SCREENING OF PHYTOCHEMICALS IN SHOOT CULTURES OF ENDANGERED HERB- *GENTIANA KURROO* ROYLE

Project Thesis submitted in fulfilment of major project of

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By

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SOLAN (H.P)

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DECLARATION

I hereby declare that the research project work entitled "SCREENING OF PHYTOCHEMICALS IN SHOOT CULTURES OF ENDANGERED HERB- *GENTIANA KURROO* ROYLE" submitted to the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology Solan (H.P), is an article on research carried out on this topic till now. The work is carried out under the guidance of Dr. Hemant Sood.

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SUPERVISOR'S CERTIFICATE

This is to certify that the content of this research project entitled "SCREENING OF PHYTOCHEMICALS IN SHOOT CULTURES OF ENDANGERED HERB- *GENTIANA KURROO* ROYLE" by Ms. Ipshita Dutta is her bona fide work carried out under my supervision, of Biotechnology and Bioinformatics branch at Jaypee University of Information Technology, Solan in the academic year 2021-22 for the fulfillment of the requirements of B.Tech Major Project. Based on the declaration made by her, I recommend this project report for evaluation.

Signature of the Supervisor

Name- Dr. Hemant Sood

Associate Professor, Department of Biotechnology and Bioinformatics, JUIT

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ABSTRACT

Gentiana kurroo Royle is a highly prized medicinal plant of the western and north-western Himalayas that is considered as critically endangered because of several reasons like overexploitation and shortfall of organized cultivation. The present study was conducted to bring out an efficient technique for alternative production of *G. kurroo* through in-vitro propagation. Culture conditions were optimized to develop a coherent shoot multiplication system of *G. kurroo* Royle. Plant tissue culture methods namely direct and indirect somatic embryogenesis were implemented on the shoots of mother plants i.e. explants of *G. kurroo* and the growth was observed after regular intervals of time. The media used was MS Media along with IBA, Kinetin and BAP for direct organogenesis, whereas for carrying out callus induction, IBA+Kinetin was used. So, the in-vitro grown tissues were multiplied for biomass generation and preparing plant extracts. The extracts were found to contain terpenoids, flavonoids and saponins. Zooming in to the medicinal uses; these phytochemicals of *G. kurroo* are valued as antibacterial, anti-inflammatory, anti-periodic, sedative, carminative, stomachic, astringent. Research is still in progress and has a lot to tell us about the pharmacological basis of these activities. This study would be further extended for quantitative analysis of the phytochemicals.

Keywords- Gentiana kurroo, overexploitation, in-vitro propagation, culture conditions

CHAPTER 1

INTRODUCTION

1.1 Introduction

Vernacular names: *Hindi*- Kuru, Kutki; *Sanskrit*- Trayamana; *Urdu*- Neelkanthi

Trade name- *Indian Gentian root*

Flowering season- *August-October*

G. kurroo Royle belongs to the family Gentianaceae (family of flowering plants). ring plants). The Gentianaceae family is represented by more than 90 genera and 1650 species; they are annual and perennial herbs or shrubs, native to northern temperate Mostly found and distributed over Indian states of Jammu and Kashmir, Himachal Pradesh and Uttarakhand and across Pakistan and Nepal specifically between altitudinal ranges of 1500 and 3500 m [1]. It is endemic to the north-western Himalayas [2]. The common name of Gentiana has been derived from "Gentius", a king of Illyria (Europe), who is believed to have discovered the medicinal value of the Gentian root [3]. More than 80% of the population decline of the species has taken place in India in a time period of ten years [4]. Therefore, the species is assessed as critically endangered [5]. India has most of the geographical range (80%) and therefore, situation in India is considered as the representative of the global population of the species.

G. kurroo was first reported in the Kashmir Himalaya by [6] at an altitude of 1850–2000 m (a.s.1) from the Pahalgam area, followed by [7] from the localities of Kangan and Wangat. 43 collections. Due to the large-scale exploitation the plant extinct from the Dachigam National Park, however, G. kurroo Royle has been rediscovered in November 2004, on the floristic expedition to the Dachigam National Park in Kashmir Himalaya after more than sixty years since it was reported earlier [8]. *G. kurroo* Royle known to be adulterated/substituted with roots of Gentiana tenella, Picrorrhiza kurroa, Gentiana decumbens, Exacum bicolor [9-12] for its high demand in international market and limited supply from natural systems. However, the purity and authenticity of crude drugs can be performed by macroscopic, microscopic and anatomical observation besides chemical and ash analysis [13-15]. Plants belonging to genus Gentiana are very popular for the bitter glycosides they contain and have been widely used in

traditional medicine for treatment of different human disorders. The dried roots and rhizomes are official in Indian pharmaceutical codex [16].

Population status:

It is a critically endangered medicinal herb. The drug plant is heavily extracted for root and rhizome. Because of its high medicinal value, the plant species has been over exploited from its natural habitat and no efforts are underway for replenishment and skilled harvesting. The red data book of Indian plants

list this as endangered and its status is critical [17]. According to IUCN this species is declared endangered, and its cultivation is not known [18].

Morphology:

The aerial part of the plant is largely made up of radical leaves that form a rosette. Only flowering branches with cauline leaves are used to symbolize the sprout. The root stock (rhizome and adventitious root) is white to brown in color, with a vertically wrinkled surface and a scaly tuft of leaf bases and blooming shoots at the end. Long, thin, simple, sessile, stipulate, lanceolate, lamina whole, apex acute, leathery texture, and basally connected in pairs form a shared sheath The radical leaves are deep green in colour and remain throughout the life cycle of the plant and gradually replaced by newer leaves. Most of the new leaves are developed upon unset of rainy season [19]. Stems are 10 – 13 cm high. Seeds are numerous and are light in weight and 1000 seed weighs 0.1275 gm [20].

Phenology:

Only flowering branches reflect the perennial herb's shoot. Stem is a rhizome that has been changed. There are two sorts of leaves: radical leaves at the plant's base and cauline leaves on the blooming stalk [21]. The radical leaves are dark green in color and last for the duration of the plant's life cycle before being gradually replaced by fresher leaves. Most fresh leaves emerge once the wet season has ended. The cauline leaves are originally green in color and gradually fade as the inflorescence matures. They shed at the same time as the flowering shoots [22].

Ecology:

This species' plants thrive on the southern open exposed slopes of mid and higher mountain ranges [23-24]. Low temperature, high insolation, strong wind speed, and being surrounded by tall grasses and scrubs, such places like to be overshadowed by them [25].

1.2 Ethnopharmacology and Phytochemistry

The leaf and root stock of *G. kurroo* is valued for presence of the most known bitter glycosidic compounds like **Iridoids** and **Secoiridoids** (Gentianine, Gentiopicrin or Gentiopicroside), xanthones, monoterpene alkaloid; polyphenol and flavones [26,27]. Gentianine possesses anti-inflammatory, analgesic, anticonvulsant, hypotensive, antipsychotic, sedative, diuretic, antimalarial, antiamoebic and antibacterial properties [28]. In Indian system of medicine, the root stock valued as bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, anthelmintic, blood purifier and carminative, antipsychotic, anti-inflammatory, sedative, anti-bacterial [29,30]. It is also medicated for curing skin diseases, leucoderma, leprosy, bronchial asthma, dyspepsia, flatulence, colic, anorexia, helminthiosis, inflammations, amenorrhea, dysmenorrheal, strangury, hemorrhoids, constipation and urinary infections [31,32]. Because of its similar properties the root stock is many times substituted for Gentiana lutea, the true or European gentian [33,34]. It is an important ingredient of many tonics for stomachic preparation too [35]. Amongst these ethnomedicinal properties, anti-inflammatory activity of flower tops [36] and analgesic activity of roots (methanolic extracts) of *Gentiana kurroo* [37] is scientifically validated.

This herb's rhizome and roots include bitter glycosides such as Gentiopicrine, Gentianamarin, and Gentianine (alkaloid) [38,39].

Leaves also contain some bitter compounds significantly higher than roots [38].

It contains iridoid glycoside 2′-(2, 3- dihydroxybenzoyloxy)-7-ketologanin [39] and 16 volatile aroma compounds [40]. Flower tops contain alkaloids, flavonoids, glycosides, free phenols and terpenes [41].

A new bitter acylated iridoid glucoside, 2'-(2,3-dihydroxybenzoyloxy)-7-ketologanin, has been isolated from the leaves of *Gentiana kurroo*. The structure of the compound was elucidated conclusively by chemical analysis, and extensive 1D and 2D NMR experiments [42]. Iridoids are vital phyto-constituents of the Gentiana genus. They are plant protectants

and markers for multiple gena in diverse plant families, including Plantago (Plantaginaceae), Galium (Rubiaceae), and Gentiana. [43].

The presence of these glycosides such as iridoids and secoiridoids can be used as marker metabolites in phytochemical analysis of *G. kurroo.*

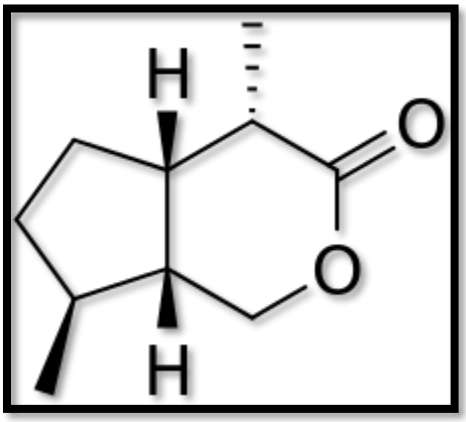


Fig.1.1
Structure of Iridoid
(Source- Wikipedia)

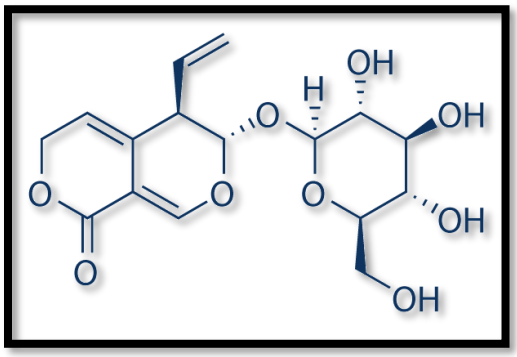


Fig.1.2
Structure of the principal component- Gentiopicroside
(Source- Wikipedia)

1.3 Need for in-vitro production

Even though this plant species reproduces by seeds, there are some bottlenecks. To begin with, the seeds take 6–9 months to germinate after being planted in soil, and the germination rate is low. Second, after 3–4 years of pollination, seeds are created [44,45]. As a result, the in vitro micropropagation approach appears to be a viable option for enhancing plant production efficiency and scale.

Organogenesis is a significant way of generating plants by large-scale vegetative propagation, induction of somatic embryogenesis, and cell selection in vitro.

CHAPTER 2

REVIEW OF LITERATURE

This chapter focuses on the overview of materials and methods of the previously published works on specific areas such as plant tissue culture and determination of the total metabolite content of *Gentiana kurroo* Royle using qualitative and quantitative techniques.

Speaking of usual cultivation practices, seeds, stem cuttings, and root divisions are all options for propagation. It needs a cool, temperate climate and well-drained soil to thrive. When developing, it demands plenty of sunlight and water. It is not cultivated on a significant scale anywhere in India [46].

2.1 In-vitro propagation

Anshu Sharma et al. in 2014 showed the in vitro morphogenic response of different explants of *Gentiana kurroo* Royle—an endangered medicinal plant.

I) Direct Regeneration:

Different leaf, petiole, and root explants from in vitro growth cultures were examined for direct regrowth without callus formation. Explants were grown on MS medium containing BA (0.25 mg/l–2.5 mg/l), TDZ (0.10 mg/l–1.0 mg/l) and NAA (0.10 mg/l) as well as other plant growth regulators. The average number of explants displaying re - growth and the proportion of shoots regenerated solely from explants were measured on numerous medium combinations.

Results- MS media containing 0.10 mg/l NAA and 0.75 mg/l TDZ was shown to be the optimum medium for direct organogenesis petiole, with a frequency of 73.3% of direct organogenesis. (Fig.2.1)

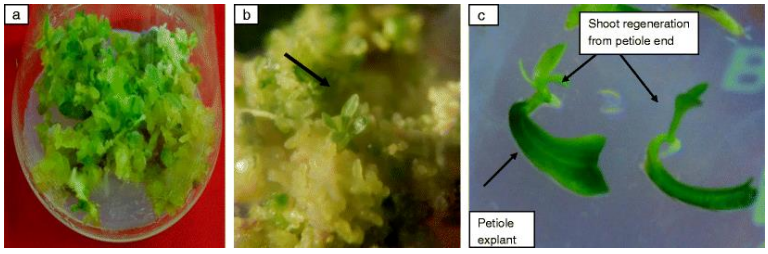


Fig.2.1

a) Regeneration of shoots from petiole derived calli on medium, b) regenerating calli, c) regeneration of shoots from petiole on MS basal after 4 weeks

II) Callus Induction and Regeneration

Explants used: Root, leaves and petioles (from in-vitro multiplying shoots). Explants were grown on MS medium treated with several growth regulator combinations [47]. Explants were incubated in both light and dark environments (incubated in dark for 1 week and the photoperiod was then changed to 16 hours of light and 8 hours of darkness. The growth pattern, kind, color of callus, and proportion of explants developing callus were all recorded daily, and the experiment was repeated three times. On green petiole derived Calli, regeneration was attempted. NAA (0.10 mg/l), kinetin (0.50 mg/l), BA (0.50 mg/l–3.0 mg/l), and TDZ (0.10 mg/l–1.0 mg/l) were cultivated on MS basal medium supplemented with various of plant growth regulators such as NAA, kinetin, BA, and TDZ.

Results- Explants of leaf, root, and petiole were cultivated on MS medium (Fig.2.2-a,b,c) with NAA and BA in various combinations. After roughly a week of growing the explants, callus formation began. On all media combinations, the leaf was the least responsive explant for callus induction, followed by the response of roots. (Fig.2.2-d,e,f)

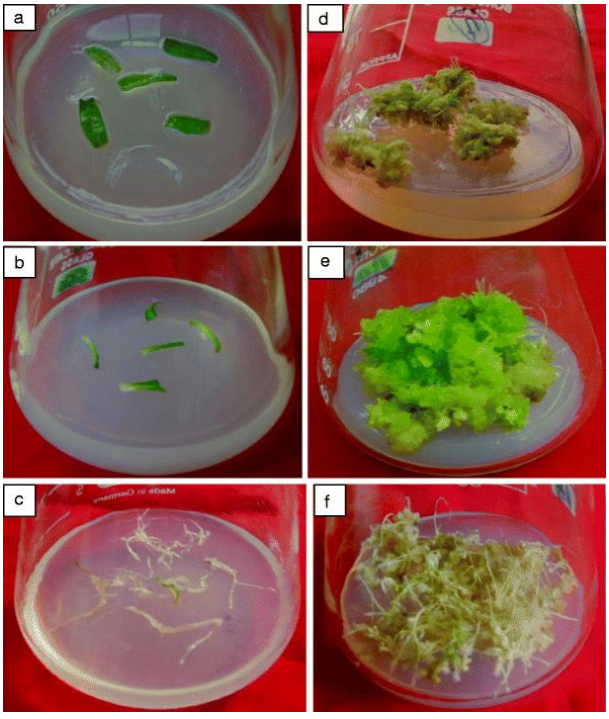


Fig.2.2 -Callus induction stages

The occurrence of certain growth regulators in the culture media, the type of explant, and the light conditions all had a substantial impact on the quality and texture of the callus (dark and light). In all the tested concentrations of NAA and BA, callus was induced. When callusing was incubated for one week in the dark, it was shown to be more vigorous. During the early stages of explant callusing, root explants produced creamish white and brownish white coloured compact hard calli when incubated under light and dark conditions, respectively, whereas leaves produced yellowish green and light green calli with profuse rooting when incubated under light and dark conditions, respectively.

III) Shoot Multiplication

Shoots were withdrawn from the parent culture and placed in a multiplication medium, after which they were shifted to MS medium for shoot elongation, which was reinforced with three distinct growth regulators.

Results- The optimum medium for shoot multiplication was MS medium enriched with 0.50 mg/l BA and 0.50 mg/l Kn. After multiplication, the shoots were switched to MS medium containing 0.50 mg/l NAA and 0.50 mg/l BA, and the largest increase in shoot length was seen when compared to other growth regulators.

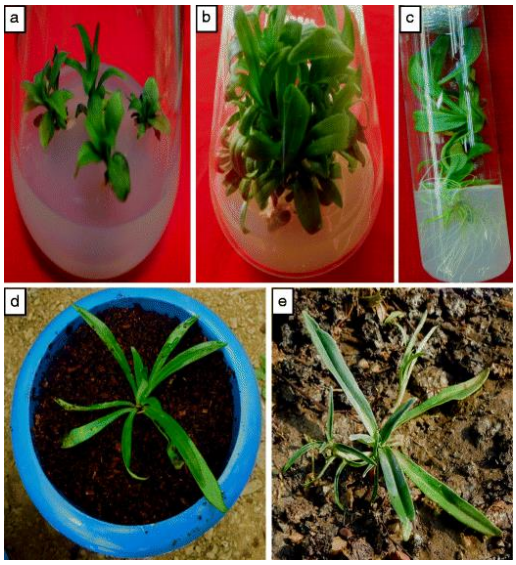


Fig.2.3- Stages of shoot multiplication after intervals of time

IV) Root Induction

Callus and petiole shoots were removed in vitro and transplanted to MS solid and liquid medium of various strengths supplemented with varying concentrations of auxins such as IBA and NAA. Root initiation started about 15 days after the shoots were cultured. (Fig.2.3). After 5 weeks of root initiation, observations were made on the proportion of shoots that regenerated roots, the average number of roots and the average root length.

V) Adjustment of regenerated plants

Plantlets with well-developed roots were removed from culture tubes, washed under running tap water for 1–2 hours to remove agar, and then treated with bavistin before being transferred to small plastic pots containing various autoclaved potting mixtures such as coco peat, sand: soil: FYM, soil: sand, vermiculite, and coco peat: perlite.

2.2 Scientific Study of *Gentiana kurroo* Royle

- The phytochemical screening depicted different groups of bioactive compounds present in different parts of the plant. [48]
  1. Leaf- [49, 50, 51]
     - Iridoid Glycoside
     - (2,3-Dihydroxybezoyloxy)-7-Ketologanin
     - Volatile Aroma Compounds
     - Dimethyl Sulphide
     - 1,8-Cincole
     - A-Terpinyl Acetate
     - Methandriol
     - 1,3-Propanediol
     - 2-Methyl Sulphide
     - 3-Methyl Butanol

2. Root and rhizome- [52,53,54,55,56,57,58]
    - Tannins
    - Alkaloids
    - Saponins
    - Glycosides (Gentiopicrine, Gentianine)
    - Terpenes
    - Flavonoids
    - Phenolics
    - Carbohydrates
    - Genianic Acid
    - Pectin

3. Phytoconstituents present in methanolic extracts of G. kurroo- [59]
    - Flavonoids
    - Phenols
    - Terpenes
    - Tannins
    - Alkaloids
    - Cardiac glycosides

Anti-inflammatory and anti-arthritic activity-

A recent study [60, 61] found that extracts of *G. kurroo* were effective in both acute and chronic anti-inflammatory tests. A rat carrageenin-induced paw edema model was used to investigate the immediate inflammatory impact. At a concentration of 250 mg/kg of body weight, various plant extracts were tested for anti-inflammatory efficacy. Methanolic extracts of *G. kurroo* showed the greatest promise for reducing the inflammatory response. The inhibitory effect on Wistar rats' paw edema was 47.62 percent, and it was significant (p -0.05) when compared to the control group (55.24 percent). However, a dose of 750 mg/kg body weight resulted in the highest level of activity (67.27 percent), which was even higher than the normal drug (56.36 percent). The results were statistically significant when compared to the control group, but non-significant when compared

to the standard group (p 0.05). Although the research on the anti-inflammatory drug obtained from a methanolic extract of *Gentiana kurroo* against rheumatoid arthritis is extensive, the likely mechanism and identification of the bioactive ingredient from *Gentiana kurroo* extract is restricted; yet *G. kurroo* is proven as a medicinal source for inflammation and rheumatoid arthritis.

2.3 Production of Phytochemicals

PRELIMINARY PHYTOCHEMICAL SCREENING AND EVALUATION OF ANALGESIC ACTIVITY OF METHANOLIC EXTRACT OF ROOTS OF *GENTIANA KURROO* ROYLE IN EXPERIMENTAL ANIMAL MODELS [70]

1. *Gentiana kurroo* Royle was gathered on field trips in the lower slopes of the Pirpanchal range of the Kashmir Himalaya at an altitude of 2150 m.
2. The plant's roots were cut, thoroughly cleaned, and dried in the shade. The material was cut and then ground to powder after drying. At 60-65°C, dry root powder (100 g) was packed in a Soxhelt apparatus and extracted with methanol. The solvent was extracted under lower pressure at 35-45°C using a Buchi rotavapor after the extract was filtered through Whatmann filter paper No.1. For experimental purposes, the dried extract was maintained in storage vials at 4°C.
3. Conventional phytochemical techniques were used to screen for main bioactive elements such as tannins, alkaloids, saponins, cardiac glycosides, terpenes, flavonoids, phenolic compounds, and carbohydrates.

- Tannins- 2 mL of 5 percent FeCl3 was added to 2 mL of ethanol extracts. The presence of tannins is indicated by the formation of a yellow brown precipitate.
- Alkaloids- 1.5 mL of 1 percent HCl was added to the 2 mL methanolic filtrate. 6 drops of Mayors reagent/reagent/Dragendroff Wagner's reagent were added to the solution after it was heated in a water bath. The presence of alkaloids is shown by the formation of orange precipitate.
- Saponins- A 2 g powder alcoholic extract was prepared and tested for foaming. Saponins were detected by the persistence of frothing. After that, a few tablespoons of olive oil were added to the foam. The presence of saponins was suggested by the formation of an emulsion.

- Cardiac glycosides- 1 ml glacial acetic acid and 1-2 drops of FeCl 3 were added to 2 ml alcoholic filtrate, followed by 1 ml concentrated H2SO4. The presence of cardiac glycosides is indicated by the appearance of a brown ring at the interface.
- Terpenes- To produce the layer, 2 ml of alcoholic extract, 5 ml chloroform, 2 ml acetic anhydride, and concentrated H 2SO4 were carefully added. The presence of terpenes is indicated by the reddish-brown coloration of the contact.
- Flavonoids- Two grams of plant material were extracted in 10 ml of alcohol or water. A few drops of strong HCl were added to 2 ml filtrate, followed by 0.5 g zinc or magnesium turnings. After 3 minutes, the existence of flavonoids was shown by a fuchsia red or pink color.
- Phenolics- 1 ml of 1 percent FeCl3 solution was added to 2 ml of alcoholic or aqueous extract. The presence of phenols is indicated by a blue or green colour.
- Carbohydrates- To validate the existence of carbs, Benedict's test (a test for decreasing sugar) and Fehling's test (a standard test for reducing sugar) were used.

4. The findings of the plant's phytochemical screening revealed that G. kurroo roots are high in a variety of active compounds (secondary plant metabolites). It backs up the plant extract's ingenuity. The plant extract was shown to be high in tannins, flavonoids, phenolics, cardiac glycosides, terpenes, and alkaloids as a result of phytochemical screening.
5. Flavonoids, alkaloids, and terpenoids found in the plant could be accountable for its analgesic, anti-malarial, and gastrointestinal disease therapy properties. The extract demonstrated considerable analgesic effectiveness at both dosage levels in analgesic trials.

CHAPTER 3

RATIONALE OF STUDY AND CONSERVATION STRATEGY

Plant-based pharmaceuticals and treatments are becoming increasingly popular around the world, resulting in increased awareness and demand. Conventionally, a variety of plants and their preparations have been used to cure a variety of ailments. Traditional medications as alternative and supplemental therapies for the treatment of many ailments need extensive investigations for scientific endorsement. Normally when the plant is sown, its roots and rhizomes are used for the extraction of secondary metabolites. But ever after it has become endangered, the leaves which are grown using artificial plant biotechnological methods can also be used for extraction of the bioactive compounds so that the plant is not over-exploited anymore.

The in-vitro culture is a viable alternative to produce useful secondary metabolites in a feasible manner. And to this, plant tissue culture has been found to produce a higher number of stable secondary metabolites [62].

Production of bioactive compounds in-vitro would save the population of *G. kurroo* from their native lands and provide quality rich herbal material to the pharmaceutical/biotech industry.

Even though very little work has been done on *G. kurroo* till date, best efforts have been made to collate all the research carried out earlier along with the self-performed methodology for artificial production including techniques like Direct and Indirect Somatic Embryogenesis and cell suspension cultures. Phytochemical analysis has also been performed for ruling out the basis for the pharmacological properties of the bioactive compounds.

Traditional medicinal plant extraction on a wide scale could be reduced to prevent these plants from being critically endangered. This could be accomplished by exploring new ecological niches with potential sources of bioactive compounds for a variety of pharmacological, agricultural, and industrial applications that are renewable, environmentally benign, and easily accessible [63]. The discovery of novel bioactive molecules played a significant part in the hunt for new medications, and they are one of the most powerful sources of novel bioactive molecules innovation. Isolation, structural elucidation, and establishing the bio-synthetic pathway of secondary metabolites are all strategies used in the discovery of natural products.

Bioprospecting of medicinal plant endophytes will be a novel strategy for drug development with minimal environmental impact and the potential to help conserve critically endangered medicinal plants [64].

Objectives

i. To carry out in-vitro propagation of *Gentiana kurroo* medicinal herb.

ii. To screen the phytochemicals in in-vitro raised plants.

CHAPTER 4

MATERIALS AND METHODS

The purpose of this chapter is to present a detailed protocol of micropropagation used for culturing *G. kurroo* under artificial lab conditions and using the cultured plants for analysis of bioactive compounds.

Plant Source- Mother plants or invitro grown shoots were obtained from culture room (25 °C) of the Plant Tissue Culture Laboratory of Jaypee University of Information Technology, Solan).

4.1 Direct Organogenesis- Plants were propagated directly from the shoots of mother plants with no intermediate callus phase.

REAGENTS AND EQIPMENTS:

For MS Media-

1. 8 stock solutions (A-H)
2. Growth hormones IBA, Kinetin and BAP
3. Distilled water
4. Sucrose
5. Agar
6. 20-30 jars
7. 1M NaOH and 1M HCl for pH adjustment

Equipment-

8. Measuring cylinders and petri plates
9. pH meter
10. Hot plate
11. Stirrer
12. Scalpel and forceps
13. Autoclave
14. Tweezers
15. Aluminum Foil paper

PROTOCOL:

4.1.1 For 1 liter MS Media preparation-

- Added stock solutions A-H as per the measurements in a large beaker

  Stock A- 100 g/L

  Stock B- 50 g/L

  Stock C- 10g/L

  Stock D- 10g/L

  Stock E- 10g/L

  Stock F- 10g/L

  Stock G- 10g/L

  Stock H- 10g/ L
- Added 3mg/L IBA, 1 mg/L BAP and 1mg/L kinetin to the above mixture of stock solutions.
- Weighed 30 gms of sucrose in a weighing balance and mixed with the above solution thoroughly while continuously stirring.
- Added distilled water to the media for making up the volume upto 1000ml.
- Adjusted the pH to 5.6-5.7 using 1M NaOH or 1M HCl as required in a pH meter while gently stirring. Weighed 9 gms of agar and added it to the media.
- Poured the media in a boiling pan and kept it for heating until the agar dissolved completely.
- After about 15-20 minutes, the still warm media was poured into 20-25 sterilized plant tissue culture jars with the lids not so tightly placed.

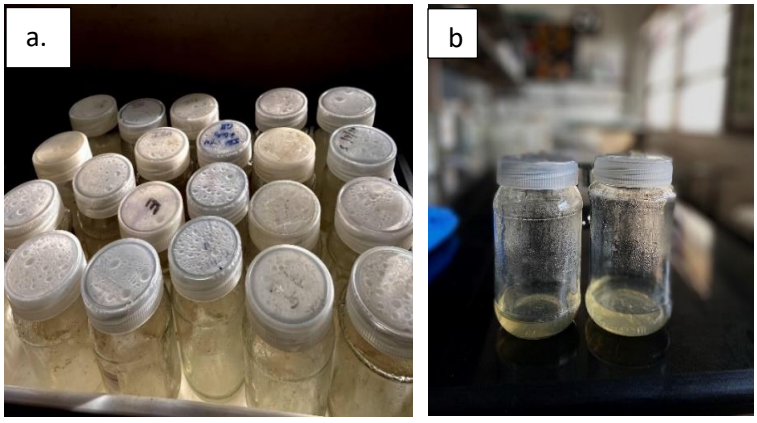


Fig 4.1 – Media jars for culturing of G. kurroo

4.1.2 Sterilization of the media

Petri plates, tweezers and forceps were covered in aluminum foil followed by autoclave bag and put inside the autoclave along with the media jars. Temperature was set at 121°C and pressure at 15 psi. Pressure was released after 15 minutes, and the media left to cool down.

4.1.3 Micropropagation of *G. kurroo* cultures

- The Laminar Air Flow was switched on and surface sterilized using ethanol wipes.
- Fully grown mother plants were brought from the culture room inside the LAF, and the lids sterilized with heat using Bunsen burner.
- Forceps, tweezers and the jars taken out from the autoclave were also heat sterilized before using.
- Shoot apices were carefully taken out from one of the mother plant samples using tweezers and placed on a petri plate, and the lid closed.
- A section of the shoot was placed carefully inside the solid media jar with sterile forceps in such a way that a small portion of the shoot were partly submerged in the media.
- The same procedure was followed for all the remaining jars too and kept inside the culture room which was well lit and a temperature of 25 degree Celsius was maintained.
- New shoots started to appear within two weeks of time.

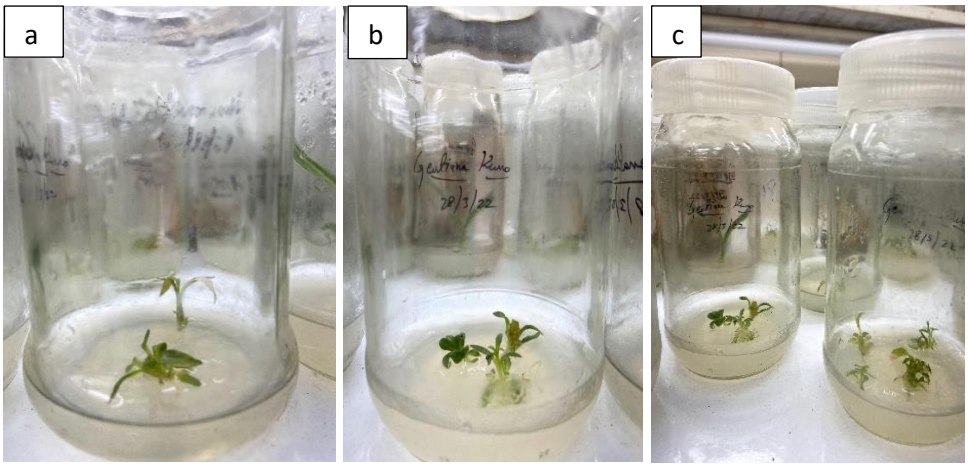


Fig.4.2
Plants kept for culturing in the optimized growth conditions (as mentioned above)

4.2 Cell suspension culture/ indirect organogenesis-

- By following the culture protocols, leaf discs were utilized for callus induction and suspension culture.
- Fully grown mother plants were brought from the culture room.
- Shoot parts were carefully taken out from one of the mother plant samples.
- They were then cut into small pieces using forceps (preferably 4), injuries were introduced and carefully submerged into the liquid media separately.
- Observations were noted after 2 weeks.

Fig.4.3- Callus induction under suspension system (a: set-up for suspension culture; b and c: leaf discs in suspension culture

4.3 Preparation of plant extract for phytochemical analysis-

Protocol followed for preparation of plant extract was reported by Abubakar et al. [71]

REAGENTS AND EQUIPMENTS

1. Plant sample
2. Flasks
3. Blotting sheets
4. Oven
5. Weighing balance
6. Shaking incubator
7. Whatman filter paper
8. Petri plates

PROTOCOL-

DAY 1:

- Fully grown G. kurroo plant samples from about 6-7 jars were obtained from the culture room.
- Shoots were plucked out from the jars carefully, washed with tap water to remove any media stuck on to the plant parts and cut into little pieces using forceps.
- Now they were transferred to a fresh blotting sheet immediately to have all the water soaked.
- The blotting paper along with the shoot parts were left for drying overnight.

Fig. 4.4- In-vitro grown *G. kurroo* shoots kept for air drying

DAY 2:

- The shoot portions left for drying overnight were now transferred to a hot oven on the same blotting paper for proper drying for about 2 hours.
- The temperature of oven was set at 40°C.

Fig.4.5- Plants kept for oven drying

- After 2 hours, the plant parts were taken out from the oven and crushed to powder form using mortar and pestle. Total weight was 1 gm.

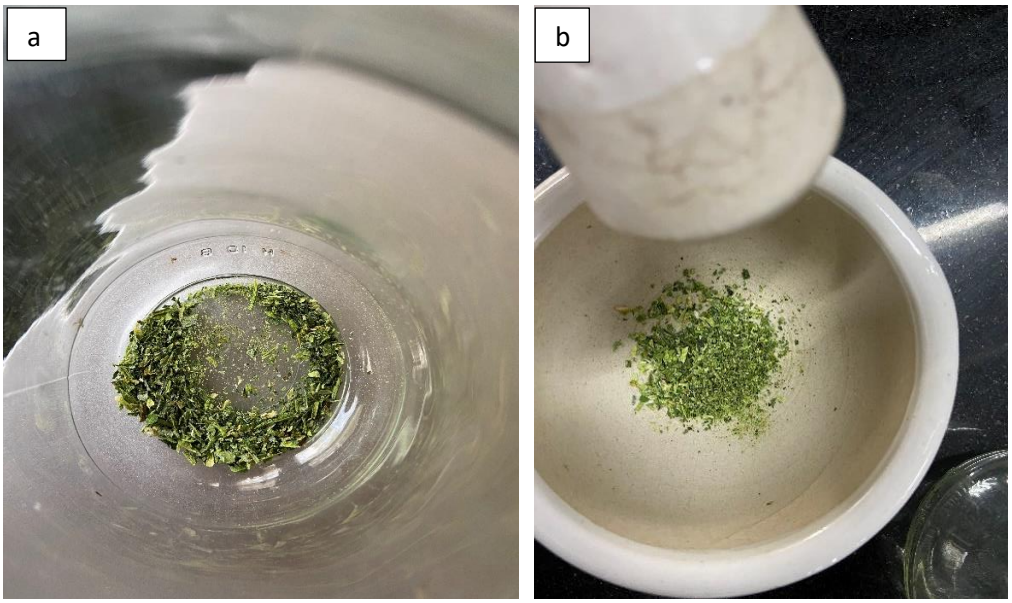


Fig.4.6- Powdered form of *G. kurroo* shoots

- 20ml of 80% methanol was prepared by adding 2ml water in 18ml methanol.
- The powdered form of the plant parts was then dissolved in the 20ml methanol prepared in a conical flask; the mouth covered using foil paper.
- The flask was then placed in the shaker for one day.

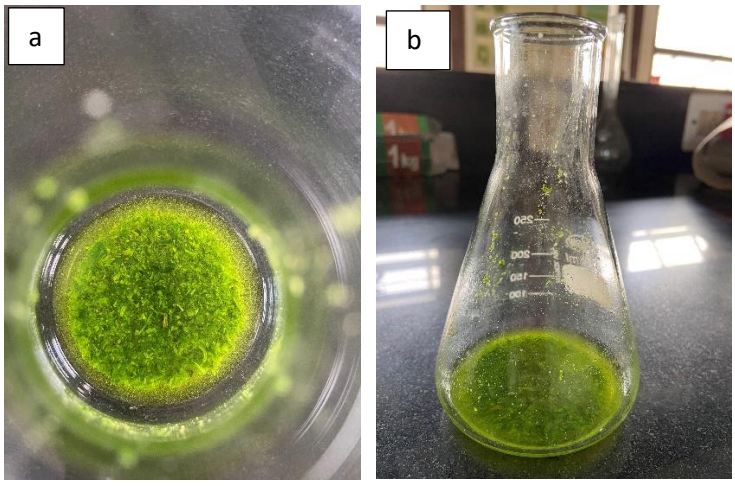


Fig.4.7 -Methanolic extract of G. kurroo

DAY 3:

- After removing the flask containing extract from shaker, the extract was filtered out using Whatman filter paper on a petri plate.
- The liquid filtrate was collected on a petri plate and left to evaporate for one day.

Fig.4.8- Extract in flask after removing from shaker

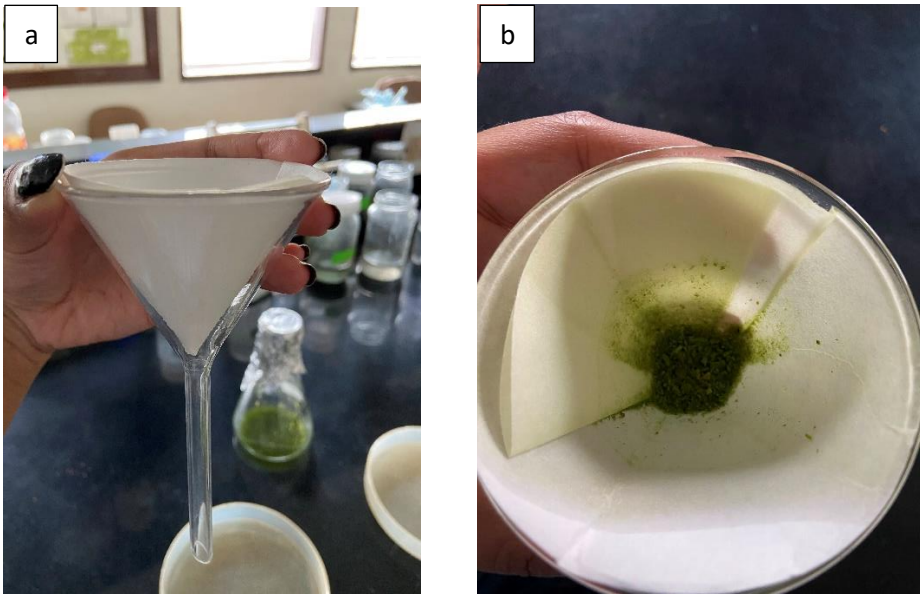


Fig.4.9- Filtration of plant extracts

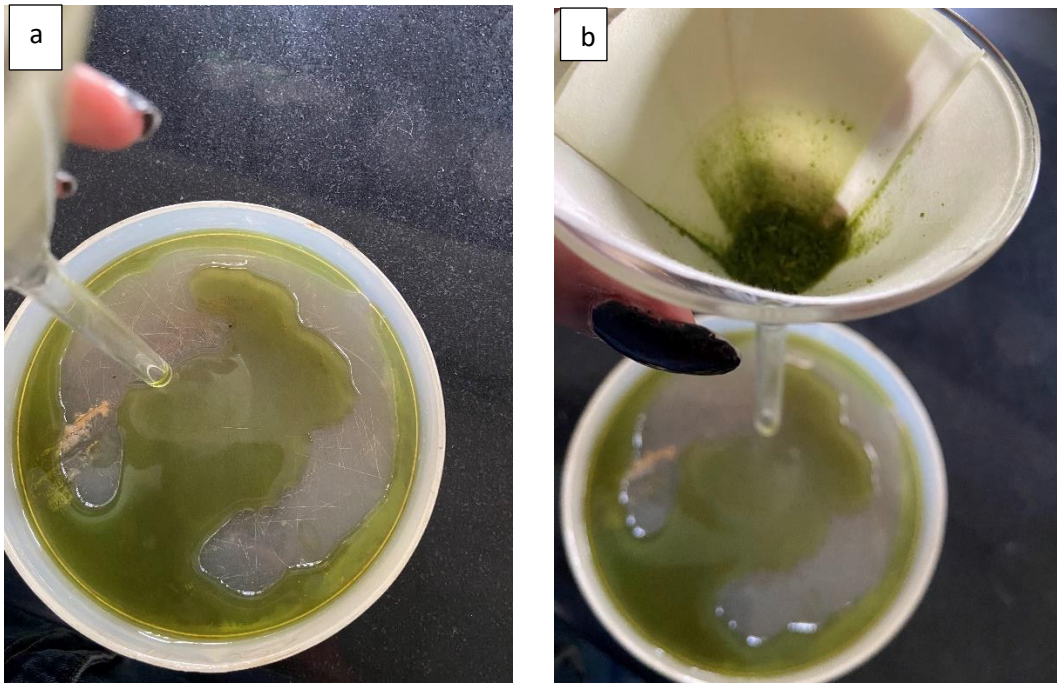


Fig.4.10- Liquid filtrate of methanolic extract of G. kurroo

DAY 4:

- Evaporated solid filtrate obtained from day 3 was scooped out using a spatula and transferred to an eppendorf vial for further analysis.

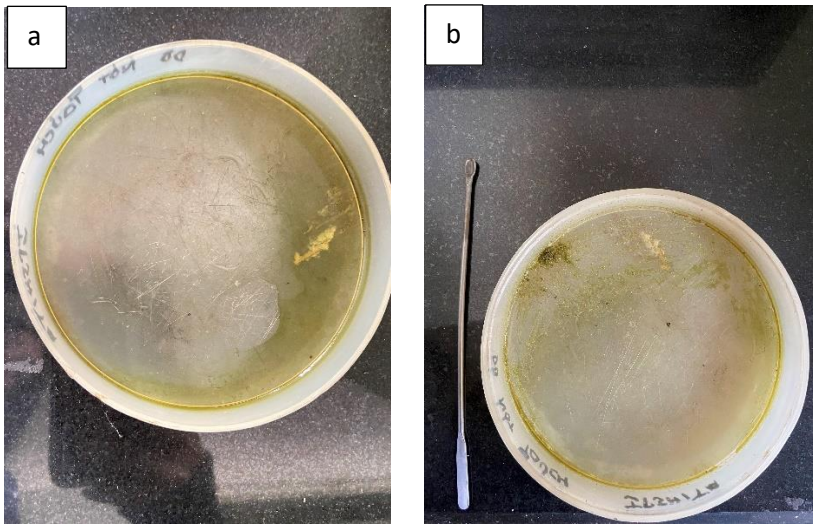


Fig.4.11- Solid powdered-like filtrate after evaporation of methanol

4.4 Qualitative analysis

Different qualitative phytochemical investigations are known that allow the determination of chemical groups or compounds in leaves extract from various plants using standard analytical techniques.

The tests mentioned below were performed for the plant species *Gentiana kurroo* Royle so as to determine the existence of some important bioactive compounds.

The solid powdered-like filtrate shown in fig-4.11 was transferred to an effendorf vial as depicted in fig-4.12.

Compounds such as saponins, tannins, terpenoids, steroids, starch, total sugar, free reducing sugars, ascorbic acid, alkaloids, phenols, flavoniods, and glycosoides can all be tested qualitatively [64].

Fig.4.12- G.kurroo extract collected in a vial

The above shown extract was again dissolved in 5ml of methanol in a test tube to obtain a solution as shown in fig-4.13. It was left to stand for 5-7 minutes before using for analysis.

Fig.4.13 -Extract dissolved in methanol

Table 4.1- Qualitative assay tests perfomed on methanolic extract of G. kurroo

Phytochemical	**Quantity of plant sample**	**Reagent 1 and quantity (and intermediate indication if any)**	**Reagent 2 and quantity**	**Final Observation if phytochemical is present**	**References**
TERPENOIDS (Salkowski test)	1 ml	2ml Chloroform	3 ml sulphuric acid	Reddish brown layer	[66]
PHENOLIC COMPOUNDS (Ferric chloride test)	1 ml	2 ml distilled water	10% ferrous chloride	Blue green appearance	[67]
SAPONINS (Foam test)	1 ml	5 ml distilled water (Froth formation)	3-4 drops of oil	Emulsion formation	[68]
FLAVONOIDS (Alkaline reagent test)	2 ml	Few drops of sodium hydroxide (yellow color seen)	Few drops of hydrochloric acid	Colorless solution	[69]

These were the 4 tests carried out on the methanolic extracts of G. kurroo to check for the presence of some important bioactive compounds like terpenoids, phenolic compounds, saponins and flavonoids.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Direct organogenesis-

- The optimum medium for shoot multiplication was MS medium supplemented with 3 mg/l IBA, 1 mg/l Kn and 1 mg/l BAP.
- It took 20–25 days for various shoots to arise.
- Results were recorded accordingly.

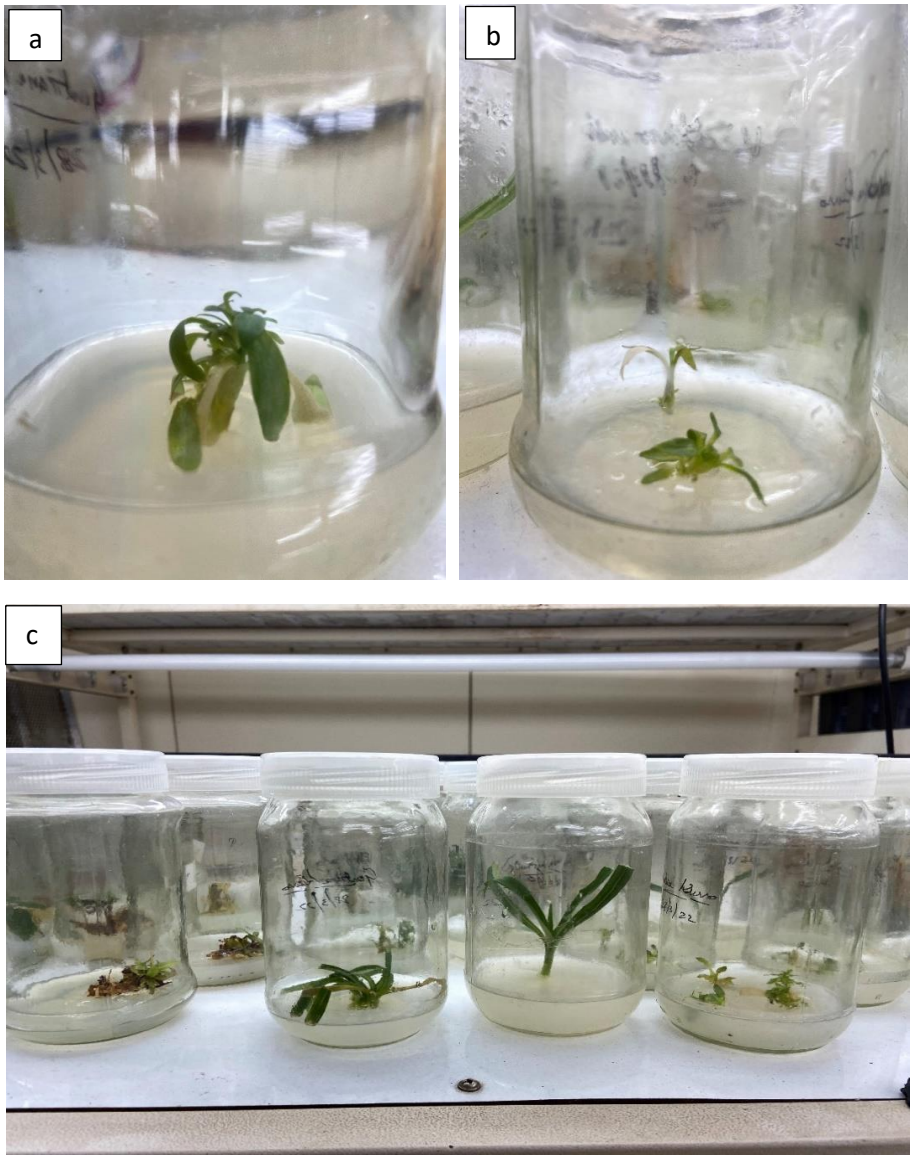


Fig. 5.1- a and b: shoots observed after 1-2 weeks of culturing; c: shoots observed after 3 weeks of culturing

- The effect of growth hormones IBA and kinetin were clearly illustrated from the above set of figures taken after intervals of time.
- *Gentiana kurroo* seedlings, regenerant organs, and callus cell suspension have a high capacity for somatic embryo development and shoot regeneration.
- In MS agar media enriched with 3 mgl-1 Kin, 1 mgl-1 IBA and 1 mgl-1 BAP, cotyledon derived cell suspension had the highest morphogenic potential among root, hypocotyls, and cotyledon in seedling explants.
- The source of suspension cultures , enzymatic treatment, aggregate fraction size, and culture medium composition all affect the efficiency and efficacy of *Gentiana kurroo* regeneration via micropropagation.

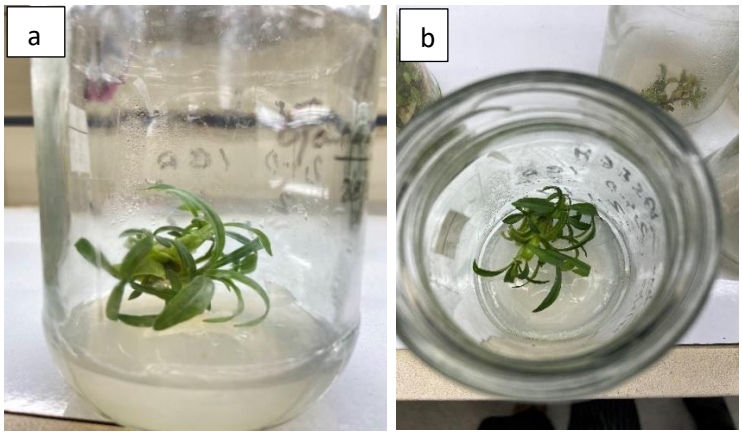


Fig.5.2- Shoots observed after 4 weeks of time

Fig.5.3- Fully grown shoot part

Table 5.1- Growth and development observed in shoots of *Gentiana kurroo*

WEEKS	NUMBER OF SHOOTS	BIOMASS (in grams)	LENGTH OF SHOOTS (in cm)
1	3-4	0.1	0.5
2	4-6	0.4	0.8
3	6-10	0.9	1
4	10-12	1.2	1.55

Hence, the fully grown shoots were observed after 4 weeks time and their characteristic features were noted down after every one week as mentioned in Table 5.1.

5.2 Observations for callus formation through cell suspension cultures-

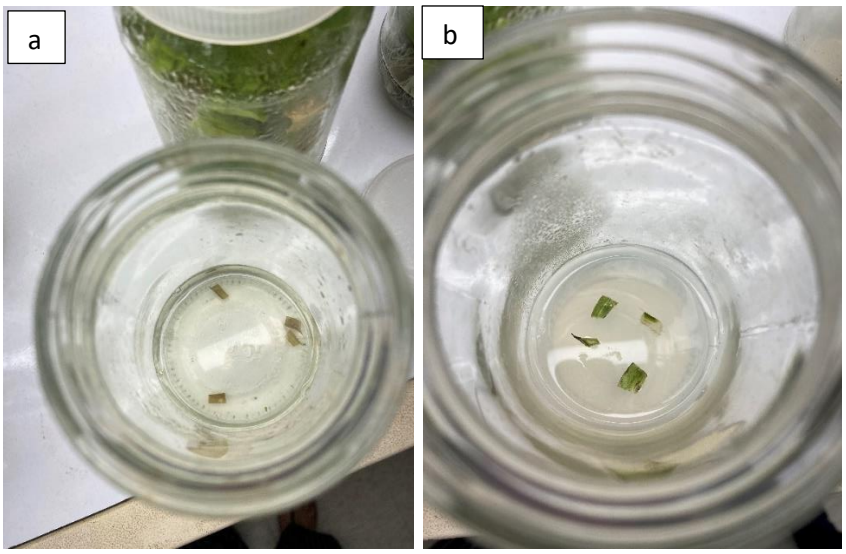


Fig.5.4- Leaf explants suspended in suspension culture/ callusing medium

After 3 repititions, no callus induction was observed under the suspension system; so other medias might be tried for further experimentations.

5.3 Qualitative analysis

As mentioned in materials and methods, the tests have been performed for identification of phytochemicals in G. kurroo and following observations have been made-

- **Terpenoids-** G. kurroo sample extract tested positive for terpenoids as a reddish brown layer was observed as shown in Fig.5.5.

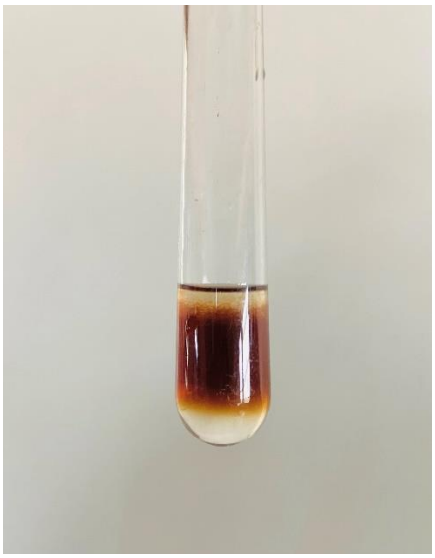
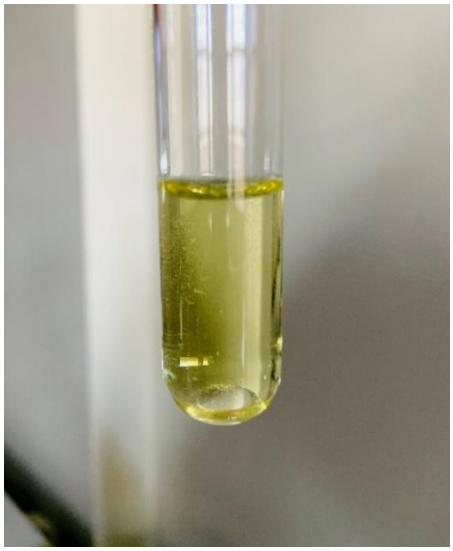


Fig.5.5- Reddish brown layer on adding reagents to plant sample

- **Phenolic compounds**- G. kurroo plant sample tested negative for phenols as there was no blue-green appearance noted.
- **Saponins**- The plant contains saponins as there was formation of emulsion observed after adding few drops of oil as shown in Fig.5.6.



Fig,5.6- Emulsion formation at the bottom of the test tube

- **Flavonoids-** The plant contains flavonoid as a phytochemical as the solution turned colorless after adding few drops of HCl as depicted in Fig.5.7.

Fig.5.7- Colorless solution indicating presence of flavonoids

Table 5.2- Results for qualitative assay of G.Kurroo extracts

TEST	OBSERVATION	INFERENCE
Salkowski test for TERPENOIDS	Reddish brown layer	**Positive; terpenoids present**
Ferric chloride test for PHENOLIC COMPOUNDS	No blue-green appearance	**Negative; phenolic compounds absent**
Foam test for SAPONINS	Formation of emulsiom	**Positive; saponins present**
Alkanine reagent test for FLAVONOIDS	Colorless solution	**Positive; flavonoids present**

DISCUSSION

So, the present study focuses on in-vitro propagation of *Gentiana kurroo* and its phytochemical analysis. The study reported that the plant can be grown artificially under optimized culture conditions both using direct and indirect methods of organogenesis. The results of phytochemical analysis showed that the in-vitro grown plant tissue extracts of G. kurroo contained terpenoids, saponins and flavonoids. These studies are in sync with those mentioned in review of literature [48, 70] whereas a more vast experimentation is required for further explorations in G. kurroo. Callus was not induced in the experiment done in the present study. As Anshu et al. in 2014 reported callus in media, in the present study, callus was not induced in leaf tissues of in-vitro grown G. kurroo. Until now, the analysis of bioactive compounds have been made on plants which are grown in the field. But this study makes use of in-vitro grown explants which are used for the phytochemical analysis. This is an effective strategy because the already endangered plant doesn't have to be exploited anymore for experimental purposes, rather grown artificially. Also, further exploration can be carried out for the production of bioactive compounds under optimized culture conditions and the phytochemicals can be assayed quantitatively.

CHAPTER 6

CONCLUSION

To sum up, the aim of this study was to establish a systematic protocol for in-vitro propagation of *Gentiana kurroo* royle, a medicinal herb put in high-risk category in terms of its survivability. The results of experiments done for micropropagation indicated that shoots are the ideal parts for artificial growth of G. kurroo. This study provides outcome in the form of significant biomass accumulation of this important medicinal herb which could be utilized directly by pharmaceutical and biotech industry as a quality rich herbal material. This study also requires further explorations for improving the production techniques of bioactive compounds via shoot cultures so that its endangered status can be rectified. The phytochemical analysis of G. kurroo extracts showed that the plant contains few important secondary metabolites like terpenoids, saponins and flavonoids. The implemented protocol can be used to produce large numbers of novel plants from related species, which can then be cryopreserved. Hence, colonization of this wild medicinal plant will rescue the species from extinction by reducing natural population pressure.

CHAPTER 7

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