



ANTIOXIDANT AND LIPID PEROXIDATION PROTECTIVE PROPERTIES OF AQUEOUS PLANT EXTRACTS IN MONONUCLEAR BLOOD CELLS UNDER HIGH OXIDANT STRESS

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ABSTRACT

Effect of hydrogen peroxide (H₂O₂) on peripheral blood mononuclear cells (PBMNCs) was determined to assess the antioxidant property of the aqueous extracts of *Nigella sativa* seeds, *Allium sativum*, and *Rosmarinus officinalis* leaves. Lipid peroxidation was measured as marker for oxidative stress. Malondialdehyde significantly increased in the presence of H₂O₂ indicating extensive lipid peroxidation. The preincubation of PBMNCs with *Nigella sativa* aqueous extract resulted in a significant reduction of lipid peroxidation in the presence of 10 mM H₂O₂ at a dose as low as 0.8 mg/ml. However, other two extracts showed a significant decrease in malondialdehyde levels at higher concentrations. Although several studies have reported antioxidant properties of many plant extracts, these studies were investigating essential oils or organic extracts. The present study provides evidence of antioxidant properties in the aqueous extracts of the used plants. The reduction of lipid peroxidation may be attributed to the presence of high levels of water-soluble antioxidant agents.

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INTRODUCTION

The biological defense system against noxious oxidant conditions depends mainly on the enzymatic detoxification processes such as superoxide dismutase, glutathione peroxidase and catalase. However, the human body also requires simpler antioxidant compounds to resist peroxidant stress. Numerous plants contain substantial quantities of natural compounds that have antioxidant activity, such as ascorbate (vitamin C), tocopherols (vitamin E), carotenoids, flavonoids, polyphenols and others, which can be utilized (1). Citrus, green vegetables and herbs products could be potential targets of future effective drugs for diseases in which oxidant or free radical damage play a pathological role.

There is increasing evidence supporting the involvement of high oxidative stress due to reactive oxygen species (ROS) in the pathogenesis of several diseases including neurodegenerative disorders such as Huntington's, Parkinson's, and Alzheimer's diseases(2, 3), in addition to a wide range of diseases such as cardiomyopathy, diabetes and

various cancers (4, 5). Moreover, ROS are generated as natural byproducts during the normal processes of oxidative phosphorylation in mitochondria, in addition to their role in the inflammatory process. Under normal conditions, cells are able to protect themselves from ROS damage. However, sometimes, the pro-oxidant conditions dominate due to the generation of ROS that exceeds the ability of cells to detoxify them causing an alteration in the cellular redox homeostasis known as oxidative stress.

The antioxidant properties of natural agents help in protecting the human cells against oxidants or free radical attacks during certain pathological conditions(6). Therefore, there is growing interest in natural antioxidants because of their cytoprotective effects against the deleterious effects of ROS. In this context, the primary objective of this study is to determine the antioxidant properties of the aqueous extracts of black seeds (*Nigella sativa*), garlic (*Allium sativum*), and Rosemary (*Rosmarinus officinalis*) leaves, which are commonly used in traditional medicine in Mediterranean countries.

MATERIALS AND METHODS

Materials

All chemicals used were obtained from commercial sources and were used as received without further purification. Malondialdehyde bis(dimethyl acetal) (1,1,3,3-tetraethoxypropane) (MDA) was obtained from Fisher Scientific (USA). All other chemicals and reagents used were of analytical grade and supplied by Sigma Chemicals (USA) unless otherwise stated.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated according to a modified method described by Böyum (7) from fresh venous blood samples given by non-smoker healthy volunteer donors aged between 17 and 28 years of age after informed consent and the approval of the institutional ethics committee. Briefly, peripheral venous blood samples were collected in sterile plastic tubes (obtained from Greiner bio-one, USA) containing 100 μ L of 10% K₂-EDTA solution as anticoagulant. A blood sample (5ml) was mixed with an equal volume of 50mM phosphate buffered saline (PBS; osmolality 290 \pm 5 mOsm/Kg water, pH 7.4). Density gradient centrifugation was performed on a Ficoll–Paque solution (density 1,077 g/mL, Pharmacia, biotech AB, Uppsala, Sweden) in a ratio of 1:2 at 900 \times g for 30 min using a centrifuge with a swinging-bucket rotor (from Centurion Scientific, UK). The upper layer was aspirated and only the middle layer containing PBMCs was harvested to a new tube. The collected PBMCs layer was washed with 14mL cold PBS and centrifuged at 400 \times g for 10 min and supernatant was discarded. To remove the erythrocytes from the isolated PBMCs, 7mL of cold isotonic NH₄Cl solution (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4) was added to the pellet and incubated for 5 min at room temperature to promote erythrocytes lysis. Cells were centrifuged at 400 \times g for 10 min, and washed twice with cold PBS. The supernatant was discarded and the pellet was resuspended in PBS (50mM) and held at room temperature for further studies. Identification of PBMCs was confirmed by counting the cells using a differentiated cell counter. The percentage and viability of the isolated cells were determined using trypan blue exclusion assay.

Exposure of PBMCs to high oxidant stress

PBMCs were resuspended in PBS containing 2mM sodium azide (catalase inhibitor) at a final concentration of 2 \times 10³ cells/ μ L. Cell suspensions were incubated at 37°C for 60 min in a shaking water bath. PBMC cells were then challenged with an equal volume of 10mM H₂O₂ as a source of oxygen radicals, and incubated for a further 30 min at 37°C.

Nitroblue tetrazolium Test

The nitroblue tetrazolium (NBT) test usually is a semi-quantitative test done by microscopic counting of the cells containing blue formazan crystals. However, in this study we employed a modified method outlined by Choi *et al.* (2006), which provides fairly more reliable distinctive results for easy statistical analysis (8). The quantitative NBT reduction was measured as the yellow colored NBT was reduced to blue formazan deposits in PBMCs. Briefly, after mixing PBMCs with NBT (21 μ g/ml) and incubating for 30 min at 37°C, the mixture was centrifuged at 450 \times g for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 2 M dimethylsulfoxide (DMSO) to dissolve the blue

formazan deposits in PBMCs. The absorbance was measured spectrophotometrically at 560 nm and results were expressed in optical density.

Lipid peroxidation measurements

Lipid peroxidation was determined according to the most prominent method currently used by measurement of MDA, a secondary product of lipid peroxidation. MDA concentrations were determined using a modified spectrophotometric Esterbauer and Cheeseman's method (9) based on the reaction between MDA and thiobarbituric acid (TBA) at 90 - 100°C for 15 min. The reaction was performed by mixture 2 ml of PBMCs suspension (2 \times 10³ cell/ μ L) preincubated with either H₂O₂ or PBS with 1ml of trichloroacetic acid (TCA)- arsenite solution (TCA 20%, sodium arsenite 0.1 M) in PBS to precipitate protein. Cell suspension was vortexed and centrifuged at 750 \times g for 15 min. To 2.0 ml of the supernatants, 0.5 ml of TBA solution (TBA 1.0%, NaOH 50 mM) in PBS was added. The samples were placed in a boiling water bath for 15 min, then rapidly cooled in cold water to room temperature. The absorbance of the formed MDA-TBA adduct was measured spectrophotometrically at a wavelength 532 nm using Shimadzu spectrophotometer (Japan). Serial dilutions of MDA (0.05–0.5 nmol/mL) were prepared and assayed along with the PBMCs suspensions for constructing a calibration curve to calculate the molar extinction coefficient of the MDA-TBA adduct ($\epsilon = 1.56 \times 10^5$ M⁻¹ cm⁻¹). MDA concentrations were expressed in nmol/2 \times 10³ cells/ μ L.

Plants used and extraction

All plants were purchased from the local market in Jordan and were botanically authenticated by a plant taxonomy specialist in biology department, at the Hashemite University, Jordan. *Nigella sativa* seeds (black seeds) and *Rosmarinus officinalis* leaves (rosemary leaves) were washed, cleaned, dried, mechanically grinded to a powder and extracted with distilled water, for 4 hours. The extracts were centrifuged at 10,000 \times g for 15 min to remove residual solid debris. The solvent was evaporated under reduced pressure with a rotatory evaporator. The yields of the aqueous extracts of *Nigella sativa* seeds and *Rosmarinus officinalis* leaves were 2.1 and 0.23% (w/w), respectively. Peeled *Allium sativum* (garlic), 30 gm were crushed with distilled water in a mortar. The crushed material was carefully decanted by pressing and 60 ml of aqueous extract was extracted. One ml of aqueous extract contained material from 500 mg of *Allium sativum*.

Statistical Analysis

Assays were performed at least three times with triplicate samples. Data are presented as mean \pm standard error of the mean of three or more independent experiments. Statistical analysis was performed using Student's t-test or one-way ANOVA followed by Dunnett's multiple comparison tests using Graphpad Prism Version 6.01 Software Package. P values < 0.05 were considered significant.

RESULTS

The isolation of PBMCs using the aforementioned methods yield (90% \pm 9.6) viable cells as determined by trypan blue exclusion assay with a purity of more than 95% PBMCs (Figure 1A). Most of the isolated cells were lymphocytes (90.7 \pm 4.3%), and to less extent monocytes (4.5 \pm 2.1%) and neutrophils (4.6 \pm 2.4%). Eosinophils and

basophils were not detectable among the isolated cells (Figure 1B).

Incubation of PBMNCs with 10 mM H₂O₂, as a source of oxidative stress for 30 min did not cause significant changes in the respiratory burst of PBMNCs, which reflects the ROS (superoxide anion) production via the respiratory burst machinery, as revealed by the NBT reduction test to form blue formazan crystals cells (Figure 2).

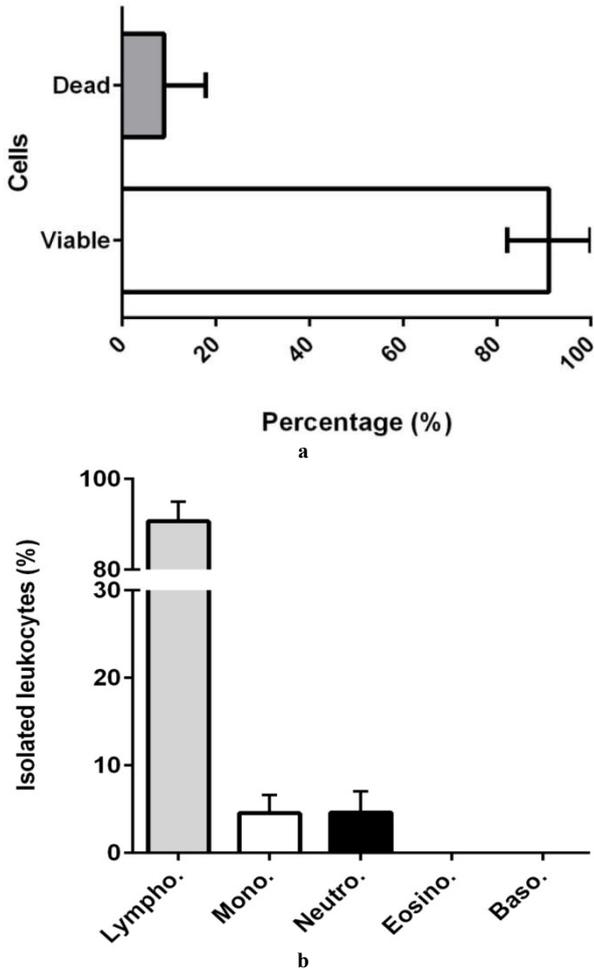


Figure 1 Viability (A) and differential (B) count (%) of isolated PBMNCs.

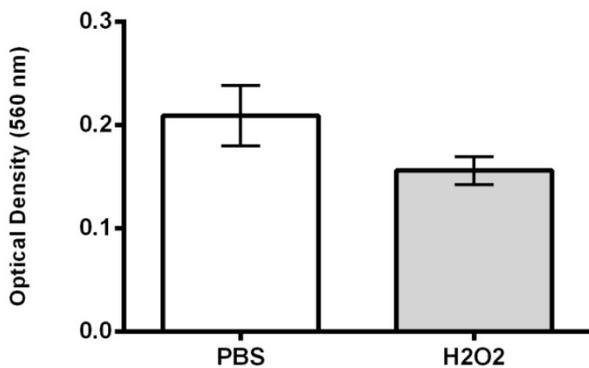


Figure 2 PBMNCs activation using nitroblue tetrazolium reduction test (p = 0.127).

The extent of lipid peroxidation was determined according to the most prominent method currently used by measurement of MDA, a secondary product of lipid peroxidation. The lipid peroxidation quantities were calculated according the established MDA standard curve (Figure 3A). Incubation of PBMNCs with 10 mM H₂O₂ for 30 min caused a significant

increase in extracellular MDA, as an increase in lipid peroxidation (Figure 3B). The H₂O₂ induced higher PBMNCs lipid peroxidation (4.13 nmol/2x10³ cells/μL) compared to the control (PBS) spontaneous lipid peroxidation (0.55 nmol/2x10³ cells/μL).

However, when PBMNCs were preincubated with different concentrations (2, 1.6, 0.8 mg/ml) of aqueous extracts of *Nigella sativa* seeds, *Allium sativum* and *Rosmarinus officinalis* leaves, the MDA concentrations were significantly reduced. Figure 4 shows the effect of 10 mM H₂O₂ on MDA concentrations of PBMNCs preincubated with aqueous plant extracts compared to the control cells. A tremendous reduction of MDA levels, as lipid peroxidation marker, in PBMNCs preincubated with an aqueous extract of *Nigella sativa* seeds at all used doses (Figure 4A). There was also a significant reduction of MDA levels in PBMNCs preincubated with an *Allium sativum* extract at 2 and 1.6 mg/mL concentrations (Figure 4B). The lowest used concentration of *Allium sativum* extract (0.8 mg/mL) caused a noticeable decrease in MDA levels; this reduction, however, was not statistically significant. Although, preincubation of PBMNCs with *Rosmarinus officinalis* leaves extract (2 mg/mL only) showed a significant decrease in the MDA level in the presence of H₂O₂ in comparison with the control value (H₂O₂ alone), MDA level was higher than that in PBMNCs preincubated with *Rosmarinus officinalis* extract without H₂O₂. However, the levels of MDA were increased as *Rosmarinus officinalis* leaves extract concentrations decreased (Figure 4C).

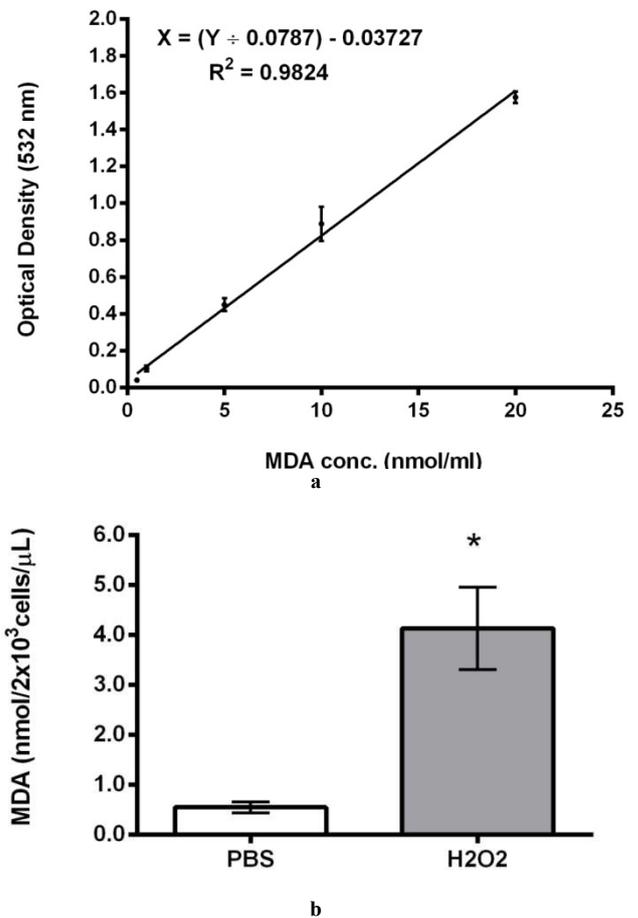


Figure 3 MDA standard curve (A), and MDA concentrations from PBMNCs after preincubation with 10 mM H₂O₂ compared to the control values (PBS), P < 0.05 (B).

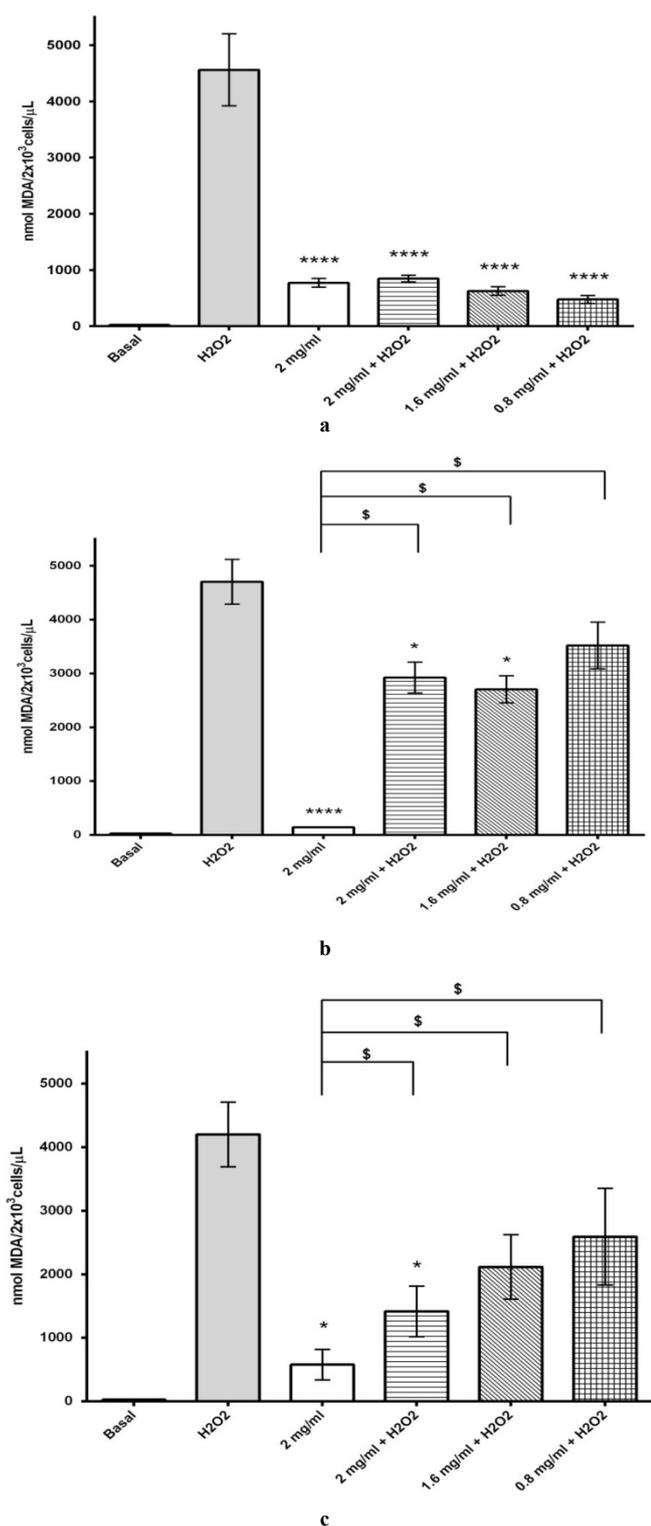


Figure 4 MDA concentrations in PBMNCs preincubated with various concentrations of aqueous extracts of A) *Nigella sativa* seeds; B) *Allium sativum*; and C) *Rosmarinus officinalis* leaves in the presence of H₂O₂. Dollar sign (\$) represent a statistical significance compared to plant extracts alone ($p < 0.05$), and asterisks (*) $p < 0.05$ and (****) $p < 0.0001$) represent a statistical significance compared to H₂O₂ control.

DISCUSSION

The use of herbal medicine to prevent or treat disease, among societies in the developing countries, has withdrawn attention in recent years as scientific evidence about the effectiveness of herbal medicine has become more widely available. It has been always recognized that the antioxidants in traditional medicine could represent an effective way to disease management. The

present study evaluated the antioxidant properties of three commonly used medicinal plants.

Nigella sativa seeds, *Allium sativum*, and *Rosmarinus officinalis* leaves are always regarded as a valuable remedy for a number of diseases. For example, Chehl *et al.* (2009) study demonstrated protective antioxidant and anti-inflammatory effects in the seed oil extract of *Nigella sativa* (10). *Allium sativum* has also been widely used as both condiment and medicine in many cultures for hundreds of years (11). The main medical uses of *Allium sativum* include treatment of hypercholesterolemia, high blood pressure, neoplastic cells, Alzheimer's disease and others (12-14). On the other hand, *Rosmarinus officinalis*, commonly known as rosemary, is usually planted as a decorative plant in gardens. *Rosmarinus officinalis* leaves are used as an aromatic seasoning or spice in foods in Mediterranean and Asian societies. *Rosmarinus officinalis* leaves have also been used traditional for their medical effects such as the use of fragrant Rosemary oil to improve memory (15), and the spasmolytic, carminative and antifatulence effects of its volatile oils in various gastrointestinal tract problems (16), in addition to analgesic and anti-inflammatory activities of the plant essential oil (17).

Hydrogen peroxide (H₂O₂) has been employed in this study as an oxygen radical generating system, to assess the antioxidant properties of the aqueous extracts of *Nigella sativa* seeds, *Allium sativum*, and *Rosmarinus officinalis* leaves in PBMNCs. H₂O₂ has been widely used as sources of ROS in *in vitro* studies to induce oxidative stress due to its peroxidant effects (18). Although H₂O₂ is not particularly reactive as an oxidizing agent, it reacts with iron and oxygen molecules to generate a variety of ROS (19). Exposure of various tissues and cells to ROS were found to cause lipid peroxidation and protein degradation, changes of cellular deformability, alteration of DNA constitution, membrane lipid perturbation, disruption of cellular enzymes and structural proteins and loss of chemotaxis (20-22).

The present results clearly demonstrate a significant cellular damage in PBMNCs as a result of H₂O₂ exposure, as indicated by the increased levels of TBA reactive substances in MDA assay due to lipid peroxidation. Although H₂O₂ caused a significant increase in lipid peroxidation, no significant difference was found in the respiratory burst activation of the H₂O₂ treated isolated PBMNCs compared to the control cells (in PBS), as evaluated by the NBT reduction test.

Preincubation of PBMNCs with *Nigella sativa* seeds aqueous extract resulted in a significant reduction of lipid peroxidation as indicated by the MDA concentrations in the presence of 10 mM H₂O₂ at a dose as low as 0.8 mg/ml. This reduction was comparable to the level of MDA in PBMNCs preincubated with *N. sativa* seeds extract only, which may be attributed to the presence of high levels of water-soluble antioxidant agents. On the other hand, *Allium sativum* extract demonstrated a significant reduction of MDA levels in PBMNCs at higher concentrations (2 and 1.6 mg/mL). Although, 0.8 mg/mL concentration of *Allium sativum* extract did not show a significant reduction of MDA level in PBMNCs exposed to H₂O₂, it did result in noticeable decrease of MDA levels in comparison to the control value (H₂O₂ alone). However, the MDA level was significantly higher than in PBMNCs preincubated with *Allium sativum* extract without H₂O₂. Similarly, *Rosmarinus officinalis* leaves extract showed a significant decrease in the MDA level in the presence of H₂O₂

only at the highest concentration used (2 mg/mL). Nevertheless, the MDA level was still significantly higher than in PBMNCs preincubated with extract alone.

There are many studies demonstrated the antioxidant effect of natural products in the organic extracts or essential oils of many plants(23, 24). However, the present results reveal the antioxidant properties of aqueous extracts of the used plants in preventing extensive cellular damage caused by lipid peroxidation due to ROS. Therefore, the aqueous plant extracts could represent a source of natural compounds with antioxidant activities, which is pivotal for retrieving the normally balance exist between the amount of free radicals generated in the body and the antioxidant defense systems.

Moreover, it has been also suggested that the excessive oxidative stress could be also caused due to depletion of the dietary antioxidants. Consequently, the potential antioxidant properties of natural agents help in protecting human cells against the burden of excessive oxidative stress and eventually ameliorating certain pathological conditions. However, the common perception of plant products as being natural and therefore harmless is somewhat misleading. Many natural compounds have a significant effect on the body and they may also interact with several pharmaceutical drugs(25). Consequently, the growing use of herbs should be confined until determining the safety and efficacy of herbal products.

CONCLUSION

The present results have demonstrated beyond doubt the importance of antioxidant effect of the aqueous extracts of investigated plants in preventing cellular damaging lipid peroxidation due to the peroxidant action of hydrogen peroxide. While, most of previous studies examined the effects of organic extracts and/or the plant essential oils, the present study has provided a tangible evidence about the antioxidant properties of the aqueous extracts of the used plant parts especially, *Nigella sativa* seeds. Therefore, the aqueous plant extracts could represent a praiseworthy source of natural compounds with antioxidant activities. These extracts are easily prepared by general public rather than the tedious organic solvent extractions. However, further studies should be conducted in searching for the active constituents of these plants possessing the antioxidant activities before drawing any definite conclusion.

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