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Short Communication

Apoptosis inducing activity of steroidal constituents from *Solanum xanthocarpum* and *Asparagus racemosus*

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ABSTRACT

A series of Sarsapogenin and Diosgenin derived steroidal constituents (**1-12**), isolated from *Solanum xanthocarpum* and *Asparagus racemosus* were screened for their ability to induce cell death and apoptosis of colon carcinoma cells. The carbohydrate moieties linked to the steroid backbones were found to strongly influence cytotoxic activity and cell death mode (apoptosis or necrosis). Compound **10,** from *A. racemosus* was found to be a potent inducer of apoptosis.

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Introduction

Plants have served as an important source of potent anti-cancer drugs for decades. The search for anti-cancer drugs from plant sources started in the 1950s, with the discovery of the vinca alkaloids (vinblastine and vincristine) and podophyllotoxin. This search spanned over four decades till 1990s, when taxanes and camptothecins were launched as anti-cancer drugs (Cragg and Newman, 2005). The success of plant based molecules still inspires researchers for searching newer anticancer agents from plants. Steroidal compounds are important class of secondary metabolites, which have been reported to exhibit wide range of pharmacological properties that include hypocholesterolemic (Sirtori et al., 2004), antioxidant (Turchan et al., 2003) and antidiabetic (Attele et al., 2002) etc. However of particular interest is the apoptosis inducing activity of steroidal compounds. Amongst the steroidal class of compounds, diosgenin has been previously reported to induce apoptosis in different human cancer cell lines (Corbiere et al., 2003). Thus, in order to identify an effective apoptosis inducing agents, we have tested several steroidal compounds, structurally related to diosgenin (including diosgenin) which were isolated from two Indian medicinal plants namely *Solanum xanthocarpum* and *Asparagus racemosus.*

S. xanthocarpum Schard. & Wendl. (Solanaceae) is a herb commonly found throughout India. The fruits are reported to contain several steroidal glyoalkaloids namely solanacarpine, solanacarpidine (Briggs, 1937), solanine and solamargine (Siddiqui and Faizi, 1983), phenolic acids like caffeic acid, methyl caffeate (Siddiqui and Faizi, 1983) and coumarins (scopoletin, esculetin and esculin (Tupkari et al., 1972). Diosgenin (Sato and Latham, 1953) and carpesterol (Tsay et al., 1970) have been isolated from the fruits. *S. xanthocarpum* has shown anti-asthmatic (Sivarajan and Balachandran, 1994), anti-nociceptive (Rahman et al., 2003), anti-fungal (Dabur et al., 2004), hypoglycaemic (Kar et al., 2006), molluscicide activity (Wei et al., 2002) and anti-bronchitis activities (Bector et al., 1971; Govindan et al., 1999).

A. racemosus Willd (Liliaceae) is an important plant used for its immunomodulatory and galactogouge effects in *Ayurveda.* The roots are used in nervous disorders, dyspepsia, inflammations, nephropathy, hepatopathy, throat infections, tumours, cough, bronchitis, hyperacidity and diarrhoea (Sharma et al., 2000). The roots are reported to contain 9, 10-dihydro-1,5-dimehtoxy-8-methyl-2,7-phenanthrenediol (Sekine et al., 1994), an alkaloid named asparagamine A (Sekine et al., 1995), and saponins like shatavarin I-IV (Hayes et al., 2006). Shatavarin-I has been shown to have anticancer activity in DMBA induces carcinogenesis in rats (Rao, 1981). It has been shown to exhibit immunoprotective effect in cancer chemotherapy (Diwanay et al., 2004).

The aim of the present study was to investigate the anti-proliferative activity of steroidal constituents isolated from *S. xanthocarpum* and *A. racemosus* on human colon carcinoma cells. Steroidal compounds were isolated from *S. xanthocarpum* and *A. racemosus* using the reported protocols established in our laboratory (Paul et al., 2008; Jadhav et al., 2006). Colon carcinoma cells were exposed to compounds at different concentrations in 96-well plates, and cell viability and apoptosis (using caspase-cleaved cytokeratin as an end-point) were determined in parallel.

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A number of compounds from *S. xanthocarpum* and *A. racemosus* were found to induce cell death of colon cancer cells. Cell death was, however, in most instances not associated with apoptosis.

Materials and methods

Plant material

S. xanthocarpum (whole plant) was purchased from local market of Chandigarh. *A. racemosus* (roots) were collected from medicinal plant garden of the institute. Both the plant materials were identified and voucher specimen (Herbarium No. NIP: 83 and 31-A-4) have been deposited in the herbarium of the Department of Natural Products. Diosgenin (**1**), diosgenone (**2**) and solasodine (**3**) were previously isolated in the laboratory and was taken from the compound repository of the department.

Extraction and isolation of the compounds

The plant material of *S. xanthocarpum* was sequentially extracted with hexane, chloroform, ethyl acetate and methanol. Compounds **4-6** were isolated from methanolic extract (Paul et al., 2008). The roots of *A. racemosus* were macerated with hexane followed by ethanol. The ethanolic extract was dried under vacuum and resuspended in water and partitioned with chloroform, ethyl acetate and *n*-butanol. Compound **7-12** were isolated from the *n*- butanolic extract as per the reported protocol (Jadhav et al., 2006).

Cell culture

HCT116 is a colon carcinoma cell line with mutant KRAS and wild-type p53 genes. Cells were maintained in McCoy's 5A modified medium/10% fetal calf serum, and FaDu head-neck carcinoma cells were maintained in Dulbecco's MEM/10% fetal calf serum at 37 °C in 5% CO_2. Tissue culture reagents were obtained from Gibco Cell Culture Products. Cisplatin was obtained from Bristol-Myers-Squibb.

Cell viability

Cell viability was analysed using the Sulforhodamine B cytotoxicity assay which measures protein content of attached cells. Medium was removed from the 96 well plates and the cells were fixed with 50 μl of 50% trichloacetic acid, and were left in fridge for 1 h. Cells were washed three times with 250 μl distilled water and then left to dry for 1 h. 100 μl of 0.4% Sulforhodamine B (dissolved in 1% acetic acid) were added per well and cells were allowed to stain for 45 min. Wells were rinsed three times with 250 μl of 1% acetic acid and then left to dry for 1 h. Sulforhodamine B dye was then solubilized in 200 μl of 10 mM Tris base solution and left for 5 min. Absorbance was measured at 565 nm with background measurement at 690 nm subtracted from the 565 nm absorbance. Viability was calculated as percent absorbance compared to the absorbance of untreated controls.

M30 CytoDeathh® ELISA apoptosis assay

The M30 CytoDeath® ELISA was used for determination of apoptosis. The M30 CytoDeath® ELISA uses the same antibodies as the M30-Apoptosense ELISA kit but is adapted for *in vitro* cell culture studies (see www.peviva.com). The ELISA measures the accumulation of a caspase-cleaved fragment of cytokeratin-18 (CK18-Asp396) in cells and tissue culture medium over time (Kramer et al., 2004). The signals obtained are blocked by addition of pancaspase-inhibitors (i.e. zVAD-fmk) to cells. In the present experiments, HCT116 cells were seeded at 10^4 cells/well in 96-well plates 24 h before treatment. At the end of the 24 h exposure period, the non-ionic detergent NP40 was added to the tissue culture medium to 0.5%. An aliquot (25 μl) of the content of each well was assayed for the CK18-Asp396 epitope as recommended by the manufacturer (Peviva AB, Bromma, Sweden).

Results

Isolation of phytochemicals

Liquid chromatography mass spectrometry of methanolic extract of *S. xanthocarpum* resulted in the isolation of compounds **4-6** (Fig. 1). Silica gel column chromatography of the butanolic extract of *A. racemosus* resulted in isolation of compounds **7-12** (Fig. 2). All the compounds (**1-12**) were characterized using physiochemical data and spectroscopic data.

Measurement of tumor cell viability and apoptosis

Cell viability was determined using the Sulforhodamine B cytotoxicity assay. The capacity of different compounds to induce apoptosis of the human colon carcinoma cell line HCT116 was assessed using the M30 CytoDeath ELISA. This assay provides an integrative measure of apoptosis based on the accumulation of a caspase-cleavage product of cytokeratin 18 (ccCK18) in cells and culture medium. Cisplatin was used at 40 μM as a reference agent in these experiments and generated an absorbance of ~1.6 A_{450} units (shown as dashed lines in Figs. 1 and 2).

Cell viability and apoptosis data for *Solanum* compounds are shown in Fig. 1. Two of the compounds (solamargine (**5**) and solasonine (**6**)) were strongly cytotoxic. There was, however, no induction of ccCK18 at cytotoxic doses > 10 μM. Cell death at high doses was therefore not due to induction of apoptosis, but to necrosis. Necrosis will remove the population of cells that undergo spontaneous apoptosis during the incubation period and ccCK18 levels are therefore background levels (below the lower dashed line). Compounds **1** and **3** (diosgenin and solasodine) were weakly cytotoxic (~ 70 – 80% cell viability at 50 μM), and these compounds induced ccCK18 to ~ 2-fold background levels. Compounds **2** and **4** were inactive.

The corresponding results for the compounds isolated from *Asparagus* are shown in Fig. 2. All compounds except **7** were cytotoxic to HCT116 cells. In general, *Asparagus* compounds did not induce signicant caspase activity. The exception to this rule was immunoside (**10**), which induced a robust increase in ccCK18 (~ 50% of the cisplatin control).

Discussion

The genus *Solanum* (*Solanaceae*) is a rich source of steroidal glycoalkaloids. The plants of this genus mainly contain steroidal alkaloids like solamargine, solasonine and solanine and steroidal sapogenins, diosgenin and diosgenone. Solanine, isolated from *S. nigrum*, has been reported to induce apoptosis in HepG2 cells, by inhibiting the expression of *Bcl*-2 protein (Ji et al., 2008). Another steroidal alkaloid, solamargine, has also been reported to exhibit the anti-tumor effects (Liu et al., 2004). It was previously reported that solamargine and solasodine are cytotoxic to Hep3B cells at 10 μM (Chang et al., 1998). Only solamargine induced apoptosis, measured as generation of cell fragments with low DNA content and the 2' rhamnose moiety of solamargine was

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