Biomedicine & Pharmacotherapy 89 (2017) 711–718

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Hepatoprotective efficacy of *Nigella sativa* seeds dietary supplementation against lead acetate-induced oxidative damage in rabbit – Purification and characterization of glutathione peroxidase

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ARTICLE INFO

Article history:
Received 7 January 2017
Received in revised form 11 February 2017
Accepted 14 February 2017

Keywords:
Nigella sativa
Lead toxicity
Oxidative stress
Antioxidants
Enzyme purification
Protein expression

ABSTRACT

Lead (Pb) is a toxic ubiquitous environmental pollutant that induces hepatotoxicity in both animals and humans. The ability of *Nigella saliva* seeds (NSS) in ameliorating lead acetate (PbAc)-induced hepatic oxidative damage was investigated using a rabbit model. Forty New Zealand rabbits were given feed and water *ad libitum*. They were allocated randomly into four groups: control; PbAc (5 g/L drinking water); NSS (20 g/kg diet) and NSS+PbAc groups. After two months, liver samples were collected and analyzed for malondialdehyde (MDA), glutathione (GSH), glutathione S-transferase (GST) and glutathione peroxidase (GPx) contents. Purification and characterization of GPx were also evaluated. PbAc exposure significantly ($p < 0.05$) increased MDA (lipid peroxidation biomarker) and reduced the GSH levels and the GST and GPx activities. Concurrently supplemented NSS significantly ($p < 0.05$) decreased MDA levels and restored the GSH, GST, and GPx contents successfully. Electrophoretically, the homogeneous GPx preparation from the liver had a specific activity of 30.44 U/mg protein and a yield of 1.31%. The K_m values for cumene hydroperoxide were 4.76 μM in control, PbAc and NSS+PbAc groups, and 4.09 μM in NSS group. The GPx reaction had a temperature optimum 40 °C, pH optimum 8 and molecular weight 21 kDa. The obtained data indicated the potent efficacy of NSS against PbAc-induced oxidative stress; that was mediated through induction and activation of antioxidants, particularly GPx and scavenging free radicals. Moreover, the purified hepatic GPx is characterized as a selenoprotein (Se-GPx).

1. Introduction

Environmental heavy metals are toxic to various organisms even at very low concentrations, which attributed to their oxidative power and ability to react with other compounds [1]. Air pollution caused by industrial emission, gasoline containing lead (Pb) compound, and food and water contamination are the primary sources of Pb exposure [2,3]. Pb is one of the most utilized metals in industries that induce a broad range of physiological, biochemical, and behavioral dysfunctions [4]. It is a potent systemic toxicant, which causes oxidative damages to the heart, liver, kidneys, reproductive organs, brain, and erythrocytes [5]. Pb can generate reactive oxygen species (ROS) in the body [6] resulting in lipid peroxidation, depletion of cellular antioxidant defense system, and DNA damage [7]. ROS such as superoxide radicals, hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) are potentially toxic to the cells and can damage biomolecules [8] that are combated by enzymatic and non-enzymatic antioxidant defenses. Pb induces alterations in enzymatic antioxidant molecules incorporating superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic antioxidant molecules, such as reduced glutathione (GSH) [9]. In this system, GPx provides detoxification of organic and inorganic peroxides by using GSH [10]. The regeneration of oxidized glutathione (GSSG) is

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http://dx.doi.org/10.1016/j.biopha.2017.02.044
0753-3322/

achieved by glutathione reductase (GR), which uses NADPH as reduced equivalents [11].

GPx is a selenoprotein, playing an important role in detoxifying the lipids and hydrogen peroxide (H_2O_2), which are formed rapidly during phagocytosis or metabolic processes [12,13]. GPx does not only depend on selenium but also on the availability of GSH for its enzymatic activity [14]. Since it scavenges H_2O_2 in the presence of GSH [15]. GPx has been purified from the cytosol (GPx1), gastrointestinal tract (GPx2), blood plasma (GPx3), phospholipid hydroperoxide (GPx4) [16], epididymis (GPx5), and olfactory system (GPx6) [17]. Therefore, the present study was undertaken to investigate the protective role of dietary supplementation of *N. sativa* seeds against Pb-induced oxidative stress via determination of the hepatic lipid peroxidation and endogenous antioxidants status. Additionally, the current study elucidated the ameliorative effect of *N. sativa* seeds against the inhibitory effect of Pb on the kinetics of the purified hepatic GPx in rabbits.

2. Methods

2.1. Nigella sativa *seeds*

N. sativa seeds (NSS) were purchased from the local market of Herbs and Medicinal Plants, Al-Manshia, Alexandria, Egypt. The identity of NSS was authenticated by Prof. Dr. Azza Shehata and Prof. Dr. Selim Heneidy – Botany Department, Faculty of Science, Alexandria University. A voucher specimen number (El-Far 009/2016) of this plant was maintained in the Department. NSS were thoroughly washed, dried and grinded to a fine powder. After that, it was added and homogeneously mixed with the rabbit's ration at a concentration of 2%.

2.2. Animals and experimentation

All procedures used were approved by the Institutional Animal Care and Use Committee of Alexandria University (Ethical Issue No: VM032/2015). Forty male White New Zealand rabbits (*Oryctolagus cuniculus*) (aged 30 ± 2 days and weighed 900 ± 50 g) were procured from the breeding colony at the Faculty of Agriculture, Alexandria University. The experimental animals were given diet and water *ad libitum*. The rabbits were housed in metal box cages at controlled temperature (28 ± 2 °C) and relative humidity 60±10% with a 12-h light/dark cycle. After two weeks of acclimatization, animals were allocated into four groups (10 rabbits/each). The control group was fed freely on basal diet and water. Lead acetate [PbAc, $Pb(CH_3COO)_2.3H_2O$] group was fed on basal diet and received water containing PbAc (5 g/L) [18]. NSS group was fed on a basal diet containing NSS (20 g/kg diet), while water offered freely. PbAc+NSS group was fed on a basal diet containing NSS and received water containing PbAc. All the treatments were daily applied for two consecutive months. The basal diet is consisting of alfa-alfa hay (33.0%), yellow corn (20.0%), barely (19.3), soya bean meal (14.0%), wheat bran (8.0%), molasses (5.0%), salt (0.5%) and vitamin (0.2%). It was formulated to meet all nutritional requirements of growing rabbits for minerals, vitamins, protein, essential amino acids and metabolizable energy [19,20].

2.3. Preparation of liver tissue homogenate

Twenty-four hours after the end of the experimental period, the rabbits of control and experimental groups ($n = 10$) were sacrificed under anesthesia with an intravenous injection of sodium pentobarbital (30 mg/kg bw), and then liver samples were immediately dissected and soaked in ice-cold saline 0.9%. They were homogenized using a motor-driven Teflon and glass Potter-Elvehjem homogenizer in 0.1 M Tris-HCl buffer of pH 7.4 containing 5 mM β-mercaptoethanol (1:4 *w*/*v*). The homogenates were centrifuged at $105{,}000 \times g$ for 60 min at 4 °C; the supernatants were divided into aliquots then stored at −20 °C for further evaluation of oxidative stress and purification of GPx enzyme.

2.4. Determination of oxidative stress parameters

The frozen aliquots of liver homogenates were utilized for the colorimetric assessment of MDA and GSH contents, as well the GST and GPx enzymes activities.

2.4.1. Determination of lipid peroxidation

Malondialdehyde (MDA) is the main aldehyde by-product of lipid peroxidation in biological systems. It was analyzed after the incubation of supernatants with thiobarbituric acid at 95 °C for 30 min (pH 3.6) to form thiobarbituric acid-reactive substances (TBARS), a pink colored compound. MDA levels were measured at 532 nm and expressed as nmol MDA/mg proteins [21].

2.4.2. Determination of reduced glutathione (GSH) levels

Reduced glutathione assay was based on the reductive cleavage of DTNB [5,5′-dithiobis (2-nitrobenzoic acid)] by compounds containing sulfhydryl groups and development of a yellow color [22]. The quantity of reduced chromogen is directly proportional to the GSH content. The absorbance was recorded at 412 nm and expressed as μmol GSH/mg proteins.

2.4.3. Determination of the glutathione S-transferase (GST) activity

The activity of GST was assayed according to the method of Vessey and Boyer [23]. This assay was based on monitoring the rate of enzyme–catalyzed conjugation of the CDNB [1-chloro-2,4-dinitrobenzene] with GSH. GST activity was measured as the increase in absorbance at 340 nm and represented as l mol CDNB/min/mg protein (A€=9.6/mM/cm).

2.4.4. Determination of the glutathione peroxidase (GPx) activity

GPx activity was evaluated according to the method of Chiu, Stults and Tappel [24]. The assay is based on measuring the oxidized glutathione (GSSG), which is produced by the reduction of organic peroxide in a reaction mixture contained GSH, GR, NADPH, and Tris–HCl. GPx activity was measured as the increase in absorbance at 340 nm and represented as GPx units/mg of protein.

2.4.5. Determination of tissue Protein

Protein concentrations in the tissue homogenates were determined using bovine serum albumin as the standard according to the method of Bradford [25].

2.5. Purification and characterization of glutathione peroxidase (GPx) enzyme

To investigate the effect of PbAc on GPx kinetics, and to monitor the degree of protection of NSS against PbAc, GPx was purified from liver tissues following the modified method of Duan, Komura, Fiszer-Szafarz, Szafarz and Yagi [26].

2.5.1. Ammonium sulfate salting out

Ammonium sulfate powder was added slowly to the supernatants of liver homogenates until 90% saturation. After overnight incubation, centrifugation at $15{,}000 \times g$ for 20 min at 4 °C was done, and the precipitate containing the GPx enzyme was collected and dissolved in 130 ml of 10 mM Tris buffer (pH 8.0) containing 5 mM β-mercaptoethanol (Buffer A) and dialyzed against the same buffer overnight.

2.5.2. Anion exchange chromatography by DEAE Sephadex A50

Column of dimensions (50 × 2 cm) of DEAE Sephadex A50 were prepared and packed using buffer A (pH 8.0). The eluent above the gel surface could drain, and then the dialyzed ammonium sulfate fraction was carefully loaded on the top of the bed. The column outlet was then opened until the samples were drained into the bed, then they were eluted by 2 L of buffer A (pH 8.0) containing potassium chloride (0.1–0.7 M KCl) in a linear gradient at a flow rate 1 ml/min fractions of 5 ml were collected. Each fraction was subjected to determination of the protein content at 280 nm and for GPx activity assay [24]. Fractions of GPx activity were collected and concentrated by buffer A (pH 8.0) containing 250 mM sucrose at 4 °C.

2.5.3. Gel filtration by Sephadex G75

Concentrated samples that were obtained by DEAE Sephadex A50 column were fractionated on a column of Sephadex G75 (50 × 2 cm), which eluted by a buffer A (pH 8.0) at a flow rate of 1 ml/min fractions of 5 ml at 4 °C. Each fraction was subjected to determination of the protein content at 280 nm and GPx activity. Fractions of GPx activity were collected, concentrated and stored at 4 °C.

2.5.4. Glutathione peroxidase assays

GPx activity was determined using cumene hydroperoxide [24]. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 7.6) (700 μl), 1 mM GSH (100 μl), the enzyme extract (100 μl) and 0.05% (*v*/*v*) cumene hydroperoxide (100 μl). The medium was stabilized at 37 °C for 5 min and 20% (*w*/*v*) TCA 1 ml was added. The samples were then centrifuged, and the supernatants were used for assessing enzyme activity. The reaction was initiated by addition of DTNB (100 μl). After 5 min, the absorbance was recorded at 412 nm against a blank of 50 mM Tris–HCl buffer (pH 7.6).

2.5.5. SDS-PAGE

SDS-PAGE was used to determine the degree of purity of crude extract, ammonium sulfate, DEAE Sephadex A50 and Sephadex G75 fractions, and to determine the molecular weight of purified GPx [27]. Briefly, SDS-PAGE was done by using 4.5% stacking gel, which overlaid on the separating gel of 10% polyacrylamide with an acrylamide: Bis ratio of 30:0.8. The running buffer consisted of 0.025 M Tris, 0.2 M glycine, and 0.1% (*w*/*v*) SDS. The imaged electrophoresis gels were analyzed by ImageJ version 1.48 for windows (NIH, MD, USA) to determine the protein expression of GPx enzyme in CTR and treated groups.

2.6. Statistical analysis

Values are showed as means ± SE. To evaluate the differences, one-way analysis of variance (ANOVA) followed by a *post hoc* Duncan's multiple range tests were used. Data analyses were performed with the use of SPSS version 22.0 for Windows (IBM, Armonk, NY, USA). The *K*m and *V*max values were determined by Graphpad 5 (Graphpad Software, La Jolla, CA) using Michaelis-Menten equation then analyzed by one-way ANOVA. The threshold for statistical significance was cited at $p < 0.05$ for all tests.

3. Results

3.1. Lipid peroxidation and antioxidants parameters

Our inquiry investigated whether NSS dietary supplementation prevents or even alleviates the oxidative hepatic damage induced by subchronic PbAc exposure. Initially, the rabbits exhibited a normal behavior with no obvious signs of toxicity and 100% survival rate throughout the entire experimental period, in comparison to the control group. The hepatic oxidative impacts of PbAc and the protective efficacy of NSS are shown in Table 1. It has been noticed that NSS supplementation evoked a significant ($p < 0.05$) depletion of MDA (3.69 nmol/mg protein), and enhancement of GSH content (65.77 μmol/mg proteins) when compared to control values. While GST and GPx activities in hepatic tissues in the control and NSS-supplemented rabbits were comparable to each other. Conversely, in relation to control, ingestion of PbAc was significantly ($p < 0.05$) enhanced lipid peroxidation bio-indicator, MDA (34.26 nmol/mg protein), and significantly ($p < 0.05$) reduced GSH content (37.67 μmol/mg proteins), GST activity (30.57 U/mg protein) and GPx activity (6.69 U/mg protein) in rabbit's liver. Furthermore, NSS ameliorated the oxidative effects of PbAc. It induced reduction of MDA (19.51 nmol/mg protein) and induction of GSH content (46.28 μmol/mg protein), and GST (33.33 U/mg protein) and GPx (8.69 U/mg protein) activities, which maintained comparable to that of control group.

3.2. Glutathione peroxidase purification

Protein of GPx enzyme was purified from a crude extract of rabbits' liver using three steps of ammonium sulfate salting out, DEAE-Sephadex A50 and Sephadex G75 columns chromatography. The crude extract's total activity, total protein, specific activity, purification factor and yield percentage were calculated (Table 2). About 110 fraction tubes were fractionated from the first DEAE Sephadex A50 chromatography column in 0.1–0.7 M KCl gradients. The GPx activity and protein content showed one peak at fraction tube number 65, while protein content showed several peaks represented many proteins other than GPx (Fig. 1A). Furthermore, the GPx activity and protein content showed one peak in Sephadex G75 chromatography step at fraction tube number 55 indicating that, this protein is the protein of purified GPx (Fig. 1B). Samples from purification steps were subjected the electrophoretic pattern analysis to determine the degree of purification purity and quantify the GPx protein level in all groups. Each band in each electrophoretic lane refers to a protein molecule in the liver. In the final purification step, only one major protein band remained on a denaturing protein gel (Figs. 2A). This band had a molecular mass of approximately 21 kDa. The data obtained by ImageJ analysis of

Table 1
Effects of *N. sativa* seeds (NSS) on the oxidative status and antioxidant parameters in rabbit's liver exposed to PbAc.

	MDA (nmol/mg protein)	GSH (μmol/mg protein)	GST (U/mg protein)	GPx (U/mg protein)
CTR	17.49 ± 3.24^{b}	51.23 ± 4.45^{b}	44.50 ± 4.86^{a}	10.53 ± 2.50^{ab}
PbAc	34.26 ± 5.24^{a}	37.67 ± 3.25^{c}	30.57 ± 4.21^{bc}	6.69 ± 1.70^{c}
NSS	3.69 ± 0.25^{c}	65.77 ± 6.55^{a}	37.25 ± 5.37^{ab}	15.87 ± 3.87^{a}
NSS+PbAc	19.51 ± 3.52^{b}	46.28 ± 3.56^{b}	33.33 ± 4.53^{b}	8.69 ± 1.70^{bc}

Different superscript letters within the same column indicate significantly different mean values ($p < 0.05$). CTR, control; PbAc, lead acetate; NSS, *N. sativa* seeds; MDA, malondialdehyde; GSH, reduced glutathione; GST, glutathione S-transferase; GPx, glutathione peroxidase.

Table 2
Purification steps of rabbit's hepatic GPx enzyme.

	Total activity (U/ml)	Total protein (mg)	Specific activity (mM/ml/mg protein/min)	Purification factor fold	Yield%
Crude extract	534	28179	0.02	1.00	100.00
Ammonium sulfate	342	308	1.11	58.59	64.05
DEAE-Sephadex A50	146	25	5.84	308.17	27.34
Sephadex G75	7	0.23	30.44	1606.03	1.31

Total activity = collected volume (ml) × GPx activity (U/ml).
Total protein = collected volume (ml) × protein (mg/ml).
Specific activity = total activity/total protein.
Purification factor = specific activity of purified enzyme/specific activity of the crude enzyme.
Yield% = (total activity of purified enzyme/total activity of the crude enzyme) ×100.

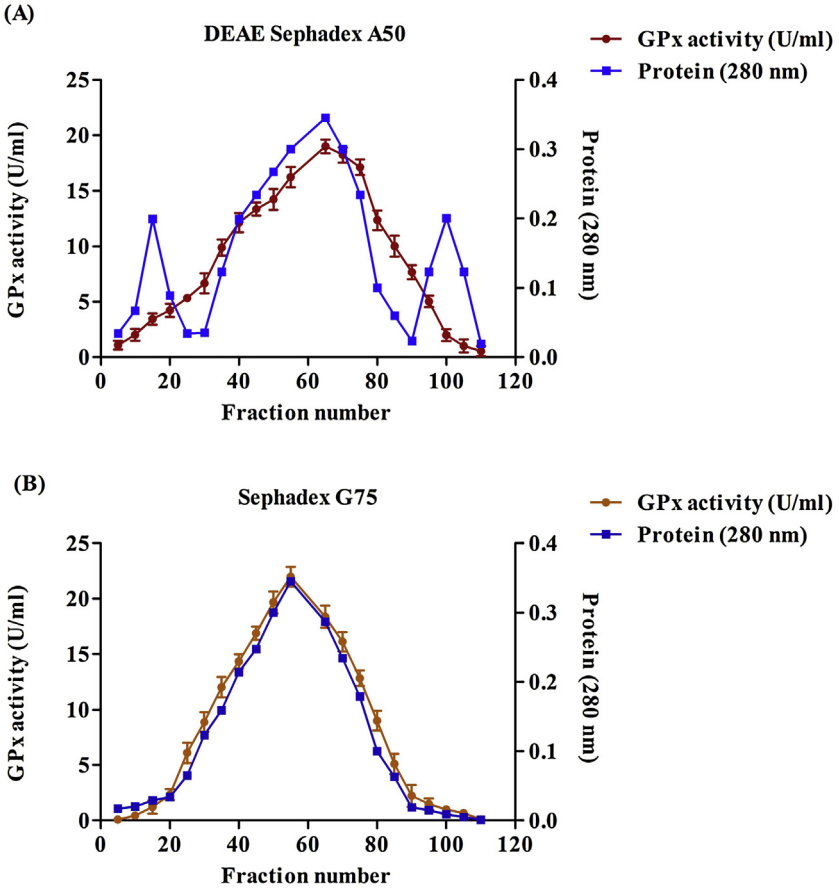

Fig. 1. The purification steps of rabbit's hepatic GPx.
(A) DEAE Sephadex A_{50} chromatography of GPx in control and treated groups. The dialyzed ammonium sulfate yield was chromatographed on DEAE Sephadex A_{50} column by elution with a linear gradient of KCl (0.1–0.7 M). The total protein contents of all fractions were monitored at 280 nm and the activities of GPx were assayed.
(B) Sephadex G75 chromatography of GPx in control and treated groups. The dialyzed DEAE Sephadex A50 fractions were chromatographed on a Sephadex G75 column. The total protein contents of all fractions were monitored at 280 nm and the activities of GPx were assayed.

GPx bands in all groups revealed a marked increase in GPx protein expression in NSS (120.92% ± 1.79) and NSS+PbAc (90.10% ± 1.93), while it drastically decreased in PbAc (55.89% ± 0.94) in comparison to CTR (Fig. 2B).

The data illustrated in Table 3 and Fig. 3 representing the factors influencing the GPx enzyme activity, which include the effect of different enzyme concentrations, optimum substrate concentration, K_m, V_{max}, temperature optimum, pH optimum, and optimum assay time. It was observed that GPx activity was increased with the increase in GPx concentration in the reaction assay showing a linear relationship (Fig. 3A). Correspondingly, the effects of different substrate concentration on the GPx activity were done by determination of GPx activity at various levels of cumene hydroperoxide from 1.5 to 18 μM revealing an optimum level at 12 μM (Fig. 3B). Both K_m and V_{max} were determined for GPx toward different concentrations of cumene hydroperoxide in all experimental animals versus 12 μM cumene hydroperoxide (Table 3). The obtained data revealed that K_m values of 2.03 ± 0.25, 2.52 ± 0.49 and 2.26 ± 0.33 μmol in control, PbAc, and NSS +PbAc-treated groups, respectively. While it was decreased to 1.80 ± 0.23 μmol in NSS-supplemented rabbits, indicating NSS increased the affinity of GPx toward cumene hydroperoxide at pH 8 and 40 °C for 15 min. However, it has been showed that there is no change in the temperature optimum 40 °C (Fig. 3C), pH optimum 8 (Fig. 3D), optimum assay time 15 (Fig. 3E) and GPx molecular weight 21 kD in all experimental animals.

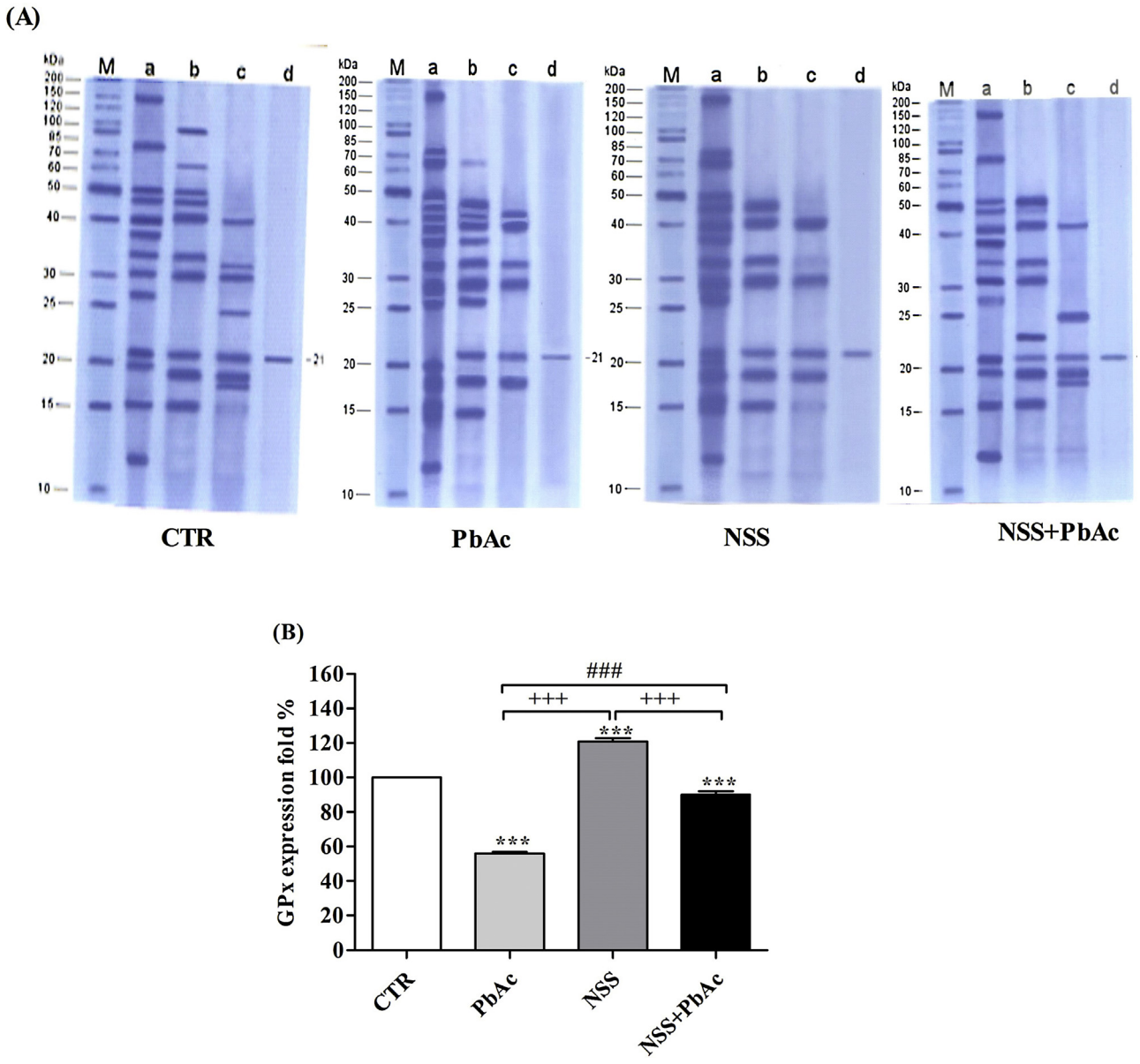

Fig. 2. SDS-PAGE of GPx separation steps and protein expression.
(A) SDS-PAGE of separation steps in CTR and treated groups (PbAc, NSS, and NSS+PbAc). M: protein ladder from 10 to 200 kDa; a: crude extraction; b: ammonium sulfate step; c: DEAE Sephadex A50 column chromatography step and Sephadex G75 gel filtration column chromatography step. (B) Percentages of GPx protein expression fold in relation to CTR. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. CTR group. $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$ vs. NSS group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. PbAc group.

Table 3
Characters of rabbit's hepatic GPx enzyme activity at different enzyme concentrations, optimum substrate concentration, Km, Vmax, optimum temperature, optimum pH and optimum assay time.

	Enzyme conc.	Opt. substrate conc. (μmol)	Km (μmol)	Vmax (U/ml)	Opt. Temp. (°C)	Opt. pH	Opt. Time (min)	MW (kDa)
CTR	Linear relationship	12	2.03 ± 0.25^{a}	17.21 ± 0.44^{b}	40	8	15	21
PbAc	Linear relationship	12	2.52 ± 0.49^{a}	9.49 ± 0.44^{c}	40	8	15	21
NSS	Linear relationship	12	1.80 ± 0.23^{a}	19.82 ± 0.51^{a}	40	8	15	21
NSS+PbAc	Linear relationship	12	2.26 ± 0.33^{a}	16.23 ± 0.53^{b}	40	8	15	21

Different superscript letters within the same column indicate significantly different values ($p < 0.05$). CTR, control; PbAc, lead acetate; NSS, *N. sativa* seeds; MDA, malondialdehyde; GSH, reduced glutathione; GST, glutathione S-transferase; GPx, glutathione peroxidase.

4. Discussion

The antioxidant system includes protective mechanisms by enzymatic (SOD, CAT, GR, GPx, and GST) and non-enzymatic (GSH, vitamin E, vitamin C, metallothionein, and phenolic compounds) antioxidant molecules [28–30]. An impaired antioxidant system can result in cell membrane damage, alterations in membrane fluidity and permeability, and oxidative stress [31]. Oxidative

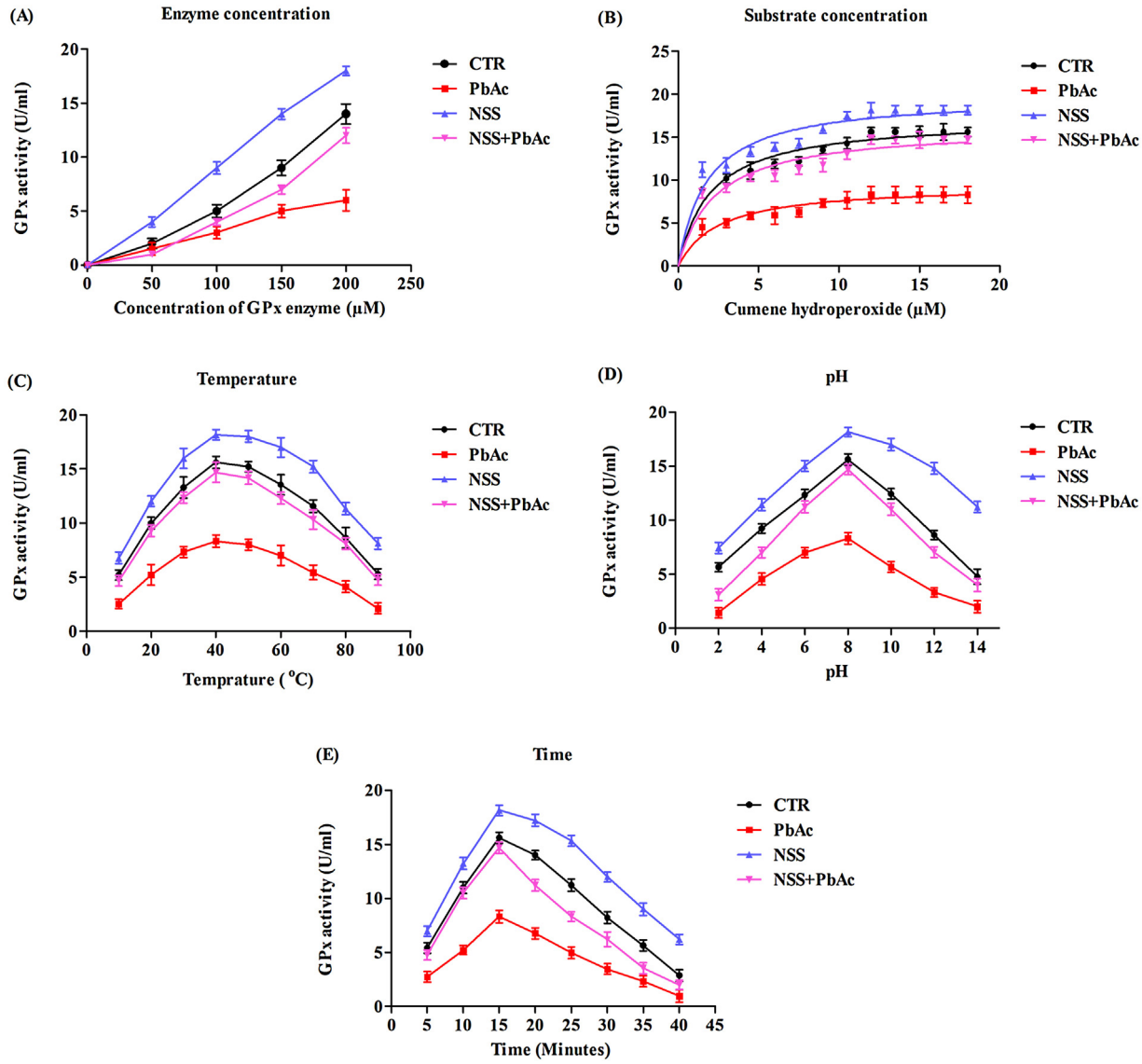

Fig. 3. The factors are affecting rabbit's hepatic GPx enzyme activity.
(A) Effect of GPx enzyme concentrations (µM), **(B)** Effect of cumene hydroperoxide (µM) concentrations (the *K*m and *V*max were determined by Graphpad prism 5, nonlinear regression, enzyme kinetic, Michaelis-Menten equation) **(C)** Effect of temperature, **(D)** Effect of pH and **(E)** different assay times on GPx activities in control and treated groups.

stress arises as a consequence of an imbalance between the antioxidant defense, and the ROS production culminates in oxidative damage to the critical cellular biomolecules such as DNA, lipids, and proteins [8]. The exposure to PbAc at a concentration of 0.5% in drinking water significantly enhanced the MDA levels and reduced GSH contents, and GST and GPx enzymes activities, which represent the extent of Pb-induced oxidative stress and generation of ROS. It has been reported that exposure of rats to PbAc evoked significant reductions in SOD, CAT and GPx activities in addition to a significant increase in MDA [32,33]. A negative relationship was observed between Pb concentrations, and the contents and regeneration rate of GSH as well the activities of GPx and GST enzymes [33,34]. The mechanisms by which the Pb-induced oxidative damage are poorly understood. However, the suggestions of the effects of Pb may be contributed to its interference with calcium inactivation of protein kinase C [35] and/or 5-aminolevulinic acid dehydratase (ALAD) in accumulating ALA [36], and thereby ROS generation. Pb-induced oxidative tissue damage could be accordingly induced as a consequence of a decreased antioxidant system, enhanced ROS production or both, which indicated by increased lipid peroxidation. Moreover, it has been found that PbAc did not only inhibited GPx enzyme activity but also it induced a drastic decrease in the protein expression of it, thus depletion of hepatocellular defense against Pb oxidative stress.

Consequently, blocking or retarding the process of oxidation development could be promising strategies for prevention of oxidative hepatic damage. Many reports indicate there is an inverse association between the nutritional antioxidants and the oxidative damage [29,33,37,38]. Dietary supplementation with natural antioxidants is considered as a type of preventive medicine; therefore, research studies investigating natural antioxidants sources are essential. Herein, it noticed that dietary NSS-supplementation significantly inhibited hepatic lipoperoxidation (LPO) in PbAc-intoxicated rabbits. In addition, the current study demonstrated that NSS-dietary supplementation significantly improved the GSH content and comparatively GSH-dependent enzymes activity, GPx, and GST. An adequate cellular content of GSH thus increased the scavenging ROS and detoxifying metabolic peroxides and hydroperoxides through the involvement of GPx and

GST [39]. In particular, it has been noticed that NSS supplementation markedly increased the hepatic Se-GPx protein expression that could be attributed to induction of its hepatocellular biosynthesis. Animals treated with *N. sativa* showed a significant reduction in the levels of hepatic lipid peroxidative markers with a concomitant improvement in the antioxidative system [39–42]. Therefore, NSS possess appreciable antioxidant and free radical scavenging properties that could be attributed to its major constituent of thymoquinone, carvacrol, 4-terpineol, dithymoquinone, and thymol, which protect against oxidative hepatic damage [43–52]. Altogether, these data indicated that Pb-induced LPO is partially due to the disruption of GSH and GSH-dependent enzymes homeostasis, and their maintaining is one of the potential mechanisms for NSS to protect against Pb-induced hepatic oxidative stress. Additionally, further investigations are required to assess the molecular mechanism ensuring the effect of Pb and NSS on Se-GPx protein expression.

The liver is known to be a rich source of GPx. Therefore, rabbit's liver in all experimental groups was subjected to a three purification steps of ammonium sulfate, DEAE-Sephadex A50, and Sephadex G75 columns chromatography. Several studies have purified GPx by DEAE-Sephadex A50. For example, the purification of phospholipid hydroperoxide GPx (PHGPx) had been accomplished during Hamster sperm capacitation [53], and from the Southern Bluefin Tuna (*Thunnus Maccoyii*) liver [13] and the red muscle of pacific Bluefin tuna (*Thunnus Orientalis*) [54]. The fraction tubes of GPx activity were collected and concentrated then loaded over a column of Sephadex G75 and fractionated after which the GPx activity and protein contents were determined. This column has been used by Cha, Kim and Kim [55] who has been purified the GPx from *Escherichia coli*. Moreover, Sephadex G-100 has been used for purification of liver monomeric GPx in chicken [56]. The purified hepatic GPx had optimum substrate values for cumene hydroperoxide of about 12 μM. Several studies investigated the K_m and V_{max} of GPx against cumene hydroperoxide as in mouse lung fibroblasts [57], C6 rat glioma cells [58] and human patients with senile cataract [59]. Studying the effects of different cumene hydroperoxide concentrations on the GPx activity revealed a significant increase in GPx activity following *N. sativa* supplementation. However, in the PbAc-intoxicated group, it shows a significant decrease in V_{max} of GPx toward cumene hydroperoxide without differences in K_m, indicating that Pb is a non-competitive inhibitor of GPx activity. In which, Pb binds GPx or GPx-cumene hydroperoxide complex other than at the catalytic site. Consequently, cumene hydroperoxide binding to GPx active site was unaltered. On the other hand, the addition of *N. sativa* counteracts the effect of Pb on GPx indicating the protective ability of *N. sativa* against Pb toxicity concerning hepatic GPx enzyme.

The temperature optimum of the purified hepatic GPx activity toward cumene hydroperoxide is 40 °C with a sharp decline in activity either side of the optimum. As well, it is the temperature optimum of GPx that is purified from *Saccharomyces cerevisiae* against cumene hydroperoxide [60]. The pH optimum of the purified hepatic GPx was pH 8 with a sharp decline in activity either side of the optimum, which similarly to that of Se-GPx purified from hamster liver [61]. Two forms of GPx have been isolated from Carp liver, one with an optimum pH of 8 and the other with an optimum pH of 9 [62]. However, it was different from the Japanese Sea Bass liver GPx, which had an optimum pH 7 [63]. The purified GPx protein from rabbit's liver has a molecular mass of approximately 21 kDa. Thus, it is similar to the GPx protein purified from human Jurkat T cells as PHGPx [64]. Western blotting allowed the detection of cytosolic GPx in all examined tissues with a molecular weight around 21 kDa, as a monomer [65]. The 21 ± 1 kDa protein refers to plasma GPx [66], but it entirely different from the GPx protein that purified from normal tissues and cultured cells with novel mouse monoclonal antibodies (24-kD) [67]. Also, a 23 kDa GPx has been purified from human blood platelets [68].

5. Conclusions

Collectively, the obtained data indicate that inclusion of NSS in diets of PbAc-intoxicated rabbits could plausibly improve PbAc-induced hepatic oxidative damage. The antioxidant role of NSS supplementation was mediated through inhibition of MDA, and induction of GSH and GSH-dependent enzymes, particularly Se-GPx. Moreover, NSS supplementation controls the non-competitive inhibition of hepatic Se-GPx activity and protein expression that induced by PbAc. Molecular studies at the level of gene and protein expressions of GPx and other antioxidant enzymes become of critical importance to understand the mechanism of NSS protective potential.

Competing interests

The authors declare that they have no competing interests related to this study.

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