Extracts of *Moringa oleifera* lack cytotoxicity and attenuate oleic acid-induced steatosis in an *in vitro* HepG2 model

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Abbreviations: $\Delta\Psi m$: mitochondrial membrane potential; DMSO: dimethyl sulfoxide; ESO: electro spray ionization; GSH: reduced glutathione; HDMS: high-definition mass spectrometer; PBS: phosphate-buffered saline; PDA: photodiode array; ROS: reactive oxygen species; UPLC: ultra-performance liquid chromatography.

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Highlights:

- Hot water and methanol extract were found to have negligible cytotoxicity.
- Although mitochondrial depolarisation was noted, it did not translate to cytotoxicity.
- Oxidative stress was not apparent.
- Cell viability was decreased at 100 µg/mL.
- The methanol extract reduced oleic acid-induced cytotoxicity and steatosis.

Abstract

Moringa oleifera Lam. has received much praise over its potential benefits to health and nutrition, however, not much literature is available to support claims of hepatic safety. The liver, as primary xenobiotic metaboliser, is subject to high levels of cytotoxicity. As such, hepatotoxicity has been a consistent hurdle during the drug discovery and development process. The aim of this study was to assess the *in vitro* hepatotoxic properties of both hot water and methanol extracts of *M. oleifera* leaves by assessing common mechanisms of cytotoxicity in the HepG2 cell line. Also, the ability of these extracts to protect against *in vitro* oleic acid-induced steatosis was investigated.

Tentative phytochemical profiling was done by ultra-performance liquid chromatography coupled to a photodiode array detector and high-definition mass spectrometer. *In vitro* cytotoxicity was assessed by sulforhodamine B staining (cell density), dihydro-dichlorofluorescein diacetate cleavage (reactive oxygen species), monochlorobimane adduct formation (reduced glutathione), JC-1 ratiometry (mitochondrial membrane potential), nile red staining (fatty acids), Ac-DEVD-AMC cleavage (caspase-3/7 activity), thiobarbituric acid reactive species production (lipid peroxidation), chemiluminescence (adenosine triphosphate; ATP), propidium iodide distribution (cell cycle) and Annexin V-FITC dual staining (mode of cell death). The protective action of the extracts was determined using an oleic acid-induced model of steatosis.

Phytochemical profiling tentatively identified several chemicals within the extracts. Viability assessment indicated slight cytotoxicity at 100 µg/mL after both the hot water and methanol extract exposure (approximately 8 – 14%), however, this was not reflected by cell density results. The cell cycle remained unperturbed, apart from a slight increase in G0/G1-phase. Although mitochondrial depolarisation was evident, especially after exposure to the methanol extract, it did not translate to cytotoxicity in the form of oxidative stress. Reactive oxygen species generation was only observed at 100 µg/mL hot water extract exposure (1.99-fold), with the rest of the values being below baseline. Slight lipid peroxidation (1.48-fold at 100 µg/mL) was observed after methanol extract exposure. The GSH was unaltered. Fatty acid levels reduced, especially after methanol extract exposure (0.43-fold at 100 µg/mL), which may be indicative of increased mitochondrial respiration. Caspase-3/7 activity and ATP were decreased slightly. Methanol extracts were

particularly active in protecting cells from oleic acid-induced cytotoxicity and fatty acid accumulation, with a decrease of up to 27% and 4.1-fold at 3.2 µg/mL, respectively.

Although the *in vitro* results suggest hepatic safety, further investigation is needed to ascertain the underlying mechanisms of cytotoxicity at concentrations above 100 µg/mL, particularly the effects on mitochondrial membrane stability. Although mitochondrial membranes were depolarised, this may assist with the reduction of fatty acid levels and offer protection against oleic acid-induced steatosis.

1. Introduction

Moringa oleifera Lam., commonly referred to as the horseradish or miracle tree (Bamishaiye *et al*., 2011; Awodele *et al*., 2012; Sinha *et al*., 2012), is a medium-sized tree traditionally used by the Greeks, Romans and Egyptians (Oluduro *et al*., 2012) against infections, cancers, hepatorenal dysfunction and cardiovascular pathologies (Paliwal *et al*., 2011). Its high nutritional content has been subject to much discussion (De Saint Sauveur, 2012; Sharma *et al*., 2012), suggesting it as a versatile plant for both medicinal and health supplementary uses. The leaves of *M. oleifera* are used in various ways medicinally, including either direct consumption of the leaf material, or boiling thereof as a tea (Stevens *et al*., 2013; Coz-Bolaños *et al*., 2018). Scientific literature primarily focuses on beneficial bioactivities, with some that have focused on potential cytotoxic effects in *in vitro* (Lipipun *et al*., 2003; Monera *et al*., 2008; Khalafalla *et al*., 2010; Varalakshmi and Nair, 2011; Asare *et al*., 2012; Waiyaput *et al*., 2012) and *in vivo* (Devaraj *et al*., 2007; Maphosa *et al*., 2008; Asare *et al*., 2012; Awodele *et al*., 2012) models, particularly with regards to hepatocellular viability. Furthermore, information regarding *M. oleifera*'s effects on cellular pathways, such as those implicating the mitochondria or reactive oxygen species (ROS) generation, are lacking, and thus a need arises to elucidate potential alterations to upstream effectors of cell growth and viability.

A common misconception within the general populace is that due to plants' natural origins, preparations thereof should be safe and effective treatments in comparison to Western medicines (Aithal, 2005). Contrary to this, toxicological and efficacy studies are not always available to support such claims. Organ-specific toxicity data, such as that of the liver, is thus also not easily obtained. The liver is well-described as the primary metabolic organ of the body, required for, among others, detoxification of exogenous substances (Brenner *et al*., 2013). As such, it is susceptible to the toxic effects of xenobiotics, including phytochemicals (Aithal, 2005). Hepatotoxicity remains an ever-present complication during drug discovery and development, as numerous pharmaceutical leads have undergone attrition due to clinically significant alterations in liver activity (van Tonder, 2011). The prevalence of herb-induced liver damage has seen an increase in recent years (Aithal, 2005), further justifying the need for thorough scientific study thereof. Unfortunately, such damage is often difficult to ascertain due to non-specific (Hayashi and Fontana, 2014) or unpredictable (Aithal, 2005) symptoms, or non-

disclosure of herbal use from patients (Steenkamp *et al*., 1999; Steenkamp *et al*., 2000; Bottenberg *et al*., 2007; Tarantino *et al*., 2009; Enbom *et al*., 2014).

Various mechanisms have been associated with the induction of hepatotoxicity, which are typically common among cell types. Reduced mitochondrial function or induction of mitochondrial toxicity is known to precipitate reactive oxygen species (ROS) generation due to leakage of electrons (Begriche *et al*., 2011), which in turn deplete reduced glutatione (GSH), an endogenous antioxidant system (Xu *et al*., 2004). As such, oxidative stress may occur, where antioxidant systems are unable to maintain redox balance due to an ever-increasing burden of free radicals (Moreira *et al*., 2011; Jaeschke *et al*., 2012). Free radicals may then induce cytotoxicity via oxidation of lipids, proteins or DNA (Moreira *et al*., 2011). Altered mitochondrial activity may also, among others, i) reduce proliferation due to decreased cellular cycling (Wallace and Starkov, 2000), ii) promote apoptosis due to leakage of pro-apoptotic factors that activate executioner caspases (such as caspase-3) (Kroemer and Reed, 2000), iii) interfere with adenosine triphosphate (ATP) production leading to reduced cellular energy (Pessayre *et al*., 2012), or iv) prevent β-oxidation of fatty acids, leading to accumulation thereof and induction of steatosis (Begriche *et al*., 2011). The latter is a condition in which fatty acids and triglycerides are accumulated in the liver, which may promote hepatocellular death (Xu *et al*., 2004).

Given the lack of information on *M. oleifera*, the study aimed to investigate potential *in vitro* hepatotoxicity of a hot water and methanol extract of *M. oleifera* leaves by assessing common cytotoxic mechanisms, as well as investigation of its anti-steatotic properties.

2. Methodology

2.1. Extraction of plant material

Leaves were provided by Prof ES du Toit (Department of Plant Production and Soil Science, University of Pretoria) from the Moringa Orchard of the Hatfield Experimental Farm. Plant material was cleaned, air-dried at ambient temperature, ground and stored in an air-tight, amber bottle. A hot water extract was prepared by brewing plant material in boiling distilled water (1:10 [w/v]) for 15 min. A methanol extract was prepared by sonicating (40 kHz, 18°C – 20°C, Bransonic 52, Branson Cleaning Equipment Co.) plant material in absolute methanol (1:10 [w/v]) for 30 min, after which it was agitated for 2 h on an orbital shaker and allowed to extract for 16 h at 4°C. Methanol was decanted, the marc re-extracted three times, and the supernatants pooled. All extracts were centrifuged (1000*g*, 5 min) and filtered (0.22 µm). Extracts were dried using lyophilization (Freezone® Freeze Dry System, Labconco) or rotary-evaporation (Büchi Rotovapor R-200, Büchi) for hot water and methanol extracts, respectively. The dried methanol extract was reconstituted in distilled water and lyophilized to yield a dry mass. Extraction yields were determined gravimetrically. Aliquots of 25 mg/mL were prepared in phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) for the hot water and methanol, respectively. The former was filter-sterilised (0.22 µm). Aliquots were stored at -80°C.

2.2. Tentative chemical profiling of extracts

Ultra-performance liquid chromatography (UPLC; Waters) coupled in tandem to a photodiode array (PDA; Waters) detector and high-definition mass spectrometer (HDMS; SYNAPT G1, Waters) was used to tentatively profile the phytochemicals within the extracts. Separation optimisation was achieved on a Waters HSS T3 C18 column (150 mm × 2.1 mm, 1.8 µm; 60ºC) with ultrapure MS grade solvents. Extracts were dissolved in methanol (10 mg/mL) and filtered (0.2 µm) prior to analysis. The solvent mixture consisted of 10 mM aqueous formic acid (pH 2.3) (solvent A) and 10 mM formic acid in acetonitrile (solvent B). The run (0.4 mL/min flow rate; 30 min; 3 µL; triplicate injections) was initiated at 99% solvent A mobile phase for 1 min, followed by a linear gradient to 5% solvent A mobile phase at 26 min, which was maintained for 1 min prior to reverting to the initial conditions. Detection using the PDA occurred between 200 and 500 nm (1.2 nm resolution), collecting 20 spectra per second. The SYNAPT G1 HDMS was used in V-optics and operated in electrospray mode to allow for phenolic and other electrospray ionization (ESI)-compatible compound detection. Reference calibration was done using leucine enkephalin (50 pg/mL) to obtain typical mass accuracies between 1 and 5 mDa. The MS was operated in both negative- and positive-mode. Capillary voltage, sampling cone and extraction cone parameters were set at 2.5 kV, 30 V and 4.0 V, respectively. The scan time was 0.1 s covering a 50 to 1 200 Da mass range. Source and desolvation temperature were set at 120°C and 450°C, respectively. Nebulisation gas (nitrogen gas) was set at a flow rate of 550 L/h, and cone gas added at 50 L/h. Analysis was done using MassLynx 4.1 (SCN 872) software.

2.3. Cellular maintenance and preparation for seeding

HepG2 (ATCC #HB-8065) cells were maintained in 10% foetal calf serum (FCS)-supplemented Eagle's Modified Dulbecco's Medium (EMEM) and grown in 75 cm^2 flasks at 37°C and 5% CO_2. Once flasks reached 90% confluence, cells were detached using trypsinisation. Detached cells were harvested by centrifugation (200*g*, 5 min) and counted using the trypan blue exclusion assay. Cells were diluted to 2 x 10^5 cells/mL or 1.7 x 10^4 cells/mL in 10% FCS-supplemented EMEM for 96- and 24-well plate experiments, respectively.

2.4. Seeding and exposure of cells to crude extracts

2.4.1. 96-well plate format

Cell density, intracellular ROS and GSH levels, $\Delta\Psi m$, fatty acid content and caspase-3/7 activity were determined using a single-plate cytotoxicity method as described by van Tonder *et al*. (2011) with minor modifications to include fatty acid accumulation and to allow for normalisation. Such a method allows for six different parameters to be assessed on a single plate from a single batch of cells. Lipid peroxidation and ATP content was assayed in individual plates. Cells (100 µL) were seeded into sterile, white 96-well plates for all assays, apart from the lipid peroxidation assay, which was in a clear plate. Plates were incubated overnight to facilitate attachment. Blanks consisted of 200 µL 5% FCS-supplemented medium alone to account

for background noise and sterility. After attachment, cells were exposed to 100 µL DMSO (0.8%; negative, vehicle control), crude extracts (2, 6.4, 20, 64 and 200 µg/mL) or the respective positive control for 72 h. Cytotoxic parameters were assessed as described below.

2.4.2. 24-well plate format

Cell cycle analysis and mode of cell death was determined in 24-well plate format. For cell cycle analysis, HepG2 cells were synchronised into the S-phase using double thymidine blocking (Chiang *et al*., 2010). Two medium types were prepared: A) 10% FCS-supplemented EMEM, and B) 3 mM thymidine- and 10% FCS-supplemented EMEM. HepG2 cells were cultured in medium A till 75% confluent. Cells were rinsed with PBS, and cultured for 16 h in medium B, rinsed and cultured for 10 h in medium A, and rinsed a final time before a further 16 h culture in medium B. Cells were detached using trypsinisation and diluted as described in Section 2.3. Synchronised and non-synchronised cells were used for cell cycle analysis and mode of cell death, respectively. Cells were seeded and exposed as described in Section 2.4.1, however, a volume of 600 µL and cell density of 1 x 10^5 cells/well was used, with exposure to the extract only at a single concentration of 100 µg/mL in-reaction, and end-point determinations done after 24 h and 72 h incubations.

2.5. Single-plate cytotoxicity parameters

2.5.1. Cellular density

Cell density was determined using sulforhodamine B (SRB) staining of fixed protein elements (Vichai and Kirtikara, 2006). Tamoxifen (10 µM in-reaction) was used as positive control. Cells were fixed with 50 µL trichloroacetic acid (50%) overnight at 4°C, and washed thrice with slow-running tap water. Fixed cells were stained with 100 µL SRB solution (0.057% in 1% acetic acid) for 30 min, after which excess dye was removed by washing with 100 µL acetic acid (1%). Plates were air-dried and the stain eluted with 200 µL Tris-buffer (10 mM, pH 7.4). Aliquots (100 µL) were transferred to a clear 96-well plate and the absorbance measured at 510 nm (reference 630 nm) (Synergy 2, Bio-Tek Instruments, Inc.). Absorbance data was blank-subtracted and cell density relative to the negative control expressed using the following equation:

$$\text{Cell density (\% relative to negative control)} = \frac{\text{As}}{\text{Ac}} \times 100$$

where, As = the blank-adjusted absorbance of the sample, and Ac = the blank-adjusted average absorbance of the negative control.

2.5.2. Intracellular reactive oxygen species

Intracellular ROS were determined by enzymatic conversion of dichloro-dihydrofluorescein diacetate (H_2-DCF-DA) to fluorescent dichlorofluoroscein (van Tonder, 2011). Potassium peroxidisulfate (150 µM in-reaction) was used as positive control.

Plates were exposed to 10 µM H_2-DCF-DA for 2 h and the fluorescent intensity measured at λ_{ex} = 485 nm and λ_{em} = 520 nm (gain 750) (FLUOstar Optima, BMG Labtech). After measurement, the SRB assay was performed to account for confoundment by altered cell density. Fluorescent intensity was adjusted by subtracting the blanks, normalised to the cell density and the percentage of intracellular ROS relative to the negative control expressed using the following equation:

$$\text{Intracellular ROS levels (fold} - \text{change relative to negative control)} = \frac{\text{FIs}}{\text{FIc}} \times 100$$

where, FIs = the normalised, blank-subtracted fluorescent intensity of the sample, and FIc = the normalised, blank- subtracted average fluorescent intensity of the negative control.

2.5.3. Intracellular reduced glutathione

Intracellular GSH was determined by the conjugation of monochlorobimane to GSH to form a fluorescent adduct (Cordier *et al*., 2013). n-Ethylmaleimide (10 µM in-reaction) was used as positive control. Plates were exposed to 16 µM monochlorobimane for 2 h and the fluorescent intensity measured at λ_{ex} = 355 nm and λ_{em} = 460 nm (gain 1250). Normalisation and expression of results was done as per Section 2.5.2.

2.5.4. Mitochondrial membrane potential

The $\Delta\Psi$m was determined using the ratio of red fluorescent J-aggregates (healthy cells) to green fluorescent JC-1 monomers (unhealthy cells) (van Tonder, 2011). Rotenone (100 nM in-reaction) was used as positive control. Plates were exposed to 10 µM JC-1 for 2 h and the fluorescent intensity measured at λ_{ex} = 492 nm and λ_{em} = 590 nm (gain 1000) and λ_{ex} = 485 nm and λ_{em} = 520 nm (gain 1750). The ratio of fluorescence of JC-1 monomers to JC-1 aggregates was calculated, and the $\Delta\Psi$m expressed relative to the negative control using the following equation:

$$\Delta\Psi\text{m (fold} - \text{change relative to negative control)} = \frac{\text{Rs}}{\text{Rc}} \times 100$$

where, Rs = the ratio of fluorescent intensity of the sample, and Rc = the ratio of average fluorescent intensity of the negative control.

2.5.5. Fatty acids

Fatty acids were determined through the cellular accumulation of nile red as a measure of potential steatosis (Kiela *et al*., 2005). Oleic acid (200 µM in-reaction) was used as positive control. Briefly, plates were exposed to 10 µM nile red for 2 h and the fluorescent intensity measured at λ_{ex} = 544 nm and λ_{em} = 590 nm (gain 1000). Normalisation and expression of data was done as per Section 2.5.2.

2.5.6. Activation of caspase-3/7

Activation of caspase-3/7 (as a measure of apoptosis) was determined through the cleavage Ac-DEVD-AMC by activated caspase-3/7 to release fluorescent amido-4-methylcoumarin (van Tonder, 2011). Staurosporine (10 µM in-reaction) was used as positive control. Plates were centrifuged (200*g*, 5 min) and the medium replaced with 25 µL cold lysis buffer for 15 min on ice. Thereafter, 100 µL caspase-3/7 substrate buffer containing Ac-DEVD-AMC was added, and plates incubated for 4 h at 37˚C and a further 16 h at 4˚C (to allow for fixation as described in Section 2.5.2 for other assays). The fluorescent intensity was measured at λ_{ex} = 355 nm and λ_{em} = 460 nm (gain 750). Fluorescent intensity was blank-subtracted, normalised to average cell density and the caspase-3/7 activity expressed relative to the negative control using the following equation:

$$\text{Caspase} - 3 \text{ activity } (\text{fold} - \text{change relative to negative control}) = \frac{\text{FIs}}{\text{FIc}} \times 100$$

where, FIs = the normalised, blank-subtracted fluorescent intensity of the sample, and FIc = the normalised, blank-subtracted average fluorescent intensity of the negative control.

2.6. Lipid peroxidation

Lipid peroxidation was determined using the thiobarbituric acid reactive species assay (Stern *et al*., 2010). 2,2'-Azobis(2-amidinopropane) dihydrochloride (500 µM in-reaction) was used as positive control. Medium (200 µL) and trypsinised cells (100 µL) were aspirated into 5 mL tubes together with 100 µL trichloroacetic acid (16.5%) and 100 µL thiobarbituric acid (2.5% in 0.1 M sodium hydroxide and 50 µM ethylenediaminetetraacetic acid). Tubes were vortex-mixed and heated in a waterbath (95°C) for 20 min. Butanol (250 µL) was added, the tubes vortex-mixed, and left to separate into an aqueous and organic layer. The organic layer (100 µL) was transferred to a white 96-well plate and the fluorescent intensity measured at λ_{ex} = 544 nm and λ_{em} = 590 nm (gain 750). The fold-change of the intracellular lipid peroxidation levels was determined as described in Section 2.5.6.

2.7. Adenosine triphosphate levels

The ATP levels were determined using the ApoSENSOR™ ATP cell viability chemiluminescence kit (Corporation, 2015). Saponin (1% in-reaction) was used as positive control. Medium was replaced with 100 µL nucleotide releasing buffer and incubated for 5 min on a shaker. After lysis, 10 µL ATP monitoring enzyme was added. Plates were measured immediately using end-point luminescent intensity. Luminescent data was blank-subtracted, normalised to relative cell density and the fold-change expressed relative to the negative control using the following equation:

$$\text{ATP level (fold} - \text{change relative to negative control)} = \frac{\text{LIs}}{\text{LIc}}$$

where, LIs = the normalised, blank-subtracted luminescent intensity of the sample, and LIc = the normalised, blank-subtracted average luminescent intensity of the negative control.

2.8. Cell cycle analysis

Three positive controls were used: FCS-deprivation (24 h, G0/G1-block), 10 µM methotrexate in-reaction (14 h; G2/M-block) and 20 µM curcumin in-reaction (14 h; S-block). Propidium iodide staining was used to assess the effects of the crude extracts on the cell cycle (Darzynkiewicz and Juan, 2001). Medium and trypsinised cells were washed with 1% FCS-supplemented PBS, and 3 mL cold ethanol (70%) added drop-wise while vortex-mixed before fixing overnight at 4°C. Cells were lysed and stained for 40 min at 37°C with propidium iodide staining solution containing 100 µg/mL RNase, 40 µg/mL prodidium idiode and 0.1% Triton X-100. Flow cytometry (FC500 Series, Beckman-Coulter) was used to determine the distribution of DNA using FL-3. Deconvolution software (Multicycle V3.0, WinCycle) was used for interpretation of the cell cycle.

2.9. Mode of cell death

The cytotoxic effect of crude extracts was assessed using the Annexin V-FITC/propidium iodide assay (Hingorani *et al*., 2011). Rotenone (50 nM in-reaction) and ethanol-sonication (3 min) was used as apoptosis and necrosis controls, respectively. Medium and trypsinised cells were washed once with 1% FCS-supplemented PBS, and stained for 15 min with 2.5 µL Annexin V-FITC solution. Prior to flow cytometric analysis, 2.5 µL propidium iodide (3 mM) was added. Quadrant analysis was used to determine the cellular distribution in either FL-1-positive (Annexin V-FITC, early apoptosis), FL-3-positive (propidium iodide, necrosis) or FL-1 and FL-3 positive (late apoptosis, or aponecrosis) quadrants.

2.10. Protection against fatty acid accumulation in an *in vitro* steatosis model

Due to the extracts impact on fatty acid content, a steatosis model was used to assess whether there may be anti-steatotic activity. Fatty acid accumulation was induced in HepG2 cells using oleic acid (Vidyashankar *et al*., 2013) with slight modifications of the method. HepG2 cells were seeded as described in Section 2.4.1. After attachment, cells were pre-treated with 50 µL EMEM (negative control) or extracts (12.8, 40 and 128 µg/mL) for 1 h, after which plates were exposed to 50 µL oleic acid (2 mM in 1% BSA-supplemented EMEM) for 72 h. The negative control received 50 µL EMEM alone. The nile red assay was performed as described in Section 2.5.5.

2.11. Statistics

All experiments were performed using at least two technical and three biological replicates. Results were captured using Microsoft Excel 2010, while statistical analyses was done with GraphPad Prism 5.0. Results were expressed as the mean ± SEM. The IC_{50} was determined using non-linear regression (normalized, variable slope). For the plate assays, significant differences were determined between the negative control and treated cells using Kruskal-Wallis and a post-hoc Dunns test. For flow cytometry results, significant difference were determined between the negative and the treated cells using two-way analysis of variance (ANOVA) and a post-hoc Bonferroni test. Significance was defined as $p < 0.05$.

3. Results

3.1. Tentative chemical profiling

Tentative chemical profiling of both extracts yielded differential results (Table 1; Figure 1). Only apigenin-6,8-C-dihexose and trigonelline was present in both extracts, while all other compounds were only present in one of the extracts. Trigonelline had a higher prevalence in the methanol extract.

3.2. Effect of extracts on cellular parameters

The hot water extract did not significantly alter cell density (Figure 2A), GSH (Figure 2D) or lipid peroxidation (Figure 2F). Intracellular ROS concentrations were decreased by approximately 0.20-fold at <100 µg/mL, however, was increased significantly ($p < 0.05$) by 1.99-fold at 100 µg/mL (Figure 2C). The $\Delta\Psi m$ was dose-dependently reduced (Figure 2B), however, was only significant ($p < 0.05$) at 100 µg/mL (0.35-fold). Fatty acid content (Figure 2E) and caspase-3/7 activity (Figure 2H) was significantly ($p < 0.05$) reduced by 0.15-fold and 0.30-fold, respectively, at 100 µg/mL. The ATP levels were reduced by 0.56-fold at ≥32 µg/mL (Figure 2G).

The methanol extract did not significantly alter cell density (Figure 3A), GSH (Figure 3D), ATP (Figure 3G) and caspase-3/7 activity (Figure 3H). However, a slight increase in cell density was noted at higher concentrations. Intracellular ROS levels were below baseline at all concentrations (Figure 3C), with a significant ($p < 0.05$) reduction at ≥32 µg/mL (0.50-fold at 100 µg/mL). The $\Delta\Psi m$ was reduced similarly to that of the hot water extract (Figure 3B), but not as much (0.28-fold). Fatty acid content decreased at concentrations ≥3.2 µg/mL (Figure 3E), where significance ($p < 0.05$) was obtained at 100 µg/mL (0.43-fold). Lipid peroxidation tended to increase dose-dependently, but non-significantly, by up to 1.48-fold (Figure 3F). Caspase-3/7 activity was not significantly altered, though a slight decrease was observed at 100 µg/mL (0.14-fold) (Figure 3H).

Table 1: Phytochemicals tentatively identified from the hot water and methanol extracts of *M. oleifera* using ultra-performance liquid chromatography coupled to a photodiode array detector and high-definition mass spectrometer.

Identified phytochemicals	Extract		Empirical formula	Monoisotopic molecular mass (Da)	Monoisotopic calculated mass (Da)	ESI mode	Fragment ions observed (Da)
	Hot water	Methanol					
3-Caffeoylquinic acid		X	$C_{16}H_{18}O_9$	354.0951	353.0873	Negative	191.0493; 179.0276; 135.0416
3-p-Coumaroylquinic acid		X	$C_{16}H_{18}O_8$	338.1002	339.1080	Negative	163.0313; 119.0452
4'-O-Acetylglucomoringin		X	$C_{29}H_{27}NO_{10}S_2$	613.1076	612.0998	Negative	370.0905; 259.0060; 96.9576
4-Caffeoylquinic acid		X	$C_{16}H_{18}O_9$	354.0951	353.0873	Negative	173.0391; 135.0412
Apigenin-6,8-C-dihexose	X	X	$C_{27}H_{30}O_{15}$	594.1585	593.1507	Negative	285.0320
Glucomoringin		X	$C_{27}H_{25}NO_9S_2$	571.0971	570.0893	Negative	424.0323; 328.0800; 259.0053; 96.9606
Hyperoside	X		$C_{21}H_{20}O_{12}$	464.0955	463.0877	Negative	301.0831
Isorhamnetin rutinoside	X		$C_{28}H_{32}O_{16}$	624.1690	623.1612	Negative	315.0465
Isorhamnetin-3-O-glucoside	X		$C_{22}H_{22}O_{12}$	478.1111	477.1033	Negative	314.0378; 300.0257; 285.0365
Kaempferol acetyl hexose	X		$C_{23}H_{22}O_{12}$	490.1111	489.1033	Negative	285.0368
Kaempferol hydroxy-methylglutaroyl hexose	X		$C_{27}H_{28}O_{15}$	592.1428	591.1350	Negative	447.0878; 285.0331
Kaempferol malonyl hexose	X		$C_{24}H_{22}O_{14}$	534.1010	533.0931	Negative	489.1057; 285.0365
Kaempferol rutinoside	X		$C_{27}H_{30}O_{15}$	594.1585	593.1507	Negative	473.1050; 353.0598
Kaempferol-3-O-glucoside	X		$C_{21}H_{20}O_{11}$	448.1006	447.0927	Negative	284.0273; 285.0931
Quercetin acetylhexose	X		$C_{23}H_{22}O_{13}$	506.1060	505.0982	Negative	301.0281
Quercetin hydroxy-methylglutaroyl hexose	X		$C_{27}H_{28}O_{16}$	608.1377	607.1299	Negative	463.0798; 301.0278
Quercetin rutinoside	X		$C_{27}H_{30}O_{16}$	610.1534	609.1456	Negative	300.024
Trigonelline	X	X	$C_7H_7NO_2$	137.0477	138.0555	Positive	94.0657

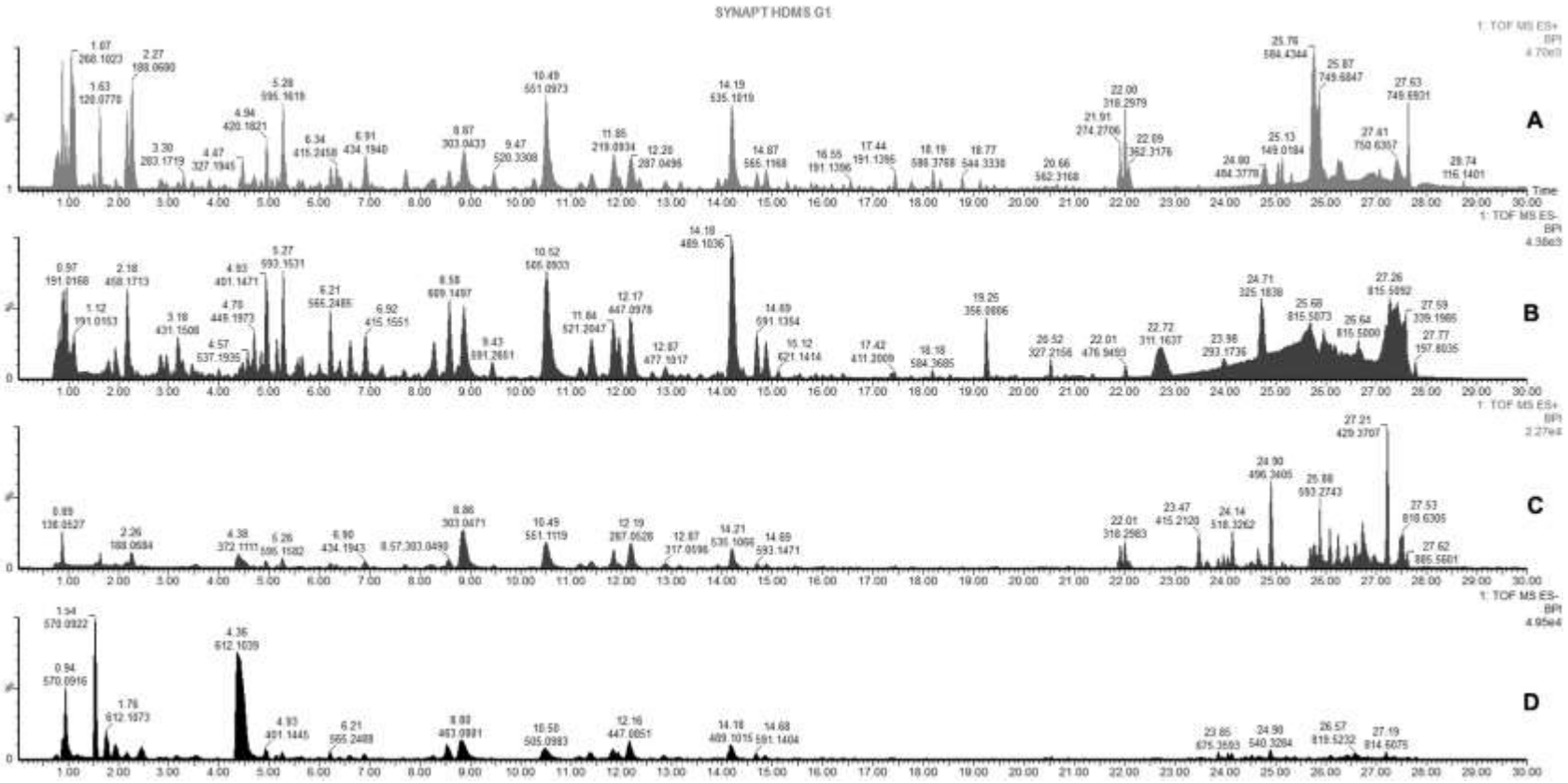

Figure 1: Representative chromatograms of the *M. oleifera* A,B) hot water and C,D) methanol extract obtained in A,C) positive and B,D) negative mode.

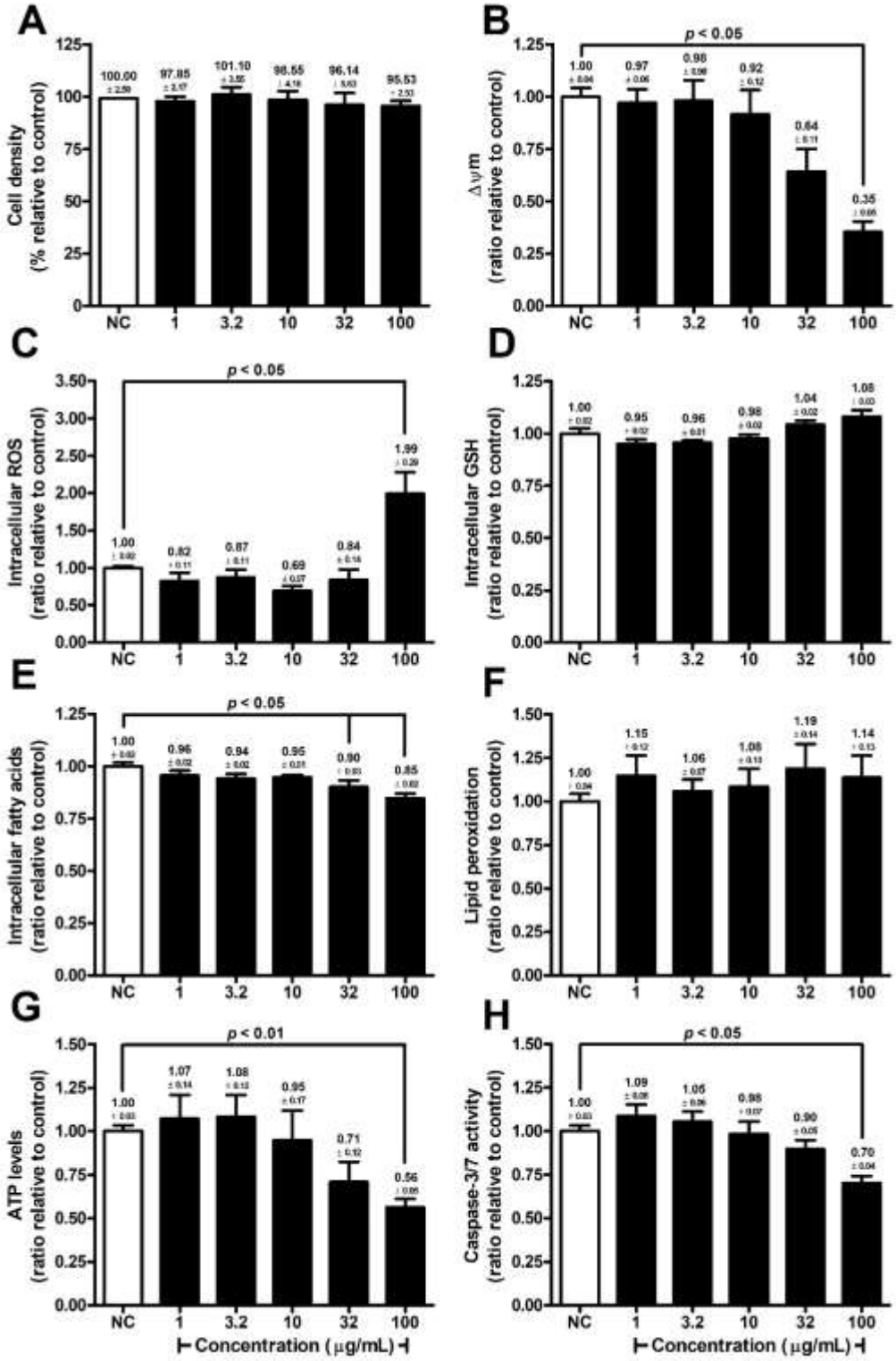

Figure 2: Cytotoxicity parameters of the *M. oleifera* hot water extract in HepG2 cells; A) cell density, B) ΔΨm, C) ROS concentration, D) GSH concentration, E) fatty acid concentration, F) lipid peroxidation, G) ATP levels and H) caspase-3/7 activity.

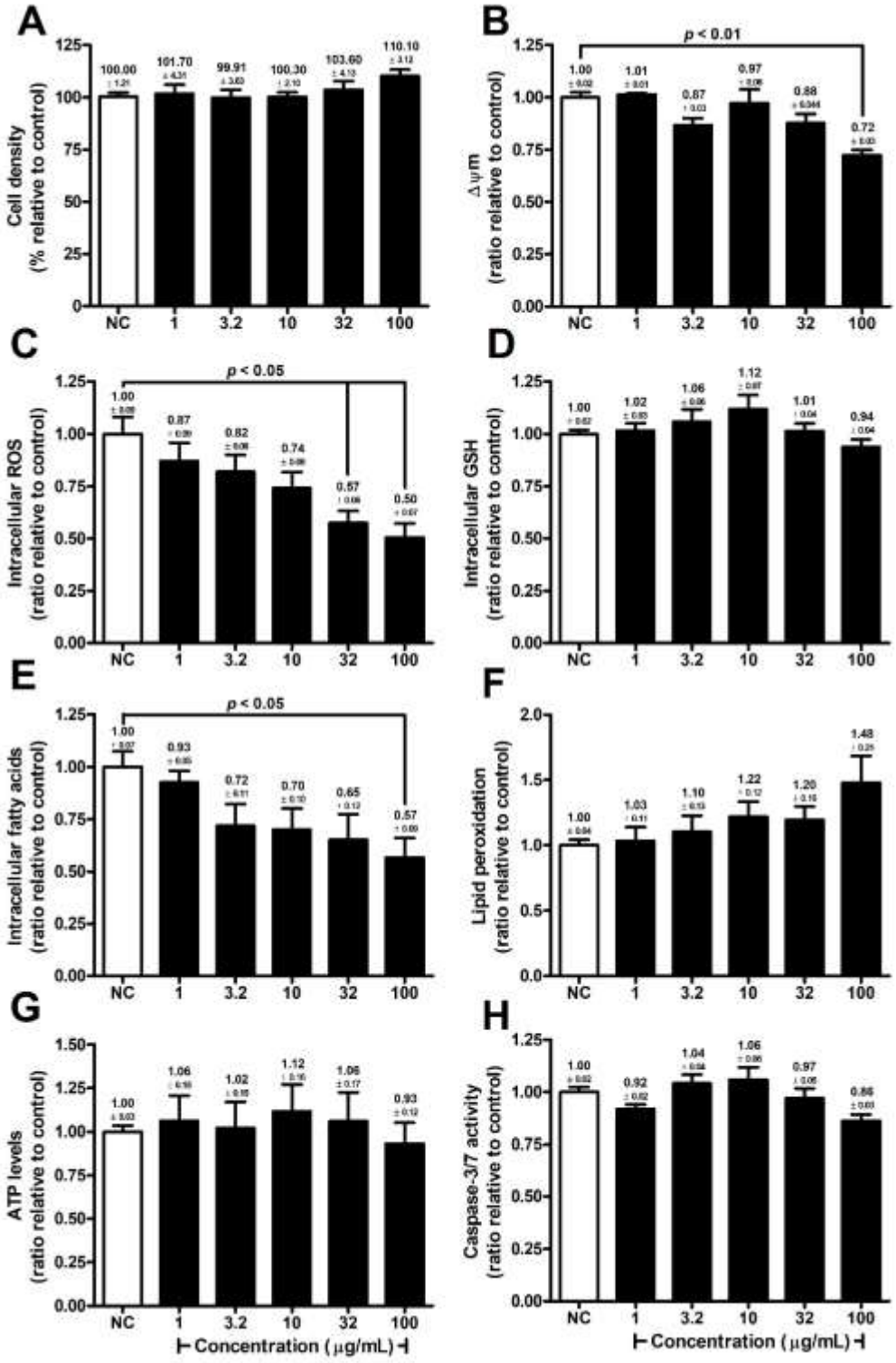

Figure 3: Cytotoxicity parameters of *M. oleifera* methanol extract in HepG2 cells; A) cell density, B) ΔΨm, C) ROS concentration, D) GSH concentration, E) fatty acid concentration, F) lipid peroxidation, G) ATP levels and H) caspase-3/7 activity.

3.3. Effect of extracts on cell cycling and cell viability

The hot water extract (100 µg/mL) did not significantly perturb the cell cycle after 24 h exposure (Figure 4B, Table 2), but either increased or decreased the amount of cells in the G0/G1-phase (6.22%) and S-phase (5.10%), respectively (Figure 4E, Table 1). The methanol extract increased the sub-G1-, G0/G1- and G2/M-phase by 1.32%, 3.53% and 1.64%, respectively, after 24 h, with a reduction of 5.17% in the S-phase (Figure 4, Table 2). After 72 h exposure, the methanol extract (100 µg/mL) increased the G0/G1- and G2/M-phase by 2.12% and 3.96%, respectively, while decreasing the S-phase by 6.08% (Figure 4, Table 2).

Table 2: HepG2 cell cycling after exposure to 100 µg/mL *M. oleifera* for 24 h and 72 h summarized as the shift in phase from the respective negative control.

Phase of cell cycle	Shift in cell cycle phase after exposure to *M. oleifera* (%)			
	Hot water extract		Methanol extract	
	24 h	72 h	24 h	72 h
Sub-G1	↑ 1.65	↓ 1.64	↑ 1.32	↓ 0.14
G1	↓ 1.77	↑ 6.22	↑ 3.53	↑ 2.12
S	↑ 0.36	↓ 5.10	↓ 5.17	↓ 6.08
G2/M	↑ 1.41	↓ 1.12	↑ 1.64	↑ 3.96

Cell viability was decreased by 8.96% ($p < 0.05$) after exposure to the hot water extract (100 µg/mL) for 24 h, with an increase in early apoptotic (5.43%) and necrotic cells (3.05%) (Figure 5B, Table 3). After 72 h exposure, viability was reduced by 8.74%, with an 2.66% and 5.62% increase in early apoptotic and necrotic cells, respectively (Figure 5E, Table 3). The methanol extract (100 µg/mL) reduced viability by 13.74% ($p < 0.001$) after 24 h exposure, while increasing early apoptotic, late apoptotic and necrotic cells by 1.31%, 1.02% and 11.41% ($p < 0.01$), respectively (Figure 5C, Table 3). After 72 h, viability was decreased by 11.08% ($p < 0.05$), with an increase of 3.97% and 6.22% for early apoptotic and necrotic cells, respectively (Figure 5F, Table 3).

Table 3: HepG2 viability after efor 24 h and 72 h summarized as the shift in viability status from the respective negative control. Significxposure to 100 µg/mL *M. oleifera* ant difference relative to the respective time points of the negative control: ★ $p < 0.05$, ★★ $p < 0.01$ and ★★★ $p < 0.001$.

State of viability	Shift in viability after exposure to *M. oleifera* (%)			
	Hot water extract		Methanol extract	
	24 h	72 h	24 h	72 h
Viable	↓ 8.96★	↓ 8.74	↓ 13.74★★★	↓ 11.08★
Early apoptosis	↑ 5.43	↑ 2.66	↑ 1.31	↑ 3.97
Late apoptosis	↑ 0.48	↑ 0.46	↑ 1.02	↑ 0.89
Necrosis	↑ 3.05	↑ 5.62	↑ 11.41★★	↑ 6.22

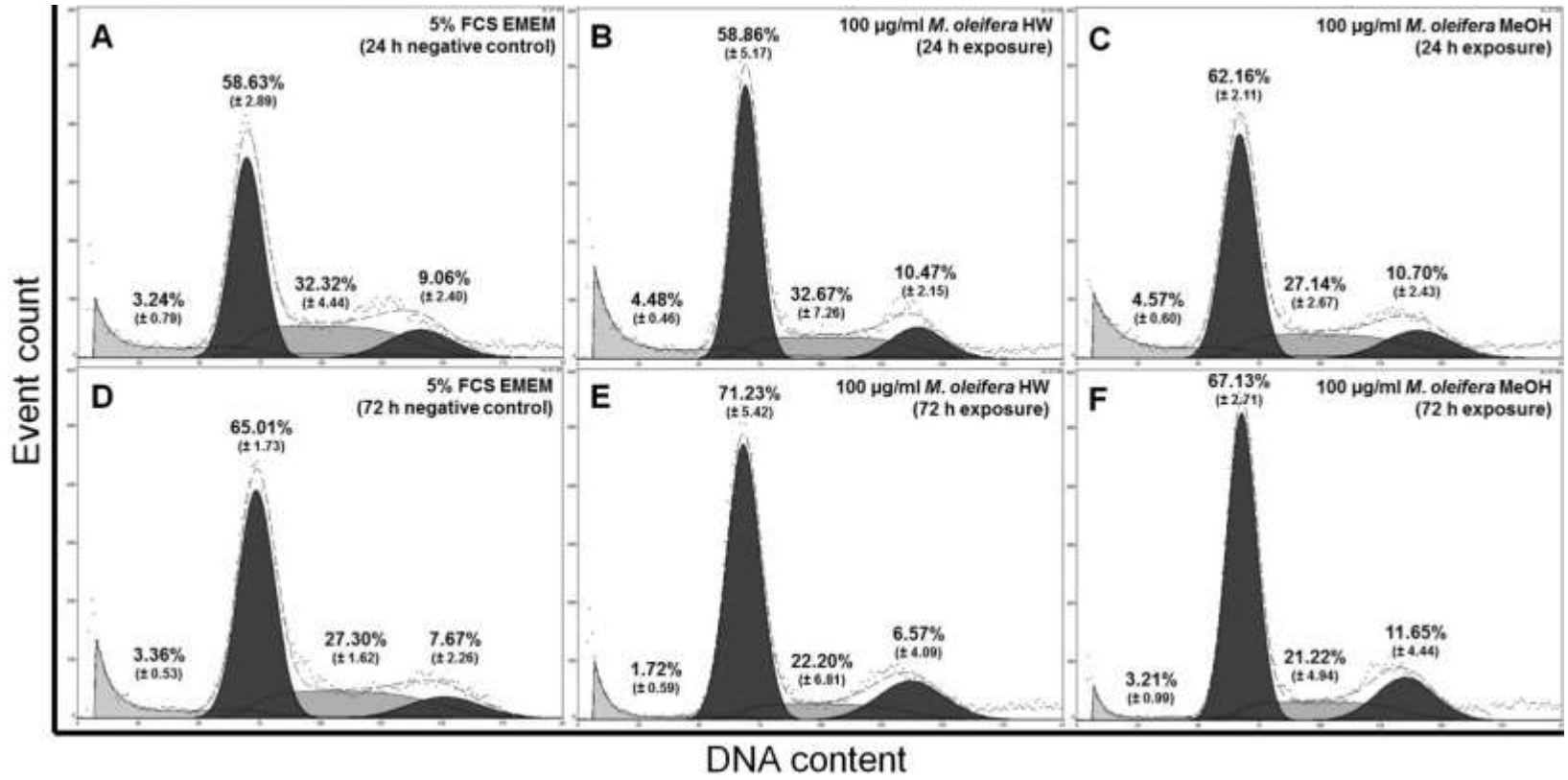

Figure 4: HepG2 cell cycling after exposure to 100 µg/mL *M. oleifera* for 24 h and 72 h; A) negative control (24 h), B) hot water extract (24 h), C) methanol extract (24 h), D) negative control (72 h), E) hot water extract (72 h), F) methanol extract (72 h).

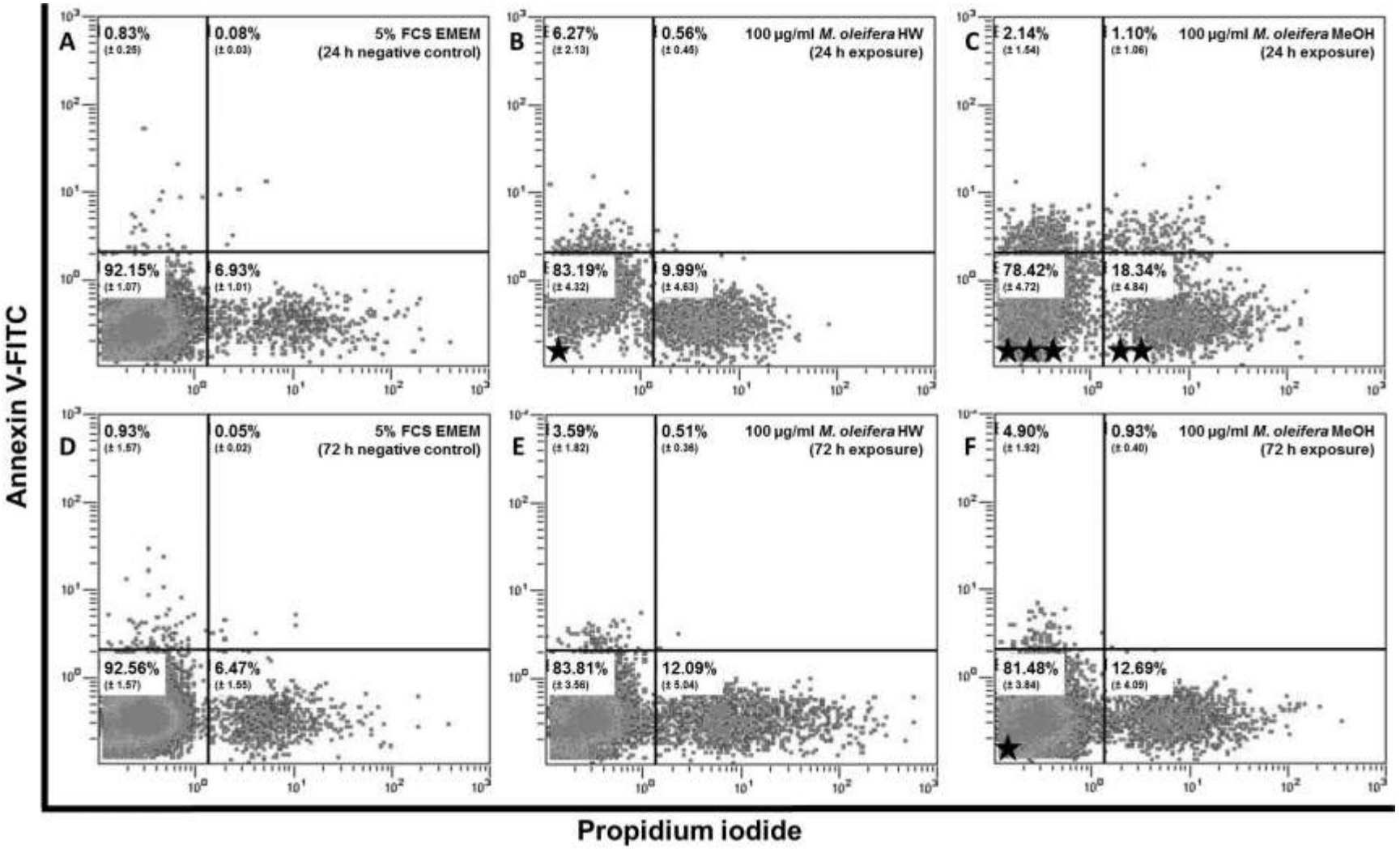

Figure 5: HepG2 viability after exposure to 100 µg/mL *M. oleifera* for 24 h and 72 h; A) negative control (24 h), B) hot water extract (24 h), C) methanol extract (24 h), D) negative control (72 h), E) hot water extract (72 h), F) methanol extract (72 h). Significant difference relative to the respective time points of the negative control: ★ $p < 0.05$, ★★ $p < 0.01$ and ★★★ $p < 0.001$.

3.4. Effect of extracts on oleic-acid induced cytotoxicity

The hot water extract decreased oleic acid-induced fatty acid accumulation, however, not dose-dependently and only significantly ($p < 0.05$) at 10 µg/mL (3.08-fold) (Figure 6B). The hot water extract's effects were paralleled by a slight reduction in cytotoxicity (10.38%), but it was not significant (Figure 6A). The methanol extract (at 100 µg/mL), reduced oleic acid-induced fatty acid accumulation and cytotoxicity by 4.1-fold and 27%, respectively, however, effects were already apparent at 3.2 µg/mL (Figure 6).

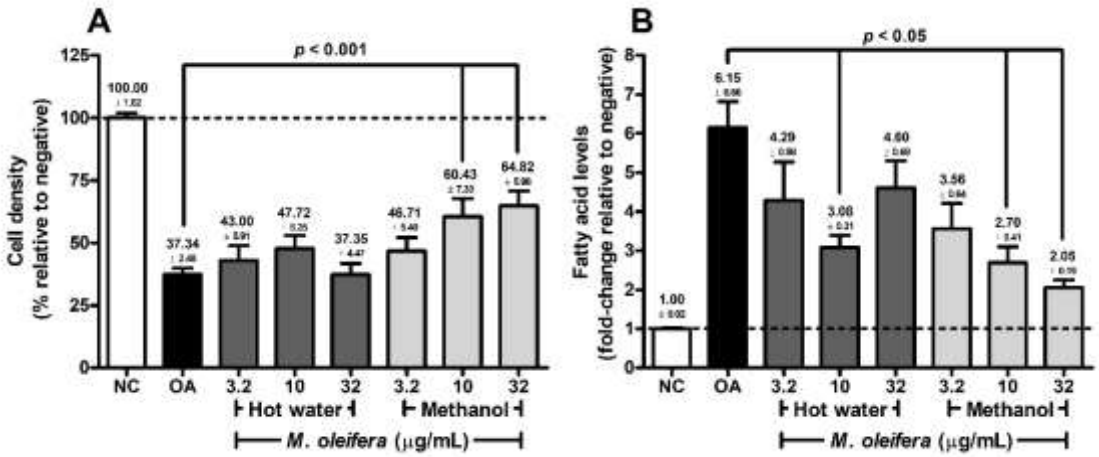

Figure 6: The protective effect of extracts against oleic acid (OA)-induced A) cytotoxicity and B) fatty acid accumulation. Significant differences were determined between oleic acid and extract-pre-treated cells

4. Discussion

Several of the tentatively identified phytochemicals in the extracts have previously been described in the leaves of *M. oleifera*: apigenin-6,8-C-dihexose (Makita *et al*., 2016), 3-caffeoylquinic acid (Bennett *et al*., 2003; Amaglo *et al*., 2010; Ramabulana *et al*., 2016), 4-caffeoylquinic acid, 3-p-coumaroylquinic acid (Ramabulana *et al*., 2016), glucomoringin, isorhamnetin-3-*O*-glucoside (Amaglo *et al*., 2010), kaempferol acetyl hexose (Ramabulana *et al*., 2016), kaempferol-3-*O*-glucoside (Bennett *et al*., 2003; Amaglo *et al*., 2010), kaempferol hydroxy-methylglutaroyl hexose (Makita *et al*., 2016), kaempferol-3-*O*-rutinoside (Amaglo *et al*., 2010), quercetin-3-acetylhexose (Ramabulana *et al*., 2016), quercetin hydroxy-methylglutaroyl hexose, quercetin rutinoside (Makita *et al*., 2016) and trigonelline (Mathur and Kamal, 2012).

Both extracts possessed minimal cytotoxicity as cell density was not decreased, however, viability was decreased by approximately 8-9% and 11-13% for the hot water and methanol extract, respectively. Although the methanol extract was shown to increase cell density marginally, this did not correlate to the mode of cell death in the assay performed, which could possibly be explained by the increased sensitivity that the latter modality offers. There is a paucity of literature available with regards to the cytotoxicity of leaf extracts, however, the few studies that are available corroborate the findings of the

present study. Little cytotoxicity was induced by aqueous (Monera *et al*., 2008; Varalakshmi and Nair, 2011; Asare *et al*., 2012; Waiyaput *et al*., 2012), ethanol (Lipipun *et al*., 2003), hot water (Lipipun *et al*., 2003), hydroethanolic (Waiyaput *et al*., 2012) and methanol (Monera *et al*., 2008; Varalakshmi and Nair, 2011) extracts when tested within similar ranges to the present study. Some cytotoxicity was apparent after exposure to hexane (Varalakshmi and Nair, 2011), hot water and ethanol extracts (Khalafalla *et al*., 2010) where IC_{50}'s were obtained at 70.00 µg/mL, 3.89 µg/mL and 6.20 µg/mL, respectively, suggesting bioactivity may be subject to geographical variation and selection of cell lines. Acute (Devaraj *et al*., 2007; Adedapo *et al*., 2009; Sudha *et al*., 2010; Chivapat *et al*., 2011; Asare *et al*., 2012) and chronic (Chivapat *et al*., 2011; Asare *et al*., 2012; Awodele *et al*., 2012) animal toxicity studies also support the lack of toxic effects of leaf extracts. Importantly, histopathologic assessment suggests little alteration to the liver apart from slight hepatocellular congestion (Awodele et al., 2012).

The marginal decrease in viability at 100 µg/mL was accompanied by an increase in both apoptosis and necrosis for the hot water extract, and primarily necrosis for the methanol extract. Although pro-apoptotic effects have been associated with aqueous extracts, this was only at high concentrations (>20 mg/mL) (Sreelatha *et al*., 2011), which supports the lack of significant apoptosis observed. Cell cycle kinetics was not perturbed, apart from a slight, non-significant increase in G0/G1-phase cells, similar to what was described in literature (Diab *et al*., 2015). Compounds such as kaempferol, trigonelline and quercetin (Baliga, 2010; Krishanamurthy *et al*., 2015; Yoo and Allred, 2016) have been described to exert antiproliferative effects, thus may contribute to the low level reduction of cell viability shown at high concentrations.

Both extracts induced mitochondrial membrane depolarisation, however, did not promote cytotoxicity. These effects suggest either an underlying mitochondrial toxicity which may manifest at a later stage, or indicates an increased mitochondrial respiration rate (Wallace and Starkov, 2000) rather than cytotoxicity. In support of the latter, fatty acid levels were decreased. Increased respiration would promote β-oxidation of fatty acids for bioenergetics (Bartlett and Eaton, 2004; Begriche *et al*., 2011; Moreira *et al*., 2011). Although the hot water extract induced a greater level of depolarisation, this did not translate to a higher level of fatty acid decline. Although it is suggested that bioenergetics may have been promoted, ATP levels did not increase, but rather were depleted. This effect suggests low-level cytotoxicity, where a balance may be perturbed between use and production of ATP.

Intracellular ROS levels were decreased below baseline for both extracts, apart from a large increase at 100 µg/mL hot water extract exposure. Such an effect is supported by literature, where an aqueous extract increased ROS in KB HeLa-contaminant carcinoma cells at ≥50 µg/mL (Sreelatha *et al*., 2011). Certain polyphenols, such as quercetin and kaempferol, have been reported to generate ROS in cancerous cell lines through pro-oxidant activity (Yordi *et al*., 2012). Reduced ROS, on the other hand, may perturb signaling pathways (Vivancos *et al*., 2010), and thus may debilitate functional processes. This, however, did not appear to translate to any significant cellular detriment apart from the highest concentration tested. Intracellular GSH concentrations

trended towards an increase after exposure to the hot water extract, however, this was not significant. The methanol extract did not alter GSH. Extracts of *M. oleifera* have been reported to increase GSH (Luqman *et al*., 2009), which may be an adaptive *de novo* response against ROS generation (Vivancos *et al*., 2010). Although no ROS was formed, lipid peroxidation appeared to increase slightly with methanol extract exposure, thus a different free radical source, such as reactive nitrogen species, may be responsible for this effect.

Caspase-3/7 activity was reduced after hot water extract exposure, though it was unaltered by the methanol extracts. An unnamed protease inhibitor has been isolated and characterised from *M. oleifera* (Bijina *et al*., 2011), and may explain the inhibitory effect displayed. The combined reduction of ATP and caspase-3/7 activity after hot water extract exposure supports a preference towards necrotic cell death, as observed in the mode of cell death assay. As these parameters were unaltered by the methanol extract, the underlying mechanism is unknown.

Oleic acid induces steatosis and cytotoxicity in HepG2 cells by increasing triglyceride levels, incurring oxidative stress through lipid peroxidation and reduced GSH levels, and ultimately promoting lipotoxicity (Vidyashankar *et al*., 2013). In the present study, oleic acid significantly decreased cell density and increase fatty acid accumulation. Although the hot water extract decreased fatty acid accumulation, it did so non-dose-dependently, and did not translate to a reduction in cytotoxicity. The methanol extract, on the other hand, attenuated cytotoxicity and fatty acid accumulation from ≥ 3.2 µg/mL. Although trigonelline does not alter fatty acid uptake in Caco-2 cells (Riedel *et al*., 2014), it does reduce lipid accumulation and inhibit fatty acid synthase in 3T3-L1 cells (Ilavenil *et al*., 2014). Trigonelline, and to a larger extent its thermal degradation product, N-methylpyridinium, promotes cellular respiration and fatty acid usage in HepG2 cells, most likely due to depolarisation of the mitochondria (Riedel *et al*., 2014). Furthermore, 3-caffeoylquinic acid has been shown to promote β-oxidation and inhibit fatty acid synthase in animal studies (Cho *et al*., 2010). This is in support of the effects observed throughout the cytotoxicity screening, where mitochondria were depolarised and fatty acid content decreased. As such, *M. oleifera* may promote fatty acid usage during bioenergetic pathways due to increased respiration, which will ultimately reduce fatty acid accumulation and downstream cytotoxicity of oleic acid.

5. Conclusion

M. oleifera does not appear to possess significant cytotoxic effects in an *in vitro* model of hepatoxicity, however, further research is needed to ascertain whether higher concentrations may precipitate mitochondrial toxicity, induce oxidative stress or promote necrosis, especially when exposed to hot water extracts. Continuous use, such as in nutrition, may allow for higher concentrations to be achieved, thus caution is needed. A risk thus arises where chronic intake of the leaf material, or extracts thereof, may alter mitochondrial function and induce a steady low level of cytotoxic effects, however, this would need to be assessed using *in vivo* chronic toxicity models in animals. Anecdotal evidence thus far does not appear to indicate a high risk, however, caution is advised.

Apart from the apparent safety, the methanol extract was shown to be a potential aid in preventing steatotic changes due to exposure to fatty acids, possibly via a mitochondrial-mediated route.

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