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Phytochemical Analysis, Antioxidant and Cytotoxicity Activities of *In Vitro* Cultures of *Gentiana Kurroo* Royle

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Abstract

Gentiana kurroo Royle is an endemic plant of the Himalaya and the roots and rhizomes of this species possess pharmaceutically important seco-iridoid glucosides. Traditionally, wild-grown plants are widely used for various ailments. Therefore, for the first time, the *in vitro* samples like callus, shoot, and adventitious roots of *G. kurroo* were analyzed for total phenolics, flavonoids, antioxidant activity, and cytotoxicity effect. The antioxidant activity of *in vitro G. kurroo* cultures was analyzed using different biochemical assays namely, diphenyl-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant potential (FRAP), and total antioxidant capacity (TAC). Among the samples tested, adventitious roots showed the highest antioxidant potential and total phenols (91.2 mg/g DW) and flavonoids (32.43 mg/g DW). The MTT assay showed the IC_{50} of 550 µg/mL for *in vitro* adventitious root extract on human umbilical cord mesenchymal stem cells (hMSCs). Therefore, the *in vitro* roots can be used as a viable option to meet its great demand and replace its natural sources.

Keywords: *Gentiana kurroo*, callus, *in vitro* shoot*, in vitro* root, phytochemicals*,* antioxidant activity, cytotoxicity

Introduction

The widely classified free radicals in the field of biology and medicine are reactive oxygen species (ROS) or reactive nitrogen species (RNS) [1]. Endogenous or exogenous sources may generate free radicals in biological systems [2]. The enzymes involved in the mitochondrial respirational process, NAD photooxidase, xanthine oxidase, and defective endothelial nitric oxide synthase are the major endogenous sources of free radicals [3], and tobacco smoke, pollutants, xenobiotics, ultraviolet, and ionizing radiation are the potential exogenous sources [4]. These free radicals are commonly believed to causes many human disorders, such as neurodegenerative conditions (Alzheimer's disease), cancer,diabetes mellitus, cardiovascular diseases (atherosclerosis and hypertension), respiratory diseases, and many other diseases by DNA damage, changing cell-signaling pathway and affecting gene expression [1,5,6]. Natural antioxidants from plant resources are essential to reduce oxidative damages and are of great attention nowadays. Several previous reports indicate that antioxidant phytochemicals like phenolic compounds, flavonoids, alpha-tocopherol, and carotenoids were attributed to the antioxidant's potential of plants [7]. The increased intakes of exogenic plant-based antioxidants can reduce the harmful effect of oxidative stress by preventing oxidative chain reactions,

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acting as free radical scavengers, oxidation-free quenchers, and reducing agents [8].

Gentiana kurroo Royle, one of the medicinally important endemic species of gentian members commonly known as Indian gentian, is found in the Himalayan region. The leaves contain iridoid glycoside markedly 20 - (2, 3-dihydroxybezoyloxy)-7-ketologanin [9]. The roots and rhizomes are rich in secondary metabolites like iridoid glycosides and alkaloids [10]. Rhizomes and roots have historically been used to treat various disorders, including bronchial, bronchial asthma, dyspepsia, flatulence, colic, anorexia, helminthiasis, inflammation, amenorrhea, dysmenorrhea, hemorrhoids, constipation, leukoderma, leprosy, and local urinary infections [11] and also have the immunomodulatory and anti-inflammatory potential [12]. This plant was extensively harvested from natural sources for their multipurpose treatments and it became endangered in India and listed under the red list category by IUCN [13]. Therefore, biotechnological methods must be used to boost its availability sustainably to meet the great need of this plant material [14]

The total phenolics and flavonoid content of *G. kurroo* were previously assessed in the naturally grown plant roots and shoots [10,15]nitroblue tetrazolium (NBT. Though few workers were standardized *in vitro* culture of *G.kurroo* [11,14,16]*hypocotyls and roots were investigated using MS (1962*, there was no study reports the phytochemical analysis and determination of the antioxidant potential of callus, *in vitro* shoot, and root culture of *G. kurroo*. Traditionally, wild-grown plants are widely used for various ailments so far. Therefore, the present study focused on the antioxidant activity of callus tissues, *in vitro* adventitious roots, and *in vitro* shoot cultures of *G. kurroo* for the first time. This study will provide new insight into the application of a crude extract of *in vitro* tissues as an antioxidant product. This present study aims i) to estimate the total phenolics and flavonoids content of callus tissues, *in vitro* adventitious roots, and *in vitro* shoot cultures of *G. kurroo*. ii) to estimate the antioxidant activity and the cytotoxicity effect of selected *in vitro* cultures.

Materials and Methods

The previously developed *in vitro* cultures in our laboratory were used for this study. The *in vitro* cultures like callus, shoot and adventitious roots was established in MS media fortified with 2,4-Dichloro-phenoxyacetic acid (2,4-D) 1 mg/L+ kinetin 0.5 mg/L, 6-Benzylaminopurine (BAP) 2 mg/L + Indoleacetic acid (IAA) 0.5 mg/L and Naphthaleneacetic acid (NAA) 1 mg/L + Indole-3-butyric acid (IBA) 2 mg/L respectively before harvesting for phytochemical analysis.

Sample extraction for biochemical analysis: The fresh roots were harvested and dried in a hot oven for two days at 55 ºC. The well-dried roots were powdered using mortar and pestle. For analyzing total phenolic and flavonoids, samples were initially sonicated for 30 minutes in 45 ºC followed by incubation at 75 ºC for 30 minutes and final sonication for 30 minutes. These extraction procedures were repeated three times to get complete metabolites using methanol. The extracts pooled and evaporated using Buchi Rotavapor and kept under a high vacuum for 20 minutes to remove excess moisture.

Phenolics estimation: The overall phenolic content in *G. kurroo in vitro* samples was estimated by the Folin–Ciocalteu method [17] with minor changes. The methanolic extract of *G. kurroo* was diluted to 1 mg/mL concentration. Briefly, 200 µL extracts were added with 2.8 mL of water to make up to 3 mL to this 0.5 mL of Folin–Ciocalteu reagent was added and mixed properly for 3 minutes. Exactly, 2 mL of 20% (w/v) sodium carbonate was mixed thoroughly to the above mixture and incubated for 60 minutes under dark conditions. The optical density was taken at 650 nm for the final reaction mixture. The authentic gallic acid (Sigma, Mumbai, India) with different concentrations was used for calibration curve preparation. The overall phe-

nolics present in the extract were expressed in gallic acid equivalents (GAE) in mg per gram dry weight (DW). All the quantification was carried out in ± standard error of nine independent *in vitro* samples.

Flavonoids estimation: The presence of overall flavonoids in *G. kurroo in vitro* samples was estimated by the aluminium chloride method previously defined [18, 19]. Briefly, 50 µL of extracts (1 mg/mL) was diluted to 1 mL by mixing 950 µL of methanol. The diluted extract was added with 4 mL of water and 300 µL of 5% $NaNO_2$ and incubated for 5 minutes. Exactly, 300 µL of 10% $AlCl_3$ solution was mixed thoroughly and kept at room temperature for about 6 minutes. Followed by 2 mL of 1 M NaOH solution was added to the above mixture and the final volume was made up to 10 mL with distilled water. This final reaction mixture was kept in room condition for 15 minutes and optical density was taken at 510 nm. The total flavonoids of the extract were expressed in terms of quercetin (Sigma, Mumbai, India) equivalents in mg per gram µg/mg dry weight. The quantifications were done in ± standard error of nine independent *in vitro* samples.

DPPH assay: Based on the proton or electron-donating capability of *in vitro G. kurroo* extracts, the free radical scavenging property was determined, which diminishes the purple DPPH free radical to a light pale-yellow color. Exactly 1 mL of 200 µM DPPH solution prepared with HPLC grade methanol was added to 3 mL of various amounts of (100–600 µg/mL) plant extracts. The reaction mixture was mixed properly and kept under dark for about 60 minutes at room condition. The Mixture's optical density was recorded at 517 nm and methanol was used as blank [20]. The antioxidant potential *of in vitro G. kurroo* extracts was evaluated using the standard ascorbic acid as a positive control. The amount of free radical inhibition was calculated in percentage with the following equation.

(%) inhibition = A_1-A_2/A_1

where A_1 is control mix absorbance and A_2 is sample absorbance.

ABTS assay: The *in vitro G. kurroo* extracts were evaluated for ABTS radical scavenging capacity using the modified protocol of Jamdar et al. [21]. The ABTS solution (7 mM) was added to potassium persulphate (2.45 mM) and incubated for 16 hrs under dark conditions at room condition to make radical cation $ABTS^+$. The working ABTS radical solution was prepared by suitably diluting the mixture with distilled water to get an absorbance of 1 ± 0.02 at 734 nm. Exactly, 0.4 mL extracts were added to 1.6 mL of working ABTS solution and incubated for 6 minutes under room condition. The reaction mixture was read at 734 nm and the percentage of scavenging activity was calculated using the following formula:

Percentage of ABTS radical-scavenging activity = ([A control – A sample]/ A control) ×100.

FRAP assay: The FRAP activity of *G. kurroo in vitro* samples was estimated by the spectrophotometer method with few changes [22]. The working FRAP assay mix was made by mixing of 0.3 M acetate buffer (pH 3.6), 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl and 0.02 M $FeCl_3.6H_2O$ in a proportion of 10:1:1 volume. Exactly, 200 µL of different concentrations of analysis extract to 800 µL of the working solution and kept in room condition for 20 minutes after mixing properly. This final reaction mix was read at 593 nm. The blank mixture was made by mixing 800 µL of FRAP solution with 200 µL of methanol. The increasing concentration of $FeSO_4$ was used for plotting the calibration curve and which intern plotted against Trolox. The results were expressed as mg of Trolox equivalent per gram of sample.

Total antioxidant capacity (TAC): The TAC was estimated based on the previous method [21]. In this assay, Mo (VI) is reduced to Mo (V) by plant extracts in acidic pH and the green phosphate/Mo (V) complex formed was measured at 695 nm. Exactly, 100 µL of different concentrations of plant extracts (0.1–1.0 mg/mL) were mixed to 1 mL of working reagent

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containing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The reaction was maintained for 90 minutes at 90 ºC in the water bath. The mixture was cooled and measured the absorbance against control containing 100 µL MiliQ water instead of the sample using 1 mL quartz cuvettes. The TAC of *G. kurroo in vitro* samples were expressed in the equivalent of α-tocopherol using the following linear equation [21]

$A = 0.011C + 0.0049$, $R^2 = 0.987$;

Where A is the absorbance at 695 nm and C is the concentration as a-tocopherol (µmol/mL).

Cytotoxicity assay: The cytotoxic effect of methanolic extracts of in vitro roots was determined by MTT (2-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide) (Sigma, USA) assay described before [4] with few modifications of [23, 24]. Briefly, hMSCs in suspension were transferred into a 96-well microtiter plate at a concentration of 1 × 104 cells per well. The seeded cells were allowed overnight at 37 °C under the 5% CO_2 environment. Then, the different concentrations (50–500 µg/mL) of in vitro adventitious root extract was mixed with the hMSCs. After 48 h,the medium was removed with a pipette, and a fresh medium was added to cells after washed with PBS. MTT assay solution (5 mg/mL PBS) was added to the cells, and the blue formazan crystals were solubilized by adding stop solution (10% SDS in 0.01N HCL). The reaction solution absorbance was taken at 570 nm. The IC_{50} levels (concentrations of the test compound to minimize cell survival by 50%) were determined from concentration-response curves and used as a measure of cell susceptibility to a specific treatment.

Statistical analysis: The average antioxidant activity and cytotoxicity measurements (MTT) were calculated as mean ± SD of three results. The total phenolics and flavanol data were subjected to one-way ANOVA using SPSS software version 16.0. Duncan's multiple range test was performed to detect the difference and results were reported as mean ± standard error of nine samples. P- values ≤ 0.05 were considered to be significantly different.

Results and Discussion

Phytochemical analysis of in vitro samples: The methanolic extracts of *in vitro* samples of *G. kurroo* (Fig.1) such as callus, root, and shoots were analyzed for total phenolics and flavanols contents (Tab.1). Among the three samples evaluated, the *in vitro* root contained significantly the highest amount of total phenolics (91.2581 ± 4.12 mg/g DW gallic acid equivalent) whereas *in vitro* shoot showed the highest concentration of GPD. A similar previous analysis of *G. kurroo* natural root methanolic extract showed 86 and 68 mg/g of total phenolics [10,15]nitroblue tetrazolium (NBT. The total flavonoid content was high in *in vitro* root (32.4349 ± 1.56 mg/g DW quercetin equivalent) compared to callus and *in vitro* shoots whereas, lower in comparison with the previous natural root [10, 15] (Table 1).

Evaluation of the antioxidant potential of in vitro samples of G. kurroo: Plants containing flavonoids, phenolics, and carotenoids demonstrate antioxidant potential [25,26]. Since the methanolic extracts of in vitro samples of G. kurroo contained a reasonable amount of total phenolics and flavanols, these samples were analyzed for their antioxidant activity. In the current study, four different antioxidant testing methods were followed. One of the reasonably considered stable free radicals was DPPH radical, and it had been commonly used for the evaluation of free radical scavenging activities of plant-based antioxidants

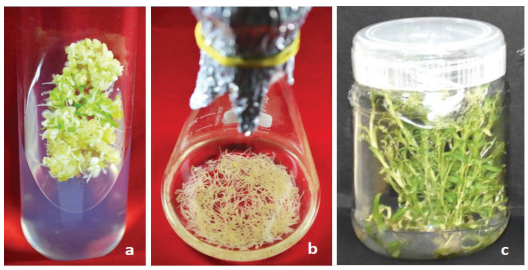

Figure 1. *G. kurroo in vitro* cultures: a-callus established on MS medium fortified 2,4-D 1 mg/L+ kinetin 0.5 mg/L; b- *in vitro* adventitious root

cultured in MS medium containing NAA 1 mg/L + IBA 2 mg/L; c- multiple shoot culture grown on MS medium containing BAP 2 mg/L + IAA 0.5 mg/L.

Table 1. Total phenolics and flavonoids content of callus, *in vitro* root and shoot methanolic extracts of *G. kurroo*

In vitro tissue	Total Phenolics (*mg/g)	Total Flavonoids (**mg/g)
Callus	41.8118 ± 2.65[a]	29.7440 ± 2.94[b]
In vitro root	91.2581 ± 4.12[b]	32.4349 ± 1.56[c]
In vitro shoot	39.3004 ± 4.36[a]	24.2094 ± 2.39[a]

Each value represented as mean standard deviation of nine independent biological replicates

*= mg GAE /g DW; **= mg quercetin equivalent/g DW.

When the DPPH free radicals were reduced by the existence of an antioxidant molecule by accepting an electron or hydrogen, a remarkable color shift from violet to yellow was observed and measured at 517 nms [27]. Therefore, the antioxidant property of the G. kurrooin vitro samples were assessed by theirability to scavenge DPPH free radical (Fig. 2a). As shown in Fig. 2a, all the in vitro samples were able to scavenge free radicals. All the extracts exhibited strong, concentration-dependent (100 – 600 µg/mL) antioxidant activity. The in vitro root possesses highest scavenging activity (81% at 100 µg/mL) than callus (29.6% - 85.4%) and in vitro shoots (30.8% – 84.3%). This present study shows that callus,in vitro shoots and roots had the highest antioxidant activity than the previously reported in field-grown shoots and roots of G. kurroo[10,15].

ABTS assay was performed to evaluate the antioxidant potential of callus, in vitro shoot, and root extracts of G. kurroo (Fig. 2b). The in vitro root extract showed the highest ABTS+ scavenging capacity (75.7% to 99.85%) than callus (25.8% to 90.1%) and in vitro shoot (15.9% to 69.1%) at the increasing concentration ranged from 50 to 300 µg/mL. The standard Trolox has been used to compare the ABTS+ scavenging activity of plant extracts. This study also demonstrated that in vitro root extract has a higher ABTS+ scavenging ability than thepreviously reported natural G. kurroo root (58.6% to 89.2%) and leaf (72. 9% to 75.2%)at the concentration varied from 50 to 400 µg/mL [15]

FRAP assay measures the capacity of plant extracts to reduce the Ferrous iron (Fe3+) to iron

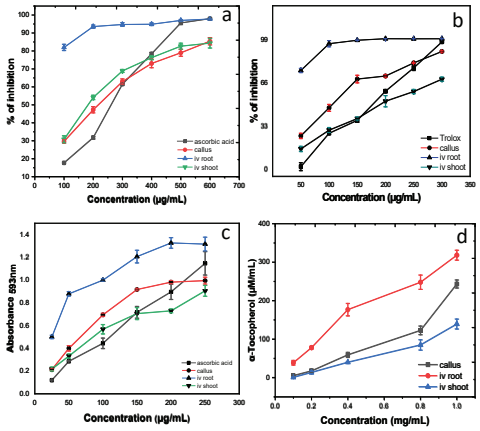

Figure 2. Antioxidant activity of in vitro cultures of G. kurroo: a- DPPH reducing power, b-ABTS activity, c-FRAP, d- total antioxidant capacity. (iv root- in vitro root, iv shoot-in vitro shoot).

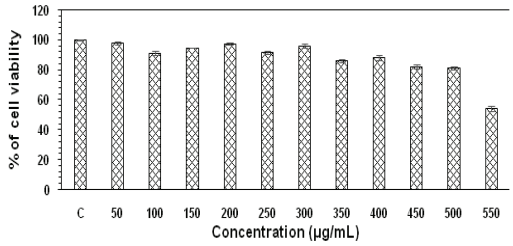

Figure 3. Cytotoxicity activity of in vitro adventitious root cultures of G. kurroo on Human umbilical cord Mesenchymal Stem Cells (hMSCs)

(Fe2+) which was shown to be an indication of antioxidant activity.

The reducing power Fe3+ to Fe2+ of in vitro samples of G. kurroo was shownin increasing concentrations Fig. 2c).

The ferric reduction power of extracts ranged from 25 µg/mL to 250 µg/mL. In comparison with callus and in vitro shoot, the in vitro root extract exhibited the highest reducing power (absorbance from 0.5 to 1.3) in a concentration-dependent manner. The in vitro shoots, roots, and callus possess much higher Fe3+ reducing activity as compared to the previous report [10] in natural G. kurroo, where the absorption value for the root extract was from 0.28 to 0.64, and the for leaf extracts were between 0.24 and 0.59 at 100 to 500 µg/mL concentrations.

TAC was used to quantify the water-soluble and fat-soluble antioxidant capability of plant extracts [28]. The ability of plant extract to reduce Mo VI to Mo V was measured based on the formation of a green phosphate complex. All three extracts tested had an antioxidant activity to increasing concentration (Fig. 2d). The highest TAC was observed in in vitro root extracts (318.2 µM/mL α-tocopherol equivalent) followed by callus (243.4 µM/mL) and in vitro shoot (139.3 µM/mL) at 1 mg/mL of extract.

Among the three in vitro samples tested, the in vitro root extract showed significantly higher total phenolics and flavanols and it also showed relatively high antioxidant activity than callus and in vitro shoot extracts. Therefore, the in-vitro root extract was assessed to find out the cytotoxic activity using hMSCs. The MTT assay results showed that the in vitro adventitiousroot extract of G. kurroo exhibits nearly 50% of inhibitory effect at 550 µg/mL concentration after 48 hrs (Fig. 3). Therefore, the in vitro adventitiousroot extract can be used as an alternative to the field roots and rhizome of G. kurroo as an antioxidant product.

Conclusion

These findings indicate that the methanolic extracts of callus, in vitro shoots, and roots of G. kurroo have antioxidant capacity and phytochemical analysis confirms the presence of phenolics and flavanols. The highest antioxidant activity, total phenolics, and flavanol content were reported in in vitroroots, and the cytotoxicity study shows less toxicity to hMSCs. Thus, the in vitro roots can be used as an alternative source of G. kurroo plant materials to meet its great demand and curb its natural source dependents.

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