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Antimutagenic Constituents from *Monanthotaxis caffra* (Sond.) Verdc.

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ABSTRACT

Objectives *Monanthotaxis caffra* (Sond.) Verdc. (Annonaceae) has been reported to possess antitumoural properties. Preliminary screening showed that the crude methanolic leaf extract had strong antimutagenic effects against aflatoxin B_1-induced mutagenicity. The aim of this study was to isolate and evaluate the antimutagenic properties of the active constituents from *M. caffra*.

Methods Different chromatographic, spectroscopic and spectrometric techniques were used for the isolation and identification of the antimutagenic constituents. The antimutagenic effect of the extract and compounds was evaluated using Ames, Vitotox and Comet assays.

Key Findings Bioassay-guided fractionation of the methanolic leaf extract yielded two antimutagenic compounds identified as (+)-crotepoxide and 5,6-diacetoxy1-benzoyloxymethyl-1,3-cyclohexadiene. Crotepoxide had strong antimutagenicity in the Vitotox assay with an IC_{50} value of 131 µg/mL. 5,6-Diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene showed strong antimutagenic activity in the Ames assay with an IC_{50} value of 348.9 µg/plate and no antimutagenic activity in the Vitotox test. Furthermore, the compound was able to inhibit, block or prevent biotransformation of aflatoxin B_1 by repressing the proteins involved in transcription.

Conclusions Crotepoxide and 5,6- diacetoxy-1-benzoyloxymethyl-1,3-cyclohexidiene have the potential to mitigate the risks arising from consumption of aflatoxin B_1 contaminated food and feed.

KEYWORDS

Monanthotaxis caffra; Annonaceae; antimutagenicity; aflatoxin B_1; *(+)-crotepoxide;* 5,6-diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major causes of mortality and morbidity among different people and animals in the world. Asians and Africans are more prone to this type of cancer due to poor food and feed storage systems, hot and humid climates.[1, 2] HCC can result from exposure to foodstuffs contaminated with aflatoxins. Aflatoxins are toxins produced by *Aspergillus flavus* and *A. parasiticus*. The four major aflatoxins are B_1, B_2, G_1 and G_2.[3, 5] However, aflatoxin B_1 is the most potent indirect mutagen of both humans and animals and has to be activated metabolically by cytochrome P450 enzymes in order to exert its effect.[6] Aflatoxin B_1 is converted to exo-aflatoxin B_1-8, 9-epoxide, a reactive species which binds to DNA and induces mutations leading to the initiation, promotion and progression of cancer and other degenerative diseases. [7, 8] Elimination of aflatoxin B_1 from foodstuff is difficult, therefore chemopreventative strategies to reduce or prevent the effect of aflatoxin B1 are required.

Medicinal plants are used world-wide in the management and treatment of many diseases including the prevention and management of cancer. Some of these plants contain antimutagens that are capable of reducing or reversing and inhibiting the mutagenicity of certain environmental mutagens.[9] Natural antimutagens from plants are beneficiary to human and animal health due to their chemopreventive and chemoprotective properties against most cancers with little undesirable effects. [10] Many antimutagens have been isolated from different plant species including the Annonaceae family. Some members of the Annonaceae contain oxygenated cyclohexane epoxide derivatives such as crotepoxide, senepoxide**,** senediol, monanthadiepoxide and pipoxide and these compounds possess tumour inhibitory properties. [11–13] Furthermore, acetogenins, caryophyllene and caryophyllene-oxide with anti-inflammatory and anti-tumour activities against various cancer cell lines were isolated from members of the Annonaceae family. [14, 16]

Monanthotaxis caffra (Sond.) Verdc., a member of the Anonnaceae family, commonly known as dwaba-berry, is a shrub or climber which is widely distributed in the evergreen forests of the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces of South Africa. This species produces a flask shaped cluster; acidulous flavor edible fruits that are red when ripe. [17] *M. caffra* is used as food and in traditional medicine to treat various diseases. The Zulu people of KwaZulu-Natal smoke roots to treat hysteria and it is also administered as an emetic. The plant extract is used to charm young women and to make cattle strong and fat. [12] Preliminary screening of methanolic leaf extracts of *M. caffra* revealed a significant activity against aflatoxin B_1-induced genotxicity in the Ames, Vitotox and Comet assays. [18] This study reported for the first time the isolation and identification of the compounds responsible for this antigenotoxic activity from the methanolic leaf extract of *M. caffra* as well as assessing their antigenotoxic activity *in vitro* using Ames, Vitotox and comet assays.

Materials and Methods

Plant Material

Leaves of *M. caffra* (Sond.) Verdc. (Annonaceae) were collected from Lowveld National Botanical Gardens (South Africa) in March 2015. The identity of the plant was confirmed by Mrs. E. Van Wyk, University of Pretoria, South Africa. A voucher specimen (Number: PRU 122761) was deposited in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

Extraction and Isolation

Powdered plant material (350 g) was extracted to exhaustion with 80% methanol by maceration at room temperature. The plant extracts were filtered and concentrated to dryness under reduced pressure.

HPLC profiling of the crude extract

HPLC profiling of the 80% methanolic crude extract of *M. caffra* (6.35 mg/mL) was performed using a Surveyor LC system equipped with a DAD detector (Thermo Fisher, San Jose, CA) and Gracesmart RP 18 column (250 x 4.6 mm, 5 µm) (Grace Vydac, Hesperia, USA). The flow rate was 1 mL/min and UV detection was carried out at 230 nm. The injection volume was 20 µL. The gradient program was as follows: solvent A: 0.1% formic acid; solvent B: acetonitrile + 0.1% Formic acid. The gradient elution Started from 10% B for 10 min; from 10% B to 100% B for 50 min and remained at 100% B for 4 min. Then from 100% B to 10% B in 1.5 min – 4.5 min 10% B. The LC system was coupled to an LXQ linear ion trap (Thermo Fisher). The conditions of the mass spectrometer were as follows: sheath gas flow, 29 arbitrary units; auxiliary gas flow, 11 arbitrary units; sweep gas flow, 2 arbitrary unit; spray voltage, + 4.0 kV; ion transfer tube temperature, 350 °C; and capillary voltage, 21 V. Mass spectral data were recorded using data-full scanning in the mass range *m/z* 50-700. All data were recorded and processed using Xcalibur software, version 2.0 (Thermo Fisher).

Isolation of the bioactive constituents

The crude extract (45 g) was dissolved in 1 L of acidified water (0.2 N HCl, pH, <3) and then partitioned with dichloromethane (3 x 300 mL). The dichloromethane fraction was dried and further partitioned between 90% methanol/ *n*-hexane (3x 300 mL) to give the 90% methanolic/ hexane fractions.

The 90% methanolic fraction of *M. caffra* obtained from liquid-liquid fractionation was the most active fraction in the Vitotox assay. An aliquot of 3 g was subjected to flash column chromatography on a 80 g pre-packed Flash Grace Reveleris® silica cartridge (40 µm particle size). The elution started with 100% dichloromethane for 50 min and then a gradient of 50% dichloromethane/ethyl acetate in 15 min, to 100% ethyl acetate in 30 min, followed by

gradient of 50% ethyl acetate/ methanol in 13 min and 100% methanol for 10 min at a flow rate of 60 mL/min. The fractions were pooled to seventeen fractions based on the UV and TLC similarities.

Fraction 3 and 4 were selected for further fractionation based on the amount of the fraction and the complexity of the chromatographic profile. About 10 mg/mL of fractions 3 (138 mg) and 4 (114 mg) obtained by flash chromatography were further separated by a repeated semi-preparative HPLC with a Quattro Micromass detector on a Luna C18 (250 x 10 mm, 5 μm) semi-preparative column. Maximum volumes were 23 ml/min, however less volumes were used when UV signals were above the threshold. As a mobile phase water + 0.05% formic acid (A) and acetonitrile (B) were used. The fractions were separated using the following gradient to isolate compound **1**: 40% to 58% B; 35 min, 90% B; 2 min, 100% B; 3 min, and remained on 100% for 8 min, decreased till 40% B in 2 min and remained on 40% B; 5 min. To isolate compound **2** a different gradient was used starting at 62% to 72% B; 20 min, 90% B; 2 min, and decreasing to 62% B; 4 min maintaining for 6 min. For both gradients the flow rate was 4.75 mL/min. Eighty percent methanol with 0.1% formic acid was used as make-up flow (0.5 mL/min). Compounds **1** (29.5 mg) and **2** (5 mg) were identified from fraction 3 and fraction 4, respectively. The structure of these compounds was elucidated using 1D and 2D NMR spectra (Bruker DRX-400 spectrometer, Rheinstetten, Germany). The chemical and physical properties of the two compounds are provided below:

*(+)-Crotepoxide (**1**).* Yellowish powder (8.2 mg); $[\alpha]_D^{20}$ +3.75 (*c* 5.2, MeOH); UV λ_{max} 197; 232 nm; ^{1}H- and ^{13}C-NMR spectroscopic data: see Table 1; MS *m/z* 385$[M+Na]^+$.

*5,6- Diacetoxy-1-benzoyloxymethyl-1,3-cyclohexidiene (**2**).* White powder (5.2 mg); $[\alpha]_D^{20}$ -5.98 (*c* 29.5, MeOH). UV λ_{max} 197; 231; 262 nm; ^{1}H- and ^{13}C-NMR spectroscopic data: see Table 1; MS *m/z* 293 $[M\text{-}CH_3COOH+Na]^+$.

Antigenotoxicity Testing

Ames Assay

The Ames assay was performed using the pre-incubation test with *Salmonella typhimurium* strain TA 98 and TA 100 as described by Maron, Ames. [20] A 0.1 mL of an overnight bacterial culture was added to 2 mL of top agar containing traces of biotin and histidine together with 0.1 mL test solution (test sample and Aflatoxin B_1 or test sample and solvent control) or the solvent control (10% DMSO) or 1µg/mL aflatoxin B1 alone and 0.5 mL of 4% rat liver S9 mix. The top agar mixture was poured over the surface of the minimal glucose agar plates and incubated at 37 ºC for 48 h. Following incubation, the number of revertant colonies in each plate were counted. All cultures were done in triplicate for all concentrations (5000, 500 and 50 µg/mL) with the exception of controls where five replicates were used. Antimutagenicity of the test sample, expressed as percentage inhibition of mutagenicity, was calculated as follows:

% Antigenotoxicity = [(1-T/M) X 100].

Where T is the number of revertant colonies in the presence of mutagen and the test solution and M is the number of revertant colonies in the presence of the mutagen alone.

Vitotox Test

The Vitotox test was performed as described by Verschaeve et al. [19] Various concentrations of crude extracts, fractions and isolated compounds were added to 10x dilutions of 16 h cultures of *S. typhimurium* TA 104 *rec* N2-4 (genox) and *S. typhimurium* TA 104 *pr* 1 (cytox) strains in the presence of rat liver S9 and 1µg/mL of aflatoxin B_1. Light production was measured every 5 min in each well for 4 h at 30 ºC using a luminometer. The signal to noise ratio (S/N) was automatically calculated for each measurement. Antigenotoxicity of the test sample expressed as percentage inhibition of mutagenicity was calculated as follows:

% Antigenotoxicity = [(1-T/M) X 100].

Where T is the S/N in the presence of mutagen and the test solution and M is the S/N in the presence of the mutagen alone.

Neutral Red Uptake (NRU) Assay

The neutral red uptake (NRU) assay is a cytotoxicity assay that measures the neutral red dye uptake by the lysosomes. [21] The assay was performed on C3A cells as described by Rashed et al. [22] After 24 h exposure of cells to six different concentrations of plant extracts, the cells were washed and treated with neutral red dye for 3 h at 37^{o}C. Thereafter, the intracellular bound dye was extracted from the cells and the optical density at 540 nm was measured. The OD values were calculated as the measured value minus the control value. Sodium dodecyl sulfate (SDS) was used as the positive control for the NRU assay. The results were expressed as percentage cell viability and the minimum inhibition of NRU (NI50) was determined from a dose response curve of SDS.

Comet Assay

The alkaline comet assay was performed according to standard methods as described by Singh et al. [23] with few modifications. The C3A cells were exposed to combinations of different concentrations of the plant extracts, S9 and aflatoxin B_1 and the control solutions for 4 h at 37^{o}C. Thereafter, the cell suspension was embedded in a low melting point agarose gel on a microscope slide and placed in a lysis buffer overnight at 4^{o}C. Following lysis, the slides were rinsed and placed in a denaturation buffer for 40 min at 4^{o}C to allow unwinding of the DNA. Then, the slides were subjected to electrophoresis for 20 min at 1 V/cm and 300 mA. The slides were washed 3 times with Tris buffer for 5 min and then placed on ice cold ethanol for 10 min and then dried. The slides were renatured with water and stained with gelred. Then, the slides were analyzed under a fluorescence microscope with metacyte and metafer software from metasystem. The percentage DNA in the tail was used as a measure of DNA damage based on the random score of 100 nuclei per slide. Aflatoxin B_1 (1µg/mL) was used

as positive control for antigenotoxicity and EMS as a positive control for the genotoxicity test.

Statistical analysis

Data were presented as means ± SD. The IC_{50} values were determined using regression analysis by plotting the concentration-response relationship curve. The Kruskal–Wallis test by ranks was performed in order to test whether samples originate from the same distribution. It was also used to compare the independent samples of equal or different sample sizes. The Dunnett's test was performed to compare each treatment with the control (aflatoxin B_1). *P* value of < 0.05 was considered to be significant. Data analysis was performed with SAS version 9.3 statistical software (SAS, 1999).

Results and Discussion

HPLC profiling of the crude extracts of *M. caffra*

The HPLC profile of the 80% methanolic crude extracts of *M. caffra* revealed the elution of the majority of the peaks between 0-30 min (Figure 1). Benzoyloxy-cyclohexane derivatives were detected between 18 and 26 min. The UV chromatogram revealed two pure compounds identified as crotepoxide at 21.6 min (*m/z* 385 $[M+Na]^+$) and 5,6-diacetoxybenzoyloxymethyl-1,3-cyclohexidiene at 25.7 min (*m/z* 293 $[M-CH_3COOH +Na]^+$). However, mixture of two benzoyloxy cyclohexadiene compounds were also identified at 20.9 min (*m/z* 311 $[M+Na]^+$ and *m/z* 269 $[M+Na]^+$). Based on the UV-chromatogram, 5,6-diacetoxybenzoyloxymethyl-1,3-cyclohexidiene was present in a rather high amount in the crude extracts. However, quantification of the isolated compounds from the crude extracts was not possible due to the limited amount of isolated compounds.

Preliminary screening revealed that the methanolic leaf extract of *M. caffra* had significant (*P* < 0.05) antigenotoxicity against aflatoxin B_1-induced mutagenicity in the Ames (TA 98 and TA 100) assay (Figure 2).[18] Two *S. typhimurium* tester strains were used in the Ames test,

TA 98 which detects frame shift mutation and the base-pair substitution detecting TA 100 strains. The two strains are widely used in mutagenicity testing because they are sensitive in detecting most mutagens and carcinogens. [24– 25] Vitotox test, another bacterial based test, was performed to confirm and compliment the antimutagenicity of *M. caffra* extracts reported in Ames assay. Vitotox test is a more rapid and sensitive high-throughput-like test, which was found to correlate well with the Ames assay. [26] The results showed concentration dependent increase in the antigenotoxicity of leaf extract of *M. caffra* (Figure 3). However, the antigenotoxic effect was only significant ($P < 0.05$) at the highest concentration tested (500 µg/mL). There was no sign of toxicity according to the criteria of the Vitotox test as indicated in the experimental section. The extracts were investigated further in the mammalian cells based Comet assay. In this assay, the extract combined with 1 µg/mL of aflatoxin B_1 at non-toxic concentrations to C3A cells (4 µg/mL+ 1 µg/mL aflatoxin B_1 and 20 µg/mL + 1 µg/mL aflatoxin B_1) induced DNA damage in these cells comparable to that caused by aflatoxin B_1 while higher concentrations significantly ($P < 0.05$) decreased the percentage DNA in a comet tail (Figure 4). The Dunnett's test revealed that non-toxic concentrations of plant extracts combined with aflatoxin B_1 were not significantly different from aflatoxin B_1 alone ($P>0.1$) while 250 µg/mL + 1 µg/mL aflatoxin B_1 was significantly different from aflatoxin B_1 alone and the other tested concentrations ($P < 0.05$). This reduction in the DNA comet tail results from toxicity rather than antigenotoxicity as shown in Figure 5 (% viability ≤ 50%).

The crude methanolic leaf extract of *M. caffra* was also investigated for potential genotoxic risk in Ames, Vitotox and Comet assays. The extract was not mutagenic in Ames test (TA 98 and TA 100) as it did not produce double the revertant colonies in both tester strains which is necessary to consider the extract mutagenic in this test. The same results were obtained in Vitotox and Comet assays (data not shown). The cytotoxicity results obtained from neutral

248 red uptake assay showed that methanolic leaf extract of *M. caffra* was not toxic at 20 µg/mL
249 (> 80% cell viability) with an IC_{50} value of 100±5 µg/mL (Figure 5).

250 Antigenotoxicity results of the fractions obtained by liquid-liquid fractionation, presented as
251 percentage inhibition of the mutagenic effect of aflatoxin B_1, showed that all fractions
252 significantly ($P < 0.05$) affected aflatoxin B1-induced genotoxicity. The 90% methanolic
253 fraction had significant ($P < 0.05$) antigenotoxicity (28– 80%) against aflatoxin B_1-induced
254 genotoxicity, followed by the *n*-hexane fraction, while the basic water and dichloromethane
255 fractions were co-mutagenic at lower concentrations when tested in the Vitotox assay (Figure
256 6) and the Ames assay (data not shown). However, methanolic and hexane fractions showed
257 toxicity at highest concentrations in the presence of aflatoxin B_1 in the Vitotox assay, but the
258 TLC and HPLC profile of these fractions indicated interesting compounds, especially the
259 methanolic fraction (Figure 1). Based on these results, the 90% methanolic fraction obtained
260 from liquid-liquid extraction was selected for further bioassay guided fractionation and
261 isolation of bioactive compounds.

262 Two compounds were isolated and identified as crotepoxide (**1**) and 5,6-diacetoxy-1-
263 benzoyloxymethyl-1,3-cyclohexadiene (**2**) using spectroscopic techniques. The ^{1}H- and ^{13}C-
264 NMR spectra of compound **1** showed the presence of two acetyl groups and a benzoyloxy
265 moiety. ^{13}C NMR chemical shifts at 48.05 (d), 52.59 (d), 53.80 (d) and 59.36 (s) ppm,
266 together with ^{1}H NMR signals at 3.09 (1H, d), 3.43 (1H, d) and 3.64 (1H, d) ppm suggested
267 the presence of 2 epoxide functionalities. In addition, two tertiary carbons carrying an
268 acetoxy substituent were observed at 69.39 (d) and 70.34 (d) ppm in ^{13}C NMR, and at 5.68
269 (1H, d) and 4.96 ppm (1H, d) in ^{1}H NMR. Finally, also an oxygen-substituted methylene
270 group was observed at 62.43 (t) ppm in ^{13}C-NMR, and at 4.21 / 4.55 (each 1H, d) in ^{1}H-
271 NMR. ^{13}C-NMR library search and comparison with published data allowed the identification
272 of compound **1** as crotepoxide (Figure 7). This was confirmed by mass spectrometry

(molecular ion observed at *m/z* 385 $[M+Na]^+$) and by the specific optical rotation determined as $[\alpha]_D^{20}$ +3.75 (*c* 5.2, MeOH).

The identity of these compounds was further confirmed by comparison of NMR data with those reported in the literature. [11,27, 28] Crotepoxide has been isolated previously from *M. caffra.* [12] It has also been reported in other members of the Annonaceae family, and was isolated from *Friesodielsia obovata* [29] and *Uvaria purpurea.* [30] Crotepoxide was also isolated from fruits of *Croton macrostachys*, *Kaempferia rotunda*, *K. pulchra, K. angustifolia*, *Boesenbergia* sp. and from members of the Piperaceae family. [27, 29, 31]

In some aspects, the ^{1}H- and ^{13}C-NMR spectra of compound 2 were similar to those of compound 1, i.e. a benzoyloxy moiety, two tertiary carbons (71.08 (d) and 72.01 (d) ppm in ^{13}C-NMR, 5.75 (1H, d) and 5.45 (1H, d) in ^{1}H-NMR) carrying an acetoxy substituent, and an oxygen-substituted methylene group (65.79 ppm in ^{13}C-NMR, 4.89 (2H, m) in ^{1}H-NMR were also observed in compound 2. The ^{1}H-NMR signals at 5.75 and 5.45 ppm were correlated with each other in the COSY spectrum. However, the epoxide functionalities were absent. Instead, 4 unsaturated carbons were observed, a quaternary one at 132.34 ppm in ^{13}C-NMR, and 3 CH-groups at 125.83, 126.76 and 126.96 ppm in ^{13}C-NMR, showing one-bond C-H correlations in the HSQC spectrum with ^{1}H-NMR signals at 5.89 1H, dd), 6.30 1H, d) and 6.18 ppm (1H, m), respectively. In the COSY spectrum a 5-spin system could be observed linking the H-2, H-3, H-4, H-5 and H-6 signals occurring at 5.75, 5.45, 5.89, 6.18 and 6.30 ppm, respectively. Careful analysis of all NMR spectral data, also including multiple bond C-H correlations in HMBC, allowed to identify compound **2** as 5,6-diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene (Figure 7). This compound has been reported before from *Uvaria ferruginea* by Kodpinid et al [32], although only partial ^{1}H-NMR assignments were reported. This is the first complete assignment of the ^{1}H- and ^{13}C-NMR spectral data of 5,6-diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene (**2**). The structure was confirmed by

298 mass spectrometry (molecular ion observed at *m/z* 293 $[M\text{-}CH_3COOH+Na]^+$) and by the
299 specific optical rotation determind as $[\alpha]_D^{20}$ -5.98 (*c* 29.5, MeOH).
300 The isolated compounds were tested using Ames and Vitotox assays to confirm their
301 antimutagenic activity (Figure 8 and 9). Crotepoxide had significant ($P < 0.05$)
302 antigenotoxicity at the highest concentration tested in the Vitotox assay with an IC_{50} value of
303 131 µg/mL. Crotepoxide induced the SOS response of the bacteria which activated SOS
304 repair mechanism. Crotepoxide was not tested in Ames assay due to the limited amount of the
305 isolated compound. The Vitotox test and Ames test correlate well and give similar results as
306 shown by Westerink et al. [33] The anti-tumour or anticarcinogenic properties of
307 polyoxygenated cyclohexane derivatives have been reported in literature. Crotepoxide
308 inhibited the expression of tumour necrosis factor (NF-kB) regulated gene products involved
309 in anti-apoptosis such as Bcl-2, Bcl-XL, cyclin D1, Cox-2, Bax, Bid, c-Myc, MMP-9 and
310 VEFG, etc. This compound potentiated chemotherapy induced apoptosis. [29] Furthermore,
311 crotepoxide also inhibited the tumours by preventing the activation of genes that are involved
312 in tumorigenesis at gene levels. [34] The compound also possessed antitumour properties
313 against various carcinoma in rats and mice. [13, 14, 35]
314 5,6-Diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene had insignificant ($P < 0.05$)
315 antigenotoxic activities in the Vitotox assay. However, it had moderate antimutagenic
316 activity, though not significantly different at $P \leq 0.05$, (37– 61%) in the Ames assay (TA100)
317 with an IC_{50} value of 348.9 µg/plate and thus capable of preventing the reversion mutation
318 induced by aflatoxin B_1 in the Ames assay with tester strain TA 100 in this study.
319 Furthermore, the presence of oxygenated cyclohexane derivatives and their tumour inhibitory
320 properties have been reported in other members of the Annonaceae family. [11, 29, 36]
321 **Conclusions** crotepoxide and 5,6- diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene were
322 isolated and identified from the methanolic leaf extract of *M. caffra*. Crotepoxide had

significant antigenotoxic activity in the Vitotox test, while 5,6-diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene had strong antimutagenic activity in the Ames assay. Therefore, crotepoxide and 5, 6-diacetoxy-1-benzoyloxymethyl-1,3-cyclohexidiene have the potential to mitigate the risks arising from consumption of aflatoxin B_1 contaminated food and feed.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure Captions

Figure 1. HPLC chromatogram of the 80% methanolic crude extract of *M. caffra.* Peaks were identified as (+)-crotepoxide, (**1**), 5,6- diacetoxy-1-benzoyloxymethyl-1,3-cyclohexadiene (**2**) and a mixture of 2 benzoyloxy cyclohexadiene components (**3**).

Figure 2. Antigenotix activity (%) of methanolic leaf extract of *M. caffra* against aflatoxin B_1- induced mutagenicity using *Salmonella typhimurium* tester strain TA 100 and TA 98.

346 Three concentrations (50, 500, 5000 µg/ mL) of the leaf extracts were used. Values are mean
347 of 9 measurements from 3 experiments.*$P \leq 0.05$ significant when compared with the
348 positive control (aflatoxin B_1) by *post hoc* Dunnett's test.

349 Figure 3. Antigenotoxic activity (%) of methanolic leaf extract of *M. caffra* against aflatoxin
350 B_1- induced mutagenicity using Vitotox assay. Three concentrations (20, 100, 500 µg/ mL) of
351 the leaf extracts were used. Values are mean of 4 measurements from 2 experiments.*$P \leq$
352 0.05 significant when compared with the positive control (aflatoxin B_1) by *post hoc* Dunnett's
353 test.

354 Figure 4. Antigenotoxic effect of methanolic leaf extract of *M. caffra* on C3A cell line against
355 aflatoxin B_1- induced mutagenicity using comet assay. Four concentrations (4, 20, 100, 250
356 µg/ mL) of the leaf extracts were used in combination with aflatoxin B1 (1µg/ mL). Ethyl
357 methane sulfonate (EMS) was used as a standard genotin. Values are mean of 400
358 measurements from 2 experiments.*$P \leq 0.05$ significant when compared with the positive
359 control (aflatoxin B_1) by *post hoc* Dunnett's test.

360 Figure 5. Percentage (%) cell viability of C3A cells exposed to six concentrations (0.8, 4, 20,
361 100, 250, 250 µg/mL) of methanolic leaf extract of *M. caffra* and the blank (0 µg/mL).
362 Values are mean of 4 measurements from 2 experiments.*$P \leq 0.05$ significant when
363 compared with the positive control (aflatoxin B_1) by *post hoc* Dunnett's test.

364 Figure 6. Antigenotoxic activity of liquid-liquid fractions against aflatoxin B_1- induced
365 mutagenicity using Vitotox assay. Three concentrations (20, 100, 500 µg/ mL) of each
366 fraction were used. Values are mean of 4 measurements from 2 experiments *$P \leq 0.05$
367 significant when compared with the positive control (aflatoxin B_1) by *post hoc* Dunnett's test.

368 Figure7. Structure of (+)-crotepoxide (**1**) and 5,6- diacetoxy-1-benzoyloxymethyl-1,3-
369 cyclohexadiene (**2**)

Figure 8. Antigenotoxic activity (%) of Compound **1** and **2** against aflatoxin B_1- induced genotoxicity using Vitotox assay with *S. typhimurium* strain TA104. Three concentrations (20, 100, 500 µg/ mL) of each compound were used. Values are mean of 4 measurements from 2 experiments *$P \leq 0.05$ significant when compared with the positive control (aflatoxin B_1) by *post hoc* Dunnett's test.

Figure 9. Antimutagenic activity (%) of compound **2** against aflatoxin B_1- induced mutagenicity using Ames assay with *S. typhimurium* strain TA100. Three concentrations (50, 500, 5000 µg/ mL) of the leaf extracts were used. Values are mean of 6 measurements from 2 experiments.

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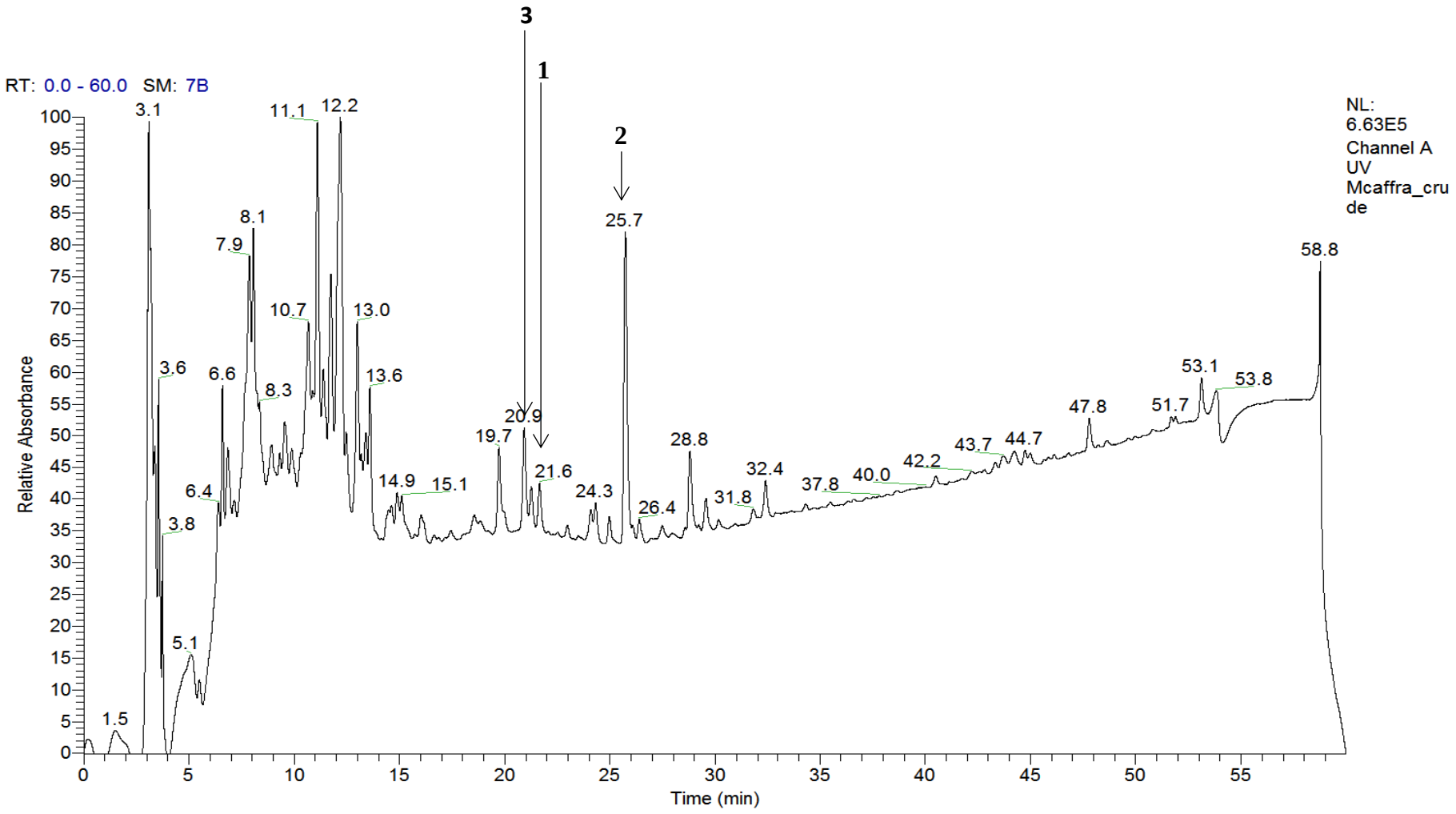
464 **Table 1.** ^{1}H- and ^{13}C-NMR assignments for (+)-crotepoxide (1) and 5,6- diacetoxy-1-
465 benzoyloxymethyl-1,3-cyclohexidiene (**2**), recorded in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz
466 (^{13}C), respectively.

467

	1		**2**	
C No.	δ_C (ppm), m	δ_H (ppm) (H, m, J in Hz)	δ_C (ppm), m	δ_H (ppm) (H, m, J in Hz)
1	59.39, s	-	132.34, s	-
2	69.39, d	5.68 (1H, d, 9.0)	71.08, d	5.75 (1H, d, 5.5)
3	70.34, d	4.96 (1H, d, 9.0)	72.01, d	5.45 (1H, m)
4	52.59, d	3.09 (1H, d, 2.4)	125.83, d	5.89 (1H, dd, 9.6, 4.2)
5	48.05, d	3.43 (1H, m)	126.96, d	6.18 (1H, m)
6	53.80, d	3.64 (1H, d, 2.5)	126.76, d	6.30 (1H, d, 5.6)
7	62.43, t	4.21 (1H, d, 12.1)	65.97, t	4.89 (2H, m)
		4.55 (1H, d, 12.1)		
8	170.04, s	-	171.84, s	-
9	20.62, q	2.01 (3H, s)	20.75, q	1.96[a]
10	169.74, s	-	171.61, s	-
11	20.66, q	2.10 (3H, s)	20.75, q	1.99[a]
1’	129.08, s	-	131.13, s	-
2’, 6’	129.79, d	8.00 (2H, br d, 7.4)	130.56, d	7.99 (2H, dd, 8.3, 1.3)
3’, 5’	128.55, d	7.44 (2H, m)	129.64, d	7.47 (2H, dd, 8.3, 7.5)
4’	133.55, s	7.57 (1H, br t, 7.3)	134.42, d	7.60 (1H, tt, 7.5, 1.3)
7’	165.78, s		167.37, s	-

468 [a] assignments may be interchanged.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NL:
6.63E5
Channel A
UV
Mcaffra_cru
de
Relative Absorbance
Time (min)
1
2
3
1.5
3.1
3.6
3.8
5.1
6.4
6.6
7.9
8.1
8.3
10.7
11.1
12.2
13.0
13.6
14.9
15.1
19.7
20.9
21.6
24.3
25.7
26.4
28.8
31.8
32.4
37.8
40.0
42.2
43.7
44.7
47.8
51.7
53.1
53.8
58.8

Figure 1

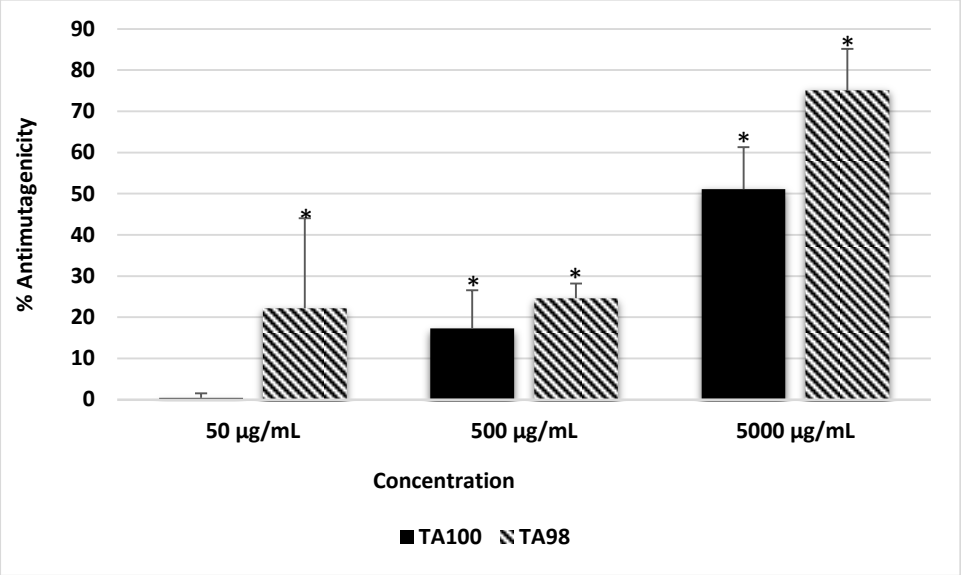

90
80
70
60
50
40
30
20
10
0
% Antimutagenicity
*
*
*
*
*
50 µg/mL
500 µg/mL
5000 µg/mL
Concentration
TA100
TA98

Figure 2

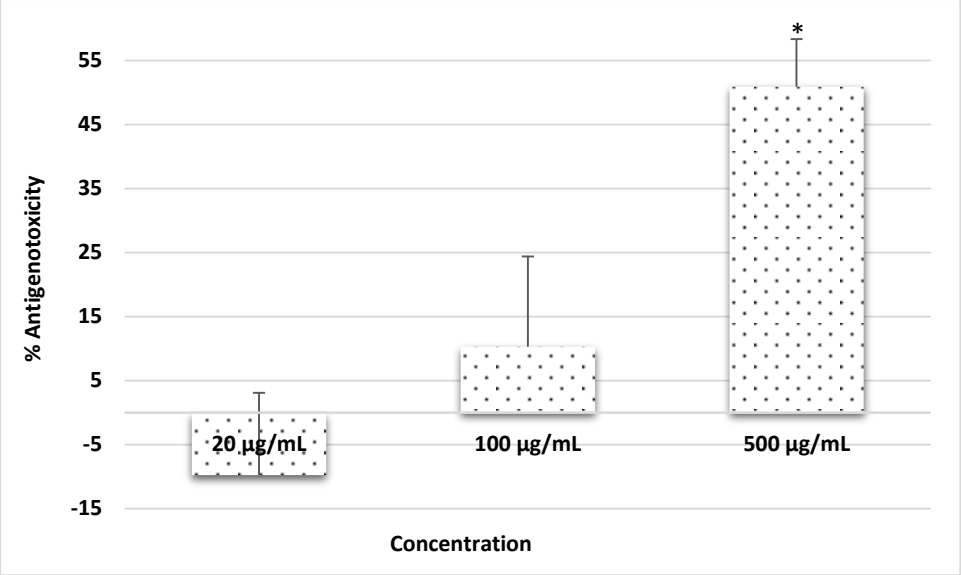

55
45
35
25
15
5
-5
-15
% Antigenotoxicity
*
20 µg/mL
100 µg/mL
500 µg/mL
Concentration

Figure 3

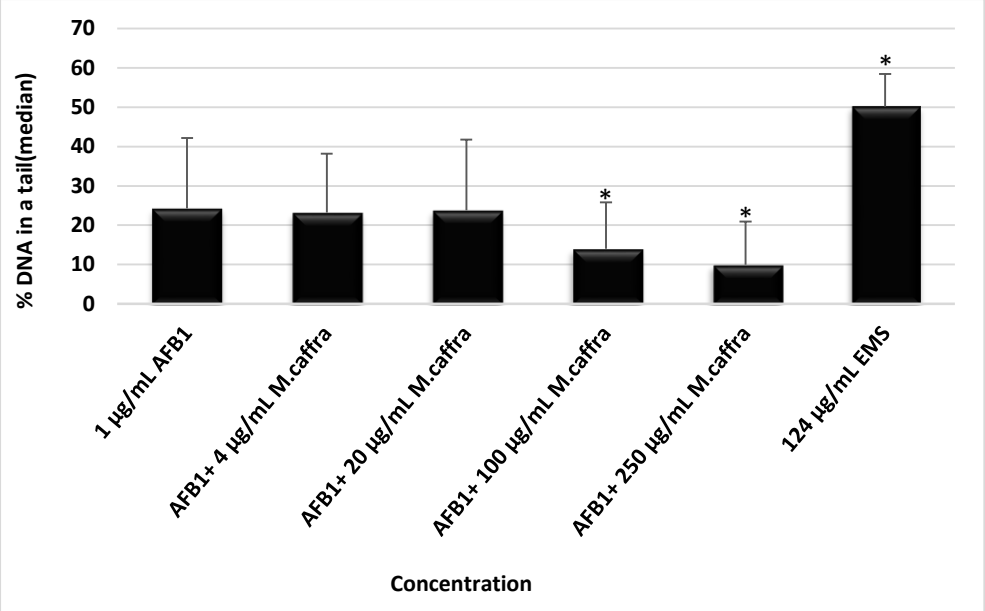

70
60
50
40
30
20
10
0
% DNA in a tail(median)
*
*
*
1 µg/mL AFB1
AFB1+ 4 µg/mL M.caffra
AFB1+ 20 µg/mL M.caffra
AFB1+ 100 µg/mL M.caffra
AFB1+ 250 µg/mL M.caffra
124 µg/mL EMS
Concentration

Figure 4

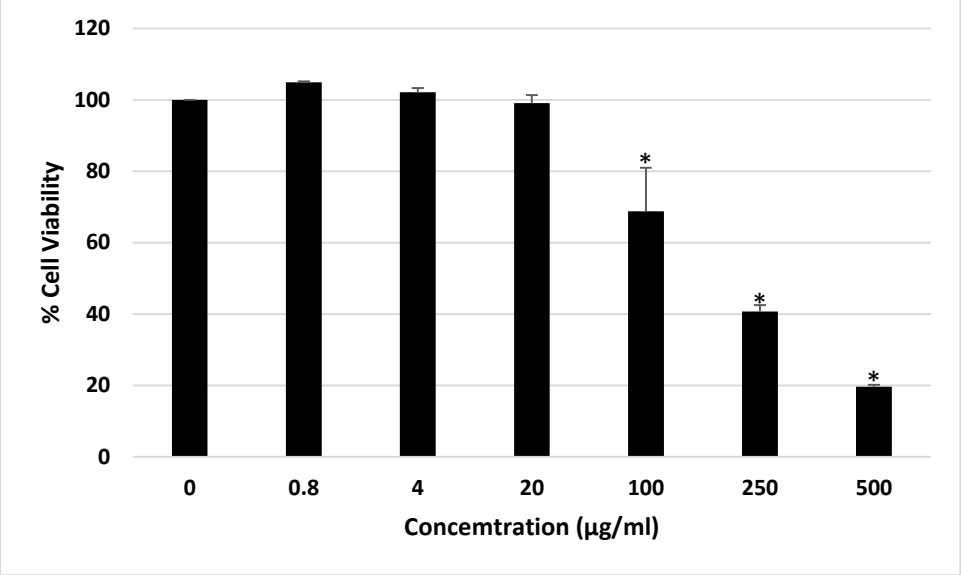

120
100
80
60
40
20
0
% Cell Viability
*
*
*
0
0.8
4
20
100
250
500
Concemtration (µg/ml)

Figure 5

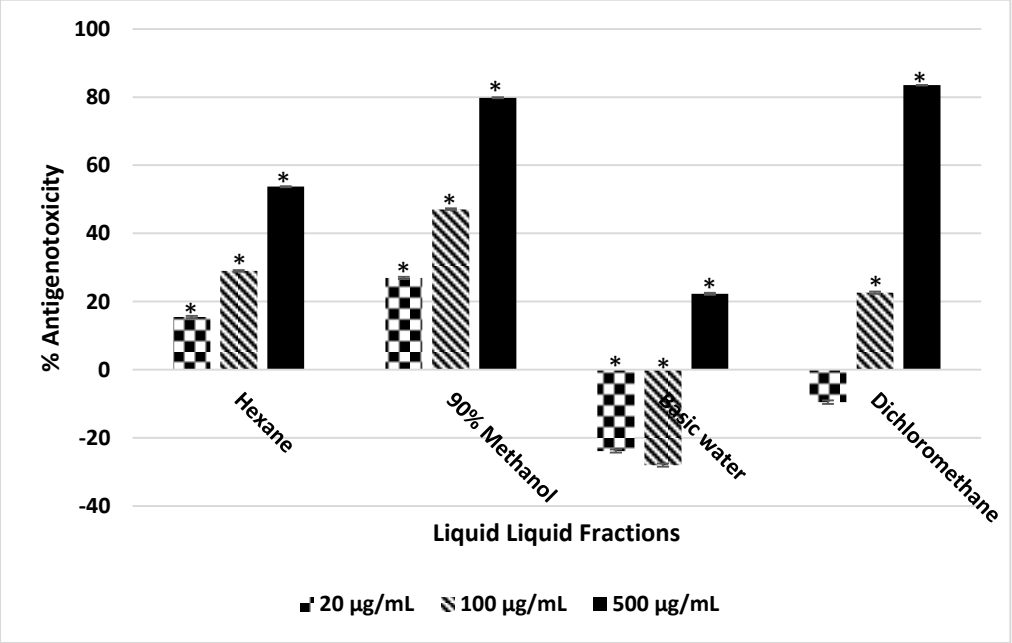
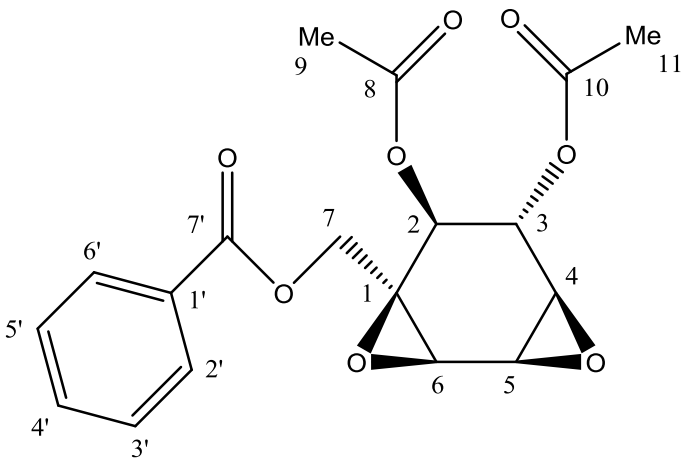
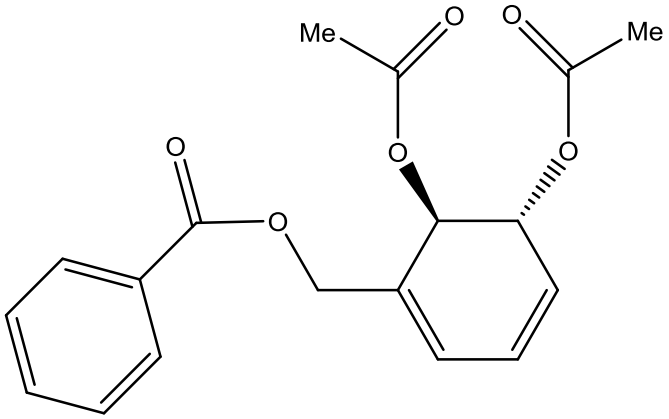

100
80
60
40
20
0
-20
-40
% Antigenotoxicity
Hexane
90% Methanol
Basic water
Dichloromethane
Liquid Liquid Fractions
20 µg/mL
100 µg/mL
500 µg/mL

Figure 6



1



2

Figure 7

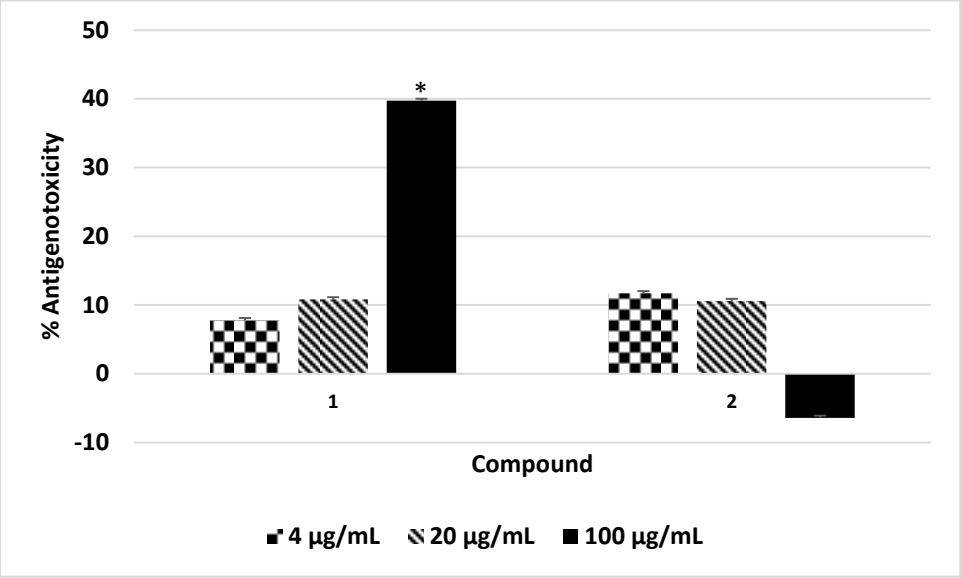

50
40
30
20
10
0
-10
% Antigenotoxicity
*
1
2
Compound
4 µg/mL
20 µg/mL
100 µg/mL

Figure 8

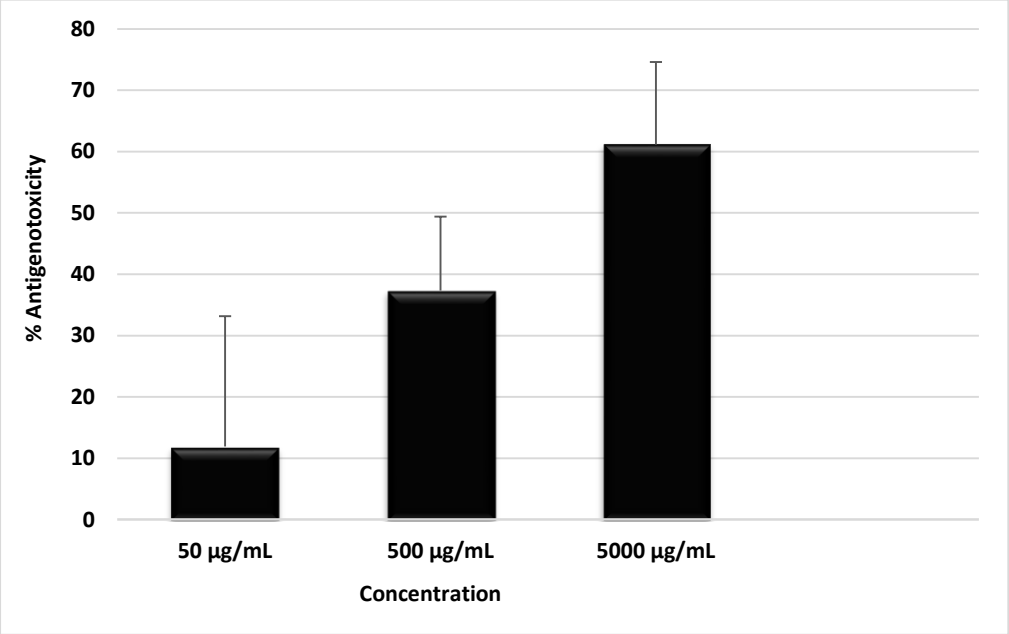

80
70
60
50
40
30
20
10
0
% Antigenotoxicity
50 µg/mL
500 µg/mL
5000 µg/mL
Concentration

Figure 9