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Cytotoxicity of *Senecio* in macrophages is mediated via its induction of oxidative stress

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ABSTRACT

In Arunachal Pradesh and other sub-Himalayan areas of India, accidental consumption of *Senecio* plants by yaks is often fatal as the plant contains toxic alkaloids like Seneciophylline. The present investigation was undertaken to demonstrate the pro-oxidant effects of an ethanolic extract of *Senecio chrysanthemoides* (S-EtOH). S-EtOH impaired viability in macrophages, the IC_{50} being 13.8 ± 1.11 μg/mL. The effect of S-EtOH (1 μg/mL) on generation of reactive oxygen species (ROS) in macrophages was measured by flow cytometry using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) where it caused a significant increase in the mean fluorescence channel (MFC) from 8.55 ± 0.03 to 47.32 ± 2.25 ($p < 0.001$). S-EtOH also effected a 3.8-fold increase in extracellular nitric oxide (NO) generation from 4.90 ± 0.72 μM to 18.79 ± 0.32 μM ($p < 0.001$), a 2.2-fold increase in intracellular NO production, the MFC increasing from 14.95 ± 0.48 to 33.34 ± 1.66 ($p < 0.001$), and concomitantly depleted non protein thiols as analyzed by flow cytometry using mercury orange, with a reduction in MFC from 632.5 ± 49.44 to 407.4 ± 12.61 ($p < 0.01$). Additionally, S-EtOH (14 μg/mL, 24 h) caused apoptosis as evident by increased Annexin V binding and terminal deoxynucleotidyl transferase mediated dUTP DNA nick end labeling. Taken together, the cytotoxicity of S-EtOH can be partly attributed to its capacity to inflict oxidative damage via generation of both reactive oxygen and nitrogen species culminating in apoptosis.

1. Introduction

Pyrrolizidine alkaloids (PA), ubiquitously present in a broad range of plants worldwide, are incriminated as a common cause of poisoning especially in livestock following consumption of these plants or their derived products. Thirteen species of flowering plants contain pyrrolizidine alkaloids and include *Senecio*, *Heliotropium*, *Crotalaria*, *Trichodesma* and *Symphytum* (WHO, 1988). Following substantial ingestion of plants rich in PA, sudden death may ensue. Reports of WHO Environmental Health Criteria have indicated PA alkaloids to be toxic mutagens and carcinogens (Robertson, 1982; WHO, 1988).

In the Himalayan region, PA poisoning in yaks following consumption of PA containing plants was first reported by Winter et al. (1994) and, in Arunachal Pradesh (India), a local plant, *Senecio chrysanthemoides* has been incriminated (Mondal et al., 1999). PAs are principally cytotoxins present in different concentrations in many plants, among which *Senecio* spp. represents the highest risk to livestock and, in turn, to consumers of livestock products.

Oxidative stress has been implicated in serious dysfunctions like Parkinson's disease, Alzheimer's disease, multiple sclerosis, aging and several mutagenic as well as teratogenic disorders. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are highly reactive molecules able to oxidize protein, nucleotide and lipid substrates, thus inducing cellular alterations and death, responsible for the development of many dysfunctions (Uchikura et al., 2004; Kizaki et al., 2006). Nitric oxide (NO), superoxide (O_2^-) and their toxic metabolite peroxynitrite ($ONOO^-$) are known to inhibit the mitochondrial respiratory chain leading to a cellular energy deficiency state (Heales et al., 1999). The development of oxidative stress with the generation of superoxides and lipid-peroxy radicals in PA (*Senecio*) toxicity was recently documented in cattle (Bondan et al., 2005). Accordingly, the present study was undertaken to test whether the plant *S. chrysanthemoides*

Abbreviations: DAF-2DA, 4,5-diaminofluorescein diacetate; H_2-DCFDA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; MFC, mean fluorescence channel; MTS, [{3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl}-2H tetrazolium, inner salt]; PMS, phenazine methosulphate; PA, pyrrolizidine alkaloids; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPMI, Roswell park memorial institute.

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mediates its toxic effects via generation of ROS and RNS, leading to apoptosis.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) except dimethyl sulfoxide (99.50%, Sisco Research Laboratory (SRL), India), ethanol (Merck, India), fluorescein isothiocyanate (FITC) conjugated Annexin V (BD Biosciences, Palo Alto, CA, USA), Cell death detection kit (Roche, Germany, Cat no: 1 684 817) and LAL test (BioWhittaker, USA).

2.2. Animals

Swiss albino mice ($n = 10$) of either sex, weighing 20–25 g were housed under standard conditions of temperature (25 ± 5 °C) with maintenance of a 12 h light/dark cycle, provided with a standard pellet diet and water *ad libitum*. The experiments were carried out under the approval from the Institutional Animal Ethical committee.

2.3. Preparation of the plant extract

The plants were collected from surrounding areas of the Nyukmadung Yak farm of the National Research Centre on Yak, Indian Council for Agricultural Research (NRC-Yak, ICAR) located in the Western Himalayan region of Arunachal Pradesh, India. The plant was identified as *Senecio chrysanthemoides* DC (Mondal et al., 1999). The leaves, stem and roots of the plants were air dried, powdered and extracted with 95% ethanol; the resultant extract was obtained after evaporation of the solvent under reduced pressure at 40 °C; the extract was dissolved in dimethyl sulfoxide (DMSO, 10 mg/mL) and stored at −20 °C before use.

2.4. Isolation of murine peritoneal macrophages

Swiss albino mice were sacrificed under ether anesthesia and peritoneal macrophages were lavaged aseptically using sterile, ice-cold, phosphate buffered saline (0.02 M, pH 7.2; PBS) as previously described (Sarkar et al., 2005). After centrifugation, (300*g* × 10 min, 4 °C), the resultant pellet was resuspended in RPMI-1640 medium (phenol red free) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 μg/mL) and referred to as medium A. Cell viability was >95%, as determined by trypan blue exclusion and cells were maintained at 37 °C, 5% CO_2.

2.5. Effect of Senecio on viability of macrophages

The viability of murine macrophages following treatment with S-EtOH was determined by the MTS assay (Ganguly et al., 2006). MTS, a tetrazolium salt, is reduced to formazan by mitochondrial dehydrogenases in the presence of the electron coupler, PMS. Macrophages (2×10^5/200 μl) were seeded in a 96-well tissue culture plate and wells were individually incubated with increasing concentrations of S-EtOH (0–25 μg/mL) for 24 h at 37 °C, 5% CO_2. After 24 h, MTS (2 mg/mL in PBS) and PMS (0.92 mg/mL in PBS) were mixed in the ratio of 20:1 and 20 μl added to each well and cells incubated for 3 h at 37 °C. Absorbances were measured at 490 nm (OD_{490}) using a microplate reader (Bio Rad Model 680). The percentage of viability was calculated as follows:

$$\frac{\text{Specific absorbance of S-EtOH } (OD_t)}{\text{Specific absorbance of control } (OD_c)} \times 100$$

where OD_t = (total OD_{490} − background OD_{490}) for each S-EtOH concentration and OD_c = (total OD_{490} − background OD_{490}) of untreated cells (background OD_{490} refers to the OD_{490} values of wells containing medium alone with/without different conc. of S-EtOH). Accordingly, the percentage of cell viability was plotted graphically vs. respective S-EtOH concentrations and the concentration of S-EtOH that caused 50% decrease in cell viability was considered as IC_{50}.

2.6. Determination of reactive oxygen species (ROS) in macrophages

Detection of ROS was measured using H_2DCFDA as previously described (Kwak et al., 2006). The assay is based on the principle that the freely cell-permeant non-fluorescent dye, H_2-DCFDA, is hydrolyzed by intracellular esterases to H_2-DCF (2′,7′-dichlorofluorescein), which is subsequently oxidized by intracellular ROS into a highly fluorescent product DCF (Kwak et al., 2006). Accordingly, murine peritoneal macrophages (1×10^6) were incubated in 6 well plates with wells receiving varying doses of S-EtOH (0–10 μg/mL) for 6 h at 37 °C, 5% CO_2. The cells were then washed with PBS, resuspended in medium A containing H_2DCFDA (20 μM) for 30 min. at 37 °C, 5% CO_2 and the reaction was terminated by placing the cells on ice for 10 min. Fluorescence intensity was measured on a FACS Calibur (Becton Dickinson, USA) and the mean fluorescence channel (MFC) value of 10,000 cells was determined upon analysis of the gated macrophage population which was defined by forward and side scatter using BD CellQuest Pro software (BD Biosciences, USA).

2.7. Measurement of extracellular nitric oxide (NO) production in macrophages

The production of NO was indirectly measured by estimation of nitrite using the Griess assay as previously described (Sarkar et al., 2005). Briefly, macrophages (1×10^6 cells/mL/well) were allowed to adhere on 6-well tissue culture plates and wells were incubated with different concentrations of S-EtOH (0–10 μg/mL) for 24 h. The supernatants (100 μl) were collected and incubated with an equal volume of Griess reagent (1:1 sulphanilamide [1% in 5% phosphoric acid] and naphthyl ethylene diamine [0.1% in double distilled water, NED]) at room temperature for 10 min and absorbances were measured at 550 nm using a microplate reader. The amount of NO production was extrapolated from corresponding absorbances plotted on the standard curve using sodium nitrite (0–100 μM).

The experiment was repeated in the presence of a LPS inhibitor, polymixin B (100 μg/mL), to eliminate the possibility of false positivity due to bacterial derived LPS contamination of the extract. The absence of endotoxin in S-EtOH incubated well plates was confirmed by limulus amoebocyte lysate (LAL) following the manufacturer's instructions.

2.8. Determination of intracellular nitric oxide generation in macrophages

The NO generated within the cells was measured using DAF-2 DA, a NO specific probe that freely enters the cells where it is cleaved by intracellular esterases to produce 4,5-diaminofluorescein or DAF-2 which is converted to the impermeable, highly fluorescent triazolofluorescein (DAF-2T) via NO dependent pathways (Rathel et al., 2003). Murine peritoneal macrophages (1×10^6/mL), were allowed to adhere to 6-well plates and incubated with different concentrations of S-EtOH (0–10 μg/mL) for 24 h at 37 °C, 5% CO_2 (Rathel et al., 2003). The cells were then washed with PBS, resuspended in medium A (1 mL) containing DAF-2 DA (10 μM) for 30 min at 37 °C, 5% CO_2 and the reaction was terminated by placing the cells on ice for 10 min. Cells were acquired on a FACS