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In vitro exposure of *Adhatoda zeylanica* to human renal cells lacks acute toxicity

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ABSTRACT

Adhatoda zeylanica is a dietary supplement ingredient present in several types of dietary supplements, including weight loss, respiratory relief, and immune regulating products. Due to its reported wide range of uses in folk medicine, it was hypothesized that it may have the potential to target multiple organs and lead to a range of toxicity features. As a preliminary evaluation of the safety of this herbal ingredient, an investigation into its effects on the kidney was sought. An *in vitro* study of its potential nephrotoxicity using the HK-2 human proximal tubule cell line in a variety of functional indicators was performed to capture both general forms of cellular toxicity as well as ones that are specific to proximal tubules. *A. zeylanica* was only capable of inducing detrimental short-term toxicity to HK-2 cells at relatively high treatment concentrations when exposed directly to the cells. The lack of acute and potent toxicity of *A. zeylanica* under our experimental conditions calls for further studies to better define its toxicant threshold and establish safe dosage levels.

1. Introduction

According to the Dietary Supplement Health and Education Act [11], supplement ingredients sold in the United States before October 15, 1994 are presumed to be safe and are therefore not required to be reviewed by the FDA. Any ingredient sold after this date is classified as a "new ingredient" and its manufacturer must show how it determined that reasonable evidence exists for safe human use. In effect, dietary supplements do not undergo stringent safety testing, leaving open the possibility of having unsafe supplement products on the market. Indeed, not only have several dietary supplements been recalled in recent years, but many even continue to be sold months after their recalls [8].

Despite the potential risk of having unsafe dietary supplements already on the market, it is reported that supplement use among American consumers has increased significantly in recent years [1,4]. Furthermore, supplements that use botanical ingredients now comprise a significant portion of the market, with current use in the United States increasing steadily [29]. This growing popularity of botanical dietary supplements may be related to a common belief among consumers that natural herbal remedies are safer alternatives to prescription drugs. One such supplement type is made from the herb *Adhatoda zeylanica* (*A. zeylanica*; also referred to as *Adhatoda vasica* or Malabar nut), which is currently used as an ingredient in a dozens of dietary supplements claiming to support weight loss, respiratory relief, or immune system health [19]. *A. zeylanica* consists of many types of alkaloids including vasicinone, vasicine, adhatonine, adhatodine, vasicinol, and vasicinolone [3,14,25], whose reported effects may help account for their use as ingredients in dietary supplements. Vasicinone and vasicine, for example, possess antiphylactic functions to possibly help control immune activation responses [25], as well as bronchodilatory and respiratory stimulant actions to help support respiratory health [18].

Although several publications have detailed the chemical composition of *A. zeylanica*, only a paucity of reports on its safety exists [2,5,7,22] and consequently, safe dosage and frequency of use levels have not been established. Furthermore, the actual amounts of *A. zeylanica* used in dietary supplements are often omitted from the product labels. These shortcomings leave consumers unaware of the potential hazards of taking dietary supplements containing *A. zeylanica* ingredients. To begin investigating the possible toxicity

Abbreviations: ROS, reactive oxygen species; MMP, mitochondrial membrane potential; B2 M, beta-2-microglobulin; KIM-1, kidney injury molecule-1.

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of *A. zeylanica*, we performed a series of *in vitro* cellular tests that specifically address the potential of *A. zeylanica* to adversely affect the kidney. The kidneys are a common target of toxicity due to their role in filtering xenobiotics from the plasma and excreting them as waste products in the urine [16]. The glomerular filtrate flows through the proximal and distal convoluted tubules before being directed to the collecting ducts, ureter and bladder for subsequent elimination from the body [16]. The proximal tubule, in particular, is vulnerable to toxins present in the glomerular filtrate, as it can concentrate solutes to levels higher than those present in the blood. To explore the possibility that *A. zeylanica* can induce proximal tubule nephrotoxicity, we exposed human proximal tubule cells directly to *A. zeylanica* and performed a series of *in vitro* cellular tests to evaluate its potential association with acute toxic effects.

2. Materials and methods

2.1. Chemical characterization of A. zeylanica leaf extract

A crude methanol-extract of *A. zeylanica* leaf was provided in lyophilized form from the University of Mississippi National Center for Natural Products Research (NCNPR, University, MS) and was stored in the dark at 4 °C in a vacuum chamber. Dried extract was dissolved in 5% acetonitrile in water to a final concentration of 0.1 mg/ml. Sample (1 μl for MS, 3 μl for MS/MS) was injected into an Agilent 1260 UPLC and chromatographed on an Agilent Poroshell 120 EC-18 column (3.0 × 50 mm, 2.7-micron particle size). Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The initial solvent was 5% B with a 3 min hold. The solvent mix was then programmed to 30% B at 7 min, then to 95%B at 10 min. Column effluent was analyzed on an Agilent 6520 QTof, high resolution mass spectrometer. Sample was analyzed in positive ESI mode with a capillary voltage of 4000 V. Compounds were identified from exact mass and MS/MS spectra by comparison with published data [9,24].

2.2. Cell culture and treatments

Human kidney HK-2 cells (ATCC, Manassas, VA) were grown in keratinocyte-SFM media supplemented with 5% FBS, 0.005 μg/ml rhEGF, and 0.05 mg/ml bovine pituitary extract (all from Invitrogen, Carlsbad, CA) at 37 °C in a 5% CO_2 humidified atmosphere. HK-2 cells are adult human proximal tubule cells that were transformed using HPV E6/E7 genes [23]. Cells were enumerated by Trypan blue dye (Invitrogen) exclusion and seeded at a density of 2×10^4 cells per 100 μl per well in 96-well plates, whose perimeter wells were filled with 100 ul of sterile water to avoid evaporation effects in the inner wells. Stock treatment solutions of *A. zeylanica*, nephrotoxicant (positive control) cis-diamineplatinum(II) dichloride (cisplatin) (Sigma, St. Louis, MO), and nephroprotectant (negative control) valproic acid (Sigma) were prepared using DMSO and diluting this mixture with media (for a final DMSO concentration of ≤0.4% in media). Following overnight incubation, cells were treated in triplicate for 24 h at the following dosages: 0, 12.3, 37, 111, 333, and 1000 μg/ml.

2.3. Cytotoxicity assay

Determination of treatment-related cytotoxicity was performed in triplicate using the established CellTiter-Glo Cell Viability Assay (Promega, Madison, WI). This assay is based on luminescence emission to quantitate cellular ATP levels, which is directly proportional to cell viability. Following the manufacturer's guidelines, treated cells seeded in black-wall, clear bottom 96-well plates were equilibrated to room temperature for 30 min. During this incubation, water in the perimeter wells were replaced with 100 μl of treatment or media only controls. Next, 100 μl of CellTiter-Glo working solution were added to each well and plates were placed on an orbital shaker for 2 min to induce cell lysis, and then incubated for an additional 10 min before being read on an OMG Fluorostar plate reader (BMG LABTECH, Ortenberg, Germany) to measure the levels of luminescence.

2.4. Reactive oxygen species assay

Reactive oxygen species (ROS) production was measured in triplicate using the ROS-Glo H_2O_2 luminescence-based detection system (Promega) and data were normalized to cell viability. Based on the manufacturer's instructions, treated cells were incubated with H_2O_2 substrate for the remaining 5 h of their 24-h treatment at 37 °C in a 5% CO_2 humidified atmosphere. Following, 'Detection Reagent' was added and samples were incubated at room temperature for at least 20 min before luminescence was read on an OMG Fluorostar plate reader.

2.5. Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) changes were evaluated in triplicate using the ratiometric dye JC-10 (Enzo, Farmingdale, NY). Lyophilized JC-10 was reconstituted using HBSS and diluted further to achieve a working solution of 20 μM. Treated cells were incubated with JC-10 working solution dye for an interval of 2 h before being rinsed twice in HBSS, and finally being overlaid with 100 μl of HBSS. Plates were read using an OMG Fluorostar plate reader to measure emission at 520 and 590 nm following excitation at 485 nm. Natural auto-fluorescence levels from treatments or media alone were insignificant.

2.6. Nephrotoxicity biomarker assays

Cell culture supernatants from HK-2 cells treated for 24 h with *A. zeylanica*, cisplatin, and valproic acid at high and low doses of 333 and 111 μg/ml, respectively, were assayed for levels of nephrotoxicity biomarkers: albumin, beta-2-microglobulin (B2 M), cystatin C, kidney injury-1 (KIM-1) using the Human Kidney Toxicity Panel 1 and 2 kits (Bio-Rad, Hercules, CA). Following the manufacturer's protocol, plates were blocked with 10 μl of blocking reagent for 1 h and washed twice using the Bioplex plate washer (Bio-Rad). Next, 30 μl of sample, standard, and controls were added to their predesignated wells and incubated for 1 h. Plates were washed and 30 μl of detection antibody were added for a final incubation of 30 min. After a final wash, the plate was read using a Luminex 200 instrument (Bio-Rad). Biomarker expression levels were normalized to cell viability.

2.7. Statistics

Data calculations and analyses were performed using Microsoft Excel and Prism (GraphPad, San Diego, CA). Student *t*-tests or 2-way ANOVAs were used to determine whether dose-matched treatment effects using *A. zeylanica*, cisplatin, and valproic acid were statistically significant at *P* values less than 0.01 or 0.001 as indicated.

3. Results

3.1. Analytical chemical characterization of A. zeylanica leaf extract

LC-high resolution MS identified 88 total molecular features of which seven were characteristic quinazoline alkaloids of *A. zeylanica* (Fig. 1). Compounds were putatively identified from exact