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

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ABSTRACT

Aspergillus fumigatus especially clinical isolate produces a series of toxic substances and proteinicios hemolysin, protease and pigment like melanin which appear to act in an additive and synergic way on cells. In this study, gliotoxin, 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), on exposure time of 24 hrs at three different concentrations of each compounds triplicate of each concentration were used, Cytotoxicity of the purified compounds are active against REF cell line under study and a toxic effect was clear with a significant difference at the level of probability ($p< 0.05$) 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, Protease, Melanin, Cytotoxic activity, *Aspergillus fumigatus.*

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]. Proteases 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 [7] and lead to a detachment of epithelium cells in the respiratory tract. [8,9,10,11,12]. Melanin conidial pigmentation, allows *A. fumigatus* to survive in phagocytes and thereby to escape from human immune effectors cells and to become a successful pathogen [13] ,Melanin, which likely enables the fungus to counteract the immune defense system and increases spore resistance [14,15,16,17]. The advantages of tissue culture technique can be achieved as cytotoxic test of new chemicals, cosmetics, food additives *in vitro*. [18, 19]. This study aimed to determine the cytotoxic activity of the virulence factors of *A.fumigaus* and comparative between them.

MATERIALS AND METHODS

Virulence factors purification: Gliotoxin, Protease, and melanin purified from virulent *A.fumigatus* strain isolated from patients with aspergillosis in previous study [20] and prepared in different concentration to study its cytotoxic activity.

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 [19].

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 depend on the nature of the compound , cells were treated and incubated with the purified extracts of gliotoxin, 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), 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 and the dead not, the plates washed by PBS to remove the excess dye, then 100µl/well of eluent solution were 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 [19, 21].

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) and Duncan test, using SAS computer program version 7.5[23] 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 was decreased gradually with the increase of Gliotoxin concentration on treated with when compared with the negative control (the same cell line without any treatment). Gliotoxin has significant differences of cytotoxic effect on REF cell line ($P<0.05$) , 81% , 63.21% and 40**%** growth inhibition was show at concentrations 100, 50 , and 25 ng/ml respectively. The biological activity of any chemical compound is based on active groups and an internal bridge, gliotoxin have an internal disulfide bridge that can bind and inactivate proteins *via* a sulfide: thiol exchange [24] therefore the cytotoxic effect of gliotoxin maybe attributed to its disulfide bridge. 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 was 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 that gliotoxin 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 [30] .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 [32] have shown by the genome-wide gene expression profile analysis that gliotoxin genes are up regulated in germination during initiation of infection in mice [33].

Cytotoxic Effect of Protease : (Figure 2) shows protease, with enzymatic activity 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 37.17 % , 47.81 % and 56.35 % , respectively Growth inhibition of REF cell line was increased gradually with the increase of enzyme concentration. 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 degradation of main compounds of the cell [7]. Our results may be agreed with the results of Balachandran *et al* [34] 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 Gregorian *et al*., [35] 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". 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 [36].

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 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 3). Results showed significant cytotoxic effect ($P < 0.05$) 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 [32]**.** Langfelder *et al*., [29] showed that melanin has no significant effect on normal cell line after 48 hours of incubation, while Oza *et al*., [13] found that Melanin from *A.niger* has slight cytotoxic effect on normal cell line.(Figure 4) indicated that there is significant difference between the effect of purified compound on normal cell line. The difference between the affects of compounds may be due to the chemical composition of each compounds, gliotoxin showed the high growth inhibition percentage when compared with the other compound.

CONCLUSION

All purified compounds showed cytotoxic effect depend on the nature of each compound, gliotoxin showed high 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 *A. fumigatus* during the infection.

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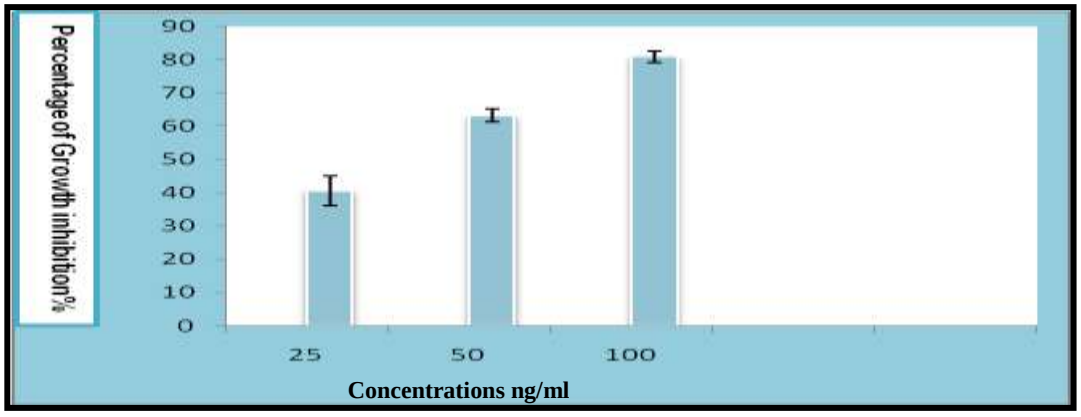

Figure 1: Cytotoxicity effect of different concentrations of purified Gliotoxin from *A.fumigatus* on REF cell line after 24 hr.

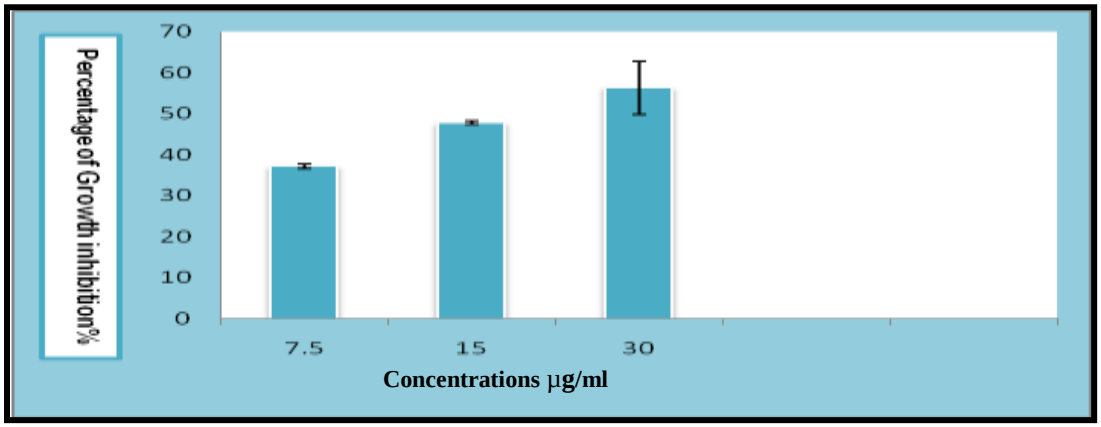

Figure 2: Cytotoxicity effect of different concentrations of purified Protease from

***A.fumigatus* on REF cell line after 24 hr.**

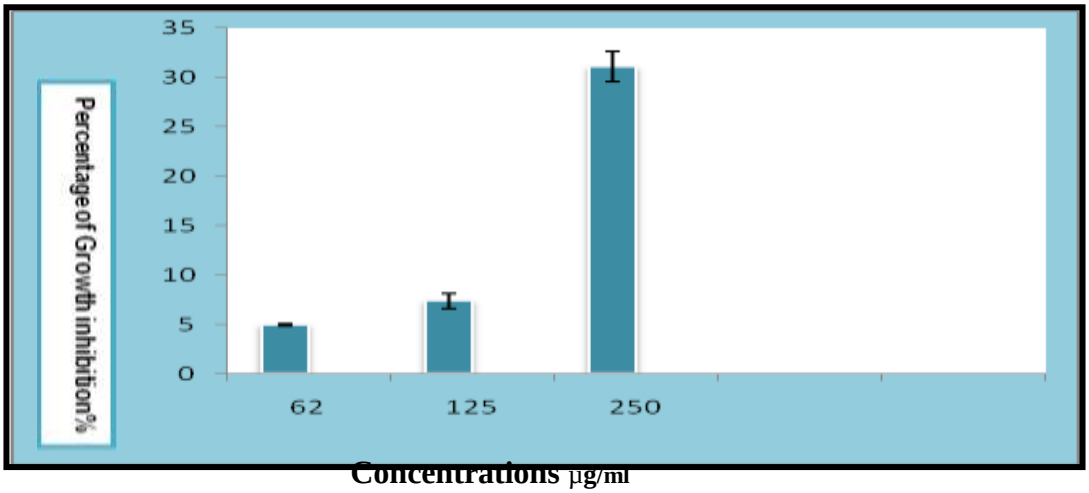

Figure 3: Cytotoxicity effect of different concentrations of purified Melanin from

***A.fumigatus* on REF cell line after 24 hr.**

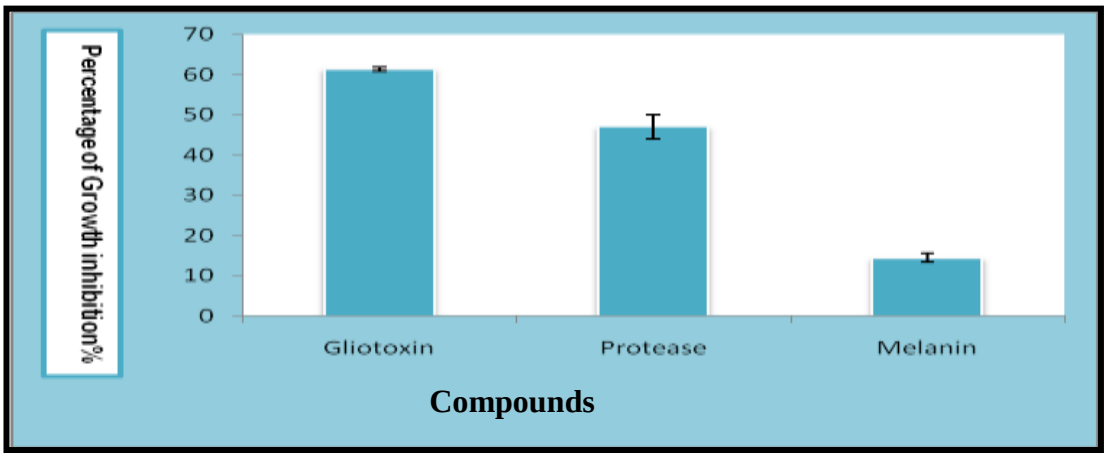

Figure 4: comparison between the cytotoxic effects of purified compound in our study.

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