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Evaluation of the anti-proliferative and anti-oxidative activities of leaf extract from in vivo and in vitro raised Ashwagandha

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Abstract

Withania somnifera (Ashwagandha) is used in Indian traditional medicine, Ayurveda and is believed to have a variety of health promoting effects. Molecular mechanisms and pathways underlying these effects have not been studied. We tried to characterize various activities of leaf extract of Ashwagandha (Lash) raised in the field and in the laboratory. We found that the Lash from field-raised plants has a significant anti-proliferative activity in human tumorigenic cells. However, it did not impart any protection against the oxidative damage caused by high glucose and hydrogen peroxide to human tumor cells suggesting that it can be used as an anti-tumor, but not as an anti-oxidant, substance.

Keywords: Ashwagandha; Leaf extract; Human tumorgenic-cells; Growth arrest; Senescence

1. Introduction

Withania somnifera (L.) Dunal (common name—Ashwagandha, family—Solanacea) is an Indian medicinal plant that grows as an evergreen shrub in dry parts of India. It is classified in the ancient Indian system of medicine (Ayurveda) as a rasayana, a group of plant-derived drugs that improve overall physical and mental health and put off diseases by rejuvenating the body in incapacitated conditions. It is used as a geriatric tonic and in traditional remedies for inflammations, conjunctivities and tuberculosis. The root extract of *W. somnifera* has been shown to have health promoting effects such as anti-stress, anti-arthiritic, anti-inflammatory, analgesic, anti-pyretic, anti-oxidant and immunomodulatory properties (Agarwal et al., 1999; al-Hindawi et al., 1992; Archana and Namasivayam, 1999; Davis and Kuttan, 2002; Dhuley, 2000; Gupta et al., 2003; Mishra et al., 2000; Panda and Kar, 1997; Prakash et al., 2002; Scartezzini and Speroni, 2000). Methanolic root extracts of *W. somnifera* includes a variety of withanolides and was shown to induce nitric oxide synthase expression that could account, at least in part, for its immunostimulant properties (Iuvone et al., 2003). Similar extracts protected rats against hepatic, renal and skin pathology induced by fungicide (carbendazim) and DMBA (dimethyl benzanthracene) (Akbarsha et al., 2000; Prakash et al., 2002). They are also found to induce growth of human neuronal cells in culture (Kuboyama et al., 2002; Tohda et al., 2000; Zhao et al., 2002). Only a few studies have investigated the effects of leaf extract of *W. somnifera*. Similar to the root

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extracts, major components of the methanol extract of leaf are withanolides. These are structurally diverse steroidal lactones and are suggested to have anti-cancer, anti-oxidative and anti-mutagenic properties (Devi, 1996). Molecular mechanisms of the health-promoting and therapeutic effects of *W. somnifera* have not been well studied.

We tested the leaf extracts of Ashwagandha (Lash) for anti-genotoxicity in *Allium cepa* root tip cells and found that it confers substantial protection against the MNNG-induced genotoxicity (Rani et al., unpublished). In the present study, we prepared Lash from field raised (in vivo) and laboratory raised (in vitro) plants and investigated their effect on the growth of human osteosarcoma and breast carcinoma cells in normal as well as stressed culture conditions. We report that Lash has anti-proliferative activity. However, it did not offer any protection against the oxidative stress caused by high glucose or H_2O_2 suggesting that Lash does not have an anti-oxidative activity.

2. Material and methods

2.1. Preparation of the leaf extract from field raised Ashwagandha plants

The leaves of *W. somnifera* were collected in the month of October from the 3-year-old seed-raised plants growing in the Botanical Garden, Guru Nanak Dev University campus, Amritsar (India). They were washed, air-dried and ground to a fine powder. The finely powdered leaf powder was extracted by Soxhlet method (Lavie et al., 1968). The Leaf powder (40 g) was exhaustively extracted with methanol (60 °C) in Soxhlet apparatus for 100–110 h. Methanol was distilled-off to concentrate the extract. It was made aqueous by adding double-distilled water and was then extracted with hexane to remove chlorophyll and other pigments. Residual aqueous methanolic solution was re-extracted with diethyl ether using separating funnel. Diethyl ether was evaporated and the extract was solubilized in sterile DMSO for use and is called in vivo Lash in this study.

2.2. Preparation of the leaf extract from in vitro raised Ashwagandha plants

For raising the in vitro cultures, seeds of glass house grown plants were germinated on Murashige and Skoog's (MS) medium without plant growth regulators (PGRs) under aseptic conditions. The shoots raised from axillary shoot base callus were multiplied on MS medium containing 6-benzyladenine (BA, 2 mg/l) or N^6-(2-isopentenyl) adenosine (2-iP, 4 mg/l) alone, or in a combination of the two (BA, 1 mg/l and 2-iP, 1 mg/l). The leaves from each concentration were harvested after an interval of 30, 60 and 90 days of inoculation and extraction was done by using similar procedure as described for in vivo plants. The extract from in vitro raised plants is called in vitro Lash.

2.3. Human cell culture and treatments

Osteogenic sarcoma (U2OS) and breast carcinoma (MCF-7) cells were cultured in Dulbecco's modified Eagle's minimal essential medium with low-glucose (5 mM, Gibco) or high-glucose (27 mM, Sigma)-supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified, 5% CO_2, 95% air atmosphere. Cells (at about 50–60 confluency) were treated with Lash (3–24 μg/ml), H_2O_2 (30–300 μM for 2 h followed by culture in normal medium for 24–72 h) as indicated in the results.

2.4. Determination of cell viability using WST-assay

To determine the effect of Lash on cell viability, WST-1 assay (Roche) was employed. The cleavage of the tetrazolium salt WST-1 by active mitochondria produces a soluble colored formazan salt that directly correlates with the viable cell number. Cells were first seeded in the 96-well plates to get 40–50% confluency and were then treated with the different concentrations of in vivo and in vitro culture derived Lash in either low (5 mM)- or high (27 mM)-glucose DMEM, H_2O_2 for 48 h. Control treatment was done with solvent alone. Cells were incubated with WST-1-supplemented medium for 1 h at 37 °C in CO_2 incubator and then assayed for formazan salt by reading absorbance at 450 nm using microplate reader.

2.5. Immunostainings

Cells were grown and treated on glass coverslips placed in 12-well culture dishes. At the end of the treatment, coverslips were washed with cold phosphate-buffered saline (PBS) and fixed with a pre-chilled methanol/acetone (1/1, v/v) mixture for 5 min on ice. Fixed cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 2% bovine serum albumin (BSA) in PBS for 20 min. Cells were stained with anti-p53 antibody (DO-1, SantaCruz), a monoclonal anti-mortalin antibody (Affinity Bioreagents #MA3-028) or a polyclonal anti-mortalin antibody (Wadhwa et al., 1993). Immunostaining was visualized by secondary staining with Alexa-594-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse (Molecular Probes). After six washings with 0.1% Triton X-100 in PBS, cells were overlaid with Fluoromount (Difco). The cells were examined on a Carl Zeiss microscope with epifluorescence optics. Images were then processed using Metamorph imaging