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Research Article

IN SILICO AND *IN VITRO* EVALUATION OF ENROFLOXACIN ON AFLATOXIN B1-INDUCED CYTOTOXICITY

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ABSTRACT: Additional therapeutics are required to minimize the toxicity of aflatoxin B1 (AFB1) in animals due to the drawbacks of mycotoxin binders. The cytochrome P450 enzyme system is necessary for the metabolic activation of AFB1 before it produces the cytotoxic AFB1-exo-8, 9-epoxide (AFBO). It is already proven that enrofloxacin (ENR) has been shown to substantially decrease the activity of the cytochrome P450 enzymes. To understand how ENR affects AFB1-induced cytotoxicity, it is important to highlight this context. In this present study, molecular docking was performed between ENR with CYP3A4 protein, apoptotic proteins (Bax, caspase 3, caspase 8, fas L, MAPK1), and catalase. To assess the effect of ENR on AFB1-induced cytotoxicity by MTT assay and trypan blue dye exclusion techniques, the mammalian simulative Vero cell lines were used in different treatment groups as AFB1 alone, ENR alone, AFB1+ENR, and AFB1+silymarin (known cell protective agent). This was done to further evaluate the *in silico* analysis. ENR interacted more positively with all of the proteins (CYP3A4, Bax, catalase, caspase 3, caspase 8, MAPK1, and fas L), according to the findings of molecular docking studies. On the other hand, cytotoxicity caused by AFB1 is successfully prevented by ENR at a dose of 25 µg/mL. The results of the present study suggest that ENR primarily protects against cytotoxicity induced by AFB1 in Vero cells as the evidence of *in silico* studies revealed that ENR may protect AFB1-induced cytotoxicity by interacting with the CYP3A4 enzyme which is primarily required for activation AFB1.

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

INTRODUCTION

Aflatoxin B1 (AFB1) is the most physiologically active form of the several aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* and is detrimental to both animal and human health when consumed over a long period in low concentrations. In many animal species, including chickens, AFB1 is the root cause of the development of hepatotoxicity, carcinogenicity, genotoxicity, immunosuppression, and other detrimental effects [1,2]. Chickens administered AFB1 showed upregulation of death receptors like fas, tnfr1, and related genes in addition to downregulation of the inhibitory apoptotic proteins XIAP and bcl-2 [3]. AFB1 therapy-induced increases in caspase-3/9 activation and Bax expression suggest that mitochondrial signaling pathways trigger apoptosis [4,5]. After AFB1-induced splenocyte death, there was an increase in the mRNA expression of fas, fasL, tnfr1, caspase-3, caspase-8, caspase-10, grp78, and grp94 [6]. AFB1 exposure may affect the tissue residues of many antibiotics that are mostly metabolized in the liver since it has been demonstrated to have an inhibitory impact on hepatic cytochrome P450 monooxygenases [7,8].

The fluorinated quinolone derivative enrofloxacin (ENR) exhibits bactericidal activity in a concentration-dependent manner [9]. Due to its favorable pharmacokinetic properties, ENR has an effective

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antimicrobial activity in chickens. Therefore, ENR has been authorized for use in poultry to treat respiratory and digestive diseases like colibacillosis, pasteurellosis, and mycoplasmal infection [10]. It is also extensively used in herbivorous animals [11]. Cytochrome P450 enzymes are known to be essential for the biotransformation of xenobiotics. Previous studies reported that ENR significantly inhibits the cytochrome P450 enzymes [12, 13, 14].

Binders or sequestering agents are routinely used to inactivate feed that has been contaminated with AFB1. Inedible adsorbents including silicates, activated carbon, complex polysaccharides, and others are examples of mycotoxin binders. No adsorbent material has received approval from the U.S. Food and Drug Administration (US FDA) to be used for the management of mycotoxicoses [15]. Because clay-based binders may contain heavy metals and dioxins, which are hazardous to animals and may cause serious adverse effects, the U.S. Food and Drug Administration (FDA) has prohibited them [16]. Due to the drawbacks of mycotoxin binders, additional treatments are needed to reduce the toxicity of AFB1 in animals. Repurposing ENR as a treatment for AFB1-induced cytotoxicity is therefore a potential initial step. The objective of this research was to determine, via molecular docking, how ENR interacts with CYP3A4, apoptotic proteins (Bax, caspase 3, caspase 8, fas L, MAPK1), and catalase proteins in AFB1-induced cell death and also to assess whether the ENR has a protective effect on the cell line exposed to AFB1.

MATERIALS AND METHODS

Molecular Docking Analysis

Bax, caspase 8, caspase 3, fas L, catalase, CYP3A4 and MAPK1 structures

Protein data bank (PDB) was used to download the three-dimensional structures of proteins, including fas L (PDB id: 4MSV), catalase (PDB id: 1QQW), CYP3A4 (PDB id: 1WOF), Bax (PDB id: 4S0O), caspase 8 (PDB id: 2C2Z), caspase 3 (PDB id: 3EDQ), and MAPK1 (PDB id: 1WZY).

Ligand preparation

From the PubChem database, the ENR structure (PubChem CID-71188) was downloaded in SDF format. utilizing a 2020 version of the Discovery Studio client program that has been converted to PDB format.

Docking steps

Using the Discovery Studio client software, docking studies were carried out to determine potential interactions between ENR and Bax, caspase 8, caspase 3, fas L, catalase, MAPK1, and CYP3A4. The target proteins and ligand molecules were imported and the water molecules in proteins were deleted except from the active site to avoid disturbances during molecular docking. Both protein and ligand were prepared using Discovery Studio client software and receptor cavities or PDB site records of target proteins were detected. Deleted the existing ligand groups from proteins before docking. Docking was done by selecting the ligand against the receptor site of various target proteins. At a time only one active site was selected for docking if more than two active sites were detected in a protein. Among all the docked poses, a better pose was selected.

Maintenance of cell lines

In 25 cm^2 flasks, the Vero cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were sub-cultured with 0.1% Trypsin once they formed the monolayer and maintained with DMEM with 10% FBS until cytotoxicity studies were carried out.

Chemicals

For this investigation, we purchased silymarin (SIL) and enrofloxacin (ENR) from Sigma-Aldrich. Aflatoxin B1 (AFB1) was extracted using *Aspergillus parasiticus* culture, which was purchased from the National Fungal Culture Collection of India (NFCCI), Pune. Stock solutions for AFB1 (500 µg/ml), ENR (3 mg/ml), and SIL (1 mg/mL) were prepared in DMSO. Each working solution was prepared using the corresponding stock solution in DMEM media.

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

To determine the cytotoxic effect of ENR, Vero cells were seeded in tissue culture grade 96 well flat bottom microplates. After 24 hours, cell monolayers were treated with ENR (25, 50, and 100 µg/mL) [17] alone for 48 hours of incubation. Cell monolayers were treated with AFB1 (30 µM) [18] to determine the protective effect of ENR. This was carried out both alone and in combination with different concentrations of ENR (25, 50, and 100 µg/mL), as well as with the well-known cytoprotective agent SIL (10 µg/mL). The plates were incubated in an incubator with 5% CO_2 for 48 hours at 37ºC. The MTT assay was performed for cell viability and proliferation

following the protocol provided by Mosmann (1983)[19]. Three independent experiments were performed for every test group. The following formula was used to determine each experiment's cell viability percentage.

Cell viability (%) = [(O.D of test - O.D of blank) / (O.D of control-O.D of blank)]×100.

Trypan blue dye exclusion test

To investigate the impact of ENR on AFB1-induced cell death, Vero cells were grown in 12-well cell culture grade plates equipped with coverslips in each well for cell adherence. ENR and AFB1 were applied to the plates in the same way as for the MTT procedure. The plates were incubated for 24 hours at 37°C in an incubator with 5% CO_2. Media containing AFB1 and ENR was discarded. After treated with 0.2% trypan blue solution for one minute and then removed. It was then fixed for ten minutes at 20°-22°C using 4% paraformaldehyde (PFA) of pH 7.5. The adherent cell protocol was carried out by the guidelines given by Perry *et al.* (1997) [20]. The following formula was used to determine the percentage of dead cells.

Percentage of dead cells = (Dead cell count/Total cell count) × 100.

Statistical analysis

All experiments were performed in triplicate. Results obtained from the experiments are shown as mean ± SEM. GraphPad Prism was used to perform a one-way analysis of variance (ANOVA) test on the data generated from various studies. Statistical significance was considered when $p \leq 0.05$ and $p \leq 0.01$.

RESULTS AND DISCUSSION

In silico molecular docking analysis

The potential interactions of ENR with the AFB1-induced cytotoxicity markers such as CYP3A4, Bax, caspase 8, caspase 3, fas L, MAPK1, and catalase were investigated using molecular docking analysis. The LibDock score, binding energy, number of hydrogen bonds involved in the interaction, and range of hydrogen bond lengths are all presented in Table 1. A more positive ligand-protein interaction has a high LibDock score (above 60) while using the least amount of binding energy. These docked molecules exhibit a high degree of stability when the energy value is low. The interaction between the ligand and protein is stronger and enables proteins to be activated as their negative binding energy value increases. All proteins (Bax, caspase 8, caspase 3, fas L, MAPK1, CYP3A4, catalase) revealed a high LibDock score and lesser binding energy value when docked with ENR (Table 1), indicating that ligand ENR strongly binds with apoptotic proteins (Bax, caspase 8, caspase 3, fas L, MAPK1), catalase and CYP3A4. The interactions between proteins and ligands are depicted in three- and two-dimensional images (Fig. 1).

Molecular docking analysis revealed that the ENR had strong interactions with apoptotic proteins such as Bax, caspase 8, caspase 3, fas L, and MAPK1. According to the study conducted by Ding *et al.* (2022) [21], ENR causes cytotoxicity in loach fin cells by inducing apoptosis that is mediated by caspase 3 and caspase 8 pathways, which is following the present study and they also reported that the ENR produced a dose-dependent inhibitory effect on catalase activity in loach cells, which is in agreement with the present study that the ENR had strong interaction with catalase.

The cytochrome P450 enzyme system, mainly CYP3A4 is necessary for the metabolic activation of AFB1 into cytotoxic and carcinogenic AFB1-exo-8, 9-epoxide (AFBO) [22]. The abundance of epoxides and other ROS generated by the buildup of AFBO also depletes GSH, which in turn causes apoptosis, cytotoxicity, and genotoxicity in cells [22]. Molecular docking analysis revealed that the ENR has a higher interaction with CYP3A4 protein. It has been reported that ENR substantially decreases the activity of the cytochrome P450 enzymes [12,13,14], which is primarily required for the activation of AFB1 and is thus one of the possible ways of preventive mechanism of ENR on AFB1-induced cell toxicity. To find out the protective effect of ENR on AFB1-induced cytotoxicity, further *in vitro* studies were conducted in the Vero cell line.

MTT assay

The MTT assay results revealed that when cells were incubated with ENR at concentrations of 50 µg/mL and 100 µg/mL, there was a significant decrease in the cell viability percentage compared to the control (untreated) group; however, no significant changes were observed in the viability percentage at the concentration of 25 µg/mL (Table 2). When the cell culture was incubated with the combination of AFB1 and ENR at the concentrations of 25 and 50 µg/mL, the MTT assay results showed a significant increase in the cell viability percentage compared to the AFB1 (30 µM) alone treated group. This increase was comparable to the cell viability percentage exhibited

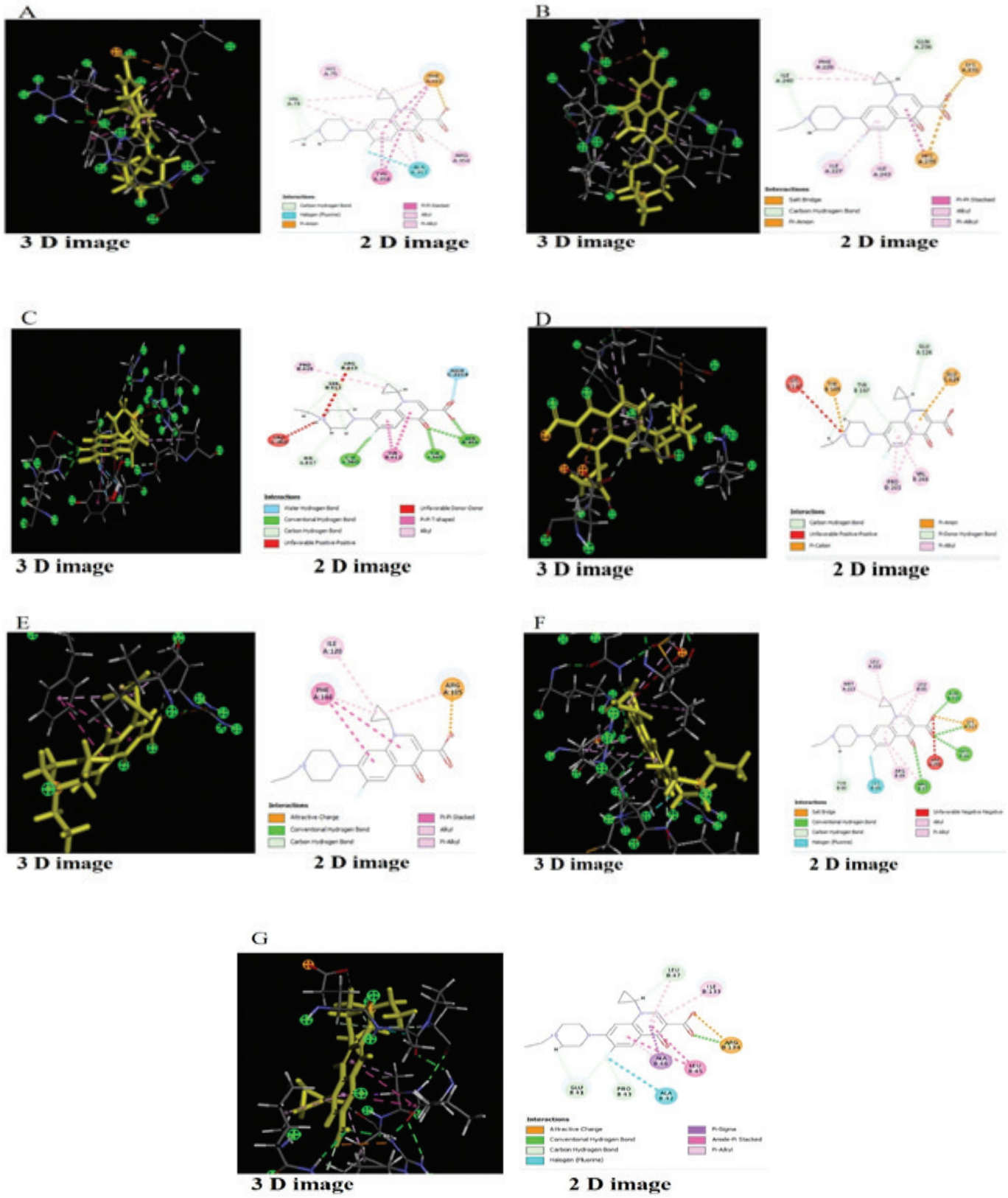

Fig. 1. Molecular docking binding features of ENR. [With (A) Catalase, (E) CYP 3A4 and apoptotic proteins such as (B) MAPK 1, (C) Caspase 8, (D) Caspase 3, (F) Fas L, (G) Bax. The ligand bound to the surrounding amino acids from the proteins is indicated by the golden color].

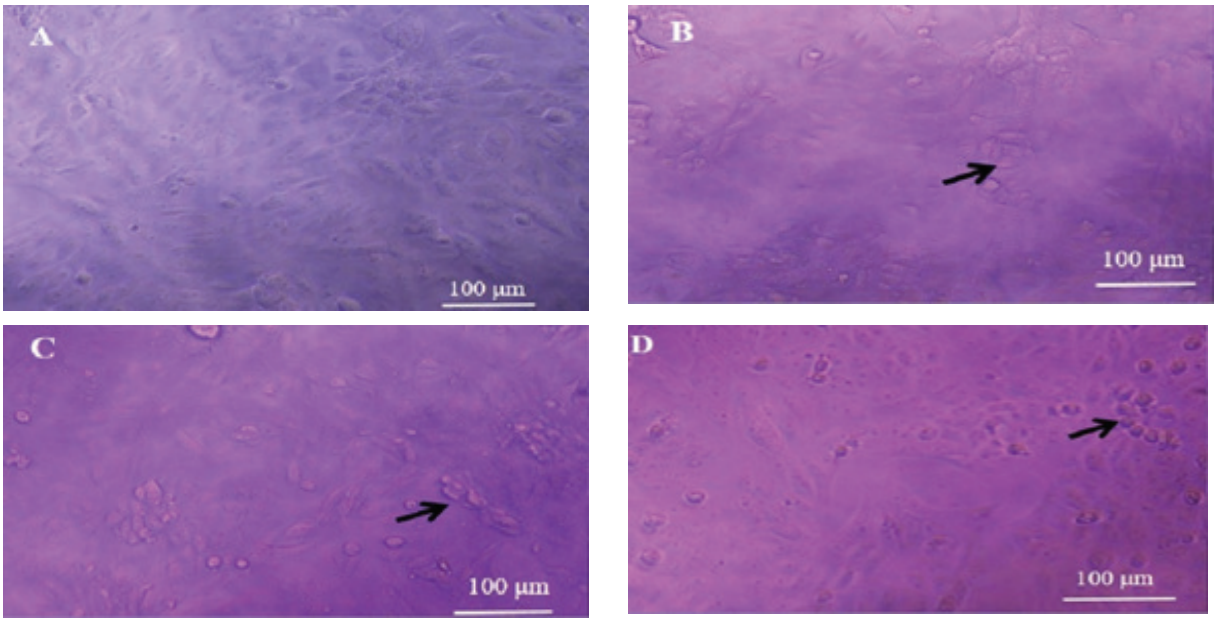

Fig. 2. Cytopathic effect of different concentrations of ENR after 48 hour of incubation. [A) Control; B) ENR (25 µg/ml); C) ENR (50 µg/ml); D) ENR (100 µg/ml). Changed morphology of the cells is indicated by arrows (20X)].

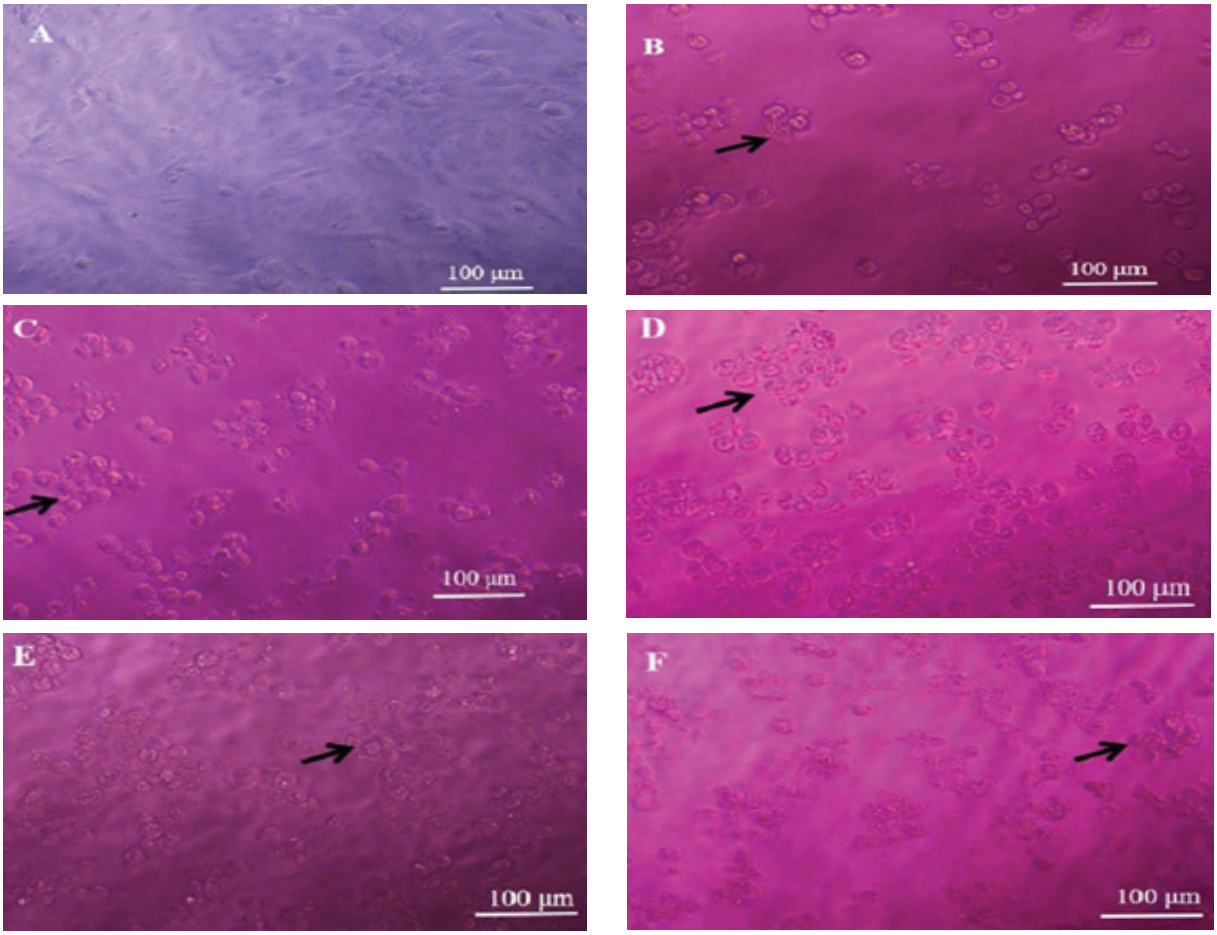

Fig. 3. Cytopathic effect of AFB1 after 48 hours of incubation, both alone and in combination with ENR. [A) Control; B) AFB1; C) AFB1+SIL; D) AFB1+ENR (25 µg/ml); E) AFB1+ENR (50 µg/ml); F) AFB1+ENR (100 µg/ml). Changed morphology of the cells is indicated by arrows (20X)].

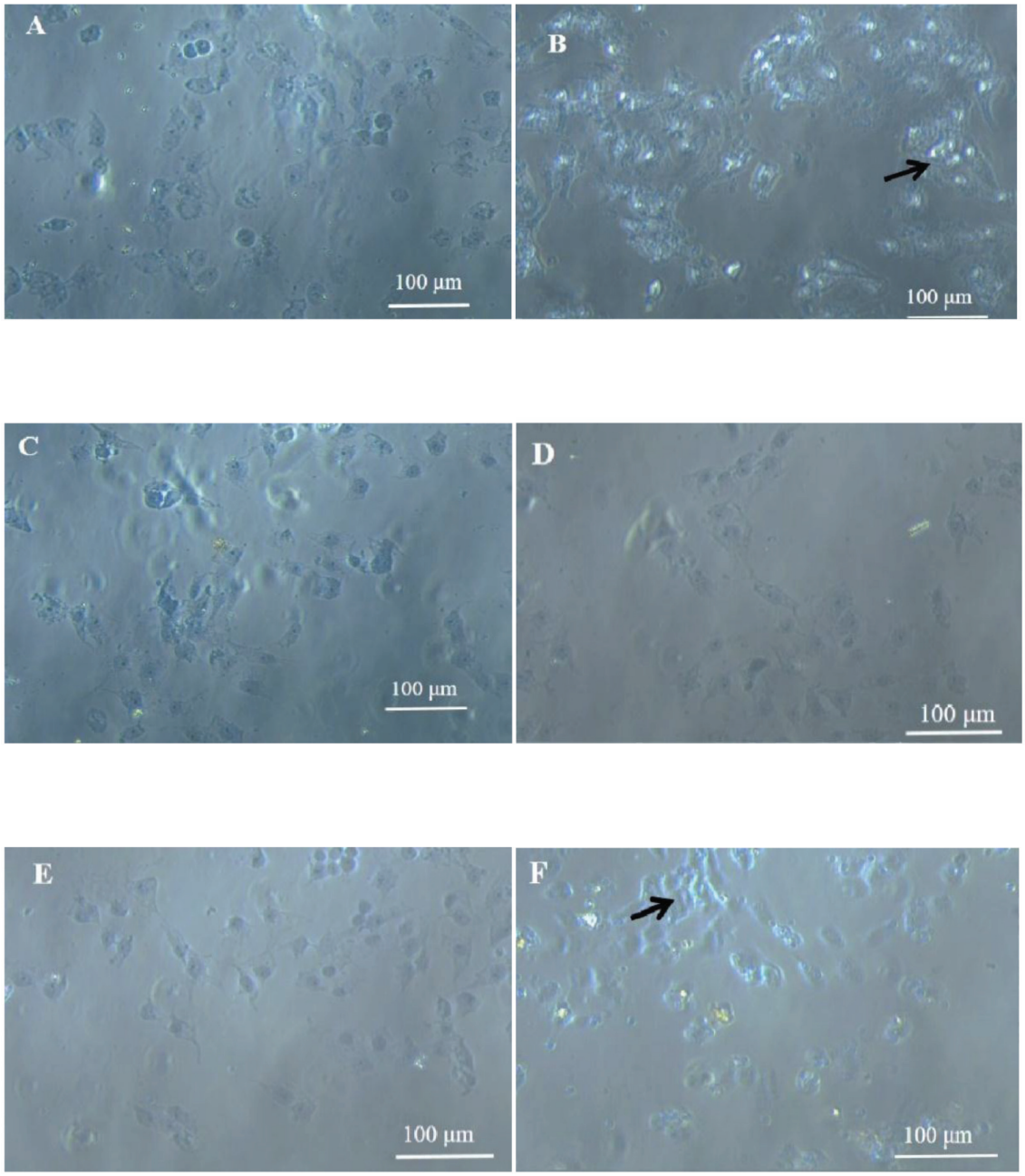

Fig. 4.The Trypan blue dye exclusion technique demonstrates dead cells treated with AFB1 both alone and in combination with ENR. [A) Control; B) AFB1; C) AFB1+SIL; D) AFB1+ENR (25 µg/ml); E) AFB1+ENR (50 µg/ml); F) AFB1+ENR (100 µg/ml). Dead cells with dark blue cytoplasm are indicated by arrows (20X)].

by the AFB1+SIL group. The current study is supported by previous studies where SIL showed a protective effect against mycotoxin-induced cytotoxicity [23, 24]. On the other hand, ENR elevated the percentage of viable cells at a concentration of 100 µg/mL, although the effect was not statistically significant (Table 3). When compared to the control group, the AFB1 alone treated group's cell viability percentage significantly decreased (Table 3). All the cell lines exposed to ENR had a decrease in cell monolayer with changed morphology, as seen in Fig. 2, whereas this change was less pronounced in the control group. When comparing the cell line exposed to the AFB1 treatment group to the control group, Fig. 3 shows a significant reduction in cell monolayer with altered morphology;

Table 1. Molecular docking results of ENR with apoptotic proteins, catalase, and CYP3A4 obtained from Discovery Studio Client software.

Proteins	LibDock Score	Binding energy (Kcal/mol)	Number of hydrogen bonds	Range of distance between hydrogen bonds
MAPK	112	-12	7	1.9 to 2.9
BAX	99	-69.6	23	1.6 to 3.1
Caspase 8	92	-14.8	16	1.9 to 3.6
Caspase 3	94	0	9	1.8 to 3.1
FAS L	87	0	11	1.7 to 3.1
CYP 3A4	76	-2.8	3	2.0 to 2.4
Catalase	94	0	9	1.8 to 3.1

Table 2. The MTT assay results show the viability percentage of ENR at different concentrations following a 48-hour incubation period.

Groups	Cell viability percentage
Control	100 ±3.73
ENR (25 µg/ml)	91.34±4.02
ENR (50 µg/ml)	74.39±3.78*
ENR (100 µg/ml)	50.12±4.06#

[The findings are presented as the mean ± SE of three independent experiments. *p<0.01 and #p<0.05, respectively, in comparison with the control group; SE stands for standard error of the mean].

Table 3. The MTT assay results show the viability percentage of AFB1 alone and in combination with ENR at different concentrations following a 48-hour incubation period.

Groups	Cell viability percentage
Control	100±3.73
AFB1 (30 µM)	9.47±1.02*
AFB1+SIL (10 µg/ml)	31.13±2.20*#
AFB1+ENR (25 µg/ml)	29.34±1.93*#
AFB1+ENR (50 µg/ml)	23.29±1.38*#
AFB1+ENR (100 µg/ml)	17.72±1.88*

[The findings are presented as the mean ± SE of three independent experiments.*p<0.05 and #p<0.05 in comparison with control and AFB1 groups respectively, SE-Standard error of mean].

however, this reduction was less pronounced in the ENR+AFB1 and SIL+AFB1 groups.

Trypan blue dye exclusion technique

Trypan blue dye exclusion technique results showed a significant decrease in the percentage of dead cells when cell culture was incubated with the combination of AFB1 and ENR at all tested concentrations when compared to the AFB1- treated group. This effect is comparable to that reported by SIL (Table 4). When comparing the AFB1-treated group to the control group, the percentage of dead cells was significantly greater (Table 4). Figure 4 illustrates that the AFB1-treated group had significantly more dead cells with dark blue cytoplasm than the ENR+AFB1 and SIL+AFB1 groups. The present *in vitro* study investigated the interaction of ENR with AFB1-induced cell toxicity. AFB1 at the dosage of 30 µM caused a cytotoxic effect on the Vero cells which is in agreement with the study conducted by Golli-Bennour *et al.* (2010) [18]. MTT assay results revealed that the ENR induced a cytotoxic effect on Vero cells in a dose-dependent manner which is in agreement with the study conducted by Liu *et al.* (2015) [17]. Among all tested concentrations, 25 µg/mL of ENR showed the highest protective effect against AFB1-induced cytotoxicity and 100 µg/mL of ENR showed the least protective effect. 25 µg/mL of ENR showed the least cytotoxic effect on Vero cells, whereas 100 µg/mL of ENR showed the most cytotoxic effect. The molecular docking study was also in agreement with the *in vitro* cytotoxic effect of ENR, where ENR exhibited good interaction with apoptotic proteins like Bax, caspase 8, caspase 3, fas L, and MAPK1. Among the three doses tested, 25 µg/mL has the least cytotoxic effect. There was an improvement in cell viability percentage in the AFB1 alone treated group at all tested concentrations of ENR after incubation of ENR with the AFB1 together. Among them, 25 µg/mL of ENR showed significantly the highest cell viability percentage against AFB1-induced cytotoxicity. In

Table 4. Trypan blue dye exclusion technique results show the dead cells percentage of AFB1 alone and in combination with ENR at different concentrations following a 24-hour of incubation.

Groups	Cell viability percentage
Control	8.00±2.31
AFB1 (30 µM)	85.33±3.53*
AFB1 + SIL (10 µg/ml)	49.33±3.53*#
AFB1 + ENR (25 µg/ml)	32.00±2.31*#
AFB1 + ENR (50 µg/ml)	44.00±4.62*#
AFB1 + ENR (100 µg/ml)	61.33±5.81*#

[The findings are presented as the mean± SE of three independent experiments.*p<0.05 and #p<0.05 in comparison with control and AFB1 groups, SE-Standard error of mean].

comparison with SIL, 25 µg/mL of ENR showed an almost similar viability percentage against AFB1-induced cytotoxicity, whereas 50 and 100 µg/mL of ENR showed a lesser viability percentage. ENR alone serves as an appropriate control for the protective effect against AFB1-induced cytotoxicity. The cell viability percentage exhibited by AFB1+ENR groups was higher than the AFB1 alone group and lesser than the ENR alone group suggesting the improvement in cell viability percentage after incubation with AFB1.

To confirm the protective effect of ENR on AFB1-induced cytotoxicity, the trypan blue dye exclusion technique was carried out. The results obtained from the trypan blue dye exclusion test revealed that AFB1 induced cell toxicity in Vero cells which is in accordance with the study conducted by Raj *et al.* (2001) [5]. The percentage of dead cells was reduced by ENR at all tested concentrations and among them, 25 µg/mL of ENR showed the highest reduction in the percentage of dead cells induced by AFB1. The molecular docking study was also in agreement with the protective effect of ENR on AFB1-induced cytotoxicity, where ENR decreased the CYP3A4 activity [12, 13, 14] by exhibiting good interaction with CYP3A4 protein, which is necessary for the activation of AFB1. In comparison with SIL, 25 and 50 µg/mL of ENR showed a lesser percentage of dead cells against AFB1-induced cell death, whereas 100 µg/mL of ENR showed a lesser percentage of dead cells.

Kalpana *et al.* (2012) reported that AFB1 reduces the conversion of ENR to its metabolite ciprofloxacin through the cytochrome P450 enzyme system, which has having higher cytotoxic effect compared to ENR [25]. Hence, it is one of the reasons for the attenuation of the cytotoxic effect exhibited by ENR, when combined with AFB1. The present study results revealed that the ENR produced cytotoxicity in a dose-dependent manner but it prevents the cytotoxicity when combined with AFB1. The interaction of both ENR and AFB1 with the cytochrome P450 enzyme results in the attenuation of cytotoxic effect induced by both molecules, predominantly AFB1-induced cytotoxicity was decreased by ENR.

CONCLUSION

The present study represents a novel attempt to investigate how ENR affects the cytotoxicity that AFB1 results in Vero cells. In Vero cells, ENR mainly protects against cytotoxicity brought on by AFB1. At 25 µg/mL, the maximum viability percentage of ENR was found to be opposed to the AFB1-induced cytotoxicity. However, additional *in vivo* and *in vitro* gene expression studies are necessary to confirm the importance of the CYP3A4 enzyme in the protective effect of ENR against cytotoxicity induced by AFB1.

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