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Occurrence of *Alternaria* Toxins, Ochratoxin A and Aflatoxins B1 in Green Arabic Coffee (*Coffea arabica*) in Saudi Arabia: Risk Assessment on HepG-2 Cell Line and Male Albino Rats

Ahmed Hashim Almusallam[1], Ahmed Mahmoud Ismail[1,2]*, Khalid Alhudaib[1], Ahlam Farouk Hamouda[3] and Eman Said Elshewy[4]

[1]Dept. of Arid Land Agriculture, College of Agricultural and Food Sciences, King Faisal University, Al-Ahsa, Saudi Arabia;
[2]Pests and Plant Diseases Unit, College of Agricultural and Food Sciences, King Faisal University, Al-Ahsa, Saudi Arabia;
[3]Dept. of Forensic Medicine and Toxicology, Teaching Hospital, Faculty of Veterinary Medicine, Benha University, Egypt;
[4]Vegetable Diseases Research Dept., Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt
*Corresponding author's e-mail: amismail@kfu.edu.sa

Arabica coffee (*Coffee arabica* L.) is among the most popular and widely consumed drinks globally today. The current study aimed to detect and quantify of potential mycotoxins in coffee beans and to investigate their potential risk on HepG-2 human liver cancer cells and male albino rats. Eighteen fully matured coffee beans samples were collected from different geographical locations in Saudi Arabia. Mycotoxins detected and quantified utilizing high-performance liquid chromatography (HPLC). Cytotoxicity of the detected toxins was investigated utilizing the MTT assay method toward HepG-2 cells and male albino rats. Cytotoxic properties of the detected mycotoxins were investigated at doses of 31.25, 62.50, 125, 250 and 500 µg/ml following exposure durations of 24 and 48 h in comparison with control. HPLC analysis revealed the detection of four *Alternaria* toxins (ALT-Ts) namely Altenuene (ALT), Tenuazonic acid (TA), Alternariol (AOH), and Altenuisol being AOH reported as the frequently detected mycotoxin in 18 coffee beans samples representing 75 % with quantity ranged 0.98 to 8.97 µg/g. OTA was frequently detected (10 samples, 55.5%) in concentrations varied from 0.75 to 9.87 µg/g. In addition, Aflatoxin B1 was also detected with low quantity ranged 0.89 to 3.22 µg/g. Treatment of HepG-2 cell line with combined *Alternaria* toxins (ALT, TeA, AOH, and Altenuisol), Aflatoxin B1 (AFB1) and Ochratoxin A (OTA) as well as their combination triggered a decline in cell viability that was proportional to the dose administered. The elevated doses (62.50, 125, 250 and 500 µg/ml) notably reduced cell viability and induced cellular damages when contrasted with the lowest dose (31.25 µg/ml) and control. The IC_{50} for the tested mycotoxins registered for ALT-Ts, AFB1 and OTA as well as their combination were 115.95, 56.68, 59.86 and 31.05 µg/ml after 48 h of exposure. Secreting inflammatory markers (Interleukin-6 (IL-6), interleukin-1β (IL-1β) and COX-2), and oxidative markers (total antioxidant capacity (TAC), glutathione (GSH) and nitrite (NO) were downregulated by all mycotoxins with the highest decrease induced by their mixture. Malonaldehydes (MDA) and TAC were significantly increased by all toxins in treated rats. Hematological results revealed that AFB1, OTA and ALT-Ts-treated rats exhibited a notable decline in hemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), and packed cell volume % (PCV). However, single and double dose-treated rats with OTA, Mix1 and Mix2 exhibited a marked increase in WBCs. The results demonstrated a notable decline in liver and kidney functions in all mycotoxins-treated rats. The studied organs (liver, kidney and spleen) exhibited significant damage, with the most severe alterations observed in the Mix1 and Mix2-treated group in comparison with the groups receiving individual treatment. This indicates that the mixture of AFB1, ALT-Ts and OTA had an enhanced toxic consequence against treated HepG-2 cell line and rats. Our results indicated the synergistic effect of the combined different mycotoxins against HepG-2 human liver cancer cells and male albino rats. This work could help fill in data gaps and enhance risk assessment for human health by addressing some of the possible risks associated with *Alternaria* toxins.
Keywords: Aflatoxin, *Alternaria*, coffee, cytotoxicity, inflammatory, ochratoxin, rats.

INTRODUCTION

The two primary species *Coffea canephora* (Robusta coffee) and *Coffea arabica* L. (Arabica coffee) produce coffee accounting 70% and 30% of the world's total commercial output of coffee, respectively. For the past four or five

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centuries, Saudi Arabia have grown Arabica coffee on small valleys and ridged mountain slopes at elevations reaching from 1200 m to 1800 m (Tounekti *et al*., 2017). Because it is primarily grown using organic cultivation methods without the use of synthetic pesticides, herbicides, or fertilizers, Saudi Arabia is consequently consistently producing some of the best coffee in the world (FAO, 2015; Lewin *et al*., 2004). For even better results, farmers frequently combine organic goat dung with stone mulching (Al-Turki, 2002). In south-western Saudi Arabia, coffee is a major socioeconomic factor and a source of revenue for mountain growers. Research on coffee cultivation in the area was increased because of growing interest in coffee production (Tounekti *et al*., 2018). Coffee producers encounter a variety of obstacles in their agricultural yields throughout tropical regions. Similar to other agricultural crops, coffee is subjected to several microbial infections throughout its cultivation, processing and storage phases in Saudi Arabia (Alhudaib and Ismail, 2024; Alhudaib *et al*., 2023; Crous *et al*., 2023). The occurrence of toxigenic *Penicillium* spp. and *Aspergillus* spp., on coffee beans was documented in both coffee-producing regions and areas where coffee is not cultivated. Under favourable environmental conditions, these fungal infections typically result in the production of mycotoxins (Bokhari *et al*., 2007). Aflatoxin B1 (AFB1) is the harmful toxin produced by various *Aspergillus* species, and its contamination is prevalent in varied food elements (Ismail *et al*., 2024; Umesha *et al*., 2017). Another common mycotoxin, ochratoxin A (OTA), is predominantly produced by *Aspergillus niger*, *Aspergillus ochraceus*, and *Penicillium verrucosum*. Foods typically contaminated with OTA include cereals, meat, cheese, coffee, tea, wine, spices, vegetables, fruits, and dried fruits (Chen *et al*., 2018). AFB1 and OTA are recognized for their toxic impacts on the liver and kidneys, respectively, and most predominantly detected in the coffee beans. European Union (EU) has determined maximum allowable limits for OTA in soluble (10 µ g/kg) and roasted (5 µ g/kg) coffee, but there are no such standards for green coffee beans (Commission Regulation, 2010). In contrast to OTA, the EU has not implemented regulations concerning aflatoxin (AF) levels in the roasted, soluble, and green coffee.

Recently, mycotoxin studies have increasingly interested in the developing category, in addition to the primary *Alternaria* toxins (Asam and Rychlik, 2015). *Alternaria* toxins are classified into five chemical classes: benzopyrones or pyranones (altenuisol (AS); altenuene (ALT); altenusin (ALN)), perylenequinones (alterperylenol, or alteichin (ALTCH), altertoxins (ATXs), and stemphyltoxin (STE)), amine/amide metabolites (altersetin (ALS); tenuazonic acid (TeA);), and anthraquinones (Altersolanol (As-A), Macrosporin A) (Escrivá *et al*., 2017). A recent investigation indicates that *Alternaria* toxins continue to be viewed as newly recognized mycotoxins, suggesting that there is a shortage of sufficient data concerning their toxic potential and human exposure to establish appropriate guidelines at this time (Aichinger, 2021). Currently, there are no laws governing *Alternaria* toxins in food and feed within Europe, and the EFSA is conducting a study to collect information on *Alternaria* toxins concentrations. Despite the small sample size, vegetarians had a greater intake of *Alternaria* toxins through their diet than the broader population (EFSA, 2019). Nonetheless, the Commission Recommendation (EU) 2022/553, issued on April 5th, 2022, sets the recommended quantities of AME, AOH, and TeA in particular food products. The threshold of toxicological concern (TTC) method was employed in assessing the potential health risks posed by these mycotoxins to humans. The computed average long-term dietary intake at the upper limit and 95th percentile for the genotoxic *Alternaria* toxins (AOH and AME) surpassed the TTC limit (2.5 ng/kg body weight (bw) per day) (EC - European Commission, 2022). This highlighted the necessity for more detailed toxicity data specific to each compound. As a result, the emergence of *Alternaria* toxins in food and feed is raising concerns in the public health sector (Mujahid *et al*., 2020). These findings indicate that coffee denotes a minor contributor to exposure to *Alternaria* toxins, and hence, safety concerns are unlikely. Consequently, consuming mycotoxins in combination may lead to more significant adverse health effects than consuming them individually. However, there is limited information regarding the toxicological risks associated with simultaneous exposure to various mycotoxins.

In this regard, the current study is designated to detect the mycotoxins in green coffee beans harvested from different geographical regions in Saudi Arabia and to investigate their individual as well as their combined risk assessment utilizing exposure data from human cell line and evidence of animal toxicity related to both single as well as combined toxins. The current study also seeks to supply further data to create a clearer understanding for the presence of mycotoxins in green coffee, where detailed information is limited.

MATERIALS AND METHODS

***Samples collection*:** A total of 18 matured cherry coffee samples comprised of Arabica species, were collected from three geographical regions in Saudi Arabia, namely Jazan, Asir, Najran and El Baha.

***Chemicals and reagents*:** Standards for AFB1, OTA and *Alternaria* toxins (ALT, AOH, TeA and Altenuisol) were obtained from Sigma Chemical Co. (St. Louis MO, USA). Fetal Bovine serum, Dimethyl sulfoxide (DMSO), trypan blue dye, and MTT were also sourced from Sigma Chemical Co. L-glutamine, gentamycin, HEPES buffer solution, RPMI-1640, and 0.25% Trypsin-EDTA were obtained from Lonza (Belgium).

***Extraction of mycotoxins*:** The extraction of mycotoxins from coffee beans was accomplished following the technique

of Masood *et al*. (2015). A one-gram sample was introduced into a 250 ml glass Erlenmeyer flask, where it was combined with 100 ml of a solution encompassing acetonitrile and water at 84:16, along with 2% sodium chloride. The flask was agitated at a medium speed of 120 rpm. Following the agitation, the mixture was filtered and acidified with 70 µL of acetic acid. The purified solution was collected after passing through a clean-up column. The solvent was then evaporated, and the resulting aflatoxin residues were derivatized with trifluoroacetic acid. These residues were re-dissolved in 2 mL of acetonitrile-water (9:1 v/v) mixture and kept at 4 °C for future chromatographic inspection.

***HPLC analysis*:** Detecting and quantifying mycotoxins in 18 coffee bean samples were done using High-performance liquid chromatography (HPLC). The analysis was accomplished utilizing HPLC-(Shimadzu) that composed of a two LC- pumps pump, a fluorescence detector, along with a C18 column (150 mm × 4.6 mm, 5 µm particle size). Chromatograms were obtained and analyzed using ChemStation. Toxins separation occurred within HPLC column with a mobile phase of Acetonitrile: methanol: water (10:40:50 v/v/v). Fluorescence detection was at an emission wavelength of 440 nm and 365 nm excitation wavelength.

Cytotoxicity Analysis

***Mammalian cell lines*:** HepG-2 cells (human Hepatocellular cancer cell line) were sourced from the American Type Culture Collection (ATCC, Rockville, MD).

***Cell line propagation*:** The cells were cultured within RPMI-1640 medium enriched with 50µg/ml gentamycin and 10% inactivated fetal calf serum. They were kept at 37 ºC within a humidified environment with 5% CO_2 and sub-cultured 2-3 times weekly.

***MTT assay*:** Cytotoxicity of the detected toxins was investigated using the MTT assay method toward human Hepatocellular cancer cell line (HepG-2). Cytotoxic properties of the detected mycotoxins were investigated at concentrations; 31.25, 62.50, 125, 250, 500 and 1000 µg/ml after exposure for 24 and 48 hr. versus control. For antitumor tests, the tumor cell lines underwent suspension within medium at $5x10^4$ cells per well within Corning® 96-well tissue culture plates, and subsequently underwent 24-hour incubation period. The compounds under investigation were subsequently introduced into 96-well plates, with three replicates for each concentration, resulting in eight different levels for every compound. Each plate included six vehicle controls using media or DMSO (0.5%). After a 24 and 48-hour incubation period, the cell viability was assessed employing the MTT assay. In summary, the media in the 96-well plate was discarded and replaced with 100 µl of fresh RPMI 1640 medium lacking phenol red. Afterwards, 10 µl of a 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) was introduced into each well, comprising the control wells. The plates were subjected to 4-hour incubation at 37 °C under 5% CO_2 conditions. Following this, 85 µl of the media was taken from each well, and 50 µl of DMSO was introduced and thoroughly mixed utilizing a pipette, then incubated at 37°C for an additional 10 minutes. Subsequently, the optical density was assessed at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) to evaluate cell viability. The viability percentage was computed using the formula [(ODt/ODc)] x 100%, where ODt represents the wells' average optical density that received the test samples, and ODc denotes the average optical density of the control wells. The relationship between the drug concentration and number of surviving cells is graphed to generate the survival curves for all tumor cell lines individually, following exposure to the designated compound. The 50% inhibitory concentration (IC_{50}), which indicates the concentration needed to induce toxicity in half of viable cells, was calculated from the graphical representations of the dose-response curves at various concentrations utilizing GraphPad Prism software (San Diego, CA. USA) (Mosmann, 1983).

***Inflammatory cytokines assay*:** Interleukin-6 (IL-6), interleukin-1β (IL-1β) and COX-2 levels in the treated HepG-2 cells exposed to tested mycotoxins at IC_{50} after 48 hr were determined utilizing the commercial ELISA kit (Elabscience, USA) according to the manufacturer's guidelines. Optical densities (OD) measured were recorded utilizing ELISA Plate Reader (BMG Labtech, FLUOstar Omega, Germany) at wavelength 450 nm. The IL-1β and IL-6 concentrations were expressed in pico gram of tissue (pg/ml), whereas the COX-2 concentration was reported in nanograms (ng/ml) and the measurements were conducted in triplicate.

Oxidative Stress Markers

***Nitric acid (NO)*:** The level of oxidative marker Nitric acid (NO) was estimated spectrophotometrically in the treated HepG-2 cells with tested mycotoxins at IC_{50} after 48 hr using a commercial Kit (Biodiagnostic, Egypt) following the manufacturer's guidelines. This technique relies on measuring the endogenous nitrite concentration as a marker for NO synthesizing. In essence, when nitrite is present in the sample within an acidic medium, nitrous acid is created, which diazotizes sulphanilamide. Afterwards, the resulting compound reacts with N-(1-naphthyl) ethylenediamine. The resultant azo dye exhibits a vibrant reddish-purple hue, with its absorbance quantified at 540 nm utilizing a plate reader (BMG Labtech, FLUOstar Omega, Germany). Measurements were performed in triplicate, and the data were recorded as micromoles per litre (µmol/L).

***Total antioxidant capacity (TAC)*:** The TAC level was estimated spectrophotometrically in the treated HepG-2 cells with tested mycotoxins at IC_{50} after 48 hr utilizing a commercial Kit (Biodiagnostic, Egypt) following the manufacturer's guidelines. In principle, a portion of the substrate, exogenously supplied hydrogen peroxide (H_2O_2), was eliminated with the total antioxidants within the sample and the chromogen (3,5, dichloro-2-hydroxy benzenesulfonate) is converted colored compound by

enzymatic reaction included the residual of H_2O_2. This colored product is measured at 505 nm utilizing a plate reader (BMG Labtech, FLUOstar Omega, Germany). The data were expressed as millimoles per litre (mM/L), and the measurements were conducted in triplicate.

***Glutathione (GSH)*:** Glutathione level was estimated spectrophotometrically in the treated HepG-2 cells with tested mycotoxins at IC_{50} after 48 hr utilizing a commercial Kit (Biodiagnostic, Egypt) according to the manufacturer's guidelines. In principle, DNTB (5, 5` dithiobis (2-nitrobenzoic acid) is reduced with glutathione (GSH) within the sample to create the yellow TNB (5-thio-2-nitrobenzoic acid). The reduced chromogen's absorbance was quantified at 405 nm utilizing a plate reader (BMG Labtech, FLUOstar Omega, Germany). The data were expressed as milligram per 100 µl (mg/100 µl), and the measurements were conducted in triplicate.

***Animals and experimental design*:** Thirty-one male albino rats, with an average body weight of 150-160gm. The rats sourced from the animal facility of the National Research Center, Giza, Egypt. The animals were housed within the animal facility at the Faculty of Veterinary Medicine, Benha university, under controlled conditions of 21-22 ºC with a12-hour dark/light cycle, and had unrestricted access water and standard food pellets during the experimental duration (20 days). To adapt to the laboratory environment, the rats underwent a one-week acclimatization episode. The experimental design adhered to ethical standards (BUFVTM 02-05-24). A total of nine groups were formed, each consisting of four rats:

Group 1: (negative control) given 0.5ml of corn oil per daily for successive 20 days.

Group 2: was received aflatoxins at dose of 10µg/kg bw, as a maximum level of AFB1 according to EC (2006).

Group 3: was received a double maximum level of AFB1(20µg/kg bw)

Group 4: was received ALT-Ts at dose of 30µg/kg bw according to EC)2023).

Group 5: was received ALT-Ts at dose of 60µg/kg bw, as double ALT-Ts maximum permissible level,

Group 6: was given maximum level of OTA at (5µg/kg bw) according to EC (2023).

Group 7: was given double maximum level of OTA at (10µg/kg bw).

Group 8: were dosed maximum level of (AFB1+ALT-Ts +OTAµg/kg bw).

Group 9: was received a double dose of mixed mycotoxins (AFB1+ ALT-Ts +OTAµg/kg bw).

All doses were dissolved in 0.5.ml of corn oil. At the experiment conclusion, blood specimens were drawn through cardiac puncture into vacutainers, some containing EDTA and others without, to perform haematological assessments and serum biochemical analyses, respectively. Finally, autopsy of specimens was drawn from spleen, kidney, and liver of rats in various groups and preserved in 10% formol saline for pathological study.

***Hematological analyses*:** Whole blood was used to measure total leukocytes count (WBCs), hemoglobin concentration (Hb), total erythrocytes count (RBCs), and packed cell volume% (PCV%). An electronic cell counter (VetScan HM5 Hematology system, Abaxis, Inc., Union City, CA, USA) was employed to assess these parameters.

***Serum biochemical analyses*:** At the experiment conclusion, collected sera were analyzed to measure the alanine transaminase (ALT) and aspartate transaminase (AST) activities **(**Reitman and Frankel, 1957**)**, and the urea concentration **(**Patton and Crouch, 1977**)** and creatinine **(**Jaffé, 1986**).** Total antioxidant concentration (TAC) **(**Erel, 2004**)** and malonaldehydes (MDA) **(**Ruiz-Larrea *et al*., 1994**).**

***Histopathological examinations*:** Tissue samples from the liver, kidney, and spleen of rats from various groups were collected during autopsy and preserved in 10% formol saline for 24-hour period. Afterward, the samples were rinsed with tap water and underwent dehydration utilizing a graded series of alcohols: methyl, ethyl, and absolute ethyl. Samples were treated with xylene for clearing and then embedded within paraffin in a hot oven at 56 °C for a 24-hour period. Paraffin beeswax was used to create tissue blocks, which were subsequently cut into 4-micron thick sections using a LEITZ rotary microtome. The prepared tissue sections were mounted onto glass slides, then underwent deparaffinization and staining with hematoxylin and eosin (Banchroft *et al*., 2013) for observation under a light microscope.

***Statistical analysis*:** All data are presented as the mean ± standard deviation (SD) of three replicates. All analyses were done in IBM SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA) utilizing one-way analysis of variance (ANOVA). The Duncan multiple comparison test was applied to identify variations between the means.

RESULTS AND DISCUSSION

HPLC analysis

***ALT-Ts amount in green coffee*:** The HPLC results of 18 coffee beans samples showed that one ALT-Ts at least was noticed in one sample. In general, ALT-Ts was the most dominant detected among other toxins. In this regard, AOH was the most frequently detected (14 samples, 77.7 %) at levels varied between 0.98 and 8.97 µg/g, followed by ALT, (10 samples, 55.5%) with levels between 1.02 to 5.10 µg/g. By contrast, in a different investigation involving 85 samples of green coffee sourced from 9 countries (Bessaire *et al*., 2019), AME was identified more often, appearing in seven samples (8%) with concentrations of 0.6-8.3 µg/g. They found also that AOH and TEN were not detected and ALT and TeA were not evaluated. However, in our study, TeA was analyzed and detected (seven samples, 38.8%) at levels ranged of 1.58-8.09 µg/g. Altenuisol was also detected less frequently (five

samples, 27.7%) at levels 0.78 to 10.29 µg/g. Based on the report of EFSA (2016), TeA emerged as the frequently recognized *Alternaria* toxin in various raw materials and finished goods (62%), and at the greatest concentrations across various food items. This was also documented by El Gobashy et al. (2018), reported that TeA was the abundant toxin produced by *A. alternata* isolates. Notably, multiple *Alternaria* toxins were often present in various samples analyzed, showing considerable differences in their levels. So far, most EU countries lack established regulations regarding permissible levels of ALT-Ts in green coffee beans. According to EFSA (2016) database, there are indicative levels of ALT-Ts for certain foods, but, no regulations for green coffee available until now. Consequently, in 2022, the European Commission issued a recommendation urging member states to track the presence of *Alternaria* toxins in food, specifically targeting AME, AOH, and TeA (European Commission 2022).

***OTA amount in green coffee*:** OTA was frequently detected (10 samples, 55.5%) in concentrations varied from 0.75 to 9.87 µg/g. Our results greatly agree with those of Al Attiya *et al*. (2021), who found out that all the tested coffee samples contained OTA, averaging 2.15 µg/g. Furthermore, Batista *et al*. (2003) indicated that, only five out of the 40 samples inspected showed contamination with OTA, with levels varying between 0.64 and 4.14 ng/g. Also, Pardo *et al.* (2004) examined 14 samples of green coffee from Asia and documented OTA levels of 1.6-31.5 ng/g, averaging 6.0 ± 7.9 ng/g. Gopinandhan *et al.* (2007) analyzed 129 green coffee specimens, collected at random, for OTA. According to their findings, 81% of the samples exhibited OTA concentrations less than 5 ng/g, while only three samples exhibited OTA concentrations exceeding this limit, with a maximum of 11.7 ng/g as reported in a sample of Robusta cherry. An examination of 80 samples of green coffee cultivated in India revealed a considerable incidence (74%) of OTA contamination (0.2–13.5 ng/g) (Gopinandhan *et al*., 2008). In Brazil, Taniwaki *et al*. (2014) documented increased OTA levels (11.3–25.7 µg/kg) in comparison with our results. The concentrations were notably increased than the levels identified in our study, which ranged from 0.75 to 9.87 µg/g (Table 1). Most EU countries lack established regulations regarding permissible OTA levels in green coffee beans (EC, 2010). Nevertheless, specific countries, including Greece, Finland, and Italy, have set legal limits for OTA at 20, 10 and 8 µg/kg, respectively, for green coffee beans (Vieira *et al*., 2015). Considering these statutory limits of the later countries, all green coffee bean samples in the current study exhibited OTA levels within the acceptable range (Table 1).

***AFB1 amount in green coffee*:** In our study, AFB1 was found in 10 samples, accounting for 55.5%, with levels fluctuating from 0.98 to 4.55 µg/g. In Saudi Arabia, Bokhari (2007) similarly documented AFB1 in coffee beans, with concentrations ranging from 2.1 to 219 µg/kg. An investigation by Humaid *et al*. (2019) in Yemen revealed that AFB1 was present in all (n = 25) green coffee bean samples (14.69 µg/kg). Additionally, a recent investigation by Ahmad *et al*. (2022) indicated that AFB1 levels in roasted coffee beans varied from 12 to 49 µg/kg. According to the latest MLs of EU (2013), Aflatoxin contamination levels in coffee are not routinely monitored globally, comprising within the EU.

Table 1. Mycotoxins detected in coffee beans using HPLC analysis.

Samples	Origin	*Alternaria* toxins (µg/g)				AFB1	OTA
		ALT	AOH	TeA	Altenuisol		
1	Al Baha	5.10	2.00	8.09	-	3.22	9.87
2	A Baha	2.35	1.42	-	-	-	-
3	Jazan	2.56	1.76	-	1.55	1.25	-
4	Jazan	3.14	6.23	5.23	-	0.98	-
5	Jazan	3.17	-	-	10.29	-	3.45
6	Asir	-	-	-	-	-	1.65
7	Asir	-	1.56	-	-	-	-
8	Najran	1.02	0.98	-	-	-	7.08
9	Jazan	-	8.97	-	-	-	-
10	Jazan	-	2.86	1.58	-	4.55	-
11	Asir	-	-	-	-	0.89	0.75
12	Najran	-	1.33	-	-	-	0.95
13	Najran	2.13	-	3.24	-	1.56	-
14	Asir	-	4.36	2.06	2.79	-	2.76
15	Asir	3.44	1.54	1.66	-	2.11	4.98
16	Al Baha	1.83	1.39	4.15	0.78	3.58	5.04
17	Asir	-	5.85	-	2.77	1.98	-
18	Asir	4.92	3.17	-	-	5.09	6.43

Altenuene=ALT; tenuazonic acid=TA; alternariol=AOH; aflatoxin B1=AFB1; and ochratoxin A=OTA

***Cytotoxicity assays*:** According to the MTT assay, the tested mycotoxins reduced the of HepG-2 cells viability in a manner dependent on both time and concentration (Fig. 2). All IC_{50} values of AFB1, ALT-Ts, OTA and their mixture on HepG-2 cells were determined from the data presented in Fig. 1 and summarized in Table 2. Notably, the exposure of HepG-2 cells after 24 and 48 hr to mycotoxins at concentrations from 2 and 3.9 µg/ml did not persuade neither cytotoxic nor inhibitory consequences (Fig. 2). The tested mycotoxins exhibited variable IC_{50} after 24 and 48 hr of incubation against HepG-2 cells (Table 2). In this regard, IC_{50} approximations of ALT-Ts, AFB1, OTA and their combination were subsequently employed for further ROS and inflammatory markers tests.

***Cytotoxicity of AFB1*:** Cell activity was markedly inhibited when the concentration of AFB1 rose from 3.9 to 1000 µg/ml (Fig. 1A). Similarly, Reddy *et al*. (2006) indicated that survival of the HepG-2 cells declined as the concentration of AFB1 rose from 11 to 33 mg/ml. Also, Zhao *et al*. (2016) indicated that increasing the concentration of AFB1 to 100 µmol/L, the viability of HepG-2 cells decreased by 65.76%. Moreover, increasing AFB1 from 1 to 100 µg/ml or exposure duration from 24 to 48 hours, the HHL-16 cell viability was reduced (Wu *et al*., 2024). The current findings along with those previously published demonstrated that AFB1 toxicity in HepG-2 cells and other tested cells varies based on both dosage and exposure duration. The IC_{50} values for HepG-2 viability, measured using the MTT assay, were found to be 204.41 µg/mL after 24 hours and 65.68 µg/mL following 48 hours of treatment (Table 2). However, a previous study reported the IC_{50} value for HepG-2 viability following 48 hours of treatment as 31.25 µg/mL (Corcuera *et al*., 2011), 6.8 µg/mL (Du *et al*., 2017) and 3.8 µg/mL (Liu *et al*., 2014), which were lower than IC_{50} values in our study. Furthermore, Chen *et al*. (2022), found out that IC_{50} value for HepG-2 viability following 24 and 48 hours of treatment were 63.6 and 3.6 µg/mL, respectively. This variation in IC_{50} value for HepG-2 cells could be attributed to the seeding density, which is a significant factor in determining IC_{50} values. This factor may impact the toxin load per cell, resulting in varying proportions of cell populations of healthy, damaged, and dead cells (Chen *et al*., 2022).

***Cytotoxicity of ALT-Ts*:** Since mycotoxins typically present in variety of food, the effects of ALT-Ts mixtures must be determined. In our study, the exposure of HepG-2 cells for the 24-hour treatment with ALT-Ts at the minimum levels (31.25 and 62.5 µg/mL) induced no cytotoxic effects (Fig. 1B, Fig. 2). These findings agree with those of Ismail *et al*. (2023). However, the HepG-2 cell viability was inhibited by ALT-Ts when the concentration rose from 15.6 to 1000 µg/ml after 48 h of treatment. Furthermore, comparable findings were also documented by den Hollander *et al*. (2022), demonstrating the synergistic consequences of *Alternaria* toxins, AME, AOH, and TeA against HepG-2 and Caco-2 cell lines. Nonetheless, some discrepancies have been observed between studies that utilized similar cell model and dosage ranges (Louro *et al*., 2024). The combined impact of frequently co-occurring *Alternaria* toxins on coffee has been inadequately researched. However, initial findings have indicated the presence of heightened health risks, warranting additional investigation.

***Cytotoxicity of OTA*:** OTA induced cell viability reduction in a manner dependent on both time and dose, within a concentration range from 7.8 to 1000 µg/ml and the IC_{50}, measured after 48 hr, attained 59.86 µg/ml (Fig. 1C). Cell viability significantly decreased when exposed to the highest concentrations (250, 500 and 1000 µg/ml), and this decrease was notable after 48 hr of incubation (Fig. 1C). These findings are coincided with those of Pinhão *et al*. (2020). After 48 hours of treatment, the IC_{50} value (59.86 µg/ml) was higher than those reported by Juan-García *et al*. (2019) (52.62 ± 0.06 µM) and lower than those (250 nM) of Shin *et al*. (2019). Furthermore, Gayathri *et al*. (2015), indicated that OTA reduced cell viability in a manner dependent on dose at concentration ranging from 0 to 300 µM, with an IC_{50} value of 210 µM. By contrast, the study of Tavares *et al*. (2015), demonstrated that OTA significantly decreased cell viability following 24 hours of exposure at concentrations exceeding 10 µM ($p<0.001$), resulting in 27.5 µM IC_{50}.

***Cytotoxicity of mixture of ALT-Ts, AFB1 and OTA*:** In natural environments, multiple mycotoxins frequently coexist, requiring the assessment of the cytotoxic impacts of various mycotoxin combinations. In this regard, the mixture ALT-Ts, AFB1 and OTA showed a synergistic toxicity against HepG-2 cells in a manner dependent on both time and dose at concentration range from 7.8 to 1000 µg/ml and the determined IC_{50} after 48 hr attained 31.05 µg/ml (Fig. 1D).

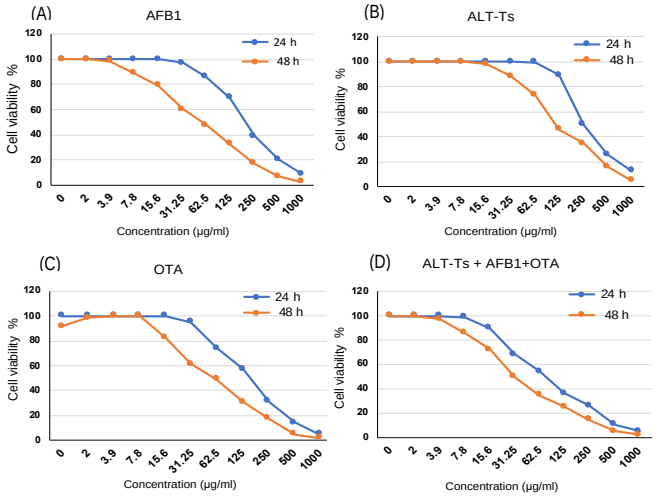

Figure 1. Cell viability of HepG-2 cells following 24 and 48 hr. of incubation with AFB1 (A). ALT-Ts (B), OTA (C) and mixture of ALT-Ts, AFB1 and OTA (D) at various concentrations.

Based on the IC_{50} value, such combination led to greater decline in cell viability in comparison with the individually tested toxins. Our findings on HepG-2 cells align with earlier research, indicating the synergistic effect of the combined different mycotoxins against various cell lines (Skrzydlewski *et al*., 2022; Ismail *et al*., 2023; Tavares *et al*., 2015; Pinhão *et al*., 2020; Juan-García *et al*., 2019; Gayathri *et al*., 2015).

Table 2. The calculated IC_{50} results of AFB1, ALT-Ts, OTA, and their mixture against HepG-2 cells.

Mycotoxins	IC_{50} ± SD (µg/ml)	
	24 h	48 h
AFB1	204.41±3.91	56.68±1.97
ALT-Ts	248.84±4.68	115.95±2.85
OTA	160.02±5.17	59.86±2.05
Mixture	76.36±2.65	31.05±0.96

IC_{50} values are reported as mean ± SE (n = 3). Aflatoxin B1=AFB1; ALT-Ts= *Alternaria* toxins; and ochratoxin A=OTA

***Microscopic examination*:** Morphological changes were observed in treated HepG-2 cells exposed to AFB1, ALT-Ts, OTA and their mixture (Fig. 2) after 48 h of incubation. In the same culture, these abnormalities appeared as reduced cell adhesion and increased intercellular spaces, rounding of cells, and cells and cell walls deterioration were also noticed. Also, cytoplasmic contraction and the emergence of blebs on cell surface were noted, leading to the formation of apoptotic bodies and subsequent cell death, agreeing with the decreased cell viability detected in the MTT test. OTA was reported to disturb cell membrane integrity and proliferation (Köhler *et al*., 2003).

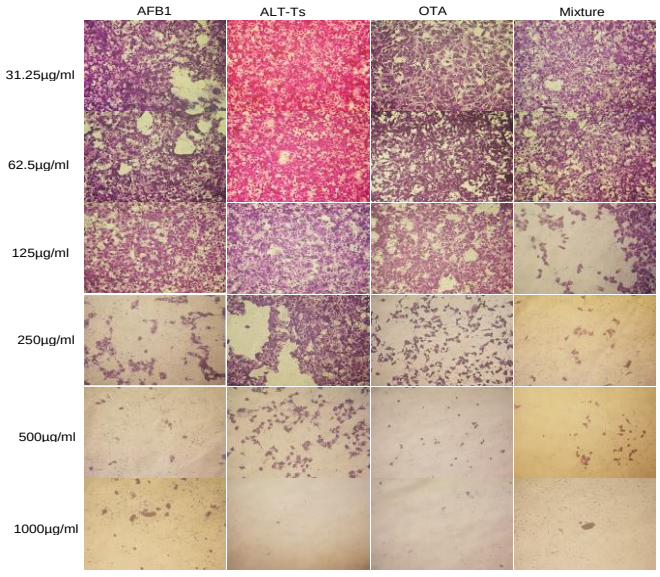

Figure 2. Morphological abnormalities in HepG-2 cells after exposure to AFB1, ALT-Ts, OTA and their mixture (ALT-Ts, AFB1 and OTA) at various concentrations for 48 hr.

The morphological alterations induced by AFB1 in our study resemble those previously documented in the literature on HepG-2 cells and other human cell lines (Kumari *et al*., 2020; Adam *et al*., 2022; Reddy *et al*., 2006). Furthermore, the recorded results of ALT-Ts agree with those of Ismail *et al*. (2023) who recognized abnormal growth patterns in PCS-200-014 and WI-38 cells treated with mixture of TeA, AOH, ALT, and AS after 24 hours. Our findings are consistent also with those int the literature of which the combined *Alternaria* toxins can cause various morphological alterations in human bronchial and colon epithelial cells after 24 hours (Grover and Lawrence, 2017; Groestlinger *et al*., 2022).

***ROS and inflammatory markers in HepG-2 cells*:** The results of ROS-related markers levels in both individual and mixture of toxin treated cells are illustrated in Fig. 3. In terms of TAC levels, the mycotoxins-treated cells revealed a significant decline in comparison with the control group ($p < 0.05$) (Fig. 3A). Concerning NO activity, a significant decrease was noted by all toxins compared to the control ($p < 0.05$) (Fig. 3B). Furthermore, GSH level was notably lowered in the HepG-2 cells treated with AFB1 compared to other toxins and their combined mixture ($p < 0.05$) (Fig. 3C), which coincided with the results of AFB1 previously reported (Singto *et al*., 2020). It was also reported, that AFB1 decrease the activity of GST, and triggering oxidative stress in HepG-2 cells (Lee *et al*., 2005; Corcuera *et al*., 2011). Furthermore, Chen *et al*. (2023), reported also significant increases in intracellular ROS levels in HepG-2 cells following exposure to AFB1 at high concentration. Our findings of OTA are supported with those of García-Pérez *et al*. (2021), of which OTA at lower doses increased the levels of intracellular GSH, while the higher doses decreased it. Contrasting to our results, Su *et al*. (2023), indicated that OTA triggered oxidative stress in Caco-2 cells and increased levels of ROS. Furthermore, in this study, ALT-Ts decreased the levels of TAC, NO and GSH in comparison with the untreated control. However, the recent study of Marin *et al*. (2024), revealed that AME increased ROS, DNA and protein oxidation. Other *Alternaria* toxins, like alternariol, have been linked to ROS production, and in vitro investigations found that they inhibit antioxidant enzyme defenses and the glutathione protection system (Fernández-Blanco *et al*., 2015; Tiessen *et al*., 2017).

Moreover, the inflammatory marker IL-6 was significantly elevated by all toxins and this elevation was significantly higher by their mixture when compared to their individual treatment and control (Fig. 3D). Conversely, Su *et al*. (2023), demonstrated that OTA markedly elevated the IL-6 level in the supernatant of Caco-2 cells. Regarding the IL-1β and COX-2 expression, the current findings specified that their levels was significantly reduced by all toxins except for OTA, which exhibited an upward trend in the IL-1β and COX-2 level when compared to control (Fig. 3E, F). The later finding was also noted by Su *et al*. (2023), of which OTA increased IL-1β content in Caco-2 cells. Additionally, ALT-Ts was able

to decrease significantly all inflammatory markers (Fig. 3D, E, F). In this regard, Groestlinger *et al.* (2022), reported similar results of which alternariol (AOH) and altertoxin II (ATX-II) downregulated the secretion of IL-8.

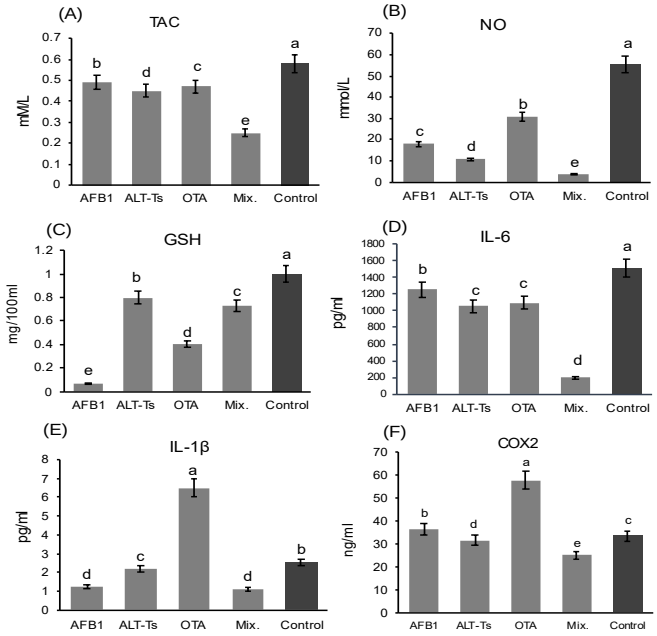

Figure 3. Levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), COX-2, GSH, TAC and NO in the HepG-2 cells treated with the IC_{50} of AFB1, ALT-Ts, OTA and their mixture after 48 hr of incubation. The values are presented as mean ± SD. Means within the same row that are followed by various letters denote significant differences ($p < 0.05$).

***Oxidative stress markers in rats*:** Fig. 4 presents the findings of MDA and TAC assessments in renal tissue. The level of MDA and TAC was significantly raised in all mycotoxins-treated groups compared to the control group. Regarding MDA, only the rats receiving the double dosage of Mix2 (AFB1, ALT-Ts and OTA) exhibited the highest ($p < 0.05$) increase with value of 97.35 Mmol/l compared to single dose of Mix1 with value of 62.17 Mmol/l (Fig. 4). The same trend of results was observed by Abdel-Wahhab *et al.* (2015), who found out that rats receiving combined mixture of AFB1 and OTA, revealed a notable increase in MDA. Similar result was noted in the rats receiving double dose (10 µg/k.bw) of OTA, which exhibited also a notable increase in MDA with value reached 80.53 Mmol/l, when compared to the single dose (5 µg/k.bw) (Ferenczi *et al.* 2020). Additionally, an increased MDA level within liver of OTA-treated rats was also noted by Damiano *et al.* (2021). Also, Ashi *et al.* (2023), documented a notable increase in serum levels of MDA and NO was noticed in the AFB1-treated rats in comparison with control. By contrast, Xiao *et al.* (2023) documented a notable decline in the MDA level in AFB1-treated rats. Furthermore, the TAC levels were found significantly ($p < 0.05$) higher in the OTA-treated rats as well as in the rats treated with the double dose of Mix2, with no statistical differences ($p < 0.05$) among them (Fig. 4). Also, the levels of MDA and TAC were considerably elevated in all double dose-treated rats compared to the single dose-treated rats for all individual mycotoxins and their two mixtures ($p < 0.05$). However, the results of Abdel-Wahhab *et al.* (2015), revealed that rats receiving individual OTA or AFB1 exhibited higher level of TAC than rats treated with their combined mixture. Consequently, this study confirmed that AFB1, ALT-Ts, and OTA can cause liver toxicity and kidney cell regeneration in rats, as evidenced by increased AFP and MDA serum levels.

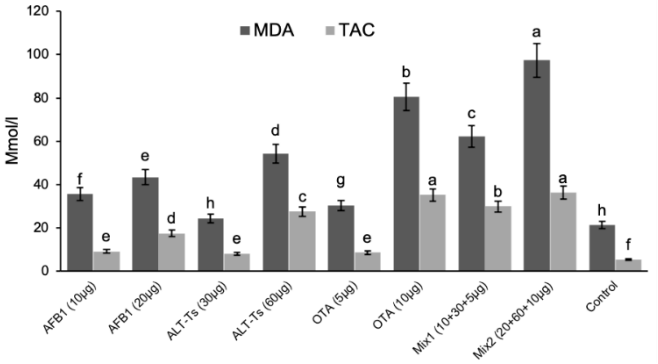

Figure 4. Effect of AFB1, ALT-Ts, OTA and their mixtures (Mix1 and Mix2) on the level of MDA and TAC in kidney tissue of Albino Rats. The values are presented as mean ± SD. Means within the same row that are followed by various letters denote significant differences ($p < 0.05$).

***Hematological analyses*:** Hematological results revealed that the double dose-treated rats with AFB1, OTA and ALT-Ts exhibited a slight increase in RBC count, and this increase being significantly different ($p < 0.05$) among them (Fig. 5). Furthermore, a significant ($p < 0.05$) increasing trend in the WBC count was also noted in the double dose-treated rats in comparison with the single dose-treated and control groups (Fig. 5A). Furthermore, all toxin-treated groups exhibited significant ($p < 0.05$) decline in the Hb and PCV %, especially in double-dose treated groups, but this decrease was appreciably noted in the double dose-treated group of Mix2 (Fig. 5C, D). The results of ALT-Ts are in agreement with those documented in prior research (Ismail *et al.*, 2023; Tang *et al.*, 2022; Chandratre *et al.*, 2014; Wannemacher, 1991) which indicate that *Alternaria* toxins could potentially impair the immune system. Regarding AFB1, our results revealed that AFB1-treated rats revealed significantly ($p<0.05$) decreased WBCs count relative to control (Fig. 5A). Conversely, Ashi *et al.* (2023), reported insignificant

differences in WBCs and RBCs counts in AFB1-treated and untreated control rats. Khaled and Thalij (2021) observed a notable rise in WBCs count in rats administered AFB1 at different concentrations (39.5, 37.9, 32.7, and 29.0 µg/kg) in comparison with the control group. Similarly, Ramamurthy and Rajakumar (2016) reported an increased WBCs in rats treated with aflatoxin relative to the controls. Our study exhibited that rats treated with single and double dose of OTA or Mix2 showed the greatest increase in WBCs (Fig. 5A).

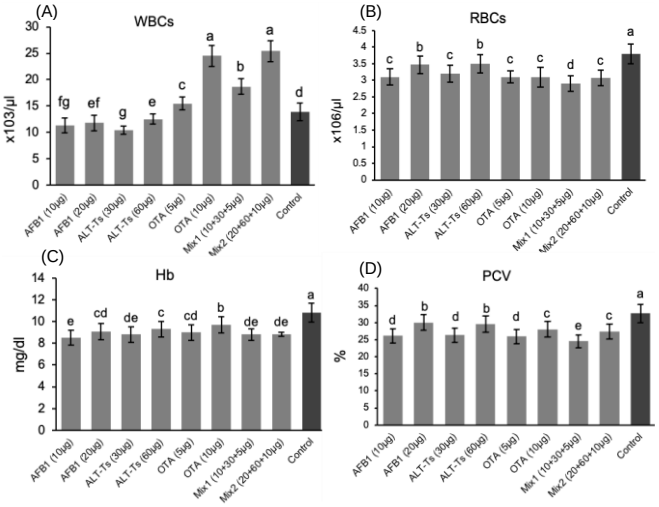

Figure 5. Effect of AFB1, ALT-Ts, OTA and their mixtures (Mix1 and Mix2) on the level of WBCs, RBCs, Hb and PCV in Albino Rats. The values are presented as mean ± SD. Means within the same row that are followed by various letters denote significant differences (p < 0.05).

These results were also recognized by Abed *et al*. (2019), who documented a significant elevation in WBCs and RBCs ($p < 0.05$) with increasing doses of OTA. Rats treated with mycotoxins exhibited a slight reduction in Hb and PCV at high doses, but still significantly ($p < 0.05$) lower than control (Fig. 5A, B). However, Abed *et al*. (2019), recorded no significant differences in Hb and PCV values between OTA-treated and untreated control rats.

***Serum biochemical analyses*:** The enzymatic activity of AST and ALT, which serves as a marker for liver function, was elevated in rats receiving either individual or combined toxins at single and double dose treatments in comparison with untreated control group (Fig. 6). The ALT and AST levels were greatly increased in the double dose-treated rats, being the highest value observed in the Mix2-treated rats; while the differences between the certain groups were statistically significant ($p < 0.05$) (Fig. 6). Additionally, serum urea and creatinine levels showed significant increases ($p < 0.05$) in rats treated with both individual and combined toxins, whether at single or double doses (Fig. 7A, B). However, there was no significant ($p < 0.05$) difference in the levels of serum urea and creatinine between some treatments (Fig. 7A, B). The results indicated that this rise was not indicative of kidney failure. The same trend of ALT-Ts results was also reported by earlier reports (Ismail *et al*., 2023; Tang *et al*., 2022; Chandratre *et al*., 2014; Meloche and Smith, 1995). Furthermore, Abdel-Wahhab *et al*. (2015), demonstrated that individual OTA or AFB1 resulted in a significantly increased ALT and AST, urea and creatinine levels, while this rise was more pronounced in the group treated with their combination. Moreover, in previous reports, a significantly increased ALT and AST levels were also detected in OTA-treated rats (Damiano *et al*., 2021; Gagliano *et al*., 2006). These findings clearly demonstrated that both AFB1 and OTA exert stress on hepatic and renal tissues, aligning with reports from existing mycotoxicosis literature (Ismail *et al*., 2023; Ashi *et al*., 2023; Abed *et al*., 2019; Abdel-Wahhab *et al*., 2015)

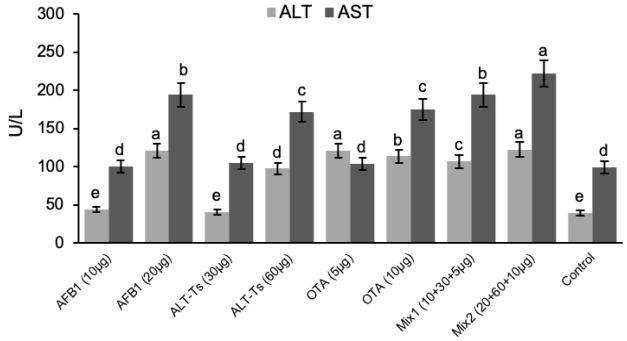

Figure 6. Effect of AFB1, ALT-Ts, OTA and their mixtures (Mix1 and Mix2) on the level of enzymatic activity of ALT and AST in liver of albino rats. The values are presented as mean ± SD. Means within the same row that are followed by various letters denote significant differences (p < 0.05).

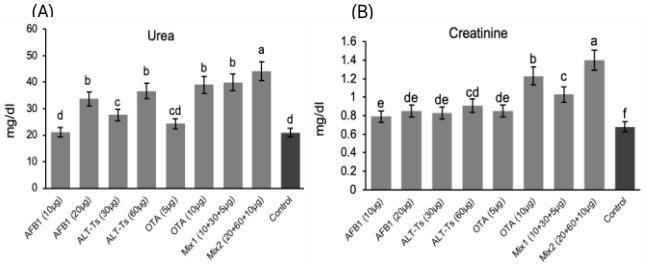

Figure 7. Effect of AFB1, ALT-Ts, OTA and their mixtures (Mix1 and Mix2) on the level of urea and creatinine in kidney of albino rats. The values are presented as mean ± SD. Means within the same row that are followed by various letters denote significant differences (p < 0.05).

***Histopathological examinations*:** Microscopic evaluation of liver tissues from the control group exhibited no

histopathological changes, revealing the normal architecture of the central vein and adjacent hepatocytes within the parenchyma (Fig. 8A). Furthermore, there was no histopathological changes in the tubules and glomeruli at the cortex of untreated rat's kidney (Fig. 9A) as well as in the white and red pulps with sinusoids (Fig. 10A). There was no histopathological alteration in the liver and kidneys of AFB1(10µg)-treated rats (Fig. 8B) and (Fig. 9B).

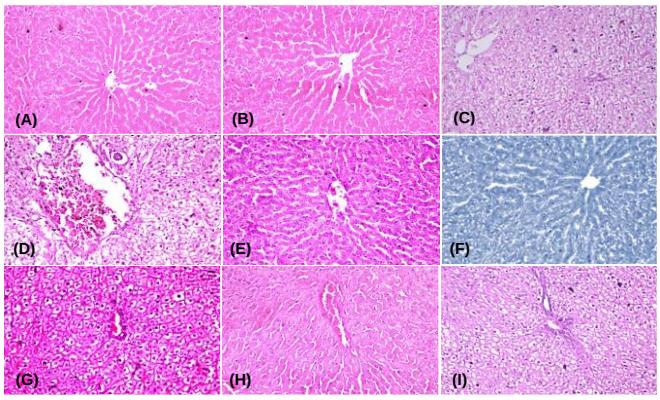

Figure 8. Histopathological features of liver of untreated control (A), AFB1-10µg (B) and OTA-5µg (F) treated rats exhibited no histopathological changes, maintaining central vein with normal histological structure and adjacent hepatocytes within the parenchyma. The hepatocytes showed vacuolar hydropic degenerative change for AFB1-20µg-treated rats (C); congested central veins for ALT-Ts 30µg-treated rats (D); and vacoular degeneration in the hepatocytes for ALT-Ts-60µg-treated rats (E); hepatocytes of OTA-10µg-treated rats showed milde vacuolar degeneration (G); congested central viens of animals treated with Mix1 (AFB1-10µg, OTA-5µg and ALT-Ts-30µg) (H); mild fibrosisin portal area associated with degeneration in hepatocytes in rats treated with Mix2 (AFB1-20µg, OTA-10µg and ALT-Ts 60µg) (I).

Moreover, the white pulps of spleen of AFB1(10µg)-treated rats showed hyperplasia (Fig. 10B). However, AFB1 (20µg)-treated rats exhibited vacuolar hydropic degenerative change of hepatocytes (Fig. 8C). On other hand, kidney of AFB1 (20µg)-treated rats showed focal infiltration of inflammatory cells between the tubules at corticomedullary junction (Fig. 9C), while spleen in AFB1 (20µg)-treated rats showed lymphoid cell depletion in the white pulp (Fig. 10C). The above-mentioned histopathological alterations in kidney and liver observed in our study were consistent with the alterations observed in previous studies indicating toxicity of AFB1to kidney and liver (Abdel-Wahhab *et al*., 2015; Albassam *et al*., 1987) lung and testes (Ashi *et al*., 2023). Additionally, mild vacuolar degeneration in the hepatocytes liver was recognized in OTA (10µg)-treated rats (Fig. 8G). Likewise, Abdel-Wahhab *et al*. (2015) stated that OTA-treated rats displayed notable liver histological changes, especially surrounding the expanded portal tracts. Moreover, kidney of OTA (10µg)-treated rats showed corticomedullary degeneration in the renal tubular epithelium (Fig. 9G). Consistent with the current findings, OTA administration also led to tubular necrosis and dilation, accompanied by pyknosis of the epithelial cells (Abdel-Wahhab *et al*., 2015). Fibrosis in renal tissue was noted in mice treated with OTA, following the administration of the toxin via intraperitoneal injection (2 mg/kg for 14 days) (Mao *et al*., 2022). Histopathological changes in the liver, characterized by significant infiltration of macrophages, were also reported in 14-days-OTA treated rats (Zhang *et al*., 2022). Further to this, OTA (10µg)-treated rats displayed depletion in lymphoid in the white and red pulps and sinusoids were congested (Fig. 10G). These findings were validated in the spleens of mice, where OTA administration resulted in tissue inflammation and histopathological damages (Liu *et al*., 2018).

The central veins of liver were congested in the Mix1-treated rats (Fig. 8H), and the cortex showed degeneration in the tubular lining epithelium (Fig. 9H). In addition, mild fibrosis was identified in the portal region, accompanied by vacuolar degeneration in the hepatocytes of rats treated with Mix2 (Fig. 8I). Likewise, kidneys of Mix2-treated rats revealed degeneration of the epithelial lining in the tubules (Fig. 9I). Both Mix1 and Mix2 treatments resulted in lymphoid depletion within the white pulp of the spleens in the rats (Fig. 10H, I). The Mix1 and Mix 2 revealed severe histological changes in the tested organs resembling those documented in the mycotoxicosis literature (Abdel-Wahhab *et al*., 2010; Abdel-Wahhab *et al*., 2015). ALT-Ts (30µg)-treated rats revealed no histopathological change within liver (Fig. 8D) and kidney (Fig. 9D). However, ALT-Ts (60µg)-treated rats exhibited noticed vacuolar degeneration in hepatocytes associated with congestion (Fig. 8E). Furthermore, a degeneration in the corticomedullary portion of tubular lining epithelium was observed in ALT-Ts (30µg) and ALT-Ts (60µg)-treated rats (Fig. 9E). Numerous researchers have found similar findings regarding the propensity of *Alternaria* toxins to induce cumulative organ damage after 28 days (Miao *et al*., 2022; Tang *et al*., 2022). A prior investigation indicated that *Alternaria* toxins could cause lesions in the spleen and liver of Swiss albino rats following 7 days of dosing (Gupta *et al*., 1981). Furthermore, a recent study of Xin and Shang (2022) revealed substantial histopathological alterations in the essential organs of mice receiving the maximum dosage of 400 mg/kg of AOH; conversely, rats receiving dosages of 50, 100, and 200 mg/kg displayed no histopathological changes in their vital organs.

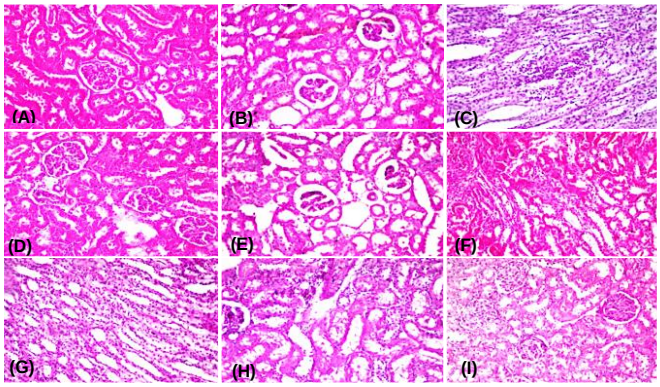

Figure 9. Histopathological features of renal tissue of untreated control group (A); AFB1-10µg (B); and ALT-Ts 30µg-treated rats (D) showed no histopathological changes, preserving the normal histological structure of the glomeruli and tubules at the cortex. AFB1-20µg-treated rats revealed focal inflammation in between the tubules at corticomedullary junction (C); lining of tubular epithelum showed degenerative changes in rats treated with ALT-Ts 60µg (E), OTA-5µg (F); OTA-10µg (G), Mix1 (AFB1-10µg, OTA-5µg and ALT-Ts 30µg) (H), and Mix2 (AFB1-20µg, OTA-10µg and ALT-Ts 60µg) (I).

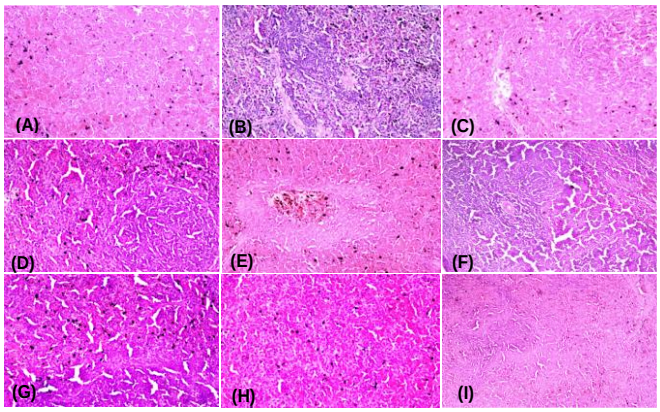

Figure 10. Histopathological features of spleen for untreated control group of rats with no histopathological changes and normal histological structure of the white and red pulps with sinusoids (A); the white pulps showed lymphoid hyperplasia in rats treated with AFB1-10µg (B), ALT-Ts 30µg (D) and OTA-5µg (F); the white pulps showed depletion in lymphoid cells in the rats treated with AFB1-20µg (C), OTA-10µg (E), Mix1 (AFB1-10µg, OTA-5µg and ALT-Ts 30µg) (G), Mix2 (AFB1-20µg, OTA-10µg and ALT-Ts 60µg) (H, I).

Conversely, Zhu *et al*. (2022) established that male Sprague Dawley rats administered altertoxin I (ATX-I) exhibited histopathological damages in the kidney, liver, and spleen, even at relatively modest doses, while showing no notable impact on hematological and serum biochemical markers. Male albino Wistar rats, unexpectedly supplied AME at a daily dosage of 10 mg kg^{-1} for 7 weeks, exhibited normal liver histology, demonstrating the non-toxic characteristics of AME (Palanichamy *et al*., 2019). Lymphoid hyperplasia was detected in the white pulps in spleen of rats treated with AFB1(10µg) (Fig. 10B), ALT-Ts (30µg) (Fig. 10D) and OTA (5µg) (Fig. 10F). Liver of OTA (5µg)-treated rats showed no histopathological alteration (Fig. 8F), while their kidney displayed degeneration changes in lining tubular epithelium (Fig. 9F). Spleen of ALT-Ts (60µg)-treated rats showed congestion in the red pulps and sinusoids (Fig. 10E).

***Conclusion*:** In summary, there is a critical need for additional *in vitro* studies on the toxicity of individual and mixed *Alternaria* toxins to better understand potential synergistic interactions, which may enhance their harmful effects on the health of humans and animals. As a future prospect, it is essential to discover new metabolites of the *Alternaria* toxins along with their metabolic pathways. Furthermore, investigations into the *in vivo* toxicity and toxicokinetic characteristics of these toxins are crucial for accurate risk evaluation. This study established significant toxicological benchmarks, specifically IC_{50} values for AFB1, ALT-Ts, OTA, and their combinations, which facilitates future in vitro cytotoxicity investigations, especially for genotoxicity tests through optimized dosing strategies. The cytotoxic impacts observed from the mixtures were notably stronger than those resulting from the individual mycotoxins, particularly regarding ALT-Ts, indicating that the effects of these toxins are enhanced when combined.

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acquisition, A.M.I. All authors have read and agreed to the published version of the manuscript."

***Conflicts of Interest*:** The authors declare no conflict of interest.

***Ethical approval*:** All procedures carried out in studies involving animals adhered to the ethical standards of the conducting institution or practice (BUFVTM 02-05-24).

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

***Informed consent*:** N/A.

***Consent to participate*:** All authors are participating in this research study.

***Consent for publication*:** All authors are giving their consent to publish this research article in JGIAS.

***SDGs Addressed*:** Zero Hunger, Good Health and Well-being

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