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Research Article

Protective Effects of *Alstonia scholaris* (L.) R.Br. Bark Extract against Oxidative Stress Induced By Hydrogen Peroxide

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ABSTRACT: Objective: To evaluate the protective effects of bark extract of *Alstonia scholaris* (L.) R.Br. against oxidative stress induced by hydrogen peroxide (H_2O_2). Materials and Methods: Study was carried out in isolated lymphocytes cultured *in vitro*. Lymphocytes were treated with extract or extract + H_2O_2 and the level of reduced glutathione (GSH) and nitric oxide (NO) as well as the activity of lactate dehydrogenase (LDH) were measured. Results: Treatment of lymphocytes with bark extract modulated the levels of GSH and NO with decreased LDH activity. Pre-treatment with bark extract also significantly increased the level of GSH in lymphocytes treated with 1% H_2O_2. But, the levels of NO and LDH activity were decreased for similar treatment condition. Conclusion: The present data suggests that the protective effects of bark extract may be via up regulation of GSH and down regulation of NO levels. The modulation of GSH levels in the lymphocytes might be due to the phytochemicals present in the hydro-alcoholic extract of *Alstonia scholaris*.

KEYWORDS: *Alstonia scholaris*, hydrogen peroxide, Lactate dehydrogenas, nitric oxide, reduced glutathione

INTRODUCTION

Hydrogen peroxide (H_2O_2) is a strong oxidizer which acts as a highly reactive oxygen species (ROS) and mediates cellular damage in biological systems, leading to disease manifestation.[1] Hydrogen peroxide is produced as a by-product of normal oxidative metabolism and a low level is essential for cellular maintenance and survivability.[2] However, under certain conditions the levels increases, generating hydroxyl radicals leading to DNA damage and alterations in alters intracellular redox state. Increased H_2O_2 levels also change the homeostasis of ions such as calcium and iron and change the mitochondrial membrane potential, which may result in cytochrome C release from the mitochondria into the cytosol.[3–6] Hydroxyl radicals are also known to induce apoptosis and cell death.[1]

Hydrogen peroxide spontaneously decomposes exothermically into water and oxygen gas in a reaction catalyzed by the enzyme catalase. The main function of catalase in the body is the removal of toxic byproducts of metabolism and the reduction of oxidative stress.[7] However, in the presence of some catalysts including as Fe^{2+} and Ti^{3+}, the decomposition may take a different path resulting in the formation of free radicals such as hydroxyl ($HO^{\cdot}$) and $HOO^{\cdot}$. Free radical production may result in peroxidation of membrane lipids.[8]

The maintenance of optimum levels of hydrogen peroxide is mandatory in order to manage a healthy cellular environment. Several naturally occurring compounds including curcumin, ascorbic acid as well as synthetic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) have been tested

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for their hydrogen peroxide scavenging activity. However, the optimum level for redox maintenance and subsequent cellular protection remains a challenge.[9–11] The present study was designed to investigate the protective effects of bark extracts of *Alstonia scholaris* (L.) R.Br. (Apocynaceae) in lymphocytes exposed to oxidative stress induced by hydrogen peroxide (H_2O_2). *A. scholaris* (L.) R.Br. (family Apocyanaceae) is an extensively used medicinal tree in traditional, Ayurvedic, and folklore systems of medicine. Decoctions of *A. scholaris* bark are well studied for various medicinal activities including antimicrobial, antiamoebic, antidiarrhoeal, hepatoprotective, immunomodulatory, anticancer, antiasthmatic, free radical scavenging, antioxidant, analgesic, antiinflammatory, antiulcer, and wound healing. Phytochemical studies have revealed the presence of ditamine, echitamine, echitin, ditain, ditamine, losbanine, picraline deacetyl, lupeol, β-sitosterol, etc.[12–14] In this study, we have evaluated the modulation of cellular reduced glutathione (GSH) and free radical nitric oxide (NO) levels and correlated with the activity of lactate dehydrogenase (LDH) released into the culture media as a marker of membrane damage.

MATERIAL AND METHODS

Chemicals and reagents

Histopaque 1077, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), tricholoroacetic acid (TCA), reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), and sodium pyruvate were obtained from Sigma Chemical Co. (St Louis, MO, USA). RPMI 1640, Fetal bovine serum (FBS), Sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) were purchased from HiMedia Laboratories (Mumbai, India). The rest of the chemicals like Bovine serum albumin (BSA), etc. were of analytical grade, obtained from local firms of India.

Preparation of Modulator

The bark of *A. scholaris* was collected from Baihata Chariali, Assam (India) during the summer season. The collected plant material was authenticated by Prof. S K Borthakur (Dept. of Botany, Gauhati University, Gauhati, Assam, India) and a voucher specimen was preserved in our laboratory. The barks were cleaned thoroughly in tape water and distilled water, dried in the shade, powdered and macerated with hydro-alcohol (80% v/v) in a shaking condition. The bark of *A. scholaris* (BEAS) thus obtained was filtered, concentrated by air drying, and stored at 4°C. The extract was dissolved in DMSO at a concentration of 2.5 mg/ml in such a way that the final concentration of DMSO doesn't exceed 1% of final treatment.

Isolation of lymphocytes

Lymphocytes were isolated from chicken blood by using Histopaque (1.077 g/ml). Briefly, blood was diluted (1:1) with 1X PBS (pH 7.4) and layered onto Histopaque, centrifuged for 30 min at 400 g and the middle buffy layer containing the lymphocytes was separated. The isolated lymphocytes were washed with 1X PBS (pH 7.4) and centrifuged for 10 min at 250 g. Finally, cell pellets were suspended in RPMI and the cell viability was checked by Trypan blue exclusion method using a hemocytometer.[15] Only the cells viability more than 90% was used for subsequent studies.

Lymphocyte culture and treatment

Isolated lymphocytes (200 µl) were cultured in RPMI supplemented with 10% heat inactivated fetal bovine serum. The lymphocytes were treated with BEAS/ BEAS + H_2O_2 and maintained at 37°C and 5% CO_2 in a CO_2 incubator. Lymphocytes were cultured for 4 h in the case of BEAS treatments. For the BEAS + H_2O_2 test, the lymphocytes were pre-treated with extract for 1 h followed by H_2O_2 treatment for 4 h. Following incubation, the lymphocytes were centrifuged, washed, and homogenized in 1 X PBS (pH 7.4). The cell supernatants were used for assaying GSH, NO, and protein levels and the cell-free media were used for assaying LDH activity.

Reduced glutathione Estimation

The level of reduced glutathione was estimated by determination of the total non-protein sulphydryl groups (–SH) by the procedure described by Moron et al.[16] Total proteins were precipitated by the addition of tricholoroacetic acid (TCA), centrifuged, and the supernatant was collected. The supernatant was mixed with 0.2 M phosphate buffer (pH 8) and 0.6 M 5,5'-dithio-bis 2-nitrobenzoic acid and allowed to stand for 8–10 min at room temperature. The absorbance was recorded at 412 nm using a spectrophotometer (Thermo Scientific, UV 10). Reduced glutathione (GSH) was used as a standard to calculate nmole of –SH content/mg protein and finally expressed as percentage change of GSH levels as compared to the control cells.

Nitric oxide Estimation

Nitric oxide level was assayed as a nitrite level in the cell homogenate by the method of Griess.[17] The reaction mixture (200 µl) containing equal volume of sample and Griess reagent (prepared by mixing equal volume of 0.2% NEDD in 95% ethanol and 2% Sulfanilamide in 10% orthophosphoric acid) were incubated at dark for 30 minutes. The absorbance was measured at 550 nm in

a microplate reader (Multiskan Ascent, Thermo Electron Corporation). Sodium nitrite was used as a standard to calculate in mM NO/mg protein and finally expressed as percentage change of NO level as compared to the control cells.

Lactate dehydrogenase

The specific activity of lactate dehydrogenase (LDH) released into the medium as a result of membrane damage was assayed by measuring the rate of oxidation of NADH at 340 nm.[18] The reaction mixture (1 ml) contained 0.5 mM sodium pyruvate, 0.1 mM NADH, and cell-free media in 50 mM potassium phosphate buffer (pH 7.5). The reaction was started at 25°C by addition of NADH and the rate of oxidation of NADH was measured at 340 nm using a spectrophotometer (Cecil Aquarius, 7000 series). The enzyme activity was calculated using extinction coefficient 6.22 $mM^{-1}Cm^{-1}$/mg protein and finally expressed as percentage change of LDH activity.

Protein determination

The protein contents were determined using bovine serum albumin (BSA) as standard.[19]

Statistical Analysis

All the data are expressed as means ± SEM. Results were statistically analyzed by Student's *t* test for significance difference between group mean using GraphPad software. The significance difference between the experimental and the control group was set as $P \leq 0.05$.

RESULTS AND DISCCUSION

Biological systems inherently possess a defense system to scavenge and/or neutralize the effects of toxicants. These defense systems mainly consist of superoxide dismutase (SOD), catalase, glutathione-*S*-transferase (GST), glutathione peroxidase (GPx), reduced glutathione (GSH). These defenses function together or separately in a cascade manner to neutralize or eliminate the ROS and their failure contributes to diseases manifestation.[20] Antioxidants are supplemented to maintain the homeostasis of cellular oxidants and antioxidant status. Several synthetic compounds and plant-derived constituents have been studied as antioxidant supplements. However, in real biological states, potent antioxidant and radical scavenger is still a challenge.[21]

Reduced glutathione (GSH) is an endogenously synthesized tripeptide which acts as a cellular antioxidant. Under pathological and stressed condition, the level of GSH may become depleted, resulting in an elevation of reactive oxygen species, which affects the normal functioning and integrity of cell and organelle membranes.[22] We have previously reported the effects of H_2O_2 in the lymphocyte.[23] In that study we reported that treatment of lymphocytes with H_2O_2 decreases the cell viability with corresponding decrease in GSH levels.[23] In the present study, bark extract treatment for 4 h increased the levels of GSH in lymphocytes compared to the untreated control [Table 1]. When lymphocytes were pretreated with bark extract (50, 100, and 200 µg/ml) for 1 h followed by H_2O_2 treatment for 4 h, increased levels of GSH were observed (Table 2). The elevation is significant at 50 and 200 µg/ml treatments in comparison to only H_2O_2-treated cells. The elevated level of GSH may play a vital role in protecting cellular proteins and other molecules from the oxidative damage either by detoxifying and/or by neutralizing reactive oxygen species.[24] This suggests for the antioxidative potentials of the hydroalcoholic extract of *A. scholaris*.

Nitric oxide (NO), as well as acting as an intercellular messenger, also functions as a free radical.[25] Inside the cells, NO reacts with inorganic and organic molecules causing lipid peroxidation and the formation of several harmful products.[26] In contrast to GSH, levels of NO increases in pathophysiological and stressed condition and its elevated level promote apoptotic cell death.[27] Oxidative stress induced by H_2O_2 also increases the NO levels and causes severe damage to the cells.[28] Bark extract of *A. scholaris* reduces the level of NO in the lymphocyte (Table 1). In the H_2O_2-treated lymphocyte, NO level also decreased when they were pretreated with bark extract

Table 1: Modulatory effects of bark extracts of *Alstonia scholaris* (BEAS)

Treatments (BEAS)	% Change of GSH Level (nMole/mg protein)	% Change of NO Level (nMole/mg protein)	% Change of LDH Activity (unit/mg protein)
Control	100 ± 5.71	100 ± 3.84	100 ± 10.24
50 µg/ml BEAS	107.44 ± 11.82	94.40 ± 0.46	118.37 ± 9.05
100 µg/ml BEAS	113.25 ± 6.12	92.98 ± 1.16	82.22 ± 4.76
200 µg/ml BEAS	110.12 ± 1.69	90.20 ± 0.08	63.41 ± 0.60 [a]

Lymphocytes were treated with the different concentrations of BEAS for 4h and GSH, NO levels and LDH activity were measured. Values are Mean ± SEM; n=3;
[a]*P* ≤0.05 compared to untreated cells.

Table 2: Protective Effects of different concentrations of bark extracts of *Alstonia scholaris* (BEAS) in 1% H_2O_2-treated lymphocytes.

Treatments	% Change of GSH Level (nMole/mg protein)	% Change of NO Level (nMole/mg protein)	% Change of LDH Activity (unit/mg protein)
Control	100±9.07	100±14.41	100±1.02
H_2O_2 (1%)	61.55±1.08[a]	157.65±13.58[a]	243.98±10.57[a]
H_2O_2 (1%)+BEAS (50 µg/ml)	77.41±1.49[b]	137.62±6.70	166.39±7.21[ab]
H_2O_2 (1%)+BEAS (100 µg/ml)	81.09±7.18	127.86±3.14	191.49±8.29[ab]
H_2O_2 (1%)+BEAS (200 µg/ml)	83.05±6.36[b]	126.38±5.11	132.78±5.75[ab]

Lymphocytes were pre-exposed to the BEAS for 1 h before H_2O_2 treatment (4h) and GSH, NO levels and LDH activity were measured. Values are Mean±SEM; n=3;

[a]*P*≤0.05 compared to untreated cells;

[b]*P*≤0.05 compared to cells treated with only H_2O_2.

(Table 2). However, the decrease in the levels of NO is non-significant in both the cases.

The specific activity of lactate dehydrogenase, a marker of membrane damage, decreases significantly on bark extract treatment (Table 1). Extrinsic or intrinsic membrane damage is known to releases large amount of LDH into the exterior.[29,30] Here, in the present study, bark extract pre-treatment reduces the LDH activity measured in the cell-free medium (Table 2). This decrease activity is highly significant in comparison to only H_2O_2 treatment at all the concentrations of bark extract pre-treatment. The observed activity suggest not only the non-toxicity of the extract, but also its protection of cells from membrane damage induced by H_2O_2.

Results of the present study demonstrate the protective effects of the bark extract of *A. scholaris* against oxidative stress induced by H_2O_2 in lymphocytes cultured *in vitro*. Protection of lymphocytes from H_2O_2-induced cellular damage by bark extract may be due to an increase of cellular antioxidant or via decrease in free radical levels. The observed protective function is possibly conferred by different phytochemicals present in the hydro-alcoholic extract. Further, hydro-alcoholic extract needs an extensive study in suitable models to identify and characterize the active principle responsible for the observed activity.

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