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Research Article

STUDY OF THE INTERACTION BETWEEN AFLATOXIN B1 AND FOSFOMYCIN USING MOLECULAR DOCKING AND *IN VITRO* CELL CULTURE SYSTEM

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ABSTRACT

The U.S. Food and Drug Administration (FDA) has not given any adsorbent product authorization to be used in the management of mycotoxicoses. Additional therapeutic agents are needed to mitigate the toxicity of aflatoxin B1 (AFB1) in animals because of the adverse effects of mycotoxin binders. Several apoptotic proteins, including bax, bcl-w, catalase, caspase 3, caspase 8, and fas L are associated with apoptosis induced by AFB1. The beneficial effects of fosfomycin in preventing cytotoxicity caused by xenobiotics are widely established. Therefore, it is important to draw attention in this context to the molecular docking analysis of fosfomycin and AFB1 with apoptotic proteins and to find out the protective effect of fosfomycin on AFB1-induced cytotoxicity in the Vero cell line. We described the molecular docking properties of fosfomycin and AFB1 with apoptotic proteins. Known mammalian simulative Vero cell lines were utilized for *in vitro* study in different treatment groups such as AFB1 alone, AFB1+fosfomycin, and AFB1+silymarin. Cytotoxicity was assessed by MTT assay, and Trypan blue dye exclusion technique. It was found that among two ligands, AFB1 interacted more positively with pro-apoptotic proteins (bax, catalase, caspase 3, caspase 8, and fas L) than with anti-apoptotic protein (bcl-w). Whereas fosfomycin showed better interaction with anti-apoptotic protein (bcl-w). 550 g/mL concentration of fosfomycin effectively protects the cytotoxicity induced by aflatoxin B1 using MTT assay, and Trypan blue dye exclusion technique. Fosfomycin primarily protects against cytotoxicity induced by AFB1 in Vero cells. Fosfomycin may protect AFB1-induced cytotoxicity by suppressing apoptotic proteins induced by AFB1. Further *in-vivo* and *in-vitro* gene expression studies are necessary to determine the exact mechanism by which fosfomycin protects against AFB1-induced cytotoxic effects.

Keywords: Aflatoxin B1, Apoptotic proteins, Fosfomycin, *In vitro* study, Molecular docking

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Both humans and animals suffer adverse health impacts from the fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. In a variety of animal species, including chicken, aflatoxin B1 (AFB1) causes hepatotoxicity, carcinogenicity, genotoxicity, immunosuppression, and other adverse effects (Richard, 2007; Rawal *et al.*, 2010). AFB1 treatment produced a significant decrease in total serum proteins, albumin, globulin, and significant increase in serum aspartate amino transaminase, alanine amino transaminase, lactate dehydrogenase and total serum bilirubin is the indication of AFB1-induced hepatotoxicity in broiler chickens (Pathak *et al.*, 2018). AFB1 intoxicated chickens showed over expression of the death receptors fas, tnfr1 and related genes as well as downregulation of the inhibitory apoptotic proteins XIAP and bcl-2 (Mughal *et al.*, 2017). Apoptosis induced by mitochondrial signalling pathways has been demonstrated by increased caspase-3/9 activation and bax expression in response to AFB1 therapy (Raj *et al.*, 2001). Following AFB1-induced splenocyte death, the mRNA expression of fas, fasL, tnf-r1, caspase-3, caspase-8, caspase-10, grp78, and grp94 increased (Zheng *et al.*, 2017).

Fosfomycin (FOS), also known as cis-1, 2-epoxyphosphonic acid, was first referred to as "phosphonomycin" (Hendlin *et al.*, 1969), is a naturally occurring, broad-spectrum antibiotic that is having bactericidal activity without being structurally associated to any other classes of antimicrobial drugs (Escolar *et al.*, 1998; Popovic *et al.*, 2010). FOS has been suggested for usage in both animals and humans due to its minimal toxicity and potential efficacy (Gallego *et al.*, 1974). According to Gobernado, FOS protects against nephrotoxicity caused by drugs like cisplatin, cyclosporine, aminoglycosides, vancomycin, teicoplanin, amphotericin B, and polymyxin (Gobernado, 2003). In addition, it had been investigated the preventive effect of FOS against aminoglycoside-induced ototoxicity in rats (Ohtani *et al.*, 1985) and protective effects on cell lines that had been exposed to deoxynivalenol (Gaudio *et al.*, 2016). Uncertainty surrounds the precise mechanism by which FOS protects cells against cytotoxicity caused by xenobiotics. Mycotoxin binders are often added to feed as a means of detoxifying feed contaminated with AFB1. Due to the adverse effects of mycotoxin binders, additional therapeutic agents are needed to mitigate the toxicity of AFB1 in animals. So repurposing FOS as a therapeutic agent for AFB1-induced cytotoxicity is a potential start. Therefore, the objective of the present study was to

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find out the interaction of FOS and AFB1 with various apoptotic proteins by molecular docking analysis and to find out the protective effect of FOS on AFB1-induced cytotoxicity in the Vero cell line.

MATERIALS AND METHODS

In Silico Study-Molecular docking analysis

Structures of bax, bcl-w, caspase 8, caspase 3, fas L, catalase: Three-dimensional structures of apoptotic proteins bcl-w (PDB id: 1O0L), bax (PDB id: 4S0O), caspase 8 (PDB id: 2C2Z), caspase 3 (PDB id: 3EDQ), fas L (PDB id: 4MSV), catalase (PDB id: 1QQW) were downloaded from protein data bank (PDB).

Ligand preparation: The structure of aflatoxin B1 (PubChem CID-186907) and fosfomycin (PubChem CID-446987) were downloaded in SDF format from the PubChem database. Using the Discovery Studio client software, it was converted into PDB format.

Molecular Docking Analysis: In order to find any possible interactions between bax, bcl-w, caspase 8, caspase 3, fas L, and catalase with aflatoxin B1 and fosfomycin, docking studies were carried out using the Discovery Studio client software.

In Vitro study- Cytotoxicity studies

Maintenance of cell lines

Vero cell lines were used in this work. Dulbecco's Modified Eagle Medium (DMEM) and 10% of fetal bovine serum (FBS) were used to culture cells in 25 cm^2-flasks. Fresh DMEM with 10% FBS was added to the medium at 48 hours to enable cell division to continue until a fully confluent monolayer had formed. Cells were then washed with Phosphate Buffer Saline (PBS) after discarding the medium. 0.1% Trypsin was added for 2-5 minutes at 37º C. Re-suspended detached cells were spun for five minutes at 1500 rpm in PBS. Re-suspending the cell mass in DMEM with 10% FBS before transferring it to fresh flasks.

Chemicals

Fosfomycin (FOS) and silymarin (SIL) were purchased from Subtle pharmaceuticals PVT. LTD. Bangalore, and Sigma-Aldrich respectively for this study. The National Fungal Culture Collection of India (NFCCI), Pune, provided the *Aspergillus parasiticus* culture that was used to extract the aflatoxin B1 (AFB1) as outlined by Shotwell *et al.* (1966). AFB1 (500 µg/mL) and SIL (1mg/mL) stock solutions were prepared in DMSO. FOS (1mg/mL) stock solution was prepared in Milli-Q water. All working solutions were prepared in DMEM media from their respective stock solutions.

Determination of cell viability and proliferation by MTT [3-(4, 5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide] assay

MTT assay for cell viability and proliferation was carried out according to the procedure given by Mosmann (1983). It is a rapid colorimetric assay based on the cleavage of the tetrazolium ring of MTT dye by dehydrogenase enzyme present in mitochondria of live cells into purple formazan crystals. Vero cells were seeded at a concentration of 1×10^5 cells per well in tissue culture grade 96 wells flat bottom microplates and after 24 h, cell monolayers were treated with FOS (400, 550, and 650 µg/mL) (Gaudio *et al.*, 2016) alone, for 48 hour incubation period to find out the cytotoxic effect of FOS. To find out the protective effect of FOS, cell monolayers were treated with AFB1 (30 µM) (Golli Bennour *et al.*, 2010), both alone and in combination with various FOS (400, 550, and 650 µg/mL) concentrations and also in combination with the well-known cytoprotective agent SIL (10 µg/mL) (Ledur and Santurio, 2020). The plates were incubated for 48 hour at 37º C in 5% CO_2 incubator. After the incubation period, the media containing toxin and drugs were completely removed and added 200 µl of DMEM media with 30 µl of MTT dye (0.5 mg/ml) to each well. The plates were incubated again at 37º C in 5% CO_2 incubator for 4 more hours, after which 100 µl of DMSO was added to each well. The wells were checked for the complete solubilization of purple formazan crystals and the absorbance of the wells were measured using ELISA reader at a wave length of 570 nm. The assay was performed in triplicate for each test group. The cell viability percentage of each experiment was calculated using the following formula. Cell viability (%) = {(O.D of test-O.D of blank)/(O.D of control-O.D of blank)}×100

Trypan blue dye exclusion technique

The dye exclusion technique is used to determine the number of viable and dead cells. It is based on the principle that viable cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, and propidium, whereas dead cells do not. Trypan blue dye exclusion technique in adherent cells was carried out according to the procedure given by Perry *et al.* (1997). Vero cells were seeded in cell culture grade 12 well plates containing coverslips in each well for cell adherence. Once the complete cell monolayer reached, treated with AFB1 (30 µM), both alone and in combination with various FOS (400, 550, and 650 µg/mL) concentrations, and also in combination with the well-known cytoprotective agent SIL (10 µg/mL). The plates were kept for incubation in an incubator with 5% CO_2 for 24 hours at 37º C. After incubation the media containing the drugs and AFB1 was

Table 1. Molecular docking results of aflatoxin B1 with apoptotic proteins

Proteins	LibDock Score	Binding energy (Kcal/mol)	Number of hydrogen bonds	Range of distance between hydrogen bonds
Catalase	125	0	9	1.7 to 3.0
Caspase 8	95	-17.9	4	2.5 to 2.8
Caspase 3	87	-54	3	2.6 to 2.9
Fas L	84	0	7	2.0 to 3.1
Bcl-W	94	71.4	11	2.3 to 3.1
Bax	69	20.9	2	1.8 to 2.5

Table 2. Molecular docking results of fosfomycin with apoptotic proteins

Proteins	LibDock Score	Binding energy (Kcal/mol)	Number of hydrogen bonds	Range of distance between hydrogen bonds
Caspase 8	67	0.1	13	1.8 to 3.5
Caspase 3	63	-1.7	9	1.6 to 2.8
Catalase	60	-30.5	7	2.1 to 3.1
Bcl-W	60	0	7	1.8 to 3.1
Fas L	58	0	7	1.8 to 2.9
Bax	52	0	4	2.2 to 2.7

Table 3. MTT assay results showing viability percentage of various concentrations of FOS after 48 hour of incubation

Groups	Cell viability percentage
Control	100.08±6.73
FOS (400 µg/ml)	96.54± 5.77
FOS (550 µg/ml)	92.72± 5.76
FOS (650 µg/ml)	63.86± 5.68*

Results are presented as mean± SEM of three independent experiments. *p<0.05 compared with control group, SEM-Standard error of mean.

discarded, and any remaining serum was removed by washing with phosphate-buffered saline (PBS) for three times. Following that, 0.2% trypan blue solution was added and kept for 1 min. Trypan blue was removed after one minute of incubation and fixed with 4% paraformaldehyde (PFA), pH 7.5, for 10 min at 20°-22° C. After fixation, washed with PBS until the PBS is clear of residual blue color. Gently lift the coverslips to free trypan blue from underneath and mount the coverslips onto glass slides with a compatible permanent mounting solution such as DPX. The percentage of dead cells was evaluated using the given formula. Percentage of dead cells= (Dead cell count/Total cell count) ×100. The assay was performed in triplicate for each test group. Viable cells with clear cytoplasm, whereas dead cells exhibit blue cytoplasm when visualized under

Table 4. MTT assay results showing viability percentage of AFB1 alone and in combination with FOS after 48 hour of incubation

Groups	Cell viability percentage
Control	100.08±6.73
AFB1 (30 µM)	9.46±1.02*
AFB1+SIL (10 µg/ml)	29.71±2.53*#
AFB1+FOS (400 µg/ml)	25.15± 2.36*
AFB1+FOS (550 µg/ml)	26.74± 2.55*#
AFB1+FOS (650 µg/ml)	20.85± 1.24*

Results are presented as mean± SEM of three independent experiments. *p<0.05 compared with control group, #p<0.05 compared with AFB1 group, SEM-Standard error of mean.

Table 5. Trypan blue dye exclusion technique showing the dead cells percentage of AFB1 alone and in combination with FOS after 24 hour of incubation

Groups	Percentage of dead cells
Control	12.00±2.31
AFB1 (30 µM)	82.66±4.81*
AFB1+SIL (10 µg/ml)	46.66±4.81*#
AFB1+FOS (400 µg/ml)	37.33±5.33*#
AFB1+FOS (550 µg/ml)	34.66±3.53*#
AFB1+FOS (650 µg/ml)	58.66±3.53*#

Results are presented as mean± SEM of three independent experiments. *p<0.05 compared with control group, #p<0.05 compared with AFB1 group, SEM-Standard error of mean.

inverted light microscope (20x).

Statistical analysis

The data generated from different parameters of the experimental study were subjected to one-way analysis of variance (ANOVA) test using GraphPad Prism software. Results are expressed as mean ± SEM performed in the triplicate experiment. Statistical significance was taken into account when p 0.05.

RESULTS AND DISCUSSION

Molecular docking analysis

Table 1 and 2 represents the molecular docking analysis and intermolecular interactions between AFB1 and apoptotic proteins as well as FOS with the same proteins, respectively. The best interaction between two ligands and apoptotic proteins is the one with the highest LibDock score while consuming the least amount of binding energy. If the energy value is low, these docked molecules tend to be highly stable. The contact between the ligand and proteins is stronger and results in the activation of proteins as their negative binding energy value increases. Kcal/mol was used to calculate the binding energy.

All proteins (bax, caspase 8, caspase 3, fas L, and

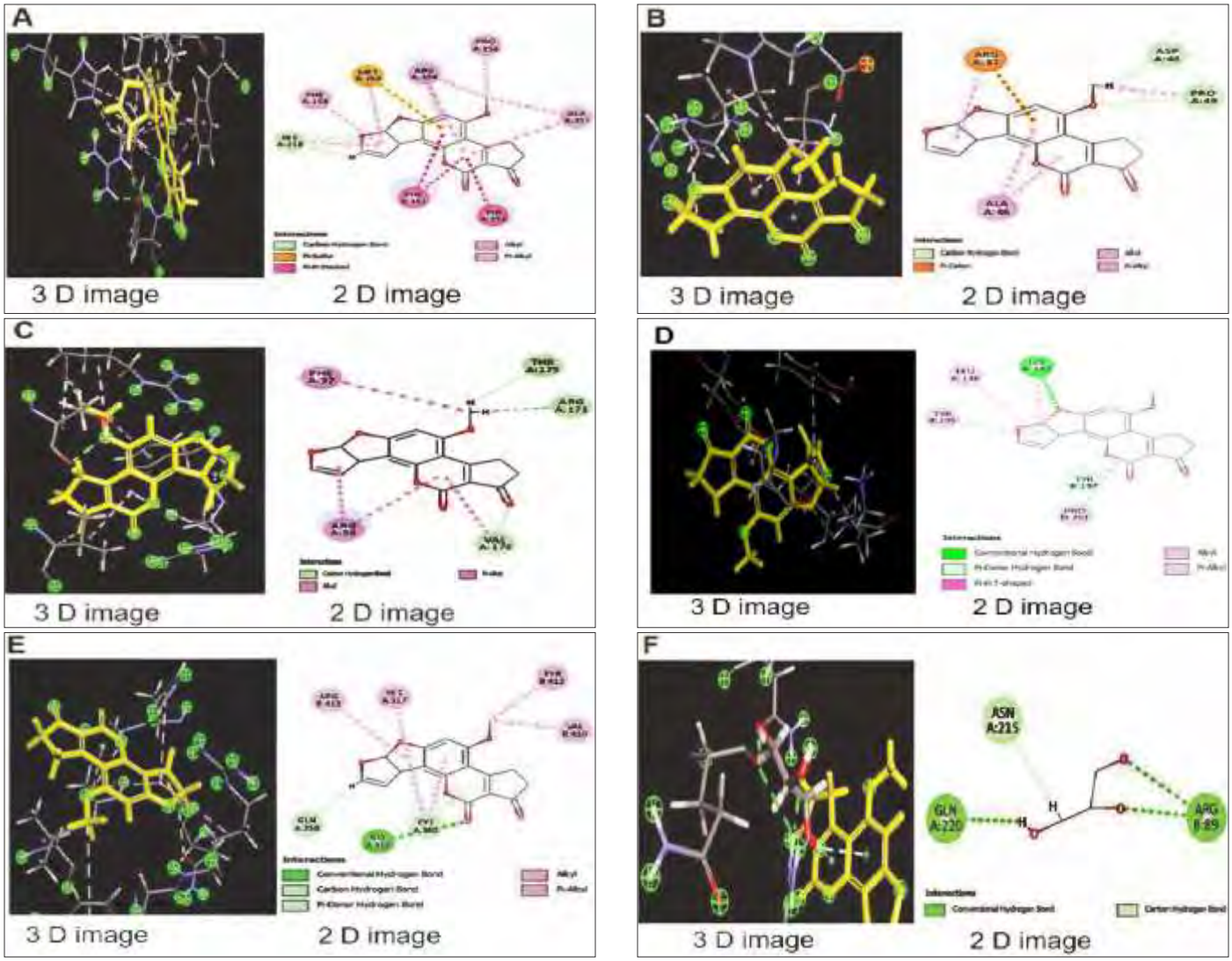
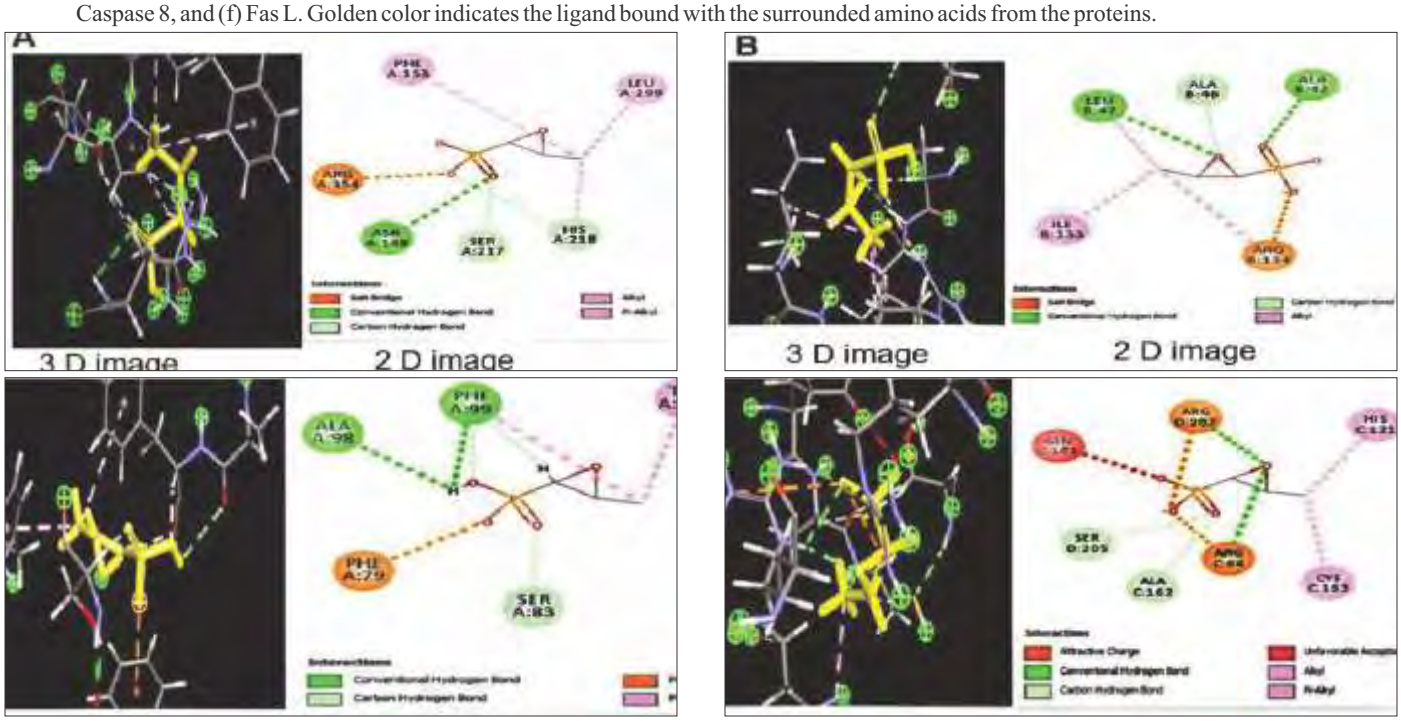


Fig. 1. Molecular docking binding features of aflatoxin B1 with apoptotic proteins such as (a) Catalase, (b) Bax, (c) Bcl-W, (d) Caspase 3, (e) Caspase 8, and (f) Fas L. Golden color indicates the ligand bound with the surrounded amino acids from the proteins.



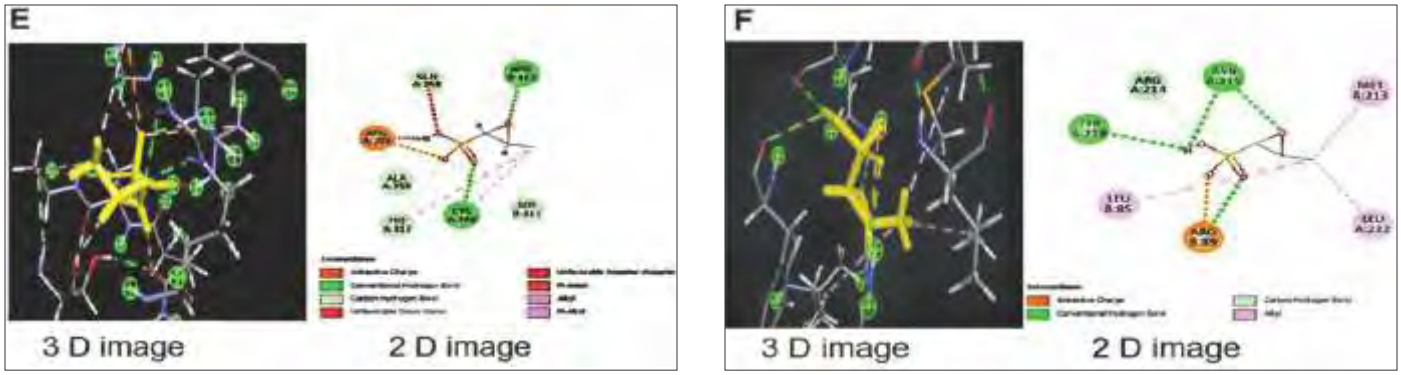


Fig. 2. Molecular docking binding features of fosfomycin with apoptotic proteins such as (a) Catalase, (b) Bax, (c) Bcl-W, (d) Caspase 3, (e) Caspase 8, and (f) Fas L. Golden color indicates the ligand bound with the surrounded amino acids from the proteins.

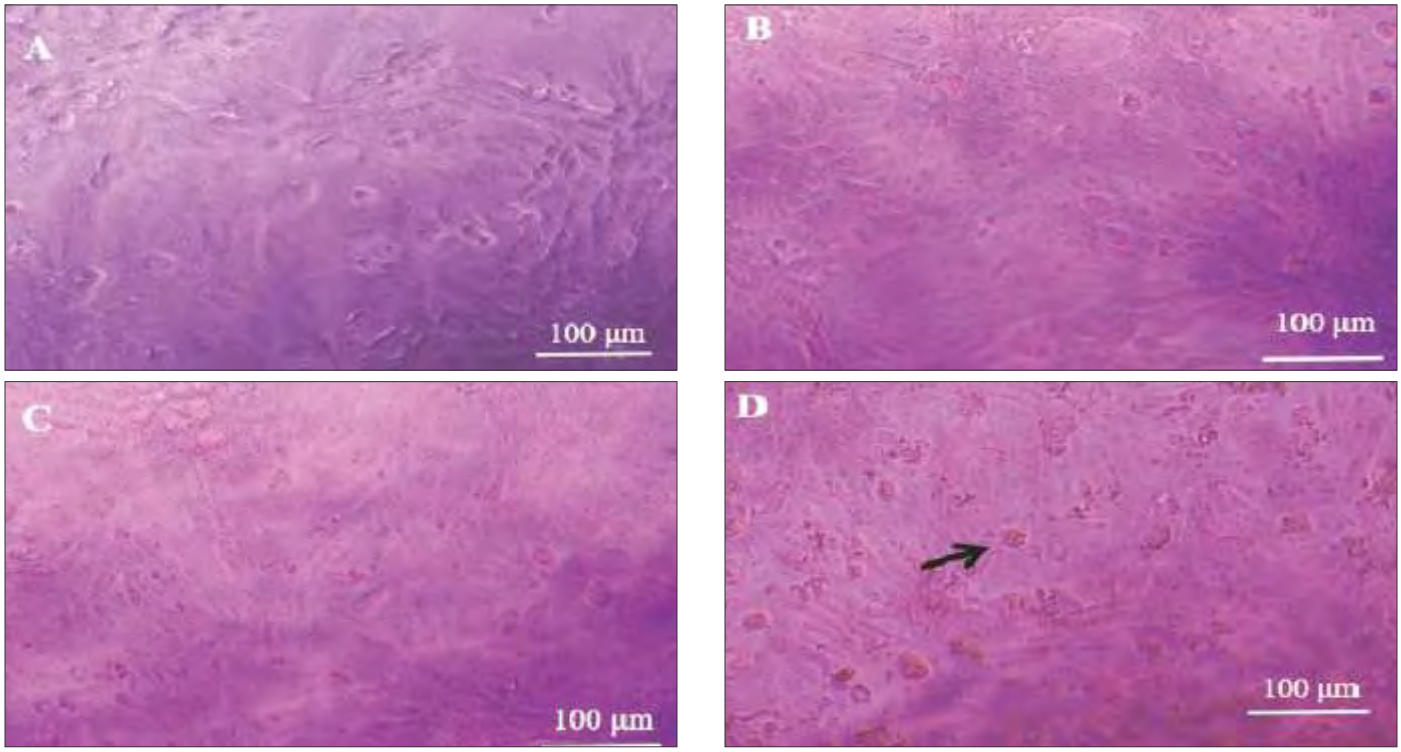


Fig. 3. Images of Vero cell line after 48 hour incubation of various FOS concentrations: A) Control; B) FOS (400 g/ml); C) FOS (550 g/ml); D) FOS (650 g/ml) (20x). Arrow indicates cells with altered morphology (20x).

catalase) except bcl-w showed the highest LibdDock score and negative binding energy value when docked with AFB1 (Table 1) than that of FOS (Table 2), indicating that ligand AFB1 strongly binds with pro-apoptotic proteins than that of FOS. FOS exhibited a LibDock score of 60 in the presence of bcl-w, which is the minimum score needed for a positive interaction, however, AFB1 demonstrated a LibDock score of 94 by utilizing 71.4 kcal/mol energy, which is an extremely high amount. Although AFB1 has a high LibDock score, FOS consumes much lesser energy (0 kcal/mol) compared to AFB1 during the interaction. FOS thus showed a greater affinity with the bcl-w protein than AFB1. Thus, it is shown that FOS interacted better with anti-apoptotic protein (bcl-w) whereas AFB1 showed better interaction with pro-apoptotic proteins. The interactions between proteins and ligands are also depicted in three and two-dimensional images (Figs. 1 and 2). To find out the protective effect of FOS on AFB1-induced cytotoxicity, further *in vitro* studies were conducted in Vero cell line.

MTT [3-(4, 5-dimethylthazolk-2-yl)-2, 5-diphenyl tetrazolium bromide] assay

In comparison to control (untreated) group, the results of MTT assay showed a significant decrease in the cell viability percentage after incubated with FOS at the concentrations of 650 µg/mL only, whereas no significant changes in viability percentage at the concentration of 550 and 400 µg/mL (Table 3). In comparison to the AFB1 (30 µM) alone treated group, the results of MTT assay showed a significant increase in the cell viability percentage when cell culture was incubated with the combination of AFB1 and FOS at the concentration of 550 µg/mL, which is

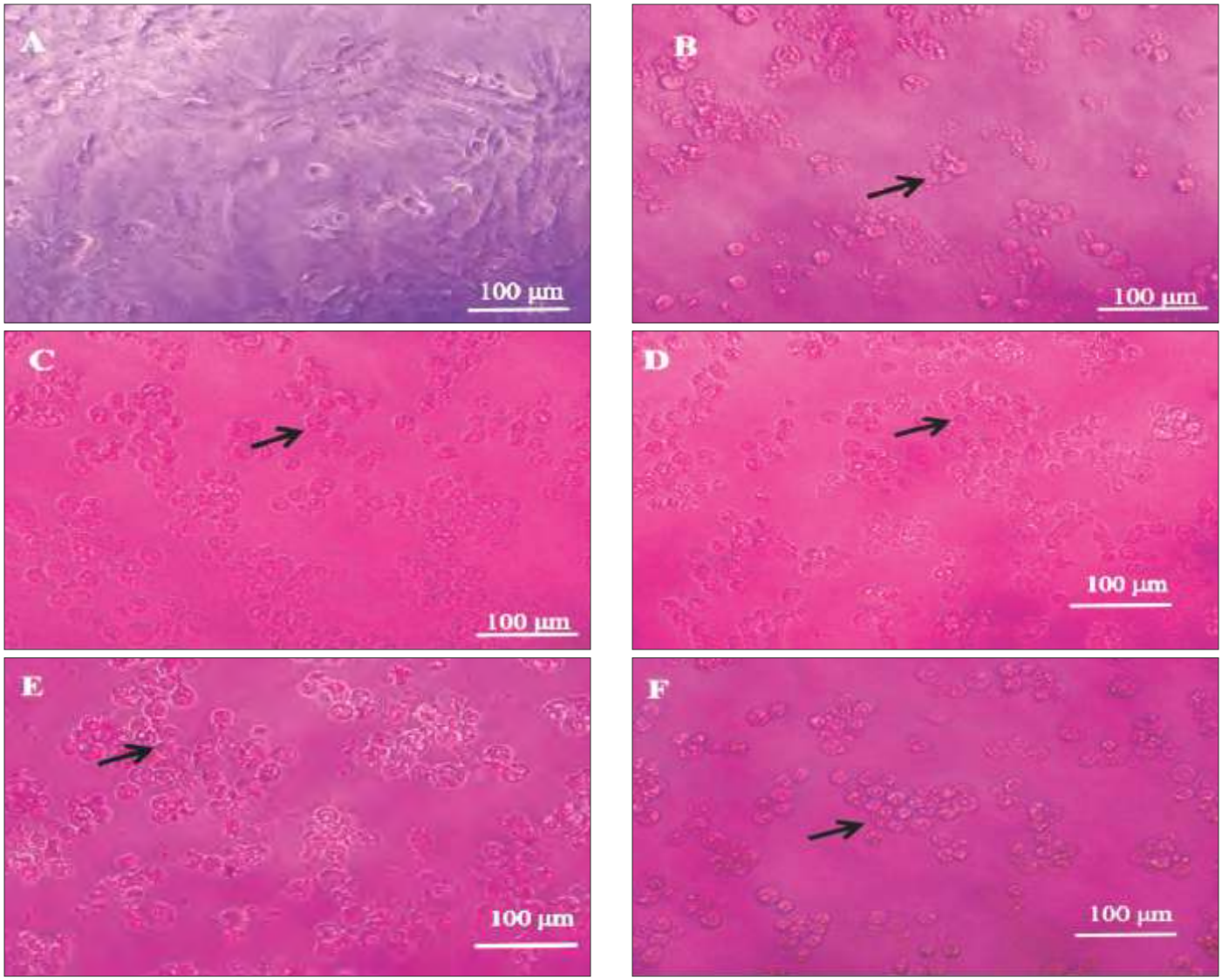


Fig. 4. Images of Vero cell line after 48 hour incubation of AFB1 alone and in combination with FOS: A) Control; B) AFB1; C) AFB1+SIL; D) AFB1+FOS (400 g/ml); E) AFB1+FOS (550 g/ml); F) AFB1+FOS (650 g/ml) (20x). Arrows indicate cells with altered morphology (20x).

comparable to the cell viability percentage exhibited by AFB1+SIL group, whereas FOS increased viability percentage at the concentrations of 400 µg/mL and 650 µg/mL also, but statistically not significant (Table 4). AFB1 alone treated group showed a substantial drop in the cell viability percentage when compared to the control group (Table 4). As shown in Fig. 3, a significant changes in cell morphology (shape of cells) was observed in cell line exposed to FOS at 650 µg/mL concentration, while this changes was less pronounced in the control group and other concentrations of FOS. Fig. 4 represents a significant reduction in cell monolayer with altered morphology observed in cell line exposed to AFB1 compared to control group, while this reduction was less pronounced in FOS+AFB1 and SIL+AFB1 groups.

Trypan blue dye exclusion technique

In comparison to the AFB1 (30 M) treated group, the trypan blue dye exclusion technique results showed a significant decrease ($p<0.05$) in the percentage of dead cells when cell culture was incubated with the combination of AFB1 and FOS at the all concentrations, which is comparable to the effect showed of SIL. Among them 550 µg/mL concentration of FOS showed a highest reduction in percentage of dead cells (Table 5). When compared to the control group, the AFB1-treated group showed a significant increase in percentage of dead cells (Table 5). As shown in Fig. 5, the number of dead cells with dark blue cytoplasm were significantly higher in the cell line exposed to AFB1 compared to the control group, whereas fewer dead cells were detected in FOS+AFB1 and SIL+AFB1 groups.

According to molecular docking studies, apoptosis induced by AFB1 may be due to its good interaction with pro-apoptotic proteins like bax, caspase 8, caspase 3, fas L, catalase, and poor interaction with anti-apoptotic protein bcl-w. The current in silico study is in accordance with the

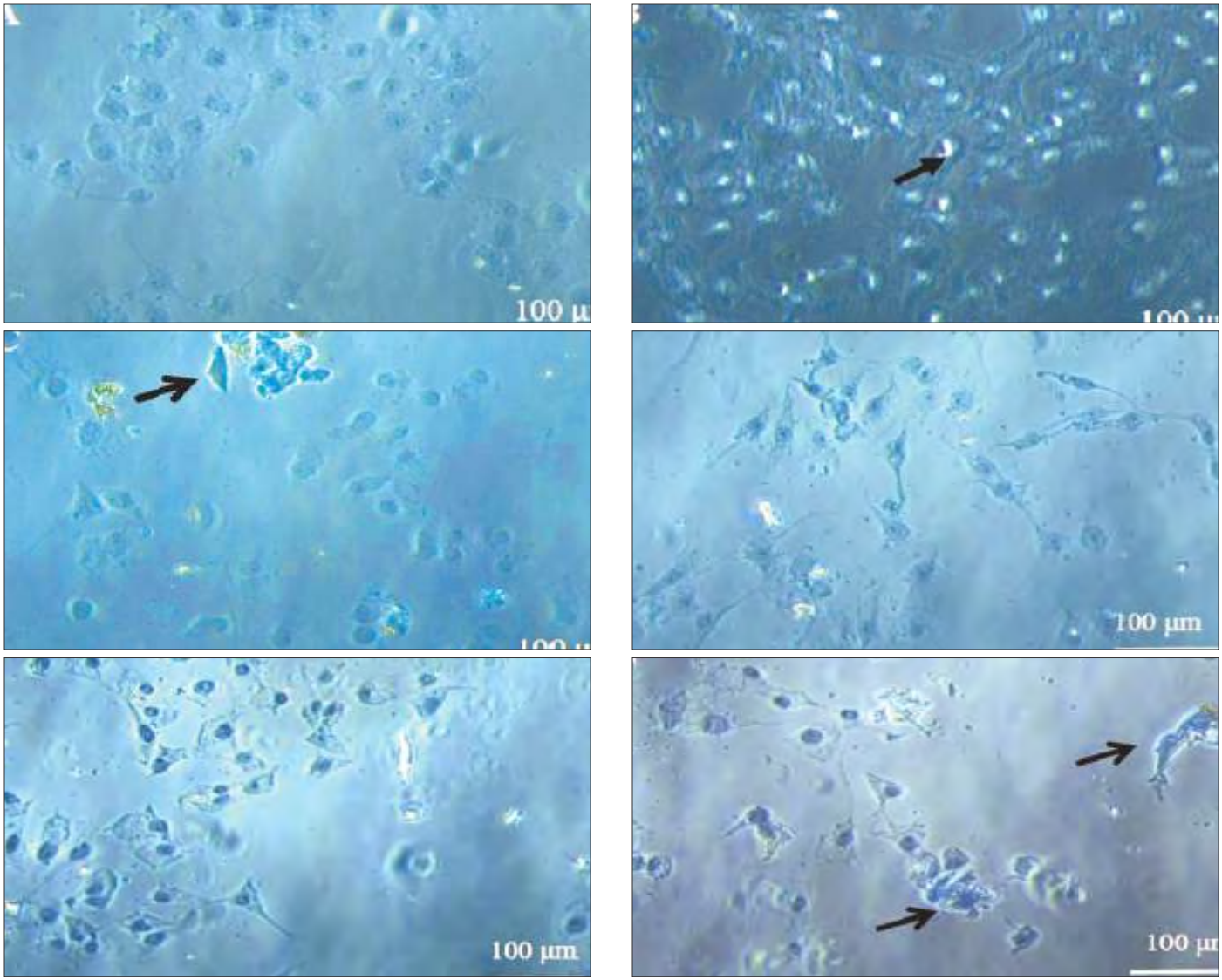


Fig. 5. Images of trypan blue dye exclusion technique showing dead cells treated with AFB1 alone and in combination with FOS: A) Control; B) AFB1; C) AFB1+SIL; D) AFB1+FOS (400 g/ml); E) AFB1+FOS (550 g/ml); F) AFB1+FOS (650 g/ml) (20x). Arrows indicate dead cells with dark blue colored cytoplasm (20x).

results demonstrated by Zheng *et al.* (2017) and Mughal *et al.* (2017). Zheng *et al.* (2017) reported that the mRNA expression of fas, fasL, TNF-R1, caspase-3, caspase-8, caspase-10, grp78, and grp94 increased following AFB1-induced splenocyte apoptosis. Mughal *et al.* (2017) reported that AFB1 intoxicated chickens showed overexpression of the death receptors fas, tnfr1 and related genes as well as downregulation of the inhibitory apoptotic proteins XIAP and bcl-2 (Mughal *et al.*, 2017). Increased caspase-3/9 activation and bax expression in response to AFB1 therapy are indicative of apoptosis brought on by the mitochondrial signalling pathways (Raj *et al.*, 2001) is also in agreement with current *in silico* study.

The current in silico study revealed that FOS may have a protective effect due to its poor interaction with pro-apoptotic proteins such as bax, caspase 8, caspase 3, fas L, and catalase and its better interaction with bcl-w (anti-apoptotic protein). The current study is in agreement with the study conducted by Kadota *et al.* (2005). He reported that FOS had no role in induction of apoptosis in human-activated peripheral lymphocytes. Gaudio *et al.* (2020) demonstrated that FOS at the concentration of 580 µg/mL significantly reduced the nuclear changes suggestive of apoptosis in intestinal cells induced by DON (2.8 µg/mL) mycotoxin is also supporting the current study.

To find out the protective effect of FOS on AFB1-induced cytotoxicity, further *in vitro* studies were conducted in Vero cell line. The results from MTT assay and trypan blue dye exclusion technique revealed that the maximum viability percentage of FOS was determined atthe concentration of 550 µg/mL against AFB1-induced cytotoxicity. 650 µg/mL concentration of FOS was cytotoxic, whereas 400 and 550 µg/mL concentrations were safe. FOS enhances cell viability percentage in a

manner similar to that of the effect showed by SIL, which has a proven protective effect against mycotoxin-induced cytotoxicity (Rastogi *et al.*, 2001; Ledur and Santurio, 2020). Therefore, it is clear that FOS protects cells from cytotoxicity caused by AFB1.

The current study is in agreement with the results demonstrated by Gaudio *et al.* (2016). He demonstrated that FOS at the concentrations of 400 µg/mL and 550 µg/mL provided 70% and 100% protection, respectively, against doses of 2.8 µg/mL deoxynivalenol with the help of TUNEL and DAPI staining techniques. Additionally, the protective effect of FOS against aminoglycoside-induced ototoxicity in rats was examined (Ohtani *et al.*, 1985; Yanagida *et al.*, 2004). An *et al.* (2019) reported the protective effect of FOS against *Staphylococcus aureus* infection *in vitro* and *in vivo* by reducing the phosphorylation levels of mitogen-activated protein kinases (ERK and p38) and expression of NLRP3 inflammasome related proteins and also he showed that the FOS inhibited the expression of mitogen-activated protein kinases are mainly responsible for the protective effect of FOS against *Staphylococcus aureus* infection.

According to Gobernado, (2003), FOS protects against nephrotoxicity brought on by drugs like cisplatin, cyclosporine, aminoglycosides, vancomycin, teicoplanin, amphotericin B, and polymyxin. The renoprotective effect of FOS in the treatment of pulmonary exacerbations in cystic fibrosis was studied, where the FOS reduces acute renal injury caused by intravenous administration of aminoglycoside in cystic fibrosis pulmonary exacerbations (Al-Aloul *et al.*, 2019). Umeki *et al.* (1988) studied the protective effects of FOS on the nephrotoxicity induced by cisplatin in lung cancer patients by inhibiting urinary N-acetyl- -D-glucosaminidase level after the anticancer therapy of cisplatin. Inouye *et al.* (1982) reported the renoprotective effect of FOS by reducing polyuria, proteinuria, and enzymuria induced by dibekacin. All these previous studies suggested that FOS is having a protective role against cytotoxicity induced by various xenobiotics, predominantly renoprotective effect, and these studies support the present study where utilized Vero cells for *in vitro* studies are also renal cells.

CONCLUSION

FOS primarily protects against cytotoxicity induced by AFB1 in Vero cells. The maximum viability percentage of FOS was determined at the concentration of 550 µg/mL against AFB1 induced cytotoxicity. FOS may protect AFB1-induced cytotoxicity by suppressing apoptotic proteins induced by AFB1. However, further *in vivo* and *in vitro* gene expression studies are warranted to confer the involvement of apoptotic genes in the protective effect of FOS on AFB1-induced cytotoxicity.

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