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Address for correspondence:
Dr. Riya
University Department of Botany, Ranchi University, Ranchi, Jharkhand, India
Biologist and Wildlife Naturalist
ORCID: https://orcid.org/0000-0002-4837-9561
Email: sheenriyas@gmail.com

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Micro propagation and phytochemical analysis of Trigonella foenum-graecum with special reference to the estimation of chlorophyll with UV spectrophotometer

Dr. Riya[1], Dr. Shweta Nag[2]
[1]University Department of Botany, Ranchi University, Ranchi, Jharkhand, India Biologist and Wildlife Naturalist
[2]Assistant Professor, University Department of Botany, Ranchi University, Ranchi, Jharkhand, India

Abstract

Trigonella foenum-graecum (Fenugreek) is an annual plant belonging to the family Fabaceae. The green leaves and seeds of fenugreek (methi) are widely used in food and medicinal applications dating back to ancient times. However, the seeds are sour in taste due to the presence of bitter saponins, which limit their acceptability in foods. Research shows that extracts from seeds and leaves, and the active compounds in them, can lower cholesterol and blood sugar, fight inflammation and ulcers, relieve pain and fever, act as antioxidants, help heal wounds, and support the immune system. The plant contains active constituents such as alkaloids, flavonoids, steroids, saponins, etc.

We performed phytochemical analysis and micropropagation, focusing especially on estimating chlorophyll with a UV spectrophotometer. We also compared roots and shoots in both in vitro and in vivo conditions. Tissue culture is the in vitro aseptic culture of cells, tissues, organs, or whole plants under controlled nutritional and environmental conditions, often used to produce plant clones. We conducted a comparative phytochemical screening of ethanol and aqueous extracts. Important phytochemicals such as alkaloids, flavonoids, phenolics, tannins, saponins, steroids, glycosides, and terpenes exhibit a wide range of biological activities. Identification of phytochemicals allows for prediction of a plant's pharmacological activity. Phytochemicals are currently identified using various modern techniques, yet conventional qualitative tests remain popular for preliminary phytochemical screening of plants. Recent research has identified fenugreek as a valuable medicinal plant with potential for treating diseases and as a source of raw materials for the pharmaceutical industry, including steroidal hormones. Chlorophylls are essential components for photosynthesis and occur as green pigments in the chloroplasts of all photosynthetic organisms. These pigments are loosely bound to proteins and can be readily extracted with organic solvents like acetone or ether. Each chlorophyll molecule consists of a porphyrin ring with a central magnesium atom and a long phytol hydrocarbon side chain linked via a carboxylic acid group. Plants contain at least five types of chlorophyll. Chlorophyll was extracted with 80% acetone, and absorbance was recorded at 663 nm, 645 nm, and 470 nm using a UV spectrophotometer. Chlorophyll content was determined from the absorption coefficients.

Keywords: *Trigonella foenum-graecum L., Fenugreek, Pharmaceutical effects, Chlorophyll estimation, UV Spectrophotometer, Micro propagation, Research.*

Introduction

Trigonella foenum-graecum L. or also commonly known as Fenugreek (Methi), is known to be one of the plants with these traits. It is from the family of Fabaceae and is a self-pollinating annual herbaceous aromatic crop, also known as bird's foot, Greek hayseed, halba, and methi.[1] Its origin is India and Northern Africa; however, it is now widely cultivated in Northern Africa, Europe, South Asia, Argentina, and Australia. Fenugreek is mainly produced in India, which accounts for 80% of the total world production.[2] In several countries, fenugreek seeds and leaves are used as spices and culinary ingredients. Fenugreek is consumed as a traditional and functional food and is applied in nutraceutical and physiological contexts. Its high fiber, protein, and gum content have led to recent use as a food stabilizer and emulsifying agent. Fenugreek is among the world's oldest medicinal herbs, with both seeds and leaves traditionally used in the treatment of various ailments.[3] Numerous studies use extracts and powders prepared from the leaves and seeds of *T. foenum-graecum* for therapeutic applications.

Preliminary studies in animals and humans show that fenugreek has hypoglycemic, hypolipidemic, and hypocholesterolemic properties. It has also been reported to exhibit antifertility, anticancer, antiparasitic, and antimicrobial effects.[4] Sown in suitable soil, fenugreek seeds sprout within three days.

Seedlings develop an erect or semi-erect habit, reaching 30 to 60 cm in height.[5] Trigonella foenum-graecum is a self-pollinating annual legume that contributes to soil nourishment through nitrogen fixation. It has slender, green stems and produces yellow-brown pods containing 10 to 20 seeds. The seeds are brownish-yellow, hard[4], and rhomboidal, with dimensions of 3–6 mm × 2–5 mm × 2 mm.[3] Fenugreek seeds possess a characteristic bitter taste with maple-like notes. Texturally, they are glutinous, fibrous, and sticky, and structurally endospermic.[6] The seed consists of a hard yellow embryo encased in a broad, corneous layer of white, translucent endosperm. Diosgenin is present in the embryonic tissue.

Phytochemical analysis of fenugreek seeds reveals the presence of alkaloids, flavonoids, and saponins. Saponins are the most abundant, occurring at 4.63 g per 10 g of seed.[7] Alkaloids constitute approximately 35% of fenugreek, with trigonelline being the major component. Fenugreek seeds further contain >10 mg flavonoids g^{-1}, as well as small quantities of volatile and fixed oils.[8] Fatty acid analysis showed the oils were predominantly linoleic acid (42.71–42.80%), linolenic acid (26.03–26.15%), and oleic acid (14.24–14.40%).[8]



Fig.1: *Trigonella foenum-graecum L.*

Origin and distribution

Fenugreek (*Trigonella foenum-graecum L.*) is named as Trigonella, meaning *'little triangle'* in the *Latin* due to its yellowish-white triangular flowers. Fenugreek is among the oldest medicinal plants, known for its exceptional medicinal and nutritional properties. Fenugreek is documented in the Ebers Papyrus (c. 1500 BC), one of the oldest extant Egyptian medical texts, where its description and therapeutic uses are recorded. Trigonella foenum-graecum exhibits moderate tolerance to salinity, drought, and heavy metal stress, and demonstrates adaptability to diverse climatic regions and marginal lands.[9]

Fenugreek presents significant potential as a sustainable income source for farmers, traders, and associated industrial sectors. Trigonella foenum-graecum is cultivated extensively across Asia, Europe, and Africa. Reported seed yields are 1675 kg ha^{-1} in India, 1400 kg ha^{-1} in Egypt, and 3700 kg ha^{-1} in the United Kingdom.

Table version for clarity:

Fenugreek is cultivated across Asia, Europe, and Africa. Reported yields include:

Country	Seed yield
India	1675 kg/ha
Egypt	1400 kg/ha
United Kingdom	3700 kg/ha

Morphology of *Trigonella foenum-graecum L.*

Under favorable soil conditions, fenugreek seeds germinate within three days. The seedlings exhibit an erect to semi-erect growth habit and attain a height of 30–60 cm.[10] Trigonella foenum-graecum is a self-pollinating annual legume that contributes to soil fertility via biological nitrogen fixation. The plant is green and slender in habit, producing yellow-brown pods containing 10–20 seeds each. The seeds are brownish-yellow, rhomboidal, and hard in texture, with dimensions of 3–6 mm in length, 2–5 mm in width, and 2 mm in thickness.[11] Raw fenugreek seeds possess a characteristically bitter flavor with maple-like notes. They exhibit a glutinous, fibrous, and sticky texture and are endospermic in nature. Anatomically, a hard central core and yellow embryo are enclosed by a broad, corneous layer of white, translucent endosperm.[12] Diosgenin has been reported to be present in the embryo of fenugreek seeds.

CLASSIFICATION

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Genus: Trigonella
Species: *T. Foenum-graecum*

Nutritional Value

Trigonella foenum-graecum serves as a rich source of dietary fiber and essential nutrients required for growth. It is further characterized by diverse phytochemicals, including alkaloids, carbohydrates, amino acids, minerals, and steroidal saponins.

Nutritional value	
Fats	0.9g
Iron	1.93g
Calcium	395mg
Carbohydrate	6.0g
Protein	4.4g
Phosphorus	51mg
Total Energy	49kcl

Benefits of consuming fenugreek:

1. **Improved digestion:** Fenugreek-infused water can help alleviate digestive issues such as indigestion, bloating, and constipation, owing to its high fiber content. It can also promote better nutrient absorption.
2. **Weight management:** Fenugreek-infused water may contribute to weight loss through appetite suppression, enhanced metabolic rate, and decreased fat accumulation.
3. **Lower cholesterol levels:** Regular intake of fenugreek-infused water has been reported to reduce LDL cholesterol levels and promote cardiovascular health.
4. **Blood sugar control:** Fenugreek-infused water has been reported to regulate glycemic levels and may therefore benefit individuals with diabetes or insulin resistance.
5. **Anti-inflammatory properties:** Fenugreek-infused water possesses bioactive compounds exhibiting anti-inflammatory properties, which may aid in alleviating inflammatory conditions including arthritis and asthma.
6. **Hormonal balance:** Fenugreek-infused water contains phytoestrogens that may assist in regulating hormonal imbalances, especially in women with menopausal symptoms.
7. **Improved skin health**: Regular intake of fenugreek-infused water has been reported to improve skin health through reduced acne, a clearer complexion, and enhanced natural radiance.
8. **Improved hair health**: Fenugreek-infused water has been reported to reduce hair loss, promote hair growth, and alleviate scalp conditions including dandruff and pruritus.
9. **Enhanced immune system:** Fenugreek-infused water is a source of antioxidants reported to enhance immune function and confer protection against infections and diseases.

Trigonella foenum-graecum infused water has been associated with multiple health benefits. Its high fiber content aid's digestive function and nutrient absorption, alleviating indigestion, bloating, and constipation. It may contribute to weight management via appetite suppression, enhanced metabolism, and reduced adipogenesis. Regular intake has been reported to decrease LDL cholesterol and support cardiovascular health, while also regulating glycemic levels in diabetic or insulin-resistant individuals. The presence of anti-inflammatory compounds may alleviate inflammatory conditions including arthritis and asthma. Its phytoestrogen content has implications for hormonal regulation, particularly during menopause. Dermatological benefits include reduced acne and improved complexion, while trichological effects involve reduced hair loss, stimulated growth, and alleviation of dandruff and pruritus. Finally, its antioxidant constituents are reported to enhance immune function and provide protection against infections.

Tissue Culture

Plant tissue culture is an in vitro technique involving the culture of plant fragments, cells, or organs under aseptic conditions and controlled nutrient media to regenerate whole plants or specific tissues. Plant tissue cultures are maintained on nutrient media formulated as liquid broth or solidified with agar. This technique is commonly referred to as micropropagation, a method used for rapid clonal multiplication of plants under in vitro conditions. The technique has demonstrated significant utility in the production of disease-free planting material and the enhancement of crop yield, particularly in developing countries. The technique requires minimal infrastructure, namely a sterile laboratory facility, greenhouse, trained personnel, and a nursery for hardening and acclimatization. Micropropagation has been successfully applied to the large-scale production of crops including Elaeis guineensis (oil palm), Musa spp. (banana), Solanum melongena (eggplant), Ananas comosus (pineapple), Hevea brasiliensis (rubber tree), Solanum lycopersicum (tomato), and Ipomoea batatas (sweet potato) in developing countries.[13] Plant tissue culture has been established as a reliable technology for the large-scale in vitro propagation of clonal, genetically uniform plants.

Two distinct in vitro morphogenic pathways are conventionally utilized for plant regeneration: organogenesis and somatic embryogenesis. Selection of the appropriate pathway is contingent upon species specificity, regeneration efficiency, cost-effectiveness, and available laboratory infrastructure.[14] Plant tissue culture

provides experimental evidence for the concept of cellular totipotency, which states that a single living plant cell possesses the genetic potential to differentiate and regenerate into a complete organism. The concept of cellular totipotency was first postulated by German botanist *Gottlieb Haberlandt* in 1902. However, he was unable to demonstrate it experimentally due to limitations in culture techniques at the time.[15] Compared to conventional propagation via cuttings or seeds, tissue culture is generally more expensive due to higher labor requirements and the need for stringent environmental control across multiple developmental stages. The high operational costs associated with tissue culture have been a significant constraint to its widespread commercial adoption.[16] The principal contribution of plant tissue culture (PTC) lies in demonstrating that plant cells possess the unique capacity for totipotency, enabling regeneration of complete plants through organogenesis or somatic embryogenesis irrespective of explant source or ploidy level.[17]

Plant tissue culture (PTC) is presently employed across diverse domains of plant biotechnology for applications including crop improvement, clonal propagation, secondary metabolite production, cryopreservation, and elucidation of the physiological, biochemical, and genetic mechanisms underlying embryogenesis, organogenesis, and in vitro growth and development.[18] Tissue culture systems have been utilized to investigate fundamental aspects of plant growth, metabolism, differentiation, and morphogenesis, and provide an ideal platform for the experimental manipulation of these processes.[19] A multidisciplinary team of researchers, technologists, and engineers has articulated how the integration of engineering disciplines enhances plant tissue culture techniques and facilitates their translation into a viable commercial technology. This volume comprises twenty-four chapters that assess the current status, state-of-the-art, strengths, and limitations of strategies applicable to in vitro systems, encompassing machine vision, bioreactor technology, mechanized micropropagation, engineering of the culture environment, and the physical parameters of plant tissue engineering.[20]

Steps of Tissue Culture

Micro propagation- Micropropagation, i.e., propagation via tissue culture, is employed to produce high-quality clonal plants (Smith 1990). Its principal advantages include rapid, large-scale multiplication of novel genotypes and minimal requirement for source germplasm.[21]

The steps of tissue culture are given below:

- **Initiation phase-** This stage involves culture initiation, wherein the selected explant is excised, surface-sterilized, and inoculated onto sterile medium under aseptic conditions to preclude microbial contamination.[22]
- **Multiplication Phase-** This stage entails inoculation of the surface-sterilized explant onto a defined culture medium containing macro- and micronutrients, vitamins, and plant growth regulators.[23] These conditions promote cellular proliferation, resulting in an undifferentiated mass of cells termed callus.[24]
- **Root Formation-** This stage involves rhizogenesis, wherein auxins are incorporated into the culture medium to induce adventitious root development, thereby producing complete plantlets. [25]
- **Shoot Formation-** Cytokinins or other shoot-inducing plant growth regulators are incorporated into the culture medium to initiate caulogenesis, with subsequent growth monitored over a seven-day period.[26]
- **Acclimatization-** Following in vitro development, plantlets undergo acclimatization in a greenhouse under controlled environmental conditions before final transfer to a nursery for growth under ambient field conditions.

Present Scenario

Over the past century, the field of plant tissue culture (PTC) has undergone substantial advancements driven by the adoption and implementation of novel technologies and concepts. From its inception, the artificial production of plants has been pursued to reduce reliance on natural resources. The growing demand for high-yielding, disease-resistant cultivars drove the need for large-scale plant production. Consequently, propelled by globalization, micropropagation was established as a platform for mass clonal multiplication, enabling thousands of plants to be regenerated from a single explant.[27] In recent years, isolated microspore culture has emerged as both a breeding tool and an experimental system for genetic manipulation. Cellular totipotency describes the inherent capacity of a plant cell to regenerate a whole plant, a competence frequently retained even after terminal differentiation within the plant body.[28] Plant tissue culture technology constitutes a seminal advance in plant science. Notably, the capacity to introduce, propagate, transfer, and conserve plant germplasm via tissue culture techniques creates substantial opportunities for researchers and commercial enterprises. While plant tissue culture broadly encompasses the in vitro cultivation of plant cells, tissues, and organs, the term callus culture specifically refers to the proliferation of unorganized cell masses.[29] In vitro culture of plant cells and tissues has become routine across diverse explant types derived from numerous economically important vegetable and fruit crops. Established technologies encompass the isolation and culture of tissues, cells, protoplasts, organs, embryos, ovules, anthers, and microspores, coupled with the regeneration of complete plantlets from these explants.[30] Although native to China, the species is also well adapted to India, Vietnam, Thailand, Taiwan, parts of Africa, Israel, Australia, and higher elevations in Mexico and Central and South America. Nevertheless, commercial cultivation remains confined to a few subtropical countries owing to its stringent climatic requirements.[31]

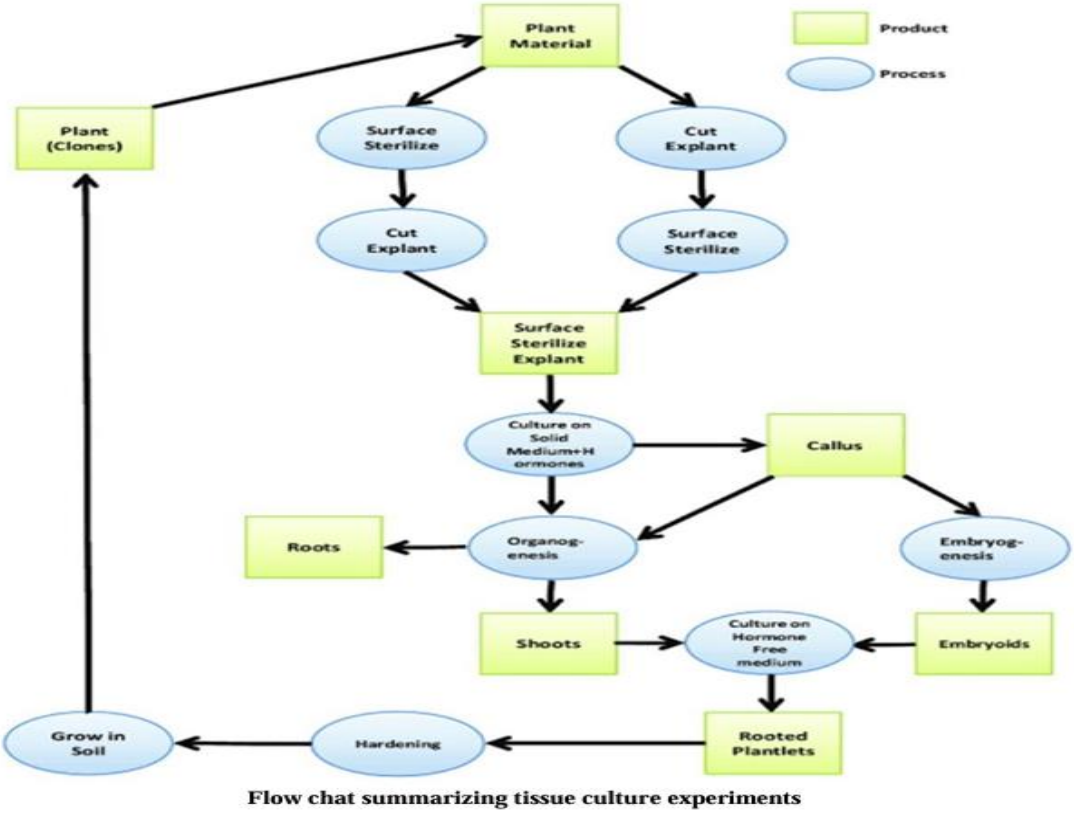


Fig.2: Showing the flow chat summarizing tissue culture experiments.

Review And Literature

Trigonella foenum-graecum L., commonly known as fenugreek, is a medicinal plant with a long history of use as both a culinary condiment and a therapeutic agent. The plant attains a height of up to 60 cm and produces golden-yellow, rhomboidal seeds. While the seeds are the most recognized component, the leaves and stems also possess documented medicinal applications. These bioactivities are attributed to diverse secondary metabolites, or phytochemicals, including alkaloids, saponins, tannins, and phenolic compounds.

According to (**Chauhan et. al., 2011**) and (**Kalaiselvi et. al., 2012**)[32] Medicinal plants have a long-standing role in health management and are increasingly gaining popularity among both rural and urban populations. Their therapeutic value is primarily attributable to bioactive constituents, including alkaloids, flavonoids, saponins, glycosides, and other metabolites of pharmaceutical relevance. Consequently, preliminary phytochemical screening facilitates the identification of plant constituents that may correlate with observed bioactivity.

Due to its rich phytochemical profile, *Trigonella foenum-graecum* has long been utilized as a food source, forage crop, and medicinal plant. (**Puri D, 1998**)[33].

Hypoglycemic activities have mainly been attributed to dietary fiber (**Neeraja A, 1996, Raghuram TC, 1994**)[34,35] and Saponin (**Lu F, 2008**)[36].

The herb fenugreek is used both in cooking and for the treatment of diabetes in many parts of the world especially in China, Egypt, India and middle eastern countries (**Kirtikar KR, 2000, Saxena A, 2004, Wang E, 2008**)[37].

Fenugreek seeds are rich in the polysaccharide galactomannan and in steroidal saponins, including diosgenin, yamogenin, gitogenin, tigogenin, and neotigogens. Additional bioactive constituents comprise mucilage, volatile oils, and alkaloids such as choline and trigonelline. **(Pribacl and Ardelean, 2008)**[38].

In vitro rooting studies primarily examine the application of phytohormones, particularly auxins, to elicit adventitious root formation. The recalcitrance of legumes to in vitro rooting has hindered the adoption of biotechnological approaches in legume improvement. (**Fratini and Ruiz, 2003**)[39].

Phytochemical screening revealed that the aqueous extract of fenugreek seeds contains alkaloids, flavonoids, phenolic compounds, tannins, carbohydrates, amino acids, and proteins, while glycosides were not detected. **(Yadav and Chowdhury)**[40].

Quantitative estimation of alkaloids was performed using the Harborne method[41], flavonoid content was determined according to procedure[42], and saponin content was estimated using established protocols. **(Obadoin and Ochuko)**[43].

Steroid content in fenugreek seeds was quantified using the Liebermann–Burchard colorimetric assay. In this assay, acetic anhydride in the Liebermann–Burchard reagent reacts with steroidal compounds to yield a green chromophore, whose absorbance was measured spectrophotometrically at 640 nm.[44]

The present results for the aqueous extract of fenugreek seeds were consistent with earlier reports[45], with the exception that tannins were not detected. Furthermore, alkaloids, steroids, phenolic compounds, and glycosides were also absent from the extract in this analysis.
These results are consistent with earlier studies reporting the presence of flavonoids, tannins, saponins, and terpenoids in aqueous extracts of fenugreek seeds. **(Chalghoume)**[46].
It is composed of proteins rich in lysine and L-tryptophan, mucilaginous fiber, and various bioactive constituents, including saponins, coumarin, nicotinic acid, sapogenins, phytic acid, scopoletin, and trigonelline, all of which exhibit therapeutic properties. **(Billaud C, 2001)**[47].
Fenugreekine, a steroidal sapogenin peptide ester isolated from fenugreek, exhibits hypoglycemic activity. **(Jellin JM, 1999)**[48].
The pharmacologically active constituents are secondary metabolites. The antimicrobial activity exhibited by plant extracts is attributable to various phytochemicals, including aldehydes and phenolic compounds. **(Lai PK, 2004)**[49].
Numerous previous investigators have conducted phytochemical analyses of Trigonella foenum-graecum. **(Mowla et al., 2009; Ahirwar et al., 2010; Yadav et al., 2010; Dande et al., 2012; Sumayya et al., 2012; and Sheikh et al., 2012)**[50-55], These studies reported alkaloids, saponins, flavonoids, bitter principles, coumarins, anthracene derivatives, and glycosides, consistent with the findings of the present investigation.
Phytochemical analysis indicated that the Trigonella seed extract contained high levels of steroidal saponins. **(Dande et al., 2012)**[56].
Mowla et al., (2009)[57] Phytochemical screening of the crude ethanol extract of T. foenum-graecum seeds was performed to detect alkaloids, steroids, flavonoids, carbohydrates, glycosides, and glucosides. Alkaloids, steroids, and carbohydrates were present, whereas flavonoids, glycosides, and glucosides were absent.
Yadav et al., (2010)[58] Phytochemical screening revealed alkaloids, flavonoids, amino acids, tannins, proteins, starch, mucilage, and saponins in both methanolic and aqueous extracts of *Trigonella foenum-graecum*.

Materials And Methodology
Plant identification: - seeds of *Trigonella foenum-graecum* is collected from local seed shop.
In-vitro culture

Basic Requirements:
- Washing, drying and storage of vessels
- Preparation, sterilization and storage of media.
- Aseptic handling of explant and Culture.
- Maintenance of Culture
- Observation of culture

Materials And Equipments:
- Analytical balance
- Autoclave
- pH meter
- Micropipette and tips
- Culture tubes
- Conical and volumetric glass
- Beaker and Petri plates
- Measuring cylinder
- Millipore water purifier
- Pure water purifier
- Pure water demineralizer
- Shakers
- Refrigerator
- Tissue culture racks
- Chemicals
- Cotton
- Brown paper
- Forceps
- Surgical blade

Nutrient Media Composition and Preparation
Components of media for the growth of plant callus and suspension cultures can be classified into following groups: -
Inorganic nutrients: - It includes **Macronutrients: -** Boron, Molybdenum, Copper, Zinc, Manganese, Iron and Chloride. **Micronutrients: -** Phosphorus, Potassium, Sulphur, Nitrogen.
Organic supplement: - Vitamins are required in trace amount as they catalyze the enzyme system of the cells.
Carbon source: - for the development of cultures.

Optimum pH: - between 5.0- 6.0
Gelling agents: - Most commonly, agar is used as a solidifying agent or chilling agent.
Growth regulators: - several growth hormones are known which stimulate the biological activity in cultured materials that promote cell division and regulate growth and development similar to kinetin. Auxin resembles indole acetic acid (IAA) shoot elongation.

Preparation Of Stock Solution
Composition of Macronutrients (strength- 10X g/l)

Compound	Chemical formula	10X g/l
Ammonium nitrate	HN4NO3	16.5
Magnesium sulphate	MgSO4.7H2O	3.7
Potassium nitrate	KNO3	19
Potassium hypophosphate	KH2PO4	1.7
Calcium chloride	Cacl2.2H2O (Stock B)	4.4

Stock C Micronutrients (strength-1000X g/100ml)

Chemical formula	1000X g/l
Kl	0.83
CuSo4.5H2O	0.025
Cacl2.6H2O	0.025
H3BO3	6.2
ZnSO4.7H2O	8.2
NaMoO4.2H2O	0.25
MnSO4.4H2O	16.9

Stock D Iron source (strength- 100X g/l)

Chemical formula	100X g/l
FeSo4.7H2O	3.73
Na2EDTA.2H20	7.28

Stock E Vitamins and Amino acids (strength- 1000X g/l)

Chemical formula	1000 g/l
Glycine	0.2
Thiamine	0.01
Myoinositol	10
Nicotinic acid	0.05
Phyrodoxin Hcl	0.05

Other requirements

Agar	8%
Sucrose	3%
pH	5.6-5.8

Preparation Of Ms Media
1. For preparation of 500ml MS media, a sterilized conical flask is required.
2. Working solutions is made using a definite amount of stock solution, by applying the formula **S1V1 = S2V2**
3. Macronutrients -12.5cc/500ml, Micronutrients -0.25cc/500ml, Cacl2.2H20 – 15cc/500ml, Iron source – 10cc/500ml, Vitamin & amino acid – 0.5cc/. 500ml, Sucrose – 15cc/ 500ml, Agar- 8%g/l – 4g/500ml, pH – 5.6 to 5.8
4. Some distilled waters were added
5. Then 15g/500ml sucrose is added in the working solution and after that media is slightly warmed and 4g agar is added by heating.
6. Final volume is brought to 500ml by adding distilled water
7. Then, the media is homogenized in water for 15-20 minutes or until the froth formation.
8. After homogenising, media is sterilized in autoclave for 20 minutes.
9. After keeping all culture tubes and racks in laminar air flow chamber, UV light is applied for 15-20 min.
10. The work surface and hand should be properly sterilized with 70% ethanol Spirits burnt and in presence of lamp the cotton plug of culture tube were carefully removed and roughly 10ml of culture media is poured into culture tube.
11. Altering media in all the culture tubes, it is placed at an angle to form slant
12. After sometime, the media solidify and after 24 hours, it is ready for the inculcation.

Surface Sterilizing Agents
- **Suthol –** It is a detergent which is used for surface sterilization of Explants. It is prepared by dissolving suthol

in distilled water.

- **Mercuric chloride –** It is most commonly used for surface sterilization of Explant. 0.1% of Hgcl2 solution was prepared by dissolving 0.1g of Hgcl2. Powder In autoclave distilled water. Precautions must be taking while handling This because it is carcinogenic.
- **Ethyl alcohol –** ethyl alcohol (70%) is prepared by dissolving 70ml of absolute Alcohol in 30 ml of distilled water.

Surface Sterilization, Inoculation And Culture Conditions

The seeds were rinsed under running tap water two to three times and subsequently with distilled water three to four times. They were then treated with a few drops of Suthol solution and rinsed again with distilled water three to four times.

Seeds were subsequently transferred to a laminar air flow cabinet. Surface sterilization was performed using 0.1% $HgCl_2$ for several minutes, followed by rinsing with autoclaved distilled water. The seeds were then treated with a few drops of 70% ethanol and rinsed with distilled water three to four times.

Following sterilization, the seeds were inoculated into basal medium and media supplemented with varying concentrations of plant growth regulators, and subsequently incubated for growth.



Fig.3: Showing inoculated tubes under Culture room

Germination in MS media

1. In the germination test carried out in MS media, the germination rate of seed was about 95% after 3- 4 days.
2. In in vitro germination, Use of nutrients media containing macro and micro-elements and sucrose, positive effect was observed
3. The fenugreek seed started germinating in 3-4 days after inoculation on MS media.
4. Epicotyl emerged from the seed.
5. From the epicotyl, tap root was observed arising from it.
6. Hypogeal type of germination.
7. The mature seedlings about 3-5cm in length was observed in 8-10 days after the germination of seeds.



Fig.4: Showing in-vitro cultivation of *Trigonella foenum-graecum*

In Vivo Condition Requirements

- Explant
- Cotton cloth
- Water
- Soil
- Pot

Methodology
Seed of fenugreek was collected from local seed shop.

Preperation
Fenugreek seeds were soaked in water for 5 min. Seeds were subsequently placed in a soil-sand mixture and lightly watered to initiate germination. Pots were maintained under mild sunlight with regular watering, and germination was recorded within 2–3 days.

Observation
In vivo germination

- In the germination test, the rate of germination of seed was about 95% in 2-3 days.
- The fenugreek seeds started germinating under the mild sunlight and epicotyl emerged out first.
- Tap roots was observed
- Epicotyl with first leaf appeared about 2-3 days.
- The mature seedlings of 4 – 5 cm in length were observed after 11- 12 days after germination.

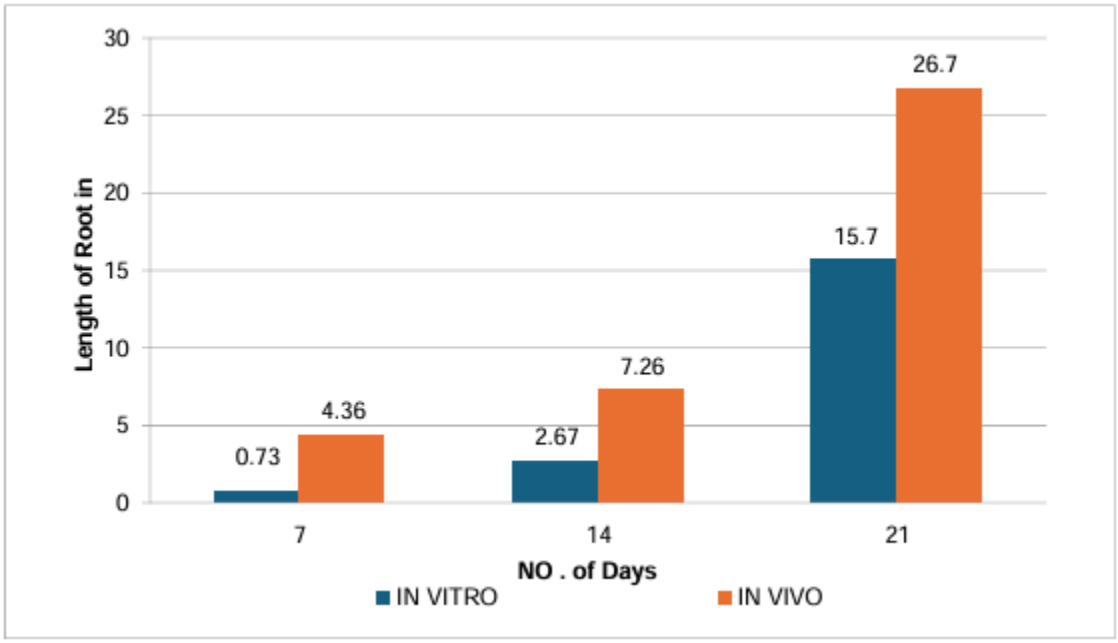


Fig.5: Showing 21 days old *Trigonella foenum-graecum*

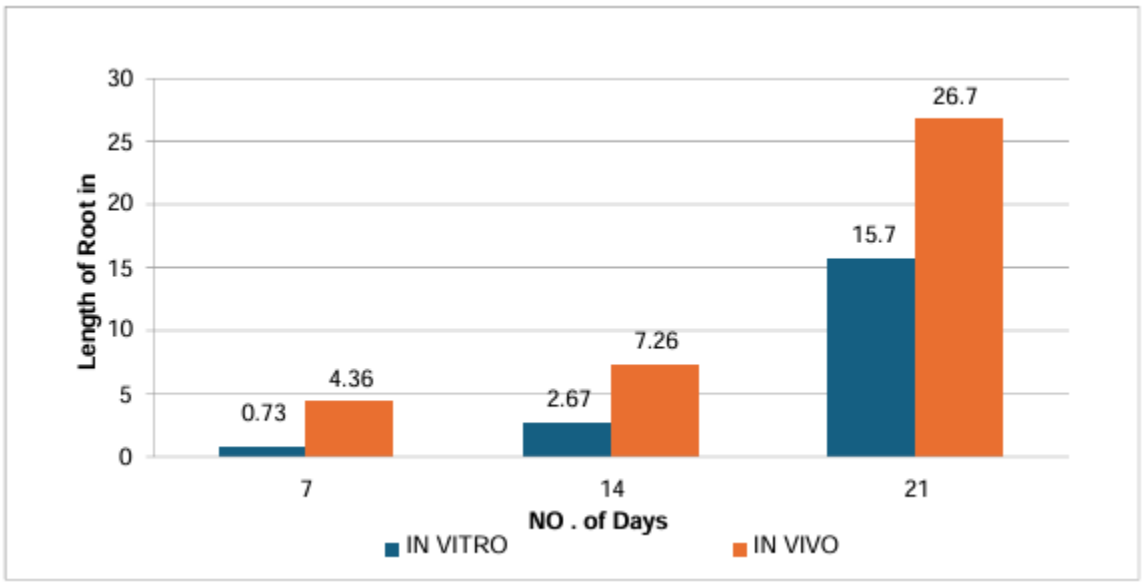
Result

No. of days	Root length		Shoot length	
	In-Vitro	In-Vivo	In-Vitro	In-Vivo
7	5.26 ± 0.011	2.43 ± 0.018	0.73 ± 0.091	4.36 ± 0.115
14	8.36 ± 0.011	4.33 ± 0.017	2.67 ± 0.0058	7.26± 0.115
21	12.43 ± 0.18	7.27 ± 0.017	15.7 ± 15.35	26.73 ± 26.321

Table.: showing in vitro and in vivo seed germination of Fenugreek Showing root and shoot Length on basal MS media after 21 days of inoculation.



Graph.1: Comparative study of length (cm) growth in vitro & in vivo condition.



Graph.2: Comparative study of shoot length (cm) growth in vitro & in vivo condition.



Fig.6: Showing root length in vivo (fig a) & in vitro (fig b)

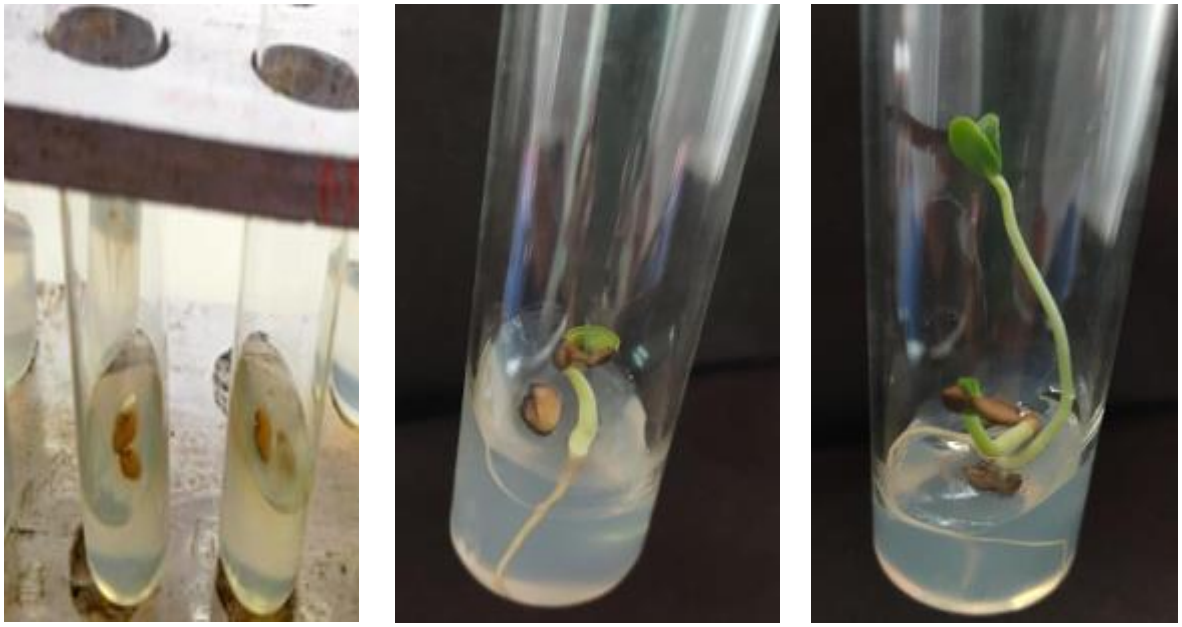


Fig.7: Showing shoot length in the interval of 7 days.



Fig.8: Showing 21 days old fenugreek seed culture on 1.5 mg/l MS media showing growth of root tap which show germination was taken for sub culture.



Fig.9: Showing 28 days old basal raised seed dried epicotyl of fenugreek on 2.5 mg/l MS + NAA showing callus formation.

Phytochemical Screening

For phytochemical screening, fully grown fresh leaf of fenugreek was taken from home garden.

Processing of plant

Fresh fenugreek leaves were washed repeatedly with water to remove dust and air-dried in the dark to eliminate residual moisture. The dried material was ground into a fine powder. Ten grams of powder were extracted with ethanol and aqueous solvents by soaking for 48–72 h in a conical flask sealed with aluminium foil, until the solution attained a thick consistency. The extract was filtered through filter paper into a clean conical flask, followed by filtration through Whatman filter paper into another conical flask.



Fig.10: Showing dry leaves of fenugreek

Extract preparation

Powdered plant material was extracted at a concentration of 10 g/100 mL using 70% ethanol and aqueous solvent for 42–78 h. After 78 h, the extract was filtered and allowed to evaporate to dryness. The dried extract was scraped and reconstituted in ethanol and aqueous solvent.

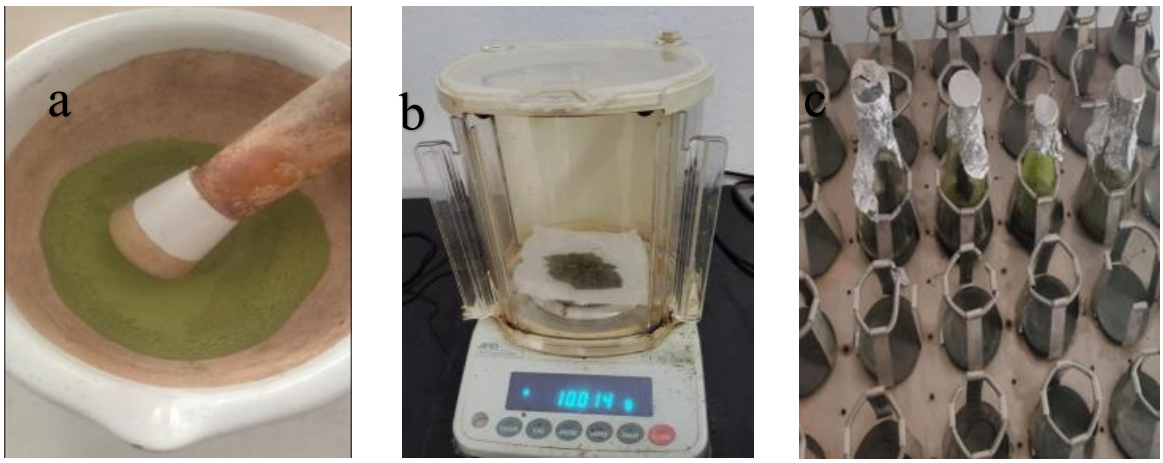
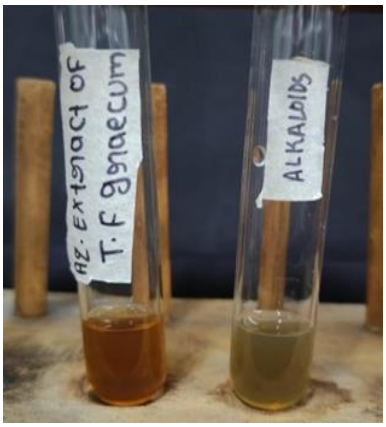


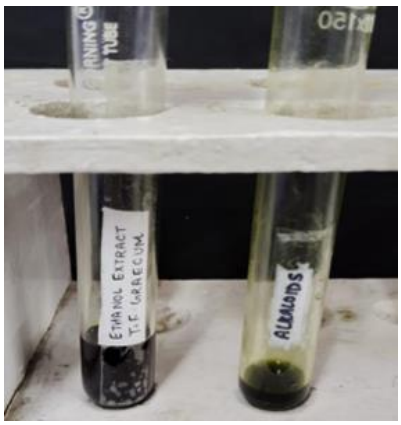
Fig.11: Showing extract powder (a), weight of leaves (b), shaker (c).



Fig.12: Showing extract filtration with aqueous and ethanol solvent.

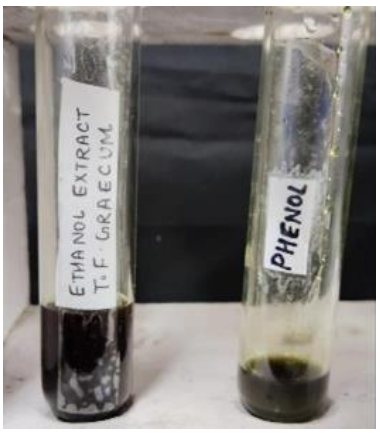
Test for Alkaloids (Mayer's):
1ml of each plant extract were taken and 3-5 drops of Mayer's reagent were added and observed for the formation of white yellowish precipitate indicates the presence of Alkaloids.



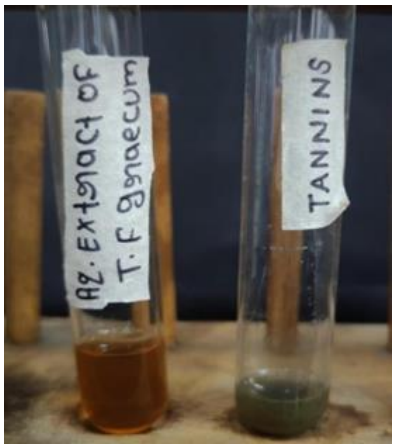


Test for Phenols:
1ml of extracts was treated with 3-4 drops of 5% ferric chloride solution. Formation of bluish color indicates the presence of phenol.



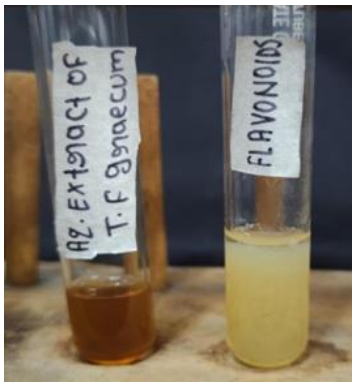


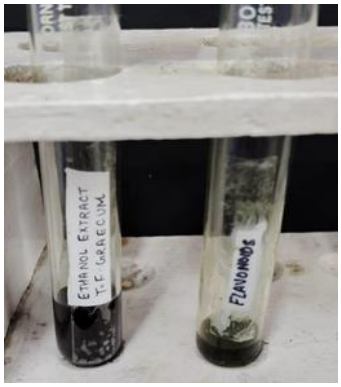
Test for Tannins:
To the extract of 1ml each 0.1% ferric chloride solution were added, formulation of a dark blue or greenish black colour showed the presence of Tannins.





Test for Flavonoids:
1ml of each extract was mixed with small amount of magnesium and HCL were added drop wise. Formation of yellow colour precipitate indicates the presence of flavonoids.

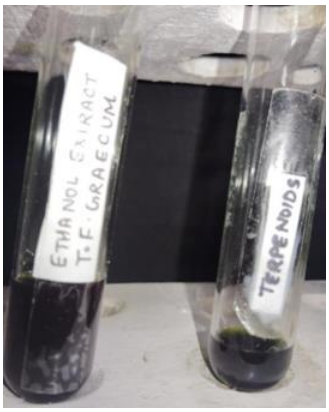




Test for Terpenoids:

1ml of each extract was added to 2ml of chloroform and 3 ml of concentrated sulphuric acid to form a monolayer of reddish-brown coloration of the interface was showed to form positive result for the terpenoids.





Test for Saponins:

1 ml of each Extract was added with magnesium turning ribbon to form the foam appearance on upper layer of the mixture. The foam layer appearance on upper layer indicates the presence of saponin.

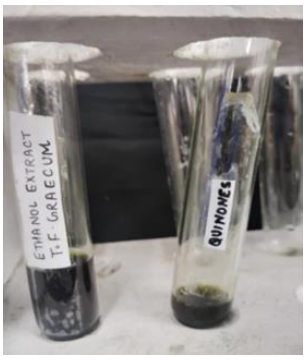




Test for Quinone:

1ml of each extract was treated with few drops of concentrated sodium hydroxide. It forms the yellowish colour appearance in aqueous solution. It indicates the appearance.

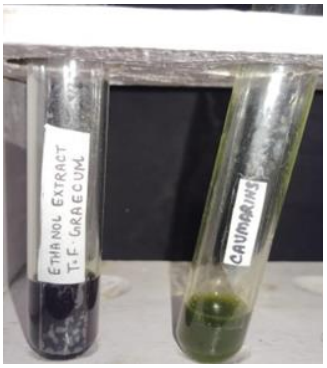




Test for Coumarin:

1ml of each plant extract and add 1.5ml of NaOH solution. Formation of yellow colour indicates the presence of coumarin.

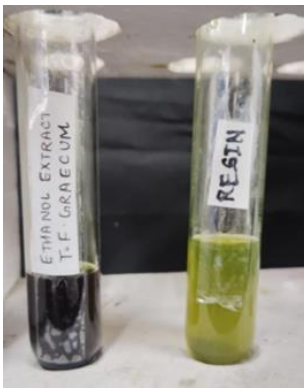




Test for Resins:

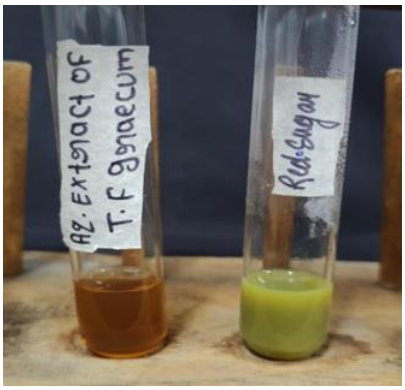
1ml of each plant extract and 5 ml distilled water. Observe turbidity.

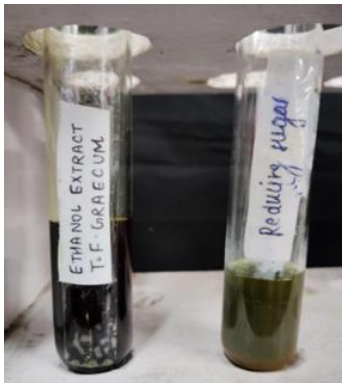




Test for Reducing sugar (Benedict's):

1ml of the analyte sample must be mixed with 2 ml of Benedict's reagent and heated in a bath of boiling water for 3 to 5 minutes.

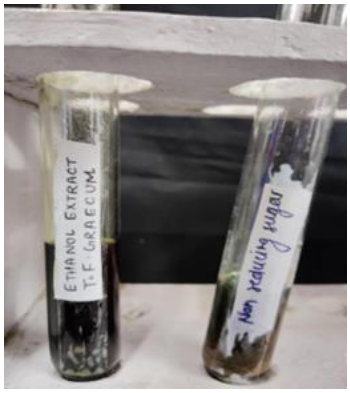




Test for Reducing sugar (Fehling's):

2ml of each extract were taken and treated with equal amount of Fehling's A & B. Reagent were added & then gently heated. Appearance of radish brown precipitated indicates the presence of non-reducing sugar.





Phytochemical screening of fenugreek			
Sl. No	**Examined test**	**Ethanolic solvent**	**Aqueous solvent**
1	Alkaloids	+	+
2	Phenols	+	+
3	Tannins	+	+
4	Flavonoids	+	+
5	Terpenoids	+	+
6	Saponins	-	+
7	Quinone	+	+
8	Coumarins	+	+
9	Resins	-	-
10	Reducing sugar (Benedict)	1.5%	1%
11	Reducing sugar (Fehling)	+	+
Present (+) Absent (-)			

Estimation Of Chlorophyll Pigments

In this method, chlorophyll is extracted with 80% acetone and absorbance is measured at 643, 645, and 663 nm using a spectrophotometer. Chlorophylls are essential photosynthetic pigments located in chloroplasts and are present in all photosynthetic plant tissues.

Plant pigments are essential to living organisms. Chlorophyll is utilized as a food additive, while carotenoids impart color to fruits and vegetables. Both photosynthetic pigments function as antioxidants, exhibit anticancer activity, and are prescribed for certain dermatological and ophthalmic conditions.

Chlorophyll and carotenoids are two major plant pigments essential to living organisms. Chlorophyll is utilized as a food additive, whereas carotenoids impart color to fruits and vegetables. Both pigments exhibit antioxidant activity, demonstrate anticancer potential, and are prescribed for certain dermatological and ophthalmic conditions.

This study aims to identify the developmental stage at which the concentrations of these two pigments peak, thereby informing the optimal harvest time for the pharmaceutical and food industries to extract these pigments for herbal formulations.

Materials And Methods

Collection of plants for Spectrophotometer: - Fresh leaves of *Trigonella foenum- graecum* grown in a pot at college campus.

Extraction Of Chlorophyll (Arnon 1949)

Fresh Trigonella foenum-graecum leaves (0.1 mg) were finely cut and homogenized in approximately 20 mL of 80% acetone. The homogenate was centrifuged at 10,000 × g for 5 min. The supernatant was collected, and extraction was repeated until the residue was colourless. The combined extracts were adjusted to a final volume of 10 mL. Absorbance was recorded at 470, 645, and 663 nm against an 80% acetone blank.

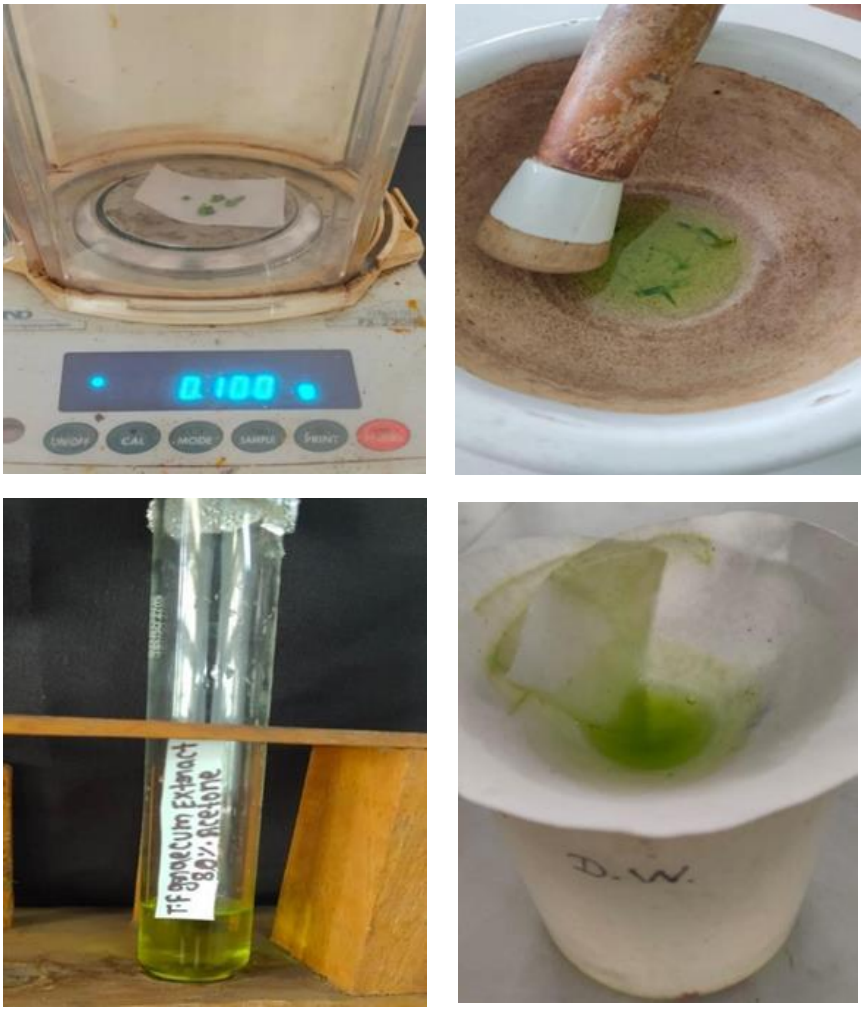


Fig.13: Showing weight of fresh leaves, leaf grinding, fenugreek with 80% acetone, and filtration process.



Fig.14: Showing centrifugation, spectrophotometer and after centrifugation

Reading Under Spectrophotometer

Chlorophyll A

For chlorophyll a quantification, absorbance was recorded at 663 nm using 80% acetone as the reference. Measurements were performed in triplicate, and the mean absorbance was used for calculation.

	Absorbance value (in triplicates)	Mean value
Chlorophyll A (A_{663})	0.710	0.712
	0.715	
	0.712	



Fig.15: Showing reading of triplicate readings of chlorophyll, A

Chlorophyll B

For chlorophyll b quantification, absorbance was recorded at 645 nm using 80% acetone as the reference. Measurements were performed in triplicate, and the mean absorbance was used for calculation.

	Absorbance value (in triplicates)	Mean value
Chlorophyll B (B_{663})	0.600	0.603
	0.610	
	0.601	



Fig.16: Showing reading of triplicate readings of chlorophyll B.

Carotenoids

For carotenoid quantification, absorbance was recorded at 470 nm using 80% acetone as the reference. Measurements were performed in triplicate, and the mean absorbance was used for calculation.

	Absorbance value (in triplicates)	Mean value
Carotenoids (A_{470})	0.816	0.811
	0.816	
	0.802	



Fig.17: Showing reading of triplicate readings of carotenoids.

Estimation Of Chlorophyll Content

The concentration of chlorophyll A, B, and total chlorophyll were calculated using the following equation (Arnon, 1949)

Chlorophyll a tissue): ⁅12.7(A tost)-2.69 (A tos)⁆ ^ * V / 1000 ^ * W (mg / g * m

Chlorophyll b (mg / g * m) tissue): ⁅22.9(A bw) 4.68(A bat)⁆ ^ * V/1000^ * W

Total Chlorophyll (mg / g * m) tissue): ⁅20.21(A {60}) + 8.02(A {563})⁆ * V/1000^ * W (a + b)

A= Absorbance of specific wavelength

W= 1 fresh weight of tissue extract

V= final volume of chlorophyll extract in 80% Acetone.

Estimation Of Carotenoids (Lichtenthaler and Wellburn method)

Carotenoid concentration was determined following the method of Lichtenthaler and Wellburn. Absorbance of the 80% acetone extract was recorded at 470 nm to calculate total carotenoids (xanthophylls + carotenes). Total carotenoids (mg/g tissue):

Lx + c = (1000A_{4m} − 1.82Ca − 85.02Cb) / 198

Where, A = Absorbance at respective wave length, Ca-Chlorophyll-a, Cb-Chlorophyll-b

Result Of Chlorophyll Estimation

Month	Chlorophyll A (mg/gm)	Chlorophyll B (mg/gm)	ChlA: ChlB	Total Chlorophyll (mg/gm)	Total Carotenoids (mg/gm)	Total Chlorophyll: total Carotenoids
May	0.242	1.047	0.231	1.789	3.642	0.491

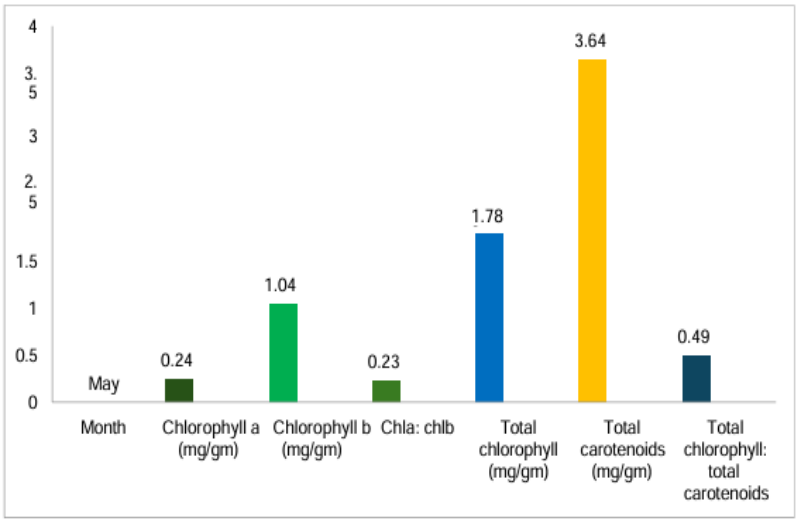


Fig.3: Graph of pigment concentration in expressed in mg/gm

Discussion

This study compared the roots and shoots of *Trigonella foenum-graecum* grown under in vitro and in vivo conditions. In vitro-grown fenugreek exhibited root lengths of approximately 12 cm, compared to approximately 5 cm for in vivo-grown seedlings. Epicotyl emergence occurred after 2 days in vivo versus 4 to 5 days in vitro. Leaves of in vitro-grown seedlings were markedly darker than those grown in vivo, whereas shoot length was greater in vivo than in vitro.

Phytochemical analysis of *Trigonella foenum-graecum* was performed using ethanol and aqueous extracts as solvents. According to the review of the literature of **Kumari** [45] Results from the aqueous extract of fenugreek seeds were consistent with prior reports, with the exception that tannins were not detected. Additionally, alkaloids, steroids, phenolic compounds, and glycosides were absent from the aqueous extract.

Mowla et al. (2009)[57] Phytochemical analysis of the crude ethanol extract of *T. foenum-graecum* seeds was performed to detect alkaloids, steroids, flavonoids, carbohydrates, glycosides, and glucosides. The extract tested positive for alkaloids, steroids, and carbohydrates, and negative for flavonoids, glycosides, and glucosides.

Yadav et al. (2010)[58] Analysis revealed alkaloids, flavonoids, amino acids, tannins, proteins, starch, mucilage, and saponins in the aqueous extracts of *Trigonella.* The presence of flavonoids, tannins, saponins, and terpenoids was consistent with previous findings from aqueous extracts of fenugreek seeds from Chalghoume.[46]

In contrast, phytochemical analysis of the ethanol extract of *Trigonella foenum-graecum* detected flavonoids, tannins, phenolic compounds, terpenoids, and coumarins. Alkaloids and tannins were present in trace amounts, whereas saponins and resins were not detected.

Phytochemical analysis of the aqueous extract of *Trigonella foenum-graecum* detected saponins, flavonoids, phenols, terpenoids, and quinones. Resins were not detected.

Medicinal properties exhibited by plants are attributed to their constituent phytochemicals, which serve as vital sources for the treatment of severe diseases. These compounds display diverse biological activities, including immune system enhancement and protection against chronic diseases and harmful pathogens. This study aimed to examine the phytochemical constituents of selected medicinal plants commonly used in Gujarat. The therapeutic value of these plants is determined by chemically active substances that elicit specific physiological effects in humans. Flavonoids, tannins, phenolic compounds, and alkaloids are key bioactive components. Standard procedures were employed to screen for various phytochemicals, and tannins, saponins, flavonoids, and phenols were detected in the tested medicinal plants.

Fenugreek (*Trigonella foenum-graecum*), a legume, is widely used as a spice to improve the sensory attributes of food. It exhibits medicinal properties, including anti-diabetic, anti-carcinogenic, hypocholesterolemic, antioxidant, and immunomodulatory activities. In addition to its therapeutic value, fenugreek functions as a food stabilizer, adhesive, and emulsifying agent in product development. It is also utilized in the formulation of healthy and nutritious extruded and bakery products. This paper reviews the nutraceutical properties of fenugreek and its applications in product development.

Conventional breeding and plant improvement strategies are increasingly insufficient to meet advancing standards and high-quality demands, leading to broader adoption of biotechnological techniques. Initially, biotechnology supplemented conventional breeding via in vitro culture methods, such as micropropagation, to accelerate multiplication and enhance uniformity. Fenugreek is recognized for its high fiber, gum, and volatile chemical content. Owing to its elevated fiber, protein, and gum levels, it serves as a food stabilizer, adhesive, and emulsifying agent. Its seeds contain approximately 25% dietary fiber, which improves the taste and texture of food products while supporting digestion.

Fenugreek proteins exhibit greater solubility under alkaline pH conditions. **(Meghwal and Goswami, 2012)**. Fenugreek contains numerous phytochemicals, such as alkaloids, carbohydrates, steroidal saponins, amino acids, and minerals, and has been employed for nutritional, nutraceutical, medicinal, and therapeutic applications. **(Aasim et al., 2018)**.

Plant chlorophyll content serves as a critical indicator of photosynthetic efficiency and overall physiological status. Elevated chlorophyll levels in fenugreek indicate effective adaptation for light capture and utilization during photosynthesis, supporting energy production for growth and metabolism. Chlorophyll and carotenoids are key bioactive molecules in plants with notable applications in herbal medicine, and chlorophyll demonstrates substantial antioxidative activity. This study utilized fresh leaves of Trigonella foenum-graecum for the extraction and quantification of the primary photosynthetic pigments, chlorophyll and carotenoids.

This study determined Chl a and Chl b concentrations to be 0.242 and 1.047, respectively, yielding a Chl a: Chl b ratio of 0.231:1.

Total chlorophyll and total carotenoid contents were 0.491 and 3.642, respectively, yielding a chlorophyll: carotenoid ratio of 0.491:1.

Conclusion

Numerous medicinal plants in nature possess both nutraceutical and pharmaceutical value. Fenugreek has been widely used in traditional medicine since antiquity for diverse applications. Current scientific reports demonstrate that it contains abundant bioactive compounds with broad therapeutic potential. Nevertheless, literature on specific pharmacological applications, such as its role in promoting hair growth, remains limited.

In vitro micropropagation studies demonstrated that *Trigonella foenum-graecum* seeds can be successfully cultured using MS medium under laboratory conditions.

This study further demonstrated that *Trigonella foenum-graecum* leaves represent an important natural source of therapeutic compounds due to their phytochemical content. Crude extracts contain diverse secondary metabolites. Phytochemical analysis indicates potential for identifying novel compounds and bioactivities, including significant antioxidant activity. The present investigation of phytochemicals and antioxidant properties in *Trigonella foenum-graecum* provides a foundation for future phytochemical and pharmacological research aimed at developing new potential compounds for various applications.
This study further confirmed that *Trigonella foenum-graecum* contains multiple pigments, including chlorophyll a, chlorophyll b, and carotenoids.

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Conflict Of Interests
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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