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Phytochemical analysis and differential *in vitro* cytotoxicity assessment of root extracts of *Inula racemosa*

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ABSTRACT

The root of *Inula racemosa* is known for its antifungal, hypolipdemic and antimicrobial properties in traditional Indian Ayurvedic and Chinese system of medicine. The biological efficacy of *Inula* species is mainly due to the presence sesquiterpene lactone (Isoalantolactone and Alantolactone), which are reported to be inducers of Nrf2 antioxidant pathway. The investigation of properties and efficacy of root extracts of *I. racemosa* and their comparison was done with a view to find most efficacious extract for use at cellular level (both normal and transformed). In the present study different extracts of root of *I. racemosa* (aqueous, ethanolic, and 50% aqueous-ethanolic) were prepared and compared for their antioxidant potential, reducing capacity, polyphenol content and flavonoid content. Our investigations suggested that the aqueous extract possess highest antioxidant capacity and reducing potential. The polyphenol content was found to be highest in aqueous extract in comparison with other two extracts. However, all the three extracts showed less flavonoid content. Further, the preliminary phytochemical screening of all the extracts revealed the presence of terpenoids, phytosterols and glycosides. The TLC profile of ethanolic and 50% aqueous-ethanolic extracts showed the presence of alantolactone while aqueous extracts did not exhibit its strong presence. This warrants the need of more stringent techniques for characterization of aqueous extract in future. The *in vitro* cell based toxicity assays revealed that the aqueous extract was less toxic to kidneys cells while ethanolic extract was toxic to cells even at low concentrations. Hence, the current investigations showed better efficacy of the aqueous extract with respect to other extracts and found to be promising for its future application at *in vitro* levels.

1. Introduction

The Indian *trans*-Himalayan region of Ladakh (Jammu & Kashmir, India) is rich in ethnobotanical wealth and is known as reservoir of diverse medicinal and aromatic plants. It is one of the global biodiversity hotspots and spans over 186,000 km^2 above the natural tree line zone [1]. Some of the known medicinal plants (*Achillea milifolium, Artemesia dracunculus, Dracocephalum heterophylum, Gallium pauciflorum, Hippophae rhamnoides, Mentha longifolia, Origanum vulgare, Rhodiola imbricata, Rhodiola heterdonta* and *Rubia cordifolia etc)* of cold desert of Ladakh are being used in traditional Amchi system of medicine for treatment of ailments related to liver, lungs, heart *etc*. [2]. These plants are also reported to exhibit anti-viral, antimicrobial, anti-inflammatory, antioxidant properties, which may play vital role in reducing a large number of diseases related to stress-induced oxidative damage [3,4]. *Inula racemosa* is one of the important medicinal herbs of Western Himalaya's cold desert area (between 5000–14,000 feet altitude) which finds wide application in Ayurveda, Amchi and Unani system of medicines.

I. racemosa is also known as *I. royleana* (C.B. Clarke), Pushkarmool, Mano (Hindi) and belongs to family Asteraceae. *Inula* species has been used for treatment of various ailments *viz.* spasm, hypotension, angina, cancer *etc* and is also used as expectorant [5,6]. The root of the plants have medicinal value and posses reportedly hypoglycemic, hypocholesterolemic and hypolipidemic properties [7]. The multi herb combination (Lipistat) of *I. racemosa* with *Terminalia* a *rjuna* and *Commiphora mukul* is reported as cardioprotective and offers protection against isoproterenol induced myocardial ischemia in rats [8]. The extracts

Abbreviations: ARE, anti-oxidant response element; HO-1, heme-oxygenase-1; NQO1, NAD(P)H-quinone oxidoreductase; GCL, glutamate cysteine ligase; GST, glutathione S-transferases; O.D., optical density; SODs, superoxide dismutase; TLC, thin layer chromatogram; TRX, thioredoxin; ROS, reactive oxygen species.

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(aqueous and methanolic) of root have been shown to protect against carbon-tetrachloride, paracetomol and rifamipicin induced hepatotoxicities in rats [9]. The aqueous extract of the roots also shown to exhibit anti-apoptotic efficacy against 4-nitroquinoline-1-oxide induced genetic mutations in mice bone marrow cells [10]. Plants from *Inula* species are known to exhibit anticancer efficacy [11]. Pal et al. reported that the active ingredients in *n*-hexane fraction of *I. racemosa* induce apoptosis in HL-60 leukemia cells through generation of ROS intermediates and dysfunctioning of mitochondria [12]. Moreover, *I. racemosa* has been used in treatment of tuberculosis by native Americans, improving stomach functions, relieving neck and shoulder pain, revitalization of spleen in traditional Chinese medicine [10,13].

The diverse medicinal value of the plant is attributed mainly to the presence of large amount of sesquiterpene lactones, especially eudesmanolides such as alantolactone and isoalantolactone [13,14]. The other sesquiterpene esters *viz.* dihydro-alantolactone, dihydro-iso alantolactone, dihydroinunolide, isoinunolide, isoallolantolactone, allolantolactone, isoinunal, inunal, isoalantodiene and alantodiene may also be present. Some of the other bioactive compounds which are known to contribute in medicinal efficacy of this plant may include B-sitosterol, daucosterol, D-mannitol, aplotaxene and phenlyacetonitirile.

The present study aimed to investigate the free radical scavenging activities, anti-oxidant capacity, reducing capacities, total poly-phenol as well as flavonoid content of aqueous, ethanolic and 50% aqueous-ethanolic extracts of *I. racemosa*. In addition, preliminary phytochemical screening along with TLC profiling was also performed in order to find the active components of extracts. Cell based *in vitro* assay was also done on normal kidney epithelial cells (NKE) as wells as on transformed kidney cells (ACHN cells and A498 cells) to assess of differential toxicity of extracts.

2. Materials and methods

2.1. Chemicals

Ascorbic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), aluminum chloride ($AlCl_3$), butylated hydroxytoulene (BHT), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), quercetin, gallic acid, methanol, potassium persulfate ($K_2S_2O_8$) potassium ferricyanide (K_3FeCN_6), trichloroacetic acid (TCA), 2,4,6-tripyridyl-s-triazine (TPTZ), ferric Chloride ($FeCl_3$), Folin-iocalteu phenol reagent, sodium bicarbonate (Na_2CO_3), glacial acetic acid, ferrous sulphate ($FeSO_4$), iodine, potassium iodide, copper sulphate, sodium hydroxide, nitric acid, hydrochloric acid, lead acetate, acetic anhydride, chloroform, tris base and absolute ethanol were obtained from Sisco Research Laboratories Pvt. Ltd, India. All chemical used during the investigations were of analytical grade. The RPMI 1640 medium, EMEM medium, heat–inactivated fetal bovine serum, penicillin, streptomycin, non-essential amino acids, β-mercaptoethanol, alantolactone, and sulphorhodamine B were obtained from Sigma-Aldrich, St Louis, MO (USA)

2.2. Preparation of root extract

The root of the plant were collected (and authentication were done by botanist) in the month of August from Leh, Jammu and Kashmir, India (1000–1100 ft). The roots were washed thoroughly, shade dried (room temperature) and were coarsely powered by using mortar-pestle. The fine powder was prepared with the help of electric mixer and thereafter extracts (aqueous and ethanolic extracts) were prepared by the process of Soxhlet extraction [15]. Briefly, 50 gm of root powder packed in the thimble was kept in Soxhlet apparatus for sequential extraction first with absolute ethanol (∼10 h) followed by double deionized water (∼40 h) at 40 °C.

The 50% aqueous-ethanolic extract was prepared by the process of Maceration [14] with minor modifications. Briefly, 50 g of the root powder was suspended in 50% ethanol (diluted in 1:10 w/v). The mixture was kept in incubator shaker (MaxQ™6000, Thermo Fisher Scientific, Massachusetts, USA) for 24 h at 25 °C; 250 rpm for extraction.

The extracts were concentrated using rotavapour evaporator (BUCHI, R-134, BUCHI Labortechnik, Flawil, Switzerland) and thereafter lyophilized (Lyophilizer (−55° C, Sub-Zero Lab Instruments, Chennai, India). The lyophilized extracts were stored in an air-tight container at −20 °C. The voucher specimen of extracts has been deposited at institutional library, INMAS [(INM/LIB/SMKDG01 (a, b, c)]

The aqueous and 50% aqueous-ethanolic extract were dissolved in 50% ethanol for all experimental studies, whereas absolute ethanol was used as solvent for ethanolic extract. The percent of ethanol administered to the cells as a vehicle was within non-toxic range (less than 1%).

2.3. Estimation of radical scavenging potential

The anti-oxidant capacity of the extracts was determined by ABTS radical scavenging assay as reported by Re et al. [16]. The ABTS radical is a stable free radical and has characteristic absorbance at 734 nm. In the presence of the antioxidant molecule, the reduction of the radical takes place which can be monitored by the decrease in its absorbance. Briefly, the ABTS radical was generated by mixing the two stock solutions [(1:1; v/v, ABTS (7 mM) and potassium persulfate (2.4 mM))] in dark for ∼12 h followed by preparation of working solution with OD of 0.70 ± 0.01 at 734 nm. Equal volume of working solution and sample (extract/standard) were mixed, incubated (10 min at room temperature) and absorbance was recorded measured at 734 nm spectrophotometrically using NanoDrop (Implen, Munich, Germany). Ascorbic acid and gallic acid were used as standards for comparison of radical scavenging activity of *Inula* extracts.

The scavenging activity of the extracts was calculated as follows:

$$\text{Radical Scavenging Capacity} = [\{A_0 - A_1\}/A_0] \times 100$$

Where, A_0 = absorbance of ABTS radical; A_1 = absorbance of ABTS radical with sample.

2.4. Estimation of reducing potential

The reducing potential of extract was determined by method as reported by Oyaizu with minor modifications [17]. The reducing capacity of extracts was examined as transformation of the Fe^{+3} (Ferric) to Fe^{+2} (Ferrous) ions, which is mediated by presence of antioxidants in the sample [18]. The presence of Fe^{+2} is monitored by measuring absorbance of Perl's Prussion Blue complex at 700 nm.

Briefly, the different concentrations of extract was mixed with phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide (1:25:2.5) and mixture was incubated for 30 min at 50 °C. After incubation, 2.5 ml of trichloroacetic acid (TCA; 10%;w/v) was added and the resulting mixture was centrifuged (3000 rpm for 10 min). The supernatant was collected and mixed with ferric chloride (1%) in equal volume and absorbance of final solution was recorded at 700 nm spectrophotometrically. The reducing power of extracts is expressed as difference in optical density with respect to control. The reducing power of extracts is compared with that of ascorbic acid as a standard.