

## Article

# Aflatoxin B1 Detoxification and Antioxidant Effect of Selected Omani Medicinal Plants against Aflatoxin B1-Induced Oxidative Stress Pathogenesis in the Mouse Liver

Rethinasamy Velazhahan <sup>1,\*</sup>, Abdullah Mohammed Al-Sadi <sup>1</sup>, Mostafa I. Waly <sup>2,\*</sup>, Sathish Babu Soundra Pandian <sup>3</sup>, Jamal Al-Sabahi <sup>4</sup> and Khalid Al-Farsi <sup>5</sup>

<sup>1</sup> Department of Plant Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, Al-Khoud, Muscat 123, Oman; alsadi@squ.edu.om

<sup>2</sup> Department of Food Science and Nutrition, College of Agricultural and Marine Sciences, Sultan Qaboos University, Al-Khoud, Muscat 123, Oman

<sup>3</sup> Central Analytical and Applied Research Unit, College of Science, Sultan Qaboos University, Al-Khoud, Muscat 123, Oman; sathish@squ.edu.om

<sup>4</sup> Central Instrumentation Laboratory, College of Agricultural and Marine Sciences, Sultan Qaboos University, Al-Khoud, Muscat 123, Oman; jamal@squ.edu.om

<sup>5</sup> Oman Botanic Garden, Muscat 123, Oman; khalid.alfarsi@omanbotanicgarden.om

\* Correspondence: velazhahan@squ.edu.om (R.V.); mostafa@squ.edu.om (M.I.W.)

**Abstract:** This study investigated the ability of aqueous leaf extracts of *Heliotropium bacciferum* (HE), *Ocimum dhofarensense* (OE), and *Zataria multiflora* (ZE) to detoxify aflatoxin B1 (AFB1) under in vitro and in vivo conditions. The results showed that HE, OE, and ZE degraded 95%, 93%, and 92% of AFB1, respectively, after 72 h incubation at 37 °C. The degradation of AFB1 was validated by liquid chromatography–mass spectrometry analysis. A molecular ion peak at *m/z* 313 specific to AFB1 (C<sub>17</sub>H<sub>12</sub>O<sub>6</sub>) was observed in the mass spectrum of untreated AFB1 (control). However, the level of AFB1 was decreased to untraceable levels in response to treatment with these plant extracts. HE, OE, and ZE effectively detoxified AFB1 in a concentration-dependent manner, resulting in mortality rates of 65, 70, and 75% of brine shrimp, respectively, in contrast to 90% in the untreated AFB1 (control). The hepatoprotective effect of HE, OE, and ZE against AFB1-induced oxidative stress pathogenesis was investigated using mice as an experimental model. Glutathione depletion, impairment of total antioxidant capacity, and increase in DNA oxidative damage were observed in liver tissues of mice treated with AFB1. However, HE, OE, and ZE extract supplementation suppressed the oxidative damage associated with AFB1 treatment. Our findings indicated that HE, OE, and ZE were highly effective in the detoxification of AFB1. In addition, HE, OE, and ZE act as potent antioxidants and combat the AFB1-associated oxidative stress and liver pathogenesis, suggesting that these plants might be valuable for the development of functional foods aimed at minimizing the toxic effects of AFB1.

**Keywords:** degraded aflatoxin B1 products; biological detoxification; food safety; herbal extracts; liquid chromatography–mass spectrometry; mycotoxins

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## 1. Introduction

Illness in humans due to foodborne microbial infection and the presence of mycotoxins has grown to be a severe public health issue over the past few years worldwide. Human foodborne illnesses are caused by different types of parasites, viruses, and bacteria [1]. Ingestion of mycotoxin-contaminated foods often results in sickness [2,3]. The filamentous fungi *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* are the major producers of foodborne mycotoxins, and aflatoxins, fumonisins, ochratoxin, patulin, deoxynivalenol, and zearalenone are the most important toxins [4]. Aflatoxin is considered the most toxic

mycotoxin. Aflatoxigenic molds attack agricultural commodities during the pre- and/or post-harvest phases and produce aflatoxins [5]. Ingestion of food contaminated with aflatoxin causes a disease called “aflatoxicosis” in humans [6].

So far, 21 varieties of aflatoxins have been detected [7]. Aflatoxin G1 (AFG1; MW 328.0578), aflatoxin G2 (AFG2; MW 330.2889), aflatoxin B1 (AFB1; MW 312.2736), and aflatoxin B2 (AFB2; MW 314.2895) are common contaminants in agricultural products [8]. Aflatoxin M1 (AFM1; MW 328.0577), the principal metabolite of AFB1, is often present in the milk of lactating animals that have ingested AFB1-contaminated feed [5]. “AFB1 (C<sub>17</sub>H<sub>12</sub>O<sub>6</sub>)” is recognized as the most serious carcinogenic mycotoxin to humans and animals [9,10]. Several nations have specified maximum tolerance limits for aflatoxins in food for human consumption to safeguard the population from the risk of aflatoxin [11]. The Food and Drug Administration in the USA and the European Union have set regulatory limits of 20 ppb and 4 ppb for foods, respectively [12].

Aflatoxins cannot be completely removed or degraded from contaminated agricultural commodities due to their stability. Currently, a few biological, physical, and chemical approaches are used to detoxify food items containing aflatoxins [13–16]. However, each process has downsides, because the treated foods must be safe for human consumption and their nutritional value must not be changed. Detoxification of aflatoxins using plant products is an effective, biologically safe, and practical approach to reduce their toxic effects on humans. Extracts of *Trachyspermum ammi* [17], *Ocimum tenuiflorum* [18], *Ocimum basilicum* [19], zimmu (*Allium sativum* × *A. cepa*) [20], *Barleria lupulina* [21], *Adhatoda vasica* [22], *Centella asiatica*, *Eclipta prostrata*, and *Hybanthus enneaspermus* [23] have been used for the detoxification of aflatoxins. In the Sultanate of Oman, the existence of more than 250 species of medicinal plants has been reported. These traditional medicinal plants may be potential tools for the biological detoxification of aflatoxins. The current study’s objectives were to (i) analyze the potential of Omani medicinal plants to detoxify AFB1 under in vitro conditions; (ii) analyze the structural changes in AFB1 molecules upon treatment with the selected medicinal plant extracts; (iii) test the toxicity of the degraded products of AFB1 using brine shrimp lethality assay, and (iv) evaluate the role of selected Omani plant extracts in prevention of aflatoxin-induced toxicity during exposure to AFB1 using mice as an experimental model.

## 2. Materials and Methods

### 2.1. Aflatoxin

AFB1 (Sigma-Aldrich, St. Louis, MO, USA) standard solution (1000 µg/mL) was prepared in 100% methanol and subsequently stored in amber-colored glass vials at 4 °C.

### 2.2. Plant Materials

Fresh leaves or stems of 57 Omani medicinal plants belonging to 27 families, viz., Amaranthaceae (1), Anacardiaceae (1), Apocynaceae (5), Asphodelaceae (3), Asteraceae (3), Bignoniaceae (1), Boraginaceae (1), Burseraceae (3), Capparaceae (3), Ephedraceae (1), Euphorbiaceae (6), Fabaceae (4), Lamiaceae (9), Leguminosae (1), Lythraceae (1), Malvaceae (2), Malpighiaceae (1), Moringaceae (1), Myrtaceae (1), Oleaceae (1), Plantaginaceae (1), Primulaceae (1), Resedaceae (1), Rhamnaceae (1), Solanaceae (1), Sterculiaceae (1), and Zygophyllaceae (2), were collected from Oman Botanic Garden, Muscat, and kept at 4 °C and processed in less than 48 h.

Aqueous extracts of the medicinal plants were prepared according to the method described by Velazhahan et al. [17] with some modifications. Briefly, leaves or stems (5 g) were homogenized in 25 mL of sterile distilled water and filtered through a sterile muslin cloth. Subsequently, the filtrate was centrifuged at 14,000× g for 15 min at 5 °C. The collected supernatant was stored at 4 °C for subsequent use.

### 2.3. Test for Detoxification of AFB1 using Medicinal Plant Extracts

An aliquot (250  $\mu$ L) of each plant extract was mixed with 50  $\mu$ L of AFB1 (50  $\mu$ g/L) in a microcentrifuge tube and incubated in a water bath at 37 °C for 72 h to degrade the toxins. Following incubation, 250  $\mu$ L of chloroform was used to extract the mixture's remaining AFB1. The chloroform fraction was collected and evaporated at 60 °C using a water bath, and the residue was dissolved in 50  $\mu$ L of 70% methanol. A RIDASCREEN Aflatoxin B1 kit was applied for AFB1 examination. For control, sterile distilled water (250  $\mu$ L) was mixed with 50  $\mu$ L of AFB1 (50  $\mu$ g/L) and processed similarly to the test samples.

For each plant extract, two replicates were used. The leaf extracts of *Heliotropium bacciferum* Forssk. (Boraginaceae; Accession No. 201600290), *Ocimum dhofarensense* (Sebald) A.J.Paton (Lamiaceae; Accession No. 202000071), and *Zataria multiflora* Boiss. (Lamiaceae; Accession No. 201100114), which showed the highest AFB1 degradation activity (above 90%), were selected for further studies.

### 2.4. Analysis of Degraded AFB1 Products

Analysis of aflatoxin B1 was carried out using an Agilent 1290 infinity liquid chromatography unit coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Basel, Switzerland). The separation of AFB1 was accomplished using a reverse-phase Symmetry C8 5  $\mu$ m, 3 mm  $\times$  150 mm column (Waters, Milford, MA, USA), and a 6460 Triple Quad MS detector was used to analyze the degraded products of AFB1 following treatment with the plant extracts as per the LC-MS conditions specified by Al-Owaisi et al. [23]. A thermostated column oven (G1316C) was maintained at 45 °C during the analysis. Mobile phase A was 0.1% formic acid in acetonitrile and mobile phase B was 0.1% formic acid in HPLC-grade water with a solvent flow rate of 0.500 mL/min using a quaternary pump (G4204A). A high-performance autosampler (G4226A) was used to inject 10.0  $\mu$ L of each sample. Mass spectrometry was operated in positive ion mode, the source temperature was set as 300 °C, and the ion spray voltage was set as 4000. The software used for data acquisition was MassHunter workstation Qualitative analysis ver 6.0.633.0.

### 2.5. Brine Shrimp Lethality Assay

Meyer et al. [24] described a brine shrimp mortality experiment for determining the toxicity of AFB1 degradation products. Briefly, 0.3 g of brine shrimp (*Artemia salina*) eggs (INVE Aquaculture Inc., Salt Lake City, UT, USA) were hatched in a container including artificially sterilized seawater (Tetra GmbH, Melle, Germany) in 1 L of distilled water. For 48 h, the vessel was kept at room temperature with continuous aeration and fluorescent light. After incubation, the newly hatched nauplii were collected in a small beaker containing freshly prepared and well-aerated seawater. Brine shrimp toxicity was tested in the presence of aqueous leaf extracts (100  $\mu$ g/mL) containing nine different AFB1 concentrations for each plant extract exposure (5, 10, 20, 30, 45, 60, 75, 90, and 100  $\mu$ g/mL). A total of 10 ml of each AFB1 concentration was added into a small Petri dish and 10 shrimp were transferred into each dish and incubated at room temperature (25  $\pm$  2 °C). After 24 h, all shrimp (living and dead) were counted, and the percentage survival of brine shrimp was calculated for each concentration of the AFB1 with plant extract present. The control was the same procedure without a plant extract.

### 2.6. Preparation of Plant Extracts

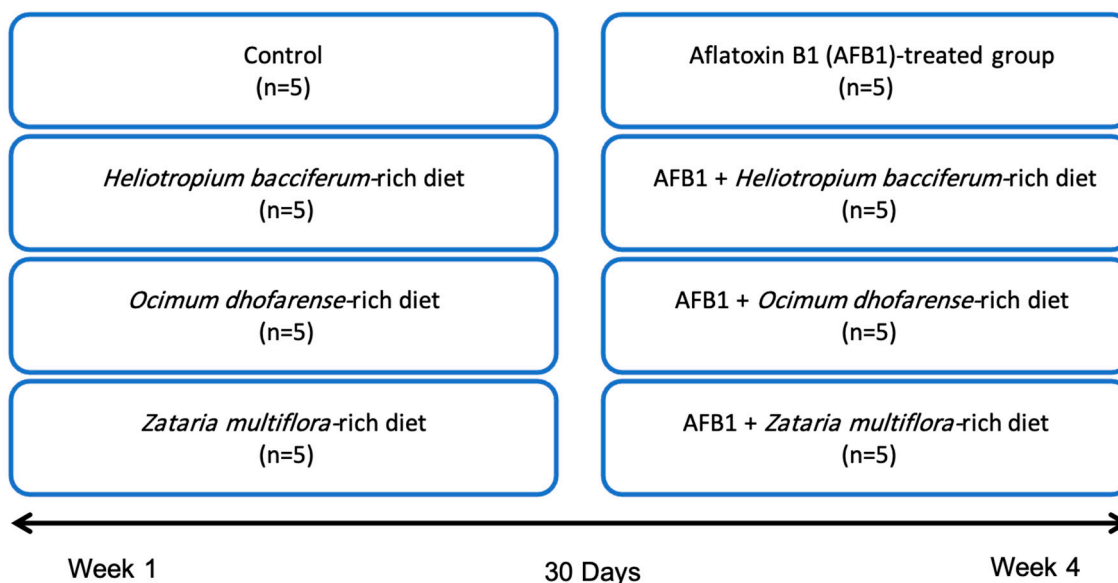
The selected plant leaves (HE, OE, and ZE) were dried for a whole day at 45 °C in an oven and ground into a powder form. A 15 mL polypropylene centrifuge tube containing one gram of the plant powder and 5 ml of sterile distilled water was left to stand at 4 °C overnight. The mixture was then centrifuged at 12,000 $\times$  g at 4 °C for 15 min and the supernatant was taken and its volume was adjusted to 5 mL with sterile distilled water.

### 2.7. Antioxidant Potential of Plant Extracts

The antioxidant ability of plant extracts was detected at concentrations from 10 to 100 µg/mL via 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay using the spectrophotometric method [25].

### 2.8. Mouse Assay Experiments

The protocol used in this study followed the guidelines established by the Sultan Qaboos University Animal Ethics Committee (Ethical approval No. SQU/ECAU/2020-21/4). Forty CD-1 mice, weighing  $50 \pm 5$  g, were obtained from the animal house facility, at Sultan Qaboos University. The animals were distributed into 8 groups ( $n = 5$ ) and housed individually in polypropylene cages under standard growing conditions ( $22 \pm 2$  °C, 60% RH, 12 h light–dark cycle). The animals were fed a standard diet and given tap water ad libitum. On the first day of the experiment, the AFB1-treated group was given 1 mL single intraperitoneal injection of AFB1 (1.5 mg/kg body weight) per mouse, while the control group received 1 mL single intraperitoneal injection of 0.9% physiological saline. The other six groups received an intragastric intubation of plant extracts in the presence or absence of AFB1 injection (Figure 1). The effective dose of plant extracts was determined based on the results of the DPPH assay. The duration of the experiment was 30 days and food consumption was recorded daily. The body weight of the animals was recorded weekly.



**Figure 1.** Experimental design.

### 2.9. Mouse Sacrifice

All mice were anesthetized at the end of the experiment using a lethal dose of a solution of 1 mg ketamine, 5 mg xylazine, and 0.2 mg acepromazine. Livers were dissected and homogenized in phosphate-buffered saline solution (PBS; pH ~ 7.4). The homogenate was centrifuged at  $6000 \times g$  for 60 min at 4 °C, and the supernatant was used for biochemical investigations.

### 2.10. Biochemical Tests

Protein concentration was detected using bovine serum albumin (BSA) as the standard, as established by Lowry et al. [26]. A glutathione (GSH) fluorometric assay kit (Bio-Vision Inc., Milpitas, CA, USA) was used to determine GSH content. Total antioxidant capacity (TAC) was assayed colorimetrically using a Randox assay kit (Randox

Laboratories Ltd., Crumlin, UK). DNA oxidative damage was measured using the Abcam DNA Damage Assay Kit (Abcam, Waltham, MA, USA).

### 2.11. Histopathological Studies

Liver tissues were kept at room temperature in 10% formalin. They were then dehydrated in graded ethanol using a Microm STP120 instrument for 12 h. Subsequently, they were cleared in xylene and embedded in paraffin using a HistoStar embedding unit. Samples were sectioned into 10 µm pieces using a rotary microtome and stained with hematoxylin and eosin. Changes in the liver tissues were examined with an Olympus BX51 microscope with an Olympus camera DP70 (Olympus Corporation, Hachioji, Tokyo, Japan) at 200 X magnification.

### 2.12. Statistical Analysis

The results from the animal studies are expressed as means ± standard deviation (SDs). Data were analyzed using GraphPad Prism 5.03 (GraphPad Software, Boston, MA, USA). The data were analyzed using a general-linear-model ANOVA and the differences ( $p < 0.05$ ) between treatment means were determined by Tukey's test.

## 3. Results

### 3.1. Screening of Medicinal Plants for AFB1 Detoxification

Aqueous extracts derived from 57 Omani medicinal plants were assessed for their potential to detoxify AFB1. Among them, the leaf extracts of *H. bacciferum*, *O. dhofarensis*, and *Z. multiflora* degraded 95%, 93% and 92% of AFB1, respectively, after incubation for 72 h at 37 °C (Table 1). The leaf extracts of *Lavandula dhofarensis* subsp. *ayunensis* A.G. Mill. (Lamiaceae), *Lavandula subnuda* Benth. (Lamiaceae), *Salvia hillcoatiae* Hedge (Lamiaceae), *Tecomella undulata* (Sm.) Seem. (Bignoniaceae), *Ephedra foliata* Fisch. & C.A.Mey. (Ephedraceae), and *Kleinia odora* (Forssk.) DC. (Asteraceae) degraded more than 80% of AFB1. Fourteen plant species' aqueous extracts failed to detoxify AFB1, while the remaining extracts demonstrated intermediate levels of potential detoxification. The leaf extracts of *H. bacciferum* (HE), *O. dhofarensis* (OE), and *Z. multiflora* (ZE) were selected to be used in further studies.

**Table 1.** Detoxification of aflatoxin B1 by Omani medicinal plants.

Name of the Plant	Family	Accession Number	AFB1 Detoxification (%)
<i>Aerva javanica</i> (Burm.f.) Juss. ex Schult.	Amaranthaceae	201800506	33
<i>Searsia aucheri</i> (Boiss.) Moffett	Anacardiaceae	201800777	0
<i>Desmidorchis adenensis</i> (Deflers) Meve & Liede	Apocynaceae	200604436	6
<i>Desmidorchis arabica</i> (N.E.Br.) Meve & Liede	Apocynaceae	201800303	0
<i>Desmidorchis flava</i> (N.E.Br.) Meve & Liede	Apocynaceae	201300308	32
<i>Desmidorchis penicillata</i> (Deflers) Plowes	Apocynaceae	201800596	0
<i>Monolluma quadrangular</i> (Forssk.) Plowes	Apocynaceae	201800011	55
<i>Aloe dhufarensis</i> Lavranos	Asphodelaceae	201000386	32
<i>Aloe praetermissa</i> T.A.McCoy & Lavranos	Asphodelaceae	201000250	0
<i>Aloe vera</i> (L.) Burm.f.	Asphodelaceae	201800270	10
<i>Kleinia odora</i> (Forssk.) DC.	Asteraceae	201000077	85
<i>Pluchea arabica</i> (Boiss.) Qaiser & Lack	Asteraceae	201000388	58
<i>Pulicaria glutinosa</i> (Boiss.) Jaub. & Spach	Asteraceae	201200418	40
<i>Tecomella undulata</i> (Sm.) Seem.	Bignoniaceae	200817492	85
<i>Heliotropium bacciferum</i> Forssk.	Boraginaceae	201600290	95
<i>Boswellia sacra</i> Flück.	Burseraceae	201300064	52

<i>Commiphora gileadensis</i> (L.) C. Christ.	Burseraceae	201000695	18
<i>Commiphora wightii</i> (Arnott) Bhandari	Burseraceae	200616808	16
<i>Capparis cartilaginea</i> Decne.	Capparaceae	201001415	42
<i>Capparis spinosa</i> L.	Capparaceae	201800536	22
<i>Maerua crassifolia</i> Forssk.	Capparaceae	200602732	24
<i>Ephedra ciliata</i> Fisch. & C.A.Mey.	Ephedraceae	201600189	88
<i>Euphorbia</i> aff. <i>Uzmuk</i> S.Carter & J.R.I.Wood	Euphorbiaceae	200604709	21
<i>Euphorbia larica</i> Boiss.	Euphorbiaceae	201800015	34
<i>Euphorbia balsamifera</i> subsp. <i>adenensis</i> (De- flers) P.R.O.Bally	Euphorbiaceae	200717381	7
<i>Euphorbia cactus</i> Ehrenb. ex Boiss.	Euphorbiaceae	201600143	0
<i>Euphorbia smithii</i> S.Carter	Euphorbiaceae	201200704	32
<i>Jatropha dhofarica</i> Radcl.-Sm.	Euphorbiaceae	201000400	10
<i>Vachellia flava</i> (Forssk.) Kyal. & Boatwr.	Fabaceae	201900813	50
<i>Prosopis cineraria</i> (L.) Druce	Fabaceae	200918235	8
<i>Tephrosia purpurea</i> subsp. <i>apollinea</i> (Delile) Hosni & El-Karemy	Fabaceae	201900065	0
<i>Tephrosia nubica</i> (Bioss.) Baker	Fabaceae	201900067	0
<i>Lavandula dhofarensis</i> subsp. <i>ayunensis</i> A.G. Mill.	Lamiaceae	201800010	86
<i>Lavandula subnuda</i> Benth.	Lamiaceae	201900964	81
<i>Ocimum dhofarensis</i> (Sebald) A.J.Paton	Lamiaceae	202000071	93
<i>Coleus barbatus</i> (Andrews) Benth. ex G.Don	Lamiaceae	201600466	70
<i>Coleus cylindraceus</i> (Hochst. ex Benth.) A.J.Paton	Lamiaceae	201800554	9
<i>Salvia hillcoatiae</i> Hedge	Lamiaceae	201900818	84
<i>Teucrium nummularifolium</i> Baker	Lamiaceae	201800146	62
<i>Teucrium stocksianum</i> subsp. <i>stenophyllum</i> R.A.King	Lamiaceae	201900997	22
<i>Zataria multiflora</i> Boiss.	Lamiaceae	201100114	92
<i>Indigofera articulata</i> Gouan	Leguminosae	201000765	60
<i>Lawsonia inermis</i> L.	Lythraceae	200917997	35
<i>Abutilon fruticosum</i> Guill. & Perr.	Malvaceae	201901182	0
<i>Senra incana</i> Cav.	Malvaceae	201000211	0
<i>Acridocarpus orientalis</i> A.Juss.	Malpighiaceae	201500463	0
<i>Moringa peregrina</i> (Forssk.) Fiori	Moringaceae	200609782	8
<i>Myrtus communis</i> L.	Myrtaceae	201500311	0
<i>Olea europaea</i> subsp. <i>cuspidate</i> (Wall. & G.Don) Cif.	Oleaceae	200717412	0
<i>Schweinfurthia spinosa</i> A.G.Mill., M.Short & D.A.Sutton	Plantaginaceae	201800698	34
<i>Dionysia mira</i> (Jaub. & Spach.) Wendelbo	Primulaceae	201800663	33
<i>Ochradenus arabicus</i> Chaudhary, Hillc. & A.G. Mill.	Resedaceae	201900726	35
<i>Ziziphus spina-christi</i> (L.) Desf.	Rhamnaceae	200817484	71
<i>Hyoscyamus gallagheri</i> A.G.Mill. & Biagi	Solanaceae	201900407	35
<i>Hermannia paniculata</i> Franch.	Sterculiaceae	201900552	15
<i>Zygophyllum luntii</i> (Baker) Christenh. & Byng	Zygophyllaceae	201901077	0

*Zygophyllum paulayanum* (J.Wagner & Vierth.) Christenh. & Byng

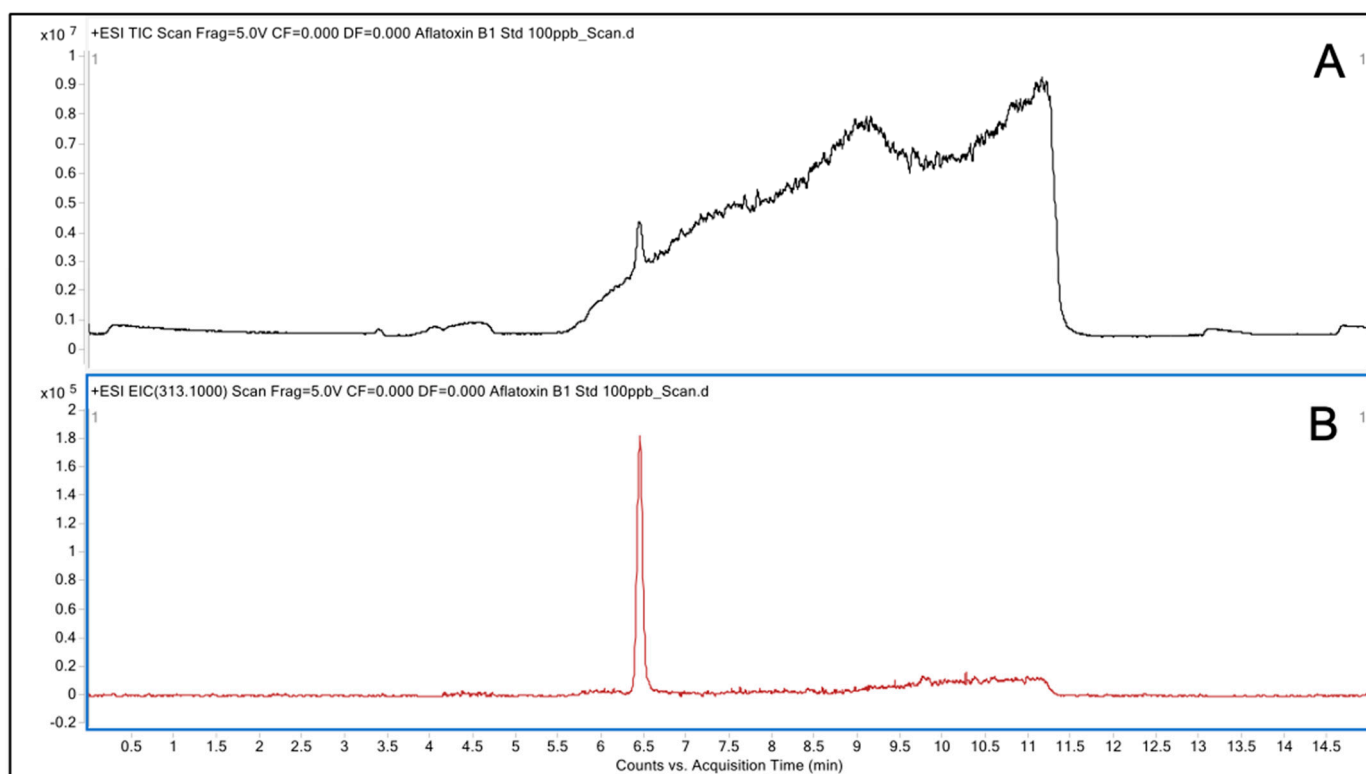
Zygophyllaceae

201800475

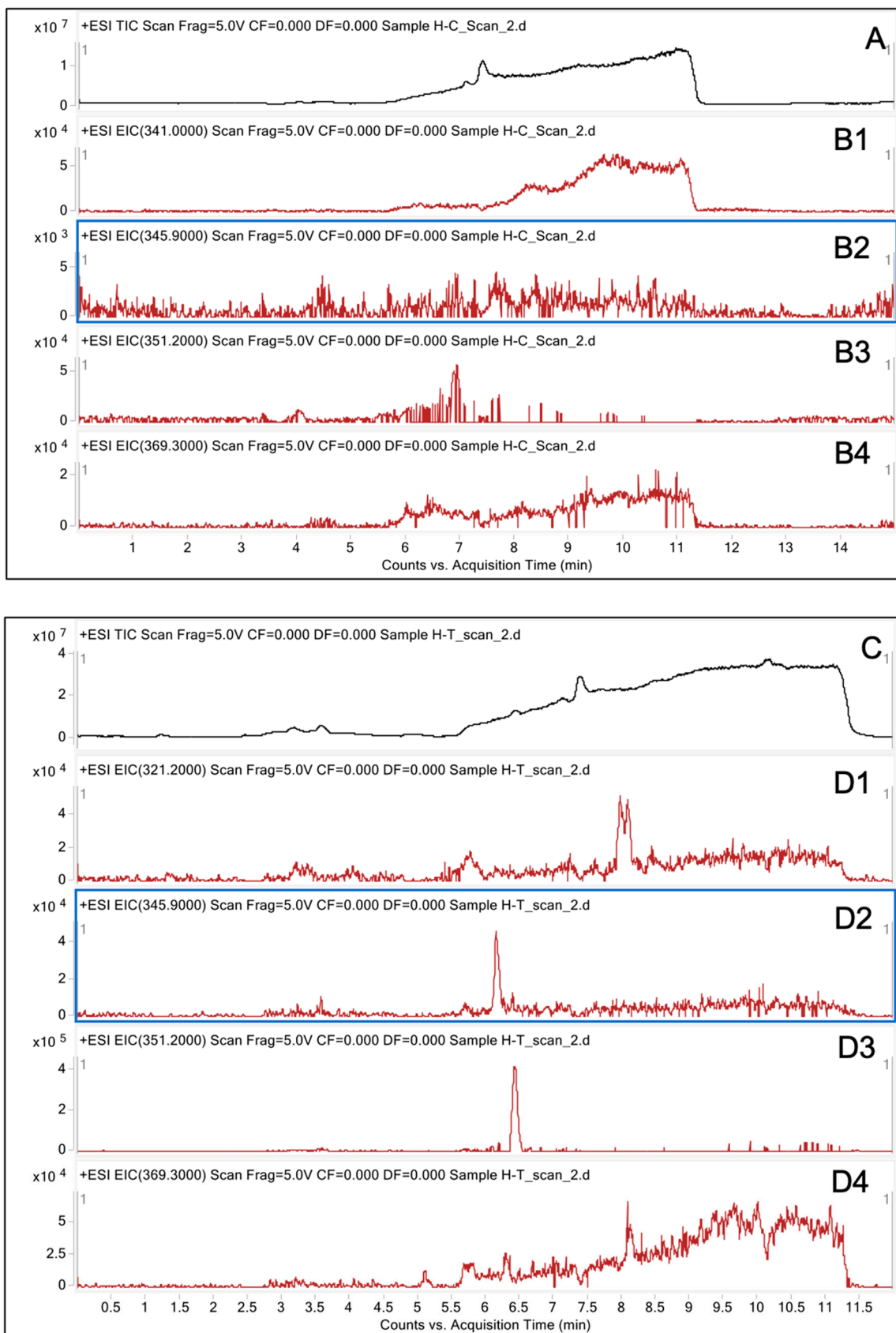
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### 3.2. LC/MS Analysis of Degraded AFB1 Products

The degradation of AFB1 following treatment with medicinal plant extracts was confirmed by LC/MS analysis. The samples were analyzed using ESI positive scan mode, and the chromatogram displayed is the total-ion chromatogram (TIC). AFB1 fragments were extracted from the TIC and are presented as peaks in the extracted-ion chromatogram (EIC). Fragments with too low intensity to show up as peaks in the TIC were confirmed by examining the mass spectrum. LC/MS chromatograms of AFB1 treated with these extracts revealed the presence of additional peaks in comparison to untreated AFB1, confirming the degradation of AFB1 (Figures 2–5). Untreated AFB1 (control) showed a molecular ion peak at  $m/z$  313 in the LC/MS analysis (Figures 6A, 7A, and 8A). This molecular ion  $m/z$  313 was not detected in the plant extracts (Figures 6B, 7B, and 8B). However, the level of AFB1 ( $m/z$  313) after treatment with the plant extracts (HE, OE, and ZE) was reduced to undetectable levels. Fragmented AFB1 products, viz.,  $m/z$  345.9, 369.3, 303.8, 351.2, 341.0, and 321.2, were detected after treatment with the plant extracts (Figures 6C, 7C, and 8C–E).

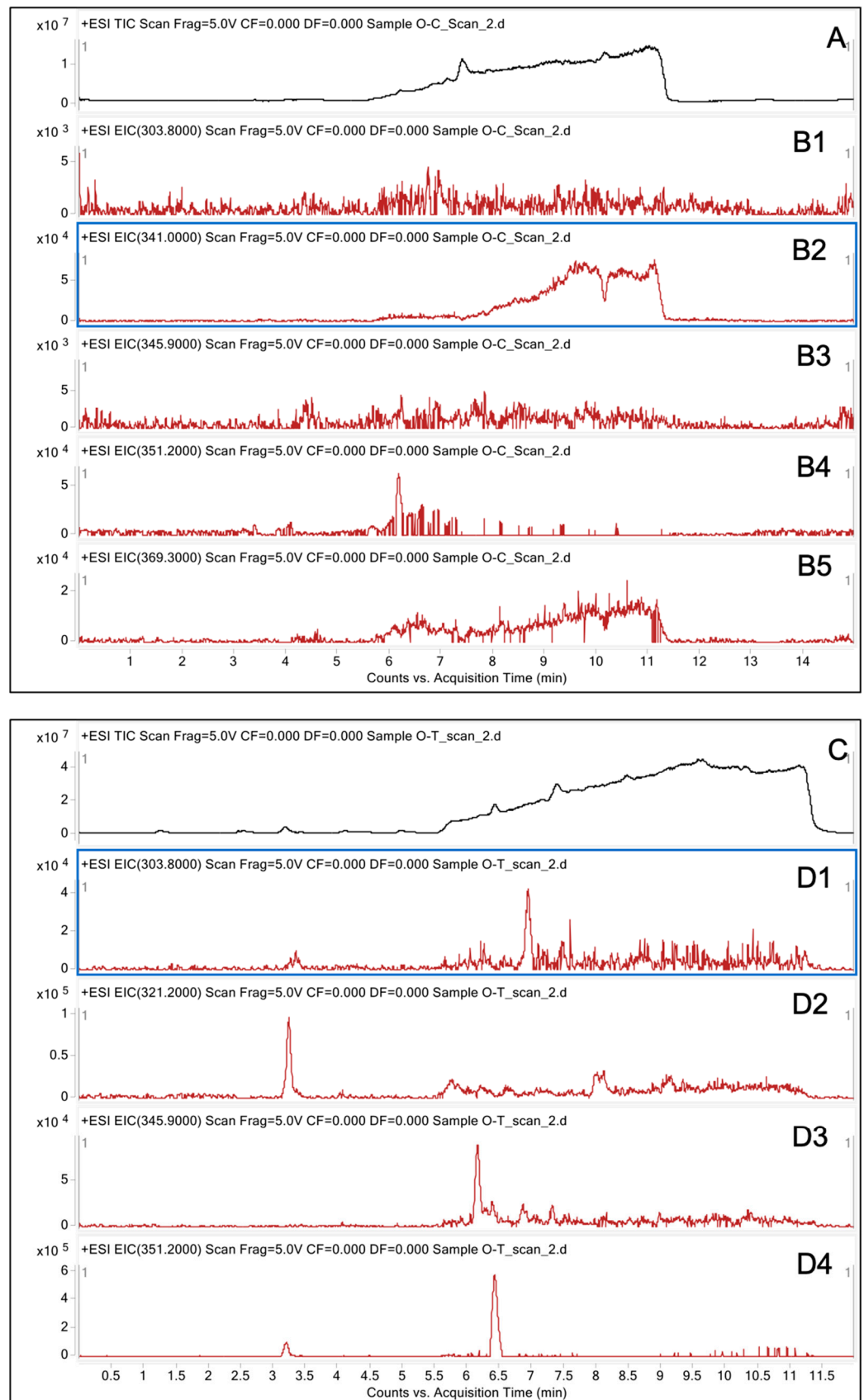


**Figure 2.** Total-ion chromatogram (A) and extracted-ion chromatogram (B) of untreated AFB1 (control).

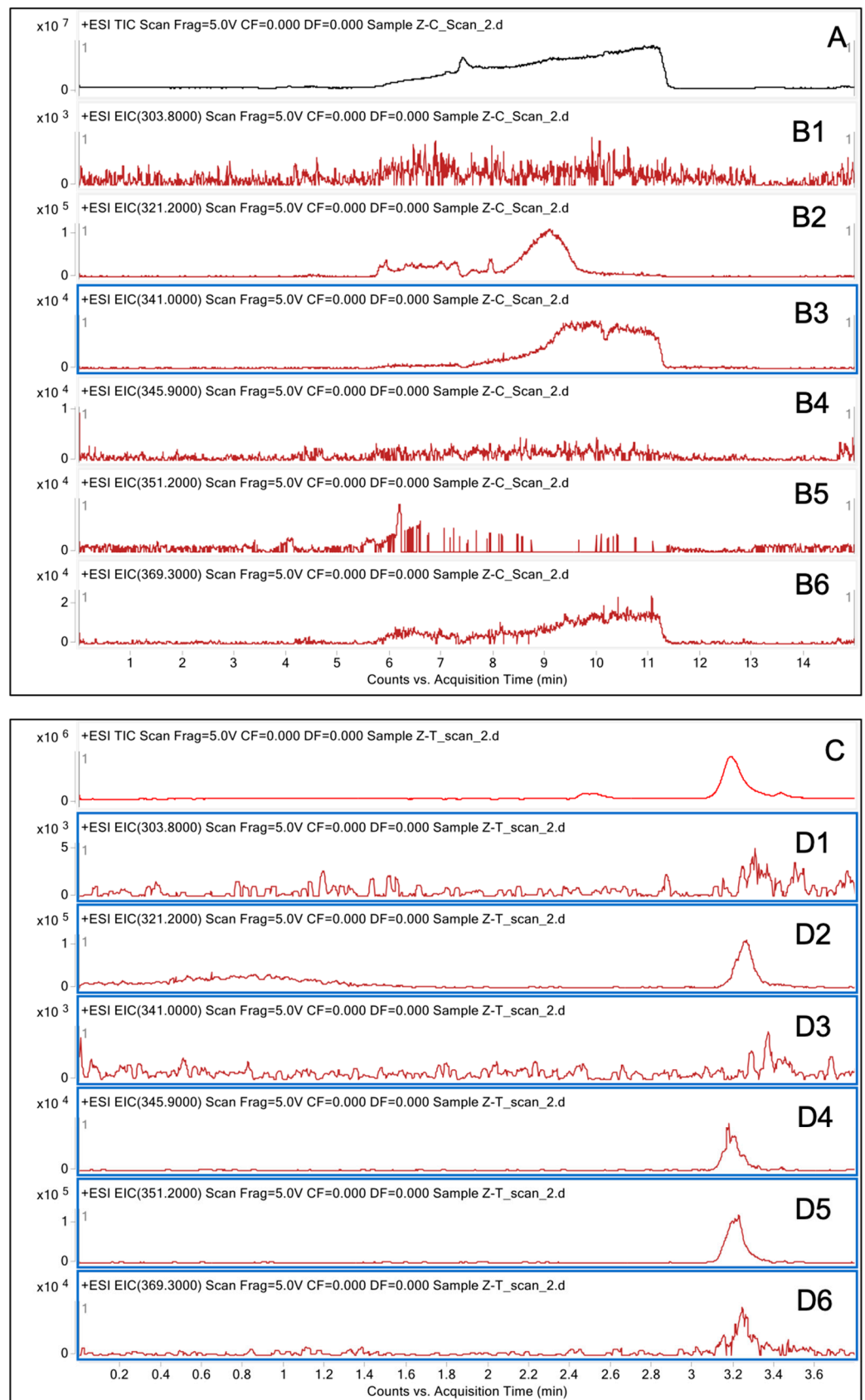


**Figure 3.** Total-ion chromatogram (TIC) and extracted-ion chromatogram (EIC) of AFB1 treated with aqueous extract of *Heliotropium bacciferum*. (A) TIC of *H. bacciferum* extract; (B<sub>1</sub>–B<sub>4</sub>) EIC of *H. bacciferum* extract; (C) TIC of AFB1 treated with *H. bacciferum* extract; (D<sub>1</sub>–D<sub>4</sub>) EIC of AFB1 treated with *H. bacciferum* extract.

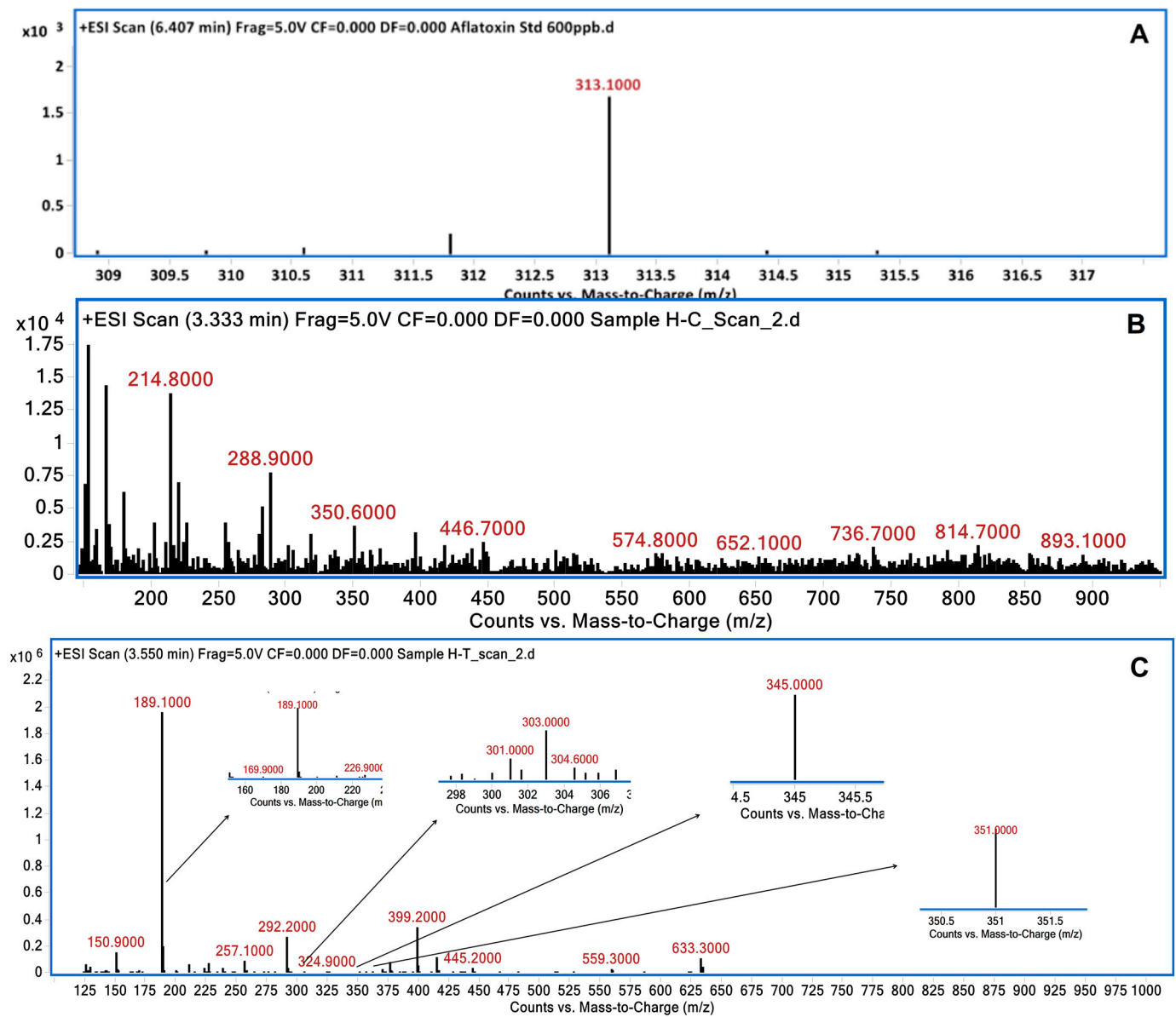




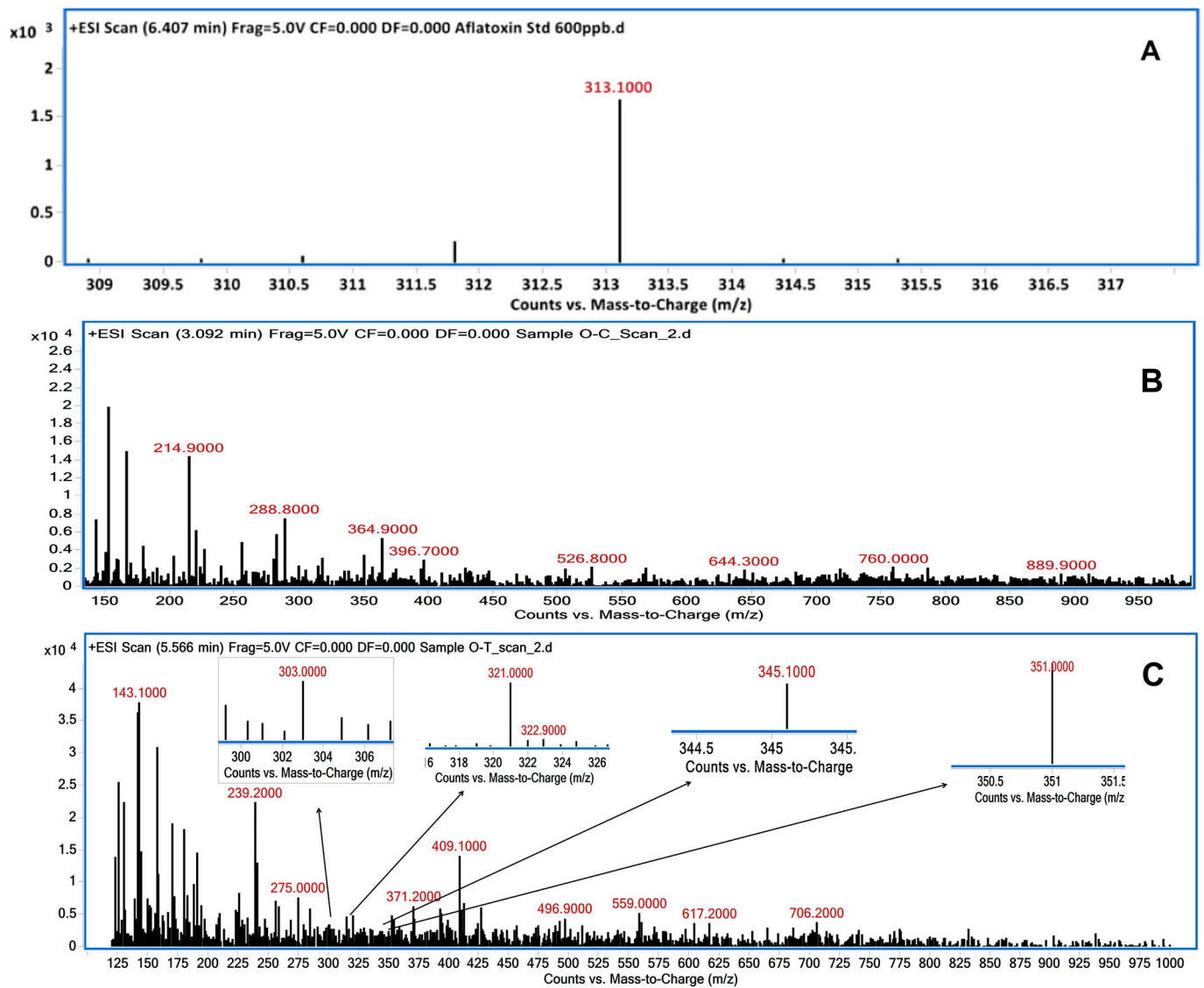
**Figure 4.** Total-ion chromatogram (TIC) and extracted-ion chromatogram (EIC) of AFB1 treated with aqueous extract of *Ocimum dhofarense*. (A) TIC of *O. dhofarense* extract; (B1–B5) EIC of *O. dhofarense* extract; (C) TIC of AFB1 treated with *O. dhofarense* extract; (D1–D4) EIC of AFB1 treated with *O. dhofarense* extract.



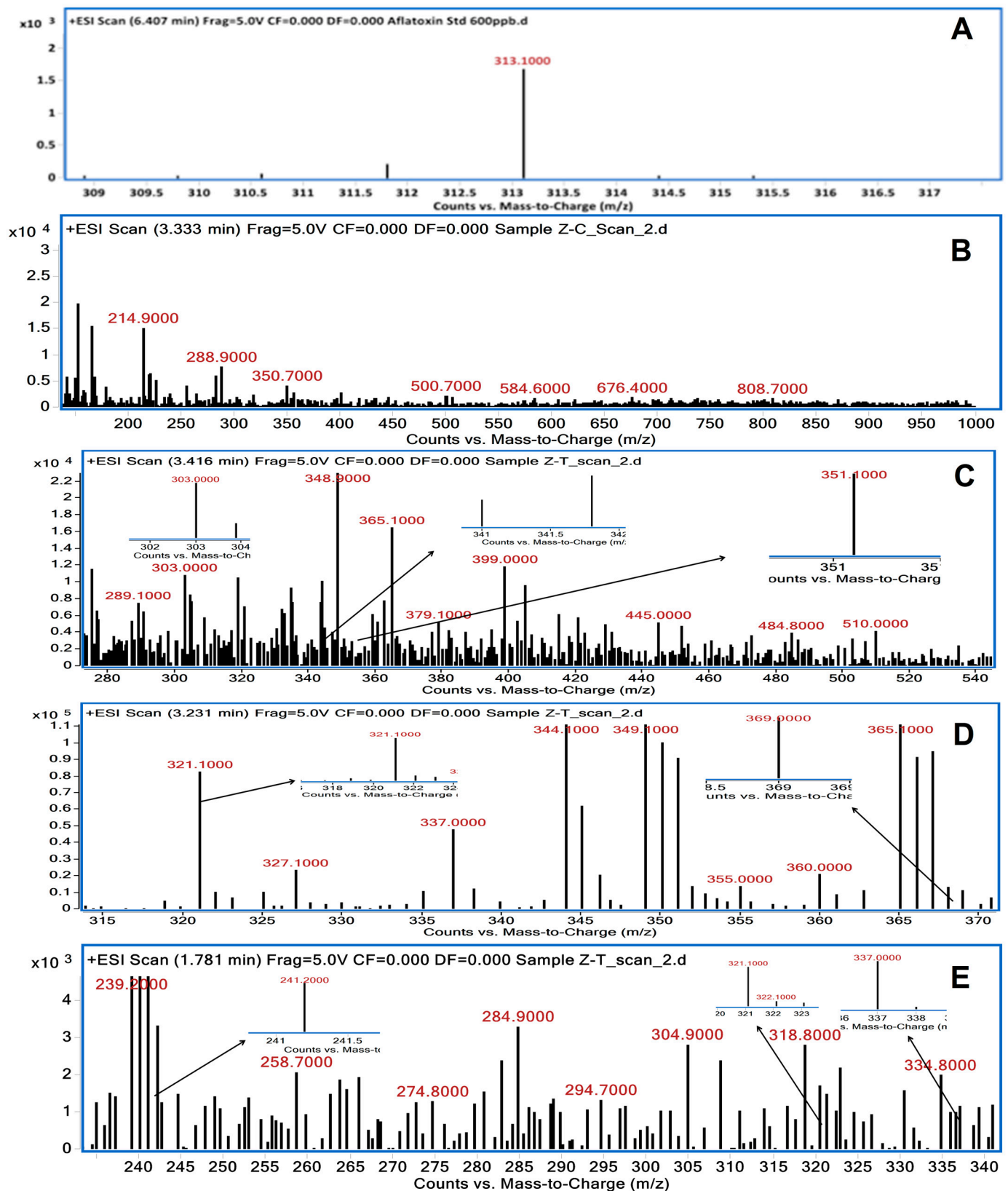
**Figure 5.** Total-ion chromatogram (TIC) and extracted-ion chromatogram (EIC) of AFB1 treated with aqueous extract of *Zataria multiflora*. (A) TIC of *Z. multiflora* extract; (B1–B6) EIC of *Z. multiflora* extract; (C) TIC of AFB1 treated with *Z. multiflora* extract; (D1–D6) EIC of AFB1 treated with *Z. multiflora* extract.



**Figure 6.** Mass spectra of AFB1 treated with aqueous extract of *Heliotropium bacciferum*. (A) Untreated AFB1 (control); (B) *H. bacciferum* extract; (C) AFB1 after treatment with *H. bacciferum* extract.



**Figure 7.** Mass spectra of AFB1 treated with aqueous extract of *Ocimum dhofarense*. (A) Untreated AFB1 (control); (B) *O. dhofarense* extract; (C) AFB1 after treatment with *O. dhofarense* extract.

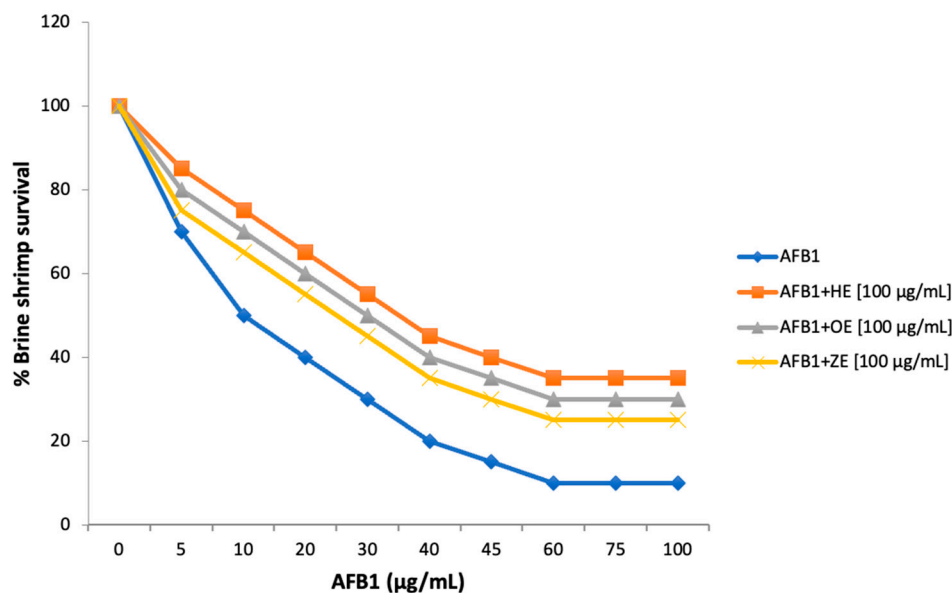


**Figure 8.** Mass spectra of AFB1 treated with aqueous extract of *Zataria multiflora*. (A) Untreated AFB1 (control); (B) *Z. multiflora* extract; (C–E) AFB1 after treatment with *Z. multiflora* extract.

### 3.3. Brine Shrimp Lethality Assay

Brine shrimp lethality bioassay was used to ascertain whether or not AFB1 was detoxified after being treated with the herbal extracts. At a concentration of 100 µg/mL, the

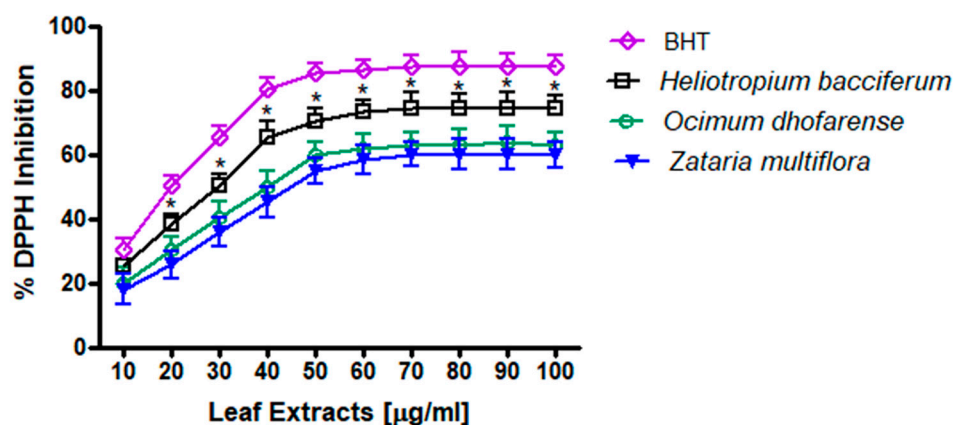
three plant extracts (HE, OE, and ZE) indicated a concentration-dependent detoxification effect on AFB1, with brine shrimp survival rates of 35, 30, and 25%, respectively, compared to 10% in the untreated AFB1 (Figure 9).



**Figure 9.** Effect of *Heliotropium bacciferum* (HE), *Ocimum dhofarens* (OE), and *Zataria multiflora* (ZE) extracts on AFB1-induced toxicity to brine shrimp.

### 3.4. DPPH Measurements

HE, OE, and ZE demonstrated a dose-dependent inhibition of DPPH formation. HE exhibited a significantly higher inhibition rate compared to OE and ZE ( $p < 0.05$ ). The effective dose, 100 µg/mL, was selected for subsequent in vivo animal studies (Figure 10).

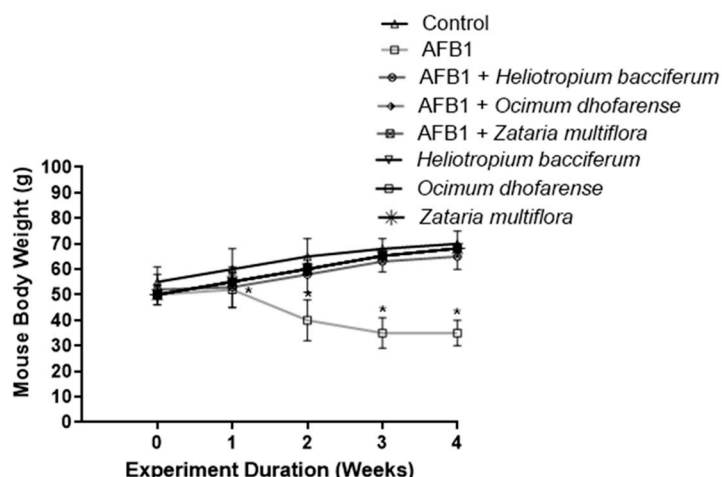


**Figure 10.** Scavenging effect of *Heliotropium bacciferum*, *Ocimum dhofarens*, and *Zataria multiflora* extracts and 2,6-di-tert-butyl-4-hydroxytoluene (BHT) against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical formation. \*Significantly higher as compared to *O. dhofarens* and *Z. multiflora* leaf extracts,  $p < 0.05$ .

### 3.5. Body Weight of Mice

A steady body weight gain was found in all the mouse groups and no mortality was noticed. However, AFB1-injected mice showed a consistent decline ( $p < 0.05$ ) in body weight between week 2 and week 4 compared to the control groups. The daily intragastric intubation of plant extracts prevented weight loss due to AFB1 treatment (Figure 11).

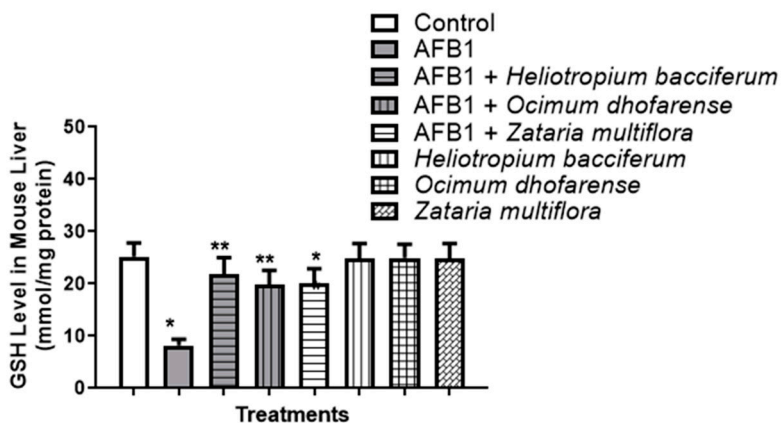




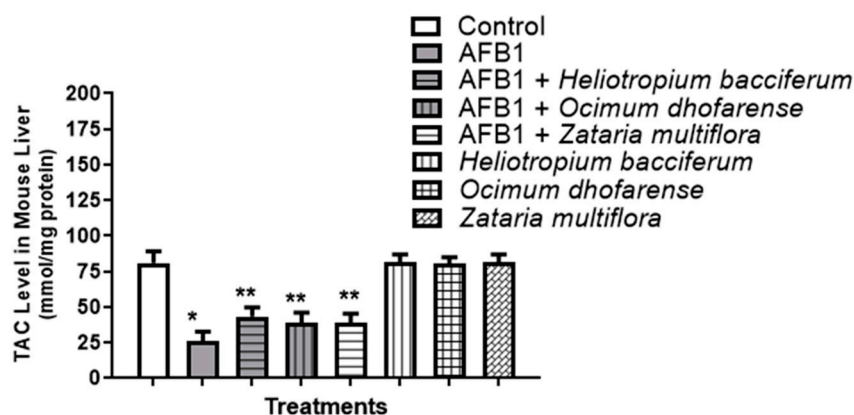
**Figure 11.** Effect of *Heliotropium bacciferum*, *Ocimum dhofarens*, and *Zataria multiflora* extracts and AFB1 on body weight of mice. Mice in the eight groups were examined for changes in their body weight every week for 4 weeks. \* Significantly lower as compared to control group,  $p < 0.05$ . Values without superscript are not significantly different as compared to control group.

### 3.6. AFB1-Induced Oxidative Stress

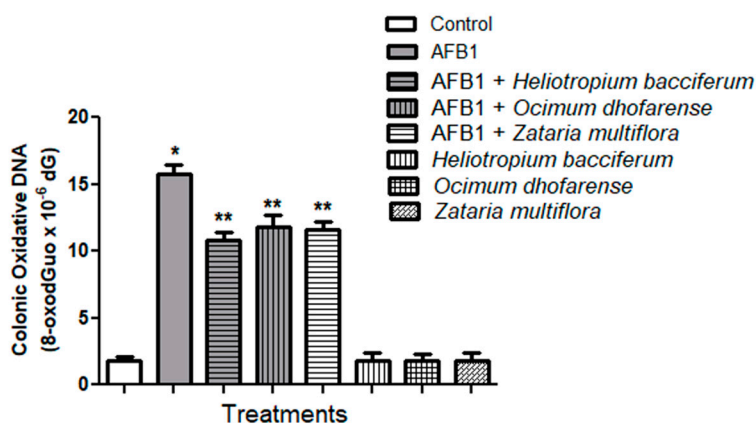
HE, OE, and ZE supplementation counteracted AFB1-induced oxidative stress in the AFB1-injected groups by reinstating the depleted GSH level to a level comparable with that of the control group ( $p > 0.05$ ) (Figure 12). The same pattern was observed for the protective effects of the three plant extracts on mitigating the AFB1-induced effect on TAC (Figure 13) and reducing DNA damage (Figure 14).



**Figure 12.** Glutathione (GSH) levels in liver tissue homogenates of mice fed with *Heliotropium bacciferum*, *Ocimum dhofarens*, and *Zataria multiflora* extracts in the presence or absence of AFB1. \* Significantly lower as compared to control group,  $p < 0.05$ . \*\* Significantly higher than AFB1-injected group,  $p < 0.05$ . Values without superscript are not significantly different as compared to control group,  $p > 0.05$ .



**Figure 13.** Total antioxidant capacity (TAC) levels in liver tissue homogenates of mice fed with *Heliotropium bacciferum*, *Ocimum dhofarens*, and *Zataria multiflora* extracts in the presence or absence of AFB1. \* Significantly lower as compared to control group,  $p < 0.05$ . \*\* Significantly higher than AFB1-injected group,  $p < 0.05$ . Values without superscript are not significantly different as compared to control group,  $p > 0.05$ .

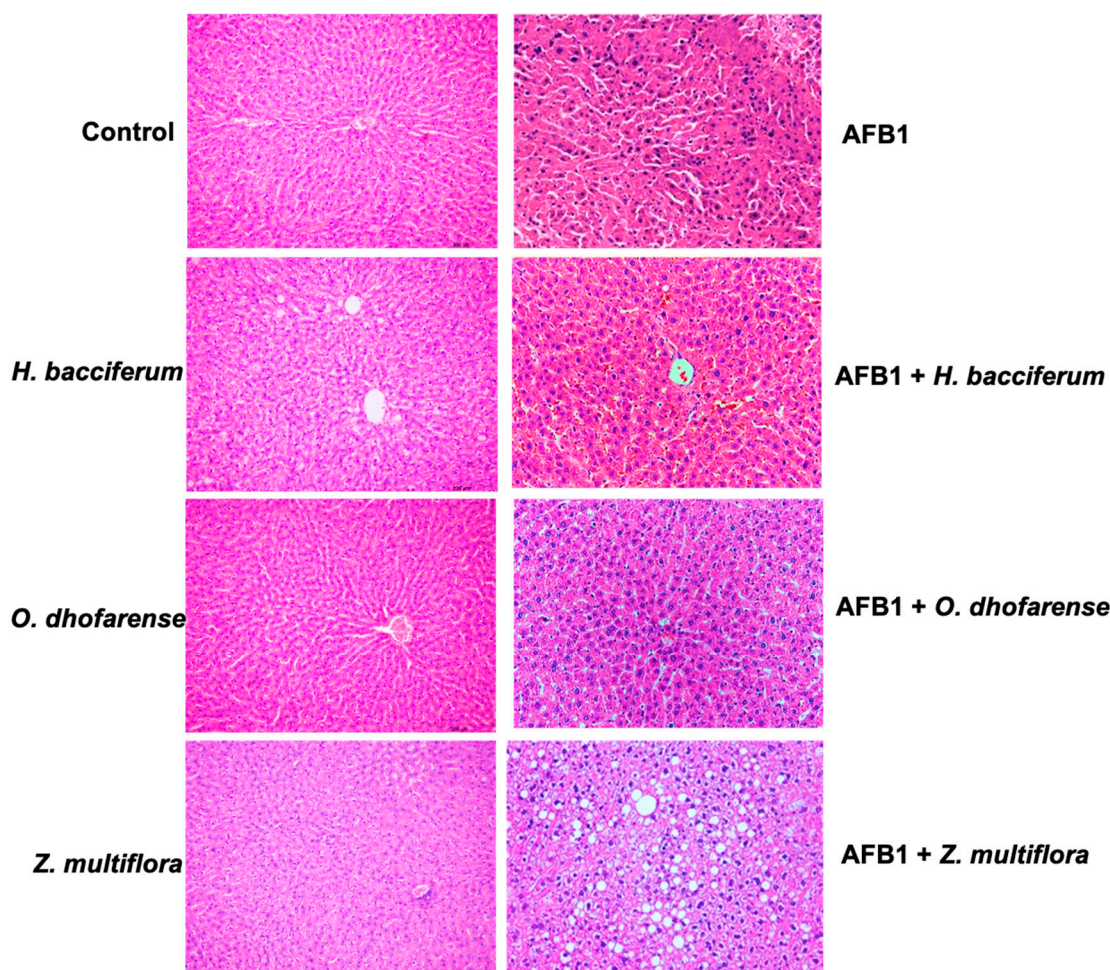


**Figure 14.** DNA oxidative damage (8-hydroxydeoxyguanosine, 8-OHdG) in liver tissue homogenates of mice fed with *Heliotropium bacciferum*, *Ocimum dhofarens*, and *Zataria multiflora* extracts in the presence or absence of AFB1. \* Significantly higher as compared to control group,  $p < 0.05$ . \*\* Significantly lower than AFB1-injected group,  $p < 0.05$ . Values without superscript are not significantly different as compared to control group,  $p > 0.05$ .

### 3.7. Histopathological Studies

Hepatocytes in the control group and in groups treated with plant extracts revealed normal liver lobular architecture and cell structure (Figure 15). In the AFB1-treated group, histopathological changes were noticed, including extensive areas of necrosis, loss of hepatocyte architecture around the blood vessels, changes in cytoplasmic acid in hepatic cells, and partial necrosis of hepatic cells with mild inflammation. HE, OE, and ZE plant extracts, on the contrary, demonstrated a decrease in liver damage linked to AFB1 treatment.





**Figure 15.** Histological changes in hepatic tissue of mice treated with AFB1 and *Heliotropium bacciferum*, *Ocimum dhofarensis*, and *Zataria multiflora* extracts.

#### 4. Discussion

It is evident from the results that the aqueous extracts of *O. dhofarensis*, *H. bacciferum*, and *Z. multiflora* effectively degraded AFB1 (above 90%) in vitro following a 72 h incubation at 37 °C. Additionally, the aqueous extracts of *Lavandula dhofarensis* subsp. *ayunensis*, *Lavandula subnuda*, *Salvia hillcoatiae*, *Tecomella undulata*, *Ephedra foliata*, and *Kleinia odora* demonstrated the capability to degrade over 80% of AFB1. The differences in the AFB1 detoxification efficacy of plant extracts may be attributed to variations in their chemical composition. Several researchers have demonstrated the aflatoxin detoxification abilities of medicinal plants under in vitro conditions [17–19,22,23,27–29]. Hajare et al. [30] found that seed extracts of *Trachyspermum ammi* could degrade 80% of aflatoxin. Velazhahan et al. [17] demonstrated that *T. ammi* seed extract resulted in more than 90% degradation of aflatoxin G1. Vijayanandraj et al. [22] reported that *Adhatoda vasica* leaf extract could detoxify AFB1 up to 98%. Kannan and Velazhahan [21] found that *Barleria lupulina* leaf extract could degrade aflatoxins and the percentages of degradation of AFB1, AFB2, AFG1, and AFG2 were 61.1%, 71.4%, 94.4%, and 58.8%, respectively. Iram et al. [19] found that aqueous leaf extracts of *Ocimum basilicum* could degrade up to 90.4% of AFB1 and up to 88.6% of AFB2.

The leaves of *H. bacciferum* have traditionally been used to treat skin disorders [31]. The antimicrobial and antioxidant activities of *H. bacciferum* have been reported [32]. Many pyrrolizidine alkaloids, such as heliotrine, europine, heleurine, and supinine have been characterized in *H. bacciferum* [33–35]. *Z. multiflora* is an aromatic perennial shrub and is often used as a flavoring component in a wide variety of dishes. Several studies

have reported its antioxidant, antimicrobial, and immunomodulatory properties [36–38]. The antifungal properties of *Z. multiflora* essential oil and its effects on the growth of *A. flavus* and aflatoxin production have been documented [39]. *Ocimum* sp. is a well-known medicinal herb and has antidiabetic and antioxidant properties [40,41]. The leaves of *Ocimum* sp. contain triterpenoids, flavonoids, tannins, and saponins [42]. *O. sanctum* essential oil was reported to suppress the growth of *A. flavus* and the production of AFB1 [43,44]. Furthermore, the aflatoxin detoxification properties of *O. basilicum* [19] and *O. tenuiflorum* [18] extracts have been reported. However, little is known about the aflatoxin detoxification potential of aqueous extract from *O. dhofarensis*. Al-Harrasi et al. [45] showed the detoxification of AFB1 by *O. dhofarensis*, *H. bacciferum*, and *Z. multiflora* essential oils. This is the first report of AFB1 detoxification by the aqueous extracts of these three medicinal plants in Oman.

The degradation of AFB1 upon treatment with HE, OE, and ZE was confirmed by LC/MS analysis. The mass spectrum of control (untreated) AFB1 showed a molecular ion peak at  $m/z$  313, which is unique to AFB1 [46], whereas the level of AFB1 ( $m/z$  313) was greatly reduced and fragmented AFB1 products, viz.,  $m/z$  345.9, 369.3, 303.8, 351.2, 341.0, and 321.2, appeared after treatment with the plant extracts. The product ions observed in the mass spectra indicate structural changes in AFB1 molecules during the degradation process. For example, the addition of a hydroxyl group to the furan ring results in a product at  $m/z$  345 ( $C_{17}H_{12}O_8$ ), while the addition of  $H_2O$  to AFB1 forms  $m/z$  369, and the elimination of  $CH_2$  from AFB1 forms  $m/z$  303. Furthermore, the addition of two hydroxyl groups to AFB1 forms  $m/z$  351 ( $C_{17}H_{18}O_8$ ), which further degrades to  $m/z$  321. The ion  $m/z$  341 is formed by the addition of two oxygen and the elimination of four hydrogen [27]. The removal of the double bond in the furan ring structure of AFB1 is evident in the product ions  $m/z$  303,  $m/z$  341, and  $m/z$  351, while the modification of the lactone group and the elimination of the double bond in the furan ring were observed in AFB1 products  $m/z$  369 and  $m/z$  321. The fragment ion  $m/z$  189, which was absent in the control plant extract, appeared only after treatment of AFB1 with plant extracts.

Several studies have described the detoxification of aflatoxins by herbal extracts and the molecular structures of aflatoxin degradation products [17,19,22,27]. Modifications to the AFG1 lactone ring structure have been suggested as the detoxification mechanism of *T. ammi* seed extract [17]. Vijayanandraj et al. [22], while studying the AFB1 detoxification effect of *Adhatoda vasica* leaf extract, also reported the appearance of a product ion at  $m/z$  189.59 and a loss of a molecular base ion at  $m/z$  313 after incubation with *A. vasica* leaf extract. Structural modifications, such as changes in the lactone group and the elimination of the double bond in the furan ring structure, have been observed in the products of AFB1 and AFB2 degradation after treatment with *T. ammi* seed extract [27]. The continuous loss of carbon monoxide and methyl and methanol losses on the methoxy group present on the side chain of benzene were observed in the products of AFB1 degraded using *O. basilicum* extract [19]. Al-Owaisi et al. [23] reported AFB1 detoxification using leaf extracts of *Centella asiatica*, *Eclipta prostrata*, and *Hybanthus enneaspermus* and the formation of a fragment ion at  $m/z$  189 due to two sequential losses of carbon monoxide. The furofuran ring and lactone ring of AFB1 are responsible for its cytotoxicity and mutagenic activity [47]. The disappearance of the ion peak at  $m/z$  313 (unique to AFB1) and the generation of new ion peaks following treatment of AFB1 with HE, OE, and ZE in this study suggest the degradation of AFB1.

The results of the brine shrimp lethality bioassay indicated that HE, OE, and ZE effectively detoxified AFB1 in a concentration-dependent manner, registering 35, 30, and 25% survival rates of brine shrimp at a concentration of 100  $\mu\text{g/mL}$ , respectively, compared to 10% in the untreated AFB1 group. These findings suggest a reduction in the toxicity of AFB1 upon treatment with these plant extracts.

AFB1-induced toxicity is associated with the accumulation of reactive oxygen species, resulting in oxidative stress [7,48]. Oxidative stress leads to membrane lipid peroxidation and oxidative DNA damage [49]. Several biologically active compounds from

plants including *Allium sativum* [50], *Ocimum sanctum* [51], rosemary [52], *Curcuma longa* [53], *Thonningia sanguinea* [54], *Adhatoda vasica* [55], grape seed proanthocyanidin extract [56], *Thymus vulgaris* oil [57], curcumin [7,58], and curcumin plus black tea [59] have been documented to decrease AFB1-induced liver damage in animal models. The present study elucidated the potential antioxidant role of HE, OE, and ZE in alleviating AFB1-induced oxidative stress in the mouse liver. It was noticed that mice treated with AFB1 developed oxidative stress in their liver tissues, as evidenced by GSH depletion, TAC reduction, and augmented DNA oxidative damage. These findings align with those of Unsal and Kurutas [60]. They reported that AFB1 injection in mice led to a decrease in the liver redox “cellular status”. However, plant extract supplementation effectively suppressed the oxidative damage associated with AFB1 injection. These findings suggest that HE, OE, and ZE had a hepatoprotective potential against AFB1-induced oxidative stress and its associated pathogenesis. These results align with the well-established role of natural plant products in treating and preventing chronic diseases, including liver disorders [61–63]. In particular, *Z. multiflora*, *O. dhofarensis*, and *H. bacciferum* have a wide medicinal application [64]. These medicinal plants are rich in phytonutrients and prevent the development of cellular oxidative stress, thus offering a novel therapeutic approach to preventing oxidative stress-induced liver pathogenesis [65].

## 5. Conclusions

Our findings indicated that the aqueous extracts of *O. dhofarensis*, *H. bacciferum*, and *Z. multiflora* were highly effective in the detoxification of AFB1. The confirmation of degradation of AFB1 by these botanical extracts through liquid chromatography–mass spectrometry analysis emphasizes their effectiveness. AFB1 administration in mice resulted in oxidative stress in liver tissues. *O. dhofarensis*, *H. bacciferum*, and *Z. multiflora* act as potent antioxidants and combat AFB1-associated oxidative stress and liver pathogenesis, suggesting that these plant extracts might be valuable for the development of functional foods aimed at minimizing the toxic effects of AFB1.

**Author Contributions:** R.V., A.M.A.-S., M.I.W. and K.A.-F. planned the study. R.V., M.I.W., S.B.S.P. and J.A.-S. conducted the experiments. R.V., M.I.W., A.M.A.-S., S.B.S.P., J.A.-S. and K.A.-F. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The experimental protocol used in this study followed the guidelines established by the Sultan Qaboos University Animal Ethics Committee (ethical approval No. SQU/ECAU/2020-21/4).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data generated or analyzed during this study are included in this article, further inquiries can be directed to the corresponding authors.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest.

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