Cytotoxicity of the Roots of Trillium govanianum Against Breast (MCF7), Liver (HepG2), Lung (A549) and Urinary Bladder (EJ138) Carcinoma Cells

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Abstract

Trillium govanianum Wall. (Melanthiaceae alt. Trilliaceae), commonly known as 'nag chhatri' or 'teen patra', is a native species of the Himalayas. It is used in various traditional medicines containing both steroids and sex hormones. In folk medicine, the rhizomes of T.govanianum are used to treat boils, dysentery, inflammation, menstrual and sexual disorders, as an antiseptic and in wound healing. With the only exception of the recent report on the isolation of a new steroidal saponin, govanoside A, together with three known steroidal compounds with antifungal property from this plant, there has been no systematic pharmacological and phytochemical work performed on T. govanianum. This paper reports, for the first time, on the cytotoxicity of the methanol extract of the roots of T. govanianum and its solid-phase extraction (SPE) fractions against four human carcinoma cell lines: breast (MCF7), liver (HEPG2), lung (A549) and urinary bladder (EJ138), using the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazoliumbromide cytotoxicity assay and liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry analysis of the SPE fractions. The methanol extract and all SPE fractions exhibited considerable levels of cytotoxicity against all cell lines, with the IC_{50} values ranging between 5 and 16μg/mL. Like other Trillium species, presence of saponins and sapogenins in the SPE fractions was evident in the liquid chromatography mass spectrometry data.

Introduction

Trillium govanianum Wall. (Melanthiaceae alt. Trilliaceae), commonly known as 'nag chhatri' in India and 'teen patra' or 'matar zela' in Pakistan, is native to the Himalayas and also distributed from Pakistan to Bhutan between the altitudinal ranges of 2500–3800m above the sea level (Uniyal and Datta, 2012; Shafiq- ur-Rahman et al., 2015). Since the report revealing the occurrence of the steroid, trillarin (Fig. 1), in this species (Chauhan, 1999), it has been extensively used in various traditional medicinal preparations that contain steroids and sex hormones, and now, it is one of the best-selling herbal products in the Indo-Pak subcontinent. However, only a few years ago, this plant was not even listed in the 960 traded medicinal plant species of India (Ved and Goraya, 2007). The rhizomes of this plant have traditionally been used in folk medicine for the treatment of boils, dysentery, inflammation, menstrual and sexual disorders, as well as an antiseptic and in wound healing (Pant and Samant, 2010; Shafiq-ur-Rahman et al., 2015). Previous studies on some other species of the genus Trillium have established that this genus is rich in steroidal saponins (Fig. 1), for example, steroidal saponins were found in Trillium erectum L. (Yokosuka and Mimaki, 2008; Hayes et al., 2009), Trillium kamtschaticum Pall. (Ono et al., 1986, 2003, 2007; Yokosuka and Mimaki, 2008; Wei et al., 2012) and Trillium tschonoskii Maxim. (Nakano et al., 1983; Man et al., 2010; Wei et al., 2012; Wang et al., 2013). The dried roots of a few Trillium species that have been used traditionally for immunoregulation, as an antiinflammatory and anti-ageing agent, were found to possess antitumour properties (Luo et al., 2006; Wang et al., 2013). However, with the only exception of the recent report on the isolation and identification of a new steroidal saponin, govanoside A, together with three known steroidal compounds, barassoside E, diosgenin and pennogenin (Fig. 1), from this plant (Shafiq-ur-Rahman et al., 2015) and showing their moderate level of antifungal property, to the best of our knowledge, there has been no systematic pharmacological and phytochemical work performed on T. govanianum. Therefore, the present study was undertaken to explore potential cytotoxicity of the methanol (MeOH) extract of he roots of T. govanianum and its solid-phase extraction (SPE) fractions against four human carcinoma cell lines: breast (MCF7), liver (HepG2), lung (A549) and urinary bladder (EJ138) using the in vitro 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazoliumbromide (MTT)

cytotoxicity/ viability assay and to carry out preliminary liquid chromatography and electrospray ionization quadrupole time- of-flight (TOF) mass spectrometry analysis of the SPE fractions.

Materials and methods

Reagents and chemicals. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Dorset, UK). Solvents were purchased form Fischer Scientific (Loughborough, UK). All cell culture reagents were purchased from Biosera (Nauaille, France).

Plant materials. Plant sample was collected from the Muzaffarabad district of Pakistan-controlled Azad Kashmir and identified as T. govanianum Wall. by Dr Muhammad Zafar, Herbarium Botanist, Department of Plant Sciences, Quaid-I-Azam University, Islamabad, Pakistan. A herbarium specimen for this collection (voucher number: Acc no. 128085) has been deposited and retained in the herbarium mentioned in the preceding texts.

Extraction and preparation of plant samples. Shade dried and finely ground roots (2.5 kg) of T. govanianum were macerated in MeOH (5 L) for 10 days at room temperature, filtered, and the solvent was evaporated under vacuum using a rotatory evaporator (<45 °C) to obtain a concentrated gummy crude extract.

Solid-phase extraction and sample purification. A portion of the dried MeOH extract (2 g) was suspended in 20 mL of HPLC grade water and loaded on to a Strata C-18 cartridge (20g), previously washed with MeOH (50 mL), followed by equilibration with water (100mL). The cartridge was eluted with MeOH–water mixture of decreasing polarity to obtain four fractions: 20, 50, 80 and 100% MeOH in water (250mL each), coded respectively as TGMF1, TGMF2, TGMF3 and TGMF4. All four fractions were evaporated to dryness using a combination of rotary evaporator and freeze- dryer, re-dissolved in MeOH (10mg/mL), centrifuged at 12000r.p.m. for 3min, filtered through 0.20μm of sterile syringe filter for injection (10 μL) into the liquid chromatography mass spectrometry (LC-MS) system.

Liquid chromatography mass spectrometry. An Alliance HPLC System 2695 (Waters) was used. Reversed-phase chromatography was performed on a Phenomenex Gemini-NX 5 μm C_{18} column (250 × 4.6 mm). The column temperature was set at 25°C. A variable wavelength ultraviolet–visible detector was set at 220 nm. An elution gradient was used with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeOH). The initial mobile phase composition was 70% of A and 30% B at 0 min, then linear gradient to 100% of B over 30min and held at that composition for 5min at a flow rate of 1 mL/min.

The LC system was connected to a quadrupole TOF mass spectrometer (Waters Micromass LCT) equipped with an electrospray ion source. Response was recorded in real time by the mass spectrometer data system (Waters MassLynx version 4.1). The tuning parameters were set as follows: electrospray interface 3000 V, rangefinder lens 250V, extraction cone set at 3V, desolvation temperature 20 °C, source temperature 100 °C, nebulizer gas flow 20L/h desolvation gas flow 760L/handTOFtube4687V. Data acquisition method was set as follows: cycle time 1 s, scan duration 0.9 s, inter-scan delay 0.1 s, mass range 100 to 1600 and centroid mode. Positive ion mode was operated with cone voltage settings of 40 V.

Cell lines, cell cultures and the MTT assay. The potential cytotoxicity of the MeOH extract of the roots of T. govanianum and its SPE fractions was studied against four human carcinoma cell lines: breast (MCF7), liver (HepG2), lung (A549) and urinary bladder (EJ138) using the MTT assay (Mosmann, 1983; Basar et al., 2015). The cells were washed by phosphate buffered sa- line and harvested by tripsinization. All cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum. The cells were cultured at 37 °C in 95% humidity and 5% CO_2. For the MTT assay, the cells were seeded into 24 well plates at a density of 1.2×10^4 cells/well in a working volume of 1 mL/well and allowed to grow for 24 h before the commencement of each experiment.

The cells were treated for 24 h with different concentrations of test samples (the MeOH

extract and SPE fractions; 5, 10, 50, 100 and 500μg/mL). Dilution of stock solutions was made in culture medium yielding final sample concentrations with a final dimethyl sulfoxide concentration of 0.1%, including the control. Each sample was used to treat four wells of cells in each 24-well plate. After the 24-h treatment period, the toxicity of the samples on each carcinoma cell line was quantified. To achieve this, the medium in each well was replaced by MTT solution (500 μg/mL in medium) and incubated for 2h. Toxicity was assessed by the ability of the cells to reduce the yellow dye MTT to a blue formazan product (Popescu et al., 2015). The MTT reagent was removed, and the formazan crystals produced by viable cells were dissolved in isopropanol, and the OD_{560} was determined with the microplate reader (CLARIO Star Microplate reader, BMG Labtech, UK). The average OD_{560} obtained from all the control wells (without test sample) on each plate was arbitrarily set at 100%, and the OD_{560} value for the average of wells of cells treated with each test samples was expressed as a percentage of this control. Each assay was performed on a minimum of three separate occasions, and the IC_{50} values for each sample on each cell line were calculated using Microsoft Excel version 2013.

Statistical analysis. All experiments were carried out in triplicate on separate occasions. Data were expressed s means ± standard error of the mean. The graphs were plotted using nonlinear regression with the use of Microsoft Excel version 2013.

Results and discussion

The MeOH extract of the roots of T.govanianum (Table 1) and its SPE fractions (TGMF1, TGMF2, TGMF3 and TGMF4) exhibited different levels of significant cytotoxicity against four human carcinoma cell lines, for example, breast (MCF7), liver (HepG2), lung (A549) and urinary bladder (EJ138), using the in vitro MTT cytotoxicity/viability assay (Table 1). The IC_{50} values of the MeOH extract and its SPE fractions are shown in Table 1. The MeOH extract displayed the highest level of cytotoxicity against the urinary bladder cell line (EJ138; IC_{50} = 5 μg/mL), but it was also considerably active against three other cell lines, MCF7, HepG2 and A549 (IC_{50} = 5, 7

and 9 μg/mL respectively). The SPE fraction TGMF1, which had the most polar components of the parent MeOH extract, showed most significant cytotoxicity against EJ138 (IC_{50} = 6 μg/mL) and considerable cytotoxicity against MCF7, HepG2 and A549, with the IC_{50} values of 9, 11 and 9μg/mL respectively. This SPE fraction was almost as potent as its parent MeOH extract in terms of cytotoxicity against the urinary bladder cell line EJ138. The SPE fraction TGMF2 showed most prominent cytotoxicity against the lung cancer cell line A549 (IC_{50} = 6 μg/mL) and was also active against other three cell lines, EJ138, MCF7 and HepG2 (IC_{50} = 11, 10 and 13 μg/mL respectively). The cytotoxicity pattern of the SPE fraction TGMF3 was quite similar to that of the TGMF1 and the MeOH extract and showed most significant cytotoxicity against the EJ138 cell line (IC_{50} = 9 μg/mL). This fraction was also cytotoxic to the MCF7, A549 and HepG2 cell lines with the IC_{50} values of 10, 13 and 16 μg/mL respectively. The SPE fraction TGMF4, which contained the least polar components of the parent MeOH extract, exhibited notable cytotoxicity against the EJ138 cell line (IC_{50} = 13 μg/mL) and was also considerably active against the HepG2, MCF7 and A549 cell lines (IC_{50} = 10, 13 and 15 μg/mL respectively). With the only exception of TGMF2, three other SPE fractions showed the highest level of cytotoxicity against the EJ138 cell line, as was observed with their parent MeOH extract. To the best of our knowledge, this is the first report on the cytotoxicity of the MeOH extract of the roots of T.govanianum and its SPE fractions against any carcinoma cell lines. The current finding is concordant with the findings of a few other previous studies on the cytotoxicity of some other species of the genus Trillium (Nooter and Herweijer, 1991; Yokosuka and Mimaki, 2008; Hayes et al., 2009).

The major bioactive components of the genus Trillium are saponins and sapogenins (Fig. 1), which are well known to exhibit cytotoxicity (Nooter and Herweijer, 1991; Yokosuka and Mimaki, 2008; Hayes et al., 2009). Most of the compounds isolated from the genus Trillium described in the literature are saponins containing mono, di, tri or tetrasacchride (Fig. 1), commonly composed of apisoe, arabinose, glucose, rhamnose and xylose, which are linked to a β-D-glucosyl moiety at C3 of the aglycone (Gao et al., 2015). Paris saponin VII, a diosgenin-based saponin isolated from T. tschonoskii Maxim showed

cytotoxicity against MCF7, human colorectal cancer cells-29 and SW-620 (IC_{50} = 9.547, IC_{50} = 1.02 ± 0.05 and 4.90 ± 0.23 μm respectively) (Li et al., 2014). Paris VII induced cell apoptosis together with caspase-3-dependent manner and cell cycle arrest in G1 Phase. Two new saponins from the underground part of T.tschonoskii displayed strong cytotoxic activity against the HepG2 cell line (IC_{50} = 0.499 mmol/L) (Chai et al., 2014). A steroidal saponin from the same species exhibited prominent potential in combating multi-drug-resistance hepatocellular carcinoma (Wang et al., 2013). Six steroidal saponins isolated from the roots of T.erratum were shown to possess considerable cytotoxicity against the HL60 human promyelocytic leukaemia cells. From the same species, spirostanol and furostanol saponins showed moderate level of cytotoxicity (IC_{50} = 1.68–8.85 μg/mL) (Yokosuka and Mimaki, 2008). Diosgenin, isolated as a major compound from Trillium species, showed potent cytotoxic effect against HepG2 and HCT116 cells in the MTT assay (Eskander et al., 2013). These are the type of compounds that could be also present in the roots of T. govanianum, and accordingly, preliminary LC-MS analysis was performed to test this hypothesis.

Liquid chromatography mass spectrometry analysis on the SPE fractions of the MeOH extract of the roots of T.govanianum was performed to obtain an insight into the possible chemical composition of the fractions, particularly to have an indication whether they contain saponins and sapogenins as possible contributors to the significant cytotoxicity of the extract and its fractions. The chromatographic conditions were optimized by method development. A linear gradient elution with water and MeOH containing 0.1% formic acid as the mobile phase offered the best resolution. Typical chromatograms of fractions with mass spectrometric detection in positive ion mode exhibited quite complex patterns of peaks (Table 2), and only the possible presence of saponins (e.g. Paris saponin VII at t_R 25.83, [M + 1] + m/z 1032) and sapogenins (e.g. diosgenin at t_R 36.62, [M + 1] + m/z 415) in SPE fractions could be suggested from the retention times and the MS spectral data of the separated peaks (Woldemichael and Wink, 2001; Madl et al., 2006; Hayes et al., 2009; Li et al., 2014; Gao et al., 2015; Shafiq-ur-Rahman et al., 2015). The presence of saponins and their aglycones in T.govanianum was in agreement with that of other Trillium species. It is reasonable to assume that the cytotoxicity of the MeOH extract and

its SPE fractions of the roots of T. govanianum might be, at least partly, owing to the presence of saponins and their aglycones. This is also the first report on the preliminary LC-MS analysis on T. govanianum.

Considering the significant cytotoxicity observed against four carcinoma cell lines in the current study and on the basis of the previously published data on the antitumour/anticancer potential of the genus Trillium as well as the presence of saponins in T.govanianum, it is reasonable to state that T. govanianum could be exploited as a good source of cytotoxic compounds with putative anticancer potential.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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