Ficus deltoidea Leaf Alters Oxidative Stress, Protein Homeostasis and Ubiquitin-Proteasome Pathways in Fatty Acid-Induced Cell Line

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SUMMARY

Research background. Ficus deltoidea (mistletoe fig) is a shrub well known among locals in Malaysia primarily for its treatment of toothaches, colds and wounds. The aim of this study is to determine the potential of leaves, sourced from three different varieties of *F. deltoidea*, to exhibit antioxidant activity, a reduction of lipid concentration, and protein expression in steatosis-induced liver cell lines.

Experimental approach. The leaves of three *F. deltoidea varieties*, namely *Ficus deltoidea* var. *angustifolia*, *Ficus deltoidea* var. *trengganuensis* and *Ficus deltoidea* var. *kunstleri*, were subjected to water extraction. The resulting crude extracts were fractionated using water and ethyl acetate. Palmitic acid was used to induce lipid accumulation (steatosis) in human liver (WRL68) cells, before all the samples were tested for their lipid-reducing activity. Several proteomic approaches were incorporated. The changes in protein expression were determined using 2-dimensional gel electrophoresis separation, whereas identification of our protein spots of interest was carried out *via* matrix-assisted laser desorption/ionization time-of-flight.

Results and conclusions. Ficus deltoidea var. *kunstleri* alone demonstrated the ability to reduce lipids at the highest tested concentration (200 µg/mL) and was, therefore, used for subsequent experiments. Treatment with *Ficus deltoidea* var. *kunstleri* was found to restore redox status by increasing superoxide dismutase and glutathione peroxidase amounts and decreasing malondialdehyde formation. Six proteins were successfully identified; these were heat shock protein beta-1 (HSPB1), proteasome subunit alpha type 1 (PSMA1), glutathione S-transferase omega 1 (GSTO1), peroxiredoxin-1 (PRDX1), histone H2B (HIST1H-2BD) and ubiquitin c-terminal hydrolase L3 (UCHL3). Through bioinformatics analysis, it was found that these proteins were significantly involved in specific pathways such as oxidative stress (PRDX1 and GSTO1), protein homeostasis (HSPB1) and degradation (UCHL3 and PSMA1).

Novelty and scientific contribution. F. deltoidea pretreatment was shown to reduce lipid accumulation, thus improving the redox status and protein homeostasis. This suggests the role of F. deltoidea as a preventive mechanism in non-alcohol fatty liver disease.

Keywords: Ficus deltoidea; steatosis; oxidative stress; ubiquitin-proteasome system; protein homeostasis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a group of liver disorders that include steatosis (excessive fat accumulation in the liver), steatohepatitis (liver inflammation), fibrosis (formation of scar tissue in the liver), cirrhosis (late stage of scarring of the liver), and the potential for progression to hepatocellular carcinoma (liver cancer). This chronic liver disease develops when there is no excessive alcohol consumption. NAFLD is a multisystem illness that affects multiple extrahepatic organs and regulatory mechanisms, according to research (*1,2*).

To date, there is very little effective treatment available for NAFLD besides dietary changes, regular exercise, mass loss and usage of lipid-lowering drugs. Statins, fibrates,

bile acid sequestrants, niacin, ezetimibe and omega-3 fatty acids represent some of the drugs that are currently able to treat NAFLD (*3,4*). Despite their function as a lipid-lowering agent, some patients may develop adverse effects, especially towards statins, such as myopathy, hepatotoxicity, diabetes mellitus, and renal and neurologic decline (*5,6*). For this reason, there is a need for a natural source of treatment, particularly derived from plants.

In Malaysia, *F. deltoidea*, commonly known as Mas cotek is a well-known medicinal herb, especially among the Malays, and is traditionally used to treat diabetes, headaches, sore throats, and colds. Previous studies have reported its glucose-lowering effect in both animal and cell culture models (*7,8*). The leaves were a promising candidate for treatment of osteoarthritis by reducing the serum level of IL-1β, PGE2, PIINP and CTX-II in osteoarthritis-induced rats (*9*), while Nurdiana *et al.* (*10*) discovered the neuroprotective effects of *F. deltoidea* by improving cortical gyrification, spatial learning and memory in streptozotocin-induced diabetic rats. In addition, our previous studies demonstrated the antioxidant activities of *F. deltoidea* leaves without toxicity towards normal liver cell lines (*11*). There is no published research on the effects of *F. deltoidea* on NAFLD, and the possibility of using it for other medical conditions has not yet been adequately assessed. This work uses a cultured cell model to investigate the potential benefits of the leaves of three *F. deltoidea* varieties on NAFLD improvement, particularly on lipid accumulation.

MATERIALS AND METHODS

Chemicals

Ethyl acetate, iron(II) sulphate, isopropanol, dimethyl sulfoxide (DMSO), methanol and chloroform were purchased from Thermo Fisher Scientific (Leicester, UK), while low-density lipoprotein (LDL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA) and thiobarbituric acid (TBA) were acquired from Merck (Darmstadt, Germany). Quercetin, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase assay kit and formaldehyde were from Sigma-Aldrich, Merck (St. Louis, MO, USA). Cell culture medium such as Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and penicillin-streptomycin were from Nacalai Tesque (Tokyo, Japan). Palmitic acid and Oil Red O (ORO) were from Acros Organics (New Jersey, NJ, USA). Lastly, phosphate buffer saline (PBS) was from Oxoid (Basingstoke, UK).

Ficus deltoidea leaf preparation

The leaves of three different variants of *Ficus deltoidea* species: *angustifolia* (FDA), *trengganuensis* (FDT) and *kunstleri* (FDK), were collected in Rembau, Negeri Sembilan, Malaysia. The Herbarium, Rimba Ilmu, Universiti Malaya assigned unique specimen numbers to each variation (KLU046467,

KLU046469 and KLU046471). The prior publication describes how the plant extracts were obtained (*11*). Briefly, *F. deltoidea* leaves were extracted with water, followed by liquid-liquid extraction using water and ethyl acetate (**Fig. S1**). For each variety, subsequent tests were carried out with crude, water (WF) and ethyl acetate (EAF) fractions.

Low-density lipoprotein oxidation assay

This assay provides a measure of the intrinsic susceptibility of the lipoproteins to be oxidised by oxidants, which in this case were Fe^{2+} ions. Using a microplate reader (Infinite M1000; Tecan, Männedorf, Switzerland), the change in the absorbance at 234 nm in 10-min intervals was monitored (Infinite M1000; Tecan) to determine the extent of LDL oxidation in relation to the generation of conjugated dienes (CD). LDL (30 g/mL) was incubated with iron(II) sulphate (10 µM) in the absence (control) or presence (0.5 g/mL) of extracts for 4 h at a constant temperature of 37 °C (*12*). The lag phase, expressed in minutes, was defined as the period when no oxidation occurred. Quercetin was used as a positive control. All analyses were performed in four replicates.

In vitro cell-free assay and kinetic studies of 3-hydroxy--3-methylglutaryl coenzyme A reductase

This assay was performed to screen and identify the most effective crude extract and its fractions with statin-like activities. The HMG-CoA reductase inhibitory activity of the samples (5 µg/mL) was measured spectrophotometrically (Infinite M1000; Tecan) at 340 nm according to the manufacturer's (Sigma-Aldrich, Merck) protocol. All analyses were done in triplicate. Results were expressed as a percentage of inhibition using the following formula:

$$\text{Inhibition} = [(A_c - A_t)/A_c] \cdot 100 \qquad /1/$$

where A_c is the absorbance of the control reaction and A_t is the absorbance in the presence of the extracts.

Cell culture and cytotoxicity of palmitic acid

Human liver cells (WRL68) were a gift of Dr Nurshamimi and Dr Shatrah from the Department of Molecular Medicine, Faculty of Medicine, Universiti Malaya. At 37.5 °C, the cells were grown in T25 flasks in DMEM supplemented with 10 % FBS and penicillin-streptomycin in 5 % CO_2.

Cells were exposed to palmitic acid to induce fatty acid overload to simulate an *in vitro* steatosis condition. Cells ($1.5 \cdot 10^4$) were seeded in a 96-well plate and exposed to different concentrations of palmitic acid (10 µL), ranging from 0 to 800 µM for 24 h. The same amount of BSA added to the cells was used as a negative control. Cytotoxicity was measured after 24 h by adding 10 µL of MTT reagent (5 mg/mL) to the cells and incubating them for an additional 4 h. Isopropanol (100 µL) was added to each well in place of the medium and MTT reagent (*11*). The percentage of inhibition was

calculated from the absorbance measured at 595 nm, using Eq. 1. Median inhibitory concentrations (IC_{50}) were also determined. Each experiment was carried out in two independent batches, each in triplicate (11).

Intracellular lipid content

Lipid absorption was quantified by using an ORO dye solution. Briefly, cells were washed with ice-cold PBS and fixed in 50 μL of 10 % formaldehyde for 1 h. After 3 changes of ice-cold PBS, 50 μL of ORO were added, incubated for another 2 h at room temperature, and rinsed with ice-cold PBS. For quantitative analysis of cellular lipids, 40 μL of DMSO were added to each well, and absorbance was monitored at 510 nm (Infinite M1000; Tecan). The examined cells were detected and quantified, and the ORO-stained cells were measured relative to the control well. This experiment was carried out in two different batches, each in triplicate. Results were expressed as absorbance at 510 nm relative to the control.

In this experiment, three different treatment groups were used: (*i*) the vehicle group (negative control, BSA), (*ii*) the palmitic acid group (positive control), and (*iii*) the treatment group, which was given the crude extract and fractions of *F. deltoidea* leaves at a final concentration of 50, 100 and 200 μg/mL for 24 h before being incubated with palmitic acid for additional 24 h. A sample that showed the most significant changes was subjected to further analysis. For the downstream experiment, treatment was carried out in a T25 flask.

Cellular antioxidant activities

The sample that showed significant changes in lipid-reducing activity was tested using superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) assays. Briefly, SOD and GPx activities were determined using the kits from Elabscience (Houston, TX, USA) and measured spectrophotometrically (Infinite M1000; Tecan) at 450 nm according to the manufacturer's protocol.

For the lipid peroxidation assay, cell suspension (0.1 mL) and TBA reagent (0.5 mL) were combined, and the mixture was heated for 20 min. After allowing the mixture to cool, it was centrifuged (Universal 32R; Hettich Instruments, Tuttlingen, Germany) at 2500×*g* for 10 min at 25 °C. The absorbance of the supernatant (0.1 mL) was measured at 550 nm (Infinite M1000; Tecan) in a 96-well plate. All experiments were done in triplicate. Results were expressed as MDA in ng/100 μg of protein (13).

2-dimensional gel electrophoresis and image analysis

Changes in the cellular response of steatosis-prone WRL68 cells with the extract were investigated using a 2DE-based proteomic approach. Cell lysate (0.1 mL) was precipitated with 0.4 mL of methanol, 0.1 mL of chloroform and 0.3 mL of water. Later, 100 μg of protein was mixed with sample buffer and incubated for 1 h at room temperature. This mixture was then applied to DryStrips (*l*=13 cm, pH=3–10; GE Healthcare, Uppsala, Sweden) and rehydrated for at least 16 h. Cell lysate proteins were isoelectrically focused at 20 °C according to the following protocol: (*i*) 500 Vh, 500 V (step-and-hold), (*ii*) 1000 Vh, 1000 V (gradient), (*iii*) 16 000 Vh, 8000 V (gradient), and (*iv*) 12 000 Vh, 8000 V (step-and-hold); and second dimension separation was carried out using a homogenous pre-cast gel (14). Gel electrophoresis was carried out at 50 V and 40 mA/gel for 30 min and then at 500 V and 40 mA/gel for 3 h (SE600 Ruby; GE Healthcare, Princeton, NJ, USA). The gels were stained using the silver staining method (15).

The stained gels were then analysed using Progenesis SameSpots v. 3.0 (Nonlinear Dynamics Ltd., Newcastle, UK). Protein spots showing at least a 1.3-fold difference in average expression level were considered statistically significant (p≤0.05).

Mass spectrometry and database search

Spots showing significant changes were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF/ToF; ABI 4800 Plus, AB Sciex, CA, USA). Proteins were identified using the MASCOT search engine (Matrix Science Ltd., London, UK). The obtained MS data were searched against *Homo sapiens* entries in the Swiss-Prot database (16) with the following parameters: one missed cleavage allowed, carbamidomethyl cysteine as the fixed modification, and oxidation of methionine as the variable modification. Proteins with a confidence level of ≥95 % were identified. Proteins with a score of more than 50 and a best ion score (MS/MS) of more than 43 indicated significant identity (p<0.05).

Bioinformatics analysis

Data from the MS analysis was combined with bioinformatics data mining techniques to show the relationship between the discovered proteins and the metabolic pathways relevant to the phenomenon under study. The discovered proteins were exposed to a web-based bioinformatics software such as PANTHER (http://pantherdb.org/) and Reactome (https://reactome.org/) to understand the mechanism underlying the lipid-reducing activity of the sample (17,18).

Statistical analysis

The Microsoft Excel statistical package (Microsoft, Redmond, WA, USA) was used to examine the results, which were presented as mean standard deviation (S.D.). The Student's *t*-test was used to determine statistical significance. In comparison to the control, differences in average values at the 95 % confidence level (p<0.05) were deemed statistically significant.

RESULTS AND DISCUSSION

Effect of Ficus deltoidea leaves on lipid-related assays

The inhibitory actions of *Ficus deltoidea* varieties were assessed in this study using quantitative techniques including LDL oxidation and HMG-CoA reductase tests. Increased LDL level was previously reported as one of the risk factors associated with NAFLD (*19*). Oxidized LDL (oxLDL) can activate hepatic stellate cells, which play a key role in the pathogenesis of non-alcoholic steatohepatitis (NASH) (*20*). Previous studies have also reported higher levels of lipid peroxidation products such as oxLDL and thiobarbituric acid reactive substances (TBARS) in patients with NASH than their respective controls (*21,22*). FDK had the longest lag phase among the samples, indicating that it has a preventive effect against LDL oxidation (**Fig. 1a**). In addition, researchers have identified HMG-CoA reductase as a viable candidate for treating NAFLD (*23*). All crude water extracts of *F. deltoidea* leaves showed favourable inhibition activity towards HMG-CoA reductase, whereas minimal or no activity was observed in the water and ethyl acetate fractions (**Fig. 1b**). The inhibitory effect of crude extracts may have been brought on by a variety of phytochemicals that worked collectively. Even though *F. deltoidea* leaves exhibited a lower inhibitory activity than pravastatin,
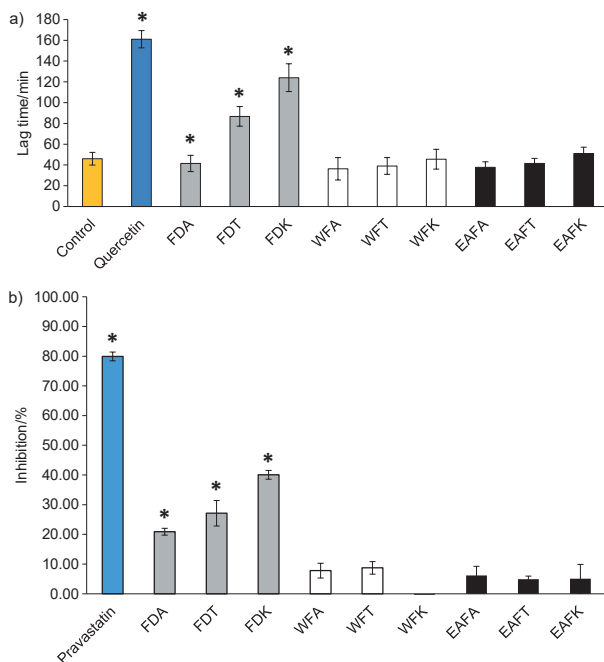
our findings may point to its potential mode of action, which involves preventing the production of endogenous cholesterol.

Inhibition of lipid accumulation (steatosis) in palmitic acid-induced cells by F. deltoidea extracts

Natural remedies have been the focus of the hunt for a NAFLD cure in recent years because of their accessibility and degree of efficacy. Since palmitic acid, a saturated free fatty acid (FFA), is the most prevalent circulating FFA in the human body, particularly in plasma and tissues, as well as in the typical diet, it was utilised in our investigation to simulate steatotic conditions (*24*).

Based on **Fig. 2a**, percentage of cell viability decreased in a dose-dependent manner. The lowest concentration of palmitic acid (100 µM) shows no sign of toxicity, whereas at higher concentrations (200, 400 and 800 µM) a decrease in the cell viability was observed. Also, morphological changes of WRL68 cells treated with different concentrations of palmitic acid are shown in **Fig. S2** (panel A). The same number of cells and concentrations of palmitic acid were tested on WRL68 cells to induce lipid accumulation and were observed using Oil Red O. **Fig. 2b** shows significant differences between the control and palmitic acid groups. The highest amount of lipid accumulation can be seen in the cells treated with 100 µM of palmitic acid compared to controls, while higher concentrations of palmitic acid (200, 400 and 800 µM) showed a
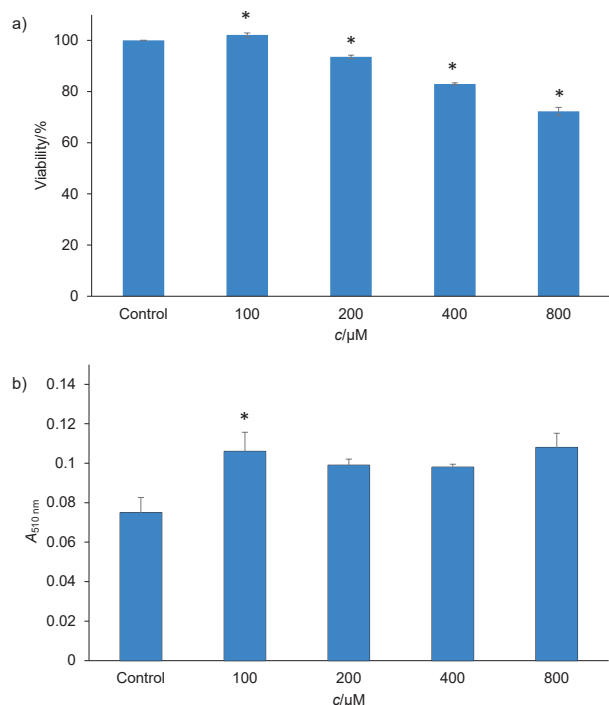
Fig. 1. The inhibitory effect of: a) crude water extracts and fractions (γ=0.5 µg/mL) of different varieties of *Ficus deltoidea* var. *angustifolia* (FDA), *Ficus deltoidea* var. *trengganuensis* (FDT) and *Ficus deltoidea* var. *kunstleri* (FDK) on Fe^{2+}-induced conjugated diene from low density lipoprotein (LDL). Values are displayed as mean±S.D. (*N*=4), and b) *F. deltoidea* (γ=5 µg/mL) on HMG-CoA reductase activity. The data were expressed as mean±S.D. (*N*=3). Negative control (orange bar), positive control (blue bar), crude extract (grey bar), water and ethyl acetate fraction of FDA, FDT and FDK (WFA, WFT and WFK, white bar and EAFA, EAFT and EAFK, black bar, respectively). Significance in comparison to the control is indicated by an asterisk (p<0.05)

Fig. 2. The results show: a) toxicity of palmitic acid (*c*=100–800 µM) on WRL68 cells after 24 h of incubation, and b) lipid accumulation in WRL68 cells after treatment with different concentrations of palmitic acid (*c*=100–800 µM). Values represent mean±S.D. (*N*=3). Significant differences from the control are denoted by asterisks (p<0.05)

reduction in lipid accumulation. Despite higher lipid accumulation, morphological changes such as shrinkage can be seen, as can empty spaces between the cells (**Fig. S2**, panel B). Thus, for this study, 100 µM of palmitic acid was chosen as the appropriate concentration to induce lipid accumulation and mimic the steatosis condition.

In **Fig. 3**, significant differences between the negative control and positive control groups were detected. Among all tested samples, only the crude extract of FDK showed a reduction in lipid accumulation. Treatment with FDK decreased significantly and in a dose-dependent manner the production of lipid droplets in WRL68 cells (**Fig. 3c**). Most of the samples, in contrast, lacked any inhibitory actions (**Figs. 3a** and **3b** and **Figs. 3d–3i**). Plants and compounds of different species, such as fenugreek and rosmarinic acid, have been reported to possess the capacity to reduce lipid accumulation in different settings (*25,26*).

FDK improves antioxidant status in steatotic palmitic acid-induced WRL68 cells

According to studies, an excess of lipids in the liver can lead to inflammation, endoplasmic reticulum (ER) stress, mitochondrial malfunction, and a decrease in the ability of hepatocytes to produce endogenous antioxidants (*27*). Thus, NAFLD may be prevented and treated by lowering the accumulation of lipids and reducing oxidative stress. Some of the crucial oxidative stress indicators in NAFLD include SOD, GPx and MDA. Based on these results (**Fig. 4a** and **Fig. 4b**), cells incubated with palmitic acid caused a reduction of both SOD and GPx antioxidant enzymes in comparison to the control group, suggesting the cells were under oxidative stress. Even though the concentration of palmitic acid used in this study was not lethal, it did influence the oxidative status of the cells. The addition of FDK to the cells increased the activity of SOD and GPx, which demonstrates that FDK could aid
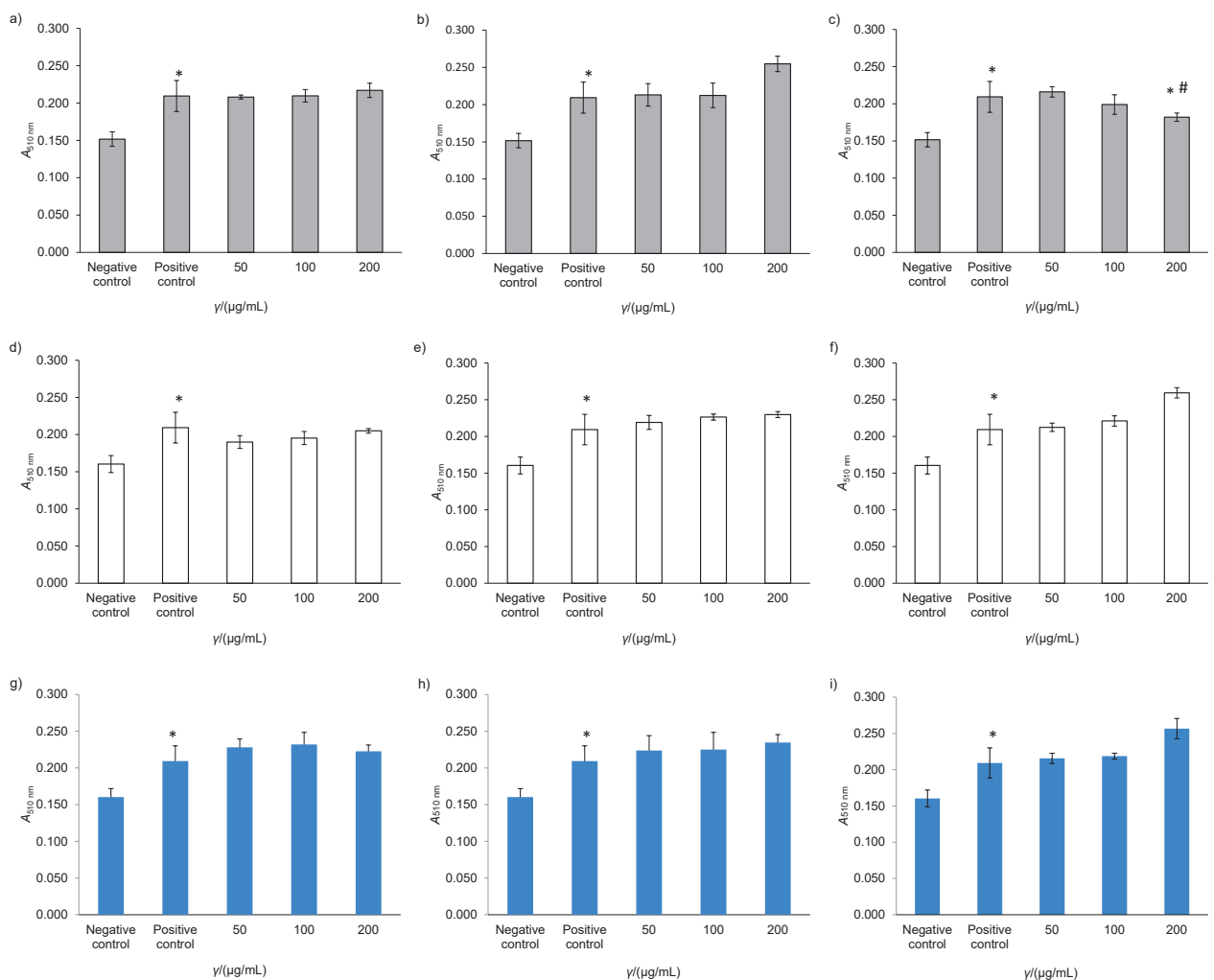

Fig. 3. Lipid-reducing activity of *Ficus deltoidea* var. *angustifolia* (FDA; graphs a, d and g), *Ficus deltoidea* var. *trengganuensis* (FDT; graphs b, e and h) and *Ficus deltoidea* var. *kunstleri* (FDK; graphs c, f and i) in steatotic WRL68 cells measured with Oil Red O assay at different concentrations (γ=50–200 µg/mL). Crude extract (grey bar), water fraction (white bar), ethyl acetate fraction (blue bar). Values represent mean±S.D. (*N*=3). Symbols indicate the significance of the treatment group compared with the negative control (*) and positive control (#) groups (p<0.05). Negative control are cells incubated without palmitic acid or FDK, and positive control are cells incubated with palmitic acid

in scavenging excess reactive oxygen species (ROS) produced by palmitic acid. Both enzymes play a critical role in first-line defences against cellular ROS accumulation.

Lipid peroxidation is another parameter of oxidative stress that has been assessed in this study. Steatosis, which occurs due to lipid accumulation, provides a potential substrate for lipid peroxidation and ROS toxicity. Excessive ROS accelerate the lipid peroxidation process, which then causes the liver to produce other reactive metabolites like MDA. Thus, inhibiting the lipid peroxidation is beneficial in the prevention and treatment of NAFLD. The addition of FDK in a pre-treatment step manages to reduce the formation of MDA in the treated cells (**Fig. 4c**). By preventing lipid peroxidation, this demonstrated the protective effect of FDK against palmitic acid lipotoxicity. Thus, based on the results, the increase of SOD and GPx activities, along with the reduction of MDA with FDK suggested an improvement of the oxidative balance in steatosis cells. Other studies have also shown that improving oxidative status in steatotic cells contributed to the lipid-reducing activities of plant extracts or compounds (*28*,*29*).

Effect of FDK on protein expressions

Using 2DE to identify changes in the protein abundance profile, the lipid-reducing effect of FDK was further studied (**Fig. 5**). Six proteins were discovered to be downregulated when pretreated with FDK and increased in the positive control group (**Fig. S3**). The protein spots HSPB1, UCHL3, GSTO1, PSMA1, HIST1H2BD and PRDX1 were successfully identified by MALDI-ToF/ToF (**Table S1**). According to the PANTHER database (*17*), most of the proteins that have been identified are cellular components that play a role in a variety of biological processes, including cellular operations, immune system functions, metabolic processes, and reactions to stimuli (**Fig. S4**). The proteins identified as being abundant in the Reactome pathway analysis are shown in **Table S2**. The proteins that have been discovered are linked to several pathways, primarily those that deal with protein homeostasis (UCH proteinases, protein ubiquitination, deubiquitination and neddylation), as well as the cellular stress response.
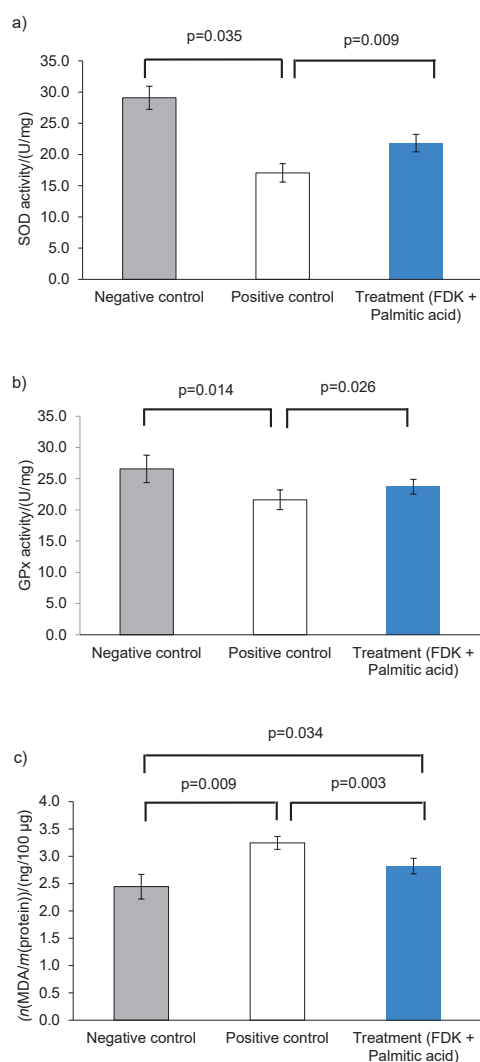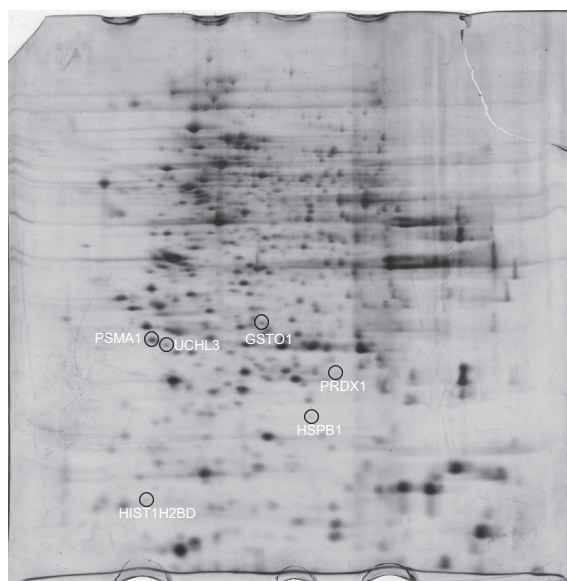

a)

b)

c)

Fig. 4. *Ficus deltoidea* var. *kunstleri* (FDK) crude extract lowers the imbalance of oxidative stress in steatotic WRL68 cells. The presence of γ(FDK)=200 μg/mL in palmitic acid-induced WRL68 cells had an impact on: a) superoxide dismutase (SOD), b) glutathione peroxidase enzymes (GPx), and c) malondialdehyde (MDA) production. Negative control (grey bar), positive control (white bar), treatment (blue bar). Values represent mean±S.D. (*N*=3). Negative control are cells incubated without the presence of palmitic acid or FDK and positive control are cells incubated with palmitic acid

Fig. 5. Proteome map of differentially expressed proteins in WRL68 cells. The protein spots were upregulated during incubation with *c*(palmitic acid)=100 μM and were downregulated when pretreated with the crude extract of γ(*Ficus deltoidea* var. *kunstleri*)=200 μg/mL (*N*=3, p≤0.05)

As previously shown, treatment with palmitic acid resulted in the downregulation of SOD and GPx and the increase of MDA in palmitic acid-induced WRL68 cells, which

disrupted the balance of the cell redox regulation and had an impact on it, particularly in the endoplasmic reticulum (ER). The formation of unfolded/misfolded proteins, which lack specific functions in cellular metabolism, can occur because of any form of ER irregularity (30). The accumulation of mis-folded proteins is toxic to the cells and results in the ER stress. Saturated fatty acids, such as palmitic acid, alter the phospholipid synthesis metabolic flux and increase the amount of saturated lipid species at the ER membrane, which may disrupt the fluidity of the membrane and encourage stiffening (31). The unfolded protein response (UPR), which is an adaptive reaction brought on by ER stress that restores protein homeostasis through intricate and parallel pathways, is brought on by an amplified state of these alterations. The activation of chaperone proteins is one of the UPR processes. This investigation discovered that palmitic acid increased the expression of HSPB1, a gene that is highly expressed in response to many environmental, physiological and pathological stressors (32). By subjecting oxidised proteins to the proteasome degradation machinery, this chaperone molecule avoids the aggregation of unfolded proteins and aids in protein breakdown (33).

Palmitic acid was also found to elevate the level of PSMA1, one of the essential protein subunits that contributes to the complex assembly of the 20S proteasome complex, involved in the ubiquitin-proteasome system (UPS). By destroying protein substrates that are no longer needed or are harmed by oxidative damage, the 20S proteasome is a molecular machine that plays a significant role in cellular activity (34). This proteasome is responsible for most of the non-lysosomal protein degradation in the cell, ensuring protein homeostasis (35). The upregulation of 20S proteasome is mediated by Nrf2 (nuclear factor erythroid 2-related factor 2), the master regulator of oxidative stress (36). The results in this study are in agreement with the findings by Soltis et al. (37), who found upregulation of PSMA1 in high fat diet-induced mice. Also, significantly altered PSMA1 level was reported and compared in the liver of healthy obese, normal control and steatosis samples (38).

UCHL3, a deubiquitinase related to UPS that removes ubiquitin from target proteins and controls their proteasomal breakdown and sub-localization, is another protein that was increased by palmitic acid. This maintains the equilibrium between ubiquitination and deubiquitination for the regulation of protein quality and homeostasis. The results of this investigation are consistent with a work by Suzuki et al. (39), who demonstrated that the reduction of UCHL3 induced an impairment in the adipogenesis that resulted in the prevention of lipid accumulation in adipocytes derived from UCHL3-/- mice. In contrast, van Beekum et al. (40) claimed that UCHL3 indirectly influenced peroxisome proliferator-activated receptor γ (PPARγ) activity by increasing β-oxidation.

Besides causing alterations in protein homeostasis, palmitic acid was found to cause changes in the levels of antioxidant enzymes. In this study, the level of PRDX1 was upregulated by palmitic acid, suggesting that it caused oxidative stress in the cells. However, pretreatment with FDK caused PRDX1 to decrease. The findings of van Greevenbroek et al. (41), who discovered that the level of PRDX1 expression in the liver of a hyperlipidaemic mouse model (HcB19) was twice as high as that of C3H mice, are supported by the current findings. Also, lipid accumulation in palmitate-induced insulin resistance HepG2 cells was reported to cause an up-regulation of PRDX1 expression (42). In contrast, PRDX1 was found to be reduced in MIN6 (mouse insulinoma) cells treated with palmitic acid (43). In response to H_2O_2, PRDX1 functions as a peroxide receptor, converting the signal into a disulfide bond that is then transmitted to ASK1 (apoptosis signalling kinase 1). This activation of the p38-MAPK (mitogen-activated protein kinase) pathway can affect various biological processes including inflammation, apoptosis, cell cycle and cell differentiation (44,45).

Another antioxidant enzyme that was affected by the palmitic acid treatment was GSTO1, which showed an increase in its activity. Overexpression of GSTO1 has been associated with increased protein glutathionylation (PG) that occurs during oxidative stress (46). By conjugating glutathione to the cysteine thiol group on the target proteins, PG is a redox-mediated post-translational modification that controls the activity of target proteins. Multiple biological processes are regulated by this mechanism, including those that control protein folding, cell signalling, inflammation and calcium homeostasis (47,48). PG is considered a defence mechanism to protect proteins from oxidative stress, a process that leads to irreversible damage (49). Also, an increase in glutathionylated proteins has been reported as part of NAFLD progression (50). In addition to PG, patients with NAFLD also had higher levels of protein carbonyls and 3-nitrotyrosine in their liver biopsies (51).

There have only been two studies on the ability of the 2DE approach to determine the decrease of lipid amount. Epigallocatechin gallate (EGCG) was investigated by Liu et al. (52) for its ability to reduce lipids in FFA-induced HepG2. The expression of proteins involved in the cytoskeleton, glycometabolism, signal transduction, DNA repair, mRNA processing, chaperone proteins, iron storage, protein phosphorylation, lipid metabolism, and antioxidant defence was found to be increased by FFA lipid build-up, but they were downregulated when EGCG was added to steatotic cells. The expression of the succinate dehydrogenase ubiquinone flavoprotein component, which is connected to cellular respiration, is downregulated by fat accumulation but upregulated by EGCG administration. In a different study, a methanol extract of Tamarindus indica was found to change the regulation of proteins in palmitic acid-induced HepG2 cells, including those involved in oxidative phosphorylation, metabolism, protein biosynthesis, cell proliferation and differentiation, and mRNA splicing (53). This change was reported to be the mechanism by which the extract lowers blood lipid levels. The number of significantly changed proteins was found to be lower than in the studies, according to the findings of this

study. Different compounds, including fatty acids such as oleic acid, palmitic acid, *etc.*, concentration, duration, treatment and cell types employed to produce steatosis, could contribute to the difference (*54*). It is well known that diverse signalling pathways are involved in the cellular metabolic programme and are altered in varying degrees in NAFLD. Thus, many plants and compounds that have been tested produced variable results (*55*).

According to our findings, lipid build-up in palmitic acid-induced WRL68 cells leads to oxidative stress, disturbs protein homeostasis and triggers the protein breakdown mechanism. We observed a decrease in lipid build-up after pre-treating these cells with FDK, which in turn helped to restore redox status and protein homeostasis (**Fig. S5**). In general, these findings may point to a protective mechanism of FDK against chronic liver disease, particularly in the early stages of NAFLD.

CONCLUSIONS

In this research, palmitic acid (100 µM) was used to imitate steatosis in WRL68 cells, and the only sample that demonstrated a decrease in lipid accumulation was the crude water extract of *Ficus deltoidea* var. *kunstleri* (FDK). Superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) – markers of oxidative stress – were used to further evaluate the impact of FDK. In palmitic acid-induced WRL68 cells, the aqueous extract of FDK (200 µg/mL) increased the SOD and GPx activities and decreased MDA, demonstrating its antioxidant properties. Palmitic acid was discovered to produce an increase of proteins associated with oxidative stress (PRDX1 and GSTO1) as well as protein homeostasis (HSPB1, PSMA1 and UCHL3) utilising a gel-based proteomics technique. A pre-treatment with FDK resulted in the downregulation of proteins, suggesting that FDK has a preventative function in NAFLD. Future research into the ability of *F. deltoidea* to reduce lipid content with a higher dose of palmitic acid to cause chronic steatosis would be intriguing.

CONFLICT OF INTEREST

The authors declare there are no competing interests.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

N. N. Abrahim performed the experiments, collected, analysed and interpreted data, drafted the article and performed review and editing. P. S. Abdul-Rahman and N. Aminudin conceived the study, provided resources, supervision and critical revision of the manuscript. All authors have read and approved the final version of the article.

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