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Research Article

Evaluation of Some Biological Effects of *Incarvillea emodi* (Royle ex Lindl.) Chatterjee and Determination of Its Active Constituents

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Abstract

This present study was an evaluation of the antioxidant and cytotoxic activities of *Incarvillea emodi* (Bignoniaceae). The aqueous extracts of different parts of *Incarvillea emodi,* collected from different places in Pakistan, were tested for radical scavenging effects on 2,2-diphenyl-1-picrylhydrazil (DPPH), nitric oxide (NO), superoxide (SO) and [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] ($ABTS^{\cdot+}$) radicals. DPPH radical scavenging effects of three polyamide column fractions of one extract were also tested. The cytotoxicity of the extracts were tested against Hep-2 (human larynx epidermoid carcinoma) cancer cell line by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. In addition, an HPLC-DAD system was used to show the presence of phenylethanoid glycosides in the most active polyamide column fraction. The free radical scavenging effects of the extracts were found comparable to that of reference antioxidants, 3-*t*-butyl-4-hydroxyanizole (BHA), quercetin and ascorbic acid (AA). Concentration dependent cytotoxic activity was observed against Hep-2 cancer cell line. Two phenylethanoid glycosides, acteoside and leucosceptoside A, were identified in the active polyamide column fraction at HPLC-DAD system. The presence of phenylethanoid glycosides in *Incarvillea emodi* was shown for the first time with this study. Antioxidant and cytotoxic activity researches are important for developing new drugs. Our results supported to use of *Incarvillea emodi* as folk medicine due to several biological effects in Pakistan. Moreover, the use of *Incarvillea* genus as a traditional ethnoveterinary medicine in dyspepsia and internal diseases were reported in previous studies.

Keywords: *Incarvillea emodi, Bignoniaceae, Radical scavenging effect, Cytotoxic activity, HPLC, Phenylethanoid glycosides*

Incarvillea emodi (Royle ex Lindl.) Chatterjee'nin Bazı Biyolojik Etkilerinin Değerlendirilmesi ve Aktif Bileşiklerinin Tayini

Öz

Bu çalışmada, *Incarvillea emodi*'nin antioksidan ve sitotoksik etkileri araştırılmıştır. Pakistan'ın farklı bölgelerinden toplanan *Incarvillea emodi*'nin farklı kısımlarından hazırlanan sulu ekstrelerin radikal süpürücü etkileri, 2,2-difenil-1-pikrilhidrazil (DPPH), nitrik oksit (NO), süperoksit (SO) ve 2,2'-azino-bis 3-etilbenzotiyazolin-6-sülfonik asit ($ABTS^{\cdot+}$) radikallerine karşı incelenmiştir. Bir ekstrenin üç poliamit kolon fraksiyonunun DPPH radikal süpürücü etkileri de araştırılmıştır. Ekstrelerin sitotoksik aktiviteleri, MTT [3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür] yöntemi kullanılarak Hep-2 (insan larinks epidermoit karsinoma) kanser hücre dizisine karşı saptanmıştır. Ayrıca, en etkili poliamit kolon fraksiyonunda, feniletanoit glikozitlerinin varlığını göstermek için bir HPLC-DAD sistemi geliştirilmiştir. Ekstrelerin serbest radikal süpürücü etkileri, referans antioksidanlar, 3-*t*-butil-4-hidroksianizol (BHA), kersetin ve askorbik asit (AA) ile karşılaştırılabilir seviyede bulunmuştur. Hep-2 kanser hücre dizisine karşı konsantrasyona bağlı sitotoksik aktivite gözlenmiştir. HPLC-DAD sistemine uygulanmış etkili poliamit kolon fraksiyonunda, akteozit ve lökoseptozit A isimli 2 feniletanoit glikozitinin bulunduğu tespit edilmiştir. *Incarvillea emodi*'de feniletanoit glikozitlerinin varlığı ilk kez bu çalışma ile gösterilmiştir. Antioksidan ve sitotoksik aktivite araştırmaları ilaç geliştirme çalışmalarında önemlidir. Çalışmamızın sonuçları, *Incarvillea emodi*'nin, Pakistan'da çeşitli biyolojik etkileri nedeniyle halk arasında kullanılışını desteklemektedir. Ayrıca, *Incarvillea* cinsinin dispepsi ve iç hastalıklarda geleneksel etnoveteriner ilaç olarak kullanımı da daha önceki çalışmalarda belirtilmektedir.

Anahtar sözcükler: *Incarvillea emodi, Bignoniaceae, Radikal süpürücü etki, Sitotoksik aktivite, HPLC, Feniletanoit glikozitleri*

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INTRODUCTION

Plants have been used as medicines by human for almost 60.000 years [1]. Different parts of plants and plant derived constituents have been used as a medicine for prevention and therapy of the diseases. For these purposes biologically active constituents that are mainly alkaloids, flavonoids, tannins and other phenolic compounds are used [2,3]. The anticancer potential of plant derived nutritive and non-nutritive constituents have been proved in different *in vitro* and *in vivo* models [4-6].

The genus *Incarvillea* is represented by 16 species in the family Bignoniaceae. *Incarvillea* species are spreading out in Central and Eastern Asia. The majority of these plants are growing in higher altitudes of the Himalaya and Tibet. Unlike the majority, other members of Bignoniaceae, which are mostly tropical woody plants, species of *Incarvillea* are herbs from temperate regions [7]. *Incarvillea* genus have been used for the treatment of hepatitis, diarrhea, febrifuge, dyspepsia and contagious diseases [8]. As well as therapeutical use of plants for human health, plants are source for the treatment of diseases in animals. Ethnoveterinary studies report the use of *Incarvillea* genus as a traditional ethnoveterinary medicine. In Ruoergai region, Sichuan province, China, *Incarvillea compacta* was reported as a medicine in dyspepsia and internal diseases of animals [9].

Herbal medicines derived from genus *Incarvillea* are used for the treatment of rheumatism and relieve pain. Previous studies on the *Incarvilllea* resulted determination of its neurotrophic, antiinflammatory, antinociceptive and anti-hepatitis activities. On this genus, isolation studies resulted with the determination of some secondary metabolites alkaloids, ceramides, iridoids, flavonoids and triterpenes [10].

Incarvillea genus is represented by 16 species of family Bignoniaceae and *I. emodi* is a rare wild attractive plant of family, distributed in Afghanistan, Pakistan, Nepal and India [11]. Bignoniaceae family is placed in super order Lamiae which are very well known for the synthesis of iridoids. In a study according to Rana *et al.*[12] two major compounds with a structure of iridoid glycoside have been isolated from the aerial parts of *I. emodi*. One of the compounds was plantarenaloside with neurotrophic effect and other one boschnaloside with an antibacterial activity [13,14]. There are only a few studies on titled plant.

The aim of this study was to determine antioxidant potential against different free radicals and cytotoxicity through human larynx epidermoid carcinoma (Hep-2) cancer cells, along with HPLC-DAD analysis of active fractions obtained from *I. emodi*.

Collection of the plant material was done from two different regions, Abbottabad and Kashmir in Pakistan. Methanolic extracts of the aerial parts (ABD-Ap) and the roots of plant (ABD-Rt) collected from Abbottabad and the aerial parts of plant collected from Muzaffarabad-Azad Jammu & Kashmir (AJK-Ap) were prepared separately. Aqueous part of each methanolic extract and polyamide column fractions of ABD-Ap were examined for their radical scavenging effects using 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), super oxide (SO) and 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) radicals spectroscopically. To determine cytotoxicity ABD-Ap and K-Ap were evaluated against Hep-2 cells. The most active fraction of polyamide column were compared by HPLC-DAD system with authentic compounds, this comparison gave information about the phenylethanoid glycoside contents.

MATERIAL and METHODS

Plant Material

Incarvillea emodi (Royle ex Lindl.) Chatterjee was collected from Himalayan regions i.e. in village Silhad-Abbottabad (ABD) and Muzaffarabad-Azad Jammu & Kashmir (AJK), Pakistan. Identification of the plant was done by Dr. Uzma Khan (Hazara University, Pakistan) and each of the plant samples were deposited at the Herbarium of Hazara University, Mansehra, Pakistan [Vouchers No. HUBOT 04707 and HUBOT 04708].

Whole plant was collected from village Silhad-Abbottabad in flowering period and seperated to aerial parts (ABD-Ap) and roots (ABD-Rt). The aerial parts were collected from Muzaffarabad-AJK in fruiting period (AJK-Ap). The plant parts were dried, powdered and then methanolic extracts were prepared.

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), ascorbic acid were obtained from Sigma-Aldrich Chem Co (St. Louis, MO). 3-*t*-butyl-4-hydroxyanizole (BHA) was purchased from Nacalai Tesque Co. (Kyoto, Japan), polyamide (50-160 µm) from Fluka (Seelze, Germany), TLC plates (60 F_{254}) from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), minimal essential medium with Earl's salts (MEM-EARLE) with nonessential amino acids, 1% antibiotic solution (penicillin and streptomycin) and trypsin solution (1:250) were purchased from Biochrom AG (Berlin, Germany). Hep-2 (human larynx epidermoid carcinoma) cell line was kindly provided by Refik Saydam Hygiene Center, Virology Laboratory, Ankara, Turkey.

Extraction of Plant Material

Extraction was done by maceration of 400 g powdered plant material in methanol and keeping this material at room temperature for seven days, followed by filtration. The residues were again macerated in methanol for

additional seven days and filtered thereafter. All collected filtrates were evaporated at 40°C under vacuum and got a greenish thick syrup material termed as "methanolic extract". Weights were noted for ABD-Ap=144.3 g, ABD-Rt=84.6 g and AJK-Ap=47.6 g (Yields: 36%, 21.1%, and 11.9%, respectively).

Preparation of Aqueous Extract

After complete drying in rotary evaporator, dissolution of crude methanolic extract carried out in distilled water (100 mL). After complete mixing, first the plant extract was filtered by cotton and then equal volume of petroleum ether was used to remove chlorophyll (3-4 times). The aqueous extract was evaporated to dryness and then lyophilized to give dry aqueous extract. Lyophilized weights of ABD-Ap, ABD-Rt and AJK-Ap were noted as 41.5 g, 68.4 g and 21.8 g respectively.

DPPH Radical Scavenging Effect

The DPPH radical scavenging effect was evaluated by the discoloration of methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectroscopically; 3-*t*-butil-4-hidroksianizol (BHA) and ascorbic acid (AA) were used as reference compounds. DPPH (50 µL) solution was added to methanol solution (200 µL) of the extracts and fractions with a concentration range of 5-200 µg/mL. After 30 min incubation the absorbance of mixture was measured at 520 nm [15,16]. Results were calculated using the following formula to give inhibition percentage.

% Radical scavenging effect = [(blank abs. - sample abs.)/ blank abs.] ×100

NO Radical Scavenging Effect

For the determination of nitric oxide (NO) radical scavenging effect of the aqueous extract, 60 µL of sample (50-1000 µg/mL) and 60 µL of 10 mM sodium nitroprusside, dissolved in phosphate buffered saline (PBS), were added to each well of the 96 well plate and incubated under light at room temperature for 150 min. To detect the nitrite content an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphtyl ethylene diamine dihydrochloride, 2.5% H_3PO_4) was added. After 10 min, absorbance was measured at 577 nm [17,18]. AA and quercetin were used as references.

SO Radical Scavenging Effect

The method of Elizabeth and Rao [19] was slightly modified by us for the assessment of superoxide radical scavenging effect of samples. Concentrations were used in the range of 10-800 µg/mL. 10 µL of nitro blue tetrazolium (NBT) (1 mg/mL solution in DMSO) and 30 µL DMSO dissolution of the samples were mixed. 100 µL of alkaline DMSO (1 mL DMSO containing 5 mM NaOH in 0.1 mL water) was added and the absorbance was measured at 560 nm. BHA and quercetin were used as references.

$ABTS^{\bullet+}$ Radical Scavenging Effect

The method of Re *et al.*[20] was used to determine trolox equivalent antioxidant capacity by using $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], with slight modification. Briefly, $ABTS^{\bullet+}$ radical cation was produced by a reaction of 7 mM ABTS with 2.45 mM potassium persulphate. The mixture was left in the dark for 16 h at room temperature to carry out the reaction and the final product was used within 48 h. Dilutions of $ABTS^{\bullet+}$ solution was continued with ethanol till to achieve the absorbance of 0.700±0.050 at 734 nm. A mixture of fifty microliters of sample and 1.9 mL of diluted $ABTS^{\bullet+}$ solution was prepared and was left at room temperature for 6 min and decolorization of $ABTS^{\bullet+}$ was measured spectrophotometrically at 734 nm. As a reference, trolox solution (0-200 µg/mL) was used. The results were given as mg Trolox/g dry weight of extract. The TEAC value (Trolox equivalent antioxidant capacity) was calculated from the standard curve of trolox.

Cytotoxic Activity

100 µL of cells (6 x 10^4 cells/mL) were cultured into 96-multi-well plates and cultured for 24 h (in a humidified 5% CO_2 incubator at 37°C) in MEM supplemented with 10% FBS, 1% penicillin-streptomycin solution. Different concentrations of the extract (0-800 µg/mL) was applied to cells and incubated for 48 h. After that, each well were washed and changed by fresh medium. 10 µL of MTT solution (5 mg/mL in PBS) was added and left to stand for 4 h. After that to dissolve formazan crystals, 100 µL of 10% SDS (Sodium dodecyl sulfate) was added to each well. The absorbance was measured at 577/655 nm. Cell viability was calculated using the comparison between the absorbance of treated and nontreated cells (Ratio expressed as percentage) [21]. Nontreated cells were accepted as negative control.

Polyamide Column Chromatography

150 g of polyamide was suspended in 1000 mL of distilled water in a large beaker and was added into the column gently. Aqueous extract of ABD-Ap (41.5 g, dry weight), the richest one in secondary metabolites among the tested extracts, was applied to polyamide column chromatography for fractionation using rising concentrations of methanol in water (0-25-50-75-100%). The fraction eluted with 50% methanol, rich in phenylethanoid glycosides (PC. Fr. 33-38) was applied to HPLC.

High Performance Liquid Chromatography (HPLC)

HPLC was used for analytical purposes. Polyamide column fraction rich in phenylethanoid glycosides (PC. Fr. 33-38) was selected for HPLC studies. For stock solution, 3 mg of fraction was taken in small bottles and dissolved in 3 mL of methanol.

HPLC-DAD system was equipped with Dionex P680 HPLC pump, Dionex ASI-100-automated sample injector, Dionex thermostatted column compartment TCC-100. Analyses were performed on Hichrom-Nucleosil 100-5 C18, 25 cm x 4.6 mm column. Detection was from 200 to 600 nm. A gradient program was used as follows: 35% B in the first 4 min, 30% B during 4-25 min, 45% B at 35 min, then B held at 45% for 20 min. The flow rate was 1 mL/min and the injection volume was 20 µL. Column temperature was at room temperature. UV chromatogram was screened at 330 nm.

Statistical Analysis

Statistical analyses have been computed by IBM SPSS Statistic 23 software. The statistical significance was determined by one-way ANOVA post hoc Dunnett's test. Results of experiments are expressed as mean±standard deviation. The inhibition values were determined using at least three independent experiments.

RESULTS

Three aqueous crude extracts of *I. emodi*, ABD-Ap, ABD-Rt and AJK-Ap were studied for DPPH radical scavenging effect using BHA and ascorbic acid as reference compounds. For extracts and references, concentrations were used in the range 5-200 µg/mL. In comparison, ABD-Ap and AJK -Ap showed 88.3% and 90.5% inhibition respectively while ABD-Rt gave 67.8% at 100 µg/mL concentration *(Table 1)*.

In view of the fact that aqueous extracts showed strong antioxidant activity, the same effects of the three polyamide fractions, rich in phenolics, were also bring into test against DPPH free radicals. At 50 µg/mL concentration, all tested fractions showed strong activity in a range of 87.3-90.2% inhibition *(Table 2)*.

Almost all aqueous extracts of *I. emodi* were showed concentration dependent nitric oxide radical scavenging effect. At 750 µg/mL, ABD-Rt extract showed 71.7% effect which is the highest activity as compared to ABD-Ap extract (63.5%) and AJK-Ap extract (33.1%) *(Table 3)*.

To determine the super oxide radical scavenging effect of *I. emodi*, the same three crude extracts of the plant were used. In this experiment, at 800 µg/mL ABD-Ap extract showed 88.0% inhibition which is the highest activity comparable to the other two extracts of the plant that is ABD-Rt (80.0%) and AJK-Ap (76.7%). All extracts showed concentration dependent radical scavenging effect in the range i.e. 10-800 µg/mL of concentrations *(Table 4)*.

Aqueous extracts of *I. emodi* were tested against $ABTS^{\cdot+}$ radical. The given results have been shown as TEAC i.e.

Table 1. *Comparative DPPH radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition			Control % Inhibition	
	ABD-Ap	ABD-Rt	AJK-Ap	AA	BHA
5	7.38±1.88	13.12±0.99	-	40.38±4.67	39.41±2.27
10	11.99±2.06	17.42±5.45	18.70±1.93	86.97±3.14	63.71±0.41
25	31.38±4.34	18.74±3.31	26.41±3.65	91.06±0.49	86.96±0.92
50	53.00±5.81	37.16±6.69	49.08±5.42	90.97±0.37	89.71±0.42
100	88.27±4.56	67.79±6.75	90.47±1.30	91.47±0.38	89.36±0.72
200	92.15±1.03[b]	90.55±1.32	91.92±0.78[b]	91.17±0.71	89.87±0.33

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir; [b] P<0.05 as compared to positive control 3-t-butyl-4-hydroxyanizole (BHA)

Table 2. *Comparative DPPH radical scavenging effect of selected polyamide column fractions from ABD-Ap[a]. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Polyamide Column Fractions % Inhibition			Control % Inhibition	
	PC. Fr. 19-27	PC. Fr. 33-38	PC. Fr. 44-46	AA	BHA
5	29.46±4.83	18.90±1.76	17.16±3.07	40.39±4.67	39.41±2.27
10	40.20±3.18	36.63±2.23	27.17±3.99	86.97±3.14	63.71±0.41
25	84.14±4.44	83.96±5.87	57.47±5.80	91.06±0.49	86.96±0.92
50	90.20±0.68	89.96±0.92	87.31±0.95	90.97±0.37	89.71±0.42
100	90.39±1.10[b]	89.68±0.37	89.00±0.44	91.47±0.38	89.36±0.72
200	90.09±0.72	90.10±0.54	89.35±0.67	91.17±0.71	89.87±0.33

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad; [b] P<0.05 as compared to positive control 3-t-butyl-4-hydroxyanizole (BHA)

Table 3. *NO radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition			Control % Inhibition	
	ABD-Ap	ABD-Rt	AJK-Ap	AA	Quercetin
50	12.24±5.04	22.05±3.32[b]	8.26±3.54	14.78±3.84	16.74±2.62
100	11.76±4.36	22.89±3.58[b]	23.59±5.37[b]	10.92±2.86	18.59±3.49
250	30.66±3.47[b]	36.70±3.55[b]	24.98±5.37[b]	15.74±2.56	41.80±6.27
500	35.52±4.63[b]	64.07±3.76[b]	36.59±5.02[b]	23.49±1.81	48.71±7.25
750	63.47±3.37[b]	71.69±0.58[b]	33.11±2.14[b]	24.25±2.20	46.71±5.37
1000	69.83±1.06[b]	72.37±0.38[b]	49.82±3.59[b]	28.08±6.55	63.63±1.84

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir; [b] P<0.05 as compared to positive control ascorbic acid (AA)

Table 4. *SO radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/ mL)	Plant Extracts[a] % Inhibition			Control % Inhibition	
	ABD-Ap	ABD-Rt	AJK-Ap	BHA	Quercetin
10	21.38±1.56	16.08±0.83	6.16±4.15	47.59±3.06	55.13±3.09
25	58.30±7.22	31.85±2.68	24.60±8.22	50.25±7.92	64.40±2.85
50	56.19±6.84	45.78±5.99	33.00±2.99	55.75±4.71	73.19±2.68
100	69.59±3.19	58.93±5.57	46.10±0.74	62.60±3.32	81.14±2.82
200	78.08±1.55[b]	66.28±5.92	56.17±0.88	63.71±5.70	87.55±1.33
400	84.51±3.05[b]	75.47±6.19[b]	64.97±0.63	63.96±4.06	91.49±1.70
800	88.02±1.99[b]	79.98±7.46[b]	76.70±1.89	69.98±3.75	91.52±1.61

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir; [b] P<0.05 as compared to positive control 3-t-butyl-4-hydroxyanizole (BHA)

Table 5. *Comparative ABTS radical scavenging effect of aqueous extracts. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition		
	ABD-Ap	ABD-Rt	AJK-Ap
25	1.70±0.43	6.34±0.46	0.24±0.92
50	5.16±0.30	6.49±1.05	2.94±0.43
200	18.99±1.10	21.31±1.26	10.09±0.62
400	41.62±0.86	45.79±2.11	20.03±0.86

[a] ABD-Ap: aerial parts of I. emodi collected from Abbottabad, ABD-Rt: roots of I. emodi collected from Abbottabad, AJK-Ap: aerial parts of I. emodi collected from Muzaffarabad-Azad Jammu & Kashmir

Table 6. *Comparative cytotoxic activity of aqueous extracts against Hep-2 cell line. Results are given as inhibition %, and expressed as mean ± S.D. (n = 3)*

Concentrations (µg/mL)	Plant Extracts[a] % Inhibition	
	ABD-Ap	AJK-Ap
20	10.44±7.78	9.18±3.78
50	12.31±10.77	15.56±4.56[b]
100	55.87±8.79[b]	12.94±8.35
200	95.77±0.74[b]	61.15±7.82[b]
400	96.12±0.69[b]	95.17±0.33[b]
800	95.62±0.40[b]	95.07±0.39[b]

[a] The aerial parts collected from Abbottabad (ABD-Ap) and Muzaffarabad-Azad Jammu & Kashmir (AJK-Ap); [b] P<0.05 as compared to negative control

Trolox Equivalent Antioxidant Capacity (mg Trolox equivalent in 1 g of extract). TEAC value was calculated for all the three aqueous extracts of the plant. ABD-Rt extract had high TEAC value and showed strong $ABTS^{\cdot+}$ radical scavenging effect as comparable to ABD-Ap extract *(Table 5)*. TEAC values of ABD-Ap, ABD-Rt and AJK-Ap were 244.5, 269.4, 115.2, respectively.

Seven concentrations of each crude extract (0-800 µg/mL) were tested for cytotoxicity against cancer cell line (Hep-2). The results of MTT assay were expressed as percent inhibition and IC_{50} values. The results indicated that the plant is significantly active against Hep-2 cell line and the activity was seen as concentration dependent manner. The IC_{50} values of ABD-Ap and AJK-Ap were found as 101.3 and 199.7 µg/mL, respectively. At 200 µg/mL, maximum inhibition calculated for ABD-Ap and AJK-Ap were 95.8% and 61.2% respectively *(Table 6)*.

Polyamide column fraction 33-38 (PC.Fr. 33-38) of aqueous

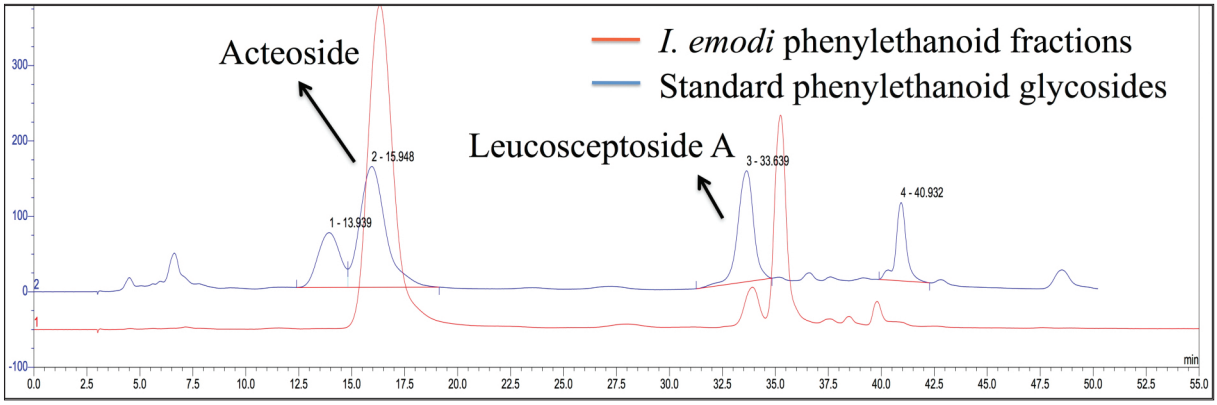

Fig 1. HPLC chromatogram of polyamide column Fr. 33-38 of *Incarvillea emodi* at 276 nm

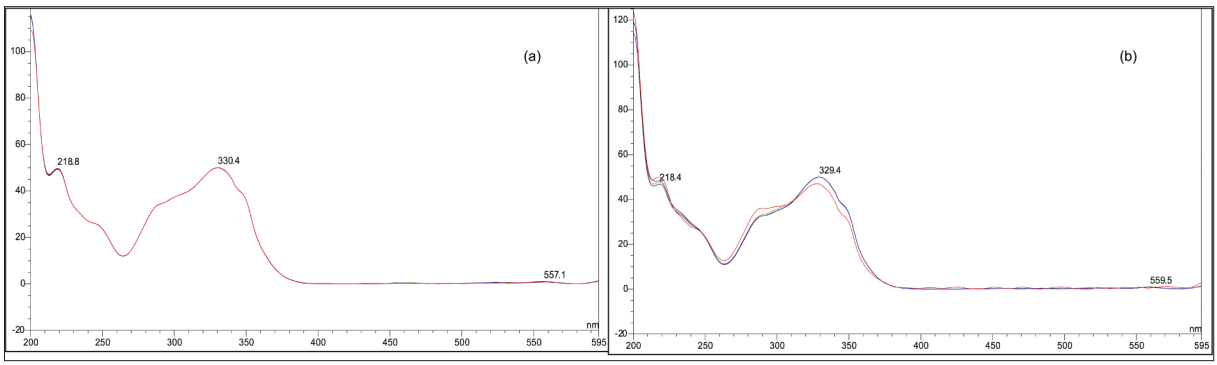

Fig 2. UV spectra of acteoside (a) and leucosceptoside A (b) of PC.Fr. 33-38 and standard compounds (Red Line: Compound of PC.Fr.33-38, Blue Line: Standard compound)

I. emodi extract was tested for the presence of phenylethanoid glycosides using HPLC-DAD system. On application of PC.Fr. 33-38 to HPLC-DAD system, 4 phenylethanoid glycosides were determined in the fraction. In comparison of phenylethanoid glycosides to the standard phenylethanoid glycosides, 2 of them were identified as acteoside and leucosceptoside A. *Fig. 1* depicts HPLC chromatogram of PC.Fr. 33-38 of *I. emodi* at 276 nm showing the presence of both compounds while *Fig. 2* showed UV spectra of acteoside and leucosceptoside A.

DISCUSSION

In countless contrasting diseases, contribution of the free radical intervened cell damage has directed us to establish the antioxidant activity of aq. extracts and three selected polyamide column fractions of *I. emodi* together with its HPLC studies. Aq. extracts were tested against DPPH, NO, SO and $ABTS^{\cdot+}$ radicals. Aerial parts of *I. emodi* aqueous extracts (ABD-Ap and AJK-Ap) were bring into being to be evidence for concentration reliant significant DPPH radical scavenging capacity, where given results were observed extremely close to that of standards (BHA and AA) at 200 µg/mL ($P<0.05$, compared to BHA). Nitric oxide (NO), and super oxide (SO) scavenging effects of aq. extracts were tested in the assortment of 50-1000 µg/mL and 10-800 µg/mL concentration respectively. ABD-Rt crude extract has given stronger NO radical scavenging effect than the standard compounds ($P<0.05$, compared to AA in all tested concentrations). However, SO scavenging effect of ABD-Ap was in much accordance to that of quercetin at 800 µg/mL while BHA showed weakest SO scavenging effect at the same concentration ($P<0.05$, compared to BHA in 200, 400 and 800 µg/mL). TEAC value was also determined for all the three crude aqueous extracts in the range of 25-400 µg/mL and ABD-Rt gave the maximum TEAC value i.e. 269.4 mg trolox/g extract. In comparison of all aqueous extracts, ABD-Ap showed the highest radical scavenging activity against DPPH and SO radicals and highest cytotoxicity. Therefore, aqueous extract of ABD-Ap was choosen for fractionation by polyamide column for further phytochemical investigations. Three polyamide column fractions (PC. Fr. 19-27 25% MeOH, PC.Fr. 33-38 50% MeOH and PC. Fr. 44-46 75% MeOH) were also bring into test of 5-200 µg/mL where PC.Fr. 19-27 and 33-38 were found to show the strongest effect (90.2% and 90.0% inhibition at 50 µg/mL, respectively) against DPPH radical. Rana *et al.*[22], performed their studies on various parts (shoots, roots and flowers) of *I. emodi* using ethyl acetate, butanol and aqueous crude extracts. Higher total antioxidant potential (253.0-384.6 mg/g as trolox equivalent) was recorded for ethyl acetate fraction in all parts which is similar to our results [22].

Cancer is still a frightful disease due to inadequate availability of efficient drugs in cancer therapy. Limitations linked with the present-day chemotherapeutic agents to treat cancer are that they are highly expensive, mutagenic and sometimes even carcinogenic. Their applications are limited [23]. Therefore, efforts are made to isolate and identify anticarcinogens that are naturally present in plants, which can effectively be used to prevent, slow or reverse cancer development. Regarding the cytotoxic activity of two aqueous crude extracts of the plant, ABD-Ap gave comparatively higher percentage growth inhibition (PGI) i.e. 95.8% at 200 µg/mL concentration ($P<0.05$, compared to negative control).

In a previous study, according to Rana *et al.*[22], *in vitro* cytotoxic effects of pure isolated iridoids- plantarenaloside and boschnaloside from *I. emodi* were studied against HCT-15 (colon), Hela (cervix), THP-1 (leukaemia), A549 (lung) and PC-3 (prostate) using sulphorhodamine B (SRB) assay. From the results, it is quite obvious that plantarenaloside is active only against THP-1 (leukaemia) cancer cells, while boschnaloside was found active against THP-1, A-549 and PC-3 [24]. This study supports our present study as we also isolated plantarenaloside from the aq. crude extract of aerial parts of *I. emodi* [24].

In the present study, polyamide column fraction 33-38 (PC. Fr. 33-38) was tested for the presence of phenylethanoid glycosides using HPLC-DAD. Two phenylethanoid glycosides, acteoside and leucosceptoside A, were detected in fraction when compared to the standard phenylethanoid glycosides. There were seen two more phenylethanoid glycosides different from acteoside and leucosceptoside A which could not be identified in our HPLC system. This is the first report for the occurrence of phenylethanoid glycosides in *I. emodi.*

Antioxidant and cytotoxic activity study results are an important basis for drug development studies. The results of our study also clearly highlight the free radical scavenging and cytotoxic secondary metabolites and supports to use of *I. emodi* as folk medicine due to several biological effects in Pakistan.

The presence of phenylethanoid glycosides in *I. emodi* was shown for the first time with this study. Biological activities of the plant may be attributed to the phenylethanoid glycosides such as acteoside and leucosceptoside A, shown in HPLC-DAD analysis.

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Conflict of Interest

No conflict of interest was declared by the authors.

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