The antioxidant and cytoprotective activity of *Ocimum gratissimum* extracts against hydrogen peroxide-induced toxicity in human HepG2 cells

Yung-Wei Chiua,b,1, Hung-Jen Loc,1, Hsin-Yu Huangd,e, Pei-Yu Chao,f,g, Jin-Ming Huangh, Pei-Yun Huangi, Shyh-Jer Huangj, Jer-Yuh Liuj,k,* Te-Jen Laib,l,**

a Emergency Department and Hyperbaric Oxygen Therapy Center, Tungs’ Taichung MetroHarbor Hospital, Taichung, Taiwan, ROC
b Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC
c Center of Teacher Education, National Taiwan University of Physical Education and Sport, Taichung, Taiwan, ROC
d Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan, ROC
e Department of Early Childhood Education, Wu Feng University, Chiayi, Taiwan, ROC
f Department of Leisure Industry Management, National Chin-Yi University of Technology, Taichung, Taiwan, ROC
g Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan, ROC
h School of Applied Chemistry, Health Care and Management College, Chung Shan Medical University, Taichung, Taiwan, ROC
i Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, ROC
j Center for Molecular Medicine, China Medical University Hospital, Taichung, Taiwan, ROC
k Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan, ROC
l Department of Psychiatry, Chung Shan Medical University Hospital, Taichung, Taiwan, ROC

**Corresponding author. Department of Psychiatry, Chung Shan Medical University Hospital, Taichung, Taiwan ROC.
**Corresponding author. Center for Molecular Medicine, China Medical University Hospital, Taichung, Taiwan ROC.

E-mail addresses: jyl@mail.cmu.edu.tw (J.-Y. Liu), ltj3123@ms2.hinet.net (T.-J. Lai).

1 These authors contributed equally to this paper.

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**Abstract**

*Ocimum gratissimum* is used as a traditional folk medicine in many countries. The objective of this study was to evaluate the antioxidant activities of an aqueous *O. gratissimum* extract (OGE) and to evaluate its cytoprotective activity against hydrogen peroxide-induced toxicity in human HepG2 cells. The results revealed that the total phenolic content of the OGE reached 20%. In the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, the OGE reduced up to 80% of the free radicals at a plateau concentration of 66.7 μg/mL. This indicates that OGE contains considerable free radical scavenging activity. In a dose-dependent manner, OGE pretreatment counteracted the decrease in cell viability in H2O2-treated HepG2 cells (**p** < 0.05), and at a concentration of 20–80 μg/mL, it effectively reduced thiobarbituric acid reactive substance (TBARS) formation. These findings indicated that the aqueous extract of...
1. Introduction

The liver is the primary organ that is responsible for metabolizing endogenous and exogenous compounds. It is therefore a target organ for the toxic action of xenobiotics or their reactive metabolites [1]. These xenobiotics can generate reactive oxygen species (ROS) or free radicals in hepatocytes during the metabolic process. Hepatocytes physiologically use ROS as secondary messengers, which plays a role in modulating normal cellular functions [2]; however, an imbalance between the production and the removal of ROS causes oxidative stress. Oxidative stress is involved in the etiology of numerous liver diseases [3,4]. Oxidative stress-mediated damage may result in inflammatory and fibrotic processes in the liver [1]. Appropriate levels of intracellular antioxidant capacity to eliminate the harmful effects of ROS, including endogenous and exogenous antioxidant systems, is crucial for maintaining normal cellular function. Oral natural antioxidant phytochemicals have been proposed as therapeutic agents in liver diseases because the liver is the primary organ that collects exogenous antioxidants from the gastrointestinal tract [4].

The genus Ocimum, which belongs to the family Lamiaceae (previously known as Labiatae), has a strong-smelling aromatic flavor and has been used as traditional herbs in many countries since ancient times. Ocimum gratissimum, which is widely distributed in tropical and warm temperate geolocations, is a well-known medicinal plant in the Ocimum genus and commonly used in folk medicine [5,6]. In its unprocessed form, it is also commonly used as a spice in dishes in most West African areas where it is locally referred to as “scent leaf” and “tree basil” [7]. O. gratissimum is known for its multiple pharmacological properties and has been prepared in a variety of forms for consumption such as “chhit-chan-than” in Taiwan [8] and “vana tulsi” in India [9,10]. This medicinal plant has shown potential anthelmintic, antibacterial, antifungal, and antiviral activities [11–15], whereas more recent research focuses on its capability in immunomodulation [16,17] and cancer chemoprevention [18]. In recent studies from our laboratory, an aqueous O. gratissimum extract (OGE) protected the heart of Sprague Dawley rats against carbon tetrachloride-induced cirrhosis-associated cardiac hypertrophy and fibrosis [19,20] and it attenuated hydrogen peroxide (H2O2)-induced chromosome damage in cardiac H9c2 cells [21]. The implication in these studies that OGE may be beneficial in the treatment of liver and heart diseases prompts further study into the mechanism of the antioxidant property of polyphenolics in OGE with regard to its protection of the liver.

Dietary antioxidants have a protective role against oxidative stress and have been proposed as therapeutic agents to counteract liver oxidative damage. To elucidate the hepatoprotective properties of O. gratissimum, an aqueous extraction was adopted to simulate food ingestion and hepatic absorption in this study. Dried O. gratissimum was extracted by boiled water, and the extract was lyophilized. The HepG2 human hepatoma cell line is considered a good model for studying in vitro xenobiotic metabolism and toxicity in the liver [22]. In the present study, the OGE was tested for its total phenolic content and effects on free radical scavenging, lipid peroxidation inhibition, and cytoprotective activity against H2O2-induced toxicity in human HepG2 cells.

2. Materials and methods

2.1. Chemicals and reagents

Sodium carbonate (Na2CO3), sodium chloride (NaCl), potassium chloride (KCl), sodium dibasic phosphate dihydrate (Na2HPO4·H2O), dibasic potassium phosphate (K2HPO4), chloric acid (HCl), methanol, ethanol, isopropanol anhydrous, and hydrogen peroxide (H2O2) were purchased from Union Chemical Works, Ltd (Hsinchu, Taiwan). Folin–Ciocalteu phenol reagent, caffeic acid, Dulbecco’s modified Eagle medium (DMEM), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), nonessential amino acid (NEAA), glucose, trypsin-EDTA, penicillin G, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material and preparation of the extract

Extraction procedures of O. gratissimum were performed in a qualified process. In brief, the leaves and stems of O. gratissimum were harvested and washed in running water, and then air-dried for 1 week to make into a coarse powder. The powdered vegetal materials (400 g) were homogenized with distilled water (1000 mL) by using a polytron. The homogenate was incubated at 95 °C for 1 hour, and then filtered through two layers of gauze. The filtrate was centrifuged at 20,000 g at 4 °C for 15 minutes to remove insoluble pellets. The supernatant was thereafter collected, lyophilized, and stored at −20 °C until use. Prior to the assays, the OGE powder was dissolved at the required concentration.

2.3. Measurement of the total phenolic content

For polyphenol content measurement, the method by Singleton et al [23] was used: Distilled H2O (10 mL), Folin–Ciocalteu reagent (0.5 mL), and the sample solution (1 mL) were mixed. After shaking the solution and placing it at room temperature for 15 minutes, 3 mL of 20% Na2CO3 was added, and was heated at 100 °C for 1 minute in a water bath. A spectrophotometer was used to measure the absorbance of the samples at 725 nm. The calibration curve was constructed by using standard methanolic solutions of caffeic acid [24].
The concentration (in mg/mL) of phenolic content in the sample solution was evaluated by interpolating the results into the calibration plot and reading them from the calibration line. Based on the concentration of the dissolved dry sample (in g/mL), the total phenolic content was calculated as milligrams of caffeic acid equivalents (CAE) per gram of dry referral materials (mg CAE/g dry sample) [25]. For proportional conception, the value was multiplied by the coefficient of $10^{-1}$ to transform "mg/g" to "mg/100 mg" and expressed as the percentage (%) of total phenols index (%PI) [26].

2.4. 2, 2-Diphenyl-1-picrylhydrazyl scavenging activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of the OGE was determined in accordance with the method by Shimada et al [27], but with some modifications. In brief, 5 mL of the OGE—along with 5 mL of α-tocopherol and butylated hydroxyanisole (BHA) methanolic solutions for reference—was mixed with 1 mL of freshly prepared α,α-diphenyl-β-picrylhydrazyl (DPPH) solution (0.1 mM, in 95% methanol). The reaction mixture, which contained varying concentrations of the extract or reference (0–140 μg/mL), was shaken well and incubated for 50 minutes at room temperature. The absorbance of the resulting solution was read at 517 nm against a blank. The radical scavenging activity was measured by the decrease in the absorbance of DPPH and was calculated by using the following equation:

\[
\text{Scavenging effect} (%) = \left[1 - \frac{A_{517\text{ of the sample}}}{A_{517\text{ of the control}}}\right] \times 100
\]

2.5. Cell culture

The human hepatoma cells HepG2 (BCRC Non, 60025) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 100 μg/mL penicillin/streptomycin (Sigma-Aldrich Chemie, Munich, Germany) at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The HepG2 cells were seeded in 24-well culture plates at an initial density of $2 \times 10^5$ cells/mL and grown to approximately 80% confluence. Oxidative stress was induced by treating the cells with freshly prepared H$_2$O$_2$. The cells were pretreated with OGE at indicated concentrations for 24 hours. A medium containing H$_2$O$_2$ was then added and the cells were incubated for indicated amounts of time. After the incubation, the cells were washed with phosphate-buffered saline (PBS; 25 mM sodium phosphate, 150 mM NaCl, pH 7.2), and then collected for subsequent analysis. For morphological analysis, the cells were observed under inverted microscope (Olympus Co., Tokyo, Japan) at 40× magnification for changes in size and number.

2.6. MTT assay

Cell viability was determined by MTT assay. The HepG2 cells were exposed to H$_2$O$_2$ or with or without pretreatment of the test samples (i.e., OGE). To determine the cytotoxicity of H$_2$O$_2$, the HepG2 cells were treated with 5 different concentrations of H$_2$O$_2$: 441 nM, 882 nM, 1764 nM, 3528 nM, and 7056 nM. More than 50% of cell death occurred after 2 hours at 882 nM of H$_2$O$_2$, compared to the untreated control cells. Thus, the concentration of 882 nM of H$_2$O$_2$ was chosen as the appropriate concentration for subsequent experiments on the effect of OGE. The HepG2 cells were starved for 12 hours and pretreated with various indicated concentrations of OGE for 24 hours. They were then treated with H$_2$O$_2$ for 24 hours. After these treatments, the medium was removed and the HepG2 cells were incubated with MTT (0.5 μg/mL) at 37 °C for 4 hours. The viable cell number was directly proportional to the production of formazan, which was dissolved in isopropanol and determined by measuring the absorbance at 570 nm by using a microplate reader.

2.7. Thiobarbituric acid-reactive substances assay

Lipid peroxidation was determined based on the amount of thiobarbituric acid-reactive substances (TBARS) [28] and with minor modifications. The results were expressed as nanomoles of malondialdehyde (MDA) per milligram of protein. In brief, equal volumes of the leaf extract and 882 nM H$_2$O$_2$ in PBS-free DMEM were added to each well and the cell plate was incubated for 24 hours. The HepG2 cells were lysed by using the freeze-thaw method. After lysis, 0.2 mL of the cell suspension was added to the TBA reagent (1.5 mL of 20% acetic acid, 1.5 mL of 8.1% sodium dodecylsulfate, and 1.5 mL of 0.8% TBA). This mixture was incubated at 90 °C for 1 hour, and then cooled. Four milliliters of a mixture of n-butanol and pyridine (15:1, v/v) was added, and the whole mixture was centrifuged (1500 g for 15 minutes). The fluorescence of the samples at an excitation wavelength of 515 nm and an emission wavelength of 555 nm was detected by a F4500 fluorescence spectrophotometer (Hitachi Co., Tokyo, Japan). The absorption spectrum in the region of 515–555 nm ($\lambda_{em} = 515$ nm, $\lambda_{em} = 555$ nm) was recorded in the upper butanol phase. Optical density (OD) was determined at 532 nm with the absorbance at 515 nm and at 555 nm used as the background value and 1,1,3,3-tetramethoxypropane (TEP) used as the TBAR standard [29].

2.8. Statistical analysis

The experimental results are expressed as the mean ± the standard deviation (SD). The data were assessed by using the analysis of variance (ANOVA). The Student t test was used to compare between groups. A p value of <0.05 was considered statistically different.

3. Results and discussion

3.1. Qualification and yields of Ocimum gratissimum extract preparation

It is generally assumed that the use of food supplements is safe and efficacious because they have been used for human consumption for centuries. In recent years, much attention has been focused on their protective function, especially the antioxidative effect of naturally occurring compounds and the mechanisms of their actions. However, understanding their mechanisms of
action as a preventive or therapeutic modality is a primary challenge for modern science. In the area of natural extracts, it is very important that different information be considered simultaneously such as agronomics, extraction process, chemical composition of the extracts, and their functional properties. The extraction process and the conditions of extraction determine the type of substances that will be extracted [30]. Thus, qualification in the whole process is necessary.

In our work, the leaves and stems of O. gratissimum were harvested in Nantou County, Taiwan, R.O.C. in the daytime during the autumn season (September 2006 to November 2006) and were identified by the Institute of Biochemistry and Biotechnology of the Chung Shan Medical University. The powdered vegetal materials were defined as dry starting material. Fig. 1 shows the extract yields of the leaves and stems. The yields of the OGE of the leaves was 18.0% (72 g/400 g) and stems was 7.0% (28 g/400 g), with reference to the dry starting material.

The genus Ocimum, a member of the Lamiaceae family, contains more than 200 species [31]. Botanical identification of the Ocimum species can be complicated because of the existence of several varieties and the variation in chemical composition. Some studies moreover report that differences exist in the biological activity of essential oils of Ocimum species obtained in different seasons of the year [32]. A previous report indicated that the chemical composition of Ocimum species varies in accordance with the time of plant collection and the preparation of the extract [33]. As a vegetable and food, blanching of Ocimum species could inactivate and wash out vitamin C, and cause a significant increase in the total phenol content [34]. Considering the factors affecting the constituents of the boiled aqueous extract, our procedures of O. gratissimum extraction were performed under a qualified process in agronomic practices, plant identification, and extraction condition.

3.2. Total phenolic content of Ocimum gratissimum extract

Polyphenols are bioactive substances widely distributed in plants and are important constituents of the human diet. The determination of total phenolic content is an important parameter to estimate the amount of antioxidants [35]. The Folin–Ciocalteu method was used in our present study because it has been proposed as a standardized method for use in routine quality control and in measuring the antioxidant capacity of food products and dietary supplements, and it is considered the best method for total phenolic determination [36]. Fig. 1 presents the results of the total phenolic content in the leaves and stems. The amount of phenolic content observed in the leaf and the stem extracts were 20% and 9%, respectively. Our data showed that the amount of phenolic components in the leaf extract were more than two-fold higher than the amount in the stem extract. To maintain a high quality of phenolic content and extraction yield, we adopted the extract of leaves in subsequent experiments.

Plants, which are a primary source of antioxidants, comprise a great diversity of compounds such as flavonoids (e.g., anthocyanins, flavonols, and flavones) and several classes of nonflavonoids (e.g., phenolic acids) as phenolics [37]. These polyphenolic compounds vary in structure and in the number of phenolic hydroxyl groups and their positions, thereby leading to variations in their antioxidative capacities. In our subsequent studies, caffeic acid (i.e., 3,4-dihydroxycinnamic acid) was a primary component of O. gratissimum extract [19]. High-performance liquid chromatography (HPLC) can detect the caffeic acid content and the difference in caffeic content beaten O. gratissimum and Ocimum basilicum [38]. The presence of the phytochemical caffeic acid is important for exerting a therapeutic function. In fact, caffeic acid has been widely used as a reference when working with the Folin–Ciocalteu method [25].

3.3. Free radical scavenging activity of Ocimum gratissimum extract

Fig. 2 illustrates the DPPH scavenging effect of the OGE with BHA and α-tocopherol as references. Concentration effects of the OGE and the references were observed: the radical-scavenging
capacity increased with increasing concentration. At a concentration of 33.33 μg/mL, α-tocopherol and BHA closely approached their plateau of radical-scavenging activities, whereas a similar ability of OGE was observed at 66.67 μg/mL. Under the same experimental conditions with positive control counterparts, the scavenging effect of OGE, BHA, or α-tocopherol (each at 100 μg/mL) on the DPPH radical was 79%, 75%, or 87%, respectively. In descending order α-tocopherol had greater activity than OGE which had greater activity than BHA. The result indicates that OGE exerts a significant effect on scavenging free radicals.

The ability of the examined extract, which acts as a donor of hydrogen atoms or electrons in the transformation of DPPH into its reduced form DPPH-H, was investigated in the DPPH assay. The DPPH radical has been widely used to test the free-radical scavenging ability of various natural products, and it has been accepted as a model compound for free radicals originating in lipids [27]. The examined extracts of O. gratissimum were able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H. Polyphenols easily transfer a hydrogen atom to the lipid peroxyl cycle and form the aryloxyl molecule, which is incapable of acting as a chain carrier and couples with another radical, thereby quenching the radical process [39]. Therefore, the content of total phenolic compounds in the extracts may explain their high antioxidant activities. The OGE, at a concentration of 66.67 μg/mL or higher, showed similar DPPH scavenging activity as that of BHA at the same concentration. Nenadis et al [40] reported that artificial antioxidants (e.g., BHA) have effective antioxidant activity, even at low levels (50 μM); however, the toxicity of BHA has to be considered when using the artificial antioxidant. Compared to the commercial antioxidants butylated hydroxytoluene (BHT) or α-tocopherol, the plant (especially leaves) can be exploited as an important source of natural antioxidants with health-protective potentials.

3.4. Cytotoxicity protection activity of Ocimum gratissimum extract on HepG2 cells

Hydrogen peroxide is regarded as a principal intermediary of oxidative stress-induced cytotoxicity [41]. Thus, the cytoprotective properties of the OGE against H2O2-induced cell death or damage can be observed by cell viability enhancement.

When HepG2 cells alone were exposed to H2O2, the cell viability significantly decreased in comparison to untreated control cells (Table 1). Fig. 3 shows that the viability of HepG2 cells was greatly reduced by approximately 75% when exposed to H2O2 (882 nM), whereas pretreatment with OGE (20–100 μg/mL) effectively increased the viability of the H2O2-assaulted HepG2 cells. Fig. 4 demonstrates the morphological change in the HepG2 cells. As Fig. 3 shows, the OGE was capable of inhibiting H2O2-induced cell death with viability rates of 34% ± 5.01%, 45% ± 4.68%, 51% ± 5.28%, 60% ± 4.85%, and 65% ± 5.70% at extract concentrations of 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, and 100 μg/mL, respectively. When compared to the H2O2-treated cells (26% ± 5.12%), all concentrations used in this study significantly abrogated in a dose-dependent manner the toxicity induced in the cells by H2O2 (p < 0.05). These results demonstrated that OGE has significant protective effects against H2O2-induced cytotoxicity in HepG2 cells.

Human HepG2 cells retain the activity of many phase I, phase II, and antioxidant enzymes; this ensures that they constitute a good tool for studying the cytoprotective, genotoxic, and antigenotoxic effects of the testing compounds [42,43]. In addition, the steady-state antioxidant defense level is higher in HepG2 cells than in other hepatic cells; this makes

| Table 1 – Non-cytotoxicity of OGE in HepG2 cells. |
|-------------------|-------------------|
| Cell viability (%) | MDA (nmol/mg of protein) |
| Control           | 100 ± 0.02        | 0.09 ± 0.05 |
| Treatment (OGE)*  |                   |           |
| 20 μg/mL          | 99 ± 0.27         | 0.11 ± 0.02 |
| 40 μg/mL          | 96 ± 2.75         | 0.143 ± 0.04 |
| 80 μg/mL          | 93 ± 2.78         | 0.12 ± 0.05 |
| H2O2, 882 nM      | 26 ± 5.12*        | 0.33 ± 0.07** |

Data are expressed as the mean ± standard deviation (n = 3).

*p < 0.01 indicates a significant difference, compared to the other groups (n = 3).

**p < 0.05 indicates a significant difference, compared to the other groups (n = 3).

* Cells were pre-cultured in 24 wells (2 × 105 cells/well in 10 mL of complete DMEM), starved for 12 hours, and then incubated at indicated concentrations of OGE or H2O2 (882 nM) alone for 24 hours.

DMEM = Dulbecco’s modified Eagle medium; H2O2 = hydrogen peroxide; MDA = malondialdehyde; OGE = Ocimum gratissimum extract.
it easier to detect variations in the responses under different conditions [44,45]. In Table 1, OGE alone did not affect the HepG2 cell viability at concentrations below 100 μg/mL. Therefore, our findings suggest that OGE is safe at the therapeutic level and has a significant ability to prevent oxidative stress. It may protect cells from oxidative stress by H2O2-mediated disruption of cellular antioxidant systems.

3.5. The effect of Ocimum gratissimum extract on lipid peroxidation

Lipid peroxidation is an autocatalytic process, which is a common consequence of cell damage and death. This process may cause peroxidative tissue damage in inflammation, cancer, toxicity of xenobiotics, and aging [46]. Hydrogen peroxide toxicity leads to severe oxidative stress in human HepG2 cells. Table 1 shows a dramatic increase in the TBARS level in the H2O2-treated group (0.33 ± 0.07 nmol/mg), compared to the control group (0.09 ± 0.05 nmol/mg). The amount of TBARS increased approximately 3.5-fold in H2O2-treated (882 nM) HepG2 cells, compared to the control (i.e., untreated) cells. We further examined whether pretreatment with the OGE contributes to TBARS decrease in H2O2-induced HepG2 cells. When HepG2 cells were treated with OGE (at concentrations of 20 μg/mL, 40 μg/mL, and 80 μg/mL) and then treated with 882 nM H2O2, the TBARS levels were measured as 0.17 ± 0.04 nmol/mg, 0.15 ± 0.05 nmol/mg, and 0.23 ± 0.03 nmol/mg, respectively. Compared to the TBARS levels in the control group, OGE treatment significantly reduced the TBARS level.

![Fig. 4](Image)

**Fig. 4** — The effect of Ocimum gratissimum L. extract (OGE) on HepG2 morphology under H2O2-induced cytotoxicity. Microphotographs (40× objective; phase contrast optics) of the HepG2 cells were obtained after treating them for 24 hours, as described in Section 2. The images show (A) the control; (B) 882 nM H2O2; (C) 40 μg/mL OGE + 882 nM H2O2; (D) 60 μg/mL OGE + 882 nM H2O2; (E) 80 μg/mL OGE + 882 nM H2O2; and (F) 100 μg/mL OGE + 882 nM H2O2. H2O2 = hydrogen peroxide; OGE = Ocimum gratissimum extract.

![Fig. 5](Image)

**Fig. 5** — Cells were precultured in a 10-cm dish (4 × 10^6 cells/dish in 10 mL of complete DMEM) for 12 hours, and then incubated for 24 hours with various concentrations of OGE prior to the addition of H2O2. The data are expressed as the mean ± standard deviation. *Indicates a significant difference, compared to the control group (n = 3); p < 0.01. **Indicates a significant difference, compared to treatment with H2O2 alone (n = 3); p < 0.05. DMEM = Dulbecco’s modified Eagle medium; H2O2 = hydrogen peroxide; OGE = Ocimum gratissimum extract; TBARS = thiobarbituric acid reactive substance.
concentration in H$_2$O$_2$-treated HepG2 cells (0.33 ± 0.07 nmol/mg), OGE pretreatment significantly decreased the concentration of TBARS (p < 0.05). The extract significantly reduced the elevated TBARS levels at dose levels of 20–80 µg/mL, which resulted in a 48.5% reduction in the TBARS level at 20 µg/mL; 54.5% reduction at 40 µg/mL; and 30.3% reduction at 80 µg/mL (Fig. 5). The protection rendered by OGE increased with increasing dose up to 40 µg/mL. A further increase in OGE (80 µg/mL) did not show more protection.

Increased MDA accumulation has been noted in response to H$_2$O$_2$ and this accumulation can induce damage to various biological macromolecules such as DNA, RNA, proteins, and lipids. In Table 1 and Fig. 5, the OGE alone did not significantly elevate the TBARS level at concentrations below 80 µg/mL. This indicates that OGE is safe at a therapeutic level. The cytotoxic effect of H$_2$O$_2$ on HepG2 cells was demonstrated by its strong inhibition of cell viability and MDA formation. Our results indicate that the OGE is capable of reducing H$_2$O$_2$-induced cytotoxicity and lipid peroxidation. Thus, the prevention of lipid peroxidation may explain its cytoprotective property on cell membrane damage caused by radicals.

4. Conclusion

These findings indicated that aqueous O. gratissimum extracts exert antioxidant and protective activities against oxidative stress induced by H$_2$O$_2$ on human HepG2 cells. The polyphenolic content and antioxidant activity proved that OGE possessed higher levels of antioxidant phytochemicals. The exhibited properties of aqueous O. gratissimum extracts may be implicated in hepatoprotective effects in oral nutraceuticals administered by dietary supplements or pharmacological preparations.

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