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Validation of ethnomedicinal potential of *Tinospora cordifolia* for anticancer and immunomodulatory activities and quantification of bioactive molecules by HPTLC ☆

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ABSTRACT

Ethnopharmacological relevance: *Tinospora cordifolia* (Willd.) Miers ex Hook. f. & Thomas. (Menispermaceae) is one of the most widely used plants in various traditional medicinal systems including "Ayurveda". The plant is used for the treatment of jaundice, rheumatism, urinary disorder, skin diseases, diabetes and anemia. The phytoconstituents present in the plant belongs to different class of compounds such as alkaloids, diterpenoids lactones, glycosides, steroids, phenol, aliphatic compounds and polysaccharides.

Aim of the study: The aim of present study was the isolation, structure elucidation, quantification and pharmacological evaluation of secondary metabolites from *T. cordifolia* for anticancer and immunomodulatory activities.

Materials and methods: Different extracts and fractions were prepared from the stem of *T. cordifolia*. Pure molecules were isolated using normal phase chromatography and characterized on the basis of NMR and mass spectroscopic techniques. The anti-cancer and immunomodulatory activities of different extracts, fractions and isolated compounds were evaluated against four different human cancer cell lines, KB (human oral squamous carcinoma), CHOK-1 (hamster ovary), HT-29 (human colon cancer) and SiHa (human cervical cancer) and murine primary cells respectively. A simple, normal phase HPTLC method was also developed for the quantification of three bioactive compounds *i.e* *N*-formylannonain (1), 11-hydroxymustakone (5) and yangambin (8) in the stem of *T. cordifolia* hosted on fifteen different plants.

Results: Chromatographic purification of different fractions led to the isolation of eight pure molecules *i.e* *N*-formylannonain **(1)**, magnoflorine **(2)**, jatrorrhizine **(3)** palmatine **(4)**, 11-hydroxymustakone **(5)**, cordifolioside A **(6)**, tinocordiside **(7)** and yangambin **(8)**. All extracts and fractions were active against KB and CHOK-1 cells whereas among the pure molecules palmatine **(4)** was found to be active against KB and HT-29; tinocordiside **(7)** against KB and CHOK-1; yangambin **(8)** against KB cells however *N*-formylannonain **(1)** and 11-hydroxymustakone **(5)**, was found active for immunomodulatory activity. HPTLC quantification of three active molecules *i.e* *N*-formylannonain **(1)**, 11-hydroxymustakone **(5)**, and yangambin **(8)** were found in highest quantity in the stem of *T. cordifolia* hosted on *Mangifera indica*, however, other two active molecules were not quantified due to their insufficient quantity.

Conclusion: Eight compounds have been isolated and characterized belonging to different classes. The pharmacological evaluation of extract, fractions and pure molecules revealed the ethnomedicinal value of *T. cordifolia* for anticancer and immunomodulatory activities.

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1. Introduction

Tinospora cordifolia (Amrita or Guduchi) is ethnomedicinally important and widely used plant in Ayurvedic system of medicine as general tonic, anticancer, antiulcer, antipyretic, immunomodulatory, hypoglycemic, anti-inflammatory,

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antiarithiritic, analgesic, diuretic and hepatoprotective potential (Srinivasan et al., 2008; Bhupindu et al., 1981; Rege et al., 1984). It has also been reported to be effective against throat cancer in humans (Chauhan, 1995). An alcoholic extract of *T. cordifolia* enhanced the differentiation of TAM (Tyro3/Axl/Mer receptors) to dendritic cells, in response to GMCSF, IL-4 and TNF-α or β (Singh et al., 2005). Evidences from animal and human studies highlight the potential anti-stress properties of *T. cordifolia* (Singh and Warrier, 2004; Patil et al., 1997). Different useful parts such as stem, root, leaves, fruits and seeds of *T. cordifolia* are having economic utility in herbal formulations; however, maximum activities are attributed to its stem. Ancient Hindu physicians prescribed the plant for gonorrhea and have also been officially recognized in Indian Pharmacopoeia. Traditionally, it is used by different tribes including "Garo" from Bangladesh (Mia et al., 2009); "Gond", "Tagin", "Korkus", and "Baiga" from different areas in India (Tambekar et al., 2009; Goswami et al., 2009; Sinha et al., 2004). Its estimated annual consumption in herbal drug industries is over 10,000 tones (Singh and Warrier, 2004). The chemical investigations of the plant have revealed the presence of different class of compounds such as alkaloids, diterpenoids lactones, glycosides, steroids, phenol, aliphatic compounds and polysaccharides. Although, the active components responsible for therapeutic effects of *T. cordifolia* are not well defined, however it may be possible that the synergistic effects of multiple constituents exhibited its high pharmacological values.

For the quality control of the pharmaceutical and herbal formulations prepared from *T. cordifolia* a rapid and validated analytical method is required. Few reports are available for the quantification of some constituents in *T. cordifolia* using reverse phase high performance liquid chromatography (RP-HPLC) (Srinivasan et al., 2008; Alam et al., 2009a; Ahmed et al., 2006), liquid chromatography (RP-LC-DAD) with mass spectrometry (MS) (Patil et al., 2010), LC-ELSD method for simultaneous determination of ten sugars (Sharma et al., 2010a) and densitometric high performance thin layer chromatography (HPTLC) method (Puratchimani and Jha, 2007; Alam et al., 2009b). In the last two decades, HPTLC is often used as an alternative to HPLC for the quantification of herbal products because of its simplicity, accuracy, and cost-effectiveness, very less amounts of sample requirement, limited solvent waste and rapidity. Due to numerous advantages, HPTLC has gained widespread interest as a favorable technique for the determination of pharmacologically interesting compounds in biological matrices, such as plants and herbal formulations. However some HPTLC methods are reported for quantification of constituent in *T. cordifolia* but have some limitations as these methods quantify only single compound.

In continuation of our studies on isolation and biological evaluation of *T. cordifolia* (Sharma et al., 2010a, 2010b, 2012a, 2012b; Bala et al., 2015), the present study described the isolation of eight pure molecules from different fractions and their characterization on the basis of NMR and mass spectroscopy. The aqueous/alcoholic extract, different fractions as well as pure molecules have been evaluated for anticancer activity against four different cancer cell lines *viz.* KB, SiHa, HT-29 and CHOK1 cells representing different cancer tissue types. However, immunomodulatory potential was tested on primary murine splenocytes which comprises T and B cell populations. Further the bioactive compounds were quantified using simple TLC densitometric method in the stem of *T. cordifolia* climbing on fifteen different plants.

2. Experimental

2.1. General

Silica gel (60–120 and 230–400 mesh), TLC silica gel 60 F_{254} plates and all other chemicals used were purchased from Merck India Ltd., Mumbai. The TLC solvents used were of analytical grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). Mass spectra were recorded on QTOF-Micro of Waters Micromass. NMR experiments were performed on Bruker Avance-300 and 600 spectrometers. HPTLC spotting device was Camag Linomat V Automatic Sample Spotter, Camag (Muttenz, Switzerland) having 100 µl syringe (Hamilton); CAMAG glass twin trough chamber (20×20 cm^2); Camag TLC Scanner 3 linked to winCATS software. Microplate reader of BioTeK Synergy H1 Hybrid Reader was used to measure the absorbance in biological assays. KB, SiHa, HT-29, CHOK-1, murine macrophages (RAW264.7 & J774A) cells were obtained from Guru Nanak Dev University, Amritsar, National Center for Cell Science (NCCS), Pune and Institute of Genomics and Integrative Biology (IGIB), New Delhi, respectively. KB, SiHa and HT-29 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Biosciences, India). CHOK-1 cells were grown in Ham's Nutrient Mixtures F-12 medium (Invitrogen Biosciences, India).

2.2. Plant material

The plant material was collected in May 2008 (PLP-12994) from *Mangifera indica* for isolation of compounds and *T. cordifolia* hosted on different trees, was collected in May 2013, from Kangra District of Himachal Pradesh. The specimen vouchers (*Lannea coromandelica*, PLP 16539; *Ziziphus jujuba*, PLP 16550; *Albizia lebbeck*, PLP 16555; *Celtis australis*, PLP 16544; *Mallotus philippensis*, PLP 16542; *Aegle marmelos*, PLP 16554; *Melia azedarach*, PLP 16549; *M. indica*, PLP 16540; *Cassia fistula*, PLP 165; *Grewia optiva*, PLP 16545; *Holarrhena pubescens*, PLP 16552; *Albizia chinensis*, PLP 16547; *Carissa opaca*, PLP 16551; *Tamarindus indica*, PLP 165; *Ficus religiosa*, PLP 16541; *Litsea monopetala*, PLP 16548) were deposited at IHBT herbarium. All the samples were dried at room temperature under shadow, grounded and stored also at room temperature till further used.

2.3. Extraction and isolation of compounds

The air dried and powdered stem of *T. cordifolia* (2.0 kg) were extracted three times with 80% ethanol in water at room temperature. The combined percolations were concentrated under reduced pressure below 45 °C to obtain dried brown extract (148.0 g). The dried brown extract was dissolved in H_2O and fractionated, sequentially, with *n*-hexane, EtOAc and *n*-BuOH. Each fraction was dried to yield *n*-hexane (3.2 g), EtOAc (22.6 g), *n*-BuOH (34.2 g) and H_2O (77.8 g) fractions. The EtOAc (22.6 g) fraction was subjected to column chromatography over silica gel (230–400 mesh) using a gradient elution with mixture of *n*-hexane and $CHCl_3$ yielded three pure compounds namely, *N*-formylannonain **(1)**, 11-hydroxymustakone **(5)** and yangambin **(8)**. Repeated column chromatography of *n*-BuOH fraction (34.2 g) over silica gel (230–400 mesh) with gradient elution of $CHCl_3$:CH_3OH:H_2O lead to the isolation of five compounds including magnoflorine **(2)**, jatrorrhizine **(3)**, palmatine **(4)**, cordifolioside A **(6)** and tinocordiside **(7)**.

2.4. Assessment of cytotoxicity on cancer cell lines

Cytotoxic activity of parent extract (**TCEWF-01**), four fractions *n*-hexane (**TCHF-02**), ethyl acetate (**TCEF-03**), *n*-butanol (**TCBF-**

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