Cytotoxic Effect of Gliotoxin, Hemolysin, Protease and Melanin purified from *Aspergillus fumigates* on REF Cell Line, *in vitro* Study

التأثيرالسمي لسم الكلايوتوكسين و البروتيين المحلل للدم وانزيم البروتييز وصبغة الميلانين المنقاة من الرشاشيات الدخناء على خط الخلايا الليفية لجنين الجرذ، دراسة خارج الجسم الحي

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مركز بحوث التقنيات الأحيائية/ جامعة النهرين *

قسم التقنيات الأحيائية / كلية العلوم/ جامعة بغداد** *

Abstract

***Aspergillus fumigates* especially clinical isolate produces a series of toxic substances and proteinaceous hemolysin, protease and pigment like melanin which appear to act in an additive and synergic way on cells. In this study, gliotoxin, hemolysin, Protease, and Melanin were used in an experimental model to study their Cytotoxic activity by evaluating their effect on REF cell line (Rat embryonic fibroblast), for exposure time of 24 hrs at three different concentrations of each compounds triplicate of each concentration were used, cytotoxicity of the purified compounds were active against REF cell line under study and a toxic effect was clear with a significant difference at the level of probability ($p \leq 0.05$) and this effect was contrasted among different concentrations for each purified compound growth inhibition of REF cell line was increase gradually with the increase of compound concentration.**

Keywords: Gliotoxin, Hemolysin, Protease, Melanin, Cytotoxic activity

الملخص

الرشاشيات الدخناء وخصوصا العزلات السريرية تنتج سلسلة من المواد السامة والبروتينات المحلله للدم وانزيم البروتييز والصبغات مثل الميلانين والتي تبدوا أنها تعمل بصورة اضافية وطريقة تآزر على الخلايا. في هذه الدراسة تم استخدام سم الكلايوتوكسين, والبروتيين المحلل للدم وانزيم البروتييز والميلانيين في نموذج تجريبي لدراسة فعاليتها السمية من خلال تقييم تأثيرها على خط الخلايا الليفية الجنينية للجرذ بوقت تعرض 24 ساعة وبثلاث تراكيز مختلفة لكل مركب وبثلاث مكررات لكل منها سمية المركبات النقية قيد الدراسة فعالة والتأثير السمي واضح بفرق معنوي عند مستوى الاحتمالية($P \leq 0.05$) تحت ظروف التجربة وكان هذا التأثير متباين بين التراكيز المختلفة لكل مركب من المركبات النقية، نسبة تثبيط نمو خط الخلايا اليفية لجنين الجرذ يزداد تدريجيا مع زيادة تركيز المركب.

الكلمات المفتاحية: الكلايوتوكسين، والبروتيين المحلل للدم، انزيم البروتييز والميلانيين، الفعالية السمية

Introduction

Aspergillus fumigatus, a pathogenic and saprophytic mould [1] causing a wide range of diseases including aspergillosis, produces a series of toxic substances and array of chemicals [2] putative virulence factors of *A. fumigatus* are toxic molecules, which are often products of secondary metabolism. Gliotoxin inhibits the phagocytosis by macrophages and can induce their apoptosis. This effect could also be seen for polymorphonuclear leucocytes (PMN) [3,4,5,6]. The hemolysin, which enables the fungus to disrupt blood cells, contains negatively charged domains that the hemolysin has toxic effects, it seems not to be a main virulence factor but a compound that increases the effects of other toxic factors involved in pathogenicity [2,7,8,9]. Protease displaying different kinds of function seem important for full virulence of *A. fumigatus*. They are needed to obtain nutrients by degradation of collagen and elastin, which constitute the main compounds of the lung [10], and lead to a detachment of epithelium cells in the respiratory tract. [11,12,13,14,15]. Melanin conidial pigmentation, allows *A. fumigatus* to survive in phagocytes and thereby to escape from human immune effector cells and to become a successful pathogen [16]. Melanin, which likely enables the fungus to counteract the immune defense system and increases spore resistance [17,18,19,20].

The advantages of tissue culture technique can be achieved as cytotoxic test of new chemicals, cosmetics, food additives *in vitro* [21,22].

This study aimed to determine the cytotoxic activity of the virulence factors of *A. fumigaus* and comparative between them.

Materials and methods

Subculture of REF cell line

Single cell suspension was prepared by treating 25 cm^3 tissue culture flask with 2 ml trypsin solution incubated for 2 min at 37°C in an incubator supplemented with (5%) CO_2 after detachment of the cells from the flask surface by gently tapping of the flask followed by the addition of 20 ml of growth medium supplemented with 10% fetal bovine serum, then the viability test of the cells was made by using trypan blue dye which stains the dead cells. Cells suspension was well mixed followed by transferring 200 µl/well to the 96 well flat bottom micro titer plate using automatic micropipette containing ($1x10^5$ cell/well). Plates were incubated at 37°C in an incubator supplemented with (5%) CO_2 until 60-70% confluence of the internal surface area of the well for REF cell line [23].

Cytotoxicity assay

To detect the growth inhibition of REF cell line, culture of this cell line was incubated with different concentrations of each compound used in our study these compounds done by different purification technique depended on the nature of the compound, cells were treated and incubated with the purified extracts of gliotoxin, hemolysin, protease, Melanin, three concentrations at triplicate form of each extracts to investigate the cytotoxic effect of these extracts respectively, the concentrations used as follows: gliotoxin 25,50,100 ng/ml, hemolysin 2.5, 5, 10µg/ml, protease 7.5, 15, 30µg/ml and melanin 62, 125, 250µg/ml triplicate form of each concentration were used. Negative Control was achieved by incubating REF cell line with only maintenance medium.

Detection of the Cytotoxic Effect

Neutral red assay used to detect the cytotoxic effect. After elapsing the incubation period, 100 µl/well of neutral red dye freshly prepared were added to each well then plates incubated for 2 hrs, viable cells will uptake the dye while the dead not, the plates washed by PBS to remove the excess dye, then 100µl/well of eluent solution was added to each well to withdraw the dye from the viable cells. Optical density of each well was measured by using ELISA reader at 492nm wave length [22,24].

Percentage of the inhibitory rate was measured according to Wang (2003) [25] as follows:

$$\text{I.R \%} = \frac{\text{O.D. of control - O.D. of test}}{\text{O.D. of control}} \times 100 \quad \text{[25]}$$

Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) fallowed by Duncan test, using SAS computer program version 7.5 [26]. Differences in results were considered significant at probability value equal or less than 0.05.

Results and discussion

Cytotoxic Effect of Gliotoxin

Results in Figure (1) show that growth inhibition of REF cell line decreased gradually when gliotoxin concentration increased. Gliotoxin has significant differences of cytotoxic effect on REF cell line ($P \leq 0.05$) , 81% , 63.21% and 40% growth inhibition was showed at concentrations 100, 50, and 25 ng/ml respectively.

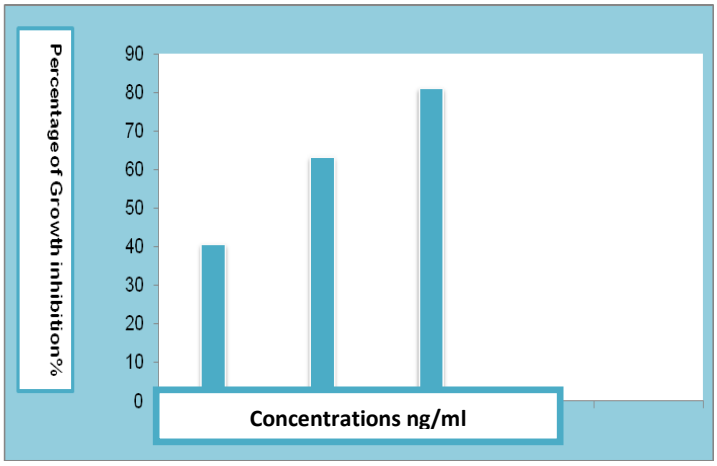

Fig. (1): Cytotoxicity effect of different concentrations of purified Gliotoxin from *A.fumigatus* on the REF cell line after 24 hr.

The biological activity of any chemical compound is based on active groups and an internal bond, gliotoxin have an internal disulfide bond that can bind and inactivate proteins *via* a sulfide:thiol exchange [27], therefore the cytotoxic effect of gliotoxin maybe attributed to its disulfide bond. The disulfide bridge allows the cross linking with proteins *via* cysteine residues and generate deleterious reactive oxygen species (ROS) through the redox cycling between the reduced and oxidized form. This mechanism of ROS generation is believed to be responsible for the toxicity of gliotoxin [28]. The ROS generated as a result reported to facilitate the release of cytochrome c and apoptosis inducing factors from mitochondria, leading to caspase activation, as well as other events that mediate cell death [29].

Cytotoxic effect of gliotoxin in our result may be attributed to the ability of gliotoxin to induce morphological changes in the cells, These changes in normal cell line are due to a loss in the adherence of the cells to their plastic container [30], which is characteristic of an apoptotic process [31].

This phenomenon has been described for gliotoxin on different cell types: thymocytes, lymphocytes, spleen cells, or macrophages [29]. Gliotoxin inhibited oxidative burst of human neutrophils, gliotoxin also causes damage to the ciliated respiratory epithelium *in vitro* and this property might assist *A. fumigatus* in the colonization of the respiratory mucosa . Furthermore, Nierman (2008) [32] have shown by the genome-wide gene expression profile analysis that gliotoxin genes are upregulated in germination during initiation of infection in mice [33] .

Cytotoxic Effect of Hemolysin

Significant cytotoxic effect ($P \leq 0.05$) was observed on the growth of REF cell line at the concentrations of 2.5, 5, and 10 µg/ml with growth inhibition percentage 33.79%, 64.44 % and 76.68%, respectively, as shown in the Figure (2).There was an increase in the inhibitory effect when compared with the control. Hemolysin is cytolytic and hemolytic protein and can induce effective permeabilization in cell [2], these activities of hemolysin help us to explain the cytotoxic effect of hemolysin in our results. Hemolysin is cytolytic and hemolytic protein and can induce effective permeabilization in the cell [2], these activities of hemolysin help us to explain the cytotoxic effect of hemolysin in our results.

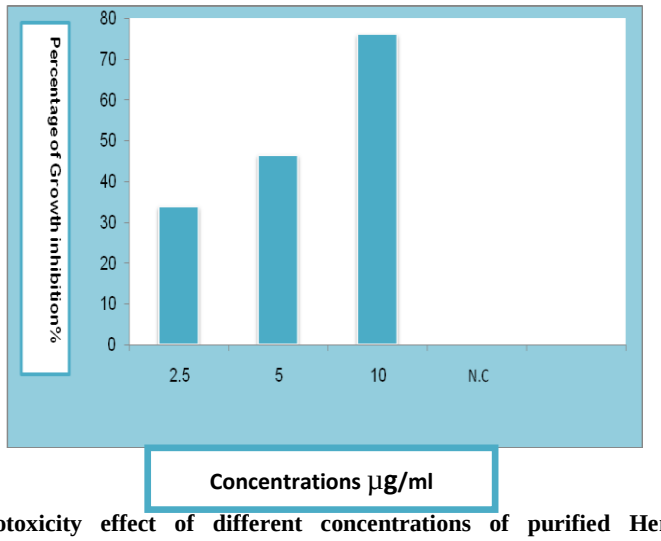

Fig.(2): Cytotoxicity effect of different concentrations of purified Hemolysin from *A.fumigatus* on REF cell line after 24 hr.

Hemolysin has an alpha helical structure, Alpha helical toxin cause damage in susceptible cells by creating pores in membranes. Donohue et al.. (2006) [34] experiments showed that specific interaction of hemolysin with plasma membrane domains occur, suggesting that hemolysin has a specific binding protein [35]. The existence of phospholipid membrane domains involved in cell signaling, endocytosis and attachment of several toxins and protein indicate that these cells membrane domains probably serve as attachment sites for hemolysin, leading to their aggregation and formation of the pore [36,37,38].

Cytotoxic Effect of Protease

Figure (3) showed protease, with enzymetic activity of 226.98U/ml purified from *A. fumigatus* had cytotoxic effect on growth of REF cell line at the concentrations of 7.5, 15, and 30 µg/ml with growth inhibition percentage of 37.17 %, 47.81 % and 56.35 %, respectively Growth inhibition of REF cell line was increased gradually with the increases of the enzyme concentration.

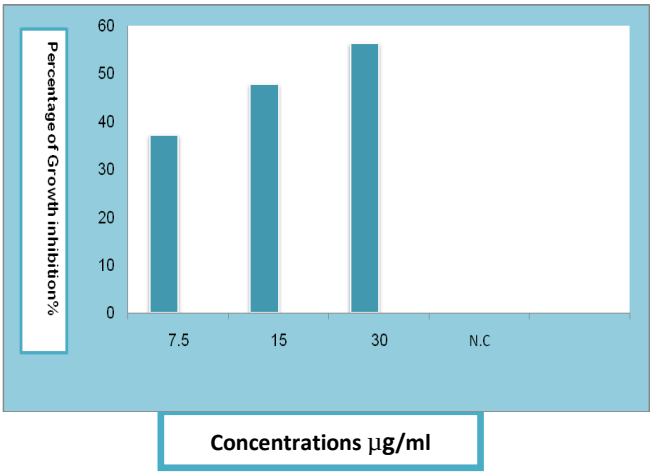

Fig. (3): Cytotoxicity effect of different concentrations of purified Protease from *A.fumigatus* on the REF cell line after 24 hr.

No significant cytotoxic effect (P≤ 0.05) between the concentrations 7.5 and 15 µg/ml, of protease and no significant cytotoxic effect between the concentrations 15 and 30 µg/ml when compared with the control.

Protease have proteolytic activity, this activity enable it to degrate the main compounds of the cell [10]. The results may be agreed with the results of Balachandran et al.. (2012) [39], who found that there is significant cytotoxic effects of protease on A549 cell line, the cytotoxicity of protease was concentration dependent, also protease inhibit growth of normal cell line. Grigoryan et al.. [40] reported that any decrease in the activity of protease accompanied by a decrease in the growth inhibition. level *in vitro* ,the mechanism of protease activity depends on a set of amino-acid residues,

typically Ser-His-Asp, known as the "catalytic triad" [41]. This set includes a nucleophilic residue (Ser), a general base (His), and an additional, acidic, residue (Asp), all connected by a chain of hydrogen bonds [42].

Cytotoxic Effect of Melanin

REF cell line treated with melanin at the concentrations of 62 ,125 , and 250 µg/ml and showed growth inhibition percentage of 4.95 % , 7.38% and 31.11% , respectively. Growth inhibition of REF cell line was increased gradually with the increase of melanin concentration as shown in the Figure (4). Results showed significant cytotoxic effect ($P \leq 0.05$) between the concentrations when compared with the control.

Our results may be agreed with the results of some studies which showed that melanin had slight cytotoxic effect, melanin revealed an elevated susceptibility to reactive oxygen intermediates (ROI) which derived from hydrogen peroxide from cells [43].

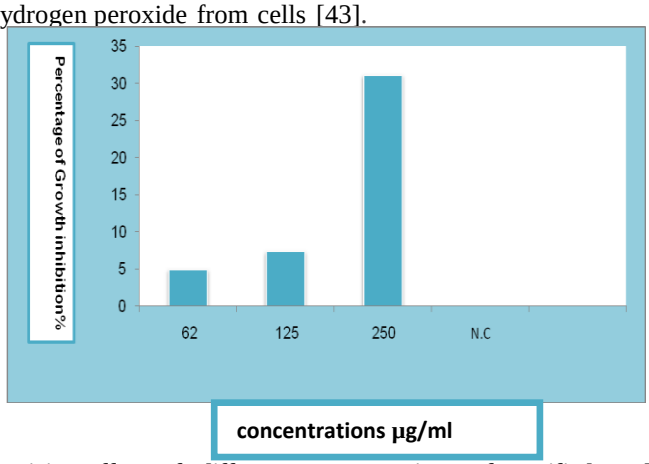

Fig.(4): Cytotoxicity effect of different concentrations of purified Melanin from *A.fumigatus* on REF cell line after 24 hr.

Langfelder et al.. (2003) [44] showed that melanin has no significant effect on normal cell line after 48 hours of incubation. Figure (5) indicated that there is significant difference between the effect of purified compound on the normal cell line.

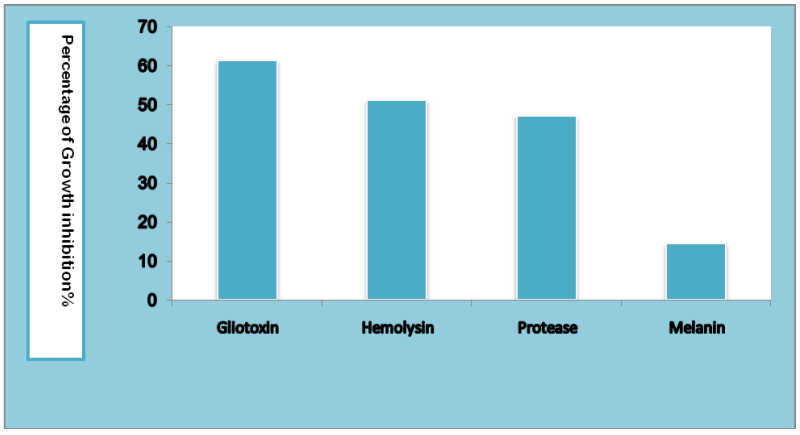

Fig. (5): Comparasion between the cytotoxic effect of purified compound in our study

The differences between the effects of compounds may be due to the chemical composition of each compounds, gliotoxin showed the highest growth inhibition percentage when compared with the other compound.

Conclusion

All purified compounds showed cytotoxic effect and depended on the nature of each compound, gliotoxin showed highest cytotoxic effect when compared with the other compounds in the study, our results indicate that gliotoxin might be considered as a possible virulence factor of *Aspergillus fumigatus* during the infection.

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