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Original Article

Effects of commonly used medicinal herbs in Jordan on erythrocyte oxidative stress markers

[Efectos de las hierbas medicinales de uso común en Jordania sobre los marcadores antioxidantes de eritrocitos]

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Abstract

Context: Much attention has been given recently to the antioxidant capacity of natural products, with particular interest on those that are frequently consumed by people.

Aims: To evaluate the commonly used and frequently consumed edible herbs in Jordan to compare their *in vitro* and *in vivo* antioxidant properties.

Methods: The *in vitro* antioxidant properties were tested by pre-incubating washed human erythrocytes with a given herb extract and then exposing these erythrocytes to H_2O_2 to induce oxidative stress, and then measuring erythrocyte malondialdehyde (MDA) as a marker for lipid peroxidation, protein carbonyl (PC) as a marker for protein oxidation, reduced glutathione (GSH) as a marker for cellular antioxidant status and the percentage of hemolysis as an indicator for the anti-hemolytic activity of the herb. The *in vivo* antioxidant properties were tested by giving orally aqueous extracts of the herbs tested *in vitro* to healthy individuals on daily bases for five days, with two blood samples being collected from each individual to measure the above-mentioned markers.

Results: Pre-incubation of human erythrocytes *in vitro* with methanolic extracts of *Zingiber officinale, Rosmarinus officinalis, Salvia triloba, Verbena triphylla, Nigella sativa* and *Origanum syriacum* significantly improved erythrocyte MDA, PC and oxidant hemolysis. Oral consumption by healthy individuals of aqueous extracts of the same herbs for 5 days significantly improved erythrocyte MDA, GSH, and superoxide dismutase (SOD) at the sixth day of administration.

Conclusions: These results indicate that aqueous extracts of medicinal herbs can be absorbed well and appear in body tissues inflecting *in vivo* antioxidant properties similar to their *in vitro* properties.

Keywords: *in vitro; in vivo*; malondialdehyde; protein carbonyl; reduced glutathione; superoxide dismutase.

Resumen

Contexto: Recientemente se ha prestado mucha atención a la capacidad antioxidante de los productos naturales, con especial interés en aquellos que son consumidos frecuentemente por las personas.

Objetivos: Evaluar las hierbas comestibles de uso común y de consumo frecuente en Jordania para comparar sus propiedades antioxidantes *in vitro* e *in vivo*.

Métodos: Las propiedades antioxidantes *in vitro* se probaron preincubando eritrocitos humanos lavados con un extracto de hierba dado y luego exponiendo estos eritrocitos a H_2O_2 para inducir estrés oxidativo, y luego midiendo el malondialdehído eritrocitario (MDA) como marcador de peroxidación de lípidos, proteína carbonilo (PC) como marcador de oxidación de proteínas, glutatión reducido (GSH) como marcador del estado antioxidante celular y el porcentaje de hemólisis como indicador de la actividad anti-hemolítica de la hierba. Las propiedades antioxidantes *in vivo* se probaron administrando, diariamente durante cinco días, extractos acuosos por vía oral de las hierbas probadas *in vitro* a individuos sanos, recogiéndose dos muestras de sangre de cada individuo para medir los marcadores mencionados anteriormente.

Resultados: La preincubación *in vitro* de eritrocitos humanos con extractos metanólicos de *Zingiber officinale*, *Rosmarinus officinalis*, *Salvia triloba*, *Verbena triphylla*, *Nigella sativa* y *Origanum syriacum* mejoró significativamente los niveles de MDA de eritrocitos, PC y hemólisis oxidante. El consumo oral por individuos sanos de extractos acuosos de las mismas hierbas durante 5 días mejoró significativamente MDA, GSH y superóxido dismutasa (SOD) de eritrocitos en el sexto día de administración.

Conclusiones: Estos resultados indican que los extractos acuosos de hierbas medicinales pueden absorberse bien y aparecer en los tejidos corporales con propiedades antioxidantes *in vivo* similares a sus propiedades *in vitro*.

Palabras Clave: carbonilo de proteína; glutatión reducido; *in vitro*; *in vivo*; malondialdehído; superóxido dismutasa.

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INTRODUCTION

Free radicals in particular reactive oxygen species (ROS) are normal by-products of oxygen metabolism, constantly produced in aerobic organisms. They include superoxide anion (O_2^-), hydroxyl radical (OH^-), and non-radical hydrogen peroxide (H_2O_2). Hydrogen peroxide can be converted into hydroxyl radicals in the presence of transition metals such as iron via Fenton reaction (Devasagayam et al., 2004). At low concentrations, ROS play key roles in normal physiological processes, including cellular life/death processes, protection from pathogens, various cellular signaling pathways, and regulation of vascular tone (Valko et al., 2007). At higher concentrations and long-term exposure, ROS can damage cellular macromolecules such as lipids, proteins, and DNA, which may lead to necrotic and apoptotic cell death (Steinbrenner and Sies, 2009).

To overcome the potential toxicity of ROS, cells developed an antioxidant system, which could be classified into two types of antioxidants known as direct and indirect antioxidants. Direct antioxidants have redox activity and short half-lives that should be supplemented or regenerated during the process. These include a non-enzymatic system involving thiol-containing small molecules such as glutathione (GSH) and thioredoxin (Txn) that neutralizes ROS via direct interactions, and an enzymatic system, including catalase, glutathione peroxidase (GPx), and peroxiredoxins (Prdx) that reduce hydrogen peroxide to water. Indirect antioxidants act through the augmentation of cellular antioxidant capacity by enhancing specific genes encoding antioxidant proteins through the key transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is known as a master regulator of the antioxidant response, so their physiological effects last longer than those of direct antioxidants (Jung and Kwak, 2010). However, excess ROS can overwhelm the capacity of the cellular antioxidant system, which could lead to an imbalance in cellular redox.

Oxidative stress is a condition of imbalance between ROS formation and cellular antioxidant capacity, which could be due to enhanced ROS generation and/or dysfunction of the antioxidant system. Biochemical markers of oxidative stress include malondialdehyde (MDA), protein carbonyls (PC), and 8-hydroxyguanosine adducts that represent ROS-mediated damage to lipids, proteins, and nucleic acids, respectively (Halliwell and Gutteridge, 2015). Biochemical alterations in these macromolecular components can lead to various pathological conditions and human diseases. The antioxidant capacity of foods, juices, teas, and medicinal herbs has been linked to *in vivo* protection from oxidative stress-related diseases in numerous studies (Lee et al., 2017). One of these studies (Prior et al., 2007) showed that the consumption of an antioxidant-poor meal results in a decrease in plasma antioxidant capacity and that adding fruits to the same meal not only prevented this decrease but also led to an increase in plasma antioxidant capacity. A number of these studies demonstrated that many dietary phytochemicals derived from various vegetables, fruits, spices, and medicinal herbs could activate Nrf2 and induce expression of antioxidant enzymes (Na and Surh, 2008; Saw et al., 2012).

Thus, much attention has been given to the antioxidant capacity of natural products, with particular interest in those that are frequently (or potentially) consumed by people. The present work, therefore, aimed to study the commonly used and frequently consumed edible herbs in Jordan to compare theirs *in vitro* and *in vivo* antioxidant properties. The *in vitro* antioxidant properties were tested by pre-incubating washed human erythrocytes with a given herb methanolic extract and then exposing these erythrocytes to H_2O_2 to induce oxidative stress, and then measuring erythrocyte MDA, PC, GSH, and the percentage of hemolysis. The *in vivo* antioxidant properties were tested by giving orally aqueous extracts of the herbs tested *in vitro* to healthy individuals on a daily basis for five days, with two blood samples being collected from each individual. The following assays were performed on these blood samples: Erythrocyte MDA, GSH and superoxide dismutase (SOD). This *in vitro* and *in vivo* comparison study was also im-

portant to address the cellular aspects of bioavailability like cellular uptake, metabolism, partitioning in cellular membranes, which are crucial to the effectiveness of the antioxidant *in vivo*.

MATERIAL AND METHODS

Chemicals

All chemicals and reagents were analytical or HPLC grade and purchased from Sigma-Aldrich.

Plant material

Leaves of *Rosmarinus officinalis* L. *(Lamiaceae)*, *Verbena triphylla* L. *(Lamiaceae)*, *Salvia triloba* L. *(Lamiaceae)*, and *Origanum syriacum* L. *(Lamiaceae)* were collected in May/June of 2017 from Al-Balqa' steppe area at 1300 m altitude, west of Jordan (GPS coordinates: 31° 58' 3.374" N 35° 36' 34.369" E), and the rhizomes of *Zingiber officinale* Roscoe *(Zingiberaceae)* and seeds of *Nigella sativa* L. *(Ranunculaceae)* were purchased from the local herbal stores in Amman. All herbs were identified by Prof. Dr. Dawood Al-Essawy (The University of Jordan). Voucher specimens were deposited in the Herbarium of Biological Sciences Department at Jordan University under the following registration numbers respectively: YYB 20120, YYB 20121, YYB 20122, YYB 20123, YYB 20125, and YYB 20175.

Preparation of methanolic extracts of tested herbs for *in vitro* studies

The methanolic extracts of the tested herbs were prepared as described elsewhere (Bilto et al., 2015).

In vitro study

In vitro experiments were performed on washed erythrocyte suspensions prepared from heparinized venous blood from 42 healthy university student volunteers of either sex, age 19-30 years. Washed erythrocyte suspensions were prepared by centrifugation of whole blood to remove the buffy coat layer and then washed the packed cells three times with cold phosphate-buffered saline. Washed erythrocytes were exposed to 10 mM H_2O_2 with and without methanolic extract of tested herbs and then used for determination of erythrocyte MDA, PC, GSH, and percentage hemolysis.

Exposure of erythrocytes to H_2O_2 (10 mM)

Washed erythrocyte suspensions with or without pre-incubation with herb extract were exposed to 10 mM H_2O_2 to induce oxidative stress for 1 h at 37°C as described elsewhere (Suboh et al., 2004). After H_2O_2 exposure, the suspensions were used to measure erythrocytes MDA, PC, GSH, and percentage hemolysis. The final concentrations of herbal extracts were 0.2, 0.4, 0.6 and 0.8 mg/mL.

In vivo study

Preparation of aqueous extracts of tested herbs for in vivo studies

These were prepared as usually used by the Jordanian public in dealing with these herbs. 250 g dried leaves of each herb were boiled in 12.50 L water for 10-15 min and then left covered soaking for 10-15 min at room temperature. An amount of 1.25 L of soaked aqueous extracts was filled in clean bottles for each individual to be consumed in the morning 250 mL dose each day for five days.

Blood samples

A number of 70 healthy volunteers were grouped into seven groups (each group n = 10). Their age and sex are shown in Table 1. Five groups drank 200-250 mL of aqueous extract from the following medicinal herbs (*Zingiber officinale, Rosmarinus officinalis, Verbena triphylla*, *Origanum syriacum, Salvia triloba*), respectively daily in the morning for five days, group six received one spoon from ground seeds of *Nigella sativa* daily for five days, group seven received 2 tablets of paracetamol (each tablet, 500 mg) as reference drug daily for 5 days. Two blood samples with heparin were collected from each healthy volunteer (sample I before drinking/taking either aqueous extract or paracetamol tablets, sample II next day following the last dose of day five. The erythrocytes were washed three times with buffered saline and then hemolyzed to measure MDA, GSH, and SOD.

Table 1. Age and sex of participants (n = 63).

No. of group	Age (mean ± SD)	Female/Male
1. Zingiber officinale	41.8 ± 7.6	7/2
2. Rosmarinus officinalis	35.4 ± 13.5	4/5
3. Verbena triphylla	34.0 ± 18.6	4/5
4. Origanum syriacum	35.8 ± 14.7	4/5
5. Salvia triloba	42.8 ± 14.6	6/3
6. Nigella sativa	36.7 ± 14.1	8/1
7. Paracetamol	30.6 ± 9.8	3/6

Determination of oxidative stress markers

Determination of erythrocyte MDA

Erythrocyte MDA was determined as a measure of lipid peroxidation according to Stocks and Dormandy's method (Stocks and Dormandy, 1971) using thiobarbituric acid (TBA) as modified elsewhere (Srour et al., 2000). All MDA concentrations were expressed in nmol/g Hb.

Determination of erythrocyte PC

Erythrocyte PC was determined as a measure of protein oxidation using Cayman's protein carbonyl assay kit (Reznick and Packer, 1994). All protein carbonyl concentrations were expressed as nmol/g Hb.

Determination of erythrocyte GSH

Erythrocyte reduced glutathione was determined using Ellman's method (Ellman, 1951) with slight modification. Briefly, to 1 mL hemolysate, 2 mL of precipitating solution was added, mixed, and allowed to stand 5 min at room temperature. Then, the mixture was centrifuged at 4200 ×g. To 1.0 mL of the supernatant, 2 mL of phosphate solution (0.3 M of Na_2HPO_4) and 0.5 mL of DTNB (40 mg/dL) were added. The assay mixture was mixed by inversion 3 times, and its absorbance was read within 4 min at 412 nm against a blank. Standard GSH dissolved in distilled water (5-20 mg/dL) was assayed as above and used for the construction of a standard curve. All GSH concentrations were expressed in mg/g Hb.

Determination of erythrocyte SOD activity

Erythrocyte SOD was measured in hemolysate using a kit from Randox (Arthur and Boyne, 1985). All SOD activities were expressed in U/g Hb.

Determination of percentage hemolysis

To induce complete hemolysis, 0.1 mL from each cell suspension was diluted and mixed with 2.9 mL distilled water. All samples before and after dilution with water were then centrifuged at 1200 ×g for 5 min, and the hemoglobin concentration of supernatants was determined spectrophotometrically at 540 nm. Percentage hemolysis was calculated from the ratio of the absorbance of pre- to post-diluted samples.

Ethical issue, inclusion and exclusion criteria

This study was approved by the Ethics Committee of the University of Jordan with reference number 1/5/2/3510/2017 and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All healthy volunteers were recruited in the study after they signed an informed consent for publication of this study. Individuals were excluded if they had (i) a disease condition, such as liver, renal, or heart dysfunction; (ii) a history of cancer; (iii) allergies to any drug or food ingredient. Furthermore, women were excluded if they were pregnant or lactating. Smokers were also excluded. This was considered in order to exclude

any results interference that might be caused by the above-mentioned conditions.

Statistical analysis

All data are reported as the mean ± SD, and statistical analysis was performed using SPSS statistics 17. The results were compared by paired *t*-test. The results with a value of $p \leq 0.05$ were considered significant.

RESULTS

In vitro results

As shown in Table 2, exposure of human erythrocytes *in vitro* to 10 mM H_2O_2 caused a significant increase in MDA (from 11.2 to 302.8 nmol/g Hb). Pre-incubation of erythrocytes with methanolic extracts of tested herbs at concentrations of 0.2, 0.4, 0.6, and 0.8 mg/mL and then exposed to H_2O_2 significantly decreased MDA production (i.e., anti-lipid-peroxidant), compared to H_2O_2 alone, in a concentration-dependent manner at all concentrations, except for *Nigella sativa* the decrease was significant only at the highest concentration of 0.8 mg/mL. There was no significant effect for the tested herbs even at the highest concentration of 0.8 mg/mL on MDA before exposure to H_2O_2.

As shown in Table 2, exposure of human erythrocytes *in vitro* to 10 mM H_2O_2 caused a significant increase in PC (from 514.4 to 1420.5 nmol/g Hb). Pre-incubation of erythrocytes with methanolic extracts of tested herbs at concentrations of 0.2, 0.4, 0.6, and 0.8 mg/mL and then exposed to H_2O_2 significantly decreased PC production (i.e., anti-protein-oxidant), compared to H_2O_2 alone, in a concentration-dependent manner at all concentrations, except for *Verbena triphylla* and *Salvia triloba* the decrease was significant only at the highest concentration of 0.8 mg/mL, whereas, *Origanum syriacum* had no significant effect at any concentration. There was no significant effect for the tested herbs even at the highest concentration of 0.8 mg/mL on PC before exposure to H_2O_2.

As shown in Table 2, exposure of human erythrocytes *in vitro* to 10 mM H_2O_2 caused a significant decrease in GSH (from 2.19-2.21 to 0.94-0.87 mg/g Hb). Pre-incubation of erythrocytes with methanolic extracts of tested herbs at concentrations of 0.2, 0.4, 0.6, and 0.8 mg/mL and then exposed to H_2O_2 had no significant effect on GSH before or after exposure to H_2O_2, by any herb or at any concentration. There was no significant effect for the tested herbs even at the highest concentration of 0.8 mg/mL on GSH before exposure to H_2O_2.

As shown in Table 2, exposure of human erythrocytes *in vitro* to 10 mM H_2O_2 caused a significant increase (from 1.7 to 13.7%) in hemolysis (i.e., oxidant hemolysis). Pre-incubation of erythrocytes with methanolic extracts of tested herbs at concentrations of 0.2, 0.4, 0.6, and 0.8 mg/mL and then exposed to H_2O_2 significantly decreased oxidant hemolysis (i.e., anti-hemolytic), compared to H_2O_2 alone, in a concentration-dependent manner at all concentrations, except for *Nigella sativa*, there was no significant effect at any concentration, but there was a significant hemolytic effect by the highest concentration before exposure to H_2O_2 (from 1.7 to 13.0%), and yet exposure to H_2O_2 did not increase it.

In vivo results

As shown in Table 3, oral administration of aqueous extracts of tested herbs for 5 days decreased erythrocyte MDA at day 6 (i.e., one day following the last dose of day five), which reached significant levels with *Zingiber officinale, Rosmarinus officinalis,* and *Verbena triphylla*, but did not reach to significant levels with *Salvia triloba, Nigella sativa,* and *Origanum syriacum* compared to 0-time of administration. Paracetamol used as a reference drug did not affect erythrocyte MDA.

As shown in Table 4, oral administration of aqueous extracts of tested herbs for 5 days increased erythrocyte SOD at day 6 (i.e., one day following the last dose of day five), which reached significant levels with *Zingiber officinale, Rosmarinus officinalis,* and *Salvia triloba* but did not reach to significant levels with, *Verbena triphylla*, *Nigella sativa*, and *Origanum syriacum* compared to 0-time of administration. Paracetamol used as a reference drug did not affect erythrocyte SOD.

Table 2. Influence of the tested medicinal herbs on some oxidative stress markers.

Species	Erythrocyte (incubations)	MDA (nmol/g Hb)	% change	PC (nmol/g Hb)	% change	GSH (mg/g Hb)	% Hemolysis	% change
Zingiber officinale	Control	11.2 ± 3.1		514.4 ± 20.6		2.19 ± 0.11	1.7 ± 0.71	
	Control + 0.8 mg/mL	17.0 ± 7.4		511.7 ± 21.1		2.17 ± 0.09	1.6 ± 0.72	
	H_2O_2 (10 mM)	302.8 ± 59.6		1420.5 ± 143.3		0.94 ± 0.16	13.7 ± 0.70	
	H_2O_2 + 0.2 mg/mL	155.3 ± 27.5*	-49%	941.0 ± 152.0*	-34%	0.95 ± 0.10	5.0 ± 0.24*	-64%
	H_2O_2 + 0.4 mg/mL	105.0 ± 16.8 *	-65%	647.8 ± 45.9*	-54%	0.95 ± 0.12	1.3 ± 0.17*	-91%
	H_2O_2 + 0.6 mg/mL	90.0 ± 9.1*	-70%	636.6 ± 52.6*	-55%	0.95 ± 0.16	1.3 ± 0.17*	-91%
	H_2O_2 + 0.8 mg/mL	72.6 ± 12.8 *	-76%	635.2 ± 66.4*	-55%	0.94 ± 0.16	1.3 ± 0.17*	-91%
Rosmarinus officinalis	Control	11.2 ± 3.1		514.4 ± 20.6		2.21 ± 0.15	1.7 ± 0.71	
	Control + 0.8 mg/mL	11.3 ± 6.1		511.7 ± 21.1		2.15 ± 0.24	2.0 ± 0.29	
	H_2O_2 (10 mM)	302.8 ± 59.6		1420.5 ± 143.3		0.87 ± 0.19	13.7 ± 0.70	
	H_2O_2 + 0.2 mg/mL	219.9 ± 33.3*	-27%	1407.0 ± 136.7	-1%	0.85 ± 0.27	9.6 ± 2.20*	-30%
	H_2O_2 + 0.4 mg/mL	191.1 ± 23.8*	-37%	1076.0 ± 136.7*	-24%	0.87 ± 0.26	8.8 ± 1.81*	-36%
	H_2O_2 + 0.6 mg/mL	160.5 ± 9.8*	-47%	988.4 ± 99.5*	-30%	0.86 ± 0.29	5.2 ± 1.46*	-62%
	H_2O_2 + 0.8 mg/mL	111.1 ± 12.1*	-63%	775.5 ± 199.2*	-45%	0.86 ± 0.21	3.4 ± 1.31*	-75%
Origanum syriacum	Control	11.2 ± 3.1		514.4 ± 20.6		2.21 ± 0.15	1.7 ± 0.71	
	Control + 0.8 mg/mL	11.2 ± 2.7		511.7 ± 21.1		2.11 ± 0.17	1.8 ± 0.21	
	H_2O_2 (10 mM)	302.8 ± 59.6		1420.5 ± 143.3		0.87 ± 0.19	13.7 ± 0.70	
	H_2O_2 + 0.2 mg/mL	228.8 ± 27.4*	-24%	1492.4 ± 171.3	0%	0.87 ± 0.16	8.8 ± 1.00*	-36%
	H_2O_2 + 0.4 mg/mL	152.2 ± 24.0*	-50%	1473.3 ± 529.3	0%	0.86 ± 0.24	3.0 ± 0.25*	-78%
	H_2O_2 + 0.6 mg/mL	103.9 ± 5.4*	-66%	1474.9 ± 431.2	-4%	0.86 ± 0.21	2.4 ± 0.78*	-82%
	H_2O_2 + 0.8 mg/mL	87.7 ± 10.9*	-71%	1252.9 ± 168.5	-12%	0.86 ± 0.22	2.0 ± 0.37*	-85%

Table 2. Influence of the tested medicinal herbs on some oxidative stress markers (continued...)

Species	Erythrocyte (incubations)	MDA (nmol/g Hb)	% change	PC (nmol/g Hb)	% change	GSH (mg/g Hb)	% Hemolysis	% change
Verbena triphylla	Control	11.2 ± 3.1		514.4 ± 20.6		2.19 ± 0.11	1.7 ± 0.71	
	Control + 0.8 mg/mL	11.8 ± 4.8		508.2 ± 14.9		2.20 ± 0.10	2.0 ± 0.48	
	H_2O_2 (10 mM)	302.8 ± 59.6		1420.5 ± 143.3		0.94 ± 0.16	13.7 ± 0.70	
	H_2O_2 + 0.2 mg/mL	285.4 ± 50.1	-6%	1414.1 ± 45.7	-1%	0.96 ± 0.20	10.8 ± 0.91*	-21%
	H_2O_2 + 0.4 mg/mL	259.2 ± 46.4*	-14%	1414.1 ± 45.7	-1%	0.96 ± 0.23	8.5 ± 1.13*	-38%
	H_2O_2 + 0.6 mg/mL	240.4 ± 44.6*	-21%	1459.5 ± 46.4	-3%	0.96 ± 0.16	7.0 ± 1.42*	-49%
	H_2O_2 + 0.8 mg/mL	230.5 ± 48.7*	-24%	1165.5 ± 114.5*	-18%	0.96 ± 0.12	6.5 ± 1.80*	-53%
Salvia triloba	Control	11.2 ± 3.1		514.4 ± 20.6		2.19 ± 0.11	1.7 ± 0.71	
	Control + 0.8 mg/mL	10.9 ± 3.8		518.3 ± 25.4		2.18 ± 0.12	1.7 ± 0.41	
	H_2O_2(10 mM)	302.8 ± 59.6		1420.5 ± 143.3		0.94 ± 0.16	13.7 ± 0.70	
	H_2O_2 + 0.2 mg/mL	283.5 ± 27.7	-6%	1426.5 ± 50.6	0%	0.92 ± 0.13	10.78 ± 0.97*	-21%
	H_2O_2 + 0.4 mg/mL	258.2 ± 22.5*	-15%	1426.5 ± 50.6	0%	0.92 ± 0.18	8.54 ± 1.81*	-38%
	H_2O_2 + 0.6 mg/mL	226.5 ± 14.9*	-25%	1318.8 ± 112.4	-7%	0.92 ± 0.16	6.28 ± 1.57*	-54%
	H_2O_2 + 0.8 mg/mL	211.8 ± 22.3*	-30%	996.3 ± 288.0*	-30%	0.92 ± 0.19	5.84 ± 1.20*	-57%
Nigella sativa	Control	11.2 ± 3.1		514.4 ± 20.6		2.19 ± 0.11	1.7 ± 0.71	
	Control + 0.8 mg/mL	17.3 ± 6.8		505.7 ± 117.1		2.15 ± 0.12	13.0 ± 1.09**	
	H_2O_2 (10 mM)	302.8 ± 59.6		1420.5 ± 143.3		0.94 ± 0.16	13.7 ± 0.70	
	H_2O_2 + 0.2 mg/mL	301.8 ± 54.7	0%	1000.1 ± 207.9*	-30%	0.95 ± 0.17	13.0 ± 1.07	0%
	H_2O_2 + 0.4 mg/mL	297.9 ± 53.0	-2%	742.0 ± 105.7*	-48%	0.95 ± 0.11	13.7 ± 0.68	0%
	H_2O_2 + 0.6 mg/mL	295.9 ± 49.7	-2%	729.0 ± 91.4*	-49%	0.94 ± 0.12	14.3 ± 0.41	+4%
	H_2O_2 + 0.8 mg/mL	259.3 ± 49.7*	-14%	727.0 ± 131.1*	-49%	0.95 ± 0.16	14.7 ± 0.32	+7%

Summary of malondialdehyde (MDA), protein carbonyl (PC), reduced glutathione (GSH) and % of hemolysis of normal erythrocytes when incubated at 37°C for 60 min in the absence or in the presence of H_2O_2, or in the presence of H_2O_2 plus different concentrations of studied herb extracts. Each result represents the mean value ± SD (n = 7). *P≤0.05, compared to erythrocytes exposed to H_2O_2 alone. ** P≤ 0.05, compared to control erythrocytes before exposure to H_2O_2.

Table 3. Erythrocyte malondialdehyde (MDA) of the humans before and after oral administration of tested medicinal herbs.

Species	MDA (nmol/g Hb)		Change (%)
	0 time	Day 6	
Zingiber officinale	22.9 ± 4.5	16.2 ± 3.7*	-29%
Rosmarinus officinalis	21.2 ± 2.0	17.7 ± 2.8*	-17%
Verbena triphylla	27.7 ± 4.8	22.9 ± 3.4*	-17%
Salvia triloba	17.9 ± 3.4	15.8 ± 3.8	-12%
Nigella sativa	14.1 ± 0.7	13.2 ± 1.5	-6 %
Origanum syriacum	17.5 ± 3.4	16.4 ± 3.5	-6 %
Paracetamol	16.1 ± 3.1	17.8 ± 4.1	

Each result represents the mean value ± SD, (n=9). *P≤0.05, compared to the 0-time administration.

Table 4. Erythrocyte superoxide dismutase (SOD) of the humans before and after oral administration of tested medicinal herbs.

Species	SOD (U/gHb)		Change (%)
	0 time	Day 6	
Zingiber officinale	11005.4 ± 298.0	1374.5 ± 160.1*	+37%
Rosmarinus officinalis	1106.6 ± 118.3	1340.5 ± 134.0*	+21%
Salvia triloba	868.0 ± 167.1	997.5 ± 192.4*	+15%
Verbena triphylla	1132.0 ± 139.0	1210.3 ± 119.2	+10%
Nigella sativa	1103.0 ± 72.0	1224.1 ± 179.7	+11%
Origanum syriacum	1037.1 ± 155.3	1098.0 ± 181.5	+6 %
Paracetamol	1014.1 ± 256.6	1091.2 ± 172.1	

Each result represents the mean value ± SD, (n =8). *P≤0.05, compared to the 0-time administration.

Table 5. Erythrocyte reduced glutathione (GSH) of the humans before and after oral administration of tested medicinal herbs.

Species	GSH (mg/g Hb)		Change (%)
	0 time	Day 6	
Zingiber officinale	0.74 ± 0.31	1.53 ± 0.37*	+107%
Rosmarinus officinalis	0.82 ± 0.13	1.41 ± 0.23*	+72%
Salvia triloba	0.54 ± 0.09	0.87 ± 0.10*	+61%
Verbena triphylla	0.80 ± 0.15	1.05 ± 0.14*	+31%
Nigella sativa	0.67 ± 0.07	0.87 ± 0.08*	+30%
Origanum syriacum	0.73 ± 0.11	0.80 ± 0.10*	+10%
Paracetamol	0.73 ± 0.12	0.75 ± 0.13	

Each result represents the mean value ± SD (n =9). *P≤0.05, compared to the 0-time administration.

As shown in Table 5, oral administration of aqueous extracts of tested herbs for 5 days caused a significant increase in erythrocyte GSH at day 6 (i.e., one day following the last dose of day five) with all herbs compared to 0-time of administration. Paracetamol used as a reference drug did not affect erythrocyte GSH.

DISCUSSION

The present *in vitro* study (Table 2) showed that pre-incubation of erythrocytes with methanolic extracts of tested herbs significantly decreased the production of MDA in erythrocytes exposed to H_2O_2, indicating anti-lipid-peroxidant activity. The studied herbs were arranged in decreasing order of their *in vitro* anti-lipid-peroxidant activity in human erythrocyte as follows: *Zingiber officinale* > *Origanum syriacum* > *Rosmarinus officinalis* > *Salvia triloba* > *Verbena triphylla* > *Nigella sativa*. The present *in vivo* study (Table 3) also showed that the tested herbs decreased erythrocyte MDA at day 6 (i.e., one day following the last dose of day five) of administration, which reached significant levels with *Zingiber officinale, Rosmarinus officinalis,* and *Verbena triphylla*, but did not reach to significant levels with *Salvia triloba, Nigella sativa,* and *Origanum syriacum*, which could be explained to be due to their being washed out quickly during the 24 h after the last dose and/or being weaker as shown in the *in vitro* study in case of *Salvia triloba* and *Nigella sativa* compared to the other herbs (Table 2). This result coincides with the findings of other researchers (Bakirel et al., 2008), who have shown that oral administration of a *Rosmarinus officinalis* extract to diabetic rabbits for one week inhibited the lipid peroxidation in erythrocytes (i.e., decreased MDA) and activated erythrocytes antioxidant enzymes. It also coincides with others (Attia et al., 2014) who showed oral administration of *Zingiber officinale* extract for 26 days to cadmium-exposed rats significantly lowered plasma MDA. It also coincides with others (Carrera-Quintanar et al., 2012) who showed that daily oral administration of *Verbena triphylla* to university students beginning a 21-day aerobic training program caused a significant decrease in plasma MDA and PC that resulted from aerobic training.

The present *in vivo* study did not show any effect for paracetamol on MDA. This drug was used as a reference drug in this study because it has been reported previously to have antioxidant activity in human erythrocytes *in vitro* (Bilto, 2016) and anti-lipid-peroxidant activity *in vivo* (Simpson et al., 2014). The lack of *in vivo* effect for paracetamol in the present study could be explained to be due to its being washed out quickly during the 24 h after the last dose since the given dose supposed to have a plasma half-life of only 1.5–2.5 h (Mazaleuskaya et al., 2015). Furthermore, previously reported effects for paracetamol were conducted under *in vitro* or *in vivo* oxidative stress conditions, whereas the present study was conducted on healthy individuals with no apparent oxidative stress conditions.

The present *in vitro* study (Table 2) showed that pre-incubation of erythrocytes with methanolic extracts of tested herbs significantly decreased the production of PC in erythrocytes exposed to H_2O_2, indicating anti-protein-oxidant activity, except for *Origanum syriacum* where the decrease did not reach a significant level. The order of studied herbs in decreasing *in vitro* anti-protein-oxidant activity in human erythrocyte was as follows: *Zingiber officinale* > *Nigella sativa* > *Rosmarinus officinalis* > *Salvia triloba* > *Verbena triphylla* > *Origanum syriacum*. This order is different from that of anti-lipid-peroxidant order, as *Nigella sativa*, which was the weakest in anti-lipid-peroxidant activity, became the second strongest in anti-protein-oxidant activity, and *Origanum syriacum*, which was the second strongest in anti-lipid-peroxidant activity, became the weakest or no effect in anti-protein-oxidant activity. However, this variation in strength between *Nigella sativa* and *Origanum syriacum* in regard to anti-lipid-peroxidation and anti-protein-oxidant excludes the possibility of being a reflection of a variation in intestinal absorption. Rather it could be due to a variation in the herb's ability to bind the metal iron that is responsible for hydroxyl radical generation via Fenton reaction, as

others (Bilto et al., 2015) showed that *Nigella sativa* extract was the strongest in iron-chelating ability and the weakest in free radical scavenging ability between various herbs in which *Origanum syriacum* was one of them, this may indicate that *Nigella sativa* by its strong ability to bind iron prevents the generation of hydroxyl radicals via Fenton reaction, whereas *Origanum syriacum* does not, and thus *Nigella sativa* was stronger in anti-protein-oxidant activity than *Origanum syriacum*.

Alternatively, the variation in strength between *Nigella sativa* and *Origanum syriacum* in anti-protein-oxidant activity could be due to their variation of the solubility in the hydrophobic medium of the heme pocket of hemoglobin in which resides the metal iron as *Nigella sativa* could be more lipid-soluble than *Origanum syriacum*. This result also indicates that the Fenton reaction could have been largely responsible for heme-protein oxidation, particularly in erythrocytes. Furthermore, our results with *Nigella sativa* are also in line with the results of others (Burits and Bucar, 2000), who found that the essential oil of *Nigella sativa* and its constituents, thymoquinone and others, have an effective hydroxyl radical (the product of Fenton reaction) scavenging activity when tested for non-enzymatic lipid peroxidation in liposomes. Also, the *in vitro* results of *Nigella sativa* coincide with others (Bilto, 2015) who showed its anti-protein-oxidant activity that was stronger than its anti-lipid-peroxidant activity in erythrocytes exposed to H_2O_2.

However, *Origanum syriacum* was shown by others (Bilto et al., 2015) of being the second weakest after *Nigella sativa* in free radical scavenging that was due to their low content in total phenols and flavonoids, which explains the *Origanum syriacum* being the weakest or no effect in the present *in vitro* anti-protein-oxidant activity (Table 2) and the *Origanum syriacum* and *Nigella sativa* being the weakest or no effect on *in vivo* antioxidant markers of erythrocyte MDA, SOD and GSH (Tables 3-5). This may indicate that the free radical scavenging property of a given herb is more important than the prevention of free radical generation, such as by Fenton reaction to exert an *in vivo* antioxidant activity.

Zingiber officinale and *Rosmarinus officinalis,* although being the weakest in iron-chelating as shown by others (Bilto et al., 2015), they were the strongest in the present *in vivo* markers of MDA, GSH, and SOD and *in vitro* markers of MDA, PC, and oxidant hemolysis. This could be explained by being them the strongest in free radical scavenging, as shown by others (Bilto et al., 2015). This result may indicate again that the free radical scavenging property of a given herb is more important than the prevention of free radical generation, such as by Fenton reaction to exert an *in vivo* antioxidant activity. These two herbs were also the strongest in serum total antioxidant status (TAS) that was found by others (Bilto et al., 2019).

The present *in vitro* study (Table 2) showed that pre-incubation of erythrocytes with methanolic extracts of tested herbs had no significant effect on erythrocyte GSH (the main intracellular antioxidant) before or after exposure to H_2O_2. This is contrary to the *in vivo* study where all tested herbs significantly increased erythrocyte GSH (Table 5). This *in vivo* result coincides with the findings of other researchers (Tülüce et al., 2009), who have shown that feeding ground *Nigella sativa* seeds to broiler chickens for 6 weeks significantly increased GSH and decreased MDA in erythrocytes. The *in vitro* results also indicate that the *in vitro* anti-lipid-peroxidant and anti-protein-oxidant activities of the tested herbs were not mediated through increasing erythrocyte GSH, nor through activating antioxidant enzymes such as GSH-reductase that is needed to regenerate GSH from GSSG, nor through external erythrocyte source for GSH. Thus, the *in vivo* increase in erythrocyte GSH could be due to the activation of antioxidant enzymes such as GSH-reductase that is needed to regenerate GSH as found by others (Carrera-Quintanar et al., 2012) in an *in vivo* study, but this enzyme was not measured in the present study, or due to external source from hepatic generation, as a result of hepatic enzymes induction by a given herb during the weeklong consumption. The *in vivo* increase in GSH also coincides with the findings of other researchers (Asnani and Verma, 2009) who have shown that oral administration of extracts of *Zingiber officinale* or *Nigella sativa* significantly in-

creased GSH or total antioxidant status in the liver and kidney tissues of mice and rats. The decreasing order of tested herbs in increasing erythrocyte GSH *in vivo* was as follows: *Zingiber officinale* > *Rosmarinus officinalis* > *Salvia triloba* > *Verbena triphylla* > *Nigella sativa* > *Origanum syriacum* (Table 5).

The present *in vivo* study (Table 4) showed that the tested herbs increased erythrocyte SOD at day 6 (i.e., one day following the last dose of day five) of administration, which reached significant levels with *Zingiber officinale, Rosmarinus officinalis,* and *Salvia triloba* but did not reach to significant levels with, *Verbena triphylla*, *Nigella sativa*, and *Origanum syriacum*, which could be explained to be due either to their being weaker in free radical scavenging ability tested by chemical-based assays as shown by others (Bilto et al., 2015), or being washed out quickly during the 24 h after the last dose of day five (Table 4). The absence of the effect of *Verbena triphylla* on erythrocyte SOD is similar to the findings of others (Carrera-Quintanar et al., 2012) who found no activation of erythrocyte SOD or GSH-peroxidase although there was an activation of GSH-reductase and catalase in university students performing aerobic training program while consuming *Verbena triphylla* extract for 21 days. They concluded that consumption of *Verbena triphylla* could specifically regulate GSH-reductase activity in erythrocytes and lymphocytes. This explains why *Verbena triphylla* in the present study increased erythrocyte GSH although it did not activate the SOD. Thus, this increase in GSH could be due to the activation GSH-reductase that was not measured in the present study. The decreasing order of tested herbs in increasing erythrocyte SOD *in vivo* was as follows: *Zingiber officinale* > *Rosmarinus officinalis* > *Salvia triloba* > *Verbena triphylla* > *Nigella sativa* > *Origanum syriacum* (Table 4).

The increase of erythrocyte SOD after administration of *Zingiber officinale*, *Rosmarinus officinalis,* or *Salvia triloba* extract coincides with other studies (Ajith et al., 2007; Bakirel et al., 2008; Asnani and Verma, 2009) that showed oral administration of extracts of *Zingiber officinale* or *Rosmarinus officinalis* significantly increased the activity of SOD in the liver of mice or in the serum of diabetic rabbits, and also the treatment of Alzheimer-induced rats with *Salvia triloba* extract significantly increased erythrocyte SOD (Mahdy et al., 2012).

The present *in vitro* study (Table 2) showed that pre-incubation of erythrocytes with methanolic extracts of tested herbs significantly decreased oxidant-hemolysis in erythrocytes exposed to H_2O_2, indicating anti-hemolytic activity. The studied herbs were arranged in decreasing order of their *in vitro* anti-hemolytic activity as follows: *Zingiber officinale* > *Origanum syriacum* > *Rosmarinus officinalis* > *Salvia triloba* > *Verbena triphylla.* This order of arrangement is, in fact, the same order obtained for anti-lipid-peroxidation, indicating that the anti-lipid peroxidant activity was probably responsible for the prevention of hemolysis and that the increased rigidity of erythrocyte membrane with consequent hemolysis was probably due to lipid-peroxidation. Furthermore, *Nigella sativa* was the weakest, hardly showing anti-lipid-peroxidant activity at the highest concentration of 0.8 mg/mL, had no anti-hemolytic effect, which supports further the conclusion that lipid-peroxidation was responsible for the oxidant-hemolysis. Unexpectedly, *Nigella sativa* methanolic extract at high concentration (0.8 mg/mL) increased the percentage of hemolysis of erythrocytes before exposure to H_2O_2, probably due to the high content of saponins (hemolyzing agents) that were reported to make the main chemical constituents of the polar fraction of methanolic extract of *Nigella sativa* (Sparg et al., 2004).

As the present findings are obtained in healthy humans with no oxidative stress induction, this indicates that medicinal herbs can improve the baseline of the defense mechanisms against possible oxidative stress, thus decreasing susceptibility to diseases related to oxidative stress. However, we could not find in the literature any similar study that dealt with the effects of the presently tested herbs on healthy humans to compare our results with.

CONCLUSIONS

Medicinal herbs commonly used in Jordan such as *Zingiber officinale, Rosmarinus officinalis, Salvia triloba, Verbena triphylla, Nigella sativa,* and *Origanum syriacum* have *in vitro* and *in vivo* antioxidant properties, which indicate that they can be absorbed well and appear in blood plasma and erythrocytes inflecting antioxidant properties similar to their *in vitro* properties. Therefore, to predict the *in vivo* antioxidant property, a given herb could be tested *in vitro* on erythrocytes exposed to H_2O_2. The following antioxidant markers were improved by the tested herbs *in vitro* and *in vivo*; erythrocyte MDA, PC, GSH, SOD, and oxidant-hemolysis.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Bilto YY	Alabdallat NG	Atoom AM	Khalaf NA
Concepts or ideas	x	x		
Design	x			
Definition of intellectual content	x	x	x	x
Literature search	x	x	x	x
Experimental studies		x	x	
Data acquisition		x	x	
Data analysis	x	x	x	x
Statistical analysis		x		
Manuscript preparation	x			
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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