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# Activation of caspases and poly (ADP-ribose) polymerase cleavage to induce apoptosis in leukemia HL-60 cells by *Inula racemosa*

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ABSTRACT

*Inula racemosa* Hook.f. commonly known as Pushkarmula (*Compositae*) has been used as a traditional drug in India, China and Europe. In the present study, 95% ethanolic extract of roots and its fractions (*n*-hexane, chloroform, *n*-butanol and aqueous) were evaluated for *in vitro* cytotoxicity against cancer cell lines of colon, ovary, prostate, lung, CNS and leukemia. The *n*-hexane fraction containing alantolactone and isoalantolactone as its major constituents was further studied for its mode of action in HL-60 cells. The lowest $IC_{50}$ value of *n*-hexane fraction was 10.25 μg/ml for Colo-205, a colon cancer cell line whereas, 17.86 μg/ml was the highest $IC_{50}$ value observed against CNS cancer cell line SF-295. Further studies on HL-60 cells treated with *n*-hexane fraction at 10, 25 and 50 μg/ml for 6 h, revealed that it induces apoptosis through intrinsic as well as extrinsic pathways by generating reactive oxygen species (ROS) intermediates. Mitochondrial dysfunction prompted the release of cytochrome c, translocation of pro-apoptotic protein (Bax), activation of caspase cascade, resulting in the cleavage of some specific substrates for caspase-3 such as poly (ADP-ribose) polymerase (PARP), which eventually leads to apoptosis. The results of present study strongly support further research and development of bioactive constituents from *Inula racemosa* as potential anticancer agent with possible therapeutic implication.



## 1. Introduction

Drug discovery from medicinal plants has played an important role in the treatment of cancer, over the last half century (Newman et al., 2003; Butler, 2004). The herbal medicines, botanicals, dietary supplements, and edible plants have been used in cancer chemoprevention and treatment (Park and Pezzuto, 2002; Kinghorn et al., 2004). *Inula racemosa* Hook.f. commonly known as Pushkarmula (*Compositae*) is a well documented Indian medicinal plant. The plant has been used as a traditional drug in India, China and Europe (Okuda, 1986). Plant extract is used for abdominal pain, acute enteritis and bacillary dysentery (Tsewang, 1994). Preparations of its roots are used in indigenous medicine as expectorant, tonic (Jabeen et al., 2007), and in folk medicine of several ethnics against a variety of ailments including asthma, (whooping) cough, bronchitis, lung disorders, indigestion, chronic enterogastritis, infectious and helminthic diseases (Cantrell et al., 1999; Dachler and Pelzmann, 1999; Konishi et al., 2002). In addition, roots of the plant are widely used in the treatment of cardiovascular diseases and tuberculosis (Patel et al., 1982; Tripathi et al., 1988; Singh et al., 1993).

*Inula racemosa* produces a wide array of sesquiterpenoid, especially sesquiterpene lactones including eudesmanolides, guaianolides, and germacranolides (Guo and Yang, 2005) as their main secondary metabolites (Bohlmann et al., 1978; Muhammad et al., 2003) and essential oils (Srivastava et al., 1971; Bohlmann and Zedero, 1977; Liu and Tan, 1994). The phytochemical investigation of plant showed the presence of alantolactone, isoalantolactone, dihydroalantolactone, dihydroisoalantolactone, sitisterol, daucosterol, inunolide, aplotaxene, phenyllacetonitile and isoinunal (Wang et al., 2000). Two major constituent alantolactone and isoalantolactone possess anti-fungal and anithelmintic activities (Satyavati et al., 1987; Tripathi et al., 1988; Tan et al., 1998). The anti-ulcer drug Alanton, consisting of a mixture of the alantolactones is well known to industry. Moreover, sesquiterpene lactones also possess a wide spectrum of biological activities including, anti-inflammatory, fungicidal (Hwang et al., 1996; Cohen et al., 2002) and anticancer properties (Cho et al., 2004; Zhang et al., 2005).

In the present study, 95% ethanolic extract of *Inula racemosa* roots and its four fractions viz, *n*-hexane, chloroform, *n*-butanol and aqueous were evaluated for their *in vitro* cytotoxic potential against human cancer cell lines of various tissues such as colon, ovary, prostate, lung, CNS and leukemia. Further studies on *n*-hexane fraction treated HL-60 cells revealed that it induces

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apoptosis through intrinsic (mitochondria-mediated) as well as extrinsic (death receptor-mediated) pathways by generating reactive oxygen species (ROS) intermediates. Mitochondrial dysfunction prompted the release of cytochrome *c*, translocation of pro-apoptotic protein (Bax), activation of caspase cascade, resulting in the cleavage of some specific substrates for caspase-3 such as poly (ADP-ribose) polymerase (PARP), which eventually leads to apoptosis.

## 2. Material and methods

### 2.1. Chemicals and antibodies

RPMI-1640 medium, 2′,7′-dichlorofluoresceine diacetate (DCFH-DA), Rhodamine-123 (Rh-123), propidium iodide (PI), sulphorhodamine B (SRB), dimethyl sulphoxide (DMSO), DNase-free RNase, proteinase-K, phenylmethanesulfonyl fluoride (PMSF), eukaryotic protease inhibitor cocktail, triton-X100, penicillin, streptomycin, L-glutamine, pyruvic acid, camptothecin, Fetal bovine serum and AnnexinV-FITC apoptosis detection kit were purchased from Sigma Aldrich, USA. Tris-base, EDTA and phosphate buffered saline (PBS) were purchased from HiMedia Laboratories Pvt. Ltd., India and trichloroacetic acid was from Merck Specialties Pvt. Ltd., India. Caspase-3, -6, -8 and -9 assay kits were from BioVision, Inc., USA. FITC mouse anti-cleaved PARP (ASP214) antibodies and mouse anti-human antibodies to cytochrome *c* were from BD Biosciences, Pharmingen, USA. Mouse anti-human antibodies to Bax, β-actin, TNF-R1, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were from Santa Cruz Biotechnology, USA. Electrophoresis reagents, protein estimation kit and protein markers were from Bio-Rad Laboratories, USA. Hyper film and ECL Plus western blotting detection kit were from Amersham Biosciences, UK.

### 2.2. Extraction, fractionation and isolation of alantolacotne and isoalantolactone

Roots of *Inula racemosa* were collected from Jammu and Kashmir State of India in the month of July after identification and authentication by Dr. S.N. Sharma, taxonomist of the Indian Institute of Integrative Medicine (CSIR), Jammu. A specimen (accession no. 17351) was also deposited in the herbarium of the institute. Dried and powdered roots were placed in a conical glass percolator, submerged with 95% ethanol and kept overnight at room temperature for 20 h. The percolate was collected and filtered. Ethanol was distilled off using rotavapour under reduced pressure at 50 °C. The final drying was done initially in a vacuum desiccator and finally in lyophilizer to get dried ethanolic extract (yield: 13% of dry wt).

Fractionation of *Inula racemosa* root extract was performed according to Li et al. (2007) with some modifications. The dried ethanolic extract (10 g) was taken in a stoppered conical flask, vigorously shaken with 500 ml *n*-hexane and allowed to stand for 30 min. The supernatant was decanted. Procedure was repeated four time using fresh *n*-hexane every time. The combined *n*-hexane soluble portion was evaporated to dryness under reduced pressure below 50 °C. The residue left after removing the *n*-hexane soluble part was further macerated with 500 ml chloroform and extraction was repeated for four times and combined chloroform soluble portion was evaporated to dryness. The residue left after removing *n*-hexane and chloroform soluble part was further suspended in 500 ml water. Suspension was taken in a separating funnel and extracted with 500 ml *n*-butanol four times. The combined *n*-butanol fraction was evaporated to dryness. Water soluble fraction was filtered, centrifuged at 1000 rpm for 20 min and finally dried by freeze dryer. All fractions (% yield: 3.55% *n*-hexane fraction, 2.40% chloroform fraction, 3.41% *n*-butanol fraction and 6.12% aqueous fraction) were transferred to air tight glass container. Nitrogen was blown in the container before capping and stored at −20 °C in desiccators unlit used.

The *n*-hexane fraction was extracted further with petrol at room temperature and concentrated under reduced pressure which, upon cooling, yielded solid alantolides. Mass spectroscopic, m.p. and NMR data were similar as reported earlier for alantolacone and isoalantolactone (Marshall and Cohen, 1964; Kaur and Kalsi, 1985; Tan et al., 1998).

### 2.3. Preparation of standards and sample

Accurately weighed 1 mg of each alantolactone and isoalantolactone were dissolved in 1 ml of HPLC grade methanol. Equal volume of the solution of alantolactone and isoalantolactone were mixed to give 0.5 mg/ml of the each of the standard in the mixture. The stock solution was stored at 4 °C. From the stock solution working solutions were prepared when required. Sample was prepared by dissolving *n*-hexane fraction in HPLC grade methanol. The solutions were filtered through a Millipore filter (0.22 μm) before injection into the HPLC system.

### 2.4. Liquid chromatography–electrospray mass spectrometry analysis

The LC separations were achieved using RP-18, Merck (4 × 250 mm, 5 μm) column. The mobile phase consisted of water:acetonitrile (45:55) delivered at a flow rate of 1 ml/min. The samples were analysed at 30 °C to provide efficiency to the peaks. The UV chromatograms were recorded at 205 nm. All the interface parameters were optimised by injecting standard solutions of the alantolactone and isoalantolactone during the LC–MS experiments. The conditions for mass spectrum analysis during the LC–MS studies were set at dry gas flow of 11 l/min, nebulizer pressure 35 psi, and drying gas temperature was at 300 °C. The mass range was from 50–500 m/z, ICC target value 8000, while the maximum accumulation time was 200 ms.

### 2.5. HPLC-DAD–MS analysis of the n-hexane fraction

Quantification of alantolactone and isoalantolactone in *n*-hexane fraction was performed as described by Huo et al. (2010). Excellent calibration curves were obtained for alantolactone ($r^2 = 0.99999$) and isoalantolactone ($r^2 = 0.99998$). The linear regression equations were: $y = 2.58305x + 0.887777$ and $y = 2.60465x - 14.53690$ for alantolactone and isoalantolactone, respectively. Fig. 1 shows the LC UV (DAD) chromatogram of alantolactone and isoalantolactone and *n*-hexane fraction at 205 nm. From the HPLC-MS results, *n*-hexane fraction from *Inula racemosa* roots extract was found to contain 19.28% alantolactone and 37.06% isoalantolactone.

### 2.6. Cell culture and treatment

The human cancer cell lines Colon (Colo-205, SW-620), ovary (IGR-OV-1, OVCAR-5), prostate (DU-145, PC-3), lung (A-549, Hop-62), CNS (SK-*n*-SH, SF-295) and acute promyelocytic leukemia cell line (HL-60) were obtained from National Cancer Institute, Frederick, USA. Cells were grown and maintained in RPMI-1640 medium, pH 7.4 supplemented with FCS (10%), penicillin (100 Units/ml) and streptomycin (100 μg/ml). The cells were grown in $CO_2$ incubator (Hera Cell, Heraeus, Germany) at 37 °C with 90% humidity and 5% $CO_2$. Human PBMC were prepared from the blood of normal human by using Sigma's Histopaque-1077 and 1119 solution as described earlier (English and Andersen, 1974; Bhushan et al., 2007). Stock solution (20 mg/ml) of ethanolic extract and its frac-