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Metabolic fingerprinting of dioecious *Tinospora cordifolia* (Thunb) Miers stem using DART TOF MS and differential pharmacological efficacy of its male and female plants

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ABSTRACT

Variations due to geographical location and dioecious nature have shown implications in the chemical and pharmacological properties of medicinal plants and their herbal products. *Tinospora cordifolia* is one of the most important dioecious plant distributed throughout India and very widely used in many herbal products and formulations. In this study a method combining direct analysis in real time (DART) ion source coupled to high-resolution time-of-flight (TOF) mass spectrometer (MS) along with multivariate analysis was developed and applied for metabolic fingerprinting and screening of the major phytochemicals in this plant. Using this approach phytodiversity in plants due to gender and geographical distribution were studied in *T. cordifolia* stem cuttings without any processing. An aqueous/ethanolic stem extracts of male and female *T. cordifolia* were also evaluated for immunomodulatory activity in inbred strain of age and sex matched BALB/c mice. A characteristic nine and sixteen marker peaks were respectively, identified as gender and geographical markers for *T. cordifolia* stem. It also discriminates the herbal and polyherbal formulations of *T. cordifolia* stem using principal component analysis. Female plant stem extract caused a significant up regulation in the pro-inflammatory and anti-inflammatory cytokines and activated the peritoneal exudate cells leading to significant release in reactive oxygen species and enhanced the in vitro lymphocyte proliferation than male stem extract. This finding underscore the importance of gender in all dioecious medicinal plants where only vegetative parts are used as a source of drug as the pharmacological activity may vary depending on the sex of the plant used.

1. Introduction

Tinospora cordifolia (Thunb.; Miers family Menispermaceae) is one of the important medicinal plant distributed throughout India, China, Burma and Sri Lanka (Singh et al., 1984). *T. cordifolia* is one of the highest commercially exploited plants in Indian system of medicine and its annual requirement is about ten thousand tons in pharmaceutical industry (Singh and Warrier, 2004). It is a main constituent of many Ayurvedic formulations, which were used for several centuries in the Indian system of medicine for the treatment of various diseases (Singh et al., 2006). Stem of *T. cordifolia* is considered as an indigenous source of medicine with antipyretic (Vedavathy and Rao, 1991), antidiabetic (Sengupta et al., 2009), hepatoprotective (Peer and Sharma, 1989), immunomodulatory (Atal et al., 1986; Maurya et al., 1996; Ghosal and Vishwakarma, 1997), anticancer (Jagetia and Rao, 2006) and antiinflammatory (Pendse et al., 1977) activities. Main phytochemicals reported from *T. cordifolia* stem are alkaloids, flavonoids, glycosides, lactones, lignans, phenolics, polysaccharides, terpenoids, and steroids (Sarma and Khosa, 1998; Singh et al., 2003).

Many factors, such as gender (Bajpai et al., 2012), geographical location (Rahimmalek et al., 2013), season (Matias et al., 2016), nutritional status, drying methodology (Teles et al., 2012; Misra

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et al., 2016) were reported to impact the secondary metabolite profile (Iacumin et al., 2009). It is one of the well-known medicinal plant with dioecy as it bears male and female flowers on different plants (Geetha et al., 2007). Recent reports have described gender-based differences in the metabolite levels in *T. cordifolia* stem (Bajpai et al., 2016; Choudhary et al., 2014).

The metabolic/chemical fingerprint is a signature of plants for the quality assessment of herbal medicines (Liu et al., 2008) which has been approved by world health organization (WHO) as a method for the assessment (Li et al., 2015). Metabolic fingerprints using direct analysis in real time – time of flight mass spectrometry (DART TOF MS) can give an overall view of the phytoconstituents in plant parts and herbal formulations to establish the extent of similarity and differences between the samples (Lesiak et al., 2015). DART is an ambient ionization mass spectrometry technique which is capable of generating ions from wide variety of analytes directly from solid surface like intact root, stem and leaf without any sample preparation (Bajpai et al., 2015). It has also advantage over other MS techniques as it is fast and does not require a chromatographic step like in GC/MS and LC/MS (Pavlovich et al., 2016). DART TOF MS spectra are very simple with minimum or no fragmentations (Jones and Fernandez, 2013; Bajpai et al., 2010).

Even though *T. cordifolia* is one of the extensively analyzed plant, however, there is lack of information on the chemical fingerprints with respect to gender and locations. This gap has implications in selection of plant material best possible pharmacological efficacy and also quality assessment of its herbal formulations. This underscores the importance of metabolic fingerprints and the pharmacological efficacy according to gender and locations in India for quality control of *T. cordifolia*.

Thus in the present study a simple DART TOF MS methodology for *T. cordifolia* stem was developed to generate the metabolic fingerprint for identification of phytochemicals/chemical markers for discrimination of the gender and geographical locations and combined with statistical analysis to infer the significance of the varaiations. Furthermore, the pharmacological efficacy of male and female plant was evaluated for its biological potential as potent immunomodulator.

2. Experimental

2.1. Collection and authentication of plant materials and herbal formulations

Male and female plants of *T. cordifolia* bearing male and female flowers were collected from three sites of naturally growing population from the banks of river Gomati, Lucknow during 2012, 2013 and 2014. Five male and female plant samples (stem) were collected each time. The voucher specimens for the male and female plants (KRA 23992 and KRA 23994, respectively) are deposited in the departmental herbarium of Botany division of CSIR-CDRI, Lucknow, India. The geographical samples were collected from the Madhya Pradesh (M.P.), Uttar Pradesh (U.P.) and West Bengal (W.B.) during 2009, 2010, 2011 and 2012. Voucher specimens were deposited in the herbarium of Central Drug Research Institute (CSIR-CDRI), Lucknow (Supplementary Table S1). Selected herbal formulations containing *T. cordifolia* stem powder/extract were purchased from the local Ayurvedic pharmacy stores at Lucknow India (Supplementary Table S2). Botanical reference standard (BRS) of *T. cordifolia* stem was purchased from Tulsi Amrit Pvt Limited Indore India (Batch No. 10TC 1438).

2.2. Reagents and chemicals

Ethanol used for phytochemical extraction was AR grade from Merck, Darmstadt, Germany. LC/MS grade methanol from the Sigma Aldrich (St. Louis, MO, USA). Water used was purified by a Milli-Q system (Millipore Bedford, MA, USA). Medium RPMI-1640 containing Phenol Red Sigma Aldrich (St. Louis, MO, USA) was used in the experiments. For in vitro cell culture, the medium was fortified with 1% antibiotic-antimycotic cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 10% Foetal bovine serum (GIBCO, USA). 2′, 7′-dichlorofluorescin diacetate (DCF-DA) and levamisole (>99% GC) were acquired from Sigma-Aldrich (St. Louis, MO, USA). All the antibodies were purchased from Becton and Dickinson (BD, San Diego, CA, USA).

2.3. Extraction and sample preparation for bioactivity evaluation

Powdered samples (500 g) of *T. cordifolia* stems were suspended in 1 L of aqueous/ethanolic (10/90 v/v) solution and the suspensions were placed in an ultrasonic bath for 30 min and left for 24 h at room temperature. Each plant material was extracted three times by aqueous/ethanol (10/90 v/v) mixture. These extracts were filtered through a 0.22-μm PVDF membrane MILLEX GV filter and concentrated under vacuum using Buchi rotavapor in reduced pressure at 20–50 kPa at 40 °C and the final yield of the dried extract was ca. 5.25%.

2.4. Preparation of samples for DART TOF MS analysis

DART TOF MS measurements were recorded by using intact *T. cordifolia* stem as samples without any sample preparation or processing. The stems were thoroughly washed with Milli-Q water in order to remove foreign particles from its surface, dried for 30 min in oven at 35 °C, and chopped in small pieces for the analysis. The crushed Tablets in the form of powder were used for each herbal formulation for the analysis.

2.5. DART TOF MS: instrumentation and analytical conditions

The mass spectrometer used was a JMS-100 TLC (AccuTof) atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART ion source. The mass spectrometer was operated in both positive ion and negative ion mode with a resolving power of 6000 (full-width at half-maximum). The orifice 1 potential was set to 28 V, resulting in minimal fragmentation. The ring lens and orifice 2 potentials were set to 13 and 5 V, respectively. Orifice 1 was set at 100 °C. The RF ion guide potential was 300 V. The DART ion source was operated with helium gas flowing at approximately 4.2 L/min. The gas heater was set to 300 °C. The potential on the discharge needle electrode of the DART source was set to 3000 V, electrode 1 was at 100 V and the grid was at 250 V. Data acquisition ranged from m/z 50–1000. All samples were analyzed in 15 repeats to check the reproducibility of spectra from DART TOF MS analysis. Mass calibration was accomplished by including a mass spectrum of neat polyethylene (PEG) glycol (1:1 mixture PEG 200 and PEG 600) in the data file. The mass calibration was accurate to within ±0.002 u. Using the Mass Centre software, the elemental composition could be determined on selected peaks.

2.6. Animals and their treatment with T. cordifolia stem extracts

Inbred strain of age and sex matched BALB/c mice (18–20 g) were used in the experiments. The Animals were housed under standard conditions of temperature (23 ± 1 °C), relative humidity (55 ± 10%), 12 h/12 h light/dark cycles at National Laboratory