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Cytotoxic effects of aqueous and methanolic extracts of *Incarvillea emodi* (Royle Ex Lindl.) Chatterjee on mammalian cells

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Abstract

The present study is designed to investigate the comparative cytotoxic activity of crude aqueous and methanolic extracts of different parts (aerial parts with flowers, roots and aerial parts with fruits) of *Incarvillea emodi* collected from different Himalayan regions in Pakistan, against CHO-K1 (Chinese hamster ovary cell line). In vitro cytotoxic assay was performed by the crystal violet assay. Cells were suspended with trypsin/EDTA in 10 % calf serum. Hemocytometer was used for cell counting. The absorbance was measured using a microplate reader at a wavelength of 562 nm and percentage growth inhibition was calculated. The crude aqueous and methanolic extract (1-200 µg/ml concentration) of *Incarvillea emodi* was taken for cytotoxic activity. At highest concentration i.e. 200µg/ml percentage growth inhibition was recorded above 90% for all the six samples of the plant. The results showed that cell growth is significantly lower in extract treated cells compared to untreated control. Based on the results it is determined that *Incarvillea emodi* is a significant source of biologically active substances that have cytotoxic activity in vitro.

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Introduction

Plants with medicinal properties are considered as vital foundation of expensive drugs. Recently, finding of novel therapeutic agents of plants origin have been targeted. The beneficial effect of compounds which have been derived from the natural sources attribute among other things to the high content of bioactive compounds (Rafter, 2002). Medicinal herbs and their derived phytoconstituents with their cytotoxic effects against different cell lines are the evidences of positive efficacy of medicinal plants for variety of ailments (Uddin *et al*., 2003; Aini *et al*., 2008; Sunilson *et al*., 2009; Siddiqui *et al*., 2010; Aisha *et al*., 2011).

Bignoniaceae is the family of flowering plants in the order Lamiales. Most of the members of this family are woody plants but few are vines, shrubs and even herbaceous plants. Herbaceous members are mostly found at high mountain habitat. One such herbaceous genus is *Incarvillea*.

Incarvillea emodi (Royle Ex Lindl.) Chatterjee is distributed in Afghanistan, Pakistan, Kashmir, Nepal and India from 600-2700 m. It is a perennial plant mostly found in rock crevice with attractive rosy-pink flowers. Among genus *Incarvillea*, *Incarvillea sinensis* is the specie studied to some extent both from phytochemical and pharmacological point of view. The class of compounds studied the most is the alkaloid and among the biological activity antinociceptive activity and antibacterial activity was done (Nakamura *et al.,* 1999a; Chi *et al.,* 1995a, 1997a, 2005a). Little work has been done on *Incarvillea delavayi* (Lu *et al*., 2007) and *Incarvillea arguta* (Luo *et al.,* 2004). *Incarvillea emodi* is being extensively used by the local people of Pakistan for different ailments and especially as anti-diabetic crude remedy.

Compounds derived from several higher plants have exhibited *in vitro* and *in vivo* antitumor activity in the field of anticancer drug research. For cytotoxic activity, plant extracts have been screened and have shown that higher plants possess active anticancer agents which can play an imperative role in chemotherapy and hormonal conduct (Anandakumar and Karmegam, 2011).

As far as the literature is concerned, the comparative cytotoxic activity of *Incarvillea emodi* aqueous and methanolic crude extracts against CHO-K1 cell line are unavailable. Hence, here we report for the first time comparative cytotoxic activity of aqueous and methanolic crude extracts of different parts i.e. aerial parts with flowers (ABD-Ap), roots (ABD-Rt) and aerial parts with fruits (K-Ap) of *Incarvillea emodi* against CHO-K1 cell line, collected from different Himalayan regions in Pakistan.

Materials and methods

Collection of plant material

Incarvillea emodi was collected from Himalayan regions i.e. in village Silhad-Abbottabad and Muzaffarabad-AJK, Pakistan. Identification of plant was done by Dr. Abdul Majid, Lecturer, Department of Botany, Hazara University, Mansehra, KPK, Pakistan and each of plant samples were deposited at the Herbarium of Hazara University with voucher number HUBOT 04707 (Kashmir) and HUBOT 04708 (Abbottabad).

Drying of plant material

Whole plant collected from village Silhad-Abbottabad and the aerial parts including fruits were collected from Muzaffarabad-AJK. Drying was done by shad dried method.

Preparation of water/aqueous extract

Extraction was done in methanol. After complete drying in rotary evaporator, crude methanolic dried plant extract was taken in round bottom flask and small amount of distilled water was added and dissolved. This slurry was then put to separating funnel and petroleum ether was added to this for complete removal of chlorophyll. After removal of chlorophyll, the aqueous extract was transferred to round bottom flask and again rotary evaporator was started for getting complete dry aqueous extract. The dried aqueous extract was then lyophilized and stored for further biological activities.

Cytotoxic activity

Preparation of stock solutions of extracts

For cytotoxicity evaluation, 5 mg of each extract was taken into a labeled sterile tube. Samples were dissolved in 62.5µl of 100% ethanol then added 62.5 µl of water (concentration= 40 mg/ml in 50% ethanol). 2 ml of sterile DME was placed in a labeled sterile plastic tubes and added 20 µl of extract solution to it (concentration= 400µg/ml in DME with 5% ethanol). Following concentrations were used i.e. 1, 2, 5, 10, 20, 50, 100 and 200 µg/ml and all experiments were done in triplicates.

Cell line

Cultures were thawed for CHO-K1 cell line and passaged it in Dulbecco's modified Eagle's (DME) medium in 10% calf serum.

Inoculum

Cells were suspended with trypsin/EDTA in 10 % calf serum in DME by washing the cultures with sterile medium, covering with sterile EDTA in Puck's saline/trypsin, drawing off the releasing solution, suspending the cells in 10 % calf serum in DME by vigorous pipetting. The cells were counted on a hemocytometer and the inoculum was diluted to add 100 µl of 2 x 10^4 cells/ml to each well.

Fixing and staining

The wells were filled with formal saline, added gently by allowing it to flow into the wells. Trays were fixed for 30 minutes and then washed under tap water. Each well was stained with 0.5 % crystal violet in 20 % aqueous methanol by adding 2-3 drops.

The trays were washed again under tap water to remove unbound stain and kept for drying in room temperature. 100 µl of DMSO was added to each well and rocked to mix. The absorbance was measured using a microplate reader at a wavelength of 562 nm. The percentage growth inhibition (PGI) was calculated using the following formula: % Growth Inhibition = 100 – {(Mean OD of individual test group / Mean OD of control group) × 100} (Shier, 1991).

Results and discussion

Whole plant of *Incarvillea emodi* except fruits was collected from village Silhad-Abbottabad and the aerial parts including fruits of the plant was collected from Muzaffarabad-Azad Jammu and Kashmir (AJK). Roots were detached from the aerial parts and processed separately. Eight concentrations of each crude aqueous and methanolic extract were prepared and tested comparatively against CHO-K1 cell line.

Comparative cytotoxic activity of crude aqueous and methanolic extracts of aerial parts including flowers of *I. emodi* collected from Abbottabad region (ABD-Ap) was evaluated against CHO-K1 cell line. Total 8 concentrations were used ranging from 1-200 µg/ml. Gradual increase in the value of PGI (percentage of growth inhibition) was recorded with increasing concentrations of extract (Fig. 1 & 2). At the highest concentration i.e. 200 µg/ml, aqueous crude extract showed comparatively higher cytotoxic effect (93.99 %) than methanolic crude extract (93.68 %) of *Incarvillea emodi*. There is not marked difference in their activities of aqueous and methanolic extracts. Hafidh *et al.,* (2009) and Bisht *et al.,* (2011) reviewed a wide range of medicinal plants which imply the scope of utilizing plant resources for anticancer drugs. To the best of our knowledge in *Incarvillea emodi* species, there is no cytotoxic work against CHO-K1 cell line. Kpoviessi *et al.,* 2014 evaluated *in vitro* cytotoxic activities of essential oils of four *Cymbopogon* species i.e. *Cymbopogon citratus*, *Cymbopogon giganteus*, *Cymbopogon nardus* and *Cymbopogon schoenantus*. The cytotoxicity tests against the Chinese Hamster Ovary (CHO) cells and the human non cancer fibroblast cell line (WI38) showed that all tested oils and components had a low cytotoxicity (IC_{50} > 50 µg/mL). The only exception was *Cymbopogon citratus* essential oil which was toxic against CHO cells (IC_{50} = 10.63 µg/mL) and moderately toxic against WI38 cells (IC_{50} = 39.77 µg/mL). Citral which is the major component of this oil was also toxic against CHO cells (IC_{50} = 20.62 µg/mL) and moderately toxic against WI38 cells (IC_{50}= 39.48 µg/mL). The second major component i.e. β-pinene was not toxic against these cells (IC_{50} > 50 µg/mL).

Here in this investigation, again the same concentrations (1-200 µg/mL) were used for the comparative cytotoxic activity of crude aqueous and methanolic root extracts (ABD-Rt) of *Incarvillea emodi* against CHO-K1 cell line. In this, highest PGI was recorded for crude methanolic root extract (94.1%) in comparison with aqueous root extract (93.99 %) at the highest concentration i.e. 200 µg/ml (Fig. 3 & 4). In the continue studies of Kpoviessi *et al.,* 2014, this time *in vitro* cytotoxic activity of essential oils and crude extracts from leaves, stems and seeds of *Ocimum gratissimum* Linn. was evaluated against Chinese hamster ovary (CHO) cells and the human non cancer fibroblast cell line (WI38) through MTT assay. Results showed that all tested oils and components had a low cytotoxicity (IC_{50} > 50 µg/mL) except leaves and seeds ethanol extracts obtained in full flowering stage which were cytotoxic against CHO cells (IC_{50} = 18.50 µg/mL and 10.25 µg/mL respectively) and WI38 cells (IC_{50} = 21.40 µg/mL and14.11 µg/mL respectively).

Aerial parts along with fruits of *Incarvillea emodi* (K-Ap) were collected from Kashmir region and tested against CHO-K1 cell line using crude aqueous and methanolic extracts in the concentrations range from 1-200 µg/ml. In comparison, crude aqueous extract of the plant gave PGI (percentage of growth inhibition) i.e. 94.1% which is comparatively higher than the methanolic crude extract which showed 92.85 % (Fig. 5 & 6). Ceschini and Campos (2006) investigated the effect of *Cochlospermum regium* (Mart & Schrank) Pilger aqueous root extract on Chinese hamster ovarian (CHO-K1) cells by the acridine orange/ ethidium bromide (AO/EB) staining method where the extract significantly decreased proliferation of CHO-K1 cells. The results confirm that the root extract of *Cochlospermum regium* has cytotoxic effects on normal cells in vitro. Durga *et al.,* 2013 also designed a study for *in vitro* cytotoxic activity of methanolic extract of whole plant of *Parthenium hysterophorus* L. against CHO-K1 (Chinese hamster ovary cell line) and A-549 (Human lung adenocarcinoma epithelial cell line) using MTT assay method. The results concluded that the plant has potent cytotoxic activity as their IC_{50} values were recorded as 183 µg/ml and 230 µg/ml for A-549 and CHO-K1 cell line respectively.

Incarvillea emodi crude extracts (aqueous and methanolic) are the multifarious mixtures of different constituents and it is hard to engage a definite constituent as reliable for the observed toxicity. According to Galati and O' Brien, 2004, some plants components like flavonoids, tannins and saponins are repeatedly reported in the literature as having hostile effects, in some illustrations acting as noxious molecules and in others being protective.

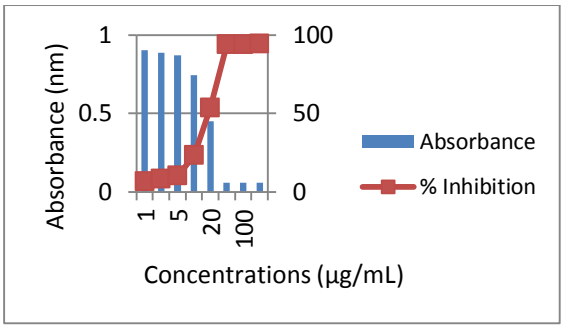

Fig. 1. Cytotoxic effect of crude aqueous extract of aerial parts (ABD-Ap).

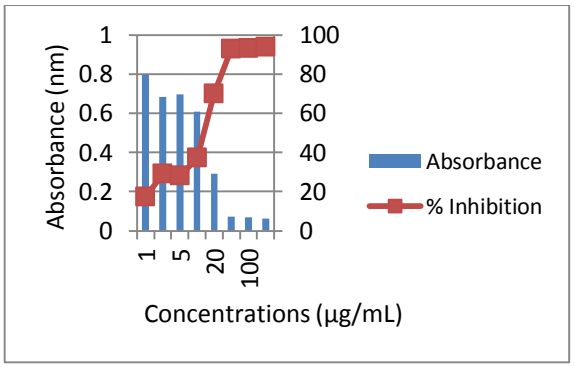

Fig. 2. Cytotoxic effect of crude methanolic extract of aerial parts (ABD-Ap).

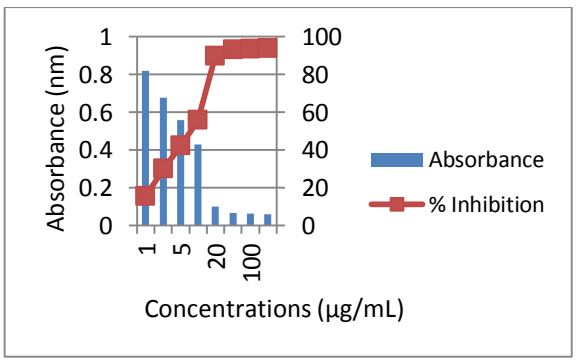

Fig. 3. Cytotoxic effect of crude aqueous extract of roots (ABD-Rt).

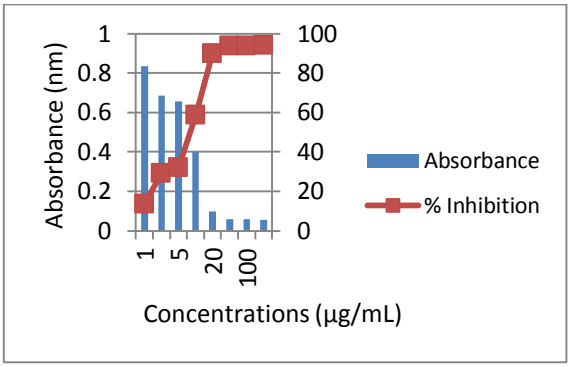

Fig. 4. Cytotoxic effect of crude methanolic extract of roots (ABD-Rt).

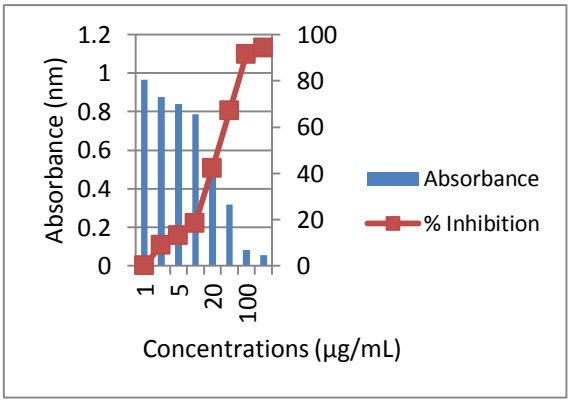

Fig. 5. Cytotoxic effect of crude aqueous extract of aerial parts (K-Ap).

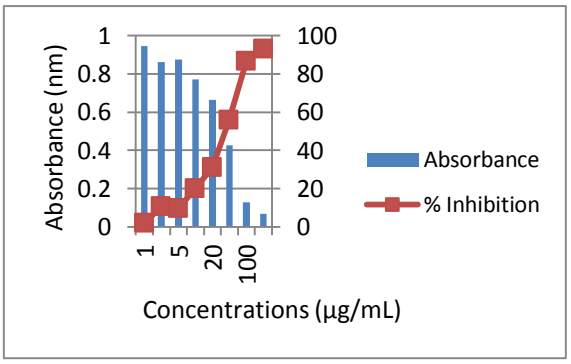

Fig. 6. Cytotoxic effect of crude methanolic extract of aerial parts (K-Ap).

Conclusion

Finally, in our conclusion, it is stated that *Incarvillea emodi* even though it exhibited cytotoxic effect to CHO-K1 cells, needs to be further investigated regarding its effect on tumor and other normal cell lines. It is also necessary to undo its diverse activities and to portray the means of action of isolated compounds.

The overall results showed that both root methanolic and aqueous extract of plant collected from Abbottabad exhibited good cytotoxic activity i.e. 94.1 % and 93.79 % respectively. The antioxidant activity and total phenolic content of plant collected from both regions were conducted. In both cases again root extract (ABD-Rt) showed good activity (PhD thesis, data not published).

All results indicate that all extracts of the plant are significantly active against CHO-K1 cell line. In our continuous studies on *Incarvillea emodi,* collected from Himalayan regions in Pakistan, polyamide column fractions and isolated compounds will be tested against CHO-K1 cell line in the near future.

Conflict of interest

The authors have no conflict of interest to disclose.

Acknowledgement

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