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Antioxidant and Antihyperlipidemic Potential of *Ficus deltoidea* Leaf Extracts: *in vitro* Inhibition of LDL Oxidation and HMG-COA Reductase, and Reduction of Lipid Accumulation in Palmitic Acid-Induced HepG2 Cells

(Potensi Antioksidan dan Antihiperlipidemik Ekstrak Daun *Ficus deltoidea*: Perencatan *in vitro* Pengoksidaan LDL dan HMG-CoA Reduktase serta Pengurangan Pengumpulan Lipid dalam Sel HepG2 Teraruh-Asid Palmitik)

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ABSTRACT

Hyperlipidemia is a growing concern due to its association with cardiovascular disease, a leading global cause of mortality. *Ficus deltoidea* (Mas Cotek), traditionally consumed in Malaysia, has demonstrated antioxidant properties and the ability to reduce cholesterol. To date, the potential of *F. deltoidea* leaves for treating hyperlipidemia remains largely unexplored. In this study, water extraction of *F. deltoidea* leaves produced a crude extract (CE) further separated into water (WF) and ethyl acetate (EAF) fractions. Antioxidant capacities were assessed through various assays including the Folin-Ciocalteu assay, as well as the 2,2-diphenyl-2-picrylhydrazyl free radical scavenging, the cupric ion reducing capacity, and the inhibition of lipid peroxidation assays. The CE, also both WF and EAF of *F. deltoidea* leaves were then evaluated for their ability to inhibit the oxidation of human low-density lipoproteins. It was discovered that CE demonstrated the highest antioxidant activities and exhibited the most potent effect in prevention of human low-density lipoprotein oxidation. The antihyperlipidemic potential of *F. deltoidea* leaves was also evaluated based on their inhibitory effect on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and their ability to reduce intracellular lipid accumulation in palmitic acid (PA)-stimulated HepG2 cells. CE demonstrated the strongest inhibition of HMG-CoA reductase activity and significantly reduced PA-induced lipid accumulation in HepG2 cells in both pre- and post-treatment protocols. Consequently, *F. deltoidea* leaves could be an excellent source of natural antioxidants and antihyperlipidemic agents, essential for maintaining healthy cardiovascular function.

Keywords: Antioxidant; cardiovascular disease; *Ficus deltoidea*; hyperlipidemia; lipid

ABSTRAK

Hiperlipidemia merupakan masalah kesihatan yang semakin meningkat kerana kaitannya yang kuat dengan penyakit kardiovaskular, salah satu punca utama kematian di seluruh dunia. *Ficus deltoidea* (Mas Cotek), sejenis tumbuhan yang secara tradisi digunakan di Malaysia, telah menunjukkan sifat antioksidan serta keupayaan menurunkan kolesterol. Namun, potensinya dalam merawat hiperlipidemia masih belum dikaji secara meluas. Dalam kajian ini, daun *F. deltoidea* diekstrak menggunakan air untuk menghasilkan ekstrak kasar (CE), yang kemudiannya dipisahkan kepada fraksi air (WF) dan etil asetat (EAF). Aktiviti antioksida dinilai menggunakan beberapa ujian termasuk kaedah Folin–Ciocalteu, ujian penyingkiran radikal bebas DPPH, ujian keupayaan penurunan ion kuprik serta ujian perencatan peroksidasi lipid. Ekstrak CE, WF dan EAF juga diuji untuk keupayaan menghalang pengoksidaan lipoprotein berketumpatan rendah (LDL) manusia. Keputusan menunjukkan bahawa CE mempunyai aktiviti antioksida yang paling tinggi serta kesan paling kuat dalam menghalang pengoksidaan LDL. Potensi antihiperlipidemia daun *F. deltoidea* turut dinilai melalui keupayaan menghalang aktiviti HMG-CoA reduktase dan mengurangkan pengumpulan lipid intrasel dalam sel HepG2 yang dirangsang dengan asid palmitik (PA). CE menunjukkan perencatan paling kuat terhadap HMG-CoA reduktase serta berjaya mengurangkan pengumpulan lipid dengan ketara dalam kedua-dua protokol sebelum dan selepas rawatan. Secara keseluruhannya, daun *F. deltoidea* berpotensi tinggi sebagai sumber antioksida semula jadi dan agen antihiperlipidemik yang penting untuk menyokong kesihatan kardiovaskular.

Kata kunci: Antioksida; *Ficus deltoidea*; hiperlipidemia; lipid; penyakit kardiovaskular

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death globally, accounting for over three-quarters of all mortalities (World Heart Organization 2021). Rising rates of hyperlipidemia, driven by poor diet and sedentary lifestyles, have significantly contributed to this trend.

Hyperlipidemia, characterized by elevated plasma lipids such as cholesterol and triglycerides, is a well-established risk factor for CVD (Aladaileh et al. 2019). While dietary modifications, lifestyle changes, and synthetic lipid-lowering drugs like bile acid sequestrants, fibrates, and statins help manage hyperlipidemia, these medications often cause side effects including gastrointestinal issues, myopathy, and rhabdomyolysis (Chuo et al. 2020). Thus, there is a growing need for safer alternatives.

The global utilization of herbal medicine as an alternative approach for treating CVD and other chronic conditions, including diabetes and hypertension, is significantly growing (Chuo et al. 2020). *Ficus deltoidea*, known locally as *Mas Cotek* and native to Peninsular Malaysia, has been traditionally used for various ailments. Its leaves, roots, bark, and fruits have been employed for conditions such as headaches, fever, toothaches, and postpartum recovery (Adam et al. 2011; Mustaffa et al. 2011). Scientific studies have reported its antioxidant, antihypertensive, anti-inflammatory, antihyperglycemic, and anticancer properties (Abdulla et al. 2020; Aminudin et al. 2007; Aris et al. 2009). It is also traditionally used as a slimming agent believed to lower blood lipids (Misbah, Abdul Aziz & Aminudin 2013; Shiau et al. 2014), although scientific evidence to substantiate these claims remains insufficient.

Given its therapeutic potential, this study aimed to evaluate the antioxidant activities of crude and fractionated (water and ethyl acetate) extracts from *F. deltoidea* leaves through several *in vitro* antioxidant assays. Additional experiments were conducted to assess their capacity to prevent human LDL oxidation and to investigate their potential antihyperlipidemic effects by evaluating HMG-CoA reductase inhibition and the reduction of intracellular lipid accumulation in palmitic acid (PA)-induced HepG2 cells.

MATERIALS AND METHODS

PLANT MATERIALS

F. deltoidea leaves were obtained from a plantation in Rembau, Negeri Sembilan. A voucher for the specimen (registration number KLU 046470) was obtained from the Herbarium of the University of Malaya.

EXTRACTION AND LIQUID-LIQUID FRACTIONATION

The *F. deltoidea* leaves were washed to remove surface contaminants and air-dried at room temperature. Following Abrahim, Abdul-Rahman and Aminudin (2018), the dried leaves were ground to a fine powder and extracted using double-distilled water to produce the crude extract (CE). For liquid-liquid fractionation, CE was dissolved into double-distilled water and transferred to a separator funnel, then partitioned with ethyl acetate by gentle shaking and allowing the layers to separate, according to Misbah, Abdul Aziz and Aminudin (2013). The upper ethyl acetate fraction (EAF) and the lower aqueous fraction (WF) were collected separately, concentrated under reduced pressure to remove solvents, and stored in airtight containers under controlled humidity until use in subsequent experiments.

ANTIOXIDANT ASSAYS

Antioxidant potential of *F. deltoidea* leaves was evaluated with different assays including Folin-ciocalteu, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), cupric ion reducing antioxidant (CUPRAC) and lipid peroxidation inhibition assays. The methods are briefly outlined herewith. Quercetin was used as a positive control in all assays.

FOLIN-CIOCALTEU ASSAY

In accordance with the procedures previously outlined by Abdullah et al. (2012), the Folin-Ciocalteu assay was conducted to estimate the total phenolic content of the CE and fractions from *F. deltoidea* leaves. In brief, 250 µL of each *F. deltoidea* leaves sample (100 µg/mL) was mixed with 250 µL of 10% (v/v) Folin-Ciocalteu reagent and incubated for 2 min at room temperature. Subsequently, 500 µL of 10% (w/v) aqueous sodium carbonate was added, and the mixture was kept in the dark for 1 h. The absorbance of the mixtures was then measured at 760 nm using a UV/Visible spectrophotometer. Results were expressed in gallic acid equivalents (GAE)/g of dry weight, utilizing a gallic acid calibration curve with concentrations ranging from 2 to 10 µg/mL.

2,2-DIPHENYL-1-PICRYL-HYDRAZYL (DPPH) ASSAY

The DPPH assay was applied to evaluate the free radical scavenging activity of the CE and fractions from *F. deltoidea* leaves, following the method outlined by Abdullah et al. (2012). In brief, 5 µL of a 100 µg/mL solution of each *F. deltoidea* leaves sample was mixed with 195 µL of 1 mM DPPH. The resulting mixtures were dispensed into a 96-well plate and allowed to incubate for 30 min in the dark at room temperature. Following incubation, absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader at 515 nm. The percentage of radical scavenging activity was calculated, and the result was expressed as the half-maximal inhibitory concentration (IC_{50}).

CUPRIC ION REDUCING ANTIOXIDANT CAPABILITY (CUPRAC) ASSAY

The CUPRAC assay was performed according to the protocol by Abdullah et al. (2012). In brief, 1 mL of each *F. deltoidea* leaves sample (100 µg/mL) was combined with 10 mM copper (II), 7.5 mM Neocuproine, and 1 M ammonium acetate buffer (pH 7.0), resulting in a final volume of 4 mL. The mixtures were then left to incubate for

30 min at room temperature. Subsequently, the absorbance of the mixtures was measured at 459 nm using a UV/Visible spectrophotometer.

LIPID PEROXIDATION INHIBITION ASSAY

The assessment of lipid peroxidation inhibition in the CE and fractions *from F. deltoidea* leaves was conducted based on the method described by Vijayakumar et al. (2013). In brief, a mixture comprising 100 µL $FeSO^4$ (1 M) and 500 µL buffered egg yolk (1%) was prepared. A volume of 100 µL of each *F. deltoidea* leaves sample (1000 µg/mL) was added to the mixture, shaken, and then incubated at 37 ºC for 1 h. Following that, the mixtures were subjected to treatment with 500 µL thiobarbituric acid (TBA) (1%) and 250 µL trichloroacetic acid (TCA) (15%). The mixtures were incubated in a 100 ºC water bath for 10 min and subsequently cooled to room temperature. After centrifugation at 3,500 g for 10 min to eliminate precipitated protein, the resulting supernatant containing thiobarbituric acid reactive substances (TBARS) was assessed at 532 nm using an ELISA microplate reader. The percentage of inhibition (%) was calculated.

INHIBITION OF HUMAN LDL OXIDATION

The inhibitory effect on the oxidation of human low-density lipoprotein (LDL) by the CE and fractions from *F. deltoidea* leaves were evaluated employing a modified version of the method established by Ahmadvand, Khosrowbeygi and Ghasemi (2011). The assay involved the measurement of crucial oxidative markers, including conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS). The methods are briefly outlined herewith. Both assays used quercetin as a positive control.

MEASUREMENT OF CONJUGATED DIENE (CD) FORMATION

To assess the formation of CD, low-density lipoprotein (LDL) was combined with 0.1 M phosphate buffer (pH 7.4) and adjusted to a final concentration of 150 µg/mL. The initiation of the LDL oxidation reaction occurred through the addition of freshly prepared 0.1 mM $FeSO_4$ to the mixture. Subsequently, each sample of *F. deltoidea* leaves (1000 µg/mL) was introduced into the reaction mixture and incubated at 37 ºC. Distilled water was used as a blank. Absorbance readings at 234 nm were recorded every 5 min using a spectrophotometer to track CD formation over a 2-h period.

MEASUREMENT OF TBARS FORMATION

To assess the TBARS formation, low-density lipoprotein (LDL) was combined with 0.1 M phosphate buffer (pH 7.4) and adjusted to a final concentration of 150 µg/mL. The initiation of the LDL oxidation reaction occurred by adding freshly prepared 0.1 mM $FeSO^4$ to the mixture. Each sample of *F. deltoidea* leaves (1000 µg/mL) was introduced into the mixture and incubated for 3 h at 37 ºC. Following this, 500 µL of 15% trichloroacetic acid and 1000 µL of 1% thiobarbituric acid were added to the reaction mixture, and the combination was incubated for 15 min at 100 ºC. The absorbance was measured at 532 nm using an ELISA plate reader to quantify the formation of TBARS. Distilled water was used as a blank. A calibration curve was estabalished using malondialdehyde (MDA) bis-(dimethyl acetal) concentrations (0 to 100 µM) as a standard. The results were expressed as µmol MDA/mg protein-LDL.

INHIBITORY EFFECT ON HMG-CoA REDUCTASE ACTIVITY

The inhibitory effect of *F. deltoidea* leaves on HMG-CoA reductase activity was determined spectrophotometrically. The HMG-CoA reductase assay kit (Sigma, St. Louis, USA) was utilized, and the experiment was carried out following the protocol provided by the manufacturer. In brief, each *F. deltoidea* leaves sample (10 mg/mL) was mixed with a reaction mixture containing HMG-CoA substrate (400 µM), NADPH (400 µM) and potassium buffer (100 mM, pH 7.4) containing DTT (mM), KCl (120 mM) and EDTA (1 mM). HMG-CoA reductase (2 µL) was then added. This reaction mixture was incubated in a 96-well plate at 37 ºC. The absorbance was measured after 10 min at 340 nm. The percentage of HMG-CoA reductase inhibition (%) was calculated. Distilled water was used as the blank, and Atorvastatin was employed as the positive control.

CELL CULTURE AND CYTOTOXICITY OF *F. deltoidea* LEAVES ON HepG2 CELLS

Human hepatoma cells (HepG2) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and other supplements including 100 units/mL penicillin, 1% L-glutamine and 100 µg/mL streptomycin. The cells were seeded at a density of 2500-5000 cells/cm^2 into T-flasks (Corning, USA) coated with 1% gelatin and incubated in a humidified 5% CO^2 atmosphere at 37 ºC. The growth medium was replaced the day after seeding and subsequently refreshed every two days.

The MTT assay was used to assess the cytotoxicity of *F. deltoidea* leaves on HepG2 cells at concentrations ranging from 31.25 to 500 µg/mL after 24 and 72 h incubation. Cell viability was measured at 570 nm, and non-cytotoxic concentrations were selected for subsequent experiments.

CYTOTOXICITY OF PALMITIC ACID ON HepG2 CELLS

HepG2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well and treated with varying concentrations of palmitic acid (PA) (0.2, 0.4, 0.6, 0.8, and 1 mM; 10 µL) for 24 and 72 h to induce lipid accumulation. Control groups were treated with 10% FFA-free, low-endotoxin

BSA. Cell viability was assessed using the MTT assay, and absorbance was measured at 570 nm. Results were expressed as a percentage relative to the control group.

INHIBITORY EFFECT ON FATTY ACID-INDUCED LIPID ACCUMULATION IN HepG2 CELLS

HepG2 cells were initially seeded and cultured in a 96-well plate. The cells underwent treatment with *F. deltoidea* leaves through two distinct protocols, namely pre-treatment and post-treatment. In the first protocol, the cells were pre-treated with the CE and fractions from *F. deltoidea* leaves for 24 and 48 h before the addition of palmitic acid (PA) for an additional 24 h. Conversely, the second protocol involved post-treatment, where the cells were exposed to PA for 24 h before being treated with the CE and fractions from *F. deltoidea* leaves for 24 and 48 h. Afterward, the cells underwent the Oil Red O staining method, following the protocol described by Liu et al. (2013). After three washes with distilled water, cells were observed using an inverted microscope. The quantification of lipid accumulation was performed by measuring absorbance at 515 nm using a microplate reader. The experiment included Atorvastatin as a positive control.

STATISTICAL ANALYSIS

The data were presented as the mean and standard deviation of triplicates. A one-way analysis of variance (ANOVA) was conducted, and statistically significant differences were determined using the Duncan test at a confidence level of 95% ($p<0.05$). The analyses were performed using STATGRAPHICS Plus for Windows 3.0. Additionally, Student's t-test was calculated using Microsoft Excel 2010.

RESULTS AND DISCUSSION

ANTIOXIDANT CAPACITY OF *F. deltoidea* LEAVES EXTRACT AND FRACTIONS

Multiple cell-free antioxidant assays were used to capture different mechanisms, including total phenolic content, radical scavenging, reducing power, and lipid peroxidation inhibition. Human LDL oxidation was also assessed to evaluate protective effects. Quercetin served as a positive control across all assays. Results are summarized in Table 1.

The total phenolic content (TPC) of the CE and fractions from *F. deltoidea* leaves was measured using the Folin-Ciocalteu assay, which quantifies phenolic compounds by forming a blue complex proportional to concentration (Norra 2011). Table 1 displays the TPC values of the CE and fractions from *F. deltoidea* leaves. The CE exhibited the highest TPC value (108.2 ± 11.2 mg GAE/g) among the tested *F. deltoidea* leaves, followed by the WF (96.2 ± 6.42 mg GAE/g) and EAF (74.8 ± 1.35 mg GAE/g), respectively. In contrast, the positive control, quercetin, recorded a significantly higher value (558.82 ± 1.54 mg GAE/g) compared to the *F. deltoidea* samples. However, a previous study by Mun et al. (2017) reported higher TPC values for *F. deltoidea* leaves, specifically 368.42 mg GAE/g (aqueous extract), 263.45 mg GAE/g (ethanol extract), and 295.03 mg GAE/g (methanol extract). The observed antioxidant activity can be attributed to the high content of non-enzymatic antioxidants in *F. deltoidea* leaves, including flavonoids, phenolic acids, proanthocyanidins, and flavan-3-ol monomers (Dzolin et al. 2016; Hakiman & Maziah 2009; Omar, Mullen & Crozier 2011), which act via redox mechanisms as reducing agents, singlet oxygen quenchers, hydrogen donors, and metal ion chelators (Mohd Dom et al. 2020). Nevertheless, direct comparison of results between different investigations can be challenging due to potential variations arising from several factors. Different extraction methods, including variations in solvent type, solvent-to-sample ratio, temperature, and extraction duration, can significantly influence the determination of TPC (Abrahim, Abdul-Rahman & Aminudin 2018).

The antioxidant activity of the CE and fractions from *F. deltoidea* leaves was evaluated using the DPPH radical scavenging assay, in which antioxidants neutralize the DPPH radical, causing a color change from deep purple to pale yellow (Mohd Dom et al. 2020; Rahman, Islam & Biswas 2015). Among the tested *F. deltoidea* leaves (Table 1), the CE demonstrated the strongest scavenging activity (IC_{50}, 5.47 ± 0.13 μg/mL), followed by the WF (IC_{50}, 6.20 ± 0.25 μg/mL) and EAF (IC_{50}, 7.35 ± 0.28 μg/mL). Lower IC_{50} values signify stronger antioxidant capacity, indicating that the CE retained the highest scavenging potency. Previous studies by Abrahim, Abdul-Rahman and Aminudin (2018) on the scavenging activity of different varieties of *F. deltoidea* leaves indicated that the EAF from large and medium leaves samples exhibited effective inhibition of DPPH radicals, with both types demonstrating a minimum inhibition of 50% of the radicals. As per the findings, the fractionation of the CE played a significant role in enhancing the scavenging activity, particularly evident in the potent activity of the EAF derived from medium and large leaves, showcasing lower IC_{50} values of 223 μg/mL and 182 μg/mL, respectively. Nevertheless, the results of DPPH radical scavenging activity observed in the present study for the CE and its fractions from *F. deltoidea* leaves exhibited a more pronounced effect compared to earlier investigations.

The CUPRAC assay relies on the redox reaction between the chromogenic oxidizing agent Cu(II)-neocuproine reagent and an antioxidant. This reaction leads to the formation of a stable Cu(I)-neocuproine complex, resulting in the enhancement of an orange-yellow color, detectable at an absorption wavelength of 450 nm. Higher absorbance values indicate a stronger reducing ability of Cu^{2+} (Sharma & Vig 2013). Table 1 illustrates the Cu^{2+} reducing ability of quercetin, as well as the CE and fractions from *F. deltoidea* leaves. The results

demonstrate that all samples of *F. deltoidea* leaves reacted with the reagent. CE exhibited the highest CUPRAC value (0.214 ± 0.01), followed by WF (0.172 ± 0.02), and EAF (0.094 ± 0.01). Abrahim, Abdul-Rahman and Aminudin (2018) documented that the EAF from small *F. deltoidea* leaves demonstrated the highest Cu^{2+} reducing ability among three sizes of *F. deltoidea* leaves tested (small, medium, and large), while the CE and WF showed lower activity. However, a consistent pattern of activity was observed for medium and large leaves, with the order of CE > WF > EAF. The higher activity of CE may reflect synergistic interactions among multiple constituents that are partially separated during fractionation.

The peroxidation of polyunsaturated fatty acids induced by free radicals leads to the formation of several by-products, including malondialdehyde (MDA). This compound can contribute to oxidative damage associated with various diseases, including atherosclerosis and cardiovascular diseases (CVDs). During the peroxidation of polyunsaturated fatty acids, free radicals attack the unsaturated sites in the structure of fatty acids, facilitating the formation of lipid radicals. This process involves the rearrangement of a double bond, leading to lipid destruction and the production of MDA. MDA reacts with thiobarbituric acid (TBA) to form thiobarbituric acid reactive substances (TBARS), a final product measured at 532 nm (Phaniendra, Jestadi & Periyasamy 2015). The detrimental effects of free radicals are mitigated by the presence of antioxidants in plant samples, leading to a reduction in MDA production. This study investigated the antioxidant protective effect of the CE and fractions from *F. deltoidea* leaves against lipid peroxidation, using fowl egg yolk homogenate as the lipid-rich medium. According to Table 1, both the CE and fractions from *F. deltoidea* leaves (1000 µg/mL) exhibited antioxidant activity against lipid peroxidation. The results show that the CE demonstrated the highest inhibition of lipid peroxidation (76.02% ± 0.01), followed by WF (68.87% ± 0.01), and EAF (58.09% ± 0.04). Meanwhile, the CE appears to show slightly higher inhibition than previously reported by Abrahim, Abdul-Rahman and Aminudin (2018). However, direct numerical comparisons cannot be made due to differences in assay conditions, sample preparation, and measurement methods.

The superior performance of the CE compared to the fractions may be due to its diverse range of phytochemicals, including both lipophilic and hydrophilic compounds, which contribute to its enhanced antioxidant properties. While our study lacked a phytochemical analysis, previous research by Abrahim, Abdul-Rahman and Aminudin (2018) provides insight into the composition of CE from *F. deltoidea* leaves. They identified p-coumaric acid and vitexin, compounds known for their antioxidant properties, which likely contribute to the CE's high antioxidative potential.

Extraction methods also play a role in the chemical composition of plant extracts. In our study, water was used to extract hydrophilic antioxidants, following traditional methods in food and nutraceutical preparation. Water extracts are nutritionally valuable and considered safer. Differences in solvents, temperatures, and extraction durations can lead to variations in the chemical profiles of extracts. It is important to note that natural products like *F. deltoidea* can vary based on factors like plant age, environmental conditions, and geographical location, which can impact the chemical composition of the extracts. Future research should include phytochemical analysis to validate and expand on these findings, helping to better understand the phytochemical composition of *F. deltoidea* leaves in different contexts.

INHIBITORY EFFECTS ON HUMAN LDL OXIDATION

Elevated LDL levels contribute to atherosclerosis, a major risk factor for CVDs. Inhibiting LDL oxidation is key to preventing atherosclerosis. Plant extracts with high antioxidant properties are believed to help reduce LDL levels, potentially due to their antioxidant capacity (Shin et al. 2016). To explore the potential inhibitory effects on LDL oxidation, we further investigated the CE and fractions from *F. deltoidea* leaves, evaluating CD formation and conducting TBARS assays during both the initial and later phases of LDL oxidation.

CD, characterized by alternating single and double bonds, marks the initial stage of LDL oxidation. CD

TABLE 1. Antioxidant capacity of the CE and fractions from *F. deltoidea* leaves

Extract/ Fraction	Folin-Ciocalteu (mg GAE/g)	DDPH (IC_{50}, µg/mL)	CUPRAC absorbance at 450 nm	Lipid peroxidation (%)
CE	108.2 ± 11.2^{b}	5.47 ± 0.13^{b}	0.214 ± 0.01^{b}	76.02 ± 0.01^{b}
WF	96.2 ± 6.42^{c}	6.20 ± 0.25^{c}	0.172 ± 0.02^{c}	68.87 ± 0.01^{c}
EA	74.8 ± 1.35^{d}	7.35 ± 0.28^{d}	0.094 ± 0.01^{d}	58.09 ± 0.04^{d}
Quercetin	558.82 ± 54^{a}	3.08 ± 0.02^{a}	0.250 ± 0.05^{a}	82.10 ± 0.02^{a}

The CE and fractions from *F. deltoidea* leaves concentration was 100 µg/mL for the Folin-Ciocalteu, DPPH, and CUPRAC assays and 1000 µg/mL for the lipid peroxidation assay. Quercetin was the positive control at 10 µg/mL. The group with different letters (a-d) show a significant difference ($p<0.05$) and the values represent mean ± standard deviation of three replicates

formation results from the rearrangement of double bonds during lipid oxidation, leading to hydroperoxide production and is commonly used as an indicator of lipid oxidative processes and cardiovascular health (Papuc, Nicorescu & Durdun 2009). According to Rahman, Abdullah and Aminudin (2014), inhibiting CD formation effectively prevents the initial oxidation of LDL. In this study, CD levels were directly measured in the solution at 234 nm. Although a lag phase typically indicates delayed oxidation initiation, it was not clearly observed in this study, possibly due to experimental variations, antioxidant presence, or LDL source characteristics (Chan, Abdullah & Aminudin 2017). Instead, we conducted a qualitative comparison of the slope pattern, specifically examining the change in CD absorbance over time. Figure 1(a) shows the effects of the CE and fractions from *F. deltoidea* leaves on CD formation. A higher absorbance increment indicated greater LDL oxidation and weaker inhibition. All samples showed a gradual absorbance increase, with quercetin (positive control) most effectively suppressing CD formation, followed by the CE, WF, and EA fraction. This finding shows a notable trend, showcasing the CE is the most potent inhibitor of CD formation among the tested *F. deltoidea* leaves, effectively preventing the initial stage of LDL oxidation. Although statistical significance was not achieved, the CE showed the strongest inhibition among *F. deltoidea* samples, suggesting potential antioxidant effects, likely due to the combine action of multiple antioxidant constituents present in the CE. These results highlight the promise of CE in mitigating LDL oxidation.

The TBARS assay is a valuable tool for assessing the final stage of LDL oxidation by measuring the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), quantified at 532 nm. In this assay, MDA formation is inversely correlated with inhibition of LDL oxidation which higher MDA levels suggest weaker inhibition, while lower levels indicate stronger inhibition. As shown in Figure 1(b), the CE demonstrated the strongest inhibition (23.08 ± 1.10 μmol MDA/protein-LDL), followed by the WF (25.30 ± 2.29 μmol MDA) and EAF (34.85 ± 2.34 μmol MDA). Although the differences were not statistically significant, the consistent trends in both CD formation and TBARS assays suggest the potential protective effects of the CE against LDL oxidation. The results imply that the CE may influence both the early and final stages of oxidative processes. While further investigation is needed, these findings highlight the promising antioxidant potential of the CE. Additionally, the use of water as the extraction solvent emphasizes the importance of solvent selection in maximizing the protective properties of *F. deltoidea*.

HMG-CoA REDUCTASE INHIBITION ACTIVITY

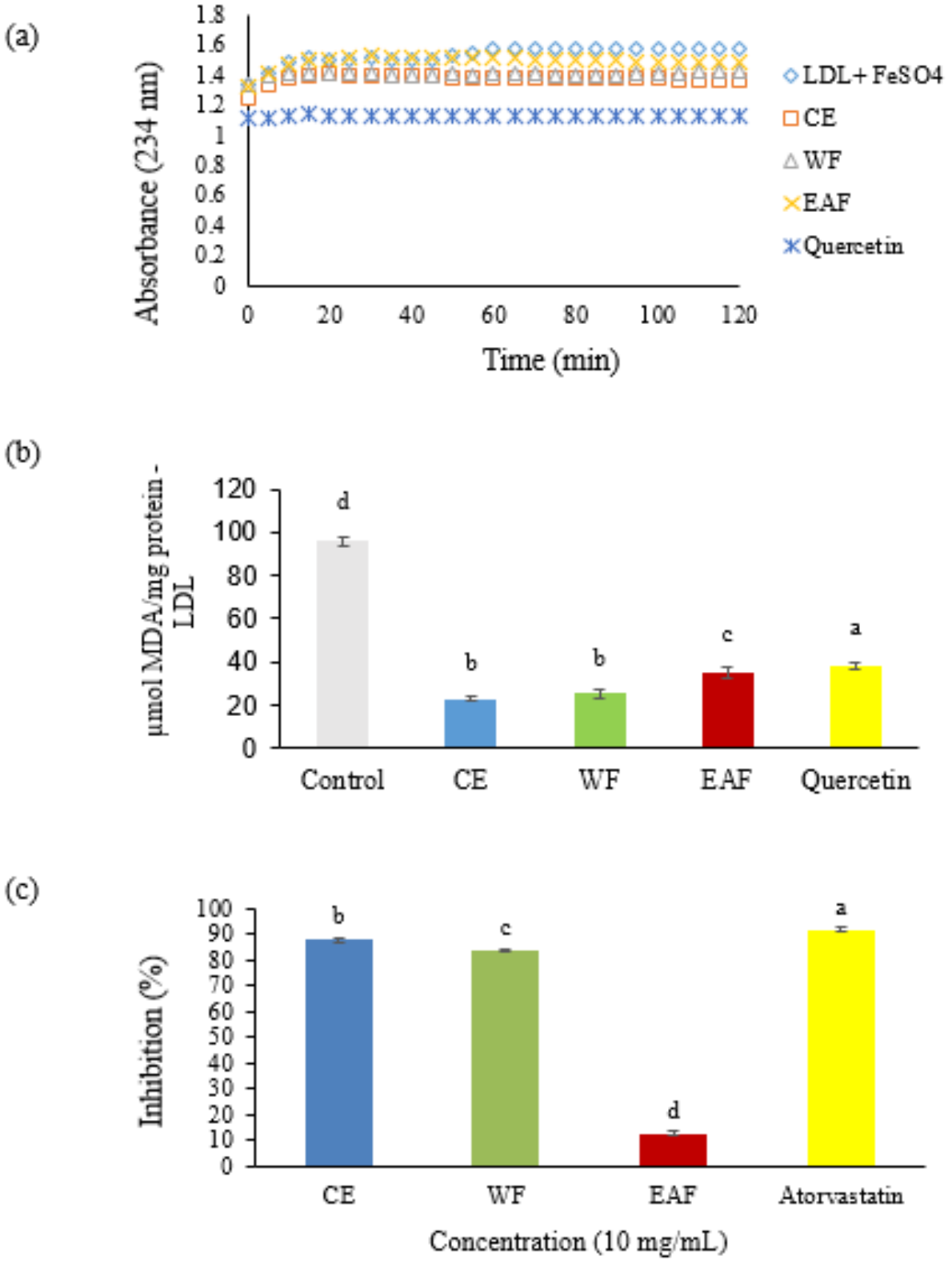
To assess the potential antihyperlipidemic properties of *F. deltoidea* leaves and their ability to reduce CVD risk, we evaluated their inhibitory effect on HMG-CoA reductase activity using a rapid assay kit. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate in cholesterol biosynthesis. Inhibiting this enzyme effectively reduces lipid levels, including triglycerides, fatty acids, and cholesterol, in both humans and animals. Inhibition activates sterol regulatory element-binding protein-2 (SREBP-2), increasing the expression of HMG-CoA reductase and LDL receptors, ultimately lowering cholesterol levels (Baskaran et al. 2015). Atorvastatin, a widely prescribed statin known for its efficacy in reducing cholesterol levels, served as the positive control. As shown in Figure 1(c), CE exhibited the highest inhibition of HMG-CoA reductase (87.65% ± 0.82), followed by WF (83.58% ± 0.57), and EAF (12.94% ± 1.14). All *F. deltoidea* samples inhibited HMG-CoA reductase activity to varying degrees. Previous research by Uzar (2015) showed that WF from *F. deltoidea* leaves inhibited HMG-CoA reductase by 88%, while ethanol and crude extracts showed about 80% inhibition at 80 μg/mL. Similarly, Abrahim, Aminudin and Abdul-Rahman (2023) reported that the CE from different *F. deltoidea* varieties (var. *angustifolia*, *trengganuensis*, and *kunstleri*) exhibited favorable inhibitory activity, whereas minimal or no activity was observed in WF and EA fractions. The strong inhibition observed for CE in both studies may be attributed to the collective action of various phytochemicals. Although *F. deltoidea* leaves showed lower inhibition compared to Atorvastatin, our findings suggest that they may reduce endogenous cholesterol production, contributing to potential cardioprotective effects.

CYTOTOXICITY OF *F. deltoidea* LEAVES EXTRACT ON HepG2 CELLS

F. deltoidea leaves have been extensively studied for their therapeutic potential against various diseases. However, evaluating their safety profile is crucial before further applications. In this study, the cytotoxicity of the CE and fractions from *F. deltoidea* leaves was assessed using the MTT assay across a concentration range of 31.25 to 500 μg/mL. Figure 2(a) and 2(b) illustrates that concentrations of *F. deltoidea* leaves at ≤125 μg/mL did not result in a significant reduction in cell viability after both 24 and 72 h. Concentrations at or below 125 μg/mL did not significantly reduce HepG2 cell viability after 24 and 72 h, whereas higher concentrations (250 and 500 μg/mL) showed a modest decrease. Based on these results, concentrations of 31.25, 62.5, and 125 μg/mL were selected for further lipid accumulation studies.

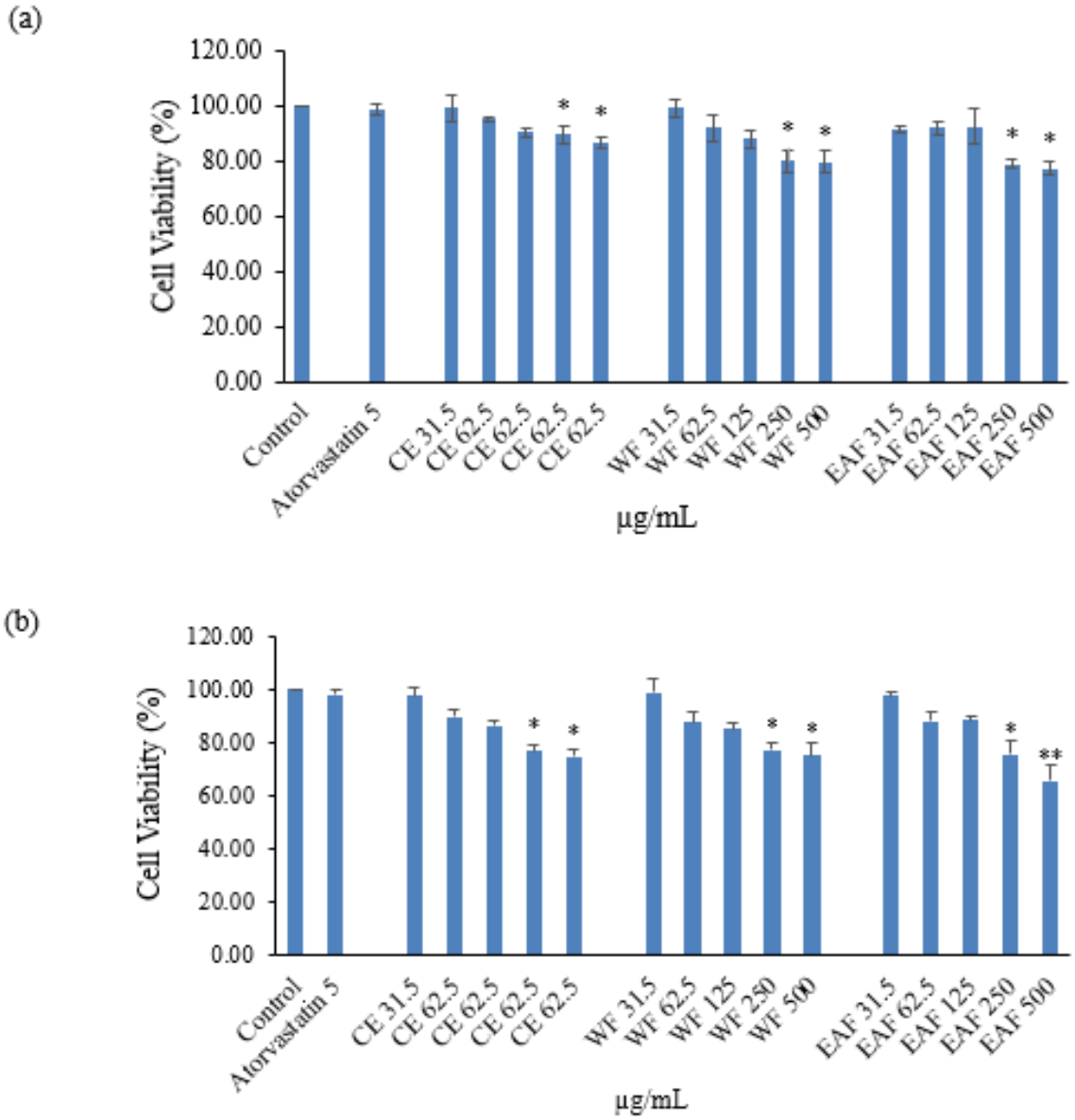
INHIBITION EFFECT OF *F. deltoidea* LEAVES ON LIPID ACCUMULATION IN PA-TREATED HepG2 CELLS

Hyperlipidemia is widely recognised as a substantial risk factor for the onset of CVD and coronary heart disorders, both firmly linked to an imbalance of lipids and



Values are expressed as the mean ± standard deviation of three replicate measurements. Groups with different letters (a-c) indicate significant differences ($p<0.05$). Significance compared to the control is indicated by an asterisk ($p<0.05$)

FIGURE 1. The effect of the CE and fractions from *F. deltoidea* leaves on (a) the formation of CD, (b) TBARS formation, and (c) inhibition of HMG-CoA reductase activity

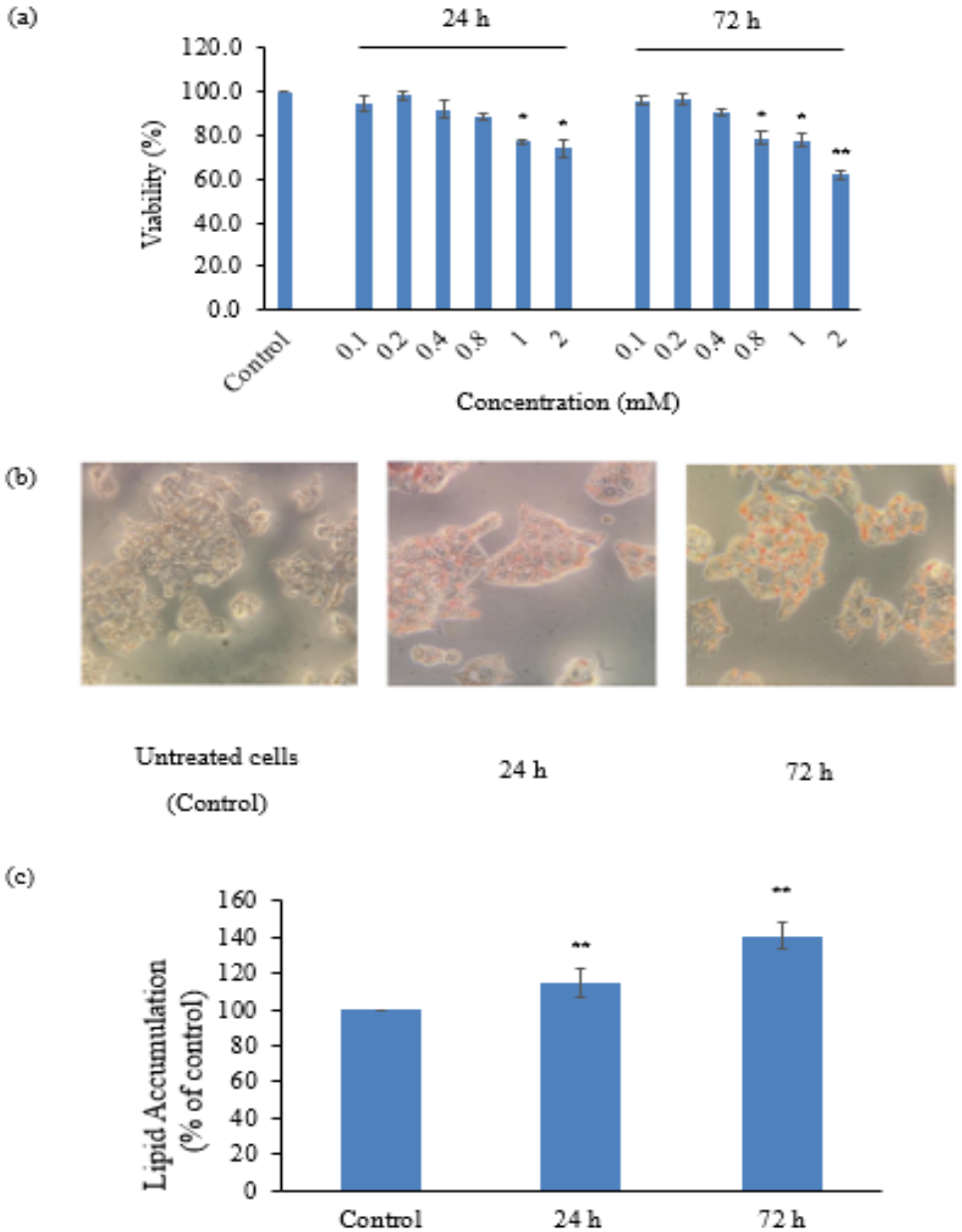


Values are expressed as the mean ± standard deviation of three replicate measurements (n = 3, error bars represent standard deviation).
* $p<0.05$ and ** $p<0.01$, compared with untreated HepG2 cells (Control)

FIGURE 2. The cytotoxicity of the CE and fractions from *F. deltoidea* leaves on HepG2 cell viability for (A) 24 h and (B) 72 h

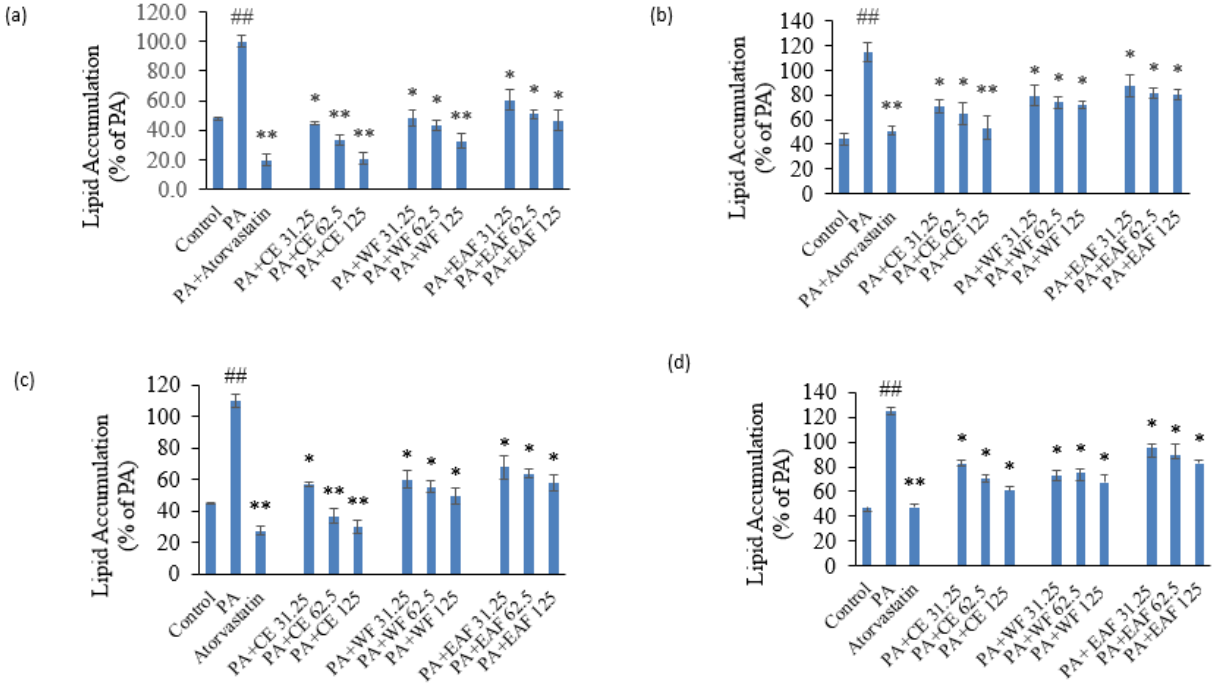
lipoproteins (Boukhalfa et al. 2018). Consequently, the lipid-lowering ability of *F. deltoidea* leaves was assessed in a cell culture system. HepG2 cells were selected due to their well-established status as a model for human liver steatosis, widely utilized to simulate the impact of various lipid-regulating agents on lipid metabolism (Limei et al. 2015). Palmitic acid (PA), a long-chain saturated free fatty acid, is abundant in human plasma and tissues and commonly found in the diet. It is known to induce lipid accumulation and inflammation (Liaqat et al. 2021). Thus, PA was employed to establish a model of hepatocyte lipid accumulation in HepG2 cells, following previous research approaches. In this study, PA at different concentrations (0.1, 0.2, 0.4, 0.8, 1, and 2 mM) was applied to HepG2 cells. Cytotoxicity was assessed using the MTT assay. Concentrations of PA below 0.8 mM showed a minor reduction in cell viability after both 24 and 72 h of incubation, as illustrated in Figure 3(a). Notably, 0.2 mM PA induced lipid accumulation without significantly affecting cell viability, and this concentration was selected for further experiments. Lipid accumulation in HepG2 cells was examined using the Oil Red O staining method, with increased intracellular lipid content visualized as a pink color under the microscope at ×400 magnification. As shown in Figure 3(b) and 3(c), HepG2 cells treated with 0.2 mM PA for 24 and 72 h exhibited significantly more lipid droplets than untreated cells ($p<0.05$). These results confirm the successful establishment of a hepatocyte lipid accumulation model using 0.2 mM PA.

The inhibitory effect on intracellular lipid accumulation was assessed, with Atorvastatin, a class of drugs known for decreasing cholesterol levels in humans, used as a positive control (Kang et al. 2013). In this study, HepG2 cells were treated with the CE and fractions from *F. deltoidea* leaves (31.25, 62.5, and 125 µg/mL) as well as Atorvastatin (5 µg/mL). Both pre- and post-treatment conditions were applied for 24 and 48 h in the presence of 0.2 mM PA. Oil Red O staining was conducted to assess intracellular lipid accumulation. Both pre- and post-treatment with *F. deltoidea* leaves and Atorvastatin significantly reduced lipid accumulation in PA-induced HepG2 cells compared to the PA group, as shown in Figure 4(a), 4(b), 4(c), and 4(d). These findings demonstrate that pre- and post-treatments with the CE and fractions from *F. deltoidea* leaves reduced intracellular lipid accumulation in a



Values are expressed as the mean ± standard deviation of three replicate measurements (n = 3, error bars represent standard deviation). * $p<0.05$ and ** $p<0.01$, compared with untreated HepG2 cells (Control)

FIGURE 3. Induction of lipid accumulation by PA in HepG2 cells. (a) The cytotoxicity of different concentrations of PA on HepG2 cells viability for 24 and 72 h, (b) Intracellular lipid droplets were stained with oil red O dye in HepG2 cells after treatment with 0.2 mM OA for 24 and 72 h, and (c) Quantitative analysis of intracellular lipid droplet accumulation in HepG2 cells



Values represent the mean ± standard deviation of three replicate measurements (n = 3, error bars represent standard deviation). ## $p<0.01$, compared with vehicle-treated control cells (Control); * $p<0.05$, ** $p<0.01$, compared with PA-treated cells (PA)

FIGURE 4. The significant reduction of lipid accumulation in PA-induced HepG2 cells through both pre- and post-treatment with *F. deltoidea* leaves and its fractions. HepG2 cells were pre-incubated with Atorvastatin (5 µg/mL) and various concentrations of *F. deltoidea* leaves (31.25, 62.5, and 125 µg/mL) for 24 h (a) and 48 h (b), followed by the addition of 0.2 mM PA. Additionally, HepG2 cells with the presence of 0.2 mM PA were incubated with the same concentrations of Atorvastatin and *F. deltoidea* leaves for the same durations, shown in (c) and (d), respectively

dose-dependent manner, with the highest concentration of CE exhibiting the most potent effect. This study is further supported by prior research from Abrahim, Aminudin and Abdul-Rahman (2023), which showed a significant dose-dependent reduction in lipid accumulation in WRL68 cells treated with *F. deltoidea* extracts, with CE displaying notable efficacy. Overall, these results underscore the potential of *F. deltoidea* leaves, particularly the CE, in mitigating intracellular lipid accumulation and present a promising avenue for further exploration in addressing lipid-related disorders.

Uzar (2015) showed that the phenolic content and lipid inhibition capacity of *F. deltoidea* leaves were responsible for reducing three key lipid profile parameters in atherogenic diet-induced rats: total cholesterol, serum triglycerides, and LDL levels. The cholesterol-lowering effect observed may be linked to specific serum proteins, including RBP4, Apo A1, Apo E, PDE1, HP, C1s, C3, PROM1, PLG, and TTR, which play crucial roles in lipid metabolism and transport, immune response and inflammation, acute-phase reactions, antioxidant defense, cholesterol homeostasis, and fatty acid catabolism (Chuo et al. 2020). Additionally, Shiau et al. (2014) investigated the anti-adipogenic effects of two variations of *F. deltoidea* extracts on 3T3-L1 adipocytes and found that both extracts exhibited similar levels of activity. Their findings suggest that extracts from both *var. deltoidea* and *var. angustifolia* may possess anti-obesity effects by inhibiting the formation of mature adipocytes. Overall, these studies provide evidence supporting the antihyperlipidemic properties of *F. deltoidea* leaves. The results align with the observed high inhibitory effect on HMG-CoA reductase activity, indicating decreased enzyme activity involved in cholesterol biosynthesis. Consequently, it is plausible that *F. deltoidea* leaves contain potential lipid-lowering agents or bioactive compounds that influence lipid metabolism. Furthermore, a phytochemical analysis conducted by Shiau et al. (2014) identified quercetin, saponins, and flavonoids in *F. deltoidea*, suggesting its potential in lowering lipid levels.

CONCLUSIONS

In conclusion, the CE from *F. deltoidea* leaves demonstrated good potential antioxidant activities in all *in vitro* antioxidant assays tested and exhibited the strongest impact on reducing human LDL oxidation and HMG-CoA reductase activity. This suggests their potential in reducing

oxidative stress on lipids, preventing LDL oxidation, and suppressing cholesterol synthesis. Furthermore, the CE from *F. deltoidea* leaves exhibited a dose-dependent antihyperlipidemic effect, with the greatest reduction in intracellular lipid accumulation observed at the highest concentration in HepG2 cells induced with PA. The consumption of *F. deltoidea* leaves may be beneficial, given their antioxidant properties and potential to restore a healthy balance in hepatic lipid accumulation, thereby contributing to the management of hyperlipidemia, atherosclerosis, and preventing further complications related to hypercholesterolemia. Nevertheless, further research is essential to identify the specific bioactive compounds present in *F. deltoidea* leaves and to comprehensively understand the underlying mechanisms responsible for their antioxidant and antihyperlipidemic properties.

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