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Original article

Phenolic constituents in the polar extracts of *Lawsonia inermis* mitigate antimycin A-induced mitochondrial degenerative cascades in Hep3B cells

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ABSTRACT

Degenerative conditions are associated with free radical-induced oxidative damages in the mitochondrial paraphernalia. Antimycin A (AMA) treatment of cells mimics such conditions *in vitro* by augmentation in ROS levels, thus causing injury to mitochondrial DNA (mtDNA), proteins and lipids, along with depolarization of mitochondrial membrane, activation of pro-apoptotic factors, resulting in apoptosis. This study investigates the potential of aqueous and methanolic extracts of *Lawsonia inermis* in prevention of such oxidative damage to the cell homeostasis. The extracts significantly mitigated membrane damages induced by peroxide, along with substantial decline in AMA-induced degeneration of mitochondrial proteins and lipids in hepatic carcinoma (Hep3B) cells. mtDNA analyzed for oxidative damage by assaying for 8-OHdG revealed considerable protective effect of both extracts against AMA-induced mtDNA damage. SQ-PCR of selected mtDNA genes confirmed that both extracts alleviated amplitudes of mtDNA injury. FACS analysis with JC-1 dye established that both extracts maintained homeostasis of mitochondrial membrane potential in AMA-treated cells. Extract treatments caused decline in AMA-induced discharge of cytochrome *c* and AIF into the cytoplasm along with consequent subjugation of apoptosis. All activities of the extracts reported in the present study significantly ($P < 0.05$) correlated to their total phenolic contents, thereby proving that polyphenolic constituents of the extracts alleviate cells from oxidative stress-induced injury.

1. Introduction

Mitochondria, the "powerhouses of cells", are the foremost generators of ATP in eukaryotic cells. Concurrently, they form the major intracellular source of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, hydroxyl radical, and singlet oxygen, under normal physiological as well as pathological conditions [1]. Simultaneously, these organelles are also the prime targets of ROS *per se* [2]. Oxidative damage to mitochondrial DNA (mtDNA) is the paramount cause of mitochondrial diseases, and has a central role in aging and cellular degeneration by apoptosis [3]. The rate of mitochondrial ROS generation has significant positive correlation to the steady-state level of oxidative stress to mtDNA [4]. Incidences of large deletions and point mutations occur at diverse sites all along the mtDNA; even in its main control region, which is decisive for its replication [4]. ROS also damage mitochondrial proteins and lipids [5,6], thereby perturbing customary mitochondrial functional and structural parameters. Such pathogenic deterioration in mitochondrial macromolecules has paramount role in induction of apoptosis and consequent tissue degeneration [4].

Antioxidants directly quench ROS or chelate the catalytic metal ions [7], thereby preventing damage to mitochondrial facets. Therefore, targeting mitochondrial ROS with various antioxidants might protect mtDNA from oxidative damage. Natural antioxidants in floral resources hold great untapped potential in this direction. *Lawsonia inermis* Linn. (Lythraceae) is a putative skin and hair coloring agent in many parts of the world. It is conventionally used as a medicinal plant by diverse ethnic/tribal groups [8–11]. Different parts of the plant are used as antirheumatic and antineuralgic formulations [10], and also as a potent antidiabetic drug [11]. There is evidence of wound healing activity of the plant [12]. Furthermore, *L. inermis* hydroalcoholic extract treatment (*in vivo*) increases levels of antioxidant enzymes, such as glutathione reductase, superoxide dismutase and catalase [13]. We have shown previously that diverse plant extracts characterized to have a multitude of phenolic

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constituents can hinder oxidative stress-induced cell degeneration, predominantly governed by the mitochondrial apoptotic cascade [14–16]. Recently, we have also characterized (by HPLC) the methanolic and aqueous extracts of *L. inermis* and have shown that the identified polyphenolic constituents hold the key to inhibit hexavalent chromium-induced oxidative toxicity to cells and DNA [17]. In the present study, we have evaluated the function of these two extracts of *L. inermis* as antithesis of free radical damage and apoptosis against AMA-induced mitochondrial oxidative stress in Hep3B (human hepatic carcinoma) cell line, and thereby, consequent recession of cellular degeneration.

2. Material and methods

2.1. Chemicals and reagents

Antimycin A from *Streptomyces* sp. (Biochemika; Fluka) and SIGMAFAST™ Protease Inhibitor tablets were obtained from Sigma Aldrich Chemical Co., WI, USA. 8-hydroxy-2′-deoxy guanosine EIA kit (# 589320) was purchased from Cayman Chemicals, MI, USA. Cellular DNA Fragmentation ELISA kit (# 11-585-045-001) was purchased from Roche Applied Science, Mannheim, Germany. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide) fluorescent dye was procured from Invitrogen Corp., CA, USA. Anti-α-tubulin mouse antibody (# CP06) and anti-AIF rabbit antibody (# PC536) were purchased from Calbiochem-EMD Chemicals, NJ, USA. Anti-cytochrome *c* rabbit antibody (# 4272) was obtained from Cell Signaling Technology, Inc., MA, USA. Horseradish peroxidase (HRP)-conjugated secondary antibodies anti-mouse (# 115-035-174) and anti-rabbit (# 211-032-171) were acquired from Jackson ImmunoResearch Laboratories, Inc., PA, USA. Polyvinylidene fluoride (PVDF) Immobilon-P^{SQ} membrane (0.2 μm) and TBS-T Wash Buffer (10X) were obtained from Millipore Corp., MA, USA. DNase I and alkaline phosphatase (calf intestinal phosphatase) were procured from Bangalore Genei (Merck Specialities Private Limited), Bangalore, India. SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Fisher Scientific, IL, USA. Hep3B cell line was procured from National Center for Cell Science, Pune, India. Thiobarbituric acid (TBA), Minimum Essential Medium (MEM) Eagle (with Earle's salts, NEAA and L-glutamine) for cell culture, and sodium pyruvate solution (100 mM) were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Fetal bovine serum was procured from Gibco-Invitrogen Corp., CA, USA. Hydrogen peroxide was purchased from Rankem, New Delhi, India. Folin and Ciocalteau's phenol reagent was procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Taq DNA polymerase enzyme, dNTPS and associated buffers were purchased from Vivantis Technologies, Inc., Selangor, Malaysia. PCR primers were synthesized by Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, India. The remaining chemicals and solvents used were of standard analytical and HPLC grades, respectively.

2.2. Plant material: collection, processing and extraction

L. inermis Linn. (whole plant) specimen were collected in May, 2010 from Vellore district (12°55′N, 79°11′E), Tamil Nadu, India, and identified at Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. Voucher specimen is maintained at our laboratory for future references (Accession No.: ACCL/10/5/09; May 9, 2010). Meticulously cleansed healthy plants were freeze-dried for 2 months at –80 °C and powdered for the preparation of extracts. Fifty grams of whole plant powder was serially extracted with methanol and water using Soxhlet apparatus [sample (g): solvent (ml) = 1:10], and the obtained extracts were concentrated at 40 °C under reduced pressure (72 mbar for aqueous extract; 337 mbar for methanolic extract) with a Rotavapor R-215 (BÜCHI Labortechnik AG, Switzerland) to yield dry extracts. Percentage yields of the methanolic and aqueous extracts were respectively 23.7% and 14.87% of dry weight.

2.3. Test for membrane integrity

Methanolic and aqueous extracts of *L. inermis* were tested for their potential to protect membranes against oxidative damage by H_2O_2 following the procedure as previously described [18]. Aliquots of human mature erythrocytes (1×10^7 RBCs) in 3 ml PBS (phosphate buffered saline) were taken in test tubes. Methanolic and aqueous extracts in diverse dosages (25, 50, 100 and 200 μg) were supplemented to each tube (except control). One hundred microliter of H_2O_2 (10 mM) was added to each test tube and incubated at room temperature with continuous shaking for 3 h. Contents of the tubes were then centrifuged ($224 \times g$) for 10 min. The supernatant was estimated for absorbance at 540 nm, using a Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA). Percentage membrane protection was calculated and compared with BHT.

2.4. Cell culture

Hep3B cells were grown in monolayer by seeding 1×10^6 cells in 25 cm^2 cell culture flasks (polystyrene coated) and maintained in MEM Eagle medium (supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 250 μg/ml amphotericin B and 100 IU/ml penicillin) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were harvested in logarithmic phase of growth for all experiments.

2.5. Test for cytotoxicity: XTT assay

In order to test whether the two extracts are cytotoxic *per se*, the method of XTT-formazan dye formation [19] was utilized. Six thousand (6×10^3) cells/well Hep3B cells were seeded in a 96-well plate and grown for 24 hours at 37 °C with 5% CO_2. The medium was removed and the cells were treated with different extract dosages: –125, 250 and 500 μg (prepared in 200 μl of medium). Extract-treated cells were re-incubated for 24 h at the same conditions. After treatment, medium containing extracts was replaced with 200 μl of fresh medium, followed by the addition of 50 μl of XTT (0.6 mg/ml in MEM) containing PMS (25 μM). The plate was further incubated for 4 h and absorbance was measured at 450 nm (reference filter: 650 nm) in a Dynex Opsys MR™ Microplate Reader (Dynex Technologies, VA, USA).

Percentage cytotoxicity was calculated as:

$$\%\ \text{cytotoxicity} = \left[\left(A_C - A_T / A_C\right)\right] \times 100$$

where A_C is the absorbance of control wells and A_T is the absorbance of test wells.

2.6. Antimycin A treatment

Antimycin A (AMA), is an inhibitor of electron transport chain at mitochondrial complex III [20]. This inhibition causes an elevation in the production of ROS, hence resulting in a collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), which opens up the mitochondrial permeability transition pores (mMTPs) and releases pro-apoptotic factors into cytoplasm, thereby inducing apoptosis [21–28]. ROS generated due to AMA treatment can damage