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In vitro toxicological investigation of *Fusarium graminearum* toxins in *Rattus norvegicus* myocardial H9c2 cells

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Mycotoxins are toxic secondary metabolites of fungi that grow on food and feed matrices and significantly harm the health of humans and farm animals. Among the reported mycotoxigenic fungi, *Fusarium graminearum* has received wide attention due to its ability to produce multiple mycotoxins, including deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA) under diverse climatic conditions. The present study focused on proving *in vitro* cytotoxicity of *F. graminearum* toxins (DON, NIV, and ZEA) in H9c2 cells (*Rattus norvegicus* heart/myocardi). The effect of *F. graminearum* toxins (FGTs) on cell viability was studied by MTT, LDH, and live/dead cell assays. The cell viability decreased with increasing the quantity of FGTs and was noticed as dose-dependent. The cell viability assays indicated that DON was significantly more toxic than NIV, and both were considerably more toxic than ZEA. The toxicity mechanism of FGTs was revealed by estimating reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and caspase-3 levels. The effect of FGTs on the generation of ROS was observed by fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA), and the ROS levels were enhanced on the treatment of FGTs. The effect of FGTs on MMP was revealed by rhodamine 123 staining, and MMP levels were depleted with exposure to FGTs. Furthermore, the expression level of caspase-3 was determined by a caspase-3 detection kit, and its levels were enhanced by exposure to FGTs. The studies conclude that FGTs have a potent cytotoxic effect on H9c2 cells and induce death by oxidative stress-mediated apoptosis.

Keywords: Caspase-3, Deoxynivalenol, *Fusarium graminearum,* Nivalenol, Zearalenone

Mycotoxins are toxic metabolites produced by filamentous fungi. The presence of mycotoxins in food and feed has become a significant concern regarding food safety due to the potential risks they pose to the health of farm animals and humans. Fungal contamination of agricultural commodities arises from improper agricultural practices, including multiple stages, such as harvesting, processing, production techniques, shipping, and packaging[1]. According to the United Nations Food and Agriculture Organisation (FAO), around 25% of agricultural goods are tainted by fungus and mycotoxins[2]. Fungal infestation of agricultural goods results in a measurable decrease in volume and weight and a decrease in the nutritional content. It also leads to undesired changes in taste, color, texture, scent, and surface qualities, contributing to rancidity in food[3]. Recent studies have increased understanding of fungi and mycotoxins in agricultural commodities, causing significant concern for consumers and the food sector[4,5]. The situation has led to the implementation of strict legal restrictions on various mycotoxins in food and feed at both national and international levels. Undoubtedly, fungi and mycotoxins in agricultural products disrupt economic trade and pose health risks to farm animals and humans[2].

The most prevalent fungal genera responsible for mycotoxins in agricultural products are *Aspergillus*, *Fusarium*, and *Penicillium*[1]. *Fusarium graminearum*, a fungal that produces mycotoxins, has attracted significant interest because of its capacity to endure various climatic conditions. *F. graminearum*, a teleomorph of *Gibberella zeae*, is the causative agent of fusarium head blight disease, a highly destructive plant disease affecting wheat, barley, and other minor grains. The Fusarium Head Blight (FHB) can substantially reduce the quantity and quality of grains,

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leading to a significant economic impact on the agriculture and food business. It is responsible for billions of dollars in annual economic losses worldwide. *Fusarium graminearum* produces mycotoxins, including deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA), which are collectively known as *F. graminearum* toxins (FGTs)[6]. The toxicological tests conducted on laboratory animals have demonstrated the intricate harmful effects of DON. These effects encompass vomiting, diarrhea, leukocytosis, anorexia, hemorrhage, and, at high doses, the induction of a shock-like reaction resembling the effects of radiation exposure, which can ultimately lead to the death of the animal. The ZEA compound interacts with oestrogen receptors, leading to changes in reproductive systems, vulvovaginitis, enlargement of gonads, and immature abortions in farm animals. It can sometimes cause hyperestrogenism in humans. The NIV molecule attaches to the ribosomal peptidyl transferase site, inhibiting the production of both protein and DNA. Consequently, cell proliferation is reduced. NIV has been found to induce apoptosis in actively proliferating cells of the bone marrow, small intestine, spleen, thymus, and testes, leading to detrimental toxic consequences. The combined biological impact of FGTs encompasses a range of unfavorable effects, varying in intensity from mild to severe. These effects include carcinogenic, mutagenic, hepatotoxic, immunotoxic, teratogenic, neurotoxic, nephrotoxic, and reproductive and developmental toxic effects[7]. Moreover, the International Agency for Research on Cancer (IARC) has demonstrated the cancer-causing characteristics of FGTs in laboratory animals and has classified them as a Group 3 carcinogen[8]. Thus far, no pharmaceuticals or antibiotics have shown efficacy in treating mycotoxicosis of FGTs. Many nations and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have established regulatory limits and implemented hazard analysis and critical control points (HACCP) protocols for FGTs in food and feed matrices[9].

In the present study, the toxic nature of FGTs has been confirmed in H9c2 cells under controlled laboratory settings. The H9c2 cells are obtained from the atria of *Rattus norvegicus*. The impact of FGTs on cell viability was assessed using MTT, LDH, and live/dead cell assays. The effect of FGTs on the production of intracellular reactive species (ROS) was assessed using the fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA). The effect of FGTs on mitochondrial membrane potential (MMP), a crucial factor for ATP generation, was assessed using the fluorescent probe rhodamine 123. Moreover, the involvement of caspase-3 activity in the cytotoxic effects caused by FGTs was revealed.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin antibiotic solution, sodium bicarbonate, and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were obtained from HiMedia (Mumbai, India). The dual staining live/dead cell assay kit was obtained from Thermo Fisher Scientific (Bengaluru, India). The solvents used in the study were fine grade and obtained from Merck Millipore Corporation (Bengaluru, India), and plasticware was obtained from Eppendorf (Bengaluru, India). Rhodamine 123, dichloro-dihydro-fluorescein diacetate (DCFH-DA), certified standards of mycotoxins (DON, NIV, and ZEA), caspase-3 assay kit, and Lactate dehydrogenase (LDH) assay kit were obtained from Sigma-Aldrich (Bengaluru, India).

Cell culture and treatments

Rattus norvegicus myocardi H9c2 cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM with 10% FBS at 37°C, 5% CO_2, and 95% air under a humidified chamber. The cells were grown in 75 cm^2 flasks, the media was changed on every alternative day, and confluent cells were used for the experiment. The stock solutions of FGTs (DON, NIV, and ZEA) were prepared in dimethyl sulfoxide (DMSO), and further test concentrations were diluted in DMEM devoid of FBS with a minor concentration of DMSO (0.001%).

MTT assay

Cell viability was determined by assessing the ability of the cell's metabolic reductase enzymes to convert MTT into formazan dyes[10,11]. A total of 5×10^3 cells were added in each well of a 96-well cell culture plate. The cells were given overnight to attach to the plate and were then exposed to various concentrations of FGTs for 24 h. The cells not exposed to FGTs were designated as the control group.

A 100 μL of MTT solution (with a concentration of 5 mg/mL in DPBS) was substituted with culture media and left to incubate for 4 h. The MTT solution was substituted with DMSO for 30 min to facilitate the dissolution of formazan crystals. The absorbance was then measured at a wavelength of 570 nm using the Biotek-H1M synergy multi-plate reader from the United States. The cell viability of FGTs treated cells was quantified as a percentage relative to the control group (100%).

LDH assay

Cell viability was quantified by assessing plasma membrane integrity by measuring LDH release[12]. The experiment was conducted in accordance with the methodology and instructions provided by the LDH assay kit manufacturer, Sigma-Aldrich. The cells were seeded at a density of 5×10^4 cells/well in a 24-well cell culture plate. After overnight settling, they were treated with various concentrations of FGTs for 24 h. Cells that were not exposed to FGTs were used as the control group. The cell culture supernatant was obtained by centrifuging at 2,500 rpm for 5 min at a temperature of 4°C. A volume of 100 μL was utilized to measure the release of LDH, and the outcomes were presented as a percentage relative to the LDH release in the control cells. The overall LDH activity of the control cells was assessed via lysis using a 2% Triton X-100 solution.

Live/dead cell assay

Cell viability was assessed using a dual staining technique that differentiates live and dead cells based on their intracellular esterase activity and plasma membrane integrity. Calcein AM is a molecule that lacks fluorescence. It can enter cells and undergo catalysis by esterase enzymes present in live cells, producing fluorescent calcein with a green color. On the other hand, ethidium homodimer can pass through the broken membrane and form a red color by binding to the cell's nucleic acids[13]. A total of 5×10^3 cells were placed in each well of a 96-well cell culture plate and left to attach overnight. Afterward, the cells were treated with various concentrations of FGTs for 24 h. Cells that were not exposed to FGTs were used as the control group. The cells were stained with a concentration of 2 μM of calcein and 4 μM of ethidium homodimer, following the instructions provided by the manufacturer (Thermo Fisher Scientific, USA). The images were acquired using the EVOS FLC inverted fluorescence microscope (Life Technologies, USA) with green and red channels representing live and dead samples, respectively. The optical density was measured at excitation and emission wavelengths of 485 nm and 530 nm for calcein AM, and 530 nm and 645 nm for ethidium homodimer-1, respectively, using a multimode plate reader (Biotek-H1M synergy, USA). The percentage of live and dead cells was then determined using the formula described by Kalagatur *et al*[13].

Determination of ROS

The impact of FGTs (DON, NIV, and ZEA) on intracellular reactive oxygen species (ROS) production was assessed using the fluorescent probe DCFH-DA[14,15]. The DCFH-DA molecules, which do not emit light, penetrate the cell's plasma membrane and undergo oxidation by the cell's ROS molecules, resulting in the formation of fluorescent dichlorofluorescein (DCF) molecules. The fluorescence properties of the DCF molecule are utilized as a metric for quantifying the reactive oxygen species (ROS) generated within cells. Cells were seeded in a 24-well cell culture plate at a density of 5×10^4 cells/well. After overnight adhesion, the cells were treated with various concentrations of FGTs for 24 h. Cells that were not treated with FGTs were used as the control group. The cells were rinsed twice with DPBS and exposed to a 20 μM concentration of DCFH-DA for 30 min. The optical density was then assessed using a multi-plate reader (Synergy H1, Bio Tek, USA) with an excitation wavelength of 485 nm and an emission wavelength of 522 nm. The quantity of ROS emitted in cells treated with FGTs was measured and reported as a percentage of the control group (100%).

Determination of MMP

The mitochondrial membrane potential (MMP) serves as an indicator of mitochondrial function and plays a crucial role in the production of ATP molecules. In brief, cells were seeded at a density of 5×10^4 cells/well in a 24-well cell culture plate. They were allowed to adhere overnight and then treated with various concentrations of FGTs for 24 h. Cells that were not treated with FGTs were used as the control group. The cells were rinsed twice with DPBS and exposed to a concentration of 5 μM of rhodamine 123 for 15 min[16]. The cells were rinsed again with DPBS, and their optical density was measured using a multi-plate reader (Synergy H1, Bio Tek, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The MMP percentage in cells

treated with FGTs was quantified relative to the control group (100%).

Determination of caspase-3 activity

Caspases are a type of proteases that play a role in the process of cell death and apoptosis. Caspase-3, belonging to the CED-3 subfamily, cleaves procaspase 2, 6, 7, and 9, which are essential enzymes involved in the apoptotic pathway[17]. The impact of FGTs on caspase-3 activity was assessed by employing a caspase-3 activity test kit in accordance with the guidelines provided by the manufacturer (Sigma-Aldrich). The cells were distributed in a 12-well plate with a density of 5 x 10^6 cells per well. They were given 6 h to settle before being exposed to various concentrations of FGTs for 24 h. Cells that were not treated served as the control group. The cells underwent trypsinization and were subsequently washed twice with DPBS. They were then lysed in a lysis buffer that had been refrigerated on ice. The lysate was obtained using centrifugation at 10,000 rpm for 5 min at 4°C. The obtained lysate was then utilized for measuring caspase-3 activity, following the manufacturer's technique. The quantification of the release of the chromophore ρ-nitroanilide (p-NA) from the labeled substrate, acetyl-Asp-Glu-Val-Asp ρ-nitroanilide, was performed using a multi-plate reader at a wavelength of 405 nm (Synergy H1, Bio Tek, USA). The concentration of p-NA liberated was determined using the calibrated standard p-NA curve. The percentage of protease activity in FGT-treated cells was expressed relative to the control[18].

Statistical analysis

The experiments were carried out independently six times (n = 6). The data was analyzed by one-way ANOVA following Dunnett's test using the software GraphPad Prism 8.0. The P-value ≤ 0.05 was considered significant.

Results and Discussion

Cell viability

The effect of FGTs on the cell viability of *R. norvegicus* myocardi H9c2 cells was determined by MTT, LDH, and live/dead cell assays. The MTT assay is widely recognized as an acceptable technique for determining cell viability and the metabolic events responsible for apoptosis or necrosis. The yellow tetrazolium salts are reduced into purple formazan crystals by the action of dehydrogenase present in metabolically active cells, and the intensity of formazan crystals is determined as an index to measure theviability of the cells[19,20]. In the present study, the cell viability of H9c2 cells was significantly (P< 0.05) decreased with increasing the dose of FGTs for 24 h (Fig. 1A). The cell viability was less in DON, followed by NIV and ZEA. The study concluded that DON has a higher cytotoxic effect on H9c2 cells than NIV and ZEA.

Cell viability was also determined by plasma membrane breakage and the release of LDH. The content of LDH release will increase with the increase in the number of dead cells[21]. The percentage of LDH release in FGTs treated cells was expressed with respect to control (100%). In the present study, the content of LDH release was significantly (P< 0.05) increased with the dose of FGTs, and the observation was dose-dependent (Fig. 1B). The results of the study were similar to the MTT assay, the study concluded that DON has a high impact on cell viability of H9c2 cells and ZEA has the least effect. The breakage of the plasma membrane could be accountable for FGTs-induced cell death.

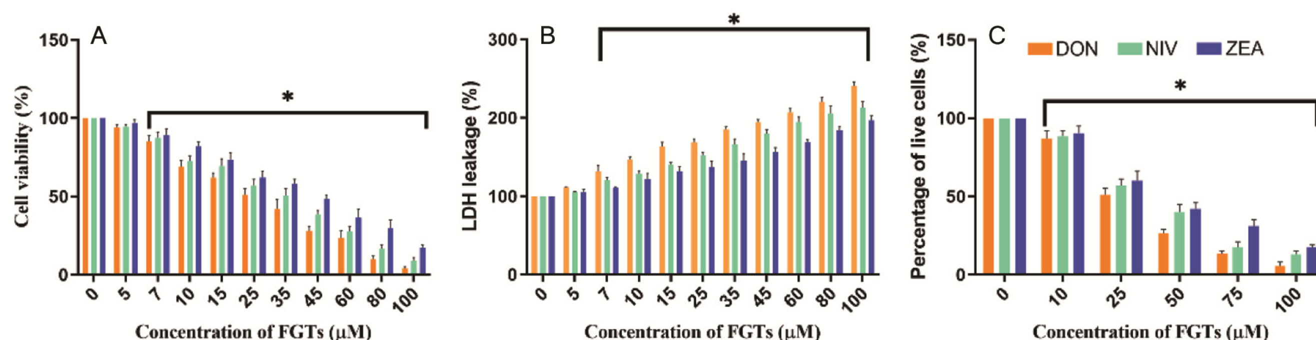

Fig. 1 — Effect of FGTs (DON, NIV, and ZEA) on cell viability of *R. norvegicus* myocardi H9c2 cells determined by (A) MTT, (B) LDH, and (C) live/dead cell assay. The data was processed by one-way ANOVA following Dunnett's test. The P-value ≤ 0.05 was significant and represented by '*'

The live/dead cell assay discriminates between the live and dead cells using two-color discrimination fluorescent reagents (calcein AM and ethidium homodimer). Calcein-AM is membrane-permeant and non-fluorescent until ubiquitous intracellular esterases eliminate ester groups and generate fluorescent molecules. Live cells appear green in color under a FITC filter or green channel. In the case of dead cells, ethidium homodimer dye crosses the damaged plasma membrane, binds to DNA with high affinity, and emits more than 30-fold red fluorescence under the red channel[22]. The percentage of live cells was significantly ($P< 0.05$) decreased with increasing the dose of FGTs (Fig. 1C). The results were that the study agreed with MTT and LDH assays, and it was prominently depicted in (Fig. 2).

In support of the present study, many reports exist on the cytotoxicity of FGTs on other kinds of cell lines. No previous reports existed on the cytotoxicity of FGTs on H9c2 cells; this is the first report. Fornelli *et al*. demonstrated the cytotoxicity of DON and NIV in the SF-9 insect cell line[23], and Venkataramana *et al*. proved the cytotoxicity of ZEA in SHSY-5Y neuroblastoma cells by MTT and LDH assays[24]. However, as far as we know, the live/dead cell assay has not been used previously to establish the cytotoxicity of FGTs. This is the first report to prove the cytotoxicity of FGTs by live/dead cell assay.

ROS and MMP analysis

The factors involved in FGTs-induced cytotoxicity of H9c2 cells were studied by assessing the ROS and MMP. ROS plays a crucial role in biological systems and acts as messenger signal transduction and cell cycle. These molecules were produced during the metal-catalyzed oxidation reactions and mitochondrial electron transport of aerobic metabolism or by means of oxidoreductase enzymes and are responsible for deleterious effects in the cell. The ROS molecules are hydroxyl ion, hydrogen peroxide, hydroxyl radical, nitric oxide, and superoxide. The ROS molecules serve as intra and intercellular messengers for gene expressions, apoptosis, and activation of cell signaling cascades. The generation of ROS molecules arrests the cell cycle, and the initiation of oxidative stress leads to the activation of the apoptosis process[14,15].

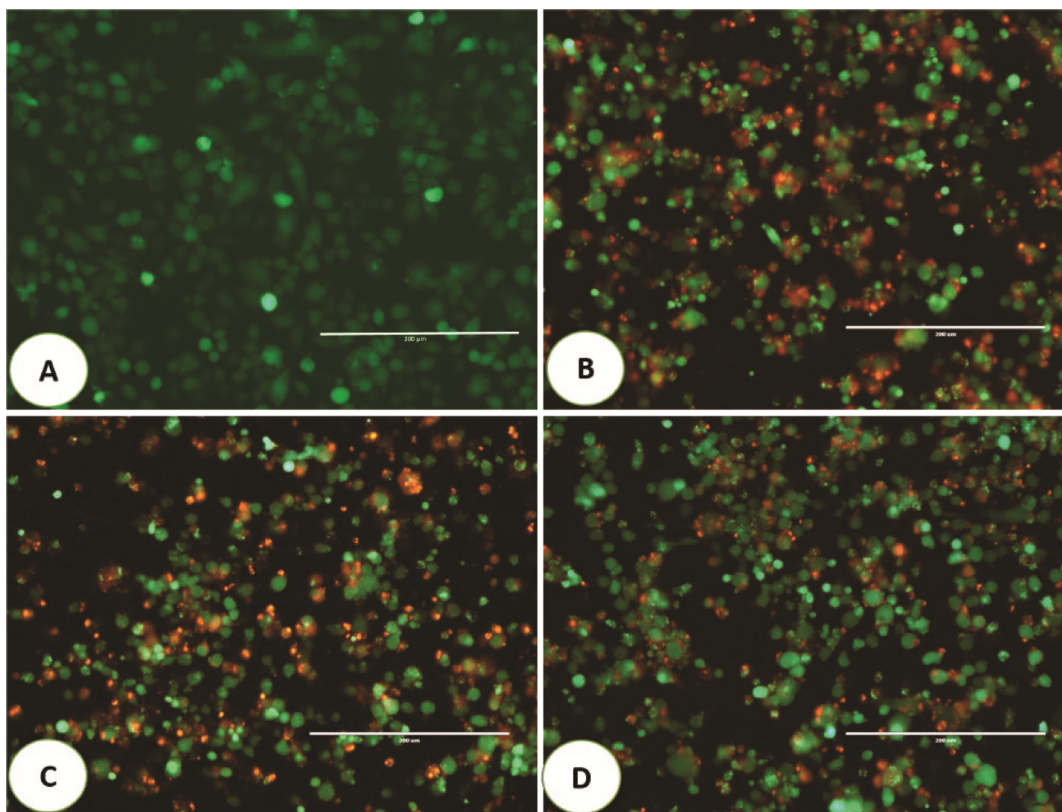

Fig. 2 — Determination of the cytotoxic activity of FGTs on H9c2 cells by live/dead dual staining technique. The live and dead cells were stained green and red color, respectively. (A) Untreated control cells. Cells (B), (C), and (D) were subjected to treatment with 25 µM of deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA), respectively

The free radicals induce alterations in the DNA sequence in the form of mutations, deletions, gene amplification, and rearrangements, which can damage DNA. These changes may activate proto-oncogenes or inactivate tumor suppressor genes of the apoptosis process and lead to the death of cells. In the present study, the content of ROS was significantly (P-value≤ 0.05) enhanced with the increasing dose of FGTs, and it was dose-dependent (Fig. 3A). The content of ROS release was high in DON and least in ZEA. The results of this study agreed with MTT, LDH, and live/dead cell assay. The enhancement of the generation of ROS and oxidative stress on exposure to FGTs could be one of the reasons for FGTs-induced cell death. In support of the study, Venkataramana *et al*. proved the role of ROS in ZEA-induced cytotoxicity in SHSY-5Y cells[24], and Zhang *et al*. documented the role of oxidative stress and ROS in DON-induced cell death in HepG2 cells[25]. Del Regno *et al*. also proved that NIV induces the death of intestinal epithelial cells through the generation of oxidative stress and ROS molecules[26]. Therefore, the present study concluded that FGTs could cause the death of H9c2 cellsby generating oxidative stress and ROS.

Rhodamine 123 is used as a selective dye for monitoring the MMP of the cell. The mitochondrial energization process brings quenching in the fluorescence of rhodamine 123, and the rate of fluorescence decay is proportional to the MMP of the cell[16]. The electrical potential between the interior and exterior membrane of the cell generates membrane potential, and it ranges from –40 mV to –80 mV. The MMP also plays a central role in the apoptotic pathway, and its disturbance leads to the induction of apoptosis. The loss of MMP may or may not be an early event in the process of apoptosis, but on the contrary, it may be an effect of the apoptosis process. Furthermore, the loss of MMP favors the release of cytochrome c and apoptosis-inducing factor (AIF) factors and leads to the activation of the apoptosis signaling pathway[27]. Kroemer & Reed also documented that MMP is crucial for the survival and maintenance of thefunctionality of cells, and its depletion leads to the obstruction in the synthesis of ATPs[28]. The MMP was significantly (P-value< 0.05) depleted with increasing the dose of FGTs, and it was highly depleted in DON (Fig. 3B). The depletion of MMP could be one of the reasons for FGTs-induced cytotoxicity in H9c2 cells.

Caspase-3 analysis

The determination of caspase-3 activity was undertaken to understand the mechanism of apoptosis involved in FGTs-induced cytotoxicity. The caspase-3 belongs to the family caspase protein, and is encoded by the CASP3 gene, and activated by upstream caspase-8 and caspase-9. Caspase 3 is implicated as an effector and hallmark of apoptosis and executes the nuclear mediating apoptosis process by DNA fragmentation, chromatin condensation, and cellular blebbing[29]. In the present study, caspase-3 levels were elevated on exposure to FGTs, and it was high in DON and lower in ZEA (Fig. 4). The rise in the levels of caspase-3 is due to the increase of ROS levels by FGTs[24]. The upsurge in caspase-3 damages the

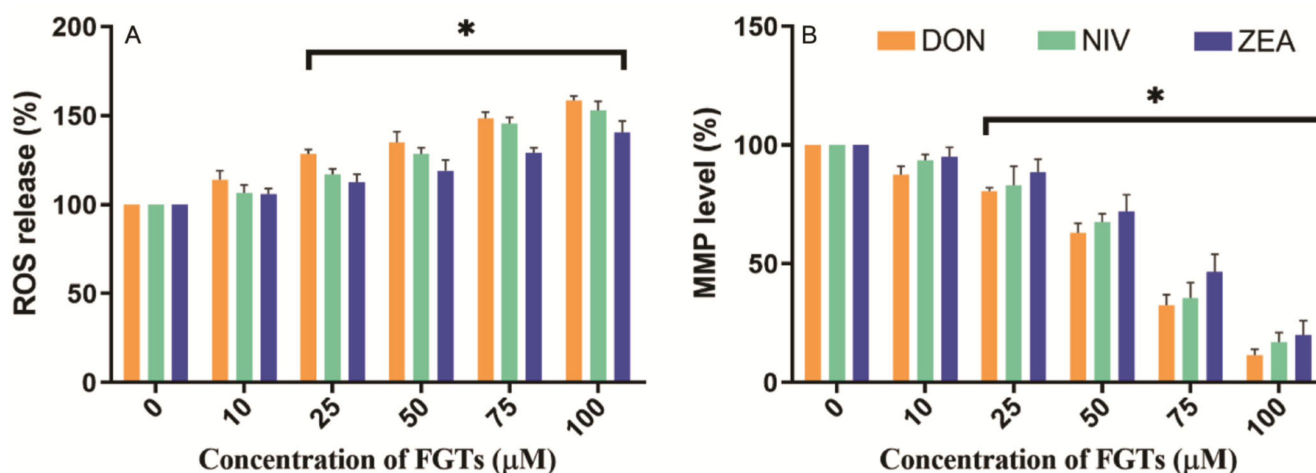

Fig. 3 — Effect of FGTs (DON, NIV, and ZEA) on (A) generation of reactive oxygen species (ROS) and (B) depletion of mitochondrial membrane potential (MMP) of H9c2 cells. The data was processed by one-way ANOVA following Dunnett's test. The P-value ≤ 0.05 was significant and represented by '*'

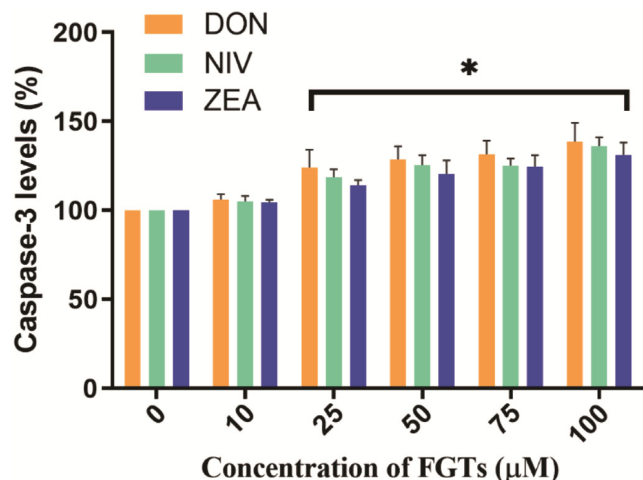

Fig. 4 — Effect of FGTs (DON, NIV, and ZEA) on the caspase-3 activity of H9c2 cells. The data was processed by one-way ANOVA following Dunnett's test. The *P*-value ≤ 0.05 was significant and represented by '*'

nuclear material of the cell and activates the apoptosis process, which could be the reason for the cytotoxicity of FGTs.

Conclusion

MTT, LDH, and live/dead cell assays showed that FGTs lowered H9c2 cell viability. Furthermore, the generation of ROS molecules was enhanced on exposure to FGTs, and it was reflected in the depletion of MMP of the cell. The rise in the levels of caspase-3 concluded that FGTs induce the death of H9c2 cells through apoptosis. The study proved the *in vitro* cytotoxicity of FGTs in *R. norvegicus* myocardi H9c2 cells and induced cell death by oxidative-stress-mediated apoptosis. The study urges monitoring and regularizing the levels of FGTs in food matrices intended for the consumption of farm animals and humans.

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Conflict of interest

All authors declare no conflict of interest.

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