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Phytoestrogenic effect of *Inula racemosa* Hook f – A cardioprotective root drug in traditional medicine

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ABSTRACT

Ethnopharmacological relevance: Roots of *Inula racemosa* are used as a cardio protective in Ayurveda in India, being prescribed as a medicine for precordial chest pain, cough and dyspnoea, both singly and as a poly herbal.
Aim: Evaluation of Phytoestrogenic activity of the root extracts of *Inula racemosa* and compounds isolated therefrom *in vivo*, *in silico* and *in vitro*.
Materials and methods: Alcohol (IrA) and hexane (IrH) extracts characterized by HPTLC/GC-MS analysis respectively and processed for compound isolation were evaluated for estrogenic activity (100 & 250 mg/kg bw) by the Immature rat uterotrophic assay using ethinylestradiol (EE −30 μg/kg bw) as standard drug. Alantolactone (ALT), Isoalantolactone (IALT) and Stigmasterolglucoside (SG) isolated from the extracts were characterized and screened *in silico* for ERα, ERβ binding affinity, assessed *in vitro* for growth modulatory effects on MCF-7 cells by MTT assay and cell cycle distribution analysis using Flow cytometry. RT-PCR analysis evaluated the mRNA expression of pS2 in these cells post exposure to ALT, IALT and SG.
Results: In the IrA treated groups there has been a statistically significant increase ($P < 0.05$) in absolute and normalised uterine weight, uterine diameter, endometrial thickness, luminal epithelial cell height,diameter of ovary and in the number of primary and secondary ovarian follicles relative to untreated controls. Presence of ciliated epithelial cells in the oviduct, elevated number of early growing follicles characterized by an increased oocyte diameter, and signs of vascularization in the cortex of ovarian sections in this group relative to EE treated group are indicative of pervasive activation of follicular growth and initiation. Virtual docking demonstrated ERα affinity for IALT, ERβ affinity for ALT, while SG showed a high binding affinity to both with a relatively greater ERβ binding affinity. Dose dependent decrease in cell viability mediated by IALT and SG in the MTT assay is corroborated by a statistically significant increase ($p < 0.05$) in sub G0-G1 cells by SG at 200 and 400 μM in cell cycle analysis and there has been an induction of pS2 by IALT and SG in the ER regulated MCF-7 cells.
Conclusions: Demonstration of classical morphological changes induced by estrogen stimulation mediated by IrA *in vivo* at both the tested doses, isolation of the antioxidant SG from IrA and its dose dependent growth inhibitory effect on estrogen sensitive MCF-7 cells through apoptotic induction and an up regulation of pS2 are suggestive of an anti-estrogenic effect through estrogen receptor binding affinity, typical of phytoestrogens that bind to ER but do not elicit a full estrogenic response. The observed estrogenic effect of IrA suggests a multi mechanistic molecular action involving antioxidant as well as redox signalling pathways acting in consonance with their anti-estrogenic effects owing to the weak estrogen like competitive receptor binding of SG.

Abbreviations: IrA, alcohol extract of *Inularacemosa*; IrH, hexane extract of *Inularacemosa*; EE, Ethinylestradiol; ER, estrogen receptor; Na-CMC, Sodium carboxy methyl cellulose; SG, Stigmasterol-3-O-β-D-glucopyranoside; ALT, Alantolactone; IALT, Isoalantolactone; RCSB-PDB, Research Collaboratory for Structural Bioinformatics - Protein Data Bank

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1. Introduction

Vascular protective effects of estrogen are well established (Farhat et al., 1996). These are reportedly mediated indirectly, by an effect on lipoprotein metabolism and by a direct effect on the vessel wall. Functionally competent estrogen receptors have been identified in vascular smooth muscle cells and specific binding sites have been demonstrated in the endothelium (Losordo et al., 1994). Estrogen is known to protect against the development of diet induced atherosclerosis in rats (Moskowitz et al., 1956) and rabbits (Constantinides et al., 1962).

Phytoestrogens are a group of plant-derived estrogen analogues whose dietary intake is associated with a reduction in risk of cardiovascular diseases (Murkies et al., 1998). In model systems they have been shown to be anti-estrogenic competing for estradiol at the receptor complex, yet fail to stimulate a full estrogenic response after binding to the nucleus. The profound physiological effects of phytosterols, isoflavones, lignans and coumestans – the known classes of phytoestrogens, have triggered an exponential expansion of literature on their possible therapeutic effects.

Inularacemosa Hook f (Asteraceae), commonly known as 'pushkarmool' in India is mentioned in the ancient Ayurvedic treatise '*Charaka samhita*' (Charak, 1941) as a medicine for precordial chest pain, cough and dyspnoea (Arora et al., 1980). The root powder reportedly reverses exercise induced ST-T changes in electrocardiographs of patients with ischaemic heart disease and possesses adrenergic beta blocking activity (Tripathy et al., 1988). The aqueous extract is anti-anginal (Tripathy et al., 1984), hypoglycaemic (Gholaps and Kar, 2003) and anti-asthmatic (Sekhar et al., 2003). Its anti-oxidant and anti-atherosclerotic activity in guinea pigs has been reported by our group (Mangathayaru et al., 2009). The plant is a rich source of sesquiterpene lactones of which alantolactone (ALT) and isoalantolactone (IALT), occurring in the ratio of 4:6 are the major alantolides. Dihydroalantolactone, dihydroisoalantolactone, alloalantolactone, inunolide, dihydroinunolide, neoalantolactone and isoalantodiene are a few of the minor alantolides. Several simple phenolics such as phenyl acetonitrile and phenyl ethanol have been reported from the roots (NISCAIR, 2002).

In view of the ethnopharmacological usage of the root as a cardio protective, reported presence of sesquiterpene lactones, known for a wide variety of biological activities, structural similarity of major alantolides to phytoestrogenic lignans and reported anti atherosclerotic activity, a vascular protective action mechanism due to a phytoestrogenic influence is hypothesized. Thus it was proposed to evaluate the phytoestrogenic activity of the root extracts and the compounds isolated from them. Estrogenicity being a determinant of their estrogen receptor (ER) binding affinity, isolated compounds were screened *in silico* for ERα and ERβ docking and assessed for their growth modulatory effects on estrogen receptor positive MCF-7 human breast cancer cell through MTT assay and cell cycle distribution analysis by flow cytometry. Being sensitive to estrogen stimulated pS2 transcription, MCF-7 cells treated with the isolated molecules were evaluated for its mRNA expression by RT-PCR.

2. Materials and methods

2.1. Plant Material and extract preparation

Authenticated roots of *I. racemosa* were obtained from, National Research Institute for Sowa Rigpa (Amchi) Research Centre, Leh-Ladakh, India in May 2005, and a voucher sample was deposited in the herbarium of Sri Ramachandra University (No: IR/17/23.05.05). They (2.5 kg) were cut into small pieces, shade dried for 7 days, extracted with hexane (4 × 1500 mL) by cold maceration and vacuum distilled to an yellowish oily mass (IrH). The hexane exhausted marc was air-dried, similarly extracted with methanol (3 × 2500 mL) and the same vacuum distilled to yield a syrupy brownish mass (IrA).

2.2. Chemicals

Ethinylestradiol (EE > 99% purity) was purchased from Sigma (St Louis, USA). Inulin (92–95% purity) was purchased from Aumgene Biosciences Pvt Ltd (Gujarat, India). Precoated silica gel plates $60F_{254}$ of 0.2 mm thickness were from E Merck (Mumbai, India). Silica gel G 60–120 mesh for column chromatography was from SISCO Research (Mumbai, India). DMEM, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS),trypsin, penicillin and streptomycin were purchased from Hi-Media (Mumbai, India). Dimethyl sulphoxide (DMSO), 3-(4,5-Dimethyl thiazol-2yl) − 2,5-diphenyltetrazolium (MTT) and Propidium Iodide were purchased from Sigma-Aldrich (St. Louis, USA). All other solvents and chemicals were of analytical grade.

2.3. Phytochemical processing and characterization

2.3.1. HPTLC analysis of IrA

The methanolic extract of *Inula racemosa* was standardized for inulin using HPTLC analysis. The sample and inulin standard solutions were applied on pre-coated silica gel G 60 F254 (10 cm × 10 cm with 250 μm thickness, E. Merck) plate in with a Hamilton 100 μL syringe using a Camag Linomat V applicator (automated spray-on applicator equipped with a 100 μL syringe and operated with the settings distance from the plate side edge 15 mm, and distance from the bottom of the plate 10 mm). The slit dimension was kept 6.00 mm × 0.45 mm. Linear ascending development was carried out in 10 cm × 10 cm, Camag twin trough glass Chamber saturated with butanol: acetic acid: water (6.3:2.7:1) as mobile phase. After development, TLC plate was completely air dried at room temperature and derivatized with 20% sulphuric acid reagent. Peak areas for samples and standard were recorded by densitometric scanning at 297 nm, using a CAMAG TLC Scanner 3 with WINCATS version 3.2.1 software. Photo documentation was performed using CAMAG REPROSTAR 3. The data of the peak areas were plotted against the corresponding concentrations. The obtained values were treated by linear regression analysis.

2.3.2. Column Chromatographic processing of IrA

IrA (30 g) was subjected to column chromatography on silica gel (54 × 4.5 cm, ILE India Pvt Ltd) using a step gradient of hexane (600 mL), hexane-$CHCl_3$, 1:1 (900 mL), $CHCl_3$(600 mL), $CHCl_3$–MeOH, 19:1 (1000 mL), 9:1 (500 mL), 4:1 (750 mL) and 1:1 (600 mL) to yield 45 fractions of 100 mL each. These were collected and monitored by TLC. Fractions 28–38 were combined, concentrated (1100 mL, 3.05 g) and separated on silica gel column (60–120 mesh, 47 × 2.7 cm), using hexane (100 mL), Toluene (75 mL), $CHCl_3$ (150 mL), $CHCl_3$-MeOH,19:1 (415 mL), 9:1 (100 mL), 4:1 (200 mL) and 1:1 (100 mL). Fractions 21–34 (350 mL) yielded an amorphous powder (Compound A) upon concentration. It gave a single spot of Rf-0.46 in $CHCl_3$-MeOH (9:1).

2.3.3. Characterization of IrH by GC-MS analysis

GC –MS analysis was carried out on Agilent HP 6890 GC/MS System with 5973 Mass Selective Detector equipped with a HP 5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness), carrier gas – helium (flow rate − 1.3 mL/min), linear velocity 25 cm/s, split ratio 1:100. Column temperature was programmed from 80 to 210 °C at a rate of 15 °C/min, 210 °C to 250 °C at a rate of 5 °C/min, 250 °C to 280 °C at a rate of 15 °C/min and for 9 min at 280 °C. Split injection mode was used for sample injection. Components of the extract were identified by matching their 70 eV mass spectra with those recorded on Wiley MS data library and by comparison with standard published data.

2.3.4. Isolation of major alantolides from IrH

IrH (50 g) dissolved in hexane and allowed to stand overnight gave a colorless crystalline substance, which on recrystallization yielded fragrant needle shaped crystalline solid (Compound B – mp107° C). It answered Nollers test (Tin and thionyl chloride) for terpenes and gave a