the proliferation of HepG2. The result showed that Cylindrocarpon candidu had cytotoxic effect more than Aspergillus fumigates in all concentration as shown in (Fig 8). Cell proliferation was determined by the cell titer 96 TM non-radioactive cell proliferation assay MTT. Each value is the mean of triplicates ± SD. Induction of apoptosis in HepG2 cells by DMD and TPP. The apoptotic effect was measured through activation of caspase enzymes. The activation of caspase 3 and caspase 9 proteases were measured at different concentrations of protein contents (25-250µg) of cell treated with graded dose of each DMD and TPP products. The results showed that highly significant increase in activity of caspase 3.
enzymes in the cell treated with DMD at concentration (400 µM) whereas the activity of enzyme was decreased at less concentration (Fig 9). The same result was obtained when monitored the activity of caspase 9 in HepG2 cells treated with different concentration of DMD (Fig 10). On the other hand, when measured the activation of caspase 3 and caspase 9 in HepG2 cells treated with TPP was lesser than that of DMD. The result showed dose dependent activity of both enzymes (Figs 11,12).

Table (1): Fractionation of Aspergillus fumigatus and Cylindrocarpon candidum crude extracts by column chromatography.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>ml</th>
<th>EtoAc: methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>100</td>
<td>100% EtoAc</td>
</tr>
<tr>
<td>F2</td>
<td>75</td>
<td>5% methanol</td>
</tr>
<tr>
<td>F3</td>
<td>150</td>
<td>10% methanol</td>
</tr>
<tr>
<td>F4</td>
<td>100</td>
<td>15% methanol</td>
</tr>
<tr>
<td>F5</td>
<td>50</td>
<td>20% methanol</td>
</tr>
<tr>
<td>F6</td>
<td>175</td>
<td>25% methanol</td>
</tr>
<tr>
<td>F7</td>
<td>100</td>
<td>30% methanol</td>
</tr>
</tbody>
</table>

Fig (4): Structure of active fraction of Aspergillus fumigatus.

Fig (3): Effect of different fraction of Aspergillus fumigatus and Cylindrocarpon candidum extract on cancer cells.
Aspergillus fumigatus and Cylindrocarpon candidum

(N-(3-4-Dichlorophenyl)2-Methyl-2,3Dihydroxypropio amide)

Fig (5): Structure of active fraction of Cylindrocarpon candidum.
(2.4.6. Triphenyl pyridine)

Fig (6): Dose response curve of DMD effect on HepG2 cells after 12 and 24 h. of exposure determined by trypan blue excusion test.

Fig (7): Dose response curve of TPP effect on Hep G2 cells after 12 and 24 h. of exposure determined by trypan blue excusion test.
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Fig (8): MTT assay for determination of proliferation of HepG2 cell line under different concentration of DMD and TPP.

Fig (9): The activity of Caspase 3 at different concentration of cell protein treated with DMD

Fig (10): The activity of Caspase 9 at different concentration of cell protein treated with DMD

Fig (11): The activity of Caspase 3 at different concentration of cell protein treated with TPP

Fig (12): The activity of Caspase 9 at different concentration of cell protein treated with TPP
Aspergillus fumigatus and Cylindrocarpon candidum

Discussion

Although, the most common fungi causes an important source of morbidity and mortality in immunocompromized hosts but its toxin may be useful to inhibit the growth of other pathogenic organisms inside the host. Some mycotoxin which produced from fungi have cytotoxic effect on cancer cells when tested in vitro on different cancer cell lines (Lewis et al., 2006). Gliotoxin is a mycotoxin produced by several moluds such as Aspergillus fumigatus produce cytotoxic effect in liver cancer (Stanzani et al., 2006). Many investigations are now being carried out to discover naturally occurring compounds and biological response modifiers (BRMs) which can suppress and prevent many diseases including cancer (Shi et al. 1992, Samaranayake et al., 2000, Thapliyal et al., 2002 and Samantha et al., 2003). In fact, many effective chemotherapeutic are agents that induced programmed of cell death (apoptosis) via activation of different pathways (Goli et al., 2002, Kaufmann and Earnshaw 2000 and Reed, 2002) and the caspase enzymes are the key executors of apoptosis. In this study, we determined the role of caspases in signaling of Aspergillus fumigates and Cylindrocarpon candidu apoptosis in liver cancer cells (HepG2) and the data revealed significant inhibition of cell growth and proliferation in both culture treated with N-(3-4-Dichlorophenyl) 2-Methyl, 2,3 Dihydroxypropio amide and 2.4.6. Triphenyl pyridine products. This inhibition was associated with the incubation time and the concentration of products. In vitro cytotoxicity and cell proliferation were measured by trypan blue exclusion and MMT assays. Similar results were reported by (Leah et al., 2006), he study in vitro cytotoxic activities of some fungal products against different cell lines including heptoma cell line HepG2 (Saint et al., 2006).

Wright et al. (2004) demonstrated that liver cells are able to react specifically to a fungal pathogen through increase expression of specific receptors. Stationary phase cultures of Aspergillus fumigates were associated with the appearance of typical markers of apoptosis including elevated proteolytic enzyme activity. In these study the activities of caspase 3 and caspase 9 were measured in HepG2 cells after treatment with different concentrations of N-(3- 4-Dichlorophenyl) 2-Methyl, 2,3 Dihydroxypropio amide and 2.4.6. Triphenyl pyridine products. The result obtained was revealed an elevation of both enzyme activities in culture treated with Aspergillus fumigates and Cylindrocarpon candidu. The elevation of caspase activity was associated with increased concentration. This result was not in agreement with Berkova (2006), who reported that , Aspergillus fumigates inhibit tumor necrosis factor (TNF) alpha, a known inducer of apoptosis he also observed that, the anti apoptotic effect of Aspergillus fumigates was associated with a significant reduction of caspase 3. In conclusion, invitro of anticancer activities of the tested components; N-(3- 4-Dichlorophenyl) 2-Methyl, 2,3 Dihydroxypropio amide and 2.4.6. Triphenyl pyridine which produced from Aspergillus fumigates and Cylindrocarpon candidum against liver cells. Also these two products were strong induced apoptotic effects through activation of caspase 3nd caspase 9 enzymes. This study needs furthermore confirmation in vivo study on experimental animal module to provide accuracy of the phase one trial on the two products of Aspergillus fumigates and Cylindrocarpon candidu.

References


تحفيز المركبات المعزولة من أسبيرجيلس فيميجاتس وسيليندروكوبونكانديلم
للموت البرمج لخلايا الكبد السرطانية عن طريق تنشيط انزيمات الكازبيزيس

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معظم الأبحاث الآن توجه أهتمامتها بالمواد الطبيعية التي أثبتت أن لها دور كبير في الوقاية والعلاج من الأمراض بما فيها السرطان. ويستخدم المواد الطبيعية الآن كعلاج إضافي وذلك للتقليل من الآثار الجانبية للعلاج الكيماوي والعلاج الإشعاعي. وقد وجد أن أكثر من 300 مركب تم عزلها من النباتات الدقيقة لها تأثير سام على الخلايا السرطانية. وفي هذه الدراسة تم عزل مركبين هما [3 و4 داي كلوروفينيل – 2 ميثيل 2 و3 داي هيدروكسي بروبيوميد] من أسبيرجيلس فيميجاتس و[2 و4 و6 ترابينين، بريدنين] من وسيليندروكوبون كانديلم. وتم دراسة تأثيرهما على خلايا الكبد السرطانية ووجدنا أن لها تأثير سمي واضح على نشاط الخلايا السرطانية. وهذا التأثير يزيد كلما زادت الجرعه وزادت فترة وجود المركب مع الخلايا. وأيضاً تم دراسة تأثير هذه المركبات على الموت البرمج لخلايا الكبد السرطانية من خلال دراسة انزيمات الكازبيزيس 3 و9 حيث أوضحت النتائج ان أعلى درجه سمية حصلنا عليها كانت عند تركيز 400 ميكروجرام/مل في كل من المركبات وهذا التركيز يزيد تأثيره السم زادت فترة الحضانة بين المركب والخلايا حيث كانت أعلى درجة سمية بعد 24 ساعة من فترة التحضير وأيضاً عند هذا التركيز وصلنا إلى أعلى نسبة للموت البرمج لخلايا الكبد حيث أظهرت النتائج نشاط ملحوظ للإنسامات كازبيزيس 3 و9 في كل من المركبين. وهذا النشاط يتسبب طردياً مع تركيز المركبات وقتة التحضير مع الخلايا. وهذه الدراسة تحتاج إلى توضيح بتجارب تأكيدية أخرى وخاصة على حيوانات التجربة ل-tsصل إلى الهدف المرغوب فيه.