

Study on hepatoprotective effect of *Moringa oleifera* Lam. leaves to prevent hepatocyte lesion induced by CCl₄ in the HepG2 cell line

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Abstract

Moringa oleifera Lam. is rich of strong antioxidant reagents, especially isoquercitrin. The present study was an attempt to prove hepatoprotective ability of isoquercitrin (IQ), ethyl acetate (EM) and 70% alcoholic extracts (AM) from *M. oleifera* leaves against CCl₄ induced hepatotoxicity in HepG2 cells. Cells were treated with 3 different samples, including IQ, EM or AM at concentrations corresponding to that of isoquercitrin at 2.5, 5, 10 µg/ml (C1, C2 and C3, respectively) alone or combined with 2 mM CCl₄ for 24h. Hepatoprotective effect was evaluated through assays including cell proliferation, lipid accumulation, necrosis, oxidative stress and apoptosis activation. Results showed that CCl₄ reduced 23.2% of HepG2 cells viability. Anti-proliferative effect was prevented in presence of IQ, EM, AM at 3 tested concentrations. Lipid accumulation was increased 53.5% due to CCl₄; however, IQ and EM at 3 tested concentrations, AM at C1 attenuated this situation. CCl₄ decreased 35.7% GSH content in cells; 3 samples enhanced GSH levels at different concentrations: IQ (C2, C3); EM (C1, C2, C3) and AM (C2, C3). Besides, CCl₄ increased 21.8% of lactate dehydrogenase, but its necrotic effect was decreased in presence of 3 samples at all concentrations. For apoptosis, CCl₄ increased 68.5% DNA fragmentation. This parameter was decreased by most concentrations of IQ, EM and AM. In sum, *M. oleifera* Lam. leaf extracts were shown to prevent HepG2 cells injury induced by CCl₄ treatment for 24h: hepatoprotective effect of IQ was better than that of EM and AM, especially at the concentration of 5-10 µg/ml.

Keyword: HepG2, *in vitro*, hepatoprotective, CCl₄, *Moringa oleifera* Lam. leaves

1. INTRODUCTION

Liver is the second largest organ that plays important roles in the metabolism of nutrients and detoxification of xenobiotics as well as drugs. Due to that central functional, it is especially susceptible to reactive oxygen species (ROS) which can reduce cell defense capacity and establish oxidative stress. Cell damage arising from this process may result in inflammation and fibrosis, even cancer and necrosis^{1,2}.

Carbon tetrachloride (CCl₄) is widely used for induce oxidative stress and liver injury, being extensively used in the evaluation of therapeutic potential of drugs and dietary antioxidants using HepG2 cells^{3, 4, 5, 6}. This

xenobiotic is undergone biotransformation by the cytochrome P450 system into a trichloromethyl free radical (CCl₃•) and transformation into a highly reactive trichloromethylperoxy free radical (CCl₃OO•), impairing critical cellular events such as lipid peroxidation and DNA damage^{7,8}. It further damages liver cell membranes and organelles and causes swelling, necrosis of hepatocytes and the release of cytosolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), into serum and eventually kills cells^{9,10,11}.

Nowadays, researches of plant-based antioxidants that retard the process of oxidative damage but with low cytotoxicity have been

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increasing. *Moringa oleifera* Lam., a tree of Moringaceae family, is a native of India and now extensively distributed throughout many southeastern Asian countries particularly in Thailand, Philippines and Vietnam etc. Traditionally, almost every part of *M. oleifera* has some nutritional, medicinal and other valuable properties. The leaves are widely utilized as a good alternative source of protein, calcium, vitamin A, C and E, β -carotene, amino acids, various polyphenolics and some natural antioxidizing agents. Apart from nutritional benefits, *M. oleifera* has been extensively used for the following applications: anti-tumor, anti-inflammatory, antibacterial, antidiyspidemia and especially hepatoprotective as a strong antioxidant activity¹². Hence, the present study was carried out in an attempt to evaluate the hepatoprotective activities of 70% alcoholic extract, ethyl acetate extract of *M. oleifera* leaves and isoquercitrin in CCl₄-injured HepG2 cell line.

2. MATERIALS AND METHODS

Chemicals and reagents

Eagle's Minimum Essential Medium (EMEM), fetal calf serum (FCS), trypsin-EDTA were purchased from Gibco, USA; L-glutamine, penicillin-streptomycine, phosphate buffer, trypan blue, ethanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), O Red oil, Brilliant G250, L-lactate dehydrogenase (LDH), carbon tetrachloride (CCl₄), glutathione (GSH), Ellman, isoquercitrin (IQ), quercetin (Q) from Sigma-Aldrich, USA; LDH detection kit from Roche, Germany; diphenylamine, from Merck, Germany. Other chemicals met cell culture standards.

Plant extracts

The 70% alcoholic (AM) and ethyl acetate (EM) extracts of *M. oleifera* leaves (harvested at An Giang, Vietnam) were standardized and provided by Ginseng and Pharmacognosy Center at Ho Chi Minh City. Isoquercitrin content was measured by UV spectrophotometric method at 2.41% and 14.09% for AM and EM respectively; moisturize content by infrared moisture balance at 19.50% and 16.18% for AM and EM respectively.

Cell culture

Human liver hepatoma cells (HepG2) (ATCC HB8065^R) were seeded and cultured in EMEM containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin in an humidified atmosphere of 5% CO₂ at 37°C to attain confluency. Cells then were trypsinized, harvested and counted using trypan blue, and seeded in 6 or 96-well-plates at 2.5×10^4 cells/cm².

Cell treatment

Cells were divided into the following groups of culture condition:

Physiological control: Cells were treated with EMEM containing DMSO (0.25%, v/v) for 24 h.

Pathological control: Cells were treated with EMEM containing DMSO (0.25%, v/v) and 2 mM CCl₄ for 24h.

Sample control: Cells were treated with EMEM containing DMSO (0.25%, v/v) and AM or EM or IQ at concentrations corresponding to that of isoquercitrin at 2.5, 5, 10 μ g/ml (C1, C2 and C3, respectively) for 24 h

Quercetin control: Cells were treated with EMEM containing DMSO (0.25%, v/v) and 10 μ M quercetin for 24 h.

Sample treatment: Cells were treated with EMEM containing DMSO (0.25%, v/v), 2 mM CCl₄ and AM or EM or IQ at concentrations of C1, C2, C3 for 24 h.

Quercetin treatment: Cells were treated with EMEM containing DMSO (0.25%, v/v), 2 mM CCl₄ and 10 μ M quercetin for 24 h.

Tested concentrations of *M. oleifera* leaf extracts and isoquercitrin were presented in Table 1.

Cell viability assay

Cell viability was evaluated as mitochondrial succinate dehydrogenase (SDH) activity, a marker of viable cells using MTT test. Briefly, SDH activity was detected after 3h incubation in culture medium without serum containing MTT (3-4,5-dimethyl-thiazol-2-yl-2,5 diphenyl-tetrazolium bromide), which is

Table 1. Concentrations of *M. oleifera* leaf extracts and isoquercitrin

	70% alcoholic extract (AM)	Ethyl acetate extract (EM)	
Isoquercitrin (% w/w)	2.41	14.09	
Moisturize content (%)	19.50	16.18	
<i>Final concentration in culture medium (µg/ml)</i>			
	Isoquercitrin (IQ)	70% ethanolic extract (AM)	Ethyl acetate extract (EM)
C1	2.5	128.9	21.2
C2	5.0	257.7	42.3
C3	10.0	515.5	84.7

converted into formazan dissolved in acidified isopropanol. The produced purple solution was spectrophotometrically measured at 570 nm on microplate reader¹³.

Intracellular lipid droplet accumulation - O red oil staining

To measure intracellular lipid droplet accumulation, HepG2 cells were stained by the O red oil method. Cells were rinsed with PBS and fixed with formalin 10%, rinsed with water followed by 60% isopropanol, stained with O red oil solution. Excess stain was removed by washing with water. The stained cells were observed under microscopy. Later, isopropanol 100% were added to dissolve stained oil droplets. The dye-triglyceride complex was spectrophotometrically measured at 500 nm¹⁴.

Determination of glutathione content

Glutathione (GSH) is an antioxidant tripeptide in hepatocyte which can be decreased in the presence of ROS. Briefly, cell lysates in cold KCl 1.15% were homogenized with Tris-HCl 25 mM pH 7.4 (2:1, v/v), then incubated in 60 min at 37 °C. Later, mixture was added TCA 10% (1:1, v/v) then was centrifuged. 100 µl the supernatant was mixed with 180 µl EDTA-phosphate buffer and 20 µl 5,5'-dithio-bis 2-nitrobenzoic acid 5 mM (Ellman reagent). GSH levels were spectrophotometrically measured at 412 nm and deduced from a calibration curve and corrected from protein level of cell

lysates. The protein content was also determined using BSA as standard¹⁵. Results were expressed as µM GSH/mg protein.

Assay of cell necrosis

Extracellular lactate dehydrogenase (LDH) was considered as an index of cell necrosis. LDH activity was measured in culture medium, using "Cytotoxicity detection LDH" kit. LDH participates in a coupled reaction which converts a yellow tetrazolium salt into a red, formazan-class dye which was measured by absorbance at 492 nm. A LDH standard curve (0 - 1000 mU/mL) was used for quantification of enzyme activity¹⁶.

Acridine orange/ethidium bromide (AO/EB) fluorescence staining

Changes in cell morphology were evaluated using AO/EB fluorescence staining. Briefly, cells were stained with dye mixture containing 100 µg/ml acridine orange and 100 µg/ml ethidium bromide in PBS, and then washed with PBS. After staining, cells were visualized immediately under a fluorescence microscope¹⁷.

Determination of percentage (%) of DNA fragmentation

Briefly, DNA from the cells was released into the lysis buffer [0.2% Triton X-100, 10 mM Tris-HCl, and 10 mM EDTA (pH 8.0)] by rupturing the nucleus. The supernatant received

after centrifugation at 13000 rpm for 10 min at 4°C, contained fragmented DNA while the intact DNA was in the pellet. The amount of DNA in both the supernatant and pellet was estimated by diphenylamine (DPA) assay. The percentage of DNA fragmentation was calculated as the ratio of DNA in supernatant and DNA in pellet¹⁸.

Detection of DNA fragmentation by agarose gel electrophoresis

Fragmented DNA was released into the lysis buffer (0.2% Triton X-100, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) by rupturing the nucleus. DNA in the supernatant was extracted using a 25:24:1 (v/v/v) equal volume of phenol: chloroform: isoamyl alcohol. DNA was precipitated overnight with absolute ethanol and 300 mM NaCl, dissolved in TE buffer [10 mM Tris-HCl pH 8.0 and 1 mM EDTA containing RNase A (0.1 mg/ml)]. The samples were analyzed electrophoretically on 2% agarose gel containing 0.01% SYBR Green¹⁷.

Evaluation of hepatoprotective effect

Hepatoprotective effect was evaluated via prevention percentage that means attenuation

of hepatocyte injury induced by 2 mM CCl₄ and calculated as the following function.

$$\text{Prevention percentage} = \frac{[(\text{CCl}_4 \text{ treatment} - \text{sample treatment}) / (\text{CCl}_4 \text{ treatment} - \text{physiological control})] \times 100\%}{}$$

Data analysis

Each assay was done twice or three times. The results were expressed as Mean + SEM of six culture of one representative experiment and statistically analyzed by Mann-Whitney test with SPSS 22.0 for Windows software. Threshold of significance was set at $p < 0.05$.

3. RESULTS

Hepatoprotective effect on the proliferation of HepG2 cells

After 24 hours, almost concentrations of AM, EM, IQ and 10 μM quercetin increased the percentage of viable cells ($p < 0.05$) (Table 2). Cell viability was found to decrease 23.2% by 2 mM CCl₄ treatment for 24h (Table 3). However, anti-proliferative effect of CCl₄ significantly attenuated in the presence of all concentrations of AM, EM, IQ and 10 μM quercetin (Table 4).

Table 2. Effect of 70% alcoholic extract (AM), ethyl acetate extract (EM), isoquercitrin (IQ) and quercetin (Q) on the proliferation, lipid accumulation, oxidative stress, necrosis and DNA fragmentation in HepG2 cells

Extract/ Concentration	Percent change (+ or -) compared to physiological control (%)				
	Cell proliferation	Lipid accumulation	Intracellular GSH content	Extracellular LDH activity	DNA fragmentation
Q	+1.35	+5.8	+66.3*	-7.2*	+57.2**
AM					
C1	+15.05*	+26.4*	-38.6**	-4.4	+29.0**
C2	+30.15**	+16.4*	+19.7**	-11.2	+8.9
C3	+21.79*	+16.8*	+135.7**	+17.5	+11.3
EM					
C1	+32.57**	+15.8*	+2.5	-13.8	+5.5
C2	+24.87**	+9.4*	+6.7	-12.0	+16.3*
C3	+30.49**	+15.7*	+93.7**	+9.6	+14.1*
IQ					
C1	+19.76*	-1.4	-51.6**	-3.3	+14.9**
C2	+25.72**	-3.2	-41.9**	-16.1*	-0.1
C3	+26.39**	+1.2	-19.1**	-17.5*	+10.1

*: $p < 0.05$ and **: $p < 0.01$: compared to physiological control

Table 3. Effect of 2 mM CCl₄ on the proliferation, lipid accumulation, oxidative stress, necrosis and DNA fragmentation in HepG2 cells

	Percent change (+ or -) compared to physiological control (%)
Cell proliferation	-23.2**
Lipid accumulation	+53.5**
Intracellular GSH content	-35.7**
Extracellular LDH activity	+21.8**
DNA fragmentation	+68.5**

** : p < 0.01: compared to physiological control

Table 4. Hepatoprotective effect of 70% alcoholic extract (AM), ethyl acetate extract (EM), isoquercitrin (IQ) and quercetin (Q) against the antiproliferation, lipid accumulation, oxidative stress, cell necrosis and DNA fragmentation in HepG2 cells induced by 2 mM CCl₄

Extract/ Concentration	Prevention percentage against CCl ₄ -induced hepatotoxicity (%)				
	Anti- proliferation	Lipid accumulation	Oxidative stress	Cell necrosis	DNA fragmentation
Q	91.3 [#]	31.3 [#]	514.0 ^{##}	104.1 ^{##}	49.8 [#]
AM					
C1	251.9 ^{##}	42.2 [#]	51.9	174.9 ^{##@@}	49.5
C2	223.3 ^{##}	14.0	102.8 ^{##}	232.5 ^{##@@}	78.3 ^{##@}
C3	111.4 [#]	-14.5	85.8 ^{##}	235.4 ^{##@@}	102.4 ^{##@}
EM					
C1	126.0 [#]	22.0 ^{##}	81.9 ^{##}	234.8 ^{##@@}	52.4 ^{##}
C2	194.2 ^{##}	27.4 ^{##}	146.8 ^{##}	202.0 ^{##@@}	53.8 [#]
C3	111.2 [#]	36.1 ^{##}	204.5 ^{##}	222.7 ^{##@@}	63.6 [#]
IQ					
C1	248.8 ^{##}	38.7 ^{##}	17.7	184.6 ^{##}	56.9 ^{##}
C2	205.6 ^{##}	35.4 ^{##}	64.5 [#]	272.4 ^{##@@}	81.6 ^{##@}
C3	220.1 ^{##}	26.7 ^{##}	80.5 [#]	273.3 ^{##@@}	91.3 ^{##@}

[#]: p < 0.05 and ^{##}: p < 0.01: compared to pathological control (CCl₄)

@: p < 0.05 and @@: p < 0.01: compared to quercetin treatment

Hepatoprotective effect against lipid accumulation in HepG2 cells

When HepG2 cell were treated alone with AM and EM, its intracellular lipid levels raised (Table 2). The same results were found in pathological control where 2 mM CCl₄ treatment for 24h increased 53.5% intracellular lipid content in comparison with physiological control (Table 3). However, in sample and quercetin

treatments, all three concentrations of IQ and EM; AM at C1; 10 μM quercetin showed a decrease in lipid accumulation. Interestingly, this effect of IQ and EM on CCl₄- injured HepG2 cells were insignificantly different from that of 10 μM quercetin. Microscopic images of O red oil-stained lipid droplet in HepG2 cells shown in Figure 1 confirmed the results found in Table 4.

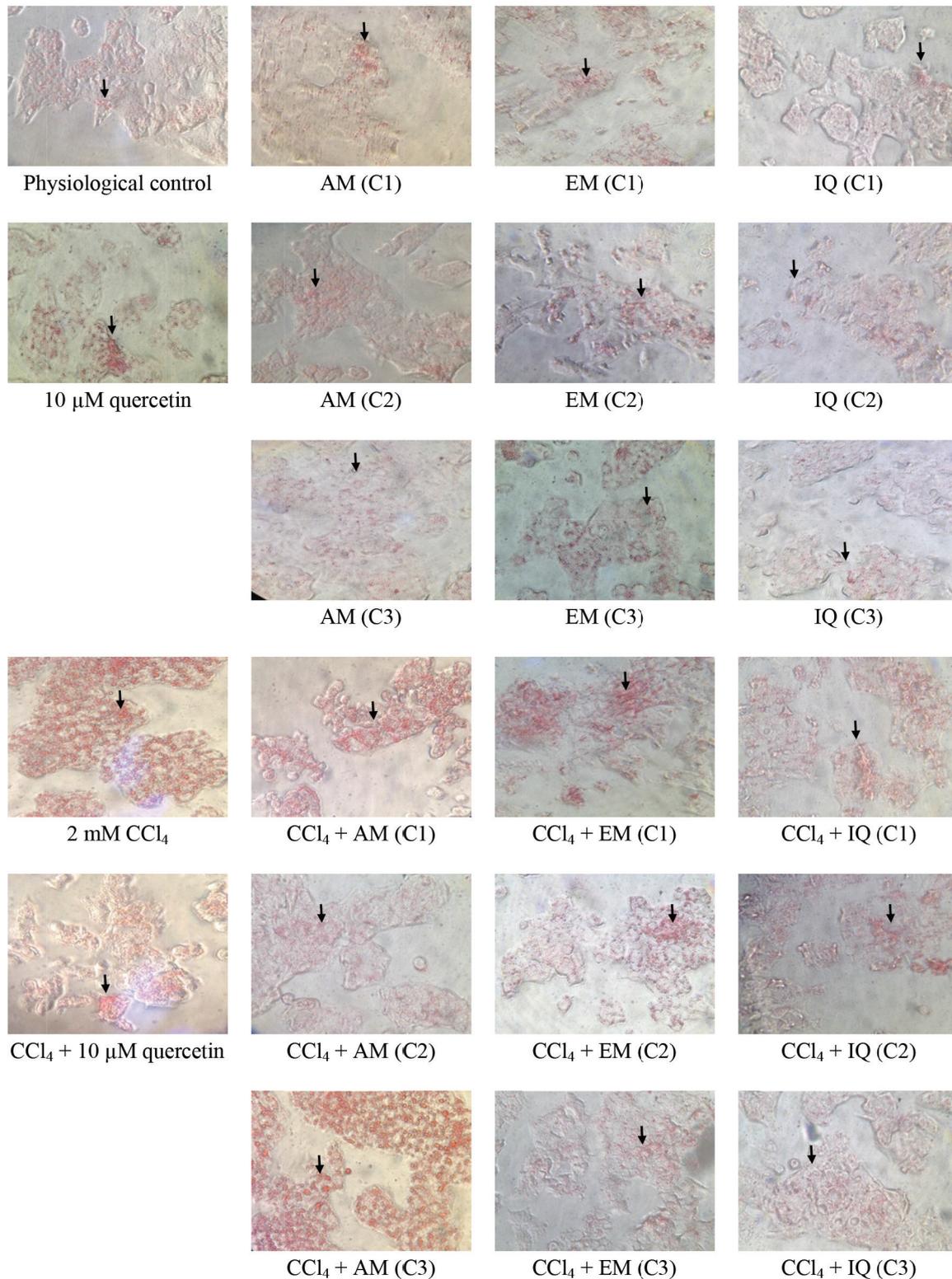


Figure 1. Images (20X) of HepG2 cells treated with CCl_4 , 70% alcoholic extract (AM), ethyl acetate extract (EM), isoquercitrin (IQ), quercetin alone or combined after Oil Red O staining (Oil Red O-stained-lipid droplet: arrow)

Hepatoprotective effect against oxidative stress in HepG2 cells

Glutathione (GSH) plays important role to neutralize oxidative species. After 24 hours of treatment, compared to physiological control, AM at C2, C3; EM at C3 and 10 μ M quercetin significantly increased GSH content ($p < 0.05$), whereas AM at C1, IQ at 3 concentrations showed the reverse effect (Table 2). In pathological control, CCl₄ decreased 35.7% GSH content ($p < 0.05$) (Table 3). The presence of AM at C2, C3; EM at 3 concentrations; IQ at C2, C3 demonstrated prevention effect from oxidative stress by enhancing GSH content 50-200% in 2 mM CCl₄-injured HepG2 cells. Quercetin at 10 μ M interestingly boosted the amount of GSH up to 514% in comparison with pathological control (Table 4).

Hepatoprotective effect against necrosis of HepG2 cells

Plasma membrane leakage from necrotic cells causes the release of intracellular contents

into extracellular milieu. Hence, the activity of extracellular LDH reflects cell necrosis status. In sample control, IQ at C2, C3 reduced significantly the amount of LDH comparing to normal control (Table 2). CCl₄ evoked necrosis process in HepG2 cells, which released 21.8% LDH comparing to normal control (Table 3). Results in Table 4 showed that all concentrations of AM, EM, IQ prevented necrosis caused by CCl₄ in HepG2 ($p < 0.05$), even better than 10 μ M quercetin did.

Hepatoprotective effect against apoptosis activation in HepG2 cells

The execution phase of apoptosis is characterized by DNA fragmentation and marked changes in cell morphology that include contraction and membrane blebbing. Those features of apoptotic cells were found in HepG2 cells treated to 2 mM CCl₄ more than in cells not treated (physiological control) or in cells treated to tested samples AM, EM and IQ (Figure 2).

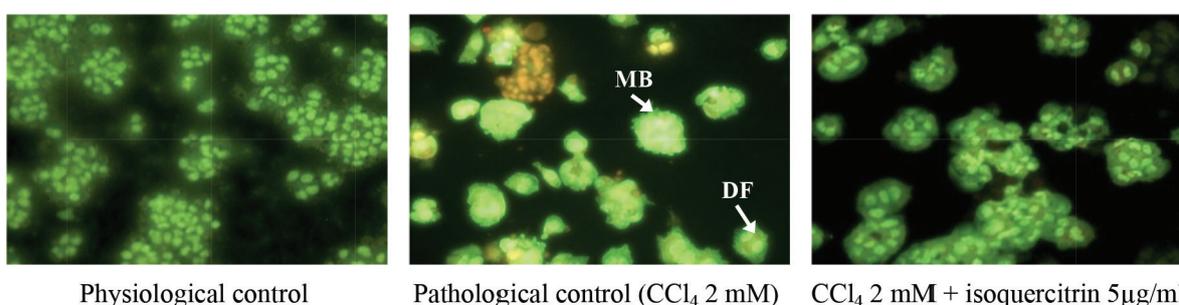


Figure 2. Hepatoprotective effect of isoquercitrin (5 μ g/ml) against apoptosis activation induced by CCl₄ in HepG2 cells (DF: DNA fragmentation, MB: membrane blebbing)

The results of DNA fragmentation analysis were presented in Table 5. In sample control, AM and IQ at C1, EM at C2 and C3 as well as quercetin 10 μ M increased the DNA fragmentation ($p < 0.05$) (Table 2). An increase of 68.5% DNA fragmentation was found in CCl₄ treated cells compared to control ($p < 0.05$) (Table 3). Incubation

HepG2 cells with 2 mM CCl₄ in the presence of most concentrations of AM, EM and IQ prevented DNA from fragmentation ($p < 0.05$). Especially, the prevention effects of EM and IQ at C2, C3 were better than that of quercetin 10 μ M (Table 4). The results were confirmed by agarose gel electrophoresis as shown in Figure 3.

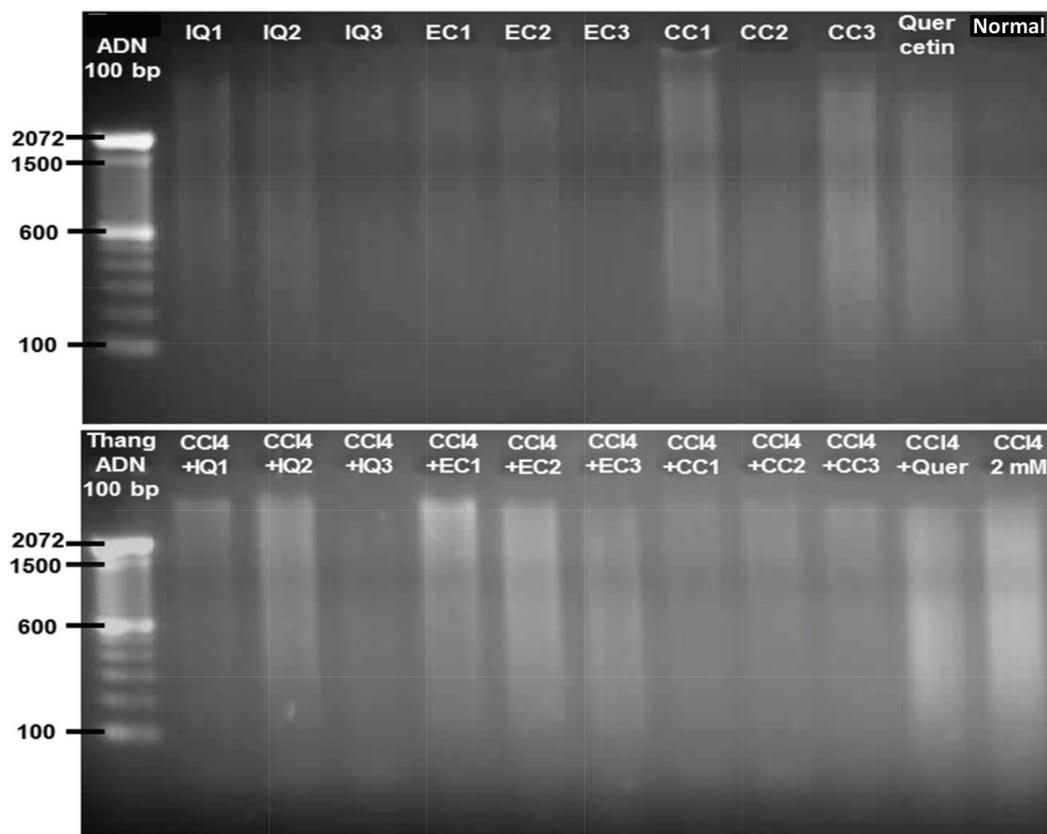


Figure 3. Images of DNA fragmentation using agarose gel electrophoresis. Upper: sample control and quercetin control; Lower: sample treatment and quercetin treatment. IQ1, IQ2, IQ3: isoquercitrin at C1, C2, C3; EC1, EC2, EC3: EM at C1, C2, C3; CC1, CC2, CC3: AM at C1, C2, C3

4. DISCUSSION

The present study was an attempt to prove hepatoprotective ability of isoquercitrin (IQ), ethyl acetate (EM) and 70% alcoholic extracts (AM) from *M. oleifera* leaf against CCl_4 induced hepatocyte lesion in HepG2 cells. Cells were treated with 3 different samples, including IQ, EM or AM from *M. oleifera* leaves at concentrations corresponding to that of isoquercitrin at 2.5, 5, 10 $\mu\text{g/ml}$ alone or combined with 2 mM CCl_4 for 24h. Hepatoprotective effect was evaluated through several assays including cell proliferation, intracellular lipid accumulation, necrosis, oxidative stress and apoptosis activation.

After 24 hours, CCl_4 decreased 23.2 % cell viability and 35.7% GSH content, on the other hand, increased 53.5% lipid accumulation, 21.8% extracellular LDH activity and 68.5%

DNA fragmentation. The results were in agreement with previous study¹⁹. CCl_4 's biotransformation compounds, $\text{CCl}_3\cdot$ and $\text{CCl}_3\text{OO}\cdot$, are capable of binding to proteins and other macromolecules of the lipid membranes with simultaneous attack on polyunsaturated fatty acids to produce lipid peroxidation²⁰. As a result, fats from the adipose tissue are translocated and accumulated in the hepatocytes²¹. GSH, a key component of the overall antioxidant defense system, decreased in the CCl_4 treated group might be due to its utilization by the excessively generated quantity of free radicals in the hepatocytes²². When the GSH stores were markedly in deplete state, necrosis begins, causing significant increase in the activities of LDH²³. The oxidative stress triggered by CCl_4 also led to apoptotic death as noted herein the morphological analysis of HepG2 cells exposed to these xenobiotics²⁴.

Those changes in CCl₄-injured HepG2 cells were shown to be minimal or absent with 10 μM quercetin treatment or AM, EM and IQ, dose-dependently. This might be due to lower fat accumulation and re-establishment of the antioxidant defense system in the liver tissue through the antioxidant and hepatoprotective effects of quercetin and *M. oleifera* leaf extracts. Quercetin was known as a strong antioxidant compound possessing hepatoprotective effect^{25,26}.

Isoquercitrin, a compound in EM, AM, IQ, was proved to regulate AMPK activation, thereby enhancing AdipoR expression, suppressing SREBP-1 and FAS expressions. AdipoRs and AMPK signaling suppress fatty acid synthesis by inactivating ACC and FAS and stimulate fatty acid oxidation. SREBP-1, a key lipogenic transcription factor, plays a major role in regulating hepatic lipid metabolism, including lipolysis and fatty acid synthesis. Inhibition of SREBP-1 expression by activating AMPK reduces lipid accumulation in hepatocytes and resulting in the regulation of lipid accumulation²⁷. In other study, 80% ethanolic extract of *Moringa oleifera* leaves showed the ability to downregulate the expression of the HMG-CoAR, PPARα1, and PPARγ genes of HepG2 cells, hereby inhibited lipid synthesis²⁸.

GSH is a tripeptide acting as an antioxidant. The intracellular GSH level is therefore often used as an indicator of the cellular redox imbalance state that is associated with oxidative stress or cellular toxicity. In the present study it was clearly shown that reduced GSH levels caused by CCl₄ were significantly counteracted by isoquercitrin. The subsequent recovery in HepG2 cells treated with *M. oleifera* leaf extracts might be explained due to the antioxidant properties in EM, AM, IQ or *de-novo* GSH synthesis or GSH regeneration (GSSG to GSH)¹².

As isoquercitrin prevented lipid accumulation as well as GSH depletion, it hereby increased HepG2 cell viability against the oxidative metabolites of CCl₄²⁹. Necrosis and apoptosis process in CCl₄-injured HepG2 cells were minimized, leading to the significant reduction in LDH leakage, cell morphological alterations and DNA fragmentation.

In conclusion, the results showed that hepatoprotective effect in HepG2 cells injured by CCl₄ were found in AM, EM and especially highest in IQ. This effect could be associated with the antioxidant activity. The result encourages further studies on evaluation of liver protective activity of *M. oleifera* leaves using animal models and clinical trials. From that, isoquercitrin and *M. oleifera* leaves can be formulated as dietary supplements and/or drugs for liver diseases.

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