

RESEARCH ARTICLE

Asparagus Racemosus Leaf Extract Inhibits Growth of UOK 146 Renal Cell Carcinoma Cell Line: Simultaneous Oncogenic PRCCTFE3 Fusion Transcript Inhibition and Apoptosis Independent Cell Death

Shiv Prakash Verma¹, Vikash Chandra Tripathi², Parimal Das^{1*}

Abstract

Aims: To evaluate anti-cancer activity of *Asparagus racemosus* (AR) leaf extract on UOK146, a renal cell carcinoma cell line, and explore its mechanism of action. **Materials and Methods:** Dried AR leaves were extracted with chloroform and dissolved in DMSO. This extract was applied to UOK146 and cell death was estimated by MTT assay. In addition PRCC-TFE3 fusion transcripts were detected by real time PCR. **Results:** Extract was found to be cytotoxic with an IC₅₀ of 0.9 mg/ml as estimated by dose response curve. Antitumor activity of the permissible doses of the extract was assessed by the down regulation of PRCC-TFE3 fusion transcript (38%) responsible for oncogenicity of the UOK146 cell line. No increment in the BAX, a proapoptotic marker level was observed. **Conclusions:** Evidence of antiproliferative effect, PRCC-TFE3 fusion transcript inhibition and static BAX level clearly indicate that AR extract provides or elicits an apoptosis independent anticancer effect on RCC cells by some specific mechanism of regulation.

Keywords: Immunomodulatory - PRCCTFE3 - phytoestrogenic - cytotoxicity - UOK 146

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Introduction

Asparagus racemosus Willd. (Family: Liliaceae) is a pharmacologically acclaimed phytoestrogenic medicinal plant used for its immunomodulatory (Diwanay et al., 2004) and galactogogue effects in Ayurveda. It is commonly known as Shatavari in Hindi and Sheetaveerya in Sanskrit growing naturally and indigenous to South Asian countries. Medicinal properties of this plant have been described in traditional medicine, such as the Ayurveda (Himalayan), Siddha and Unani system of medicine (Gautam et al., 2004). The main chemical constituents of *A. racemosus* identified so far include 9, 10-dihydro-1, 5-dimethoxy-8-methyl-2, 7-phenanthrenediol (Sekine et al., 1997), steroidal saponins named shatavarin I-IV (Hayes et al., 2006; Hayes et al., 2008) and an alkaloid asparagamine A (Sekine et al., 1995). Ethanolic leaf extract of *A. racemosus* display anti-inflammatory effect in rats (Battu et al., 2010). *A. racemosus* root extract possess antioxidant activity (Kamat et al., 2000; Wiboonpun et al., 2004; Bhatnagar et al., 2005), protective against Lipofectamine-induced apoptosis in human lung epithelial H460 cells (Kongkaneramt et al., 2011) and prevent hepatocarcinogenesis induced by diethylnitrosamine treatment (Agrawal et al., 2008). Immunoside was the only *Asparagus* compound which induces apoptosis amongst

all the compounds tested in HCT116 human colon carcinoma cell (Bhutani et al., 2010). The carbohydrate moieties linked to the steroid backbones were found to strongly influence cytotoxic activity and cell death mode (apoptosis or necrosis) (Bhutani et al., 2010). *A. racemosus* root extract competitively inhibits the acetylcholine and monoamine metabolizing enzymes *in vitro* explaining its neuropharmacological activities (Meena et al., 2011). *A. racemosus* extract was found to be active against Kainic acid induced hippocampal and striatal neuronal damage in mice and reduces Fe³⁺ and oxidative damage, indicating that plant extract has a primary role in reducing free radical induced oxidative damage (Parihar et al., 2004). Aqueous root extract of *A. racemosus* is a potential immuno adjuvant that offers a direct therapeutic benefit resulting in less morbidity and mortality (Gautam et al., 2004) and demonstrates mixed Th1 and Th2 adjuvant and immuno pharmacological properties under different biological stimuli (Gautam et al., 2009). Ethanol extract and five partition fractions (aqueous, butanol, ethyl acetate, chloroform and hexane) of *A. racemosus* root has insulinotropic effects as assessed using perfused rat pancreas, isolated rat islets and a clonal rat insulin secreting cell line (Hannan et al., 2007). Polysaccharides of *A. racemosus*, which are fructo-oligosaccharides (FOS) with a neo-ketose backbone, solely responsible for the

¹Centre for Genetic Disorders, Faculty of Science, Banaras Hindu University, Varanasi, ² University of Calicut, Kerala, India *For correspondence: parimal@bhu.ac.in and hellow_parimal@yahoo.com

augmentation of Natural Killer (NK) cell activity, thus it can be comprehended that the FOS from *A. racemosus* may be an important factor in rejuvenating the immune system (Thakur et al., 2012). The crude saponins extracted from the edible part of *Asparagus officinalis* were found to have anti-tumor activity which inhibited the growth of HL-60 human leukemia cells in dose and time dependent manner (Shao et al., 1996). Methanolic shoot extract of *A. officinalis* activates TRAIL apoptotic pathway in human colon carcinoma cells and inhibits carcinogenesis in rat preclinical model (Bousserouel et al., 2013). Anti-cancerous property of Asparagus extract was studied earlier at cytotoxicity level. The capacity of the steroidal constituents of *A. racemosus* in inducing tumor cell death indicates its potential attribute to cancer therapeutics. The renal cell carcinoma (RCC) make up only 3% of all adult malignancies which constitute 90% of kidney cancers and it is among the most resistant to cytotoxic chemotherapy (Mohan et al., 2009; Tanriverdi, 2013). Here we have studied its effects at molecular level using UOK146, a human Renal Cell Carcinoma cell line as model system for better understanding of anti-tumor action of *A. racemosus* leaf extract *in vitro*. UOK146 cell line has chromosomal translocation leading to synthesis of PRCCTFE3 fusion protein which is responsible for oncogenesis (Sidhar et al., 1996; Weterman et al., 2001). So the level of fusion protein/transcript was measured for evaluating the anti-cancerous effect of *A. racemosus* leaf extract.

Materials and Methods

Plant material

A. racemosus leaves were collected from Calicut University Botanical garden and identified by Dr. A K Pradeep, Department of Botany, Calicut University, Kerala, India. *A. racemosus* leaves specimen CU No. 290408 is preserved at the Calicut University campus landscaping, University of Calicut for future reference.

Crude extract preparation

A. racemosus leaves were detached from the plant and kept at room temperature in shade for drying. After drying these leaves were grinded by mortar and pestle. The grinded leaf (3.3 g) was taken, mixed with organic solvent chloroform (33 ml) in the ratio of 1:10 and kept overnight on shaker at 32°C and 160 rpm. Extract was filtered with Whatman filter paper no. 1 and kept in water bath at 40°C for evaporation of chloroform. Crude extract was dissolved in Dimethyl sulfoxide (DMSO).

Preliminary phytochemical screening

The chemical tests were carried out on the AR to find the presence of phytochemical constituents by the method described by Sofowora, Harborne, Trease and Evans with some modifications (Harborne et al., 1973; Trease et al., 1989; Sofowora et al., 1993).

Alkaloid: AR extract was evaporated to dryness and the residue was heated on boiling water bath with 2% HCl, reaction mixture was cooled, filtered, treated with a few drops of 5% Sodium Hydroxide and observed for

the presence of turbidity or yellow precipitation.

Glycoside: 5 mg AR extract treated with 0.5 ml glacial acetic acid, few drops of ferric chloride; to this conc. Sulphuric acid was added and observed for a reddish brown colour at the junction of two layers and the upper layer for bluish green colour.

Terpenoid and steroid: 4 mg of AR extract was treated with 0.5 ml of acetic anhydride, 0.5 ml of chloroform, conc. solution of sulphuric acid was added slowly and red violet colour was observed for terpenoid and bluish green colour for presence of steroid.

Flavonoid: 1 ml of AR extract solution was treated with 0.5 ml of lead acetate solution and white colour precipitation was observed for the presence of flavonoids.

Tannins: To 0.5 ml of extract solution 1 ml of water and 1-2 drops of ferric chloride solution was added and observed for green precipitate an indication for the presence of tannins.

Cell culture

UOK 146, a renal cell carcinoma cell line with specific chromosomal translocation t(X;1)(p11.2;q21.2) generating a chimeric PRCCTFE3 gene which is responsible for oncogenesis, was maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and grown in 5% (v/v) CO₂ in a humidified incubator.

Cell viability

Exponentially growing 2×10⁴ Cells were seeded in 96-well plate in complete DMEM medium. After 24h cells were incubated with varying concentrations of extract in triplicates for further 24h. After that DMEM with 0.5 mg/ml 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added and incubated for 4h at 37°C. After that, media was removed and blue coloured formazan was extracted in 100 µl of DMSO for 15 min at room temperature. The purple color end products were quantified by measuring absorbance (OD 570 nm) with a microplate reader (Bio-Rad).

Microscopy

Phase contrast microscopy (Motic, China) was performed to see the effect of AR treatment at the level of morphological characteristics in UOK 146 cells. Cells cultured in 96 well plate for cytotoxicity assay were used for the microscopic observation before MTT assay.

Flow cytometry

To determine fractions of cell cycle stages, cells were harvested and washed in PBS, fixed/permeabilized in 70% ethanol at -20°C for 45 min and stained with 50 µg/ml Propidium Iodide (PI) in PBS containing 50 µg/ml RNase A. The DNA content was measured on FACS Calibur (Becton Dickinson) flow cytometer using CellQuest Pro software (BD Biosciences).

Real time PCR

Total cellular RNA was extracted using the Trizol reagent (Sigma, USA) and treated with DNaseI (Ambion,

USA) to remove any DNA contamination and quantified by NanoDrop 2000 (Thermo Scientific, USA). cDNA was synthesized from 2 μ g of total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) in 20 μ l of reaction solution according to the manufacturer's instructions. Real time PCR was performed in the ABI 7500 Real Time PCR instrument using the SYBR Green Real time PCR master mix (Thermo Scientific, USA). Primers used for the study are listed in the table.

Results

AR extract components

Among different phytochemical screening reactions for Alkaloid, Glycoside, Terpenoid, steroid, Flavonoid and Tannin, AR extract was found to be steroid positive. Development of bluish green colour upon reaction of the extract with acetic anhydride and chloroform confirms the presence of steroid in the extract (Figure 1C). Many other biologically active components were also identified from the extract by HPLC-ESI-MS/MS analysis like Hydrocortisone, Hydrogen succinate, Ellagic acid, Amfetamin, Quercetin, Malvidin-3-glucoside (MS/MS data not shown) and many more to be identified.

Morphology

Effect of AR on UOK 146 cells was evaluated by phase contrast microscopy. Cells were incubated for 24 h after treatment with 1 mg/ml AR. Cell became rounded, detached from the surface and started floating into the growth media which is a characteristic feature of cell death (Figure 2A).

Cytotoxicity

Effect of AR on UOK 146 cells was evaluated by MTT cell viability assay. AR treated UOK 146 cells displayed a dose dependent decrease in cell viability. Addition of AR extract of different concentrations at 0.5, 1 or 1.5 mg/ml to UOK146 cells for 24 h inhibited the growth by 28%, 54% or 72% respectively (Figure 3A). IC_{50} was estimated to be ≈ 0.9 mg/ml of crude extract by dose response curve plotted between the % inhibition of cell viability as analysed by the MTT assay at different doses concentration (Figure 3B).

Quantitative RT-PCR for BAX

We were interested to examine the mode of cell death induced by AR treatment at molecular level. BAX, a proapoptotic marker gene was used for quantitative RT-PCR to see the apoptotic mode of cell death. In apoptotic condition BAX is upregulated indicating apoptotic cell

death. Quantitative RT-PCR for BAX showed that AR treatment reduces the level of BAX transcripts up to 49% as compared to vehicle control (Figure 4). This result indicates apoptosis independent cell death.

Flow cytometry

Effect of AR on cell cycle stages were analyzed by flow cytometry. UOK 146 cells were treated with 1 mg/ml AR and compared with vehicle control (DMSO) treated cells. Change in cell cycle stages were not observed, absence

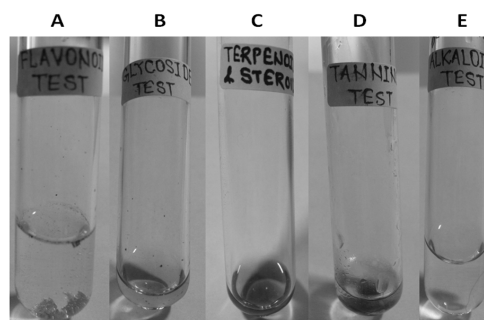


Figure 1. Phytochemical Screening Test of AR: Tube C is the Test for Terpenoid and Steroid showing the Steroid Positive Result. Tube A, B, D and E are the test for flavonoid, glycoside tannin and alkaloid respectively showing the negative test result

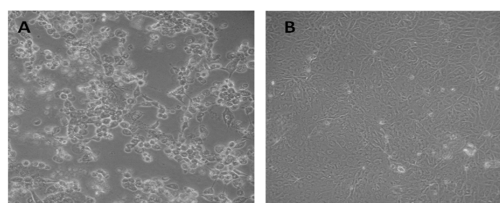


Figure 2. Phase Contrast Microscopy Image of UOK 146 Cells (A) After 24h of 1 mg/ml AR Treatment (B) Vehicle (DMSO) Control

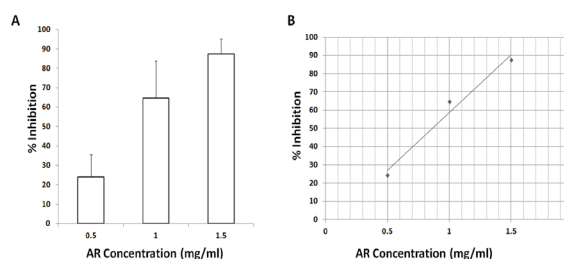


Figure 3. Dose Response Graph: (A) Graph showing the Dose Dependent % Cell Death of UOK 146 Cells (B) Dose Response Curve to Estimate the IC_{50} Value

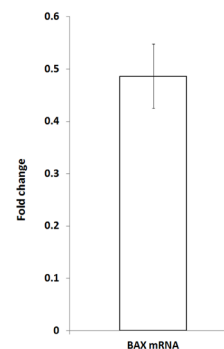


Figure 4. Real Time PCR Analysis of BAX Transcripts

Table 1. Oligonucleotide

Oligonucleotide name	Oligonucleotide sequence	Reference
PRCCTFE3*	5'-CCAAGCCAAAGAAGAGGAAA-3'	Macher-Goeppinger
PRCCTFE3**	5'-CGAGTGTGGTGGACAGGTACT-3'	et al., 2012
BAX*	5'-CCCTTTTGCTTCAGGGTTTC-3'	Liu et al., 2008
BAX**	5'-TGTTACTGTCCAGTTCGTCC-3'	
GAPDH*	5'-AGGGCTGCTTTAACTCTGGT-3'	Ito et al., 2007
GAPDH**	5'-CCCCACTGATTTGGAGGGA-3'	

*Forward; **Reverse

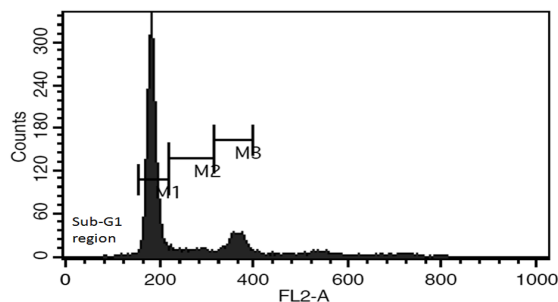


Figure 5. Flow Cytometry Analysis. M1, M2 and M3 in the histogram denote the cells in G1, S and G2+M phase respectively and there is no sub-G1 peak

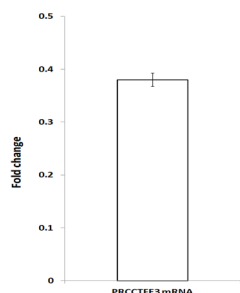


Figure 6. Real Time PCR Analysis of PRCCTFE3 Fusion Transcript after AR Treatment

of any sub-G1 peak which denotes DNA fragmentation also indicates absence of DNA fragmentation leading to apoptosis and thus supports quantitative RT-PCR result (Figure 5).

PRCCTFE3 fusion transcript inhibition

For determining the role of AR's anti-cancerous property and its impact on PRCCTFE3 fusion transcript, real time PCR was performed. PRCCTFE3 fusion transcript level was reduced up to 38% after AR treatment compared to vehicle control treatment (Figure 6). Since PRCCTFE3 fusion protein is responsible for the oncogenicity of UOK146 cell line, inhibition of PRCCTFE3 fusion transcript displayed anti-cancerous property of AR at molecular and/or transcription level.

Discussion

Asparagus racemosus is widely used in Ayurveda for its medicinal properties and its anticancerous property has been implicated in the regulation of cell proliferation and apoptotic gene products. Change in the control of the cell death processes like apoptosis and autophagy may extend the life span of cells and favour neoplastic expansion. Herbal components having anticancerous and immunomodulatory activity are required to be identified for cancer treatment. Many ayurvedic formulations are recognized for their ability as anti-cancer (Cragg et al., 2005), anti-inflammatory immunomodulatory and many other therapeutic uses. *A. racemosus* being a renowned Ayurvedic drug and its shoot extract has shown anti-tumor activity, it's of much interest to see the *A. racemosus* leaf components and its activity as a potent anticancer drug. Asparagus root is a rich source of steroidal saponins which are important secondary metabolites with cytotoxic activity. We have found through phytochemical screening

that *A. racemosus* leaves are good source for steroids and those could be potent source for developing cancer therapeutics. Present investigation demonstrated that AR inhibited the growth of Renal Cell Carcinoma UOK146 cells and thus it can be reasonably predicted that AR has some active components which have antitumor activity specifically for Xp11.2 translocation derived malignancies. We therefore, investigated the chloroformic crude extract for constituent components by phytochemical screening and HPLC-ESI-MS/MS and found that steroid was the main constituent. Asparagus root and shoot possess saponins as major bioactive compound and all the saponins are chemically steroids. 1mg/ml crude extract was found to be sufficiently $\approx 50\%$ cytotoxic but the mode of cell death is unknown. After treatment BAX was found to be downregulated and no sub-G1 peak in flow cytometry data was observed, both these results support each other and confirms apoptosis independent mode of cell death. PRCCTFE3 fusion transcript/protein is responsible for oncogenesis of UOK 146 cells therefore targeted molecular inhibitors of PRCCTFE3 are required to cope with its oncogenic property. AR inhibited the cell growth along with downregulating the PRCCTFE3 transcripts, therefore it could be a good targeted inhibitor of PRCCTFE3 and many more Xp11.2 translocation generated fusion transcripts.

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