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Evaluation of prominent *Isoflavonoids* of *Iris plant* as futuristic cancer drug components

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Abstract
Three important constituents of *Iris* plant - *Iridin*, *Irigenin* and *Tectorigenin*, belonging to the isoflavanoid class of compounds were evaluated for their effectiveness in modulating growth of epithelia cancer cell lines A549 and Caco-2 and their possible effect on signalling molecules affecting cancer progression-EGFR, AKT and K-Ras. While all the compounds were able to limit cancer growth in a dose dependent manner in both A549 and Caco-2 cells, *Tectorigenin* was most effective among them (IC50 *(tectorigenin)* = 7.15µM for A549 and 23.39µM for Caco-2). *Iridin* and *Tectorigenin* were also observed to cause a dose-dependent downregulation of KRAS expression in A549 cells supporting their anti-cancer capabilities. With future evaluation of these compound(s) in a highly specific co-culture model there is tremendous scope of these compounds in drug design and futuristic use.

Keywords: *Iris* plant, isoflavanoid, EGFR, AKT, K-Ras

Introduction
Medicinal plants have played significant as important therapeutic aids for alleviating ailments of humankind [1]. India is endowed with a treasure of world's oldest traditional systems of healthcare. The Indian system of medicine (ISM) is of great antiquity and dates back to about 5000 years B.C. with extensive documentation and an impressive record of safety and efficacy throughout and Kashmir valley in particular [2, 3]. About 40 percent of medicinal plants growing in Kashmir are used in the Indian pharmaceutical industries alone, some of them fetching high prices in the International market [4]. With continued onslaught of killer diseases like cancer, aids and nibbling disease like diabetes and arthritis coupled with side effects of synthetic drugs more and more plants are been screened for their therapeutic aids [5]. Recent drug discovery studies have focused on flavonoids and isoflavonoids of various plants. These are important secondary metabolites with immense medicinal value [6]. These have high structural diversity and are consumed by human as dietary constituents. The role of isoflavones in cancer [7]. Osteoporosis, cardiovascular diseases, menopausal symptoms in addition to their antioxidant [8] antimicrobial [9] anti-inflammatory and estrogenic activities [10] are well documented. It is anticipated that plants can act as potential source of bioactive compounds that can be used in near future for the development of new 'leads' to combat against dread full diseases such as cancer.
Iris plant, a member of the family *Iridaceae* is represented by several wild, cultivated and some endemic species [12]. *Iris* species have been extensively used in traditional medicine for treatment of cancer, inflammation, bacterial and viral infections [13]. While its various constituents have been identified to possess different activities such as antiulcer, antibacterial, anti-inflammatory, piscicidal, antineoplastic, antioxidant, hypolipidemic, and antitubercolosis [13-19]. The phytochemistry of this genus has been the subject of extensive investigation and found to be a rich source of flavones, isoflavones [20, 21] and quinones [22] These classes of compounds have attracted considerable attention because of their antioxidant [23] cytotoxic [24] anti-microbial, anti-inflammatory and phytoesterogenic properties [25, 26] The preventive role of isoflavones in cancer, cardiovascular diseases, osteoporosis, and menopausal symptoms is well documented [27, 28, 29]. In traditional Chinese medicine, it was used as a bitter medicine to treat disorders described as *Zheng Jia Jie Ju*, which are similar to modern descriptions of tumors [30, 31].
Cancer develops when one or more cells lose their ability to control cell division and begin to proliferate in an uncontrolled fashion. The origin of cancer lies in the genetic material of the cell and is a result of the accumulation of mutations that promote clonal selection of cells with an aggressive phenotype. This phenotype is underlined by a rapid proliferation rate and alterations in cell morphology. All oncogenic proteins participate in cellular functions that involve the transduction of signals from the extracellular environment through the membrane receptors into the cytoplasm and towards the nucleus, where transcription is initiated to generate proteins that will eventually contribute to the oncogenic phenotype [32]. Similar to genetic causes, external stimuli is equally important for the initiation and progression of cancers. If environmental factors such as radiation and exposure to carcinogens are excluded, a potential candidate for cancer causation is chronic inflammation [33] Chronic inflammation accounts for 20% of all types of human cancers [34] Population studies have

demonstrated the increase susceptibility of cells to become cancerous when exposed to chronic inflammation.

Taking leads from the work that has already been done in relation to the medicinal properties of plant, anti-neoplastic in particular; the present study aimed to investigate the molecular changes that occur in cancer cells due to the pharmacologically potential compounds purified from selected indigenous species of *Iris* plant from Kashmir valley. The results generated as such are promising and with future evaluation of these compound(s) in a highly specific co-culture model there is tremendous scope of these compounds in drug design and futuristic use.

Material and Methods

Cell lines. Cell lines (A549, Caco2) were kindly provided by Hybridoma Lab., National Institute of Immunology, New Delhi. Cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% heat inactivated fetal calf serum and supplemented with antibiotics penicillin-streptomycin (100U/ml) at 37°C in 5% CO2. The medium was changed every 2 days or until the cells became confluent and then the cells were used for the experimentation.

Buffers and Reagents

Cell culture materials like DMEM, FBS, Antibiotics (Gentamycin) were obtained from PAA, Germany. All other reagents were of molecular biology/ cell culture grade.

Anti-proliferation assay using MTT method

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (e.g. isopropanol) and the released solubilized formazan is measured spectro-photometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. A549 & Caco-2 cells were seeded in 24-well plates (1 × 104 cells/ well). After 24 hrs, various concentrations of pure compounds were added to wells in fresh media. The cells were allowed to grow for 24hrs and 48 hrs after drug treatment. All experiments were performed at least in triplicate.

The Cell growth was evaluated by MTT assay by adding 80µl of MTT solution followed by incubation for 4h in 37^0C in dark. Media was then removed from wells and added with 200µl acidic isopropanol and mixed well. The plate was placed in dark on shaking rotor. 50µl of solution was harvested from 24-well plate and transferred to 96 well plate and read at 570nm (background wavelength is 630nm).

Evaluation of mRNA expression by reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells after 24hrs treatment with *Iridin*, *Irigenin* or *Tectorigenin* using RNazol method (Merck, USA) and quality of RNA was verified by 260/280-nm ratio. 1 µg of RNA was reverse transcribed to cDNA using First Strand cDNA Synthesis kit for RT-PCR (Novagen, USA). The amplification by polymerase chain reaction (PCR) was carried out using 1 µl of cDNA, and 2 µl each of 10 mM oligonucleotides primers for *EGFR, K-Ras, AKT* and *GAPDH*. The PCR products were resolved in 1.5% agarose gel electrophoresis, ethidium bromide stained specific bands were visualised under UV light and photographed. The densitometric analysis of the specific bands was made using Quantity One® 1-D Analysis Software (BioRad, USA) and data are represented as the ratio of the specific gene to GAPDH (Table 1).

Statistical Analysis

Statistical analyses were performed by using statistical program Prism from GraphPad Software, Inc. (San Diego, CA USA). Each of the experiments was performed in triplicates. The results are expressed as Mean ± SD. p value of < 0.05 was considered significant. The inhibitory concentration (IC50) was calculated from dose–response curve obtained by plotting the percentage of inhibition versus the concentrations.

Table 1: Primer sequences for amplification of EGFR, KRAS, AKT and GAPDH

Gene	Primer	Sequence (5'->3')	Length (bp)	Tm (OC)
EGFR	Forward	CTACAACCCCACCACGTACC	1125	61.0
	Reverse	GGGATCTTAGGCCCATTCGT		
KRAS	Forward	CTTGTGGTAGTTGGAGCTGGT	800	61.1
	Reverse	CTAACAGTCTGCATGGAGCAGG		
AKT	Forward	CAGGATGTGGACCAACGTGA	934	62.7
	Reverse	GGTCCTGGTTGTAGAAGGGC		
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC	138	65.3
	Reverse	TGGTGAAGACGCCAGTGGA		

Results

Effect of characterized pure molecules isolated from *Iris* plant rhizomes on the proliferation of A549 and Caco2 cell lines

The pure molecules viz. *Irigenin*, *Iridin* and *Tectorigenin* isolated from *Iris* plant rhizomes were bioassayed for their possible effect on cell proliferation of A549 and Caco2 cell lines. The effect was evaluated on 24hr and 48 hr time points. Each of the three molecules showed a dose dependent effect inhibitory effect on proliferation of A549 and Caco-2. Tectorigenin was most effective with IC50 values of 7.15µM for A549 and 23.39µM for Caco-2 respectively, followed by *Irigenin* (A549: 22.57µM; Caco-2: 39.88µM) and *Iridin* (A549: 68.02; Caco-2 68.62) (Table 2 to 4)

Table 2: Percentage inhibition of A549 and Caco-2 cells treated with *Iridin* at various doses w.r.t control group

DOSE (per ml)	A549						Caco-2					
	24 hour			48 hour			24 hour			48 hour		
Iridin 15µM	7.42	±	1.75	11.73	±	1.88	14.59	±	1.23	20.08	±	1.10
Iridin 30µM	18.53	±	1.69	24.34	±	1.56	22.97	±	1.13	32.11	±	1.26
Iridin 60µM	20.34	±	1.58	43.37	±	1.70	35.16	±	1.89	42.45	±	1.76

N=3. Data is presented as Mean± SEM percentage inhibition w.r.t control group (DMSO treated). Data is analyzed using one-way ANOVA with multiple comparisons using Dunnetts test vs. control group. Values were considered as significant for $p < 0.05$. (Cell conc: 2x103 seeded in 96well plate, PDT: 24hrs)

Table 3: Percentage inhibition of A549 and Caco-2 cells treated with *irigenin* at various doses w.r.t control group

DOSE (per ml)	A549						Caco-2					
	24 hour			48 hour			24 hour			48 hour		
Irigenin15µM	14.43	±	1.78	45.80	±	1.90	18.54	±	1.99	24.32	±	2.11
Irigenin 30µM	37.36	±	1.79	53.69	±	1.67	25.08	±	1.96	42.45	±	1.83
Irigenin 60µM	51.34	±	1.08	75.84	±	1.20	42.82	±	2.85	62.34	±	2.98

N=3. Data is presented as Mean± SEM percentage inhibition w.r.t control group (DMSO treated). Data is analyzed using one-way ANOVA with multiple comparisons using Dunnetts test vs. control group. Values were considered as significant for $p < 0.05$. (Cell conc: 2x103 seeded in 96well plate, PDT: 24hrs)

Table 4: Percentage inhibition of A549 and Caco-2 cells treated with *Tectorigenin* at various doses w.r.t control group

DOSE (per ml)	A549						Caco-2					
	24 hour			48 hour			24 hour			48 hour		
Tectorigenin (15µM)	29.19	±	1.20	50.49	±	1.08	25.06	±	1.36	29.19	±	1.48
Tectorigenin (30µM)	54.87	±	0.94	68.27	±	1.07	44.79	±	2.03	54.87	±	1.91
Tectorigenin (60µM)	66.95	±	2.20	77.07	±	2.08	58.96	±	2.18	66.95	±	2.30

N=3. Data is presented as Mean± SEM percentage inhibition w.r.t control group (DMSO treated). Data is analyzed using one-way ANOVA with multiple comparisons using Dunnetts test vs. control group. Values were considered as significant for $p < 0.05$. (Cell conc: 2x103 seeded in 96well plate, PDT: 24hrs)

Effect of *Iridin*, *Irigenin* & *Tectorigenin* on EGFR and its downstream targets

Majority of the isoflavones derivatives have been reported to alter EGFR signaling thereby modulating growth and survival of cancer cells. Gene expression studies were carried out to study possible modulation of EGFR signaling by the characterized molecules viz. *Iridin*, *Irigenin* & *Tectorigenin*. mRNA levels of EGFR and its downstream targets viz. *AKT* and *K-RAS* were analyzed by Reverse Transcriptase PCR using gene specific primers as described in materials and methods. The effect on gene expression was quantified by densitometry of target genes against GAPDH serving as control. The integrity of RNA was checked before proceeding for gene expression analysis (Figure 1). The effect of *Iridin, Irigenin* & *Tectorigenin* is summarized as below:

- *Iridin* showed a dose dependent inhibition of K-RAS without affecting expression of EGFR or AKT (Figure 2).
- *Irigenin* induced expression of EGFR, AKT and KRAS (Figure 3).
- *Tectoregenin* also induced expression EGFR and AKT while inhibiting KRAS levels (Figure 4)

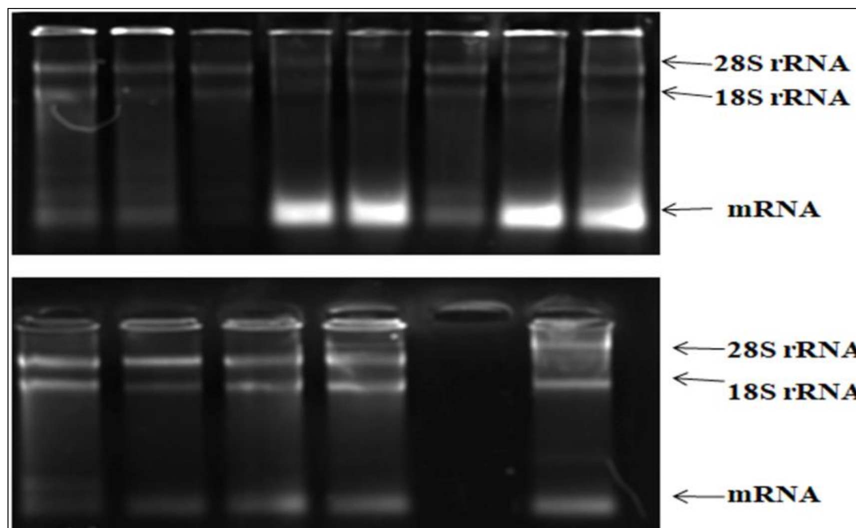

Fig 1: RNA isolation form A549 cells Using from A549 CELLS USING RNAZOL method. Represemttive gel picture showing intergrity of total RNA isolated from A549 cell after 48 hrs of drug treatment

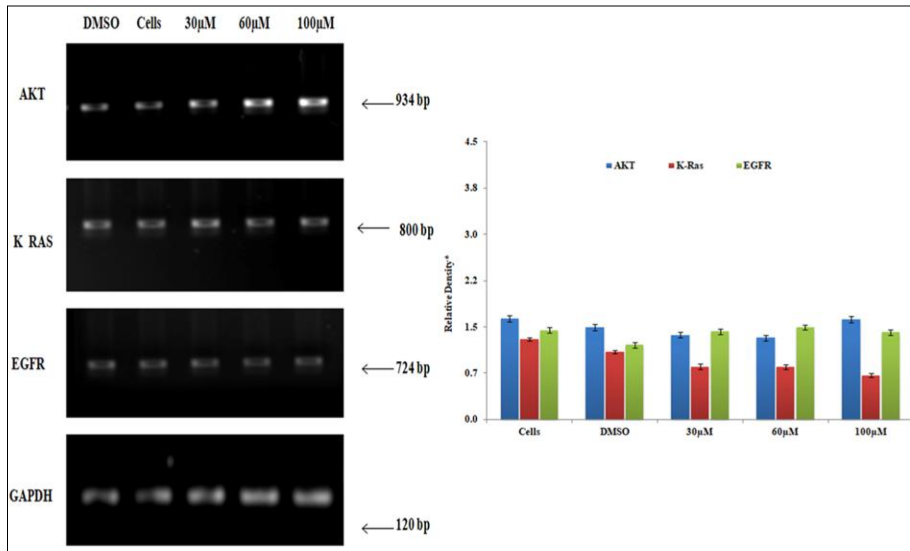

Fig 2: Effect of Iridin on gene expression of EGFR, AKT and K-Ras at specified concentration

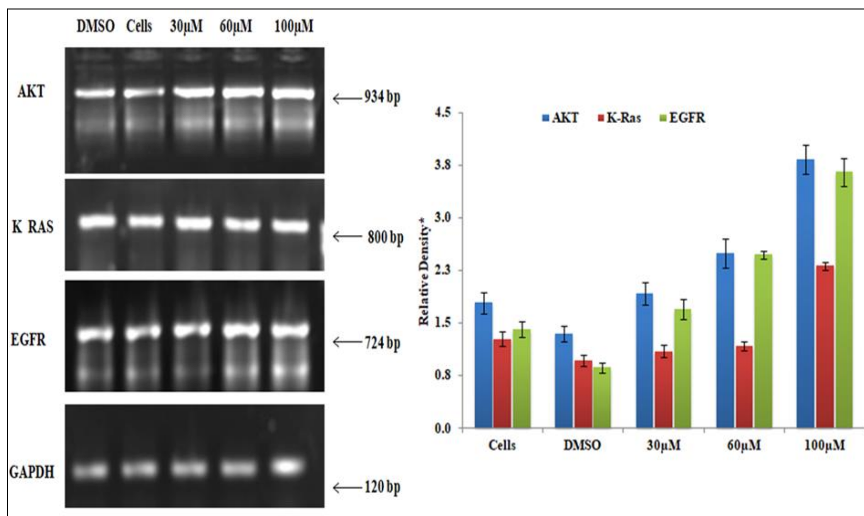

Fig 3: Effect of Irigenin on gene expression of EGFR, AKT and K-Ras AKT specified concentration

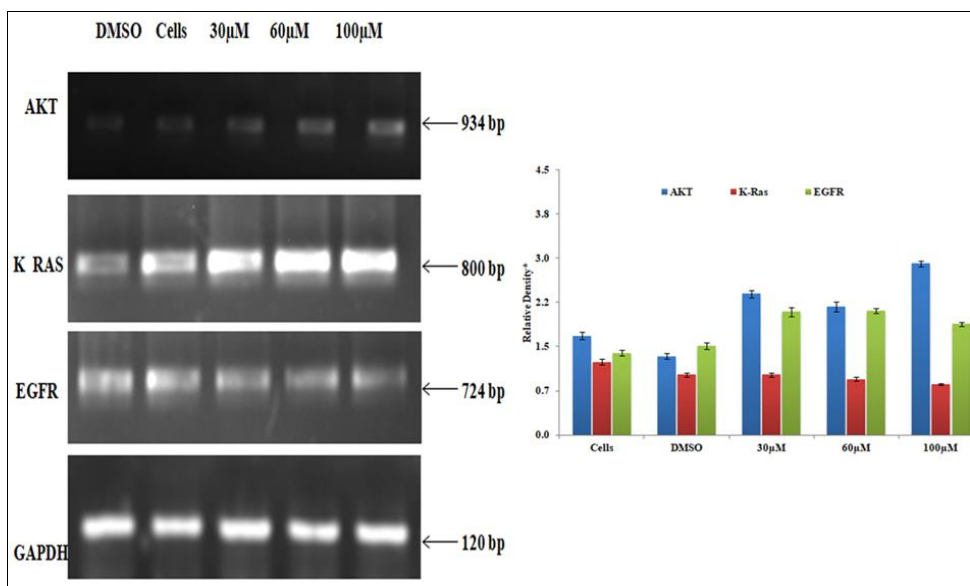

Fig 4: Effect of Tectorigenin on gene expression of EGFR, AKT and K-Ras at specified concentration

Discussion

Iris species are considered as one of the most important medicinal plants owing to their huge reserves of secondary metabolites viz. flavanoids, isoflavanoids, quinones, triterpenoids, flavones and xanthones. The most essential class among these secondary metabolites are those of flavanoids and isoflavanoids-the entities known to have varied medicinal properties ranging from anti-bacterial or anti-inflammatory to anti-cancer activities. The three important secondary metabolites of *Iris* plant rhizome namely *Iridin, Irigenin and Tectorigenin* are well known for their differential activities in various signaling circuits, yet substantial evidences are still vague as far as their mechanism of action is concerned. *Iridin, Irigenin and Tectorigenin,* the naturally occurring *Phytoestrogens* have been reported in modulating EGFR mediated signaling [35]. EGFR has been implicated in many cancers including breast and lung cancers and is regarded as the most important target for cancer prevention. Over-activation and/or dysregulation of EGFR promotes processes involved in tumor progression, including invasion, angiogenesis, metastasis, and resistance to anticancer treatment with blocking apoptosis [36, 37], Activated EGFR leads to activation of the oncogene KRAS, which in turn activates the oncogene BRAF, mitogen-activated protein kinase kinase (MEK), and mitogen-activated protein kinase (MAPK), and leads to expression of growth-promoting genes. In addition to activation of KRAS, EGFR activates the oncogene PIK3CA which phosphorylates phosphatidylinositol-2-phosphate (PIP2) to phosphatidylinositol-3-phosphate (PIP3), which in turn activates AKT and several downstream effectors, leading to cell growth and survival, proliferation, migration, and angiogenesis [38]. Traditionally growth factors, hormones and cytokine receptor signaling networks display PI3K/Akt and Ras/MAPK as two independent, parallel pathways. However, there are multiple crosstalk points between these two pathways, whose coordinated action determines the cell fate. All the three compounds viz. *Iridin, Irigenin and Tectorigenin* showed differential effects on EGFR signaling. While both *Irigenin* and *Tectorigenin* stimulated EGFR and AKT expression, *Iridin* and *Tectorigenin* caused KRAS inhibition. This differential activity of the molecules points towards their differential targets that influence cellular signaling and hence cancer cell survival. Cancer cell survival not only depends on the tumor cell phenotype but is majorly defined by communication between the tumor cells and the surrounding cells—the microenvironment. The tumor microenvironment consists of tumor cells, fibroblasts, leukocytes, bone marrow-derived cells, blood and lymphatic vascular endothelial cells. The interplay between these cell types determines the fate of tumor cell survival and progression. Recent evidence indicates that the microenvironment provides essential cues to the maintenance of cancer initiating cells and to promote the seeding of cancer cells at metastatic sites.

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