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# IMMUNOMODULATORY ACTIVITY OF *IN VITRO* PROPAGATED TISSUES OF *GENTIANA KURROO* ROYLE

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## ABSTRACT

*Gentiana kurroo* Royle is a critically endangered medicinal herb belonging to the family Gentianaceae. This study evaluates the effect of *in vitro* propagated tissues of *G. kurroo* on the proliferation of human peripheral mononuclear cells (PBMC) under *in vitro* conditions. Embryogenic compact callus was induced from leaf explants and the maximum induction frequency of 86 % was obtained on Murashige and Skoog (MS) medium supplemented with 2, 4-D (1.5mg/l) + BAP (0.5mg/l) + TDZ (0.5 mg/l). The embryogenic calli showed differentiation into green buds after 35 days on MS medium supplemented with BAP (2.0 mg/l) and KN (1.0 mg/l). *In vitro* regenerated shoots developed roots on half strength MS+ IBA (0.5 mg/l) after 6 weeks of culture with a survival rate of 91.6 %. Lymphocyte proliferation was carried out using human PBMC with *in vitro* propagated leaves, roots and callus extracts at concentrations ranging from 50 µg/ml to 200 µg/ml. The results showed that the methanolic extracts of *in vitro* grown tissues of *Gentiana kurroo* significantly enhanced PBMC proliferation as compared to DMSO control. The PBMC incubated with leaf extract described maximum proliferation of 87.6% followed by roots and callus extract (50.6 and 16%) respectively. Our results suggested that the *in vitro* propagated leaves could be clinically useful for stimulating immune response, thereby protecting the natural stands and conserving this medicinal plant.

**Keywords: *Gentiana kurroo;* Plant Tissue Culture; Immunomodulation**

## INTRODUCTION

*Gentiana kurroo Royle* (karu, kutki) is a critically endangered medicinal plant of Gentianaceae family. It is mainly endemic in northwestern Himalayas found mainly on south-facing steeper slopes along dry and rocky grasslands **[1]**. In traditional and modern medicine, roots and rhizomes of this plant are used in the treatment of leucoderma, urinary tract infections, bronchial asthma, anorexia, gastric infections, exhaustion from chronic diseases, and loss of appetite. It is also valued as a bitter tonic, antiperiodic, antibilious, anthelmintic, astringent, antipsychotic, sedative, stomachic and carminative **[2]**. The roots of this plant are a source of iridoid glycosides like gentiopicrine and gentiamarin and the alkaloid, gentianin **[3]**. Their biological function is the treatment of digestive disorders, urinary infections, leucoderma and bronchial asthma. Unfortunately, the pharmaceutical industries are largely dependent on natural population of *G. kurroo* to fulfill their demands, which is depleting the wild stands of this plant. Therefore, this plant has been listed as critically endangered by Government of India and in the negative list of exports vide Notification no. 2 (RE-98) 1997–2002 dated 13 April 1998 **[2]**. Many *in vitro* studies have been carried out on propagation of *G. kurroo* using shoot tips, nodal segments **[3]**, seedlings and leaves as explants **[4]**. It was found that *G. kurroo* can be propagated through rhizome cuttings; shoot nodal segments, seeds and somatic embryogenesis. Recently indirect and direct organogenesis has been carried out using leaf, roots and petioles as explants with maximum response (86.6%) of shoot regeneration from callus cultures **[5]**.

Roots and rhizomes of *G. kurroo* were reported to have antioxidant, antiproliferative and anticancer activity on human pancreatic cell line MiaPaCa-2 **[6, 7]**. In recent times modulation of immune response to cure various diseases has been a very interesting concept in the field of natural products emphasizing on strengthening host defenses against different diseases. To the best of our knowledge, there is no report on immunomodulatory activity on human peripheral blood mononuclear cells (PBMC) using *in vitro* grown tissues (leaves, callus and roots) of *G. kurroo*. The present communication provides the first report on immunomodulatory effect of *in vitro* grown tissues of *G. kurroo* on human PBMC and also an improved method of plant regeneration through callus cultures. So far, phytochemical studies of *G.kurroo* are based on destructive collection of the plant from its

native sites, whereas in present study efforts have been made to follow multipronged conservation strategies.

## MATERIAL AND METHODS

### Plant material and culture conditions

Plant cultures of *G. kurroo* were obtained from Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan and was maintained in Murashige and Skoog medium supplemented with KN and BAP (0.5 mg/l) each under controlled temperature (25°C), humidity (70-75%) and light (10 h dark and 14 h light) conditions in a growth chamber. Leaf cuttings were used as explants for callus initiation. MS medium containing 3% sucrose gelled with 0.8 % agar supplemented with varying concentrations and combinations of BAP (0.5-2.0 mg/l), KN (0.5-2.0 mg/l), IAA (0.5-1.0 mg/l), IBA (0.5-1.0 mg/l), TDZ (0.1-0.5 mg/l), NAA (0.5-2.0 mg/l), $GA_3$ (0.5-1.0 mg/l) and 2,4-D (0.5-1.5 mg/l) were used as shown in the Table 1. The pH of the medium was adjusted to 5.8 ± 0.1 and was autoclaved at 121º C and 1.05 Kg $cm^{-2}$ for 15 min.

### Plant regeneration from organogenic cultures and acclimatization

For plant regeneration, organogenic calli were transferred to the shoot induction media; i.e., MS medium supplemented with different concentrations of BAP (1.0-3.0 mg/l), KN (1.0-3.0 mg/l), NAA (0.5-2.0 mg/l), 2, 4-D (1.0-2.0 mg/l) and IAA (0.5-2.0 mg/l). Regenerated plantlets from the embryogenic calli were subcultured in the shoot multiplication media for further shoot proliferation. Fully developed shoots with healthy roots were then acclimatized and transferred to the greenhouse for hardening.

### Preparation of Crude extracts

The parts of plant (leaves, roots and callus) left after repeated subculturing of *G. kurroo* were thoroughly washed with distilled water and air dried at ambient temperature. After complete drying, all the parts were ground to yield coarse powder. The powdered roots, leaves and callus were extracted with methanol by cold maceration for 5 days. The extracts were filtered using whatmann filter paper no. 1 and were concentrated using rotary evaporator. All the extracts were preserved at 4°C in airtight bottles for further analysis.

### Preparation and cultivation of PBMC

Blood was collected from healthy human donors. This work was carried out in compliance with the ethical committee guidelines of Shoolini University, Solan. PBMC were isolated from blood using Histopaque (Himedia) according to manufacturer's instructions. The blood sample was diluted with the same volume of

PBS. The diluted blood sample was carefully layered on Histopaque. The mixture was centrifuged at 400×*g* for 30 min at 18-20°C. The undisturbed lymphocyte layer was carefully transferred out. The lymphocytes were washed and pelleted down with three volumes of PBS twice and resuspended in RPMI-1640 media supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (100µg/ml penicillin and streptomycin). Cell counting was performed to determine the PBMC number with equal volume of trypan blue.

**Cell viability assay**

The effect of plant extracts on cell viability was assessed by MTT assay. MTT is a pale yellow substance reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. PBMC ($5 \times 10^5$ cells/ ml) were seeded in 10% RPMI-1640 medium in a 96-well plate (in triplicate). After overnight incubation, the medium was replaced with fresh 10% RPMI-1640 medium containing fractions isolated from *G. kurroo*. Concanavalin A and DMSO treated PBMC were used as positive and negative controls respectively. All plates were incubated in 37°C, 5% $CO_2$ for selected time period. For MTT assay, 10µl MTT (5mg/ml) was added into each well to generate formazan, and then cells were incubated in humidified atmosphere with 5% $CO_2$ at 37°C for 4 h. After removing the supernatant, 100 µl DMSO was added to dissolve the purple crystal. The optical density of each well was measured at 570 nm by a microplate reader. The percentage of proliferation was calculated by the following formula:

**%Proliferation = (OD sample – OD control) / OD control × 100 %**

**Data Analysis**

The data for the percentage of explants initiating calli, number of days for initiation, texture and morphology, frequency of shoots developed on embryogenic callus and percentage of survival were determined after 6 weeks of subculture. A minimum of 12 cultures were raised in each set and each experiment was repeated thrice. Means and standard errors were carried for each experiment. The significance of the results was calculated using Graph pad prism 5.02 software. The overall variation in a set of data was analysed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

**RESULTS AND DISCUSSIONS**

**Callus induction**

A total of 68 various concentrations of cytokinins, auxins and gibberellins either

alone or in combinations were tested using leaves as explants of *G. kurroo.* There was no callus formation when explants were cultured in MS media without auxin or cytokinin (data not shown). Frequency of compact callus formation was observed highest (75%) in MS medium supplemented with 2, 4-D (1.5 mg/l) + BAP (0.5 mg/l) + TDZ (0.5 mg/l) and 72.2% callus induction frequency was observed in MS medium supplemented with 2, 4-D (0.8 mg/l) + KN (1.0 mg/l) + BAP (1.0 mg/l) and these concentrations were further used for plant regeneration **(Table 1 and Figure.1c and 1d)**. Callus initiation was observed at the $10^{th}$ and $8^{th}$ day on the former (**Figure 1b**) and latter concentrations. Of the various concentrations of NAA (0.3 mg/l - 2.0 mg/l) tested, with BAP, the maximum callus production from leaf explant was 91.6 % in media having NAA (2.0 mg/l) and BAP (1.0 mg/l) **(Figure 1e)**. When NAA at (1.0 mg/l) was tested with KN (0.5 mg/l) we got 61.1% of callus formation after 30 days of culture **(Figure 1f)**. Following subculturing in the light, callus grew rapidly into greenish-yellow nodules after 2 weeks. Recent report on callus induction was observed on different explant cultures of *G.kurroo* using BAP (1.0 mg/l) and NAA (3.0 mg/l) where maximum response was 86.6% using petioles as explants **[5]**.

**Plantlet regeneration and acclimatization of *G. kurroo***

After 5 weeks of callus multiplication the calli were aseptically transferred to the shoot regeneration medium. For the indirect organogenesis, 25 various combinations of growth regulators were tested (**Table 2**). Response of shoot regeneration from callus was maximum on combination of BAP (2.0 mg/l) & KN (1.0 mg/l) with 3 shoots/ callus after 35 days of subculture **(Figure 2 a-d)** followed by IAA (4.0 mg/l) and KN (2.5 mg/l) with 1- 2 shoots/ callus explant **(Figure 3)**. Many reports indicated that *in vitro* organogenesis required a high cytokinin/ auxin ratio in several plant systems like *Eucalyptus grandis, Coffea Arabica and Eleusine indica* **[8, 9, and 10]**. Similar report on indirect organogenesis was observed on different explant cultures of *G. kurroo* using 0.1 mg/l NAA + 0.75 mg/l TDZ where 3 shoots/ callus were obtained **[5]**.

*In vitro* shoots (5-8 cm) were aseptically excised and transferred to the half strength MS medium supplemented with various concentrations of IBA, IAA and NAA (0.1-0.5 mg/l) for rooting. Among different concentrations of auxins, IAA and IBA at 0.5 mg/l were found to be better than NAA for producing roots. After 45 days of culture best rooting was observed with half strength of

MS medium supplemented with 0.5mg/l IBA, with an average number of roots being 23.4± 0.4 with root length of 8.2± 0.32 **(Figure 2e, 4a and 4b)**. Similar results have been reported, where the addition of IBA promotes the induction of roots in several plant systems including *Bixa orellana* and *Dioscorea zingiberensis* **[11, 12]**. More than 75% of plantlets survival was observed on hardening in sterile mixture of clay soil: farmyard manure (1:1) (w/w) for six weeks without any somaclonal variations **(Figure 2f)**.

**Lymphocyte proliferation assay**

The methanolic extracts of callus, leaves and roots of *G. kurroo* were tested for their ability to proliferate human lymphocytes. The results revealed that PBMC stimulation was significantly elevated with the *G. kurroo* leaves extract followed by roots and callus at concentration ranging from 50 µg/ml to 200 µg/ml **(Figure 5a & 5b)**. The methanolic extract of leaves showed higher percentage proliferation (87.6%) as compared to callus and root extract (16% & 50.6%) respectively. After 24 h of incubation, the lymphocytes that have been treated with Concanavalin A (positive control) showed 51% proliferation. Previously, it has been reported that two triterpenoids lupeol and ursolic acid isolated from the roots and rhizomes of *G. kurroo* were able to activate humoral function in Swiss albino mice **[13]**. Also the methanolic plant extracts were found to have anti-inflammatory activity when tested in paw edema model of Wistar rats with the inhibitory potential of 47.6% **[7]**. The cases where the vegetative organs (root, rhizome) or reproductive organs (fruit, flower seeds) are used, the species are much more in danger of extinction in comparison to a species from which only leaves have been collected. However, this is the first report on immunostimulation of human peripheral blood mononuclear cells by *in vitro* propagated *G. kurroo* leaves which suggest that *in vitro* propagated plant organs can be utilized for therapeutic purposes thereby conserving medicinal plants.

**CONCLUSION**

The present study established the immunostimulation effect of *G. kurroo in vitro* propagated leaves on human PBMC and plant regeneration from callus cultures of *G. kurroo* which is highly valuable, as it is a critically endangered medicinal herb thereby ruling out the dependence on natural stands to fulfill the growing demands for this species.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**ACKNOWLEDGMENT**

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**Table 1: Effect of growth regulators on callus induction and morphology of *G. kurroo***

| Growth regulators(mg/l) | Initiation time (d) | Intensity of callus formation | % Response | Morphological characteristics |
|---|---|---|---|---|
| **NAA alone** | | | | |
| **0.5** | **20** | **c** | **11.1** | **C + Y W** |
| **1.0** | **10** | **c** | **16.6** | **C + Y W** |
| **NAA + BAP** | | | | |
| **0.3 + 0.8** | **10** | **c** | **25.0** | **C + Cr B** |
| **0.5 + 0.5** | **13** | **a** | **38.8** | **F + Rz + Y W** |
| **1.0 +1.0** | **14** | **b** | **41.6** | **F + Rz + O W** |
| **2.0 + 1.0** | **15** | **a** | **91.6** | **F + Rz + O W** |
| **NAA + KN** | | | | |
| **0.5 + o.5** | **17** | **b** | **44.4** | **F + Rz +Y W** |
| **1.0 + 0.5** | **15** | **b** | **61.1** | **F + Rz + O W** |
| **IAA + KN** | | | | |
| **0.5 + 0.5** | **26** | **c** | **27.7** | **L + Y W** |
| **1.0 + 0.5** | **21** | **c** | **30.5** | **F + Y W** |
| **2,4-D + KN + BAP** | | | | |
| **0.8 + 0.8 + 0.8** | **12** | **b** | **63.8** | **C + O W** |
| **0.8 + 1.0 + 1.0** | **8** | **a** | **72.2** | **C + Cr B** |
| **2, 4-D + BAP +TDZ** | | | | |
| **0.5 + 0.5 + 0.5** | **13** | **b** | **66.6** | **C + P G** |
| **1.5 + 0.5 + 0.5** | **10** | **b** | **75.0** | **C + O W** |

**W: White; Cr: Creamy; PG: Pale Green; OW: Off-white; B: Brown; Y: Yellowish; F: Friable; C: Compact; L: Loose; Rz: Rhizogenic; % Response: [a] profuse callusing, [b] moderate callusing, [c] slight callusing**

**Table 2: Effects of different concentrations and combination of growth regulators on indirect organogenesis**

| Growth regulators(mg/l) | Average mean no.of shoots/ callus | Percentage of regeneneration of shoots from callus explant (%) |
|---|---|---|
| **NAA + KN** | | |
| **0.25 + 0.25** | **0.0 ± 0.0** | **0.00** |
| **0.25 + 0.75** | **0.0 ± 0.00** | **0.00** |
| **0.5 + 1.0** | **0.08 ± 0.08** | **2.7** |
| **NAA + BAP** | | |
| **0.25 + 0.25** | **0.0 ± 0.0** | **0.00** |
| **0.25 + 0.75** | **0.08 ± 0.08** | **2.7** |
| **0.5 + 1.0** | **0.33 ± 0.11** | **11.1** |
| **BAP + KN** | | |
| **1.0 + 0.25** | **0.08 ± 0.08** | **5.5** |
| **1.0 + 0.5** | **0.16 ± 0.11** | **8.3** |
| **1.0 + 0.75** | **0.125 ± 0.1** | **16.6** |
| **1.0 + 1.0** | **0.16 ± 0.15** | **22.2** |
| **1.5 + 1.0** | **0.41 ± 0.12** | **44.4** |
| **1.5 + 1.0** | **1.9 ± 0.10** | **47.2** |
| **2.0 + 1.0** | **3.0 ± 0.11** | **80.5** |
| **IAA + BAP** | | |
| **1.0 + 0.25** | **0.00 ± 0.00** | **0.00** |
| **1.0 + 0.5** | **0.08 ± 0.08** | **2.7** |
| **1.5 + 0.75** | **0.16 ± 0.15** | **5.5** |
| **2.0 + 1.0** | **0.41 ± 0.12** | **16.6** |
| **2.0 + 1.5** | **0.75 ± 0.14** | **22.2** |
| **IAA + KN** | | |
| **1.0 + 0.25** | **0.00 ± 0.00** | **0.00** |
| **1.0 + 0.5** | **0.08 ± 0.08** | **2.7** |
| **1.5 + 0.75** | **0.25 ± 0.13** | **8.3** |
| **2.0 + 1.0** | **0.5 ± 0.15** | **16.6** |
| **2.5 + 1.0** | **1.08 ± 0.19** | **36.1** |
| **3.0 + 2.5** | **1.3 ± 0.14** | **41.6** |
| **4.0 + 2.5** | **1.8 ± 0.16** | **66.6** |

**Each value represents mean ± SE of 12 replicates per treatment**

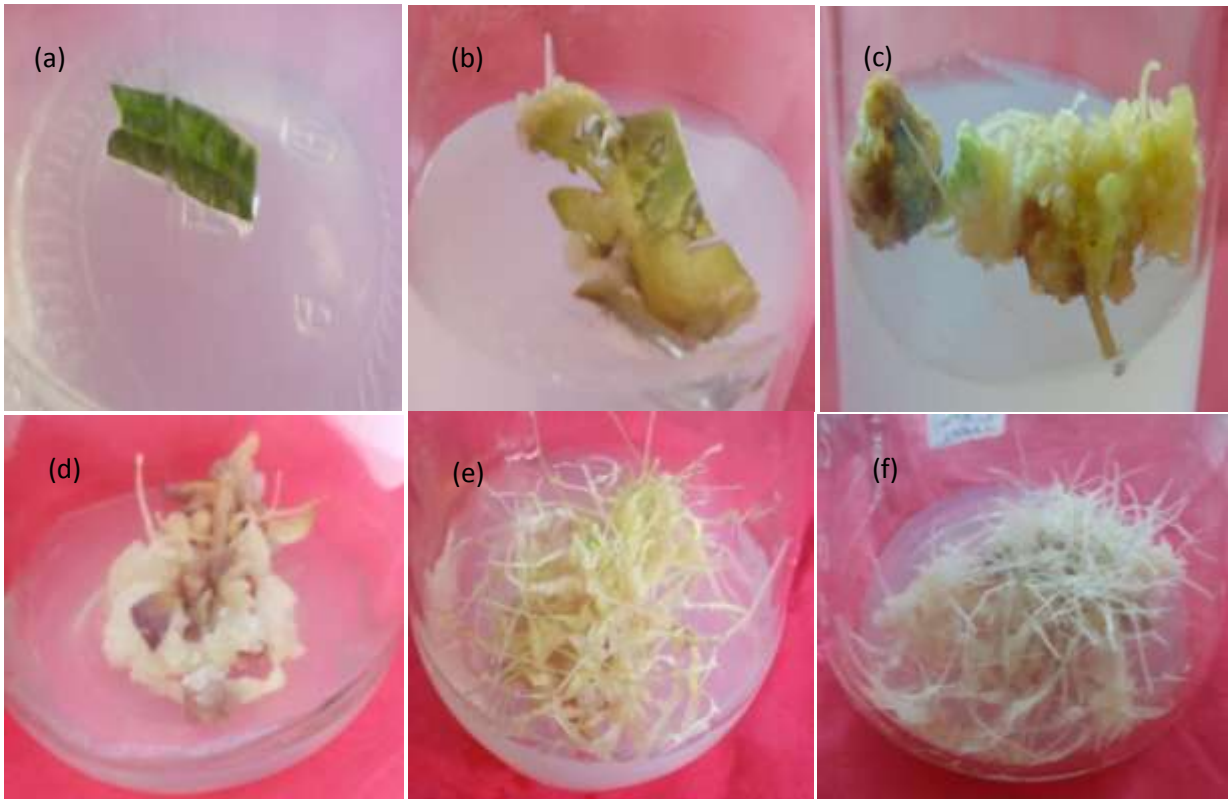


**Figure 1: Callus induction and morphology of *G. kurroo* after 60 days of inoculation. Leaf explant was inoculated on MS media supplemented with growth regulators and incubated in dark at 27±2 °C : (a) zero day (b) callus initiation after 8 days; Compact callus formation on: (c) 2, 4-D (0.8 mg/l) + KN (1.0 mg/l) + BAP (1.0 mg/l); (d) 2,4-D (1.5 mg/l) + BAP (0.5 mg/l)+TDZ (0.5mg/l); Friable callus formation on: (e) NAA (1.0mg/l) + KN (0.5mg/l); (f) BAP (1.0 mg/l) + NAA (2.0 mg/l).**

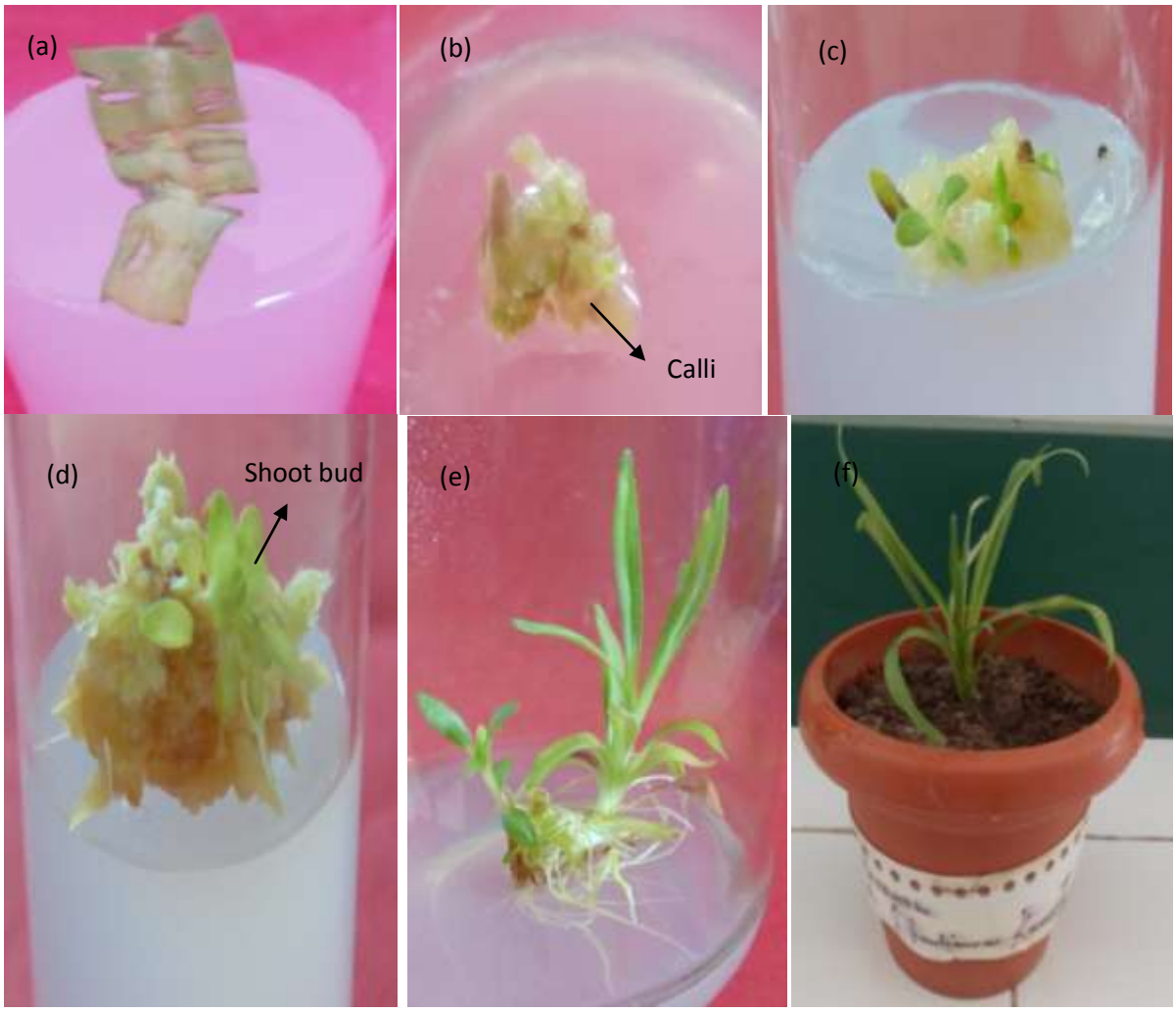


**Figure 2: Plant regeneration through callus cultures of *G. kurroo* (a & b) Establishment of embryogenic callus from the leaf explant on MS+ 2,4-D(1.5 mg/l)+ BAP (0.5 mg/l)+TDZ (0.5mg/l) after 8 days and 4 weeks respectively; (c) Calli differentiating into green shoot buds after 2 weeks; (d) Development of shoots from callus on MS+ BAP (2.0 mg/l)+ KN (1.0 mg/l) after 45 days of culture; (e) Elongated shoots with well developed roots on half strength MS+ IBA (0.5mg/l) after 45 days; (f) Establishment of *in vitro* grown plantlets after six weeks.**

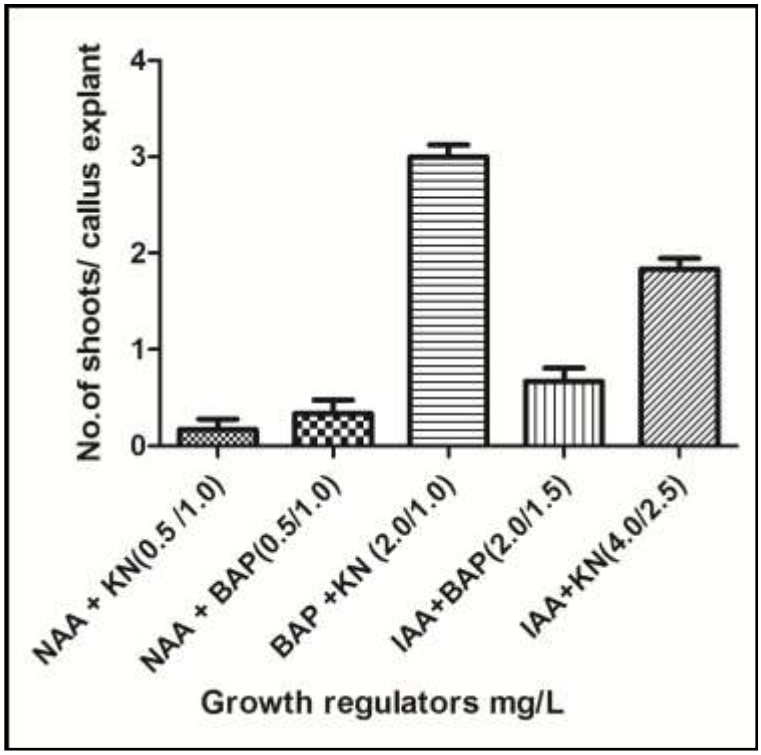


**Figure 3: Effect of different concentrations of growth regulators on average number of shoot bud formation from organogenic calli derived from leaves of *G. kurroo* after 45 days of culture**

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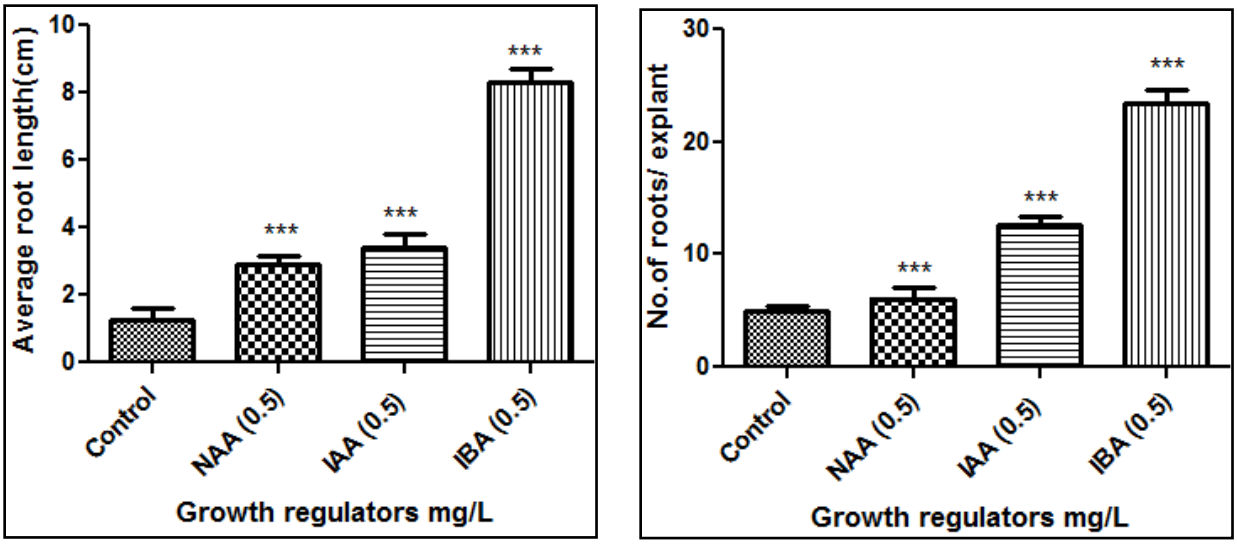


**Figure 4a and 4b: Effect of IBA, IAA and NAA on rooting and growth of *G. kurroo* in half strength MS media after 45 days. Results presented as the average and standard error of experiments performed in triplicate; $^{***}p < 0.005$**

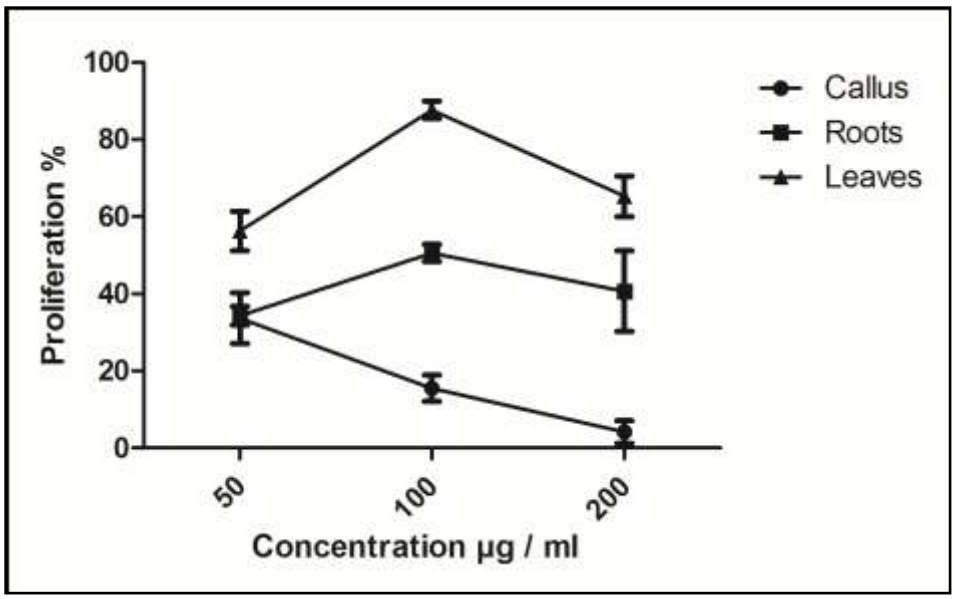


**Figure 5a: Percentage of (PBMC) cell viability after treatment of in vitro grown tissues of *G. kurroo* at various concentrations**

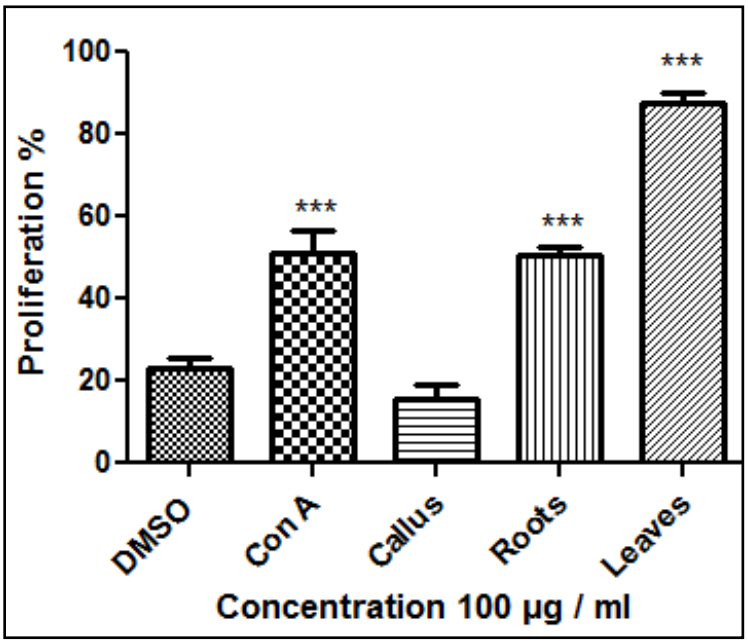


**Figure 5b: Percentage of (PBMC) cell viability after treatment of in vitro grown tissues of *G. kurroo* compared to Control DMSO and Mitogen Con A. Response of the leaves and root extract as a result is highly significant as indicated by ANOVA test., ***p < 0.005**