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Induction of Oxidative stress response in *Aspergillus fumigatus* when exposed to *Malva parviflora* ethyl acetate extract

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ABSTRACT

The *filamentous fungus Aspergillus fumigatus* is capable of causing a range of diseases in immunocompromised individuals and is responsible for up to 3% of all hospital-based deaths in the EU. Exposure of A. fumigatus hyphae to antifungal drugs leads to a release of amino acids and gliotoxin. *Aspergillus spp*. poses a life threat to society, and particularly immunocompromised patients, since many strains have shown resistance towards the existing antifungal agents. Medicinal plants provide new hope in the fight against Aspergillus infections.

The *Malva parviflora* ethyl acetate extract have anti-fungal activity which have been recently introduced and shows excellent in vitro activity against Candida and Aspergillus species. The aim of the work presented here was to characterise the response of *A.fumigatus* to *Malva parviflora* ethyl acetate extract. The results obtained demonstrated that extract has potent anti-fungal activity and retards fungal growth. Exposure of A. fumigatus hyphae to this extract leads to a release of amino acids and proteins as demonstrated by 1- and 2-dimensional gel electrophoresis. Mass spectrometry was employed to identify a number of escaped peptides were found upon exposure to the ethyl acetate extract. LC-MS was employed to identify a number of escaped peptides that were found to have change in intensity upon exposure to the extract; several released e.g [IgE binding protein] and synthesised protein e.g [Allergen Asp F3] were identified. The result presented in this study demonstrate that extract have an effect on the release of protein which synthesised in the cells and could lead to improve the immune response of the aspergillus in vivo. This study illustrate that, in addition to activity on the enzyme responsible for synthesis of glucan in cell wall, these extract may also interfere with the permeability of the cell wall of A. fumigatus.

KEYWORDS: *Aspergillus*, *Aspergillosis*, *Malva parviflora* ethyl acetate extract, Hyphae, Oxidative stress response

1. INTRODUCTION

Opportunistic fungal infections occur in susceptible patients who have weakened defense systems. Fungal-related characteristics such as dimorphism and phenotypic switching may play important roles in initiating and establishing infections by several fungi (1). *Aspergillosis* is a group of diseases caused by fungus of the genus *Aspergillus* and usually occurs in patients with lung diseases or weakened immune systems. The diseases include invasive aspergillosis, allergic bronchopulmonary aspergillosis, and aspergilloma. Some individuals with very severe asthma may also be sensitised to fungi such as Aspergillus (2).

Previous work has established that exposure of growth arrested *C. albicans* to caspofungin leads to the leakage of amino acids and proteins (3) and the induction of oxidative and osmotic stress responses as evidenced by the activation of the Cap and Hog pathways (4). It is estimated that close to a half-million plant species exist on earth. Besides being partly used by humans as food, an increasing surge in use in pharmaceuticals, including antimicrobial is taking place (5). The multidrug resistant of microorganisms to antimicrobial agents is rising globally and appearance of strains with reduced susceptibility to antibiotics is increasing, which is of great concern as a worldwide problem. This increase has been attributed to extensive use of broad-spectrum antibiotics and its related toxicity issues that limit the effective use of antimicrobial agents. Medicinal plants are being used as alternative for the control of these antibiotic resistant strains due to comparable safety and efficacy (6)

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MATERIALS AND METHODS

M PARVIFLORA STOCK SOLUTION:

M parviflora ethyl acetate extract stock solution: was dissolved in phosphate buffet saline to give a stock concentration of 1.0 mg/ml prior to diluting in sterile water to working concentrations. Stock solutions were stored in 50µl aliquots at -70°C.

SUSCEPTIBILITY ASSAY OF *A. FUMIGATUS:*

Susceptibility testing was performed using a NCCLS-based broth microdilution technique for filamentous fungi. The surface of the agar plate was washed with 10 mL of PBS 0.05% Tween 80, and the conidial suspensions were washed twice and counted manually with a haemocytometer. The conidia were diluted in media used to produce a working suspension of 5 × 105 cells/ml. The final inoculum was 2.5 x 105 conidia/mL. The MICs for isolates were determined in triplicate. Micro-plates were incubated at 37°C for 48 h, and the growth in each well was compared with that of the growth control.

ASPERGILLUS FUMIGATUS CULTURE CONDITIONS:

Stocks of *A. fumigatus* ATCC 26933 were grown on malt extract agar (Oxoid Ltd) plates at 37 oC and maintained on the same medium at 4 oC for short term storage. *A. fumigatus* liquid cultures were grown in RPMI 1640 (GIBCO) medium supplemented with 5% (v/v) fetal bovine serum (FBS, Sigma Aldrich) at 37°C and 200 rpm, for up to 4 days.

EVALUATION OF PROTEIN RELEASE:

Exposure of *A. fumigatus* to 0.1 or 1.0 mg/ml *M parviflora* ethyl acetate extract stock solution for 4 hours resulted in protein leakage from hyphae and the increased expression of a variety of proteins involved in the oxidative stress response (e.g. catalase, antibiotic response protein), virulence (e.g. Asp F3, 18kDa antigen) and homeostasis (e.g. glyceraldehyde 3-phosphate, translation elongation factor, ATP synthase F1). In addition there was an increase in the activity of catalase, glutathione reductase and superoxide dismutase in hyphae exposed to 1.0 mg/ml extract for 1 hour.

EXTRACTION OF PROTEINS FROM HYPHAE:

Cultures were grown for 96 hours at 37oC, hyphae were harvested as described and washed with PBS. Hyphae (1.5g) were exposed to extract (0.1 or 1.0 µg ml-1) for 4 hours. Hyphae were harvested, washed with PBS and ground to a fine powder using a pestle and mortar under liquid nitrogen. Protein extraction buffer (4 mls, 0.4 M NaCl, 10mM Tris HCl, 2 mM EDTA) was added. All samples were centrifuged at 1500 g, 4 oC for 5 min. Supernatant was retained and the pellets were discarded. Protein was precipitated from the supernatant and retained.

LC-MS ANALYSIS:

In gel digestion of peptides was performed according to the method of Schevchenko (7). Resulting data were analysed using the mascot search engine, (www.matrixscience.com). Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot, (www.uniprot.org), and NCBI, (www.ncbi.nlm.nih.gov), websites. All gels were performed in triplicate on independent occasions and the average fold change in protein abundance was calculated.

ASSESSMENT OF ENZYMATIC ACTIVITY:

A. fumigatus hyphae (96 hr culture, 1g) were exposed to extract (1.0 µg ml-1) for 15, 30 or 60 minutes or to hydrogen peroxide (0.5 mM) for 15 minutes, as a positive control. Superoxide dismutase (SOD) activity was measured using a SOD Assay Kit (Fluka Biochemika) as described (8). For analysis of the glutathione reductase activity of cells the method described previously (9) was used. Catalase activity was measured as described (3).

STATISTICAL ANALYSIS:

All assays were performed on three independent occasions. Results presented are the mean ± standard error. Statistical analysis were performed using a Student's two tailed t-test with values of $p < 0.05$ considered statistically significant.

RESULTS:

Identity of proteins released from *A. fumigatus* following exposure to *M parviflora* ethyl acetate extract. *A. fumigatus* hyphae were exposed to *M parviflora* ethyl acetate extract (0.1 or 1.0 µg ml-1) for up to 240 minutes and the quantity of protein released was evaluated as described. Protein was released from hyphae throughout the incubation period but the greatest release occurred from hyphae exposed to 1.0 µg extract ml-1 for 240 min (593±14.2 µg ml-1) (Figure 1). Protein released from *A. fumigatus* hyphae following exposure to 0.1 or 1.0 µg extract ml-1 for 240 minutes was precipitated and resolved by 2D SDS-PAGE as described. The results indicate the increased abundance of a variety of proteins following exposure of A. fumigatus to 1.0 µg *M parviflora* extract ml-1 (Table 1).

Table 1: Identified leaked peptides from A. fumigatus, plus fold change in response to M parviflora extracts

BAND	Identified protein	Accession No	Coverage [%]	Score	Source	Fold increase			
						PBS	0.5 DMSO	0.1 µg ml-1	1.0 µg ml-1
1	IgE-binding protein	CAA12162	11%	72	Aspergillus fumigatus	1	1.28	1.3	3.6
2	aldehyde dehydrogenase	XP_746831	20%	276	Aspergillus fumigatus	1	1.28	1.4	3.6
3	alpha1 antiproteinase precursor	XP_615250	5%	175	Aspergillus fumigatus	1	0.75	0.7	1.9
4	secreted dipeptidylpeptidase	AAB67282	14%	397	Aspergillus fumigatus	1	2.8	1.2	6.9
5	ATPB_NEUCR ATPsynthase beta chain, metochodrial precursor	XP_659919	10%	202	Aspergillus nidulans FGSC A4	1	1.1	1.5	2.4

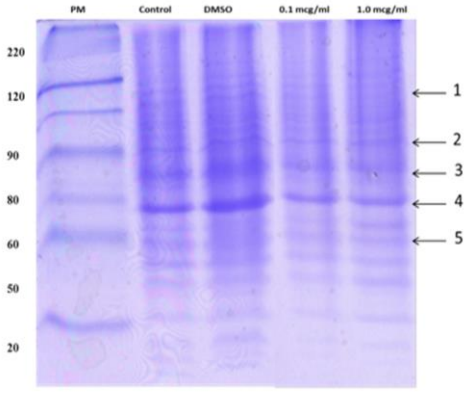

Figure 1: 1D Leaked proteins from A. fumigatus when exposed to *M parviflora* extracts

EFFECT OF *M PARVIFLORA* ETHYL ACETATE EXTRACT ON PROTEIN EXPRESSION IN *A. FUMIGATUS:*

Hyphae (96 hour old) were harvested, exposed to 0.1 or 1.0 µg extract ml-1 (for 4 hour), and the proteome was extracted and resolved by 2D SDS-PAGE (Figure 2). Proteins showing alteration in intensity were excised and identified by LC/MS (Table 2). A range of proteins showed

homology to proteins involved in stress responses (e.g. spot B18 (catalase), spot B16 (Vacuolar protease A), spot B19 (Hsp70), spot B2 (Cip-like antibiotic response protein), and spot B12 (thioredoxin reductase). A number of spots showed homology to proteins associated with the virulence of *A. fumigatus* (e.g. spot B1 (18kDa antigen), spot B3 (allergen Asp F3), and with cell homeostasis (e.g. spot B7 (glyceraldehyde 3-phosphate), spot B9 (translation elongation factor), and spot B17 (ATP synthase F1).

Table 1: Identified leaked peptides from A. fumigatus, plus fold change in response to *M parviflora* extracts

spot	Score	Name of protein	Accession no	Seq. Cov	Source	Molecular function	Fold increase			
							PBS	0.5 DMSO	0.1 µg ml-1	1.0 µg ml-1
B1	84	18-kDa antigen	CAA41217	11%	*A.fumigatus*	Fungal type ribonuclease	1	2.1	1.8	1.5
B2	270	CipC-like antibiotic response	XP_753706	40%	*A.fumigatus AF293*	CipC-like antibiotic response protien	1	1.3	4.7	0.8
B3	278	Allergen Asp F3	XP_747849	48%	*A.fumigatus AF293*	Oxidoreductase activity	1	1.5	1.4	1.8
B4	175	Chitosanase partial	AAD26111	26%	*A.fumigatus*	Chitosanase activity	1	1.7	1.9	0.9
B5	148	Dienelactone hydrolase family protein	XP_751152	10%	*A.fumigatus AF293*	hydrolase activity	1	1.4	1.5	1.5
B6	276	FG-GAP repeat protein	XP_750162	30%	*A.fumigatus AF293*	Calcium ion binding	1	0.6	0.7	0.8
B7	250	Glyceraldehyde 3-phosphate dehydrogenase	XP_748283	27%	*A.fumigatus AF293*	Oxidoreductase process	1	0.7	1.2	1.4
B8	57	fructosylamino acid oxidase	XP_747733	12%	*A.fumigatus AF293*	FAD dependent Oxidoreductase	1	1.5	1.2	0.8
B9	219	Translation elongation factor alpha	ABF50913	19%	*Zugozymma suomiensis*	Promotes the GTP-dependent binding	1	1	1.1	1.3
B10	84	Actin-dehydrogenase (AtsC)	XP_002420345	21%	*C. dubliniensis*	polypeptide binding	1	1	2	2.1
B11	68	ATP synthase delta chain	XP_750060	9%	*A.fumigatus AF293*	ATP synthase mitochondrial precursor	1	1.5	1.8	1.2
B12	88	Thioredoxin reductase Glit	XP_750863	10%	*A.fumigatus AF293*	Posttranslational protein turnover	1	1.1	1.2	1.2
B13	73	Short chain dehydrogenase (AtsC)	XP_748339	6%	*A.fumigatus AF293*	NADP binding site	1	0.9	1.1	1.3
B14	130	GLIN	AAW03301	11%	*A.fumigatus*	methyltransferase domain	1	0.9	1.8	1.3
B15	148	Aspartic endo peptidase Pep70	XP_754479	11%	*A.fumigatus AF293*	Eukaryotic Aspartic proteinase	1	0.9	0.8	0.8
B16	90	Vocoular protease A	XP_001399855	11%	*Aspergillus niger*	Fungal Proteinase Aspartic proteinase	1	3	0.8	0.9
B17	291	ATP synthase F1, beta subunit	XP_753589	17%	*A.fumigatus AF293*	F1 ATP synthase beta subunit	1	1.3	1.2	1.4
B18	724	catalase	AAB71223	24%	*A.fumigatus*	Inorganic ion transport and metabolism	1	1.9	1.3	1.4
B19	322	Molecular chaperone Hsp 70	XP_750490	13%	*A.fumigatus AF293*	Nucleotide binding site	1	0.9	2	1

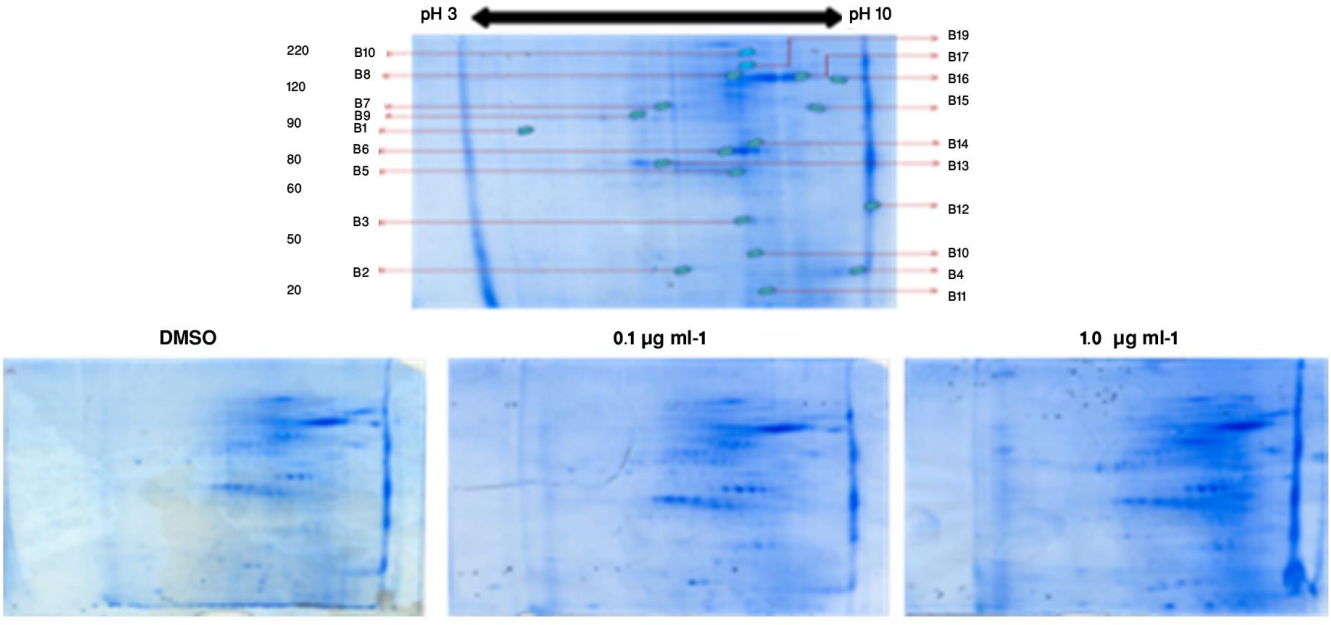

Figure 2: Change in internal proteins in A. fumigatus exposed to *Malva parviflora* extracts

EXPOSURE TO *M PARVIFLORA* ETHYL ACETATE EXTRACT INDUCES AN OXIDATIVE STRESS RESPONSE IN *A. FUMIGATUS.*

Exposure of *A. fumigatus* to 0.1 µg *M parviflora* ethyl acetate extract ml-1 for 30 minutes induced a six-fold increase in catalase activity while exposure to the drug for 60 minutes induced an eight-fold increase in activity (Figure 3A). Exposure of *A. fumigatus* to 0.1 µg extract ml-1 for 15 – 60 minutes resulted in a twofold increase in GLR activity (Figure 3B). SOD activity was increased significantly when *A. fumigatus* was exposed to hydrogen peroxide. SOD activity was increased by approximately twofold following exposure of *A. fumigatus* to extract for 15 – 60 minutes (Figure 3C).

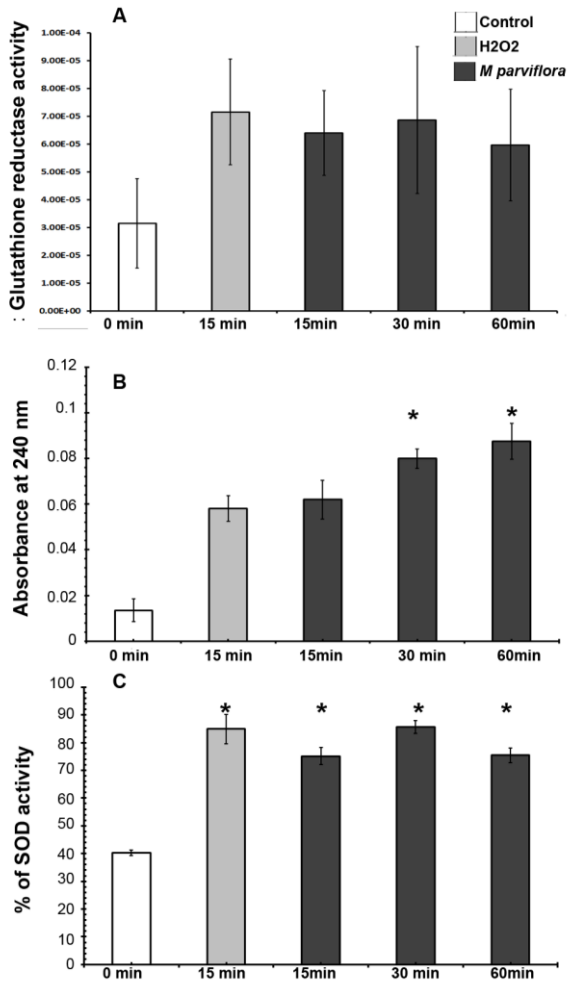

Figure 3: Catalase activity **A,** Glutathione reductase activity **B** and Superoxide dismutase activity **C** in 0.1 µg/ml *M parviflora* extract treated *A. fumigatus*

DISCUSSION:

The results presented here indicate that exposure of non-growing *A. fumig*atus hyphae to *Malva parviflora* extracts leads to the release of protein from various locations within the cell and the induction of an oxidative stress response as indicated by the increased expression and activity of a number of stress–response proteins and enzymes. Some of the released proteins (e.g. allergen Asp F3), and those elevated in expression (18kDa antigen, allergen Asp F3, Hsp 70) display antigenic properties (2, 3) thus raising the possibility of increased inflammation at the site of infection and an enhanced ability of *A. fumigatus* to counteract some of the effects of the antifungal.

Protein release may be indicative of altered cell permeability and it appears the cell mounts an oxidative stress response to this by increasing the expression of stress response proteins and the elevated activity of a number of detoxifying enzymes.

The results presented here indicate that exposure to extract induced the release of a variety of proteins but that the fungus mounts a stress response to reduce the effect of the extract. Exposure of *A. fumigatus* to *Malva parviflora* extracts resulted in the elevated activity of catalase, glutathione reductase and superoxide dismutase which are associated with the oxidative stress response. Increased activity of these enzymes has also been observed in C. albicans exposed to caspofungin or hydrogen peroxide (6). It can be concluded from this work that *Malva parviflora* extracts results in the activation of an oxidative stress response in *A. fumigatus*. As shown in a previous study with caspofungin antifungal agent (10), this extract caused leakage of intracellular component from the *Aspergillu*s it may lead to changes in the membrane permeability or cell wall organization, which possibly results in the oxidative stress within the cell. Interestingly, another conclusion that can be made is that plant extract used in this study resulted in the leakage of proteins from different locations within the cell including cell wall, cytoplasm and mitochondria.

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