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Characterization of the aqueous extract of the root of *Aristolochia indica*: Evaluation of its traditional use as an antidote for snake bites

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ABSTRACT

Ethnopharmacological relevance: The aqueous extract of the roots of *Aristolochia indica* is used as a decoction for the ailment of a number of diseases including snake bite treatment. Though the alcoholic extract of the different parts of the plant are well studied, information on the aqueous extract is limited. We have estimated aristolochic acid, different enzymes, enzyme inhibitors and anti-snake venom potency of its root extract.

Materials and methods: Reverse phase–HPLC was used to quantify aristolochic acid. Zymography, DQ-gelatin assay and atomic force microscopy were done to demonstrate gelatinase and collagenase activities of the extract. SDS-PAGE followed by MS/MS analysis revealed the identity of major protein components. Toxicity of the extract was estimated on animal model. Interaction of the extract with Russell's viper venom components was followed by Rayleigh scattering and enzyme assay.

Results: The aristolochic acid content of the root extract is $3.08 \pm 1.88 \times 10^{-3}$ mg/ml. The extract possesses strong gelatinolytic, collagenase, peroxidase and nuclease activities together with L-amino acid oxidase and protease inhibitory potencies. Partial proteomic studies indicated presence of starch branching enzymes as major protein constituent of the extract. The extract did not show any acute and sub-chronic toxicity in animals at lower doses, but high dose causes liver and kidney damage. The extract elongated duration of survival of animals after application of Russell's viper venom.

Conclusions: Considering the low aristolochic acid content of the extract, its consumption for a short time at moderate dose does not appear to cause serious toxicity. Strong inhibition of L-amino acid oxidase may give partial relief from snake bite after topical application of the extract.

1. Introduction

Aristolochia indica (Indian Birthwort) is widely grown in the alluvial, arid and semi-arid regions of the Indian subcontinent. Application of different parts of the plant like leaf, stem, bark, seed, rhizome and the plant as a whole have been described in regional language (Chatterjee and Pakrashi, 1994) as well as in the national compendium (Wealth of India, 2003). It is used for curing ailments like skin diseases, rheumatism, wound, diarrhea, inflammations, leprosy, leucoderma, dyspepsia, intestinal worms, cough, dysmenorrhea, etc. As a decoction, the root finds maximum application to heal ulcers, asthma, bronchitis and also against reptile and insect bites. An updated compilation of references of all *Aristolochia* species along with their usage is available (Heinrich et al., 2009).

Alkaloids, essential oils, bitter principles (aristolochin), fixed oil, tannic acid, resin, gum and sugar are the major constituents of the root (The Ayurvedic Pharmacopoeia of India, 1999–2011). Aristolochic acids I and II (derivatives of nitrophenanthrene carboxylic acids) are found in virtually all plants of the *Aristolochia* genus (Nolin and Himmelfarb, 2010). *Aristolochia indica*, however, contains only aristolochic acid I (Che et al., 1984). During metabolism, aristolochic acids are activated by cytosolic enzymes to aristolactams (AL) (Stiborova et al., 2003). These products react with DNA to form covalent adducts dA-AL and dG-AL (Arlt et al., 2002). The dA-AL adduct is a potent carcinogen and nephrotoxin that persists for an extended period and classified

Abbreviations: AFM, atomic force microscopy; ALP, alkaline phosphatase; APTES, 3-aminopropyltriethoxysilane; ATA, aurintricarboxylic acid; BSA, bovine serum albumin; CHAPS, 3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid; CHCA, α-cyano-4-hydroxycinnamic acid; CT-DNA, calf thymus DNA; dA-AL, deoxyadenosine-aristolactam; dG-AL, deoxyguanosine-aristolactam; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); DTPC, diheptanoyl-thio-phosphatidylcholine; H_2O_2, hydrogen peroxide; HRP, horse radish peroxidase; i.p., intra peritoneal; i.v., intra veneous; LAAO, L-amino acid oxidase; L-Phe, L-phenyl alanine; MLD, minimum lethal dose; NEM, N-ethylmaleimide; ODA, *o*-dianisidine; PBS, 0.9% phosphate buffer saline, pH 7.5; PHMB, *p*-hydroxymercuribenzoate; PLA_2, phospholipase A_2; PMSF, phenylmethylsulphonyl fluoride; RP-HPLC, reverse-phase high performance liquid chromatography; RVV, Russell's viper venom; SGOT or AST, aspartate aminotransferase; SGPT or ALT, alanine aminotransferase; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin layer chromatography

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as Group 1 carcinogens (Bieler et al., 1997; Vanherweghem, 1998; IARC , 2012).

In spite of this knowledge, the usage of the aqueous extract of the root of *Aristolochia indica* is continuing in different parts of South–East Asian countries like Bangladesh, India, Myanmar, Nepal, Philippines, Sri Lanka, Thailand, Tibet etc. Its use as an antidote against snakebites, especially Russell's viper bites is common (Heinrich et al., 2009). Snake envenomation is a WHO identified occupational hazard for the paddy growers of the tropical and subtropical countries (Warrell, 1995). Worldwide, India is the country most affected by venomous snakes with 35–50,000 fatalities/year (Alirol et al., 2010). For the registered medical practitioners, application of the 'antivenom' is the only mode of treatment against snake bites. However, adequate 'antivenom' is neither available nor could be properly preserved in the remote places and people depend on traditional medicines. Survey reports and *in vitro* experiments confirmed the presence of antivenom property of *Aristolochia indica* (Selvanayagam et al., 1995; Samy et al., 2008; Dey and De, 2012). However, biochemical characterization of the aqueous extract remains pending. In parallel, doubt exists about the efficacy of similar plant products against snake bites as expressed by the noted toxinologist Mebs (2000).

Our laboratory is involved in the characterization of toxins from Russell's viper (*Daboia russelli russelli*) venom that include hemorrhagins (Chakrabarty et al., 1993, 2000; Kole et al., 2000), LAAO (Mandal and Bhattacharyya, 2008), PLA_2 (Maiti et al., 2007), nucleases (unpublished results) etc. A methanolic extract of the plant can neutralize *Daboia russelli* venom induced hemorrhagic, edema and fibrinolytic activities (Meenatchisundaram et al., 2009). There is a wealth of information on the composition of alcoholic extract of the root (Kupchan and Doskotsch, 1962; Pakrashi et al., 1976; Pakrashi and Pakrashi, 1977; Pakrashi and Chakrabarty, 1978a; Pakrashi and Shaha, 1978b; Che et al., 1984; Shafi et al., 2002). Except the antibacterial property, information on the aqueous extract is limiting (Ravikumar et al., 2005; Sini and Malathy, 2005). Our aim is to evaluate the potential of the root extract as an ethnomedicine in terms of its safety, toxicity and biochemical characters.

2. Materials and methods

2.1. Materials

Aristolochic acid, azoalbumin, BSA, collagen Type III (acid soluble), CT-DNA, gelatin (from bovine skin), H_2O_2, HRP, 5'-nucleotide monophosphates, 5'-nucleotide diphosphates, 5'-nucleotide triphosphates and dialysis tubing of cut off range <12 kDa were from Sigma-Aldrich (St. Louis, USA). Purity of aristolochic acid was over 98% as observed from RP-HPLC and mass analysis. Other fine chemicals were procured as follows: agarose powder, SRL (India); EnzChek Gelatinase/Collagenase Assay kit, Molecular Probes, Invitrogen, (USA); pre-stained protein Mw marker for SDS-PAGE (PageRuler), Fermentas (USA) and trypsin gold, Promega (Madison, USA). RVV was procured from Mr. Dipak Mitra, a licensed trophy of Calcutta Snake Park, Kolkata, India as desiccated crystals that contain ~60% of protein (w/w). The venom was suspended in 20 mM Tris–HCl, pH 7.5 at 25 °C for 30 min and the insoluble materials were removed by centrifugation (Chakraborty et al., 2000).

2.2. Plant material

Fresh *Aristolochia indica* roots were procured from local vendors. One flowering plant was identified by The Botanical Survey of India, Shibpur, Howrah. The herbarium sheet (No. 18/10) has been deposited at the institute repository. Before extraction, roots were weighed and kept at 4 °C overnight in 20 mM Tris–HCl, pH 7.5 (1:1, w/v). Wet roots were cut, macerated in mortar-pestle, centrifuged and the supernatant served as the working solution. The extract was dialyzed against the same buffer as required. The protein concentration was determined with Bio-Rad Protein Assay Reagent (Catalog no. 10044, Bio-Rad Laboratories) using BSA as reference.

2.3. Enzyme activities

2.3.1. Protease activity

Zymography was performed after incorporating 1.5 mg/ml of gelatin into 10% polyacrylamide containing 0.1% SDS at pH 8.8 (Ratnikov et al., 2000). For 2-D gelatin zymography, the electrophoresis was carried out as mentioned in the Supplementary materials section except that no detergent, denaturant, reducing and alkylating agents were used. For collagen zymography, the substrate (1.5 mg/ml) replaced gelatin.

For quantification, EnzChek® Gelatinase/Collagenase assay kit was used. Enzymatic hydrolysis of DQ-gelatin was followed fluorimetrically (ex: 495 nm; em: 515 nm) as per manufacturer's guideline. Collagenase (Class II, *Clostridium histolyticum*, 436 units/mg, $\varepsilon_{280\ nm}=1.576\ mM^{-1}\ cm^{-1}$) served as control. A linear dependency correlating the amount of collagenase (1–20 ng) and the reaction rates was observed ($R^2=0.9857$). Proteolytic activity was also measured using 1% azoalbumin or azocasein in presence of 20 Tris–HCl, pH 7.5 for 1 h at 37 °C. The generation of TCA-soluble peptides was estimated under alkaline conditions at 440 nm (Beynon and Bond, 1989).

2.3.2. Nucleotidase/nuclease activity

It was estimated after incubation of varying amount of the extract with 10 mM of mono-, di- or tri-phosphates of 5'- nucleotide (adenosine, guanosine, cytosine, uridine) in 50 mM Tris–HCl, pH 7.5 containing 10 mM $MgCl_2$, 50 mM NaCl and 10 mM KCl in 1 ml at 37 °C. Generation of phosphomolybdate complex was estimated at 660 nm (Avruch and Wallach, 1971). The DNase activity was detected by incubating 40 ng of CT-DNA or pTRC99A vector DNA with varying amount of the extract for 30 min at 25 °C. The products were analyzed using 0.8% agarose gel electrophoresis.

2.3.3. Peroxidase activity

Conversion of H_2O_2 by peroxidase was followed optically for 2 min in presence of ODA ($\varepsilon_{436\ nm}=8.3\ mM^{-1}\ cm^{-1}$) (Mandal and Bhattacharyya, 2008). In all set of enzymes, one U of enzyme was defined as the amount that converts 1 μmole of the substrate/min at 25 °C.

2.3.4. LAAO activity

The conversion of L-Phe by LAAO to the corresponding keto-acid and H_2O_2 was followed spectrophotometrically at 436 nm and at 25 °C by the coupling enzyme HRP and the chromophoric group ODA (Mandal and Bhattacharyya, 2008).

2.3.5. Hyaluronidase activity

The extract containing 25–1000 μg of protein was incubated with 50 μg of hyaluronic acid in 1 ml of 0.2 M Na-acetate, pH 5.0 containing 0.15 M NaCl for 25 min at 37 °C. After 30 min, absorbance at 400 nm was read against a blank devoid of the substrate. Turbidity-reducing activity was expressed as a percentage of the remaining hyaluronic acid where absorbance of the solution devoid of enzyme was considered as 100% (Yingprasertchai et al., 2003).

2.3.6. PLA_2 activity

PLA_2 activity was measured using Cayman's secretory PLA_2 assay kit (Cayman Chemical Company, USA). Free thiols originated from the hydrolysis of DTPC by PLA_2 were detected using DTNB at 405 nm for 10 min. PLA_2 from bee venom served as