BIOCHEMICAL STUDIES OF *Clitoria ternatea* AND ITS USE AS BIOGEL

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DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

By

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APPROVAL SHEET

This thesis entitled "**Biochemical studies of *Clitoria ternatea* and its use as Biogel" by Kiran** (Admission No. 19SBAS3060001) is approved for the degree of Doctor of Philosophy in Biochemistry.

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis, entitled **"Biochemical studies of *Clitoria ternatea* and its use as Biogel"** in the fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Biochemistry, School of Biological and Life Science, Galgotias University, Greater Noida (Uttar Pradesh) is an authentic record of my own work carried out during a period from 2019 to 2024 Jan. under the supervision of Dr. Anuradha Singh, and Dr. A.K. Jain**.**

The matter embodied in this thesis has not been submitted by me for the award of anyother degree of this or any other University/ Institute.

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This is to certify that the above statement made by the candidate is correct to the best ofour knowledge.

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The Ph.D Viva-Voice examination of Kiran, Research Scholar, has been held on----------

Sign. of Supervisor Sign. of Co-Supervisor Sign. of External Examiner

Dedicated

to

My Parents and Family

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Place: Greater Noida, U.P.
Date: ***(Kiran)***

STATEMENT OF THESIS PREPARATION

1. Thesis title: **"Biochemical studies of *Clitoria ternatea* and its use as Biogel"**
2. Degree for which the thesis is submitted: **Doctor of Philosophy**
3. Thesis Guide was referred to for preparing the thesis.
4. Specifications regarding thesis format have been closely followed.
5. The contents of the thesis have been organized based on the guidelines.
6. The thesis has been prepared without resorting to plagiarism.
7. All sources used have been cited appropriately.
8. The thesis has not been submitted elsewhere for a degree.

(**Kiran**)

Admission No. 19SBAS3060001

ABSTRACT

Clitoria ternatea (CT), often known as butterfly pea, is a perennial herbaceous plant that grows around the world, but is most common in tropical areas. It is a member of the *Fabaceae family.* The plants' genotypes are generally identified by flower colours like blue, white, and pink. The plant has great quality of numerous medicinal properties presented in every part of plant like root, leaf, flower, and seed. The plant that haslong been employed in conventional herbal remedies to treat a wide range of illnesses do so because they contain a large number of bioactive chemicals and antioxidant enzymes. The present research objectives were to evaluate of its phytochemicals and different antioxidant properties through qualitative and quantitative analysis by 60% ethanol extraction. The selection of plant for the research study was the blue flower genotype of root, leaf, flower, and seed and compared its bioactive compound. The formation of biogel from *Clitoria ternatea's* blue flower component was the final objective of this study. The formulated bio gel was evaluated by its physical evaluation, P^H measurement, spreadability, stability, viscosity, skin sensitivity test, toxicity, and inhibitory effect of microbes.

The qualitative properties of *C. ternatea* were tested by appropriate methods to assess secondary metabolites or bioactive compounds such as total carbohydrates, alkaloids, amino acids and proteins, flavonoids, glycosides, tannins, phenols, saponin, sterols, terpenoids, quinones, oxalates and resins based on deep to light colour present or absent in four different part of the root, leaf flower and the seed of CT plant.

The qualitative test of the above secondary metabolites revealed that the carbohydrates were present in all tissues. In seeds tennins, amino acids, proteins, sterols, and terpenoids were strongly present whereas resins, phenols, flavonoids, glycosides, and alkaloids were more present, while quinones were less present. The oxalates and saponins were absent in the seed. However, the flowers contain highly present alkaloids, flavonoids, phenols, tannins, terpenoids, and quinones but more present resins, and less present oxalates, sterols, amino acids, and proteins, glycosides and found absent of saponin. Flavonoids, amino acids, proteins, and terpenoids were less prevalent in the root section of *Clitoria ternatea*, but alkaloids,

glycosides, phenols, tannins, sterols, quinones, oxalates, and resins were not present. Although phenols were abundant in the leaves, *Clitoria ternatea* lacked alkaloids, saponin, and oxalates, and had more flavonoids, tannins, sterols, and quinones. Terpenoids, resins, amino acids, proteins, and glycosides were less prevalent.

The quantitative analysis was estimated through selected parameters such as total carbohydrates, total phenols, flavonoids, tannins, and anthocyanin in different parts (seeds, flowers, leaves, and roots) of *Clitoria ternatae*. The biochemical study revealed through quantitative analysis that the total carbohydrate was found (89.01 mg Glc/g fresh wt) to be higher in leaf tissue, and the average mean value was obtained 76.75. Similarly, total flavonoids (469.34 Catechine mg/g fresh wt.), the total phenols (93.00 mg GAE/g fresh wt.), total tannins (72.00 mg TAE/g fresh wt.), and total anthocyanins (178.33 mg CGE/g fresh wt.) were presented highest in flower tissue of *Clitoria ternetea* in comparison to other parts of the plant.

Clitoria ternatea's antioxidant activity was evaluated utilizing the DPPH, FRAP, ABTs, and superoxide radical scavenging tests. The highest DPPH, ABTS, and superoxide activities were observed maximum in flowers i.e. 92.43 mg ascorbic acid/ g FW, 89.36 mgGAE/g FW, and 89 mg ascorbic acid/g FW respectively against standard. Similarly, FRAP activity was maximum in leaves (399 mg BHT/g FW). The observation of DPPH, ABTs, FRAP, and superoxide activities was also recorded in roots, leaves, seeds, and flowers on different concentrations like 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml through inhibition percentages against standard. The results showed that maximum activity was recorded at 1.0 mg/ml concentration in comparison to other concentrations.

Plant sections of *Clitoria ternatea* were subjected to an analysis of the antioxidant enzymes superoxide dismutase, peroxidase, polyphenol oxidase, and guaiacol peroxidase. The peroxidase activity was observed highest in flower (391.20 U/ml), followed by leaves (376.33 U/ml), lowest in seed (289.14 U/ml), and the mean value was recorded 340.23 U/ml.

The biogel was formulated with different chemical ingredients with dried CT extract (0.1% and 0.2%) with two formulations against control. It was observed at par in all tests acquired but 0.2% was better than 0.1% formulation of biogel. The results showed that the formulated biogel was deep blue colour, clear, transparent, had good viscosity (2200 cps), good spreadability (25.4 gm. cm/sec), good PH (6-7.04), and homogenous with smooth texture. The skin sensitivity test of biogel was continued for up to seven days regularly, three times a day on the different skin of human beings. It was observed that biogel has no side effects on the skin like irritation, allergy, edema, and rashes after using it. The storage capability of biogel was also very good under various temperatures (20^{O}C, 25^{O}C, and 30^{O}C) and humidity conditions (RH 60%) for duration of three months. The formulated biogel was evaluated through one antifungal (*Candida albicans*) and three antibacterial (*Staphylococcus aureus, Escherichia coli,* and *Bacillus cereus*) activities based on the zone of inhibitions (diameter mm) at three concentrations i.e. 10%, 20% and 30% v/w for 2 to 3 days against control. The highest inhibition zone against positive control obtained from *Escherichia coli* (14.5 mm/16.5 mm) followed by *Staphylococcus aureus* (13.2 mm/16.0 mm) and *Bacillus cereus* (13.1mm/15.1 mm) at 30% concentration of biogel in 42 hours whereas antifungal activity (*Candida albicans*) was observed (13.3 mm/14.8 mm) at 30% concentration of biogel in 72 hours. The maximum inhibitory zone was seen at a 30% of biogel concentration, as compared to 10% and 20% in specific microbial activity. For the toxicity test biogel of *Clitoria ternatea* was analyzed through an ICP-MS instrument for the detection of heavy metals like Arsenic and Lead. It was found that 0.01 mg/kg in each could be ignored.

Clitoria ternatea exhibits remarkable antioxidant properties observed in whole plant parts in the present research work. However, flower extract exhibits more antioxidant potential than seed, leaf, and root. All part of plant extract contains many phytochemicals and antioxidant enzymes like Peroxidase, gluaiacol peroxidase, superoxide dismutase, phenolic antioxidant, flavonoids, etc. which is beneficial for disease removal. The overall observation of the present research study concluded that *Clitoria ternatea* could be used safely for medicated purposes as well as industrial production of herbal medicines.

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TABLE OF CONTENTS

LIST OF CONTENT

LIST OF TABLE

LIST OF FIGURES

LIST OF PUBLICATIONS

A. Research Papers:

1. **Kiran**, Singh, A., and Jain, A. K. (2020). Biochemical and pharmacological aspects of *Clitoria ternatea*-A review. *Indian Journal of Agricultural Biochemistry*, *33*(2), 115- 124 (Scopus and UGC care list 92)
2. **Kiran**, Singh, A., and Jain, A. K. (2022). Qualitative and quantitative analysis of phytochemical constituents in *Clitoria ternatea* L. *Indian Journal of Agricultural Biochemistry*, *35*(1), 51-57(Scopus and UGC care list 16)
3. **Kiran,** Anuradha Singh, A. K Jain (2023). Formulation, Evaluation and antimicrobial activity of biogel of *Clitoria ternatea* blue flower extract (Communicated).
4. **Kiran**, Singh Anuradha, and Jain, A. K. (2023). Study of Antioxidant activities of *Clitoria ternatea* (Under Communication).

B. Awards:

1. Awarded '**Outstanding women Scientist Award'** in recognition of valuable contribution and achievements, given by Pragatishil Krishak Vikas Sewa Santhan-An Overview" during three days 3rd International Conference on 24th – 26th Decenber-2021 @ Holiday Home, Ranchi, Jharkhand. Dec-2021, organized at Ranchi, Jharkhand.

2. Awarded '**Best Oral Presentation Award'** for presentation entitled "Profitable Agribusiness for Indian Farmers - An Overview" during three days 3rd International Conference (Hybrid Mode) on food Agriculture and Innovations (3rd ICFAI) on 24th – 26th Decenber-2021 @ Holiday Home, Ranchi, Jharkhand. Dec-2021, organized at Ranchi, Jharkhand.

C. Conferences/ Seminars/ webinars

1. Certificate of Presentation in the 'International Conference on Advanced Materials for Next Generation, Application, and AMNGA-2021' held on 29th – 30th Sept., 2021, organized by SBAS, Galgotias University, Greater Noida, U P.

2. International webinars on Basics of ICP-MS and Analysis of Heavy Metals in Herbal Drugs organised by Life Sciences Research, Perkin Elmar, Messachusetts, USA on 15.6.2020.
3. National webinar on National Learning Practical Research Skills and Techniques organized by University Centre for Research And Development (UCRD), 10-14 Aug,2020, Galgotias University, Greater Noida, U P.
4. National webinar on How to write and publish paper in any 5000+ web of science & Scopus, indexed journals conference paper effectively & efficiently using TYPESET Research studio organized by Research and Development Cell, Annasaheb Dange College and Engg. Astha Sangli, Maharashtra on 16.07.2020.
5. Abstract published title Role of Herbal Medicine for Prevention, Protection, and Management of Pre & Post Covid-19, in Disha-2022 on 11^{th}-12^{th} June 2022, Kamla Nehru Instituteof Physical and Social Sciences, Sultanpur, U P.
6. Pradeep Kumar, Simran Kirti, and **Kiran**, Identification and characterization of micro Proteins and their potential uses in Biotechnology, Ensuring Food Safty, Security, and Sustainability through crop protection at International web conference at Bihar Agricultural University, Sabour, Bhagalpur, India on 5^{th} & 6^{th} August 2020.(ISBN:978-81-950908-4-6).
7. 'Abstract Proceedings Book' and 'Keynotes/ Lead Proceeding Book' were published in the 5th National Conference of Doubling Farmers Income for Sustainable & Harmonious Agriculture on 11^{th}-12^{th} June-2022, vol.5, organized by KNIPSS, Sultanpur, UP.

D. Workshops:

1. IPR workshop, organized by Galgotias University, Greater Noida, U P. from Institutions Innovation Council, MoE's, GOI, on 23^{rd} to 27^{th} October, (one week)2021(Online)
2. Indo-Muscat One-week Virtual Workshop on Research Writing Skills-Workshop Series -01 organized by University Center of Research and Development(UCRD) Galgotias University, Greater Noida, U P. from Institutions on 26-30, May, 2020 (online).

ABBREVIATION

SFC	Supercritical Fluid Chromatography	TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography	CC	Column Chromatography
LC	Liquid Chromatography	NP	Normal Phase
CSP	Chiral Stationary Phase	RP	Reverse Phase
USD	United State Dollar	bn	Billion
Rf	Retention factor	MeOH	Methanol
EtOH	Ethanol	CO_2	Carbon dioxide
-NH2	Primary amine	CH_3OH	Methanol
IPA	Isopropyl alcohol	ml	Milli liter
Mg	Milli-gram	kg	Kilogram
PEEK	Polyether ether ketone	UV-Vis	Ultra violet-visible
MS	Mass Detector	PDA	Photo diode array
R&D	Research and Development	K	Retention or Capacity factor
Rs	Resolution factor	A	Separation factor
NH3	Ammonia	H_2O	Water
N_2O	Nitrous Oxide	THF	Tetrahydrofuran
TEA	Triethylamine	FA	Formic acid
ABTs	2,2′-Azino-bis-(3- ethylbenzothiazoline-6-sulfonate)radical cation (ABTS•+)	SOD	Superoxidase
FRAP	Ferric reducing antioxidant power	POX	Peroxidase
DPPH	2,2-Diphenyl-1-picryl-hydrazyl-Hydrate	ROS	Reactive Oxygen Species
GPO	Glutathione peroxidase (GPx) oxidizes	mg	Milli-gram
Nm	Nano-meter	°C	Degree Celsius
µl	Micro-liter	µg	Micro-gram

CHAPTER-I

INTRODUCTION

CHAPTER-I
INTRODUCTION

1.1. Introduction*:*

Ayurveda is the depository of available Medical knowledge in the India, as the only surviving system of medicine all over the world, being currently practiced. As evidenced available in Ayurveda, India has a rich tradition of herbal medicine. Ayurveda means knowledge (Veda) of life (Ayur) had its beginning in Atharvaveda. The Rigveda also have a collection of Hindu sacred verses, contains most aspects of Vedic science such as yoga, meditation, mantra, and Ayurveda, which are still widely practiced today [1]. Since ancient times, Ayurvedic medicines had an important role in human healthcare. In the last few decades, Ayurvedic medicines have become very popular.

Ayurvedic medicines are herbal, mineral, and herbomineral compounds, which are basically parts of Bhaishjya Kalpna and Rasshastra. The beneficial effects on health and other uses of Ayurvedic medicines have been proven over time. Given their nutritional richness and the possibility of healing, they come highly recommended. Their approach is to revitalize the entire body rather than concentrating on a single organ or bodily portion. They help with appropriate digestion and absorption and take a holistic approach [2].

Herbal products have been more popular in recent years. The herbal medicines involve the integration of several therapeutic experiences and practices of indigenous systems of medicine that may span many previous generations, which often provides valuable guidelines to the selection, preparation, and application of herbal formulation for the treatment, control, and management of a variety of ailments [3].

The indigenous system of medicine is the best-accepted method of treatment and it is widely practiced in villages of whole world. By enhancing the immune system, they function as a preventive antibiotic that improves general health and wellbeing rather than being disease-specific. They are comparable to allopathic medications have occasionally shown promise in the treatment of autoimmune disorders. They are non-

toxic and safe because they are self-sufficient and nourishing holistic in nature. It addresses general wellbeing and seeks to achieve mental, physical, and spiritual balance [4].

Medicinal plants can produce specific compounds or secondary metabolites that are non-nutritive but helpful in defense mechanisms to defend themselves from dangerous insects, pathogenic microbes, and bad environmental changes. These are referred to as essential oils and phytochemicals. In addition to shielding plants against some diseases brought on by bacteria or their poisons, it can also shield people and animals from these illnesses. Its antibacterial nature is the reason for this. Phytochemicals may be employed as chemo-preventive agents in the future. Numerous phytochemicals have been identified to far and categorized into broad classes based on differences in their chemical structures. Phytosterols, flavonoids, terpenoids, saponins, alkaloids, carotenoids, aromatic and organic acids, essential oils, and protease inhibitors are the main categories of phytochemicals. The metabolites can act as a direct or indirect defense mechanism against pathogens or hazardous illnesses because of their various qualities, which include antibacterial, anti-inflammatory, anthelmintic, anticarcinogenic, antigenotoxic, antiproliferative, antimutagenic, and antioxidative.

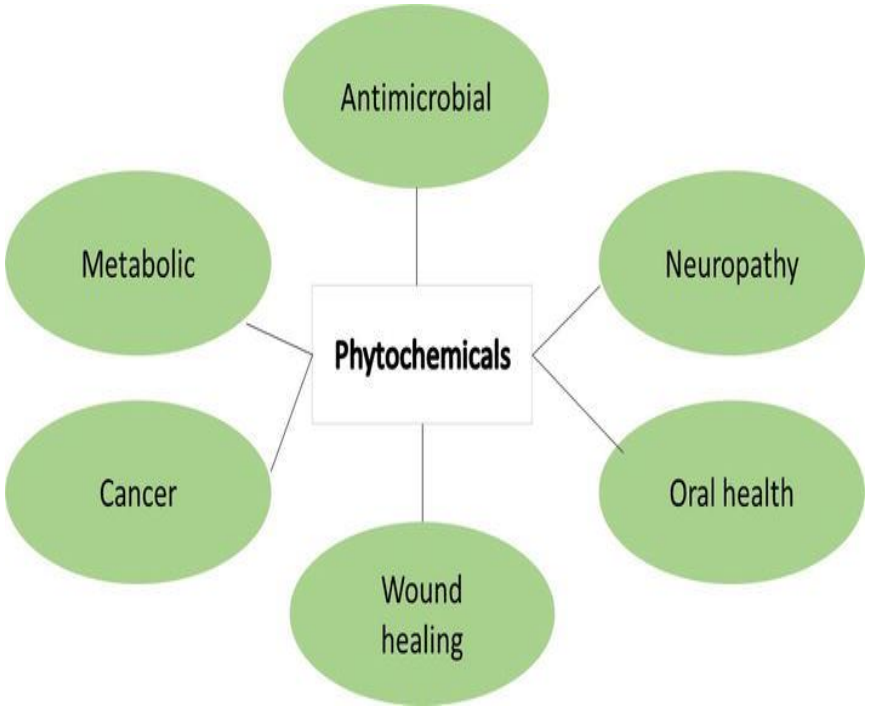

Figure 1.1: Medicinal Properties of Phytochemicals

Because they scavenge free radicals, preserve dietary antioxidants like vitamins A and C, and chelate pro-oxidant metal ions, antioxidant enzyme cofactors in humans, natural antioxidants like polyphenols in plant play a significant role in maintaining human health. Due to the antioxidant activity of phenolic compounds found in medicinal plants, epidemiological studies have shown a positive link between plant material and a decrease in the pace of aging, cardiovascular disease, and other degenerative disorders. Lipid peroxidation, radical scavengers, and other activities caused by free radicals are inhibited by antioxidants. As a result, it shields the body from numerous illnesses linked to the effects of free radicals. Numerous phenolic antioxidants, including tannins, flavonoids, xanthenes, coumarins, and procyanidins, have demonstrated the ability to scavenge radicals in a manner that is dependent on dosage, making them attractive therapeutic candidates for the treatment of free radical diseases [5].

Aprajita plant is also utilized medicinally. In the Ayurveda, its function in boosting Medhya (intellect), chakshuya (BIO tonic), svasa-kasa (respiratory system), shoolaghna (pain killer), vishaghna (antipoisonous), Balya (strength, particularly mind) are documented. Aprajita's function in the treatment of Kustha Roga (leprosy/skin disease), Asra-vikara (blood disorders), Kasa (cough), Visa (poison), Sopha (oedema), Jwara (fever), and Unmada (insanity) has been extensively documented over the ages. As a result, Aprajita has a lot to offer the medical community [6].

1.2. Common Names:

The word 'Aprajita' is used to describe the dish 'Aishanidisha.' Vish-vaidya which is referred to as ‘Aprajita' in the Shaunkiya Atharvaveda Samhita. According to traditional beliefs, its flower is associated with the element of water, which bestows upon it qualities such as love, emotion, calmness, and protection. Venus is also thought to be the flower's governing planet. This flower's name in India comes from the Hindu goddess Aparajita, which means "one who cannot be conquered or defeated." Also the name "shankhpushpi" in Sanskrit means "the plant with conch-shaped flowers" is similar to Lord Shiva's holy instruments the Shankha. Hence, it is commonly known as “Aprajita(Hindi)” and “Shankhpushpi” (Sanskrit). Botanically, Aprajita is named as *Clitoria ternatea Linn(*CT) belongs to Fabaceae family. In North

America and Australia, it is known as Butterfly pea. It is also known as “Blue-pea” and “Blue bell vine” in English [7].

1.3. Taxonomic Classification of *Clitoria ternatea* (CT) [8}:

Kingdom	:	Plantae
Subkingdom	:	Viridaeplanta
Infrakingdom	:	Streptophyta
Division	:	Tracheophyta
Subdivision	:	Spermatophytina
Infrodivision	:	Angiospermae
Class	:	Magnoliopsida
Superorder	:	Rosanae
Order	:	Fabales
Family	:	Fabaceae
Genus	:	*Clitoria*
Species	:	*ternatea*

A. Whole plant of *Clitoria ternatea* **B.** Leaves of *C. ternatea*

Figure: 1.2. *Clitoria ternatea* Plant

1.4. Distribution of Plant:

The plant is mostly found in the tropical regions of the Philippine Islands, Burma, India, Malaysia, and Sri Lanka. Later, it spread far over Africa, especially South Africa. Additionally, the USA and the Northwestern Pacific region are home to it. Due of its fast growth rate and drought tolerance, this well-known ornamental is often found growing in gardens and landscapes or growing wild in its native Asian habitat [9].

Clitoria and Centrosema are both Clitorinae sub-tribes with 180° downward rotated flowers. In Africa, China, Sudan, Southeast Asia, East and West Indies, South and Central America, and Sudan, CT is one of the most significant forage legumes. Around the world, populations of CT have become naturalized in tropical and subtropical areas [10].

Beautiful flowers of this plant hue find their way into gardens and homes, as well as decorative crops, giving value to them. The butterfly pea flower is the main component of herbal tea in used by native of Southeast Asia. Because of its slender stem and big leaves, as well as its non-toxic nature, it serves as a multi-purpose feed legume that is extremely appealing to cattle and it is a good source of livestock in India and South East Asia [11].

1.5. Description of Plant:

Clitoria ternatea requires little care; low maintenance grows as a vine or creeper and does well in neutral, moist soil. The herb is perennial. It has five elliptical to oblong-shaped leaves with impaired pinnate. Leaflets measure up to 6.5 × 4 cm, ovate to elliptic-oblong, and typically hairy on the bottom and hairless on top. Its stem is slender, thin and downy. The flower of *C. ternatea* is solitary with deep blue, pink, and white colours. It has axillary, solitary or in pairs, resupinate, big and spectacular, brilliant blue flowers which is 6-12 cm long, flattened, mucronate at the apex, hairless or finely pubescent pod linear-oblong. The flower of *C. ternatea* is self-pollinated by nature and also found in cross-pollination in the genotype segregation. Its seeds pods contain six to eight black or brown –coloured (Figure 1.3) [12].

Root Leaves Flowers Seeds

Figure 1.3: Plant Parts of *Clitoria ternatea*

1.6. Background of Study:

Apart from its several culinary uses, the blue butterfly pea vine has been used for thousands of years in Ayurvedic and traditional Middle Eastern and Asian healing. *Clitoria ternatea* have been utilised in the rejuvenating herbal preparation ―Medhya Rasayana is commonly used as a brain tonic in the Ayurvedic system of traditional Indian herbal medicine to ameliorate intelligence and intensify the memory function, prevents convulsions as in epilepsy (anticonvulsant) and also as relaxing agent. In USA also, alternative forms of medical treatments are enjoying great popularity and *Clitoria ternatea* plant is used as one of the popular herbal medicine there [13].

Across the globe, people utilized medicinal plants and herbs to improve their health. As a result, scientific examination of their medicinal potential, physiological, biological characteristics and safety will aid in making informed choices regarding their usage. The medicinal qualities of *Clitoria ternatea* plant have been widely documented in traditional medicine, but they have yet to be completely utilised. Based on potential sources of active components the analysis's findings provided information for edible flower screening and identification, such as flavonoids, phenols, anthocyanins, antioxidant enzymes, and others, with strong antioxidant properties that may be of interest to both users and healthcare providers.

Antioxidant-rich beverages protect the body from infections and are also beneficial to diabetes control, heart health, hair, and skin. It is also used in culinary, colouring, cosmetics, and traditional medicine [14]. In the sketch study done by several others, it is revealed that *Clitoria ternatea* possesses a variety of pharmacological characteristics, including antiparasitic, antibacterial, antidiabetic, and anti-inflammatory effects [15].

Clitoria. ternatea is very helpful in the development of medications and treatments due to its medicinal, biological, and pharmacological value. Hence, being well aceepted medicinal plant it was selected for further research study.

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CHAPTER-II

REVIEW OF LITERATURE

CHAPTER-II
REVIEW OF LITERATURE

2.1. Introduction:

Numerous researchers and scientists have studied a variety of therapeutic plants, including *Clitoria ternatea*. *C. ternatea* is a garden plant native to India that has been used in traditional and folklore medicine to treat a variety of diseases. Its anti-inflammatory, antipyretic, analgesic, insecticidal, larvicidal, antimicrobial, anxiolytic, hepatoprotective, tranquilizing, and sedative properties have all been scientifically assessed. The goal of this chapter is to provide a thorough description and review of the literature regarding *Clitoria ternatea* and other pertinent medicinal plants. Through these studies, it helps in examines the pharmacological activity, traditional uses, ornamental value, phytochemical constituents, pharmacognocal description, and phytonomic traits of butterfly pea.

The ornamental plant CT is frequently seen growing in Asian environment or grown in gardens and landscapes because of its drought tolerance and rapid growth rate. CT has a long history as a medical plant as well. Mainly three popular varieties such as blue, pink, and white of *Clitoria ternatea* are everywhere found. All these varieties of flowers are based on ratio of anthocyanidins /anthocyanin in flowers. Various diseases such as allergies, arthritis, nerve disease, liver disease, diabetes, inflammatory disease, cancer etc. are cured through these colored pigments of flowers [16]. This contains healthy anthocyanins (blue pigments) and has a number of health advantages, like; Proanthocyanidin, an anti-oxidant, enhances vision by boosting blood flow in the capillaries which improves vision.

2.2. Conventional Collection and Extraction of CT:

Many southern Asian villagers preserve extract in the powder form of the plant by simple extraction process. They gathered fresh blue flowers in the months of June and July from their home garden. The flowers are washed in basic (soft) water and sun-dried or dried in the shade at home for two to three days. Fifty grams of dried flowers are added to 2 litres of boiling water and simmered until the amount was reduced to a quarter or 500 ml. Nylon fabric is used to filter the soluble extract. They use this as tea, supplement or health drink added with hot water, milk, and juice [17].

Mainly polyphenolic compounds are extracted from the flower, seed, root, and leaf from the plant. The best traditional methods applied for the extraction of polyphenolic compounds are maceration, hydro-distillation, and Soxhlet extraction. However, all these methods are not authentic and have some drawbacks like taking more time, consuming more solvent, and needing high temperatures [18]. The result of all drawbacks is polyphenolic degradation. So to overcome this drawback, some novel techniques such as ultrasound-assisted (US), enzyme-assisted, and microwave-assisted have been employed for the extraction of phytochemicals from plant materials without the loss of bioactive compounds. The easy method is ultrasound-assisted which gained high efficiency. In the US extraction method, temperatures are also lower, less time consumed, less use of organic solvent, and lower energy input [19].

2.3. **Best Solvent for Maximum Extraction of CT:**

The maximum extraction means the maximum amount of polyphenolic compounds and antioxidants come in the solvent. There are many various organic/ solvents like ethanol, methanol, petroleum ether, etc. Water is also a good solvent for extraction they can use it safely and easily because of uses herbal instant medicine for home remedies. Water mixed ethanolic solvent of CT flowers showed a much higher scavenging free radical activity and antioxidants both maximum present in the extract as compared to methanol-based extracts. The maximum antioxidant activity shown of the blue flower in ethanolic aqueous extractions [20].

The extraction of *C. ternatea* flower isolated and identified the structure of various phytochemicals mainly anthocyanins by utilizing aqueous solvent mixtures. However, other studies on the flavonol constituents found that extractions through aqueous solvent mixtures of ethanol or methanol rather than water alone with heating to investigate its potential bioactivities and phytochemical content [21]. It is found that the concentrations of extracts were increased, the percentage of the extracts to scavenge off free radicals also increased. According to their findings, when the concentration of samples both water and ethanol were 25.00 μg/mL, 50.00 μg/mL, 100.00 μg/mL, and 125.00 μg/mL while the scavenging activity was 32.67±1.16%, 353.33±3.06%, 411.33±1.16%, and 422.67±3.06%, respectively in ethanol. The water

content was 390.67±2.31%, 401.33±3.06%, 449.33±2.31% and 490.67±4.62%, respectively [21].

The extraction efficiency of anthocyanins was found maximum by using various solvents (low to high polarity). However, the lowest efficiency was found in ethyl ether. The study revealed that higher efficiency was found even in polar compounds (anthocyanin) with higher polarity (ethanol). The differences in the content of phytochemicals of the flowers of CT using ethanol (hydrophilic) and ethyl acetate and hexane; 1:1(hydrophobic) extraction was analysed and found that the various anthocyanins, quercetin glycosides, and kaempferol are hydrophilic however, fatty acids, phytosterols, and tocopherols are hydrophobic in nature which is dependent on the polarity of the compound [22].

2.4. Ash Residue of *Clitoria ternate:*

Measurements of the ash content (mg/100g dry weight) of various plant sections (stem, flower, leaves, seeds, and root) in *Clitoria ternatea* were made. This included total ash content (5%); Insoluble acidic ash content (2%); Insoluble alcoholic ash(5%), water soluble extracts(8%) and others like crude fiber and protein; soluble minerals and carbohydrates; and total lipid in less amount. The dry weight range of butterfly pea flowers is 3.8 ± 0.42 mg/100 gram to 10.93 ± 0.29 mg/100 gram. The overall amount of ash in the leaves was found higher than that in the seed. There was no insoluble ash in the stem or seed, and the largest concentration of insoluble ash was found in the leaves, followed by the root and the flowers. The stem had the highest concentration of soluble minerals, followed by flowers, leaves, and seeds [2 3] .

2.5. Horticultural Review of *Clitoria ternatea*:

Clitoria is presently divided into three subgenera, with *Clitoria ternatea* serving as the holotype [24]. From *Clitoria ternatea L*., which is distinguished by zygomorphic blooms or flowers with five uniform petals, both *Clitoria ternatea* var. *pleniflora Fantz* and *Clitoria ternatea var. ternatea* [25] are direct ancestors. A biologically active family of flavonoids, CT blooms are usually white, pale blue, dark blue, or mauve in color and contain high levels of flavonoids [26]. Under ideal conditions, the blooms bloom practically all year round and, in contrast to common perception, are not magnets for butterflies. Conversely, CT is required for the winged form of the

bloom [27]. Malaysian delicacies like as "nasi kerabu" and "kuihtekan" are blue in color because natural plant pigments made from fresh or dried CT flowers or flower petal extracts are used. [28].

To prepare “un-chan juice powder” or “doknamanchan” (hot or iced tea) in Thailand, the extract of the blue flower of CT is dried and combined with sugar [29]. Neutral and acidic environments cause many anthocyanins to lose their color; however, organically produced CT flower extracts, which last longer and are easier to use than other blue colorants, manage to keep their blue-purple color in these conditions. In addition, anthocyanin extracts have a high chrom and color density, which are crucial characteristics for application in aqueous food systems [30].

Furthermore, current health issues linked with synthetic food colourant intake and usage will certainly increase the popularity of CT in food and beverage production[31]. It is evident that this plant is gaining popularity in the United States, where reports of its potential applications as an environmentally friendly insecticide, nutraceutical, pH-dependent food coloring, and cosmetic have already surfaced [32]. Primarily grown as a decorative perennial climber, trailer, or scrambler, CT reaches maximum heights of 3 to 5 meters (10 to 15 feet) vertically and 1 to 2 meters (3 to 6 feet) laterally [33].

2.6. Photosynthetic Aspect of *Clitoria ternatea:*

The Scientist found their result of light is the primary source of energy for plants via photosynthetic synthesis; light amount, quality, and photoperiod have a major effect on growth and development. The entire amount of light received by the plant is described as light quantity, which relates to the intensity of the light. Within the PAR range, quality is mainly determined by the wavelength and colour of the light [33].
Environmental and management variables such as competing vegetation shadowing, placement in a greenhouse, usage of shade fabric, and so on may influence both the amount and qualityof light. Plant photoperiod, or the amount of time they are exposed to light, is directly tied to latitude; it is highest in the summer and lowest in the winter. The overall amount of light that a plant can receive depends on these three elements combined, and this amount is associated with the number of photosynthetic processes the plant can perform. [34].

It was shown that the CT tends to be grown in full sun, but due to its wide range of climatic conditions, it can withstand minor shade. Because CT is a nitrogen-fixing forage legume that can improve soil fertility, there has been interest in using it to restore formerly overfarmed agricultural areas. The authors came to the conclusion that the ideal planting density (D10) to maximize the nutritional value and output of feed [35].

2.7. Effect of Soil pH on *Clitoria ternatea:*

The study revealed that three main factors that affect adaptation are soil salinity, pH, and aeration/compaction. Nutrients needed for plant growth and development are absorbed by the roots of plant; the pH of the soil greatly influences the availability and solubility of certain nutrients. Elevated levels of acidity combined with excessive iron, manganese, and aluminum content can be hazardous, whereas alkaline soils decrease nutrient bioavailability and may result in plant nutritional deficits. The amount of nutrients available may be decreased by soil acidity, which can also affect microbial activity. Most plant thrive in a pH range of 6-7 since CT is typically grown on soils with a pH range of 5.5–8.9 [36].

Subsequent research showed that inadequate soil porosity may considerably reduce the soil's ability to exchange carbon dioxide for oxygen, which would make root respiration challenging. Reduced microbial activity, restricted and irregular root growth, and inadequate root penetration and development could result from this. When these variables are combined, they may have a substantial impact on nutritional Bioavailability and absorption, as well as contribute to disease development. It has been found that CT has adapted to a broad variety of soil types and densities, including as calcareous soils, heavy clays, sandy soils, and deep alluvial loams. These adaptations may help explain why the species is able to withstand both chronic drought and excessive rainfall [36].

2.8. Effect of Soil Moisture on CT:

The quantity of accessible water in the soil is measured by soil moisture content (SMC) which is very important to agricultural producers. Like other legumes, CT can become stressed by a lack of water. Sushmita & Deeksha et al. discovered that mild drought exposure resulted in the maximum germination, increasing it by 6.25 percent

in comparison to the control, after exposing CT seed to various levels of drought stress. Under extreme drought stress, shoot length, root length, and fresh weight all arose by 1.6%, 2.4%, and 3.8% in comparison to the control [37].

Drought stress is one of the most hazardous environmental stressors affecting crop output globally; it gets worse with rising temperatures and results in significant losses in the agricultural sector. A reduction in transpiration, chlorophyll concentration, photosynthesis, stomatal closure, and osmotic stress are among the physiological and developmental processes in plants that it impacts [38].

2.9. *Clitoria ternatea* as Natural Colorant:

Flowers, leaves, young shoots, and delicate pods are all edible, and the leaves may also be used as a green colorant [39]. This provides a reference for the extraction, processing, and storage of the color-changing natural colorants present in butterfly pea flower extract, recognizing the necessity for dependable supplies of these natural colorants (BPFE) [40].

The pH of the surrounding solution has a significant impact on BPFE and its related anthocyanins, particularly when it comes to the colour of the extract. The deep blue to purple hue is produced by a nearly equal combination of flavylium (red) and quinoidal (blue) types of anthocyanin at its usual pH (6.0–8.0), however, stepping outside of that typical pH range may induce unwanted colour changes in the final product. With a greater PC than other natural colorants evaluated, BPFE has a lot of promise in applications where it will be subjected to pH fluctuations during storage and processing [41]. The blue, purple, pink, red, and orange hues seen in plant is caused by the anthocyanin.

The Anthocyanins is the biggest category of phenolic pigments, are mainly linked to the antioxidant and colorant qualities of the flower or fruit in which they are found Anthocyanins are found in blue form, the anionic quinoidal base, in aqueous and alkaline solutions. The deep blue-to-purple hue of the blooms is produced by a nearly equal combination of flavylium (red) and quinoidal (blue) forms [42]. However, owing to the large percentage of the colourless hemiacetal form, the colour fades quickly and is usually of low intensity. In the food industry, value addition is

important. These blossoms are often used as a culinary colour or dipped in batter and deep-fried as fritters in Southeast Asia (Indonesia and Malaysia) and Madagascar. Beautiful blue flowers may be used to make herbal beverages and teas, as well as ready-to-drink products that are high in antioxidants and phytonutrients (Figure 2.1). Flowers are used as a natural food colourant in sweets and even cereals like rice and porridge to add appeal to the meal [43]. Delphinidin is the primary anthocyanin responsible for the rich blue to purple colour of butterfly pea blooms [44].

Natural dyes may create unique visual characteristics, which, when coupled with the ethical value of an ecologically friendly product, add value to textile manufacturing as a craft and as an industry. Plant that produce colour, such as butterfly pea blossoms, have been used to investigate colour extraction and may be utilised as a dyeing material in the colouring business. Natural dyes have risen to prominence as viable alternatives to synthetic colours. Natural colours have been resurrected as a result of increasing environmental awareness of the health risks posed by synthetic dyes [45].

Figure: 2.1-Product samples of Three Mocktail Drinks

2.10. *Clitoria ternatea* use as Animal Feed:

Because of its superior yields over other legumes, quicker regrowth after grazing, and good palatability to livestock animals, CT was commonly used to replenish badly cultivated regions with natural grasslands and as a fodder crop. Up to eight cuttings may be made each year, spaced forty-five days apart. The cuts should be made 10 cm above the ground to allow the plant 25 days to regrow [46]. CT is excellent for fresh fodder, forage, and silage because of its high leaf protein content, lower acid digestible fiber level, non-toxicity, and reduced bloat. Planting and harvesting

schedules that are appropriate for the climate ensure that the growth phases of the plant occur at the appropriate periods. This may have a major effect on yield, growth, and development. The best time to plant and harvest depends on the cultivar, growing area, and growing season [47].

2.11. Availability of Bioactive Compound:

Roots, stem, leaves, seeds, and flowers are the reported plant part used as an herbal medicine from ancient times of any medicinal plant. Any Medicinal plant parts contains many phytochemicals compounds like carbohydrates, proteins, volatile oils, flavanoids, flavonol, cardiac glycosides, phlobatannin, phenols, saponins, tannins, alkaloids, triterpenoids, anthocyanins, antharaquinone, and steroids.

Rich amounts of fatty acids, including oleic, palmitic, stearic, and linolenic acids, can be found in CT seeds. The seeds also included water soluble mucilage, anthoxanthin glucoside, beta-sitosterol, cinnamic acid, finotin, a highly basic small protein, and delphinidin 3,3',5'-triglucoside [48]. The seeds of CT contains nucleoprotein with its amino-acid sequence similar to insulin, delphinidin-3,3,5-triglucoside, essential amino-acids, pentosan, water-soluble mucilage, adenosine, an anthoxanthin glucoside, greenish-yellow fixed oil, a phenol glycoside, 3,5,7,4-tetrahydroxy-flavone-3-rhamoglycoside, an alkaloid, ethyl D-galactopyranoside, p-hydroxy cinnamic acid polypeptide, a highly basic protein-finotin, a bitter acid resin, tannic acid, 6% ash and a toxic alkaloid [49].

There are four types of kaempferol glycosides I II, III, and IV was isolated from the leaves of *Clitoria ternatea* Linn. Kaempferol-3- glucoside (I), kaempferol-3 - rutinoside (II) and kaempferol-3-neohesperidoside (III) were identified by Ultra Violet, Protein Magnetic Resonance and Mass Spectrometry. (IV), C33H40O19, mp: 198, was characterized as Kaempferol-3-orhamnosyl glucoside from spectral data and was named Clitorin [50]. The presence of flavonoid glycosides such as rutin, delphinidin, kaempferol, quercetin, and malvidin are available in *Clitoria ternatea*, and it has been documented that its leaves contain δ-lactone of 2-methyl-4-hydroxy-n-pentacosanoic acid [51].

The maximum phytoconstituents present in blue flowers are pentacyclic triterpenoids such as taraxerone and taraxerol. The petals of *C. ternatea* were used to isolate three flavonol glycosides: myricetin 3-O-(2”,6”-di-O-α-rhamnosyl)-β-glucoside, quercetin 3-O-(2”-O-α-rhamnosyl-6”-O-malonyl)-β-glucoside, and kaempferol 3-O-(2”-O-α-rhamnosyl-6”-O-malonyl)-β-glucoside. [51]. The compounds are found as quercetin 3-(2(G)-rhamnosylrutinosides, kaempferol, myricetin 3-neohesperidosides, 3-rutinosides, and 3-glucosides. Consistent with the LC/MS/MS results for crude petal extracts, myricetin 3-O-(2"-O-alpha-rhamnosyl-6"-O-malonyl)-beta-glucoside was also detected [52]. All of the blue petal lines contained ternatins, a group of 15 (poly) acylated delphinidin glucosides. Anthocyanins were not found in the white petal line Mome inositol (33.6%), cyclohexen, 1-methyl-4- (1-methylethylideme) (7.1%), acetic acid, cyano- (6.5%), and hirsutene (5.7%) were extracted from the aqueous extract of *Clitoria ternatea* flowers, whereas mome inositol (33.6%), cyclohexen, 1-methyl-4-(1-methylethylideme) [53].

Clitoria termite petal extracts, are found in a huge amount of poly-phenolic bioactive compounds, such as caffeoylmalic acid (137.59 mg/100 g), kaempferol 3-(2G-rhamnosylrutinoside) (129.28 mg/100 g), and kaempferol-3-neohesperidoside (462.63 mg/100g), and which defense against lipid oxidation. The assessment of the solvents used to get the CT petal extracts after 6 hours, 12 hours, and 24 hours of soaking times, including dH_2O, CH_3OH, and/or a mixture of dH_2O: CH_3OH (1:1) ratio, total anthocyanin, phenolic, and total enzymatic antioxidant activity. The CT petal extracts may enhance health benefits and lengthen product shelf life. [54].

Tannins were present in plant and to be responsible for high immunomodulatory activity in earlier research and *C. ternatea* confirmed a considerable presence of tannins [55]. Anthocyanin pigments of this plant are notable for their chemical components which produce cyclotides, and ultra-stable macrocyclic peptides, found in all tissues of this plant, give *C. ternatea* flowers their distinctive blue hue. The study provides a description of peptides' genetic origins, which include the co-option of an ancient albumin gene to generate the cyclotide precursor protein. An asparaginyl endopeptidase, which is found in *C. elegans*, is involved in the biosynthesis phase that results in the formation of the cyclic peptide backbone. Butelase-1 is an enzyme that has been the subject of several recent researches on peptide ligation and

cyclization for biotechnological applications due to its great efficiency in peptide ligation [56].

2.12. Antioxidant of *Clitoria ternatea:*

The water-soluble pigments known as anthocyanins give *C. ternatea* blooms their blue and purple hues. These substances, which are flavonoid group members, have anti-inflammatory and antioxidant qualities. The potential medical advantages of anthocyanins have garnered a great deal of attention. In *C. ternatea*, anthocyanins contribute to its antioxidant activity and have been associated with various health benefits. It has been demonstrated that they guard against oxidative stress, lessen inflammation, improve cardiovascular health, sharpen cognitive function, and encourage good aging. Additionally, anthocyanins may have anticancer effects by stopping the spread of cancer cells and encouraging apoptosis. [57].

Butterfly peas have a lot of promise for use and establishment as a functional food ingredient due to their high anthocyanin proportion. Because of their high anthocyanin content, colored flowers have been shown to have antioxidant potential [58]. Although there may be problems with their oral bioavailability, several anthocyanins are known to have antioxidant properties that may be essential in the protection of numerous diseases, including cancer, cardiovascular diseases, and skin aging. [59].

The powder of butter pea fly has a reputation for promoting energy and a healthy ageing process. It's high in antioxidants, such as pro-anthocyanidin, which helps skin collagen and suppleness; and anthocyanin, which helps skin glow, supports hair and BIO health. Both of these substances aid in cells' overall healthy life cycle. Blue Butterfly powder is a well-known nerve tonic that helps the circulatory and neurological systems [60].

The yeast cell was used to investigate the antioxidant properties and apoptotic studies of *Clitoria ternatea* leaves. The yeast cells and yeast cell DNA were recovered from the effluents of a sugar plant. In a dot plot fast screening assay technique, the leaves extract from various solvents were evaluated for their scavenging ability against the stable free radical DPPH (2, 2'-diphenyl-1-picryl hydrazyl) and measured using a

spectrophotometric assay method. In order to investigate the effects of the leaf extracts, oxidative damage was produced in vitro by treating yeast DNA. YBD broth culture was used to isolate genomic DNA samples. The methanol extract of *Clitoria ternatea* generated a lot of DPPH scavenging activity. The treatment with *Clitoria ternatea* leaf extracts significantly reduced the amount of DNA damage [61].

Clitoria ternatea's root is used as a laxative, purgative, diuretic, anti-inflammatory, indigestion, constipation, fever, arthritis, Bio problems, sore throat, and anthelmintic. Kirtikar and Basu also said that CT may be used to treat severe bronchitis, asthma, and fever. Local tribes also utilise CT to induce abortion and treat abdominal swelling, sore throat, mucus problems, and fever. In chronic bronchitis, CT root juice is mixed with cold milk to help decrease phlegm [62]

The study of antioxidants found naturally in plant is becoming more and more popular. *Clitoria ternatea* is one plant that merits consideration. The in vitro antioxidant characteristics of the ethanolic extract of *Clitoria ternatea Linn* were evaluated using the DPPH free radical technique. When present at room temperature, ethanol is turned violet by the free radical diphenyl picryl hydrazine, or DPPH. When an antioxidant molecule is present, it is reduced, resulting in the formation of the uncolored solution [63].

A plant's flower is a vital component that contains a wide range of naturally occurring antioxidants, including anthocyanin, flavonoids, and phenolic acids, among many other phenolic compounds. The presence of different concentrations of polyphenolic substances (polyphenols, flavonols, flavonoids, anthocyanins, etc.) is responsible for the preventive effects [64]. Studies on the leaves and flowers of two species of *Clitoria ternatea*—one with blue flowers and the other with white flowers—found considerable concentrations of several non-enzymic antioxidants, such as ascorbic acid, reduced glutathione, and total carotenoids. The floral extracts have natural antioxidant properties, enhance blood circulation, and help prevent hair graying and hair loss [65].

According to research, flavonoids—a class of naturally occurring plant phenolic chemicals available in CT plant that includes isoflavones, chalcones, flavones, and flavonols—are significant antioxidants. The distinctive C6–C3–C6 structure of

flavonoids is characterized by a free hydroxyl group joined to aromatic rings. By scavenging free radicals or through additional mechanisms including metal chelation, singlet oxygen quenching, and lipoxygenase inhibition, these prevent lipid oxidation [66].

Mice exposed to acetaminophen-induced liver toxicity were used to assess the hepato-protective and antioxidant properties of *C. ternatea* flower extract. The hepato-protective effect was verified against acetaminophen as a model hepato-toxicant. [67]. It is capable of effectively controlling the biochemical indices linked to diabetes mellitus and has a significant ability to preserve the liver [68].

2.13. Reviews of Pharmacological Properties of *Clitoria ternatea*:

Flower extracts of *C. termatea* possess a wide range of pharmacological activities including antimicrobial, antipyretic, anti-inflammatory, anti-asthmatic, hepatoprotective analgesic, diuretic, local anesthetic, antidiabetic, insecticidal, blood platelet aggregation-inhibiting and for use as a vascular smooth muscle relaxing properties. Aqueous and ethanol extracts of roots, leaves, and flowers have been studied for their anti-diabetic potential [69,70]. *Clitoria ternatea* seeds contain a variety of fatty acids, including linoleic, stearic, oleic, and palmitic acids. [71].

2.13.1. Anti-inflammatory, Anti-pyretic and Analgesic properties:

A normal reaction of the body to damage is inflammation, which is commonly accompanied by symptoms like heat, redness, swelling, and joint discomfort. On the other hand, inflammation can prevent the body from mending. It has been discovered that *Clitoria ternatea* extarcts contains anti-inflammatory qualities that not only relieve pain and discomfort but also hasten the healing process. [72].

Clitoria ternatea's root of the plant is traditionally used to cure ascetics, swelling of the abdominal viscera, sore throats, and skin disorders. The root is used as a general tonic for children, along with honey and ghee, to improve their intellectual abilities and physical strength. The seeds and leaves of this plant are extensively utilised as a brain tonic and to improve cognitive abilities. Flowers and juice of it are used as antidotes for snake bites. Swollen joints are treated with seeds, while urinary issues are treated with crushed seeds and cold or boiling water [73].

The deep blue blooms of a butterfly have a lot of Anti-inflammatory advantages. It contains flavonoid pigments that protect the plant, comparable to blueberries, acai berries, mangosteen rinds, and so on. It has an anti-inflammatory effect that is very comparable to other of the plant available in nature. It helps in diabetes by reducing dietary glucose consumption. The availability of cyclotides, which induce cell death by breaking cell membrane integrity, is known to have anti-cancer and anti-tumor properties [74].

Clitoria ternatea flower extract was tested for analgesic and anti-inflammatory effects in rats (carrageenan paw edoema) and mice (hot plate). The anti-inflammatory and analgesic effects of the petroleum ether (60-80^{o}C) extract were considerable. *Clitoria ternatea* Linn's methanolic extract has analgesic properties. On mice, leaves were tested at dosages of 200 and 400 mg/kg body weight. The acetic acid-induced writhing test was used to evaluate the analgesic properties. The plant extract's depressive action in the Central Nervous System (CNS) was assessed by utilizing hole cross and open field tests. In an acetic acid-induced writhing test, the extract reduced 82.67 percent of writhing at the lower dosage and 87.87 percent at the higher dose, which is similar to the reference medication diclofenac sodium. The extract reduced the dose-dependent motor activity and exploratory behavior of mice in hole cross and open field tests, indicating that it has CNS depressive action. As time passed, the number of fields traversed in the open field test and holes crossed in the hole cross test dropped [75].

It has the antipyretic effect which lowers fever via the dilation of blood vessels immediately under the skin, increasing blood flow near the skin's surface, where it may be more readily cooled by the air. The high availability of antioxidants boosts collagen synthesis which prevents premature ageing. It is thought to regulate blood sugar levels and has been used in ancient Ayurveda medicine as a memory booster, stress reliever, and mood lifter. Hair is strengthened to promote healthy hair development and to prevent hair loss and greying [76].

The anti-pyretic potential of a methanol extract of the blue flowered type of *Clitoria ternatea* root (MECTR) was tested in albino rats with normal body temperature and yeast-induced pyrexia. After 19 hours of subcutaneous injection, yeast suspension (10

ml/kg bw) raised rectal temperature. The extract reduced normal body temperature and yeast- provoked high temperature in a dose-dependent manner at dosages of (200, 300, and 400 mg/kg bw, po). The impact lasted for up to 5 hours after the medication was given. The extract has an antipyretic activity similar to paracetamol (150 mg/kg bw, po) [77].

Using various antinociception models, the potential mechanism behind the antinociceptive effect of methanolic extracts of *Clitoria ternatea* leaf and root was investigated. The antinociceptive activity of both leaf and root extracts was determined using several antinociception models such as the hot plate, tail-flick, and formalin tests, as well as naloxone (a non-selective opioid antagonist). In experimental animals, both leaf and root extracts of *Clitoria ternatea* showed significant anti-nociceptive activity. The antinociceptive action of the extracts may be mediated at both the central and peripheral levels, according to the results of the formalin test. Furthermore, heat plate and tail-flick experiments revealed that *Clitoria ternatea* root extract mediated antinociceptive activity centrally at supraspinal and spinal levels, while *Clitoria ternatea* leaf extract mediated antinociceptive activity centrally only at supraspinal level. The opioid receptors are thought to be involved in the antinociceptive action of both *Clitoria ternatea* root extracts, according to scientists [78].

2.13.2. Anti-Microbial Properties:

Antibacterial activity against the studied microbiological pathogens was found to be promising. When compared to the other extracts, methanol extract was shown to have a more powerful inhibitory action than petroleum ether and ethyl acetate [79].

Clitoria ternatea's antibacterial activities were examined using agar disc and well diffusion techniques. Extracts from the leaves of *Clitoria ternatea were* evaluated against *K lebsiella pneumoniae, Proteus vulgaris,* and *Salmonella typhi* using organic solvents (petroleum ether, ethyl acetate, and methanol [80].

Clitoria ternatea seeds were used to extract a single protein (finotin). Finotin, a protein, has a wide and strong inhibitory impact on the development of a variety of significant fungal plant diseases (*Rhizoctonia solani, Fusarium solani, Colletotrichum*

lindemuthianum, Lasiodiplodia theobromae, Pyricularia grisea, Bipolarisoryzae and *Colletotrichum gloeosporioides*). It also prevented the pathogen *Xanthomonas axonopodispv*, which causes common bean bacterial blight. Finotin is also an effective inhibitor of the bean *bruchids Zabrotessub fasciatus* and *Acanthoscelides obtectus* [81].

Clitoria ternatea leaves extract has demonstrated potential inhibitory effects against certain microorganisms, including *Str. pyogenes, Shigellaflexneri*, and *Klebsiella sp*. Gram-positive bacteria are known to cause food poisoning and a host of other illnesses in both people and animals, therefore, this inhibitory function is especially noteworthy. Anxiolytic and antidepressant properties of the extract of the plant parts (bark, flowers, leaves, and seeds) have a variety of effects on the rat or mouse CNS. Numerous investigations on the *Clitoria ternatea* plant have examined the seed extracts' insecticidal and antibacterial qualities, which may be therapeutic in nature, especially as antioxidants [82].

Research on the antibacterial qualities of methanol, petroleum ether, and ethyl acetate extracts from *C. ternatea* leaves revealed that the methanol extract had stronger antibacterial activity than the other two solvent extracts, as well as a more potent inhibitory activity effect. Higher antibacterial activity against a variety of fish infections was demonstrated by *Clitoria ternatea* extracts prepared with ethyl acetone, acetate, ethanol, and petroleum ether compared to those prepared with water [83].

Flower extracts were tested against a range of urinary and intestinal infections that were identified from patients using flowers. The findings demonstrated that whereas aqueous, methanol, and chloroform extracts were effective against *Typhimurium, Klebsiella pneumonia, Pseudomonas aureginosa,* and *E. coli* of uropathogenic, enteropathogenic, and enterotoxigenic germs, hexane extracts and petroleum ether had no effect when compared to standard antibiotics. They developed a procedure for inducing many shoots by cultivating *C. ternatea* nodes and using callogenesis and organogenesis. [84].

The development of a protocol aimed at achieving rapid clonal propagation of CT involved tissue culture of embryo explants in vitro using callogenesis and

organogenesis. Additionally, an antibacterial study was conducted on the ethanolic extract of in vitro raised plant and callus mass against *Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli,* and *Klebsiella pneumonia* [85].

Clitoria ternatea tissue-cultured and wild plant was examined for their antibacterial qualities, and it was discovered that the former showed antibacterial action against specific pathogenic bacteria. [86]

2.13.3. Antiparasitic and Insecticidal Properties:

The Indian earthworm (*Pheritima posthuma)* is paralysed in 15-20 minutes and dies in 28-30 minutes after being exposed to an ethanolic extract of *Clitoria ternatea* (100 mg/ml). Adult Indian earthworms *Pheretima posthuma* were used to test the anti- helmintic activity of ethanolic extracts of *Clitoria ternatea* flowers, leaves, stems, and roots. *Clitoria ternatea* roots require less time to paralyze and kill earthworms [87].

The roots were also extracted in solvents like petroleum ether, chloroform, ethyl acetate, and methanol, and the extracts were tested for anti-helmintic efficacy. The methanol extract of *Clitoria ternatea* root was found to be the most powerful. The anti-helmintic activity of aqueous and ethanolic extracts of *Clitoria ternatea* leaves against *Eisenia foetida* was tested in vitro at three distinct doses (100, 50, and 25 mg/ml). The researchers wanted to know when the worms were paralysed and when they died. When compared to the reference medication, levamisole (0.55 mg/ml), both the ethanolic and aqueous extracts exhibited extremely substantial anti-helmintic activity at 100 mg/ml. The time of paralysis and death in the aqueous extract was 18 1.57 minutes and 53.33 0.33 minutes, respectively, whereas the time of paralysis and death in the ethanolic extract was 12.33 0.80 minutes and 32.33 0.71 minutes [88].

The methanolic extract of *Clitoria ternatea* leaves was found to have anthelmintic activity, whereas the ethanolic extract showed no such activity in adult Indian earthworm Pheritimaposthuma, which is anatomically and physiologically similar to the intestinal roundworm parasite that infects humans [89].

Clitoria ternatea was tested for mosquito larvicidal efficacy against three main mosquito vectors: *Aedes aegypti*, *Culex quinque fasciatus*, and Anopheles stephensi. The seed extract from *Clitoria ternatea* leaves, roots, flowers, and seeds was effective against the larvae of all three species, with LC50 values of 65.2, 154.5, and 54.4 ppmfor A, B, and C, respectively. *A. Stephensi, A. Stephensi, A. Stephensi, A. Stephen C. aegypti*, and *C. aegypti quinquefasciatus* and *quinquefasciatus*, respectively. *Clitoria ternatea*, out of the three plant species examined, has the most promising mosquito larvicidal efficacy [90].

2.13.4. Anti-Cancer Property:

Using the trypan blue dye exclusion technique, the in vitro cytotoxic impact of petroleum ether and ethanolic floral extracts (10, 50, 100, 200, 500 g/ml) of *Clitoria ternatea* was investigated. Both extracts had a strong dose-dependent cytotoxic effect on cells. In the case of petroleum ether extract, a concentration of 10 g/ml resulted in and 8 percent decrease in cell count, while a concentration of 500 g/ml resulted in a 100 percent loss in cell count. In the instance of ethanolic extract, the 10g/ml concentration resulted in a 1.33 percent decrease in cell count, while the 500g/ml concentration resulted in an 80 percent reduction in cell count [91].

On six types of normal and cancer-origin cell lines, the cytotoxicity of aqueous and methanol extracts of *Clitoria ternatea* flowers was tested. Human ovarian cancer cell line (Caov-3), human cervical cancer cell line (Hela), human liver cancer cell line (HepG2), and human foreskin fibroblast cell line were among them (Hs27). Colorimetric MTT (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide) assays were used to assess the extracts' anti-proliferative effects across time periods of 24, 48, and 72 hours. The water derived from *Clitoria ternatea* demonstrated a significant (p0.05) inhibitory impact on MCF-7, with an IC50 value of 175.35 g/ml [92].

2.13.5. Antidiabetic Properties:

Methanol, water, petroleum ether, and chloroform extract of *Clitoria ternatea* leaves were tested for acute and subacute hypoglycemic effects in Streptozotocin-induced diabetic rats. In Streptozotocin-induced diabetic rats, *Clitoria ternatea* extract (200 and 400 mg/kg) substantially decreased blood glucose levels. 400 mg/kg had a substantial hypoglycemic impact; whereas 200 mg/kg reduced glucose levels but not

as much as 400 mg/kg. The acute impact of the methanol extract indicated that 200 and 400 mg/kg had a very comparable effect, although 200 mg/kg exhibited a fine reduction inblood glucose level at the first stage of 30 minutes. The subacute activity revealed that when using the extract for a long time, a dosage of 200 mg/kg is significantly more effective in controlling blood glucose levels than a dose of 400 mg/kg [93].

In alloxan-induced diabetic rats, the hypoglycemic effects of methanol extract of *Clitoria ternatea* leaves (200 and 400 mg/kg) were studied. Twelve hours after treatment, the extract of *Clitoria ternatea* substantially (P0.001) decreased blood glucose levels in alloxan-induced diabetic rats. In alloxan-induced diabetes in rats, the hypoglycemic effects of an aqueous extract of *Clitoria ternatea* leaves and flowers (50- 500mg/kg) were studied. The aqueous extracts of CT leaves and flowers (400 mg/kg bw) decreased serum glucose, glycosylated haemoglobin, and the activities of the gluconeogenic enzyme glucose-6-phosphatase, but increased serum insulin, liver and skeletal muscle glycogen, and the activity of the glycolytic enzyme glucokinase. In all of the biochemical assays, the leaf extract-treated rats had virtually the same profile as the floral extract-treated rats [94].

2.13.6. Effect on Central Nervous System:

Clitoria ternatea seeds and leaves have long been used as a brain tonic and are said to improve memory and intellect. *Clitoria ternatea's* activity in Alzheimer's diseasewas investigated in order to determine its effectiveness and to discover the main bioactive component responsible for the activity. The findings revealed that *Clitoria ternatea* aqueous extract was helpful in Alzheimer's disease via a variety of methods. The separated chemicals may serve as a starting point for discovering novel derivatives that could be used to improve memory [95].

C. ternatea is widely used for a variety of central nervous system (CNS) benefits, including memory improvement. The anxiolytic, antidepressant, and CNS-depressant effects were also assessed and compared in CT plant. The elevated plus-maze (EPM) and step-down models were used to assess the no otropic activity of each plant's aqueous methanol extract. EPM, Porsolts swim despair, and actophotometer models were used to assess anxiolytic, antidepressant, and CNS-depressant research. At 200

and 100 mg/kg, respectively, *Clitoria ternatea* extract (CTE) exhibited maximal memory-enhancing and anxiolytic effects (p0.001). *Clitoria ternatea* extract (CTE) was exhibited an antidepressant effect (p0.05) [96].

2.13.7. Gastrointestinal Property:

In various experimentally generated ulcer models in rats, the antiulcer potential of aqueous and ethanolic extracts of *Clitoria ternatea* was investigated. Pylorus ligation and indomethacin-caused stomach ulcers in rats were studied using ethanolic extract (200 and 400 mg/kg) and aqueous extract (200 and 400 mg/kg) of the entire plant. Following ulcer induction, several parameters such as volume of stomach acid secretion, pH, total acidity, ulcer index, and antioxidant parameters were measured and compared between extracts, standard, and vehicle control groups. In pylorus ligation and indomethacin-induced ulceration, the high dosage of alcoholic extract exhibited substantial antiulcer action [97].

2.13.8. Hypo-Lipidemic Property:

Clitoria ternatea has an anti-hyperlipidemic action. Rats with artificially induced hyperlipidemia were examined. The poloxamer 407-induced acutehyperlipidemia and diet-induced hyperlipidemia models were utilized for study. Oral treatment of a hydroalcoholic extract of *Clitoria ternatea* roots and seeds reduced blood total cholesterol, triglycerides, very low-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels significantly (p 0.05). In diet-induced hyperlipidemic rats, the atherogenic index and HDL/LDL ratio were likewise corrected following therapy. The effects of atorvastatin (50 mg/kg, po) and gemfibrozil (50 mg/kg, po) were compared [98].

2.13.9. Anti-histaminic and Asthmatic Property:

Milk-induced leucocytosis and eosinophilia in mice, egg albumin-induced mast cell degranulation in rats, and passive cutaneous anaphylaxis in rats were used to test the antiasthmatic efficacy of an ethanol extract of *Clitoria ternatea* root (ECTR) at dosages (100-150 mg/kg ip). The findings revealed that ECTR reduces milk-induced leucocytosis and eosinophilia in mice, protects against egg albumin-induced mast cell degranulations in rats, and inhibits the area of blue dye leaking in passive cutaneous anaphylaxis in rats [99].

In wister rats, the antiasthmatic effect of an ethanol extract of *Clitoria ternatea* roots was tested in bronchospasm produced by histamine aerosol. *Clitoria ternatea* ethanolic extract (400 mg/kg, po) protected rats against histamine-induced bronchoconstriction by 47.45 percent. The findings revealed that the aqueous extract of *C. tenatea* not only possesses bronchodilating properties, but also reduces bronchial hyperreactivity by reducing inflammatory cell infiltration in the airway and inhibiting the release of histamine-like mediators from mast cells by stabilising them [100]**.**

2.13.10. Anti-acne properties:

Acne is a common skin condition that can cause difficulties during adolescence and persist into adulthood. Traditionally, CT plant extract has been used to be very successful in treating acne and lowering inflammation in a number of skin regions due to availability of vitamins, minerals, flavonoids, and hormones. Additionally, it possesses moisturizing properties that help protect the skin from excessive dryness, which can be detrimental to acne-prone skin. Most importantly, it doesn't cause skin irritation and helps extract to absorb deeply into the skin, increasing its efficacy. This is because of its amazing moisturising qualities and the fact that it contains organic vitamins like A and C. It increases the skin's capacity to hold onto moisture, helps eliminate dead skin cells, and encourages the synthesis of collagen and elastin fibres. Thus, degenerative skin changes are effectively reversed and the skin becomes more elastic and less wrinkled [101]. To find out how CT plant extract affect skin sensitivity, more research is necessary.

2.13.11. UV Protection Property:

The anti-oxidant protein metallothionein is essential to the skin because it scavenges hydroxyl radicals, which keeps the skin's glutathione peroxidase and superoxide dismutase from becoming inhibited which is available in CT also. In doing so, it effectively lowers the synthesis and release of epidrmal keratinocyte-derived immunosuppressive cytokines, such as interleukin-10. As a result, it helps avoid the suppression of delayed-type hypersensitivity, a kind of immunological response, caused by UV radiation [102].

2.13.12. Wound Healing properties:

Mannose-6-phosphate is a chemical found in the mucilaginous plant extract of *Clitoria ternatea* that is thought to be the active ingredient that promotes wound healing. Tannic acid and a particular polysaccharide are potential key components in facilitating the healing of wounds. Wound healing is an essential mechanism that aids in the restoration of damaged tissues within the human body. To evaluate the effectiveness of *C. ternatea*, similar to other medicinal plants, parameters such as collagen thickness and the presence of fibroblasts can be examined. Because it may reduce the risk of ulcers and speed healing from a variety of dermal injuries, such as pressure sores, burns, surgical wounds, inflammation, frostbite, infections, herpes ulcers, chronic wounds, and diabetic foot ulcers, the use of *Clitoria ternatea* may have positive effects on wound healing and prevention [103].

2.14. Objective of Present Research Study:

After a thorough study of multiple published research articles and books, it was found that *Clitoria ternatea* is an important ayurvedic medicinal and therapeautic herb. Various research studies have mainly been done for medicinal and phamacological aspects of CT either taking one part of the plant or taking plant as a whole. However, it was observed that the comparative study related to qualitative and quanatitative estimation of phytochemicals, antioxidants and enzymes of each parts of CT plant are still lacking.

The aim of the study was to compare the phytochemical contents present in leaves, roots, seeds, and flowers of *Clitoria ternatea* by assessments of different analytical assays. Such analytical studies will help in revealing the maximum active ingredients present in different parts of the plant. The last objective was to formulate biogel from blue flowers of CT which was furthers studies for toxicity and antimicrobial activity.

Therefore, the following objectives were selected for the study:

i. Qualitative test of alkaloids, carbohydrates, glycosides, flavonoids, phenols, Tannins, Amino acids and Proteins, saponin, Sterols, terpenoids, Quinones and Oxalates and Resins.

ii. Quantitative estimation of total carbohydrates, total phenols, flavonoids, tannins, and anthocyanin.

iii. Evaluation of antioxidant activities of *C. ternatea* by DPPH (diphenyl picryl hydazyl) assay, FRAP assay (ferric reducing antioxidant power), ABT assay, and superoxide radical scavenging activity.

iv. Evaluation of antioxidant enzymes v*iz.* Peroxidase, Superoxide dismutase, Guaiacol peroxidase and Polyphenol oxidase.

v. To formulate Biogel from the blue flower of *Clitoria ternatea.*

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CHAPTER - III

QUALITATIVE TEST OF ALKALOIDS, CARBOHYDRATES, GLYCOSIDES, FLAVONOIDS, PHENOLS, TANNINS, AMINO ACIDS AND PROTEINS, SAPONIN, STEROLS, TERPENOIDS, QUINONES AND OXALATES AND RESINS

CHAPTER-III

QUALITATIVE TEST OF ALKALOIDS, CARBOHYDRATES, GLYCOSIDES, FLAVONOIDS, PHENOLS, TANNINS, AMINO ACIDS AND PROTEINS, SAPONIN, STEROLS, TERPENOIDS, QUINONES AND OXALATES AND RESINS

3.1. Introduction:

Bioactives such as alkaloids, carbohydrates, flavonol, tannins, phenols, proteins, phlobatannin, saponins, triterpenoids, flavonoids, anthraquinone, glycosides, Stigmast-4-ene-3,6-dione, cardiac glycosides, volatile oils, anthocyanins, and steroids are all present in most of the medicinal plant. These possess the analgesic, anti-inflammatory, antipyretic, antidiabetic, anticancer, hypolipidemic, central nervous system, gastrointestinal tract, antiparasitic, antibacterial, and insecticidal qualities of herbal remedies [104]. Biologically active compounds or secondary metabolites are used as traditional, Ayurvedic, homeopathic, and Chinese herbal remedies, and also influence modern healthcare [105].

In this chapter, the first objective of the study has been taken to screen out secondary metabolites from root. leaf, flower and seed of *Clitoria ternatea,* assessed by qualitative method.

3.2. Materials and Methods:

Effective solvents, like aqueous ethanol, were employed to extract the phytochemicals from CT plant. The many research studies found that maximum extraction of phytochemicals are depends upon its solubility of phytochemicals in the organic solvents. Anthocyanin and other complex compounds are less or difficult to dissolved in water and others organic solvents like benzene, petroleum ether, methanol etc. It is observed that the maximum polarity of the solvent shows maximum extraction like ethanol. Since ethanol was shown to be the most potent extract, aqueous ethanol (60%) was chosen as the extraction method's solvent in the current research investigation. The next most effective extracts were found to be those containing methanol, chloroform, and petroleum ether.

3.2.1. Collection of Plant Sample:

From the nursery of the Horticulture Wing of the Central Public Works Department, located in Mehrauli, Delhi, the healthy and diseases free plant parts of *Clitoria ternatea* were sourced. All analytical work was performed in the well-developed laboratory of the National Institute of Food Technology Enterprises and Management (NIFTEM) at Sonipat, Haryana. The blue variety blooming plant's roots, leaves, seeds, and flowers were taken for further analysis as it was easily available than the white and pink genotype.

A. ROOT B. SEED C. LEAF D. FLOWER

Figure 3.1. Different Parts of C. *tertnea* Plant

3.2.2. Extracts Preparation:

From the nursery, the mature, robust, and fresh *Clitoria ternatea* leaves, roots, seeds, and flowers were gathered. Five minutes were spent cleaning them in fresh water. After that, distilled water was used to rinse it. All parts such as leaves, roots, seeds, and flowers were crushed into small pieces. They were stored separately in bowls.

The solvent used to prepare the extract was ethanol; one gram of fresh material was dissolved in one hundred milliliters of a 60% ethanol solution [106]. After being kept at room temperature for three hours, the flasks were put in a rotary shaker with a speed setting of 100 rpm. For the qualitative test of secondary metabolites, samples were treated with charcoal powder and left for 10 minutes for removing colour of the solvents. Extracts were then centrifuged separately in ethanol at 5000 rpm for 15 minutes. After that samples were kept in the refrigerator at 4^0C.

3.2.3. Qualitative Screening of Secondary Metabolites:

The presence of several secondary metabolites, such as oxalate, alkaloids, glycosides, flavonoids, proteins, saponins, sterols, quinones, terpenoids, phenols, and tannins, was analized thrice in each extracted sample with the opted methods of Harbone JB. (1998) method [107] which are given below. All tests were performed in the triplicate and selected best result, according to colour intensity of product. And the all chemicals were used in Analytical grade and distilled water was used as double distilled.

3.2.3.1. Wagner's Test for Alkaloids:

Natural organic substances known as alkaloids have one or more nitrogen atoms that are attached to a heterocyclic ring in their basic form. In natural environments, alkaloids—many of which are weak bases with oxygen in their structures—usually crystallize as colorless substances.

Method:

The most popular test to determine if alkaloids are present or not in the partially purified fractions is Wagner's test. A 0.5 ml plant sample was added to 2 drops of diluted hydrochloric acid (HCl), 4–5 drops of Wagner's reagent (2g of potassium iodide and 1.27g of iodine taken in 100 ml of distilled water), mixed, and then filtered. The development of a reddish-brown precipitate using Wagner's reagents shows the presence of alkaloids in the sample. The production of colorful precipitate was noticed after testing various alkaloid reagents on the filtrate.

3.2.3.2. Molisch's Test for Carbohydrates:

In Molisch's test, the presence of strong sulfuric acid (H_2SO_4) causes the carbohydrate to dehydrate, forming an aldehyde (-CHO group) as a result. This aldehyde experiences condensation with the reagent, naphthol, and during the reaction, a purple or reddish-purple complex is created.

Method:

2 ml of the extracted sample treated with 4-5 drops of Molisch's reagent (3.75 grams of 1α naphthol in 25 ml of 99% ethanol). Then, 2 ml conc. H_2SO_4 was added inside the wall of the test tube. The sample was left for 2-3 minutes. A dull red or purple

formation in the midpoint of the two layers showed an affirmative test that sugar is present.

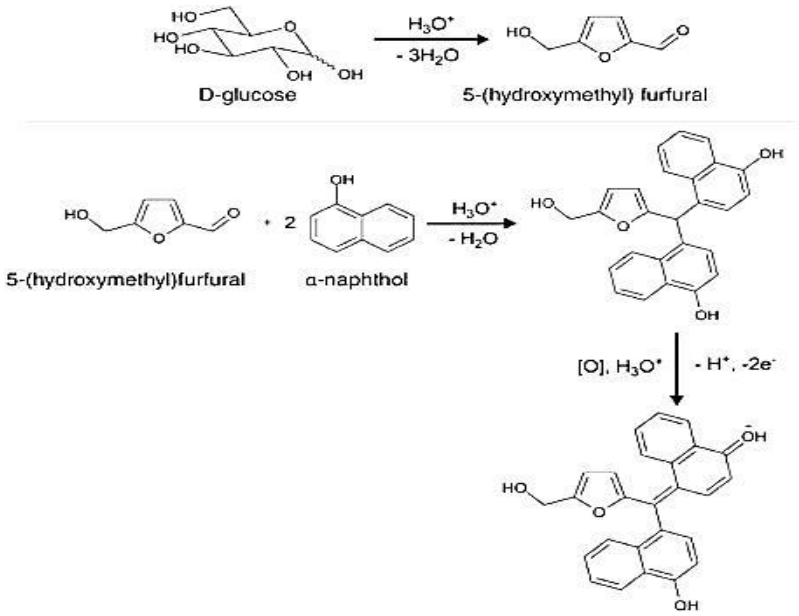


Figure 3.2: Reaction of carbohydrates with Molisch reagent indicates the formation of complex coloured compounds.

3.2.3.3. Keller Kelliani's Test for Glycosides:

Glycosides play key roles in living organisms. These are stored in plant and plants' parts form an inactive phase, for the activation of glycosides requires the enzyme hydrolysis. Glycosides are categorized based on linked molecules such as O (O-glycoside), N (glycoaylamine), S (thioglycoside), and C (C-glycoside) glycosidic linkages.

Method:

4 ml of glacial acetic acid, a few drops of 2.0% ferric chloride ($FeCl_3$), and roughly 10 ml of the extracts were put to a test tube. 1 ml of strong sulfuric acid (H_2SO_4) was cautiously added to the mixture. The presence of cardiac steroidal glycosides observed through a brown ring appeared on test tube interfaces.

3.2.3.4. Alkaline Reagent Test for Flavonoids:

Flavonoids are plant-synthesized secondary metabolites. The flavonoid's chemical structure is made up of a 15-carbon skeleton and two benzene rings joined by a connecting chain of three carbons. The degree of oxidation and unsaturation of the

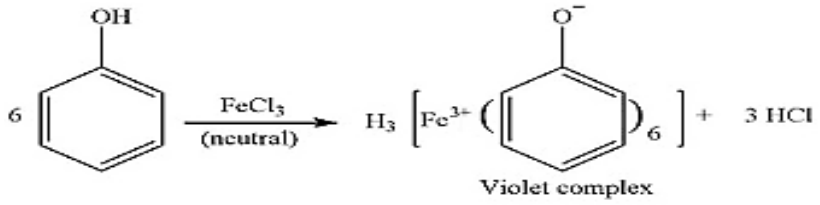
connecting chain allows for the classification of flavonoids into a variety of classes, such as isoflavonoids, chalcones, anthocyanidins, flavonols, flavones, and flavan-3-ol. Sometimes may work as physiological regulators, chemical messengers, and inhibitors of cell cycles.

Method:

A few drops of a 20% sodium hydroxide solution were added to the CT plant parts extracts, which amounted to 2.0 ml. The production of a bright yellow tint that turns colorless when diluted hydrochloric acid was added demonstrated the presence of flavonoids.

3.2.3.5. Ferric chloride Test for Phenols:

Phenols are more acidic as compared to alcohols. The simplest form is phenol (C_6H_5OH). Phenols are synthesized industrially and produced by microorganisms as well as plant. Phenols are reactive species just before oxidation. The phenolic functional group can be detected using a number of procedures, such as the ferric chloride test, the Libermann's test, the Litmus test, and the phthalein dye test.



Method:

The addition of 5% aqueous ferric chloride solution with a small amount of CT plant parts extract, which had a vivid blue or black color, resulted in a positive phenol test.

3.2.3.6. Precipitate Test for Tannins:

Much of the plant material is likely composed of tannins, or chemicals that mimic tannins in nature. There are two primary classifications for these polyphenolic compounds: hydrolyzable and condensed. Gallic acids or hexa hydroxydiphenic acids esterify glucose most of the time, although not always, in hydrosable tannins. Condensed tannins are not easily broken down into simpler building elements since the majority of them are flavonols, which are most likely polymers of flavan-3-ol (catechin).

Method:

Given that most condensed Tannins can be detected by the red precipitate that appears when 2 ml of CT plant parts extract are boiled with 1 ml of 1% hydrochloric acid solution. Next, 0.5 gm of the powdered components were combined in a 250 ml conical flask along with 75 ml of distilled water. After gently boiling the flask for thirty minutes, centrifuging it for twenty minutes at 2000 rpm, and pouring the supernatant into a 100 ml volumetric flask, the required volume was added. To confirm the presence of tannins, place 2.0 ml of the sample extract in tubes and mix it with 1.0 ml of hydrochloric acid to create a crimson precipitate. The same resulted in confirmation of the presence of tannins.

3.2.3.7. Ninhydrin Test for Amino acids and Proteins:

It is a basic test of all amino acids. Ninhydrin is a powerful oxidizing reagent, the reaction between an amino functional group and produced blue colour, in the case of proline has an imino group and formed bright yellow colour.

Method:

Acid Ninhydrin: Warm 1.25 grams with stirring until dissolved in 30 ml of glacial acetic acid and 20 ml of 6M phosphoric acid. It was stored at 4^0C and used within 24 hours. Homogenize 0.5 g of plant material in 10 mL of 3% sulphosalicylic acid or phosphoric buffer to obtain the extract. Put Whatman filter sheets through the homogenate to filter it. Add 2 mL of glacial acetic acid to 2 mL of filtrate in the test tubes was treated for one to two minutes in a warm water bath with 2 mL of ninhydrin solution (1% ninhydrin solution in acetone), which caused test tubes to become purple and show the presence of proteins and amino acids.

3.2.3.8. Foam Test for Saponins:

Saponins are complex glycoside compounds that resemble steroids. Use a foam test to find out if saponins are present or not. The method of modest alteration was applied to identify saponin.

Method:

A small amount of extract about 2.0 mL was diluted in 80 mL of distilled water and thoroughly mixed before being shaken continuously for 15 minutes . When heated,

foam started to form, indicating the presence of saponins. As a screening test, the ability of saponins to create foam in aqueous solutions was applied to the plant sample.

3.2.3.9. Liebermann-Burchard Test for Sterols:

Sterols, sometimes referred to as phytosterols, are based on the cyclopentane ring structure and are made from triterpenes. using the Liebermann-Burchard technique, phytosterol secondary metabolites from plant extract can be found.

Method:

1 ml of concentrated sulfuric acid (H_2SO_4), 2 mL of chloroform ($CHCl_3$), 1 mL of acetic anhydride, and 1 mL of plant extract were all mixed together fully through the test tube's side. A dark pink or brown-red colour ring that forms at the intersection of two layer is indicative of the presence of steroids. Sterol is present when a reddish-pink colour begins to appear.

3.2.3.10. Salkowki's test for Terpenoids:

In order to protect itself from biotic and abiotic stress, plants need terpenoids. They are treated as a signal molecule to attract insects for pollination. Terpenes are abundant in higher plants, citrus, conifer, eucalyptus, and aromatic medicinal plants.

Method:

2 ml of each extract, 1 ml of chloroform, and 2-3 drops of concentrated H_2SO_4 were added. A reddish-brown precipitate indicated the presence of terpenoids.

3.2.3.11. Quinones Test:

The secondary metabolites that are present in plant stems are called quinones. It is a white, crystalline alkaloid with a bitter flavour that has antipyretic, antimalarial, analgesic, and anti-inflammatory properties.

Method:

A few drops of extract and 1ml of concentrated HCl were added and yellow colour of the precipitate was found.

3.2.3.12. Oxalate Test:

The high content of oxalic acid is found in the root & and leaf of plant like spinaches, buckwheat, and rhubarb where it is synthesized by incomplete oxidation of saccharides.

Method:

4 drops of glacial acetic acid were applied to 3mL of extract sample in a test tube. The green-black hue indicates that *Clitoria ternatea* plant parts contain oxalates.

3.2.3.13. Resins Test:

Resins are important secondary metabolites produced when an injury occurs to the plant to give mechanical support and to save from environmental damage. It also protects the plant from insects and pathogens. It can be produced through the special resin cell available in bark, flower stems, etc.

Method:

Extract sample (2ml) added with 4 ml of H_2O. Turbidity shows the confirmation of resin in the sample

3.3. Result:

After being tested in the laboratory with the methods cited above, the results were noted based on the intensity of colour of the product i.e absent (colourless), light, medium and bright /deep colour. The findings were tabulated, which is at Table 3.1 and Figure 3.1 below:

Secondary Metabolites in CT Roots: The findings indicated (Table 3.1) that the CT plant's root includes a number of physiologically significant low molecular weight secondary metabolites. The root extract showed deep purple ring indicated maximum availity of carbohydrates (+++), but less availabilities (+) of amino acids, proteins, saponins, and terponoids. Several secondary metabolites, such as quinines, oxalates, phenols, glycosides, tannins, sterols, and resins, were absent (-) from the root of *C. ternatea*, as indicated no colour change.

Table 3.1. Availability of Secondary Metabolites in the Plant Parts of *Clitoria ternatea*

Secondary Metabolites	Observations	Intensity of Colour in Plant Parts*			
		Roots	**Flowers**	**Leaves**	**Seeds**
Alkaloids	Reddish-brown	-	+++	-	++
Carbohydrates	Purple ring	+++	+++	+++	+++
Glycosides	Brown ring	-	+	+	++
Flavonoids	Bright yellow convert to colourless	+	+++	++	++
Phenols	Blackish Blue	-	+++	+++	++
Tannins	Red ppt.	-	+++	++	+++
Amino acids and Proteins	Purple	+	+	+	+++
Saponin	Foam formation	+	-	-	-
Sterols	Dark pink	-	+	++	+++
Terpenoids	Red –brown ppt.	+	+++	+	+++
Quinones	Yellow ppt.	-	+++	++	+
Oxalates	Green-black	-	+	-	-
Resins	Turbidity shown	-	++	+	++

*[Absent/Colourless (-), Light colour (+), Medium (++), Bright /Deep Colour(+++)]

Secondary Metabolites in CT Leaves: The results showed (Table 3.1) that plant leaves contain strongly present (+++) numerous secondary metabolites such as carbohydrates and phenols, whereas more present (++) in flavonoids, tannins, Sterols, and quinines. However, less present (+) in glycosides, amino acids and proteins, terpenoids, and resins. Some secondary metabolites such as alkaloids, Saponin, and Oxalates were absent (-) in the leaves of *C. ternatea.*

Secondary Metabolites in CT Flowers: Through the research study, the result shows (Table 3.1) that secondary metabolites of CT flowers like alkaloids, carbohydrates, flavonoids, phenols, tannins, terponoids, and quinones were highly present (+++). Other metabolites such as resins was more present (++) in the CT flowers. The less presence (+) of metabolites shown in CT flowers were oxalates, sterols, amino acids and proteins, and glycosides.

Secondary Metabolites in CT Seeds: The findings indicated (Table 3.1) that seeds of CT plant contained a highly presence (+++) of carbohydrates, Tannins, sterols, terpenoids, amino acid and proteins. Alkaloids, glycosides, flavonoids, phenols and resins were showed more present (++). Less presence (+) of quinones was found and oxalates was observed absent (-) in seeds of *Clitoria ternatea.*

3.4. Discussion:

CT flowers showed more alkaloids as compared to seeds but were absent in leaves and root tissue. It indicates that the CT plant has more capability to protect itself from pathogens and regulate their growth. The availability of rich alkaloids will help in pharmaceutical activities for the formation of drugs such as anti-malarial anticancer, antiasthma, analgesic, etc.

The main function of carbohydrates in plant is cellular metabolism and their abundance presence indicates plant defense against disease. Current research indicates that the leaves, roots, flower, and seed tissue of *Clitoria ternatea* plant contain significant amounts of carbohydrates.

Flavonoids are found in greater concentrations in the flowers of *Clitoria ternatea* in the current study than in the leaves, seeds, or roots. Here also, flavonoids are available in the whole tissues of the plant of *C. ternatea*. This natural source of flavonoids attracts the pharma industries.

Phenols are the most common type of secondary metabolite, and they comprise polymers like tannins and lignins as well as simple molecules with a single aromatic ring. In the present research study, the phenols are strongly present in leaves and flower tissue followed by seeds and absent in root of *Clitoria ternatea* plant.

Tannins did not play themselves in the cell as a primary antioxidant but donated hydrogen atoms or electrons to the phenol group. This activity is possible due to acting as oxidizable substrates and free radical scavengers to protect against pathogens. The tannins are strongly present in flowers and seeds and more present in leaves but absent in root tissue of the *Clitoria ternatea* plant. The results showed that CT plant is highly effective at scavenging free radical activity with the avaibility of tannins.

The fundamental components of the organism and the primary drivers of plant growth and development are proteins and amino acids. They are various precursors of the intermediate metabolites and amino acid metabolism. Compared to the roots, leaves, and flower tissue of *Clitoria ternatea*, amino acids and proteins are highly significant components that are concentrated in seeds. Plant sterol is considered an important dietary component for reducing bad cholesterol or LDL. It supports the management of blood cholesterol levels in the human body and lowers the risk of stroke. It was observed that the sterols were present in descending order in seeds, leaves, and flowers but absent in the root tissue of CT. Hence, CT plant extract can be used as one of the best Ayurvedic medicines to reduce bad cholesterol.

Terpenoids are recognised to mediate plant-insect interactions, including pollinators, predators, parasitoids, and herbivores. The result shoed that flowers and seeds of CT plant are also rich in Terpenoids whereas less presence in root and leaf.

Saponin, quinones, and resins provide the defense in plant against pathogens and diseases. The result indicated that the status of saponin is present in root tissue only and absent in leaves, flowers, and seeds of CT plant. Opposite to it, quinones are absent in root tissue but strongly present in flowers, more present in leaves, and present in seeds in CT plant.

The Oxalates are least present only in flower tissue and absent in leaves, roots, and seed tissue in *Clitoria ternatea* plant. The present results also indicated that more present resins in flowers and seeds but absent in root tissue of *Clitoria ternatea* plant. The result shows that CT plant have capacity to fight against pathogens and diseases.

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CHAPTER-IV

QUANTITATIVE ESTIMATION OF TOTAL CARBOHYDRATES, TOTAL PHENOLS, FLAVONOIDS, TANNINS AND ANTHOCYANIN

CHAPTER-IV

QUANTITATIVE ESTIMATION OF TOTAL CARBOHYDRATES, TOTAL PHENOLS, FLAVONOIDS, TANNINS AND ANTHOCYANIN

4.1. Introduction:

Both qualitative and quantitative analysis was used in phytochemical analysis. Qualitative analysis focuses on whether a phytochemical is present or absent, whereas quantitative analysis considers the amount or concentration of the phytochemical in the plant sample. In this study, Chapter III presents a qualitative analysis of the various secondary metabolites identified in the plant parts of *Clitoria ternatea*. The results of qualitative analysis of secondary metabolites were observed that only strongly present parameters will be go for the further analysis of quantitative estimation. Now, based on laboratory studies, a thorough quantitative estimation of the total amount of carbohydrates, anthocyanin, flavonoids, tannins, and phenols present in the root, leaves, flowers, and seed of *Clitoria ternatea* has been covered under this chapter.

Accurate quantitative estimation of bioactive compounds in *C. ternatea* is essential for assessing its quality and determining its potential therapeutic applications. The quantity of nutrients, the amount of light, and the temperature may have an impact on how these compounds are synthesised and concentrated within the plant. Genetic factors, including the plant's genotype and the presence of specific genes, can also influence the levels of these compounds. Additionally, post-harvest handling and processing techniques can impact the levels of bioactive compounds in *C. ternatea*. Factors such as drying methods, storage conditions, extraction techniques, and instrument sensitivity can also affect the stability and preservation of those compounds. Therefore, considering of these factors is necessary to ensure the optimal levels of bioactive compounds in CT extracts.

The amounts of flavonoids, tannins, anthocyanin, total phenols, and total carbohydrates in *C. ternatea* extracts were measured by using different suitable methods which are detailed in this chapter. The results of all analysis data were

statistically analysed in triplicate, performed by the Microsoft Excel and also calculated the p value of mean. And further all data of results were checked for statistically significant exit or not at 5%.

4.2. Preparation of Extracts:

The extraction method of root leaf, flower and seed were followed as per method and procedure detailed at Para 3.2.2. of Chapter III.

4.3. Quantitative Estimation of Phytochemicals:

All samples were analyzed in triplicate. The all chemicals used for methods were of the analytical grade. The double distilled water was used for every analysis. The standard curve was plotted mg per mililiter concentration against absorbance by the linear graph. Afterthat the obtained results mg/ml were converted to mg/gm fresh weight of the sample which was obtained value from the standard curve. The equations of every analysis were expressed by R^2 which was mentioned in the result.

4.3.1. Estimation of Total Carbohydrates:

For the quantitative analysis of carbohydrates, following reagents were prepared in the laboratory. The estimation of carbohydrates of the CT plant parts were analysed through the Anthrone method as described by Yemm, E. W., & Willis, A. (1954) with slight modification [108].

Chemical Used:

- Anthrone Reagent: 200 mg of anthrone is dissolved in 100 ml of ice-cold conc H_2SO_4 (Freshly Preapared).
- Ethanol: 60% (For extract preparation)
- Sulphuric acid: Concentrate H_2SO_4
- HCl: 2.5 N HCl
- Sodium Carbonate: one pinch
- Glucose: mg/ml (for standard)

Procedure:

100 mg of the extract was weighed in a boiling tube. After that, the sample was hydrolyzed for three hours in a boiling water bath with 5.0 ml of 2.5N HCl and then allowed to cool at room temperature. Test tubes for use were placed in an ice bath.

Solid Na_2CO_3 was used to neutralize it until the effervescence stopped. The volume was made up to 100 mL and centrifuged for a quarter-hour at 10,000 rpm. The 0.5 and 1.0 ml aliquots were used for further analysis of the reaction mixture. Glucose (mg/ml) concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 ml were used as standard. By adding distilled water to all of the tubes, the volume was made up of 1.0 ml. After that, 4.0 ml of Anthrone reagent was added. The mixture was heated in a boiling water bath for 10 minutes. Then the mixture was cooled rapidly and measured dark green color at 630 nm. Blank was also made in the same way, except instead of a sample, distilled water was used. The standard was used as glucose and expressed as mg glucose/g fresh weight.

Result:

Anthrone reagent was used to determine carbohydrates and glucose was used as a standard within the tested plant extract. It was calculated glucose ($y = 0.806x + 0.046$; $R^2 = 0.9945$) using the standard curve equation. As per observartion, the finding of total carohydrates was noteworthy and are tabulated in Table 4.1 (Figure 4.1). According to the study, the maximum amount of carbohydrates found in *C. ternatea* leaves was 89.01 mgGlc/g fresh weights, compared to a total mean value of 76.75 mg Glc/g fresh weight followed by seeds, root and flowers. The data of table 4.1 was significantly higher (#P 0.0041) at 5%.

Table 4.1: Total Carbohydrates Content in *Clitoria ternatea*

Unit: (mg Glc/g Fresh weight)

Plant Parts of CT	**Total Carbohydrates**
Root	71.00±0.60
Leaves	**89.01±0.23**
Flowers	68.00±0.42
Seeds	79.30±0.87
Mean	76.75
C.D.	3.84
SE(m)	1.15
SE(d)	1.63
C.V.	2.60
p value	0.0041(< 5%)

Values are presented as mean±S.D,n=4. (Experiments were made as 3 parallel)

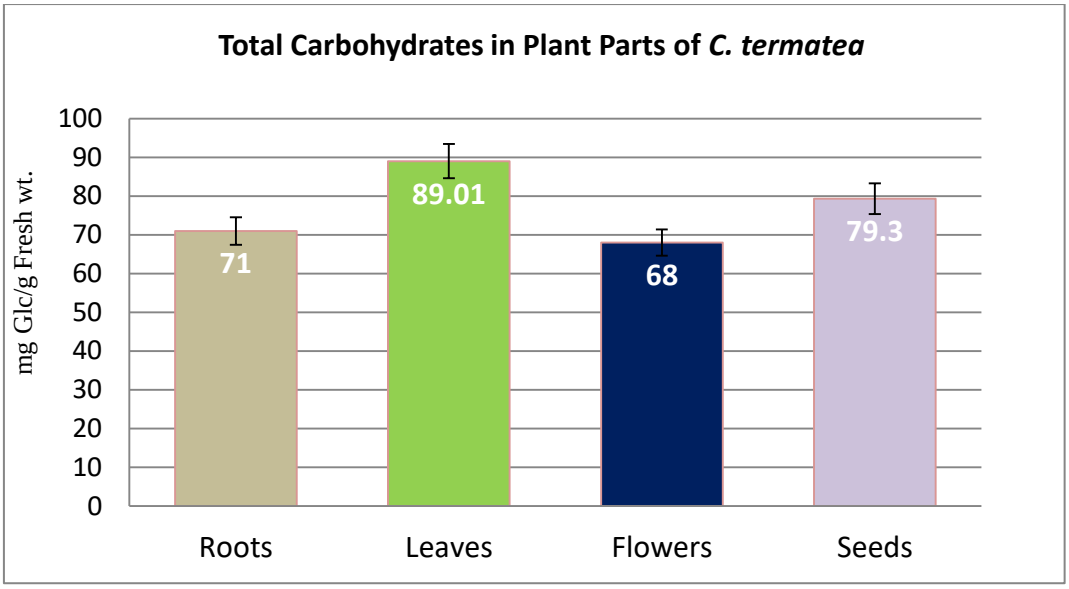

Figure 4.1: Total Carbohydrates present in *C. ternatea*.

4.3.2. Estimation of Total Phenol:

The method of Singleton and Rossi (1965) was opted for determination the total phenol content [109] in plant parts of CT:

Chemicals Used:

- Folin-Ciocalteu reagent (2N): Mix the reagent with an equivalent volume of water to dilute it.
- Na_2CO_3: 20% (w/v)
- Ethanol: 50%
- Gallic acid: mg/ml(Standard)

Procedure:

1g freshly isolated plant tissue samples of leaves, roots, flowers, and seeds were crushed in mortar-pestle in 2 ml of 50% ethanol and allowed to stand for 15 minutes. Centrifuge it at 8000 rpm for 15 minutes and supernatant was used for assay. All the samples were treated with 0.1 ml Folin-Ciocalteu (2N) reagent along with 2.5 ml of 0.2N Na_2CO_3 and allowed to stand at 30ºC for 30 minutes. Absorbance was read at 760 nm in a spectrophotometer. Gallic acid was used(mg/ml) as a reference and was measured in milligrams of gallic acid equivalents per gram of sample fresh weight (mg GAE/g Fwt).

Result:

Folin-Ciocalteu reagent was used to determine phenols and gallic acid was used as a standard within the tested plant extract. They were calculated in gallic acid equivalents ($y = 0.979x$; $R^2 = 0.996$) using the standard curve equation. The findings of total phenols showed that the total phenol evaluated under the control plant, and recorded total mean. i.e. 70.50 in C*litoria ternatea.* In the results, the highest mean value was 93.00±0.95mg GAE/g fresh wt. of the total phenol observed in flowers followed by 78.00±0.70mg GAE/g fresh wt. in seeds, 69.00±0.96mg mg GAE/g fresh wt. in leaves. The root was recorded lowest i.e. 42.00±0.38mg GAE/g fresh wt. (Table 4.2 and Figure 4.2). The results of total mean P value was 0.0049 showed that the phenol concentration was significantly higher at 5%.

Table 4.2: Total Phenols content present in *Clitoria ternatea*

Unit: (mg GAE/g Fresh wt.)

Plant parts of *C. ternatea*	**Total Phenols**
Roots	42.00±0.38
Leaves	69.00±0.96
Flowers	**93.00±0.95**
Seeds	78.00±0.70
Mean	70.50
C.D	1.98
SE (m)	0.59
SE (d)	0.85
C.V.	1.48
p Value	0.0049(<5%)

Values are presented as mean ±S.D, n=4.(Experiments were made as 3 parallel)

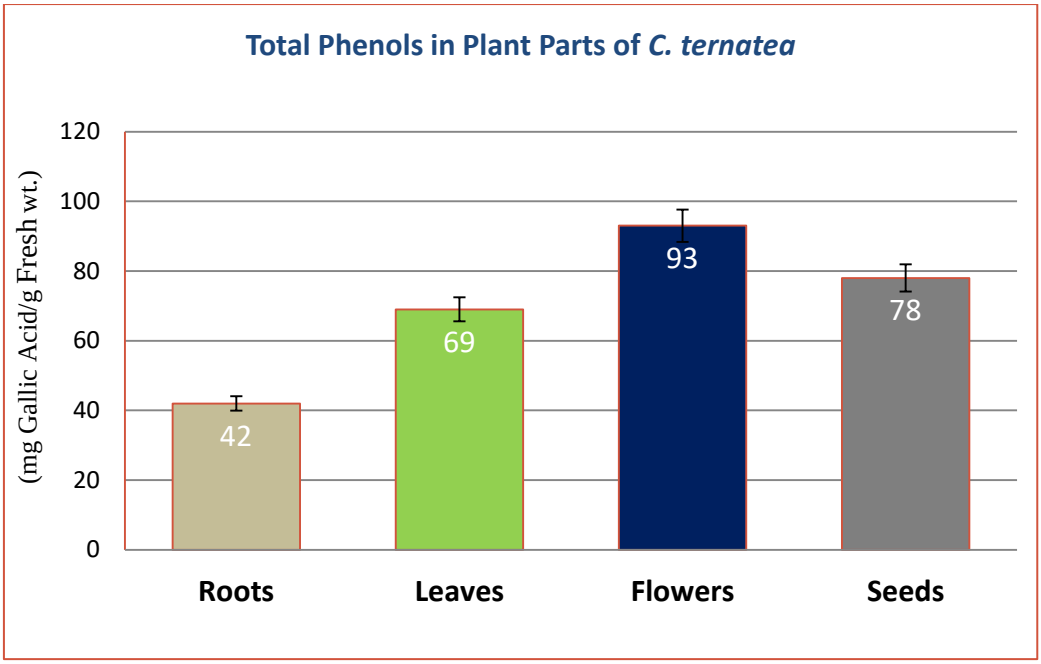

Figure 4.2: Total Phenol present in *C. ternatae*

4.3.3. Estimation of Total Flavonoids:

The total flavonoid was estimated through the method of Kumaran *et al*., (2005) with the following chemicals and laboratory technique [110]:

Chemicals Used:

- $NaNO_2$: 0.5 M
- Methanol: 30%
- $AlCl_3$. $6H_2O$: 0.3M
- Catechine: mg/ml(Standard)
- NaOH

Procedure:

The aluminum chloride test determines the total flavonoid content. In a test tube, the samples of a plant amounting to 0.3 ml were added to 0.15 ml of $NaNO_2$ (0.5 M), and 0.15 ml of $AlCl_3$. $6H_2O$ (0.3M) and 3.4 ml of 30% methanol. After 5 minutes, add 1 ml of NaOH. Then absorbance was read at 506 nm. The total flavonoids of the sample were calculated by the standard curve of catechine mg/ml.The total flavonoid content was expressed in catechine equivalents mg/g fresh wt.

Total flavonoid content mg/g = Sample(ml) from calibration curve X volume of extract
weight of plant(g)

Result:

The present status of flavonoids is showed significant (Table 4.3 and Figure 4.3). The total flavonoids were evaluated under the control plant and recorded the total mean. The results of flavonoid were presented on straight line of standard curve of the equation (**y** = 0.8995x + 0.0137, R^2 = 0.9953). The mean value of total flavonoids was observed at 273.75 in control plant of C*litoria ternatea.* The results showed that the CT flower was found highest mean value of 469±0.61catechine mg/g fresh wt., followed by 68.30±0.55 catechine mg/g fresh wt. in leaves, 181.0±1.30 catechine mg/g fresh wt. in seeds and minimum flavonoids were found in 177.00±0.68 catechine mg/g fresh wt.in roots. This shows that total flavonoid which are responsible for the colour, flavours, and fragrance, are present in abundant form in flowers of *Clitoria ternatea.* The p value (0.0027) of flavonoid results were obseved highly significant (<5%).

Table 4.3: Total Flavonoids content in *Clitoria ternatea*
(Catechine mg/g Fresh wt.)

Plant parts of *C. ternatea*	**Total Flavonoids**
Roots	177.16±1.01
Leaves	268.00±1.04
Flowers	**469.34±0.61**
Seeds	181.03±1.30
Mean	273.75
CD	3.76
SE (m)	1.16
SE (d)	1.67
C.V.	0.79
p value	0.0027 (<5%)

Values are presented as mean±S.D, n=4 (Experiments were made as 3 parallel)

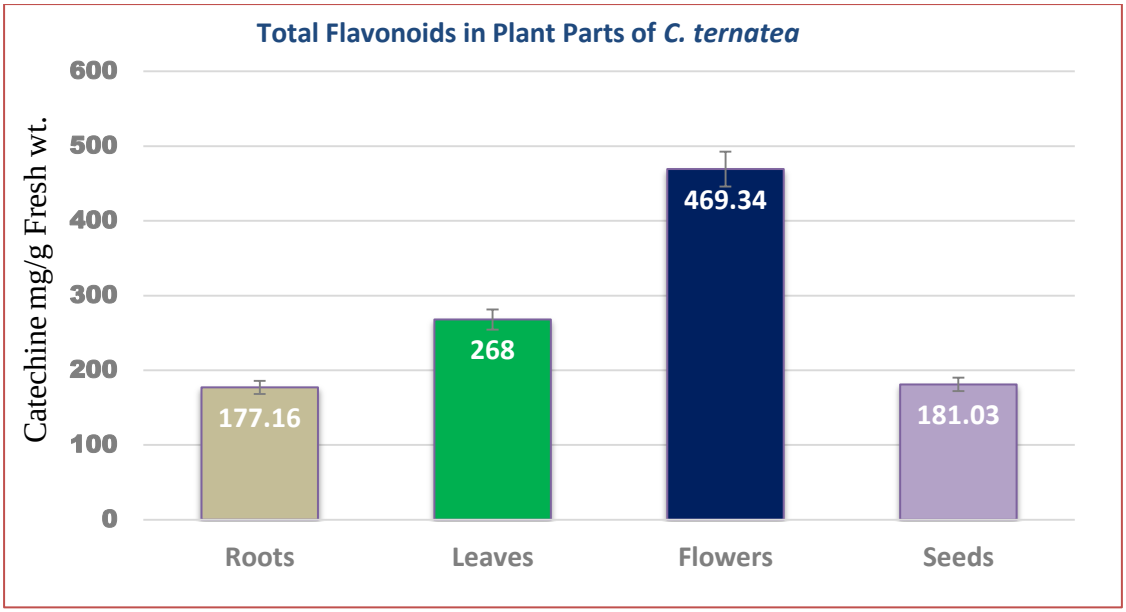

Figure 4.3: Total flavonoids present in *Clitoria ternatea*

4.3.4. Estimation of Total Tannin:

The determination of total tannin was done using Folin-Denis regent with the procedure adopted as under [111]:

Chemical Used:

- Folin-Denis reagent: Dissolved 100 g of sodium tungstate and 20 g of phosphomolybdic acid in 750 ml of distilled water in a beaker. And added 50 ml of phosphoric acid. Shake well, leave for two hours and made the volume 1L.
- Sodium carbonate
- Tannic acid: TEA(standard)

Procedure:

1ml sample extract of the 1: 100 weakening was mixed with 5ml Folin-Denis reagent. The mixture was continuously shaken and allowed to leave for three minutes. A 10 ml solution of sodium carbonate was mixed well. The sample was left for 2 hours. After that, the sample was centrifuged until all the elements were settled down at the bottom of the centrifuge tube. Only supernatant was used to measure OD at 700 nm. The same method was used to create a blank. A standard curve for tannic acid was produced for milligram equivalent tannic acid (mg TEA/g Fwt.) using several dilutions of the tannin solutions (1: 250, 1: 50, 1: 40, 1: 25, and 1: 10).

Table 4.4: Total Tannin content in *Clitoria ternatea*

Unit: (mg TAE/g Fresh weight)

Plant parts of *C. ternatea*	**Total Tannins**
Roots	29.67±1.76
Leaves	25.33±0.88
Flowers	**72.00±0.11**
Seeds	38.33±0.85
Mean	41.00
C.D	4.05
SE (m)	1.22
SE (d)	1.73
C.V.	5.04
p Value	0.018(<5%)

Values are presented as mean±S.D,n=4.(Experiments were made as 3 parallel)

Result:

According to Table 4.4 and Figure 4.4 of the current investigation, the total tannin content results were noteworthy. The results of total tannins (tannic acid equivalent) were plotted at straight line on the standard curve equation ($y = 0.82x - 0.004$, $R^2 = 0.9994$). The sample of each parts of CT were taken in triplicate. The total tannins content was evaluated under the control and recorded overall mean. The mean value of total tannin content was observed 41.00 in control plant of C*litoria ternatea.* In the present study, a flower had the highest mean value 72.00±0.11mg TAE/g fresh wt., of the tannins content followed by 38.00±0.45mg TAE/g fresh wt., in seeds, 29.67±1.76 mg TAE/g fresh wt., in roots and minimum was present in 25.33±0.88mg TAE/g fresh wt., in leaves. The results of total tannin were expressed higher significant (p= 0.018<5%).

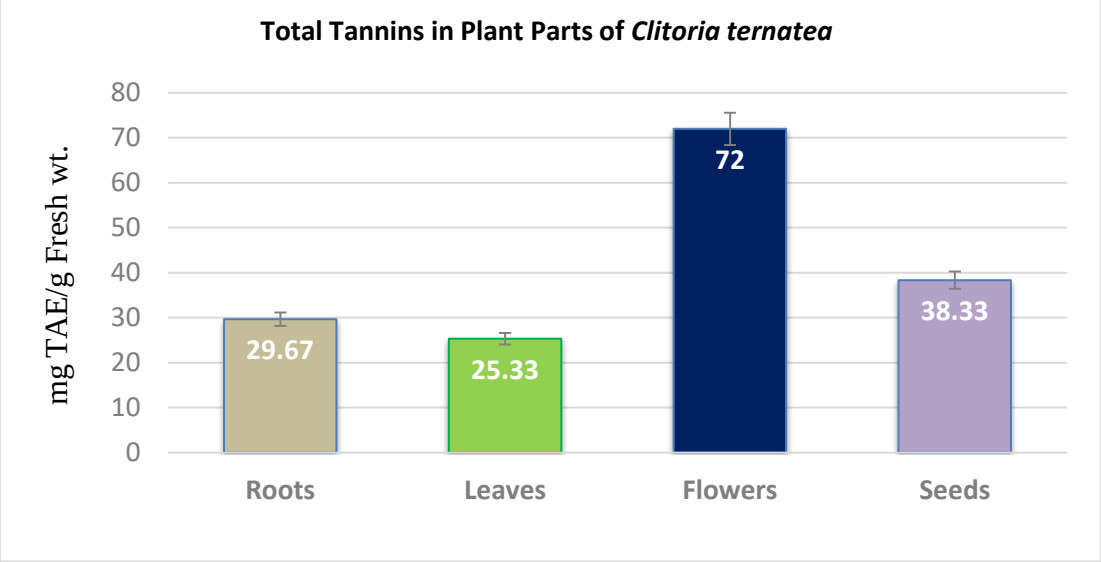

Figure 4.4: Total Tannins present in *Clitoria ternatea*

4.3.5. Estimation of Total Anthocyanins:

Determination of total anthocyanin pigments content in the plant samples (leaves, roots, flowers, and seeds) was estimated by the pH differential technique [112].

Chemicals Used:

- Sodium acetate (CH_3COONa) buffer: 0.4 M, pH 4.5
- Potassium Chloride (KCl) buffer: 0.025 M, pH 1.0
- Cyanidin-3-glucoside equivalents: mg CGE/ml (Standard)

Procedure:

Determination of total anthocyanin pigments content in the plant samples (leaves, roots, flowers, and seeds) by the pH differential technique. The anthocyanin pigment content was determined using this technique, in which two types of buffers included KCl buffer (0.025 M, pH 1.0) and sodium acetate (CH_3COONa) buffer (0.4 M, pH 4.5). The 1.0 ml aliquot of the ethanolic extract was mixed with 4.0 ml of Potassium chloride (KCl) buffer and 4.0 ml sodium acetate buffer and the test tube was incubated at 28 ˚C to 29 ˚C for 15 to 20 minutes approximately. The blank was used with deionized water. The absorbance was read at 510 nm and 700 nm. Anthocyanin was represented as milligrams of cyanidin-3-glucoside equivalents (CGE) per gram of fresh weight. The absorbance value was determined by using the equation:

Absorbance (A) = [(A520nm - A700nm) P^H 1.0 - (A520nm - A700nm) P^H 4.5]

Total anthocyanin mg/g = $\frac{A \times MW \times DF \times 1000}{(e \times l)}$

Where:

MW = Molecular weight (449.2 g/mol for cyanidin-3-glucoside)

DF = Dilution factor

e = Molar extinction coefficient (26900 molar extinction coefficient in L/mol/cm for cyanidin-3- glucoside)

l = path of length(1cm)

Result: The results of anthocyanins for standard used CGE mg/ml concentration were expressed in linear graph by the equation (y=0.226+0.014x, R^2 0.996) of *C. ternatea.* Table 4.5 and Figure 4.5 shows that the total mean value of total anthocyanins was observed 87.75 in the control which was significant. The results indicated that the flower had the highest value 178.00±0.37mg CGE/g fresh wt. of the anthocyanins content followed by 98.00±0.49mg CGE/g fresh wt., in leaves, 43.00±0.19 mg CGE/g fresh wt.in roots and 32.56±0.57 mg CGE/g fresh wt. in seeds, found lowest. The results of anthocyanins of each part of C T were higher significants (p 0.011 <5%).

Table 4.5 Total Anthocyanins present in *Clitoria ternatea*

Unit- (mg CGE/g Fresh wt.)

Plant parts of *C. ternatea*	**Total Anthocyanins**
Roots	43.5±0.76
Leaves	98.16±1.09
Flowers	**178.33±0.88**
Seeds	32.56±0.57
Mean	87.75
CD	2.73
SE (m)	0.83
SE (d)	1.17
C.V.	1.62
p Value	0.011(<5%)

Values are presented as mean ±S.D,n=4.(Experiments were made as 3 parallel)

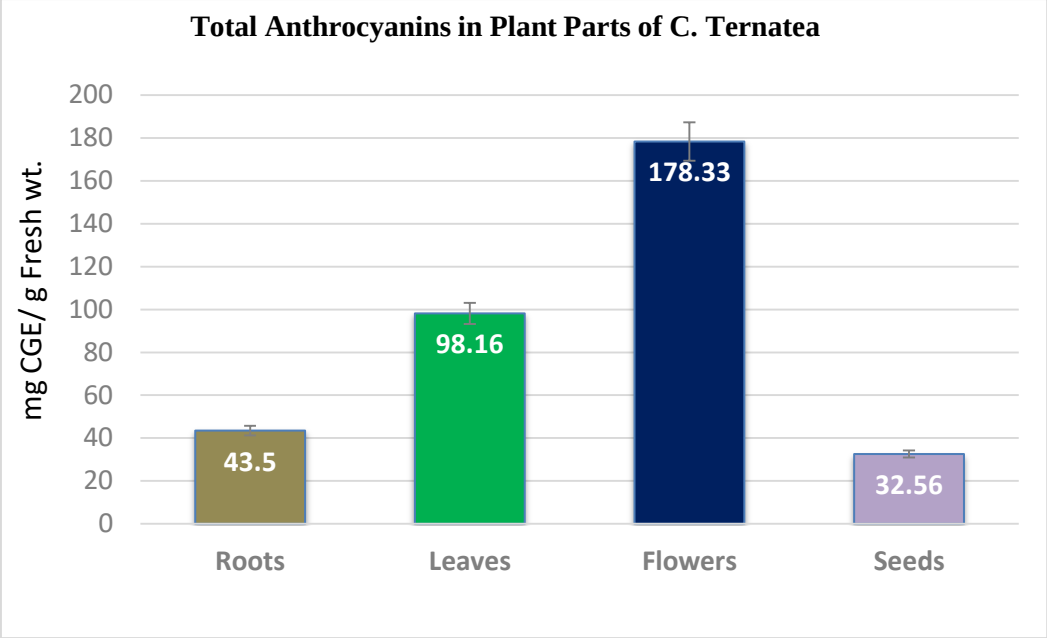

Figure 4.5: Total Anthocyanin present in *Clitoria ternate*

4.4. Discussion:

This objective involves the quantitative evaluation of the plant *Clitoria ternetea's* total carbohydrates, phenols, flavonoids, tannins, and anthocyanins.

Among the main constituents of *C. ternatea*, carbohydrates are essential to its physiological functions. The main source of energy and the fuel for numerous metabolic processes is glucose. As per result of the study, the largest amount of carbohydrates found in *C. ternatea* leaves was 89.01 mgGlc/g fresh weights, compared to the other plant parts.

The therapeutic activities of *C. ternatea* are attributed to a family of secondary metabolites known as total phenols. Because of their well-known antioxidant qualities, phenols are essential for shielding plant from oxidative stress brought on by external variables. The total phenol content of *C. ternatea* has been linked to several health advantages. Folin-Ciocalteu method was used to quantify total phenols. In this technique phenolic compounds reacts with particular reagents to generate blue coloured complexes that be measured using spectrophotometer. The findings showed that the total phenols content was highly present (93 mg GAE/g fresh wt.) in the flowers of *C. ternatea* compared to other plant parts.

The primary roles of flavonoids include controlling cell development, drawing pollinators, and providing defence against biotic and abiotic stressors. Sodium nitrite

reduction method and the aluminium chloride spectrophotometric assay was used to quantitatively measure flavonoids. It observed that the total flavonoids in flowers was 469.34 mg catechine/g fresh wt. against mean value of 273.75 was maximum than other plant parts.

Tannins belongs to secondary metabolites, a family of polyphenolic compound. The main role of tannins is defence mechanisms of plant against pests and pathogens. Their antioxidant qualities aid in the fight against oxidative stress and lower the chance of developing chronic illnesses. The Folin-Denis method was used for tannins estimation. Spectrophotometric measurements of the coloured complexes or precipitates that are formed as a result of tannins reacting with particular chemicals are the basis for these techniques. The results showed that flowers had the highest tannins (72 mg TAE/g fresh wt.) than other parts against mean (41 mg TAE/g fresh wt.) (Figure 4.6).

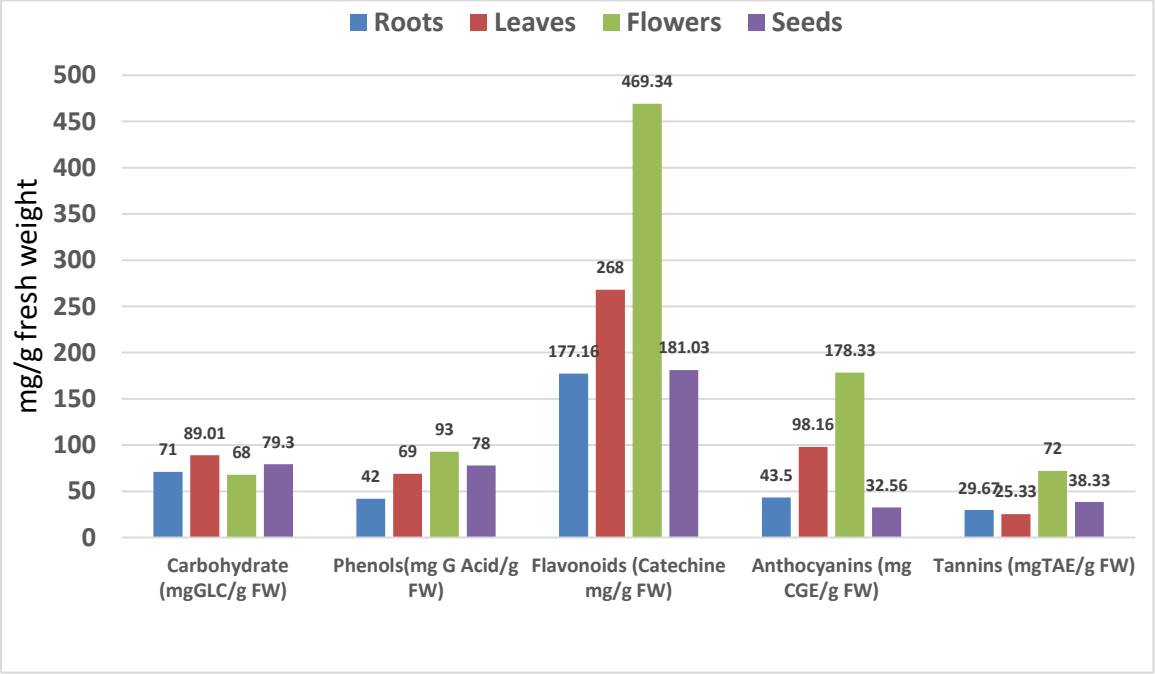

Figure 4.6: Comparision of Phytochemicals in CT Plant Parts.

Anthocyanins are also secondary metabolites, belong to major group of polyphenols which are actually flavonoids. They are water soluble colour pigments present in the flowers, fruits, stem and colour leaves as a colour like red, purple and blue. The main role of anthocyanins in the plant is that to scavengs free radical and prevents oxidative stress. Anthocyanins act as H+ donor in the reaction. Findings observed that the

flowers had the highest value of anthocyanins i.e.178.00±0.37mg CGE/g fresh weight whereas minimum anthocyanins present in seeds (32.56±0.57 mg CGE/g Fresh wt.).

Rich in flavonoids, tannins, anthocyanins, total carbohydrates, total phenols, and other bioactive components, *C. ternatea* is a notable medicinal plant. The results of all data of above all selected parameters of *C ternatea* of each parts were observed highly significant at 5%. Its p values of data were observed less than 0.05. These substances have important effects on health and support their therapeutic qualities. These compounds have been found to possess antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, making *Clitoria ternatea* a promising plant for therapeutic applications.

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CHAPTER-V

EVALUATION OF ANTIOXIDANT ACTIVITIES OF *CLITORIA TERNATEA* BY DPPH ASSAY, FRAP ASSAY, ABTs ASSAY AND SUPEROXIDE ACTIVITY

CHAPTER-V

EVALUATION OF ANTIOXIDANT ACTIVITIES OF *CLITORIA TERNATEA* BY DPPH ASSAY, FRAP ASSAY, ABTs ASSAY AND SUPEROXIDE ACTIVITY

5.1. Introduction:

In this chapter, the third objective of the research has been studied. The third objective of the research was as under:

"Evaluation of antioxidant activities of *C. ternatea* by DPPH (Diphenyl Picryl Hydazyl) assay, FRAP assay (Ferric Reducing Antioxidant Power), ABTs assay, and superoxide radical scavenging activity."

Plant products have been recognized for their potential to provide a broad choice of health welfare due to their bioactive compounds having antioxidant properties. Free radicals scavenge and prevent cellular damage, which is how antioxidants mitigate the damaging effects of ROS. *Clitoria ternatea* or butterfly pea plant has gained attention for its antioxidant properties. The bioactive substances, such as carbohydrates, flavonoids, phenolic acids, tannins and anthocyanins as antioxidant were quantitatively evaluated in previous chapter IV. In this chapter *C. ternatea's* antioxidant scavenging activity has been assessed by using a number of assays, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, superoxide radical scavenging activity assay, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay and ferric reducing antioxidant power (FRAP) assay and superoxide assay. The comparative study of these antioxidants assay of root, leaf, flower and seed of CT was not done in previous research study. These method is generally used for assessing the antioxidants activities of foods, food additives, and natural items [107]. For the study, collection of plant and extraction method was adopted same as detailed at Para 3.2.1 and 3.2.2 of the Chapter III. The results of all data were statistically analysed in triplicate, performed by the Microsoft Excel and also calculated the p value of mean. And further all data of results were also checked for statistically significant exit or not.

All parameters of the above objectives were analyzed for antioxidant enzymes present in the root, leaf, seed, and flower of *C. ternatea*. The extraction and analytical procedures were performed in an ice box or at 0^{O}C. To prevent the denaturation of enzymes, care was must taken. After extraction, extract samples were stored in the freezer.

5.2. Evaluation of Antioxidants:

For evaluation of antioxidants the extracted samples were analized thrice as per methods given against each aasay. All chemicals as analytical grade was used for the procedure of analysis. Double distilled water was also used in every method.

5.2.1. DPPH scavenging assay:

DPPH assay is a commonly used protocol to determine the scavenging activity of free radicals in plant extracts. DPPH, a stable free radical, reacts with antioxidants by donating an electron, leading to a color change from purple to yellow, which is measured spectrophotometrically. The degree of discoloration is compared to the scavenging capacity of the antioxidant. In this assay, the lower the half-maximal inhibitory concentration (IC50) value, the higher the antioxidant activity of the sample.

Chemicals Used:

- Ethanol: 50%
- DPPH: 0.1 mM
- Ascorbic acid: mg/ml(Standard)

Method:

Chan *et al.* [113] method was used for DPPH radical scavenging activity with the slight modification. The 0.5 g sample was homogenised in mortar-pestle with 50% ethanol in an ice box. The sample was centrifuged at 5000 rpm for 15 minutes and the final volume of the supernatant was made 4.0 ml which was adjusted with 50% ethanol. Finally, extracts of 1.0 ml ml aliqute and 1.0 ml DPPH solution (0.1 mM) was added. For blank equal amount of ethanol and DPPH was used. The mixture was shaken vigorously and was left to stand in dark for 30 min and the absorbance was read at 517 nm. DPPH radical scavenging assay was standardized

against mg/ml of ascorbic acid and expressed as mg ascorbic acid/g of fresh weight of sample and also calculated by inhibition percentage.

% Inhibition = [(A control - A sample) / A control] x 100%

The extract's capacity to scavenge DPPH radicals was also quantified as IC50 values. The effective concentration of the extract (IC50) needed for 50% DPPH radical scavenging was determined by plotting the scavenging activity against sample concentration. DPPH activity was also calculated in mg ascorbic acid/gm fresh wt. in the sample.

Result:

The results data were calculated in mg ascorbic acid per gram equivalents by using ($y = 0.895x + 0.051$, $R^2 = 0.9979$) the standard curve equation. The outcome reveals that the *C. ternatea* plant's total mean activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is 85.81 mg ascorbic acid/g fresh wt. The flower had the highest DPPH activity with 92.43 mg of ascorbic acid/g fresh wt. followed by roots and leaves i.e. 89.40 and 82.80 mg/g fresh wt. respectively. However, seeds of the *C. ternatea* had the lowest DPPH activity, measuring 78.63 mg ascorbic acid/g fresh wt. The results of DPPH activity of selected parts of C T were highly significant (p 0.0037) at 5% (Table 5.1 and Figure 5.1).

5.2.2. ABTs radical scavenging assay:

A well-known compound utilised in biochemical experiments, specifically in the assessment of antioxidant activity, is 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTs). The ABTs assay evaluates antioxidants' capacity to scavenge free radicals and prevent oxidative damage. ABTs is a synthetic compound that generates a blue-green radical when oxidized, and the scavenging capacity of the antioxidant enzymes is inversely proportional to reducing the color intensity. This blue-green colour change can be quantitatively measured spectrophotometrically, providing information about the antioxidant capacity of a sample. ABTs assay is widely used in antioxidant research, screening natural products, evaluating the efficacy of antioxidants in foods, and studying oxidative stress-related diseases.

Chemicals Used:

- Phosphate buffer: 1Mm P^H 7.4
- ABTs solution: 7Mm
- Potassium Persulphate: 4.95Mm
- Gallic acid: mg/ml(standard)

Method:

Stratil *et al.* [114] method used for the procedure for measuring ABTS (2, 2'-azino-bis -3- ethyl benzothiazoline 6-sulphonic acid) was determined. 0.5 g of fresh sample was homogenized in mortar-pestle with 2.0 ml of phosphate buffer and centrifuged at 5,000 rpm for 10 minutes at 4^oC and the supernatant was only used for the assay. Adjust the volume to 4.0 ml with phosphate buffer. For the ABTs solution, made mixture of ABTs (7mM) and potassium persulfate (4.95mM) in 1:1 ratio (v/v) was allowed to stand overnight at room temperature in the dark to form radical cations ABTs+. In the reaction mixture, 1.0 ml of extract was mixed with 3.9 ml ABTS solution and 0.1 ml phosphate buffer (P^H7.4), and a decrease in the absorbance was measured at 745 nm. The blank was prepared in the same procedure without the use of extract. ABTs assay was standardized against mg/ml of Gallic acid and expressed as (GAE) mg/g fresh weight and also calculated by inhibition percentage by using the following formula:

% Inhibition =

[(A control - A sample) / A control] x 100%

The extract's ABT radical scavenging activity was represented as IC50 values. A graph that shows the sample concentration needed to scavenge 50% of the free radicals produced by 50% ABT was used to compute the IC50 values which expressed in percentage value. ABTs antioxidants activity were also calculated in mg gallic acid/g fresh wt of sample.

Result:

The result of ABTs was determine by the gallic acid equivalent through the standard curve equation ($y = 0.308x - 0.03$, $R^2 = 0.9887$). The total mean activity of gallic acid equivalent antioxidant capacity (ABTs) in *C. ternatea* plant was observed 81.92 (GAE)mg/g fresh wt.. The highest ABTs activity recorded in flower was 89.36

(GAE)mg/g fresh wt. followed by 85.65 (GAE)mg/g F.W., 81.47 (GAE)mg/g F.W. in seeds and leaves respectively. The lowest ABTs activity was 71.26 (GAE)mg/g F.W. in the roots of *C. ternatea* (Table 5.1 and Figure 5.1). The resut of the ABTs activity was observed highly significant (p 0.0039 <5%).

5.2.3. Ferric Reducing Antioxidant Power (FRAP) assay:

Antioxidants' capacity to convert ferric ions (Fe3+) into ferrous ions (Fe2+) is the basis for the FRAP assay. Iron in the oxidised form, FeIII, is reduced to FeII using this process at an acidic pH. The final product is a yellow core iron atom complex (FeIII) with two ligands (2 molecules of 2,4,6-tris(2-pyridyl)-S-triazine, TPTZ) in an acidic aqueous buffer. An iron ion (FeIII) turns blue when it is converted to a ferrous ion (FeII) by being in the presence of antioxidants. The intensity of the blue colour, read at 593 nm absorbance. The reduction potential of antioxidants is determined by measuring the change in absorbance caused by the formation of the blue ferrous-tripyridyltriazine complex. more absorbance indicates more reducing power and, thus, higher levels of antioxidant activity.

Chemicals Used:

- Acetone: 70%
- Phosphate buffer: 200mM, P^H 6.6
- Potassium Ferricyanide: 30 mM
- $FeCl_3$: 6 mM
- BHT: mg/ml(standard)

Method:

FRAP activity was determined by the modified method of Athukorala *et al*. [115]. 0.5g of fresh sample was homogenized in mortar-pestle with 4.0 ml of 70% acetone. After centrifugation at 5,000 rpm for 10 min, the final volume of the supernatant was made upto 10.0 ml with 70% acetone. In the reaction mixture, 1.0 ml of extract was mixed with 2.5 ml phosphate buffer and 2.5 ml potassium ferricyanide, incubated for 20 min at 50^0C, and then added 2.5 ml $FeCl_{3.}$ After that again centrifuge for 10 minutes at 3000 rpm. The upper layer of solution was pull out and 2.5 ml was mixed with 2.5 ml distilled water and 0.5 ml $Fecl_3$. Absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

The ferric reducing antioxidant power assay was calibrated against mg per ml of BHT (Butylated Hydroxy Toluene) and was expressed as mg of BHT per gram of fresh weight of sample.

The extract's of FRAP radical scavenging activity was quantified in terms of IC50 values. A graph that shows the sample concentration needed to scavenge 50% of the FRAP free radicals was used to calculate the IC50 values. The inhibition percentage was also calculated by using the following formula:

% Inhibition =

[(A control - A sample) / A control] x 100%

Result:

As per result, he total mean value of FRAP activity in different tissues of plant extract was 308.86 mg(BHT)/g fresh wt. The resultant of samples were calculated by standard curve equation ($y = 0.28x + 0.017$, $R^2 = 0.9987$). The outcome demonstrated that seeds had the lowest FRAP activity and leaves had the highest activity of FRAP.. The increasing order of activity of FRAP was observed in 268.50 mg(BHT)/g fresh wt., 272.43 mg(BHT)/g fresh wt., 294.80v and 399.73 mg(BHT)/g fresh wt. in seeds, roots, flowers and leaves respectively as in Table 5.1 and Figure 5.1. The data of result were found highly significants (p 0.0042) at 5%.

5.2.4. Superoxide Radical Scavenging Activity Assay:

Superoxide radicals ($O2^-$) represent very reactive oxygen species that possess the potential to inflict harm on cells. The efficacy of antioxidants to neutralise these radicals is assessed using the superoxide radical scavenging activity assay. Nitro-blue-tetrazolium (NBT) is spontaneously reduced by superoxide radicals in this assay, producing a blue formazan product as a byproduct. The extent of color reduction indicates the superoxide-free radicle scavenging activity of the plant extract of plant samples. Superoxide radicals are produced by the nonenzymatic phenazine methosulfate nicotinamide adenine dinucleotide (PMS/NADH) system, which then reduces to nitroblue tetrazolium (NBT) to produce purple formazan. Superoxide also activates receptors that are confined to membranes, which is essential for cell survival and signaling. To encourage apoptosis, they might also change the permeability of the mitochondrial membrane.

Chemical Used:

- Hydroxylamine hydrochloride: 1mM
- NBT (nitroblue tetrazolium): 24mM
- Ethylene diamine tetra acetic acid (EDTA): 0.1 millimolar
- Ascorbic acid: mg/ml (standard)

Method:

The activities of the ethanolic extracts of *Clitoria ternatea* were measured using nitroblue tetrazolium (NBT), according to a reported method by Sunil *et al*. [116]. Briefly, 24 mM of NBT and 1 mM of hydroxylamine hydrochloride were prepared. A total of 100 μL of NBT (24 mM) was added into the reaction mixtures, which contained 0.2 μL of 0.1 mM of ethylene diamine tetra acetic acid (EDTA) solution, 1 mL of distilled water, and the ethanol extract (50–250 μL). Then, approximately 0.1 mL of hydroxylamine hydrochloride (1mM) was added to initiate the reaction. After incubation at 25°C for 20 minutes, the NBT reduction was measured at 560 nm using a reaction mixture.Without extract as the control was follow same above procedure. Standard as ascorbic acid 0.1mg/gm was plotted through standard graph and value of sample was also expressed mg ascorbic acid/g fresh weight of sample. Superoxide radical scavenging activity was calculated as inhibition percentage by following the formula:

% superoxide scavenging activity =

[(A control - A sample) / A control] x 100

Result:

The results of superoxide anion activity was observed through standard curve equation ($y = 1.935x + 0.079$, $R^2 = 0.9959$). The total mean value of superoxide activity observed in different tissues of C. ternatea was 67.73 mg ascorbic acid/g fresh wt. The highest superoxide value was observed in flower tissue (89.33 mg ascorbic acid/g fresh wt.) and the minimum value was observed at 52.60 mg ascorbic acid/g fresh wt. in the root tissue of *C. ternatea*. The results of data were highly significant (p 0.0195) which was less than 0.05 (Table 5.1 and Figure 5.1).

Table 5.1: DPPH, ABTs, FRAP, and Superoxides Radical Activities in *C. ternatea*

Plant parts *C. ternatea*	DPPH (mg Ascorbic ácid/g F.W.)	ABTs (GAE mg/g F.W.)	FRAP (mg BHT/g F.W.)	Superoxide (mg ascorbic acid/g F.W.)
Roots	89.40±0.43	71.26±0.66	272.43±1.16	52.60±0.64
Leaves	82.80±0.78	81.47±0.56	**399.73±1.42**	69.43±1.04
Flowers	**92.43±0.49**	**89.36±0.67**	294.8±0.74	**89.33±1.21**
Seeds	78.63±0.94	85.65±0.75	268.5±0.83	59.57±1.18
Mean	85.81	81.92	308.86	67.73
C.D.	2.29	2.2	3.55	3.46
SE(m)	0.69	0.66	1.07	1.06
SE(d)	0.98	0.94	1.52	1.5
C.V.	1.39	1.41	1.65	2.67
P < 0.05	0.0037	0.0039	0.0042	0.0195

Values are presented as mean ± S.D n=4. (Experiments were made as 3 parallel)

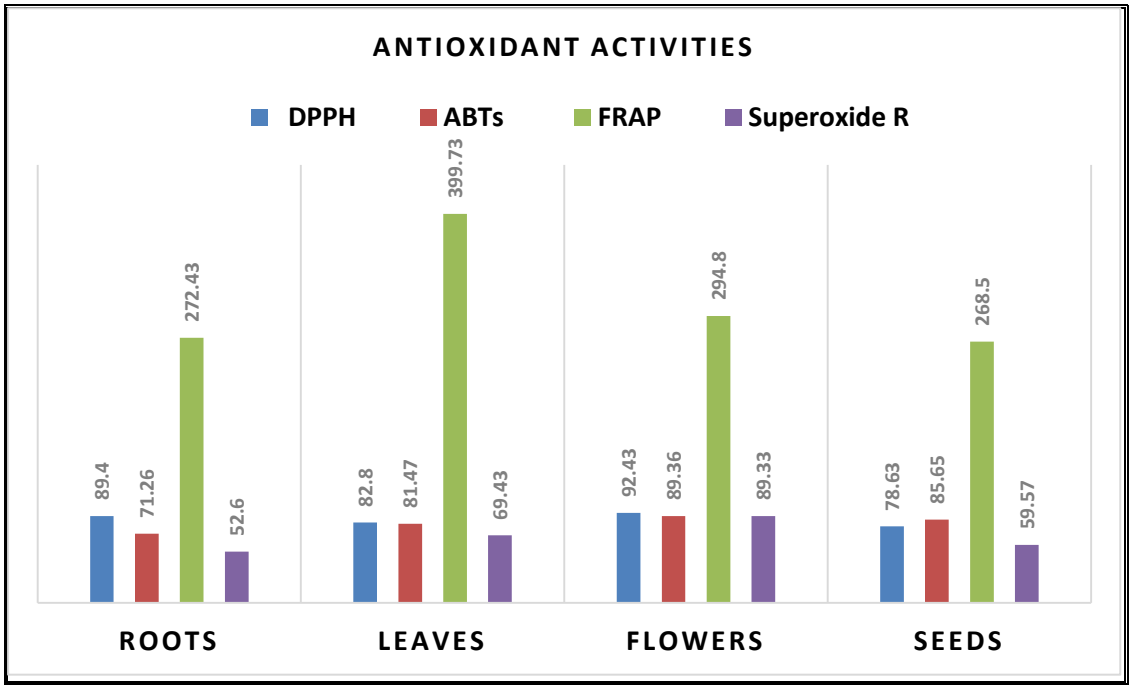

Figure 5.1: DPPH, ABTs, FRAP, and Superoxide activities in *C. ternatea*

5.3. Inhibition percentage of DPPH Activity in CT at different concentrations:

Ethanol based extract of root, leaf, flower and seed tissue of *C. ternatea* plant was taken at five distinct concentrations like 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml, respectively and observed by inhibition percentage value. These were analysed for DPPH activity with the method mentined at para 5.2.1 above. It was used to analysed the inhibition % of DPPH's scavenging activity. These activities were recorded as per Table 5.2 and Figure 5.2 below:

Table 5.2: DPPH activity of plant parts of *Clitoria ternatea* on different concentrations:

Concentration (mg/ml)	**Inhibition %**(Unit: mg Ascorbicacid /g Fresh wt.)			
	Root	**Leaf**	**Flower**	**Seed**
0.2	25.53±0.61	35.80±0.72	53.57±1.13	23.28±0.57
0.4	28.96±0.67	43.16±0.74	58.96±1.27	27.50±1.09
0.6	35.77±0.55	53.93±0.83	68.40±0.63	34.50±0.69
0.8	39.90±0.59	65.26±1.01	79.33±0.66	38.87±1.13
1.0	**48.8±0.72**	**79.73±1.15**	**88.43±0.69**	**48.20±1.06**
Mean	35.79	55.57	69.74	34.47
C.D.	1.973	2.89	2.91	2.997
SE(m)	0.618	0.91	0.912	0.939
SE(d)	0.874	1.28	1.29	1.328
C.V.	3.002	2.82	2.265	4.718
P value at 5%	0.0091	0.0061	0.0046	0.0098

Values are presented as mean±S.D, n=4.(Experiments were made as 3 parallel)

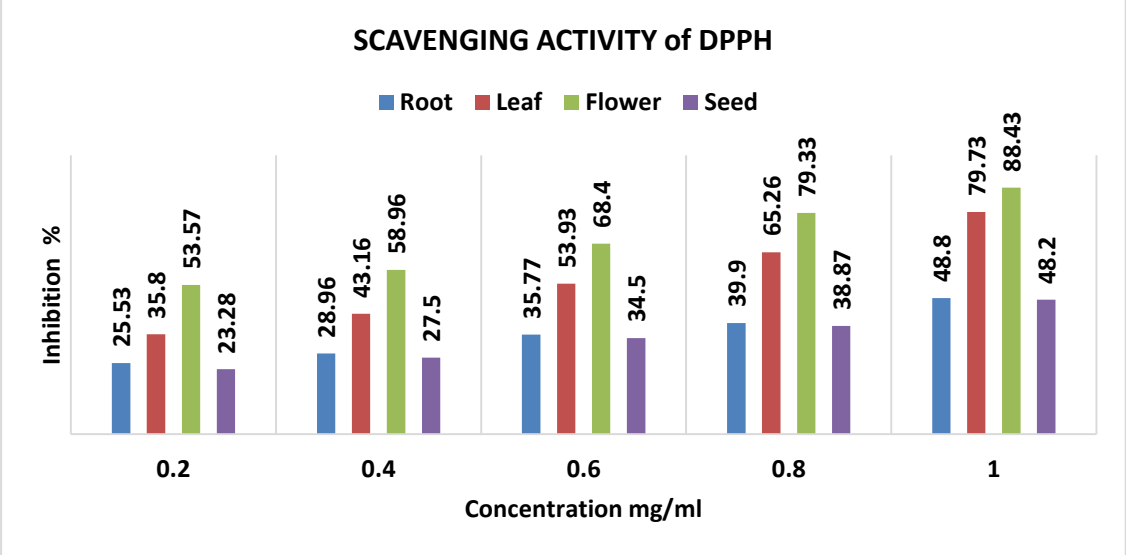

Figure 5.2: DPPH Scavenging Activity in parts of *C. ternatea* at different concentration

The result showed that scavenge free radicals are found in increasing order with increase in concentration of extracted sample of leaf, flower, root, and seeds of CT plant. The maximum inhibition percentage was observed in flowers with all concentrations followed by leaf, root, and seeds respectively. The flower with the highest DPPH scavenging activity was measured at 86.43% at a concentration of 1.0 mg/ml in the CT extract whereas leaf (79.73%), root (48.8%), and seed (48.2%) respectively. The results of all scavenging activity of DPPH were statistically highly significant at 5%.

5.4. Inhibition percentage of ABTs activity in CT at different concentration:

The basis for ABTS assays is the extract contents' capacity to transfer an electron to the preformed radical, which lowers absorbance. ABTs activity in different parts of CT plant ethnolic extract was evaluated on the different five concentrations i.e. 1.0, 0.8, 0.6, 0.4 and 0.2 respectively. The method mentioned at para 5.2.2 were adopted for ABTs activity at different concentration of extract sample of CT plant parts. The obserbation were recorded in Table 5.3 below and inhibition percentage were plotted at figure 5.3.

Clitoria ternatea activities of ethanolic extract against ABTs free radicals showed concentration-dependent effects and maximum inhibition percentage found in flower at 1mg/ml concentration i.e. 96.7 mgGAE/g FW followed by leaf- 85.76 mgGAE/g FW, root- 68.73 mgGAE/g FW and seed -68.5 mgGAE/g FW. Although the potency of the CT extract was less than that of the positive control (GAE), these assays demonstrated the extract's ability to scavenge free radicals. As per result, the increasing order of ABTs activity in extracts were recorded with increasing concentration.

Table 5.3: ABTs activity in plant parts of *C. ternatea* at different concentrations

Concentration (mg/ml)	**Inhibition %** (Unit: (GAE) mg/g Fresh wt.)			
	Root	**Leaf**	**Flower**	**Seed**
0.2	33.26±1.13	55.46±1.24	65.36±1.09	35.20±1.18
0.4	46.83±0.63	58.87±0.55	78.87±0.60	48.40±0.60
0.6	53.63±0.54	64.20±0.60	85.59±0.66	55.30±0.68
0.8	59.17±1.09	73.43±1.12	89.53±0.69	59.20±1.12
1.0	**68.73±0.60**	**85.40±1.15**	**96.70±1.21**	**68.50±0.52**
Mean	52.32	67.54	83.21	53.32
C.D.	2.69	3.14	2.823	2.739
SE(m)	0.843	0.92	0.884	0.858
SE(d)	1.192	1.39	1.251	1.214
C.V.	2.79	2.58	1.841	2.788
P <0.05	0.0112	0.0048	0.00384	0.0062

Value are presented as mean±S.D,n=4. (Experiments were made as 3 parallel)

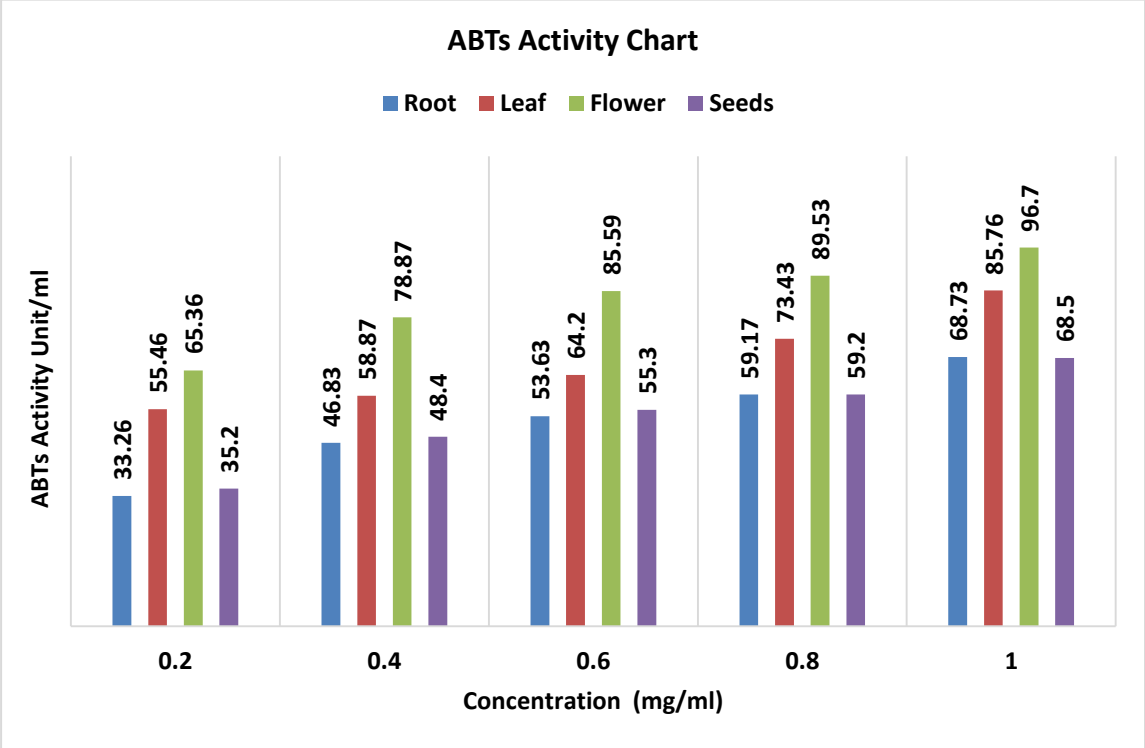

Figure 5.3: ABTs activity at different concentrations in *C.ternatea*

5.5. Inhibition percentage of FRAP activity in CT at different concentration:

The inhibition percentage of FRAP activity was evaluated on the different concentration i.e. 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of roots, seeds, flowers and leaves of CT plant. The method adopted for FRAP activity was as mentioned in para 5.2.3 above. The observations were recorded as per Table 5.4 and inhibition percentage was plotted in Figure 5.4below:

Table 5.4: FRAP activity on different concentration in Plant tissue of CT

Concentration (mg/ml)	**Inhibition %** (mg BHT/g F.W.)			
	Root	**Leaf**	**Flowers**	**Seeds**
0.2	22.70±0.63	37.43±0.61	42.70±0.46	32.67±0.66
0.4	29.87±0.61	41.80±0.53	59.87±0.55	39.77±1.09
0.6	37.41±0.87	53.80±1.21	67.43±0.56	47.43±0.67
0.8	43.40±0.74	61.37±0.62	73.26±1.18	53.33±0.56
1.0	**56.67±1.18**	**69.87±0.60**	**86.63±0.63**	**66.67±0.69**
Mean	38.01	52.85	65.98	47.97
C.D.	2.66	2. 41	2.321	2.42
SE(m)	0.834	0.75	0.727	0.77
SE(d)	1.179	1.06	1.028	1.07
C.V.	3.798	2.47	1.909	2.73
P<0.05	0.009	0.006	0.005	0.007

Values are presented as mean±S.D, n=4.(Experiments were made as 3 parallel)

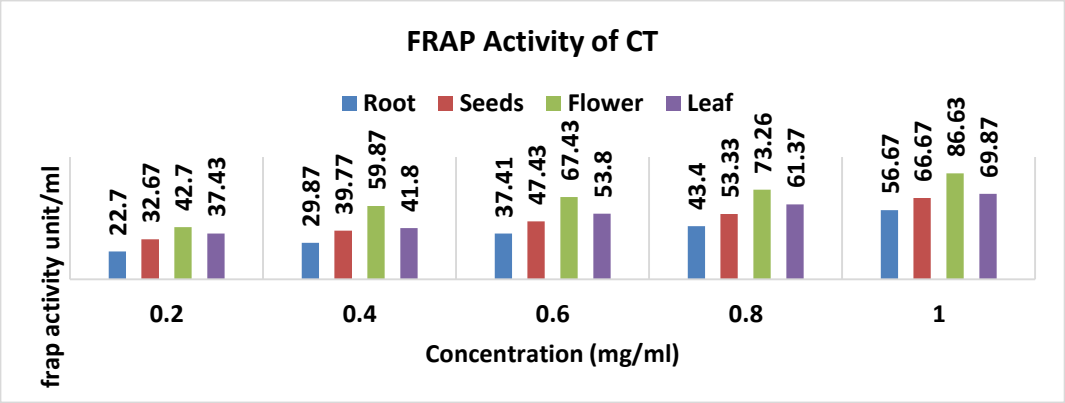

Figure 5.4: FRAP activity at different concentration in *C. ternatea*

The table 5.4 shows that gradually increased in concentration of the ethanolic extract of all parts, the mean value of FRAP was also increased. The study revealed that the concentration of 1mg/ml, the flower showed the maximum FRAP activity i.e. (86.63±0.63) followed by leaf (69.87±0.60), seeds (66.67±0.69) and root (56.67±1.18). The same trend was observed in all other concentration (Figure 5.4).

5.6. Inhibition % of Superoxide activity in CT at different concentration

Superoxide radical activity was evaluated through inhibition percentage on the different concentration of leaf, root, flowers and seeds of CT plant. The extract sample concentration was taken at 0.2, 0.4,.0.6, 0.8 and 1.0 mg/ml. The method was adopted as per para 5.2.4 above for superoxide activity of these extracted samples of CT. The results were recorded and is tabled at Table 5.5 and percentage inhibition is plotted at Figure 5.5. The result shows that at 1mg/ml superoxide scavenging activity was 72.37% for root, 85.76% for leaf, 97.56% for flower and 90.23% for seed. The same trend was found in all other concentration (Figure 5.5).

Table 5.5: Superoxide activity on different concentration in Plant tissue of *C. ternatea*

Concentration (mg/ml)	**Inhibition %** (Unit: mg ascorbic acid/g FW)			
	Root	**Leaf**	**Flower**	**Seeds**
0.2	52.67±0.64	59.47±0.55	62.37±0.76	59.57±0.25
0.4	55.87±0.66	66.23±0.54	69.07±0.69	67.11±0.16
0.6	61.47±0.64	71.43±0.64	76.70±0.52	72.15±0.56
0.8	69.43±0.78	79.60±0.65	89.86±0.60	82.33±0.34
1.0	**72.37±1.18**	**85.76±0.47**	**97.56±0.52**	**90.23±0.47**
Mean	62.36	72.42	79.11	74.28
C.D.	2.557	1.81	2.004	2.23
SE(m)	0.801	0.56	0.628	0.71
SE(d)	1.133	0.84	0.888	0.94
C.V.	2.225	1.35	1.374	2.14
P <0.05	0.005	0.004	0.004	0.004

Values are presented as mean±S.D, n=4.(Experiments were made as 3 parallel)

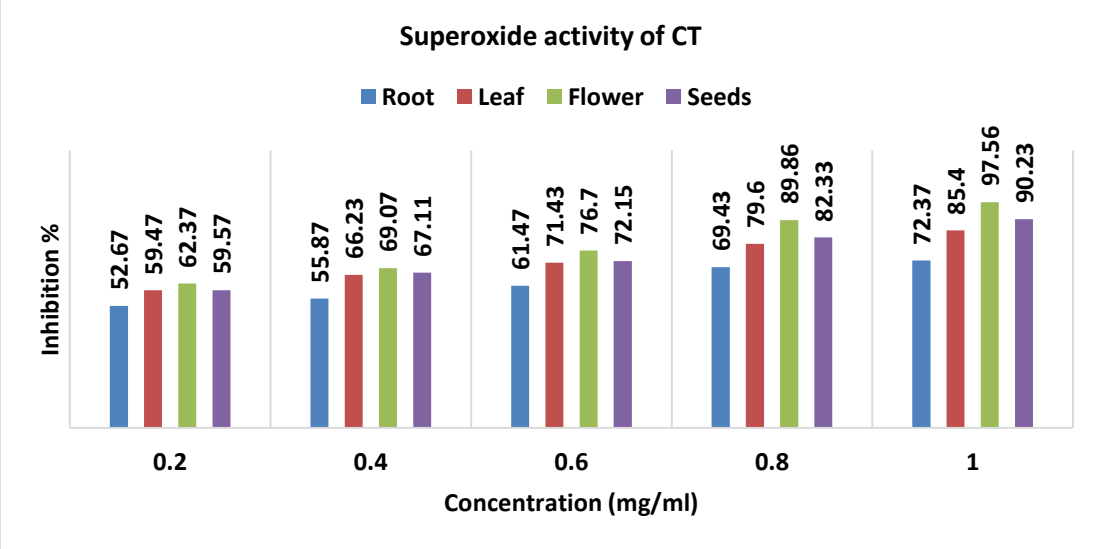

Figure 5.5. Superoxide activity at different concentration in *C. ternatea*

The result shows the increasing trend after increase in superoxide activity on increase of concentration of the extract, the mean value of superoxide is also increased. The study revealed that on concentration of 1mg/ml, the flower showed the maximum superoxide activity and their inhibition percentage was 97.56% followed by seeds (90.23%), leaf Overall, according to results of current research, *C. ternatea* flowers showed high antioxidant contents at 1.0mg/ml concentration, as per the given Table 5.6 (Figure 5.6) below:

Table 5.6: Comparative study of Inhibition % for scavenging activity at 1.0 mg/ml concentration

	Root	**Leaf**	**Flower**	**Seed**
DPPH	48.8±0.72	79.73±1.15	88.43±0.69	48.20±1.06
ABTs	68.73±0.60	85.40±1.15	96.70±1.21	68.50±0.52
FRAP	56.67±1.18	69.87±0.60	86.63±0.63	66.67±0.69
Superoxide	**72.37±1.18**	**85.76±0.47**	**97.56±0.52**	**90.23±0.47**

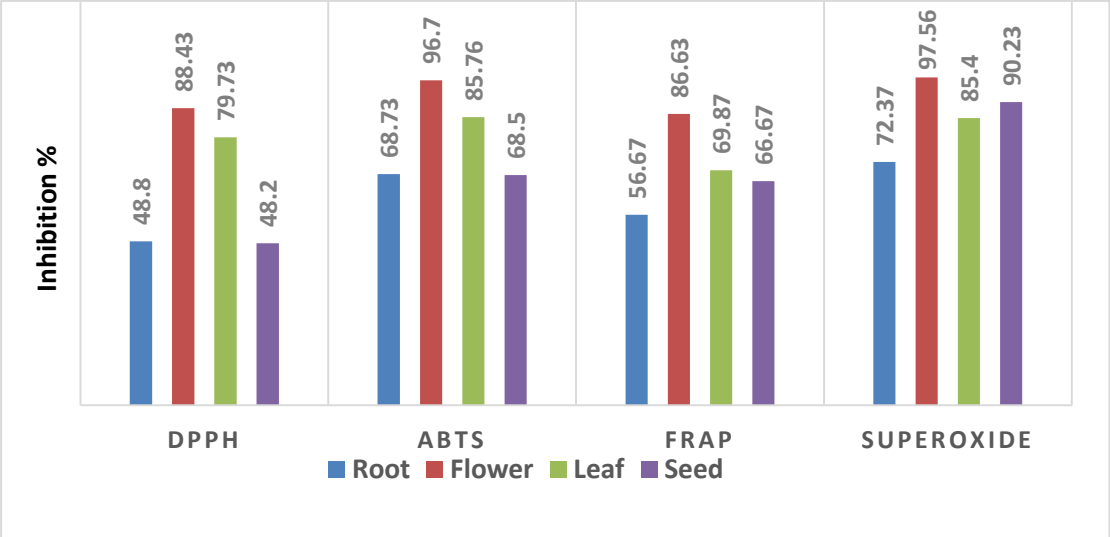

Figure: 5.6. Inhibition % of scavenging activity at 1.0 mg/ml concentration of C.T plant

5.7. Discussion:

Copper reducing power assays, ferric reducing antioxidant power (FRAP), ferrous ion chelating power, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging, and 2,2 diphenyl-1-picrylhydrazyl radical (DPPH) radical scavenging are examples of antioxidant assays. All results of antioxidants were statistically highly significant at 5%. By employing these complementary assays, the study intended to comprehensively characterized the antioxidant potential of *C.*

ternatea and shed light on the mechanisms underlying its scavenging free radical molecules.

Research investigations have shown that the FRAP and DPPH assays exhibit quick and robust suppression of free radicals, indicating antioxidants activity. The current study determined the maximal antioxidant activity for the DPPH assay at 1mg/ml. Free radical scavenging is accomplished by phytochemical components, which also positively correlate with antioxidant activity. Using the DPPH assay, the anthocyanin-rich fraction of the flower exhibited the highest antioxidant activity of inhibition % at a concentration of 1 mg/ml, or 88.43 ± 0.69 mg/mL, for radical scavenging, while ABTS of inhibition % was determined at 96.70 mg/mL. The results showed that the extracts were more powerful at 1.0 mg/ml concentration for all part of CT. The anthocyanin-rich fraction of *C. ternatea* flowers was shown to be more potent in the crude extracts, with an average GAE equivalent mg/ml extract fresh weight of 294.8 mgBTH, according to the FRAP result. Therefore, the ethanolic extracts of *C. ternatea* flowers that had IC50 values ranging from 0.2 to 1.0 mg/mL in the DPPH assay were the only ones whose antioxidant activity was determined by the experimental results. For FRAP assay and superoxide, results showed the maximum free radical inhibition and possess higher flavonoid contents.

Findings of above results in Table 5.6, the superoxide scavenging activity was showed maximum in root (72.37 mg/ml) at 1.0 mg/mg in comparision to other antioxidants activity like DPPH, ABTs and FRAP. However, the activities of ABTs and super anions were showed highest in leaves and found value (85.40 and 85.76) in comparision to DPPH and FRAP activities at 1mg/ml concentration. While, ABTS and superoxide anion activities observed highest in flowers and found approxmate same value (97.56±0.52 and 96.70±1.21) at 1mg/ml in comparion to others selected antioxidants like DPPH and FRAP. The results of superoxide scavenging activity was observed highest in seed (90.23±0.47) than FRAP, ABTs and DPPH activity. The results of DPPH and superoxide activity were observed 48.20±1.06 and 90.23±0.47 in seeds whereas the FRAP shows 66.67±0.69 at 1mg/ml. Overall, among the all selected antioxidant parameters, superoxide scavenging activity was observed highest in all part of *C. ternatea* plant (Table 5.6).

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CHAPTER-VI

EVALUATION OF ANTIOXIDANT ENZYMES *VIZ*. PEROXIDASE, SUPEROXIDE DISMUTASE, GUAIACOL PEROXIDASE, AND POLYPHENOL OXIDASE

CHAPTER-VI

EVALUATION OF ANTIOXIDANT ENZYMES *VIZ*. PEROXIDASE, SUPEROXIDE DISMUTASE, GUAIACOL PEROXIDASE, AND POLYPHENOL OXIDASE

6.1. Introduction:

Medicinal plants and herbs are used by homeopathic, Ayurveda, and folk medicine systems for the treatment of innumerable types of encumbrance. Medicinal plant, *Clitoria ternatea* is a widely available plant that can be easily cultivated. Today, due to oxidative stress in the run of daily life, it has become common to have diseases like neurodegenerative disease, heart attack or cardiovascular disease, autoimmune disorders, and cancer [117]. The discovery of natural sources of antioxidants to prevent these diseases is proving helpful for human health. Antioxidants are molecules that protect the enzymes as well as cell organelles from oxidative damage, and they are used as a defense mechanism in removing or repairing damaged molecules. Natural antioxidant compounds have become very important. And the medicinal plants are becoming important natural sources of antioxidant enzymes [118].

The aim of the current study was to examine the biochemistry of the main antioxidant enzymes found in *Clitoria ternatea's* root, leaf, flower, and seed. These enzymes also require co-factors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity. The fourth objective of the current study was to assess the presence of antioxidant enzymes in *Clitoria ternatea* plant components, specifically peroxidase, superoxide dismutase, guaiacol peroxidase, and polyphenol oxidedase.

For the study of assay of enzymes, collection of plant and extraction method was adopted same as detailed at Para 3.2.1 and 3.2.2 of the Chapter III.

The parameters of the above objectives were analyzed very carefully for antioxidant enzymes present in the different parts of the CT such as the root, leaf, seed, and flower. The extraction and analytical procedures were performed at an ice box or at

0^{O}C and stored in refrigerator. To prevent the denaturation of enzymes, samples were analyzed very carefully.

6.2. Evaluation of Antioxident Enzymes:

The extrat sample was taken in triplicates for each antioxidant enzymatic analysis. Analytical grade chemicals and double distilled water were used for analysis as per given methods of each assays following below:

6.2.1. Assay of Peroxidase (POX):

A broad class of enzymes known as peroxidases or peroxide reductases (EC number 1.11. 1. x) are involved in many different biological processes. Through the use of free radicals, the enzyme peroxidase catalyses oxidation-reduction processes, converting a variety of substances into oxidised or polymerized products. Peroxidases are glycoproteins that are widely distributed throughout the kingdom of plants. They can reduce hydrogen peroxide and other hydroperoxides to water and are found in a variety of isoenzyme forms. Peroxidase oxidises a range of substrates while reducing HO^{-} to water. Peroxidases are hence oxidoreductases that catalyse various oxidative processes using HO-as an electron acceptor.

Chemicals Used:

- Phosphate Buffer 0.1 M (pH 7.0).
- Guaiacol Solution (20 mM)
- Hydrogen peroxide solution (0.042%, 12.3 mM) (freshly prepared).

Method:

The Peroxidase activity was determined by the method of Putter *et al.* (1974). An accurate amount 1.0 g of freshly isolated leaves, seeds, roots, and flowers of *C. ternetea* was homogenized with the help of mortar-pestle in 3.0 ml of chilled 0.1 M phosphate buffer in an ice box. After centrifugation at 5,000 rpm at 4ºC for 15 minutes, the final volume of the supernatant was made up to 3.0 ml with phosphate buffer and stored on ice. For the assay mixture pipette out 3.0 ml buffer solution, 0.05 ml guaiacol solution, 0.10 ml extract, and 0.03 ml hydrogen peroxide solution in a cuvette, and the absorbance was measured at 430 nm using a UV-VIS spectrophotometer. Blank was prepared as same procedure without taking the

extract. The spectrophotometer was adjusted to zero reading at 430 nm wavelength and taking reading at 20 seconds of sample. The peroxidase activity was expressed as Unit/ml or micromole / g /min of fresh weight of *C. ternetea*. The following formula was used to determine the peroxidase activity:

$$\text{Unit/ml} = \frac{[(\text{A } 430/20 \text{ sec Test sample} - \text{A}430/ 20 \text{ sec Blank}) \times \text{total volume} \times \text{D. factor}}{6.39 \times \text{volume (ml) of enzyme used in the reaction mixture}}$$

where,

6.39 = Guaiacol dehydrogenase product's extinction coefficient at 436 nm

$$\text{Units/ mg Protein} = \frac{\text{Unit/ml enzyme}}{\text{mg protein/ml enzyme}}$$

D.F = dilution Factor

Result:

In this current study, the antioxidant peroxidase enzyme was calculated by the formula mentioned above by using extinction coefficient value 6.39 at 436 nm.

(Table 6.1 and Figure 6.1). The overall mean value was observed 340.23 Unit/ml in *C. ternatea* plant. The result showed the highest mean value was for flowers (391.20 Unit/ml) followed by leaves (376.33 Unit/ml), roots (304.20 Unit/ml), and the lowest mean performance was recorded for seeds (289.14 Unit/ml). The resultant data of peroxidase enzyme activity was obtained highly significant at 5% p value (0.0009).

6.2.2. Assay of Superoxide Dismutase (SOD):

All aerobic and some anaerobic species have superoxide dismutase enzyme (EC 1.15.1.11), the fastest known enzyme and the initial enzymatic antioxidant barrier of aerobic organisms. Superoxide ions have a high degree of conversion to hydrogen peroxide, which is made up of the Ferron reaction, which can become catalytic in the presence of oxygen, and the creation of OH from H_2O_2 in the presence of transition metals like Fe or Cu. Nitric oxide (NO), which NO inactivates to create peroxy-nitrite, is the superoxide anion that SOD competes with NO for. Thus, SOD increases the activity of nitrous oxide (NO) by scavenging superoxide anions.

Chemicals used:

- Sodium pyrophosphate buffer: 0.025M, pH 8.3
- Potassium Phosphate buffer: 50mM, pH 6.4
- Phenazine Methosulphate (PMS): 186µM
- Nitroblue tetrazolium (NBT): 300µM
- NADH: 780µM
- Glacial acetic
- acid n-butanol

Method:

The SOD was assayed by the method of Kakkar *et al.,* [120]. The blue variety of *C. tertnea* blooming plant's roots, leaves, seeds, and flowers were cut into small part and washed with distilled water for further analysis. 1.0 g of freshly *C. tertnea* of leaves, roots, seeds, and flowers were crushed with the help of a mortar pestle in 3.0 ml of potassium phosphate buffer at icebox. The crushed sample was centrifuge at 5000 rpm for 10 min at 4^0C. The reaction mixture had 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, and 0.2 ml of extract made up for assay combination. In total volume of reaction mixture, 0.2 ml of NADH was added lastly for started the reaction. After that the mixture was incubated for 90 seconds at 30°C and then added 1ml of glacial acetic acid for stopped the reaction. Then adding 4.0 ml of n-butanol and reaction mixture was shakened well and left for 10 minutes. The chromogenic intensity of butanol layer was separated and it read at 560 nm. For the purpose of standardizing, butanol was utilised as the blank. The quantity of enzyme that produced a 50% inhibition of NBT reduction in a minute is known as one unit of enzyme activity. Start the reading of blank and sample OD at 560 nm noted the value upto minute in spectrophotometer.

NBT reduction % =

[(A control/min - A sample/min) / A control/min] x 100 %

Based on the inhibition % of each SOD standard, the inhibition % vs log (SOD concentration) was plotted on the SOD standard Inhibition curve.

Result:

The conversion of superoxide anions to hydrogen peroxide is catalysed by SOD. It is essential for cells to protect themselves against the harmful effects of oxygen radicals. SOD enzyme activity was calculated Unit /ml by using standard curve equation ($y = 0.106x + 0.127$, $R^2 = 0.9646$). It was observed in the result that seeds had the lowest activity of superoxide dismutase (510.30 U/ml) whereas flower had the highest activity (772.33 U/ml). SOD had a mean value of 658.92 U/ml was presented in table 6.1. The p value was observed less than 5% (p 0.0004).

6.2.3. Assay of Guaiacol Peroxidase (GPO):

One significant type of peroxidase is guaiacol peroxidase (GPO), which oxidises methoxyphenol, a common reducing substrate. They are present in cytoplasm and appoplasm fractions of cells and have a variety of functions linked to the growth and development of plant.

Chemical Used:

- Phosphate buffer: 50 mM pH 7.0 and 40 mM, pH-6.1
- Guaiacol: 9 mM
- H_2O_2: 2mM

Method:

The Guaiacol Peroxidase was measured using the Egley *et al*. [121] method. The 0.1 gm of sample was homogenized with 2.0 ml of extraction buffer (50 mM pH-7.0 phosphate buffer combined with 9 mM of guaiacol) and then it centrifuges at 5,000 rpm for 15 min. at 4^0C. The final amount of the supernatant was made to 3.0 ml and stored at 0^oC. The assay combination included 0.05 ml sample extract, 2ml phosphate buffer (40 mM, pH-6.1), and 2 mM of 0.2 ml H_2O_2 in a total volume of 5.0 ml. At 30-second intervals up to one minute, a rise in absorbance was recorded at 420 nm (Extinction coefficient of 26.6 mM-1cm-1). One way to express enzyme-specific activity was as µmol of H_2O_2 decreased in a minute.

Result:

Antioxidant guaiacol peroxidase enzymes were evaluated from standard curve microgram per ml of linear equation on standard curve (y = 0.008x- 0.001, R2 0.999)

of the *C. ternatea*. The result was significant (p 0.0006) (Table 6.1). The overall mean value was observed 487.45 Units/ml in *C. ternatea* plant. The highest mean value was found for leaves (552.90 Unit/ml) and followed by flower (513.40 Unit/ml), and roots (471.90 Unit/ml), whereas the lowest value performance was recorded for seeds (411.60 Unit/ml).

6.2.4. Assay of Polyphenol Oxidase (PPO):

The metallic group known as polyphenol oxidases (PPOs) (EC.1.10.3.2) is composed of copper-containing oxygen-dependent enzymes that catalyze either one or two-electron oxidation of phenols. The polyphenols oxidase enzymes convert o-diphenols to quinones in the presence of oxygen. PPO enzymes are ubiquitous in higher plants, animals, fungi, and bacteria. Plants polyphenol oxidases are 40-72 kDa proteins. Several are associated with root plastids, tubers, leaves, fruits, and floral parts.

Chemicals Requirement:

- Sorbitol: 0.4M
- NaCl: 10mM
- Catechol solution: 0.01M catechol dissolved in Phosphate buffer (0.1M, pH 6.5)
- Tris-HCL buffer: 50mM, pH-7.2

Method:

The Esterbauer *et al*. method [122] was used to measure the activity of polyphenol oxidase. Using spectrophotometry, the activities of laccase and catechol oxidase were simultaneously measured in this approach. 0.5 gm of extract of leaves, roots, flowers, and seeds were homogenized separately in 4.0 ml of the extraction medium comprising Tris HCl, sorbitol, and NaCl. The homogenate was centrifuged at 5000 g for 10 minutes at 4^0C, and extract was made to 5.0 ml final volume. The cuvette was filled with 2.5 ml of 0.1 M phosphate buffer, 0.3 ml of catechol solution, and the spectrophotometer was calibrated to measure at 495 nm. In a spectrophotometer, the enzyme extract (0.2 ml) was added, and the absorbance change was noted every 0 to 5 minutes. The quantity of enzyme required to convert one micromole of di-hydrophenol into 1.0 micromole of quinone per minute under test conditions is known as one unit of PPO. The activity of PPO was expressed as Unit/ml. The activity of PPO was calculated by using following formula:

Enzyme Unit =

K × Absorbance per minute

Where,

K for Catechol Oxidase = 0.272

K for Laccase = 0.242

Result:

Table 6.1 and Figure 6.1, indicated that the polyphenol oxidase (PPO) activity significantly decreased in the leaves, roots, flowers, and seeds respectively. PPO activity was calculated through standard curve by linear equation (y = 0.420, R^2 0.995). The mean value of polyphenol oxidase activity is 827.94 Units/ml in the *C. ternatea* plant. In the present study, the minimum polyphenol oxidase activity recorded in seeds was 779.00 Unit/ml. The maximum PPO activity recorded in leaves was 879.93 Unit/ml followed by roots (840.67 Unit/ml) and flower (812.16 Unit/ml). The data was observed statistically significant at 5% (p 0.0003).

Table 6.1: The antioxidant enzymes of *C. ternatea* (Unit/ml)

Plant parts of *C. ternatea*	**Peroxidase**	**Superoxide dismutase**	**Guaiacol Peroxidase**	**Polyphenol Oxidase**
Roots	304.30±1.19	612.16±1.18	471.90±0.38	840.67±1.53
Leaves	376.33±1.08	740.86±0.55	**552.90±0.89**	**879.93±1.34**
Flowers	**391.20±0.70**	**772.33±0.67**	513.40±1.05	812.16±1.29
Seeds	289.14±1.01	510.30±0.67	411.60±1.02	779.00±1.32
Mean	340.23	658.92	487.45	827.94
C.D.	3.36	2.67	2.92	4.58
SE(m)	1.01	0.81	0.88	1.38
SE(d)	1.43	1.14	1.25	1.96
C.V.	0.52	0.21	0.31	0.29
P value <0.05	0.003	0.0004	0.0006	0.0009

Values are presented as mean±S.D, n=4.(Experiments were made as 3 parallel)

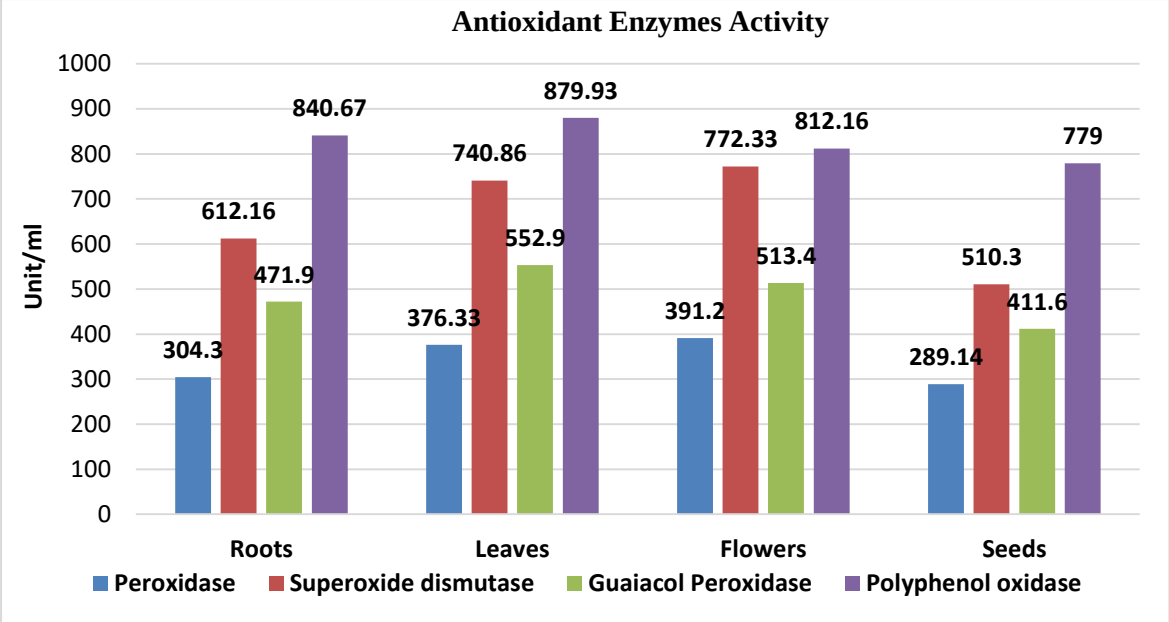

Figure **6.1:** The antioxidant Enzymes of the plant *C. ternatea*

6.3. Discussion:

CT's leaves, seeds, flowers, and roots obtained from plant were analyzed for enzymatic antioxidant activities. Peroxidase (POX), Superoxide dismutase (SOD), Guaiacol peroxidase (GPX), and Polyphenol peroxidase (PPO) activities change in various tissue as well as environmental factors. Enzyme antioxidant peroxidase can be used as a metabolic marker in relation to various stresses. Peroxidases are widely used as research enzymes in plant, and it is believed that they are involved in many physiological processes.

The result of the antioxidant enzymes i.e. Peroxidase, Superoxide dismutase, Guaiacol Peroxidase and Polyphenol oxidase activities in different plant tissue of *C. ternatea* are tabulated in Table 6.1 and Figure 6.1 above. The maximumactivity of peroxidase and superoxide dismutase were observed in flowers of CT i.e 391.20±0.70 and 772.33±0.67 rspectively, whereas in leaves antioxidant enzymes like Guaiacol Peroxidase and Polyphenol oxidase activities were found maximum i.e. 552.90±0.89 and 879.93±1.34 respectively. Herein, overall polyphenol oxidase activity was observed in leaves than to others.

The results showed that all selected anti-oxidant activities were observed lowest in seeds (i.e. peroxidase 289.14±1.01, Superoxide dismutase 510.30±0.67, Guaiacol

Peroxidase 411.60±1.02, and Polyphenol oxidase 779.00±1.320) in comparision to enzymatic activities of root, leaves and flowers. A major group of peroxidase, including SOD, catalase, PPO and GPOs, provides defence against ROS activity [123]. Methanolic leaves extract of *Ginkgo biloba* methanolic, and *Lawsonia alba* were significantly increased of guaiacol peroxidase and PPO activities [124]. These enzymes that catalyses the transformation of O^- radicals into O_2 and H_2O_2. The all peroxidase enzymes and non-specific peroxidase enzymes are mainly involved in ROS scavenging. The main role of these enzymes are cellular detoxification by trapping free radicals and sustains intracellular hydrogen peroxide (H_2O_2) levels.

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CHAPTER-VII

FORMULATION OF BIOGEL FROM THE BLUE FLOWER OF *CLITORIA TERNATEA*

CHAPTER- VII

FORMULATION OF BIOGEL FROM THE BLUE FLOWER OF *CLITORIA TERNATEA*

7.1. Introduction:

Clitoria ternatea is an extremely nutrient-rich plant that is utilised medicinally around the world. Medicinal plant components, including seeds, flowers, petals, leaves, stems, and roots, as well as occasionally entire plant, are utilised in medicine. [125]. Flavonoids, tannins, coumarins, xanthenes, and procyanidins are examples of phenolic antioxidants that have been shown to have the capacity to eradicate radicals in a way that is dependent on dosage. As a result, they have potential as medicinal medications for diseases linked to free radicals. These include improving cognitive function and reducing dementia, treating respiratory conditions such as asthma and bronchitis, reducing inflammation, and exhibiting laxative and diuretic actions [126]

In the previous chapter, the qualitative and quantitative properties of various plant parts were analyzed and discussed that blue flower of *Clitoria ternatea* have maximum presence of secondary metabolites and anti-oxidant potential than the other plant parts. The flowers of *Clitoria ternatea* were used as a natural coloring agent in food, tea, decorations, and other applications [127]. However, biogel or gel formulation from the flower extract is still lacking in the previous research study.

The genotype of blue flower of CT plant is easily avavilable in tropical reason. From the findings in previous chapters, it was observed that flower has more content of secondary metabolites (flavonoids, phenols, tannins, anthocyanin etc.) [128], antioxidants (DPPH, ABTs, Super Oxide anions and FRAP) and antioxidant enzymes (peroxidase, SOD, PPO and Guaiacol peroxidase). Therefore, this attributed to choose blue flowers for biogel preparation rather choosing the other parts of the plant or taking whole plant for this purpose.

The study therefore formulated a biogel with an ideal consistency and assessed it according to several criteria in order to investigate the possible applications of floral extract in a gel basis for therapeutic, pharmacological, and cosmetic purposes. Thus

the objective of present study was formulation of biogel from blue flowers of *Clitoria ternatea* with good consistency, viscosity, transparency, and evaluated it by different physical parameters at different temperature and relative humanity. Besides these investigation, biogel was also tested by its antimicrobial property and toxicity.

7.2. Sample collection:

The flowers of *C. ternatea* were obtained from the nursery of the Central Public Works Department, Mehrauli, Delhi. All analytical work was performed in the Department of Biochemistry, NIFTEM, Sonipat, Haryana. This laboratory was used for extraction process of *C. ternatea* to upto biogel formulation and its test of evaluation. The another laboratory was also used for further test of antimicrobial activity and toxicity of formulated biogel i.e AGSS Analytical and Research Lab (P) Ltd. Delhi (An ISO 9001:2015, 14001: 2015, 45001:2018 Certified Company).

Chemicals Used:

- Disodium EDTA
- Carbopol 940
- Methyl Paraben
- Glycerin
- Triethanolamine
- Citric acid
- Ethanol

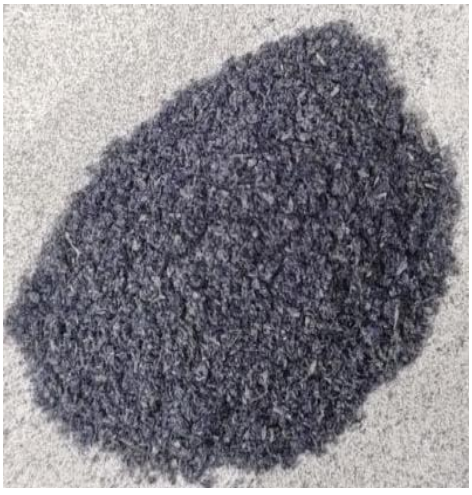
7.2.1. Method of Extraction:

The petals of the blue flower were dried naturally in shade at room temperature (37°C). Once dried, 200 mg of the dried petals were homogenized with 100 ml of 95% ethanol in an Erlenmeyer flask. The mixture was kept using an automatic shaker at room temperature (37°C) for 15 minutes at 250 rpm. Afterward, the crude mixture was centrifuged at 5000 rpm for 10 minutes. Collect the supernatant in the beaker and cover it with aluminum foil. To concentrate the extract, reduced pressure was applied using a rotary vacuum evaporator. The resulting product was a crude extract weighing dry matter of flower extract. This dry extract was stored at 20°C for further observation.

A. Flower blooming of *Clitoria ternetea*

B. Picking the fresh flower of CT

C. Grinding flowers during preparation of Biogel



D. Dry Powder of *Clitoria ternatea* flower

Figure 7.1: Processing of CT blue flowers for preparation of Biogel

7.3. Formulation of Biogel of *Clitoria ternatea:*

Method:

The method for preparation of biogel was followed as establish on different medicinal plant by Jamadar M.J. *et al*. (2017) with modification [129]. For the preparation of biogel, a gel base was mixing by adding 1.0 g of Carbopol 940 to 40 mL of distilled water. The mixture was left to stand for 1 hour. After that, 2 mL of triethanolamine and 100 mg and 200mg dry extract of flower weigh separately and were mixed with 30 ml of distilled water. The mixture was the content of 30 mL of flower extract, 0.1 g of citric acid, 0.01 g of disodium EDTA, 0.1 g of concentrated methylparaben and 15 g of propylene glycol. The solution was then cooled, and then 1g of glycerin was added and mixed thoroughly. The volume was brought to 100 mL by adding distilled

water. It was important to ensure that Carbopol 940 was properly mixed with the mixture by continuously stirring, and triethanolamine was added gradually to adjust the pH of the formulation to the desired range of 6.8-7 and achieve the desired consistency of the biogel. (Table 7.1 and Figure 7.2).

Table 7.1: Chemical Ingredients used for formulation of Biogel:

Formulation	Ingredients	Quantity
Control	Carbopol 940	1.0 gm
	Triethanolamine	2.0 ml
	Citric acid	0.1 gm
	Disodium EDTA	0.01 gm
	Methylparaben	0.1gm
	Propylene glycol	15.0 gm
	Glycerine	1.0 gm
	Distilled water	Up to 100 ml

7.4. Methods of Evaluation of Biogel:

A. Physical Evaluation:

The color and appearance of the biogel are checked by physical parameters.

B. **pH Measurement:**

The gel of pH was measured by using a pH meter.

Figure: A

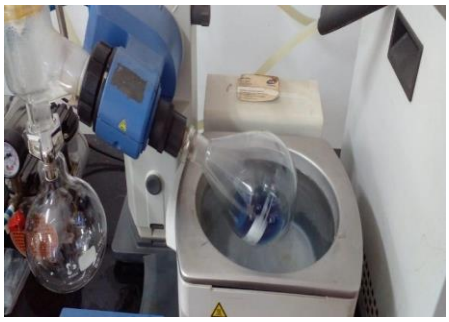


Figure: B

Figure 7.2 : A and B: Method of extraction of *Clitoria ternatea*

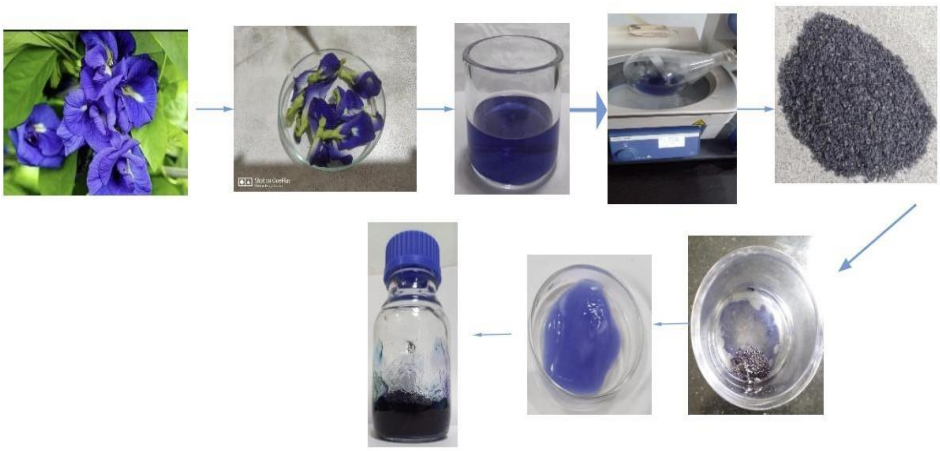


Figure 7.3: Processing and preparation of Biogel of *Clitoria ternatea*

C. Spreadability Test:

Method:

The spreadability of the gel was determined using an apparatus consisting of a wooden block attached to a pulley at one end [130]. This method measured spreadability based on the slip and drag characteristics of the gels. Approximately 0.5 g of the gel under study was placed on a ground slide. The gel was then sandwiched between this slide and another glass slide, which had the same dimensions as the fixed ground slide and was equipped with a hook. A 1kg weight was placed on top of the two slides for 5 minutes to remove air and ensure a uniform film of gel between the slides. The excess gel was removed from the edges. The top slide was then pulled with a force of 80 grams using a string attached to the hook. The time (in seconds) taken by the top slide to cover a distance of 7.5 cm was recorded. A shorter time interval indicated better spreadability of the gel. Spreadability was calculated using the following formula:

$$S = W \times L / T$$

Where,

S = Spreadability,

W = Weight in the pan (tied to the upper slide)

L = Length Stretch by a glass slide

T = Time (sec.) interval of separation of the two slides.

D. Test of Stability

The test of the stability of the formulated biogel was conducted following the guidelines set by the International Council for Harmonisation for Pharmaceuticals for Human Use (ICH) [131]. The gel was filled into collapsible tubes and stored under various temperature and humidity conditions for a duration of three months. The storage conditions included 20°C ± 10°C/60% ± 5% RH, 25°C ± 10°C/60% ± 5% RH, and 30°C ± 10°C/60% ± 5% RH. During these three months, the biogel was tested for its appearance, colour, viscosity, pH, and spreadability to assess its stability

7.5. Skin Sensitivity Test

Method: For the testing process, 0.5 gm of the herbal biogel was used as the test sample and applied to an approximate area of 15 cm^2 of human skin. The applied gel was covered with a gauze patch, which was lightly secured in place using a semi-occlusive dressing for a duration of 1 hour. After 1 hour, the gauze patch was removed, and any remaining test substance was gently wiped away without causing any disruption to the skin's integrity or any existing responses. Observations were then recorded after the patch removal. A control group was also established using the same procedure, where 0.5 gm of the gel base (gel formulated with all ingredients except the flower extract) was applied, and observations were made in a similar manner to the test group. The gel was applied to the skin once a day for a period of 7 days, and any sensitivity or reactions observed were graded accordingly.

Result: The formulated biogel was evaluated by further different test like physical evaluation and skin sensitivity test. The physical test of biogel was evaluated by the parameters like appearance, pH, spreadability, viscosity, stability, skin and sensitivity test for a period of three months under varying temperature (20^OC, 25^OC and 30^OC) and humidity levels (RH 60%). The results observed that the formulated biogel was exhibited superior stability with standard PH (6.9-7.04). The parameters were also tested with two formulations of biogel i.e. 0.1% and 0.2%. However, the spreadability and viscosity of biogel were also observed good with range (18.2 and 25.4 gm.cm/sec against control) and (2100 and 2200 cps(centipoise) against the control) respectively (Table 7.2, 7.3 and 7.4).

Additionally, the biogel was also tested on human skin up to 7 days of regular used with two time a day (Table 7.5). The result was showed very good on skin. There was not found any signs of skin irritation, edema and patches. It was notice that the regular use of the biogel resulted in smoother and more radiant skin texture. The appearance of biogel was obtained dark blue colour with good clarity, transparent, homogenous and smooth (Table 7.2, 7.3 and 7.4).

Table 7. 2: Physical Evaluation of biogel during formation: First Month (20°C ±10°C/60% ± 5% RH),

Formulation	Color	Clarity	Appearance	Spreadability (gm.cm/sec)	pH	Viscosity (cps)	Appearance
Control	White	+++	Clear, Transparent	10.5	7.04	1830	Homogeneous
F1 (0.1%)	Blue	+++	Clear, Transparent	18.2	7.04	2100	Homogeneous
F2 (0.2%)	Blue	+++	Clear, Transparent	25.4	7.04	2200	Homogeneous

Table 7.3: Physical Evaluation of biogel during formation: Second Month (25°C ± 10°C/60% ± 5% RH)

Formulation	Color	Clarity	Appearance	Spreadability (gm.cm/sec)	pH	Viscosity (cps)	Appearance
Control	White	+++	Clear, Transparent	10.8	7.02	1830	Homogeneous
F1 (0.1%)	Blue	+++	Clear, Transparent	19.6	7.01	2150	Homogeneous
F2 (0.2%)	Blue	+++	Clear, Transparent	24.7	7.0	2240	Homogeneous

Table 7.4: Physical Evaluation of biogel during formation: Third Month (30°C ± 10°C/60% ± 5% RH)

Formulation	Color	Clarity	Appearance	Spreadability (gm.cm/sec)	pH	Viscosity (cps)	Appearance
Control	White	+++	Clear, Transparent	11.1	7.0	1830	Homogeneous
F1 (0.1%)	Blue	+++	Clear, Transparent	19.1	7.0	2180	Homogeneous
F2 (0.2%)	Blue	+++	Clear, Transparent	23.5	6.9	2260	Homogeneous

Where:

F1= 1st concentration (100 mg dried extract),

F2 = 2nd concentration (200 mg dried extract) of *C. ternatea*

(Turbid: +, Clear: ++, Very Clear (Glassy): +++).

Table 7.5: Result of Skin Sensitivity Test:

Treatment	1st day	2nd day	3rd day	4th day	5 day	6th day	7th day
Control	N	N	N	N	N	N	N
F1 (0.1%)	N	N	N	N	N	N	N
F2 (0.2%)	N	N	N	N	N	N	N

Where:

N – No reaction and non irritent,

A – Slightly patchy erythema,

B – moderate erythema,

C-Severe erythema

7.6. Antimicrobial activity test of *C. ternatea's* Biogel:

Method:

The study was to investigate about the antibacterial and antifungal activity performed by Agar diffusion methods by the zone of inhibition at three different biogel concentration i.e. 10%, 20% and 30% w/v. For antibacterial activity, three microorganism like *Staphylococcus aureus*(NCIM-2079), *Escherichia coli* (NCIM-2065) and *Bacillus cereus* (MTCC-430) were selected. Also, for the antifungal activity, one microorganism was selected as *Candida albicans* (NCIM-3100) for the present study. For the culture preparation, taken 5ml Nutrient Broth transfered loopful culture to prepared fresh culture media for the *S. aureus, E.coli* and *Bacillus cereus*. And all were incubated at 37^0C for 24 hour. Whereas, for the fungus culture preparation, taken 5ml Soyabean Casein Digest Medium transfer loopful culture to prepared fresh culture media of *Candida albicans.* It also incubated at 37^0C for upto 72 hrs. For the preparation of plate, Plate count agar by inoculating the 100 micro liter freshly prepared culture from Nutrient broth and pouring 15-20 ml molten plate count agar and allow to stand for 30 minute till solidification. And made four 6 mm wells with the help of borrer and transfer the 100 ul of prepared sample concentration at w/v

(10%, 20%, and 30%) in each well and 10% DMSO as positive control and let it stand for upto 30 minutes and incubated at 37^0C for 48 hours.

For the fungal plate preparation, plate count agar was prepared by inoculating the 100 micro liter freshly prepared culture of Soyabean Casein Digest Medium with the same procedure. The biogel was prepared in three different concentrations like 10%, 20% and 30%. Biogel was taken 10 gm, 20 gm and 30 gm in separate each and transferred to 90 ml, 80 ml and 30 ml sterile saline water in their respective for the concentration of 10%, 20% and 30%.

The of procedure of bacterial and fungal activity, the plates of Plate count agar made four 6 mm wells with the help of borrer and transfered the 100 ul of prepared sample concentration at w/v (10%, 20%, and 30%) in each well and 10% DMSO (dimethyl sulfoxide) used as positive control and let it stand for upto 30 minutes and incubated at 37^0C for 72 hours for fungus.

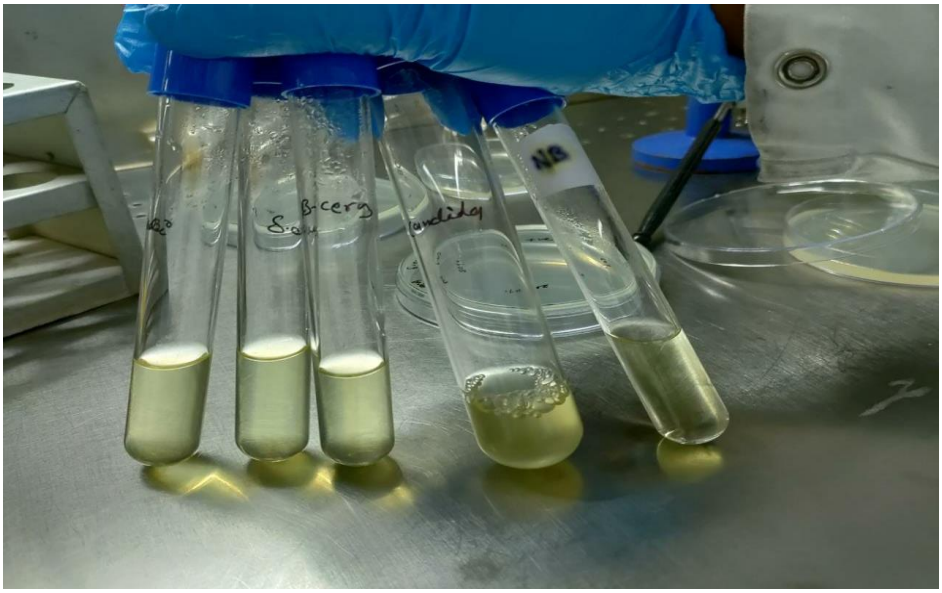


Figure 7.4: Culture Broth of microbes

Result:

The effect of antibacterial and antifungal activities of biogel against control was measured by the zone of inhibition at three different biogel concentration i.e. 10%, 20%, 30% w/v. The observation was given below in the table 7.6.

Table 7.6: Observation of Antimicrobial activity of Biogel (*Clitoria ternatea* Blue flower)

S. No	Category	Microorganisms	Biogel -Sample Concentration (%) (Diameter mm)			Positive control DSMO
			10%	20%	30%	
1	Antibacterial activity	*Staphloccocus aureus*	9.0	11.1	13.2	16.0
2		*Escherichia coli*	7.8	12.0	14.5	16.5
3		*Bacillus cereus*	7.1	11.3	13.1	15.1
4	Antifungal Activity	*Candida albicans*	7.5	11.5	13.3	14.8

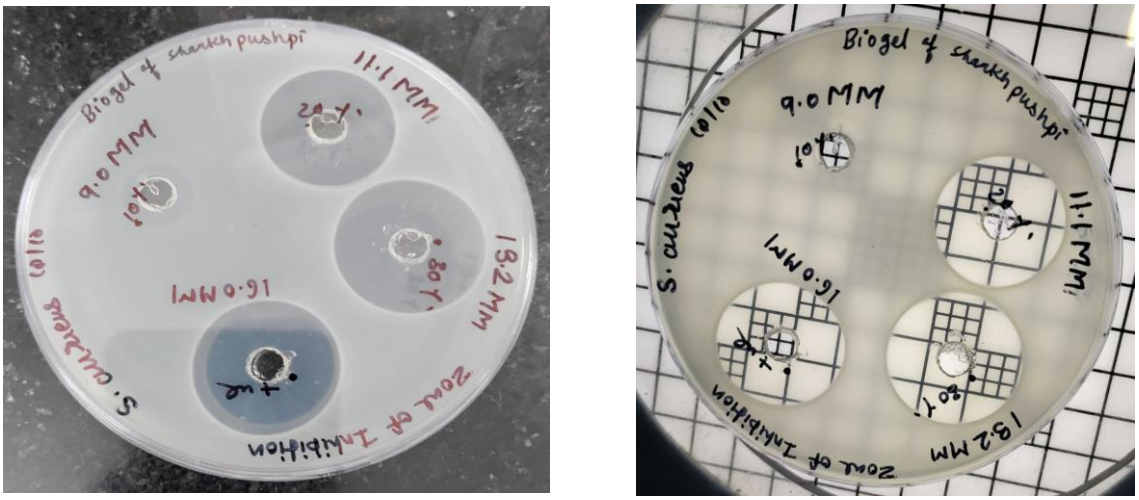


Figure 7.5: Inhibitory effect of *S. aureus* at different concentration of Biogel (dia. mm)

From the Table 7.6 and Figure 7.5, 7.6, and 7.7 had observed that the antibacterial activity of biogel of *C. ternatea* blue flower against *Staphylococcus aureus, Escherichia coli and Bacillus cereus* at different concentration of biogel (10%, 20% and 30%) through inhibition zone. The highest inhibition zone against positive control obtained from *Escherichia coli* (14.5 mm/16.5 mm) followed by *Staphylococcus aureus* (13.2 mm/16.0 mm) *and Bacillus cereus* (13.1mm/15.1 mm) at 30% concentration of biogel.

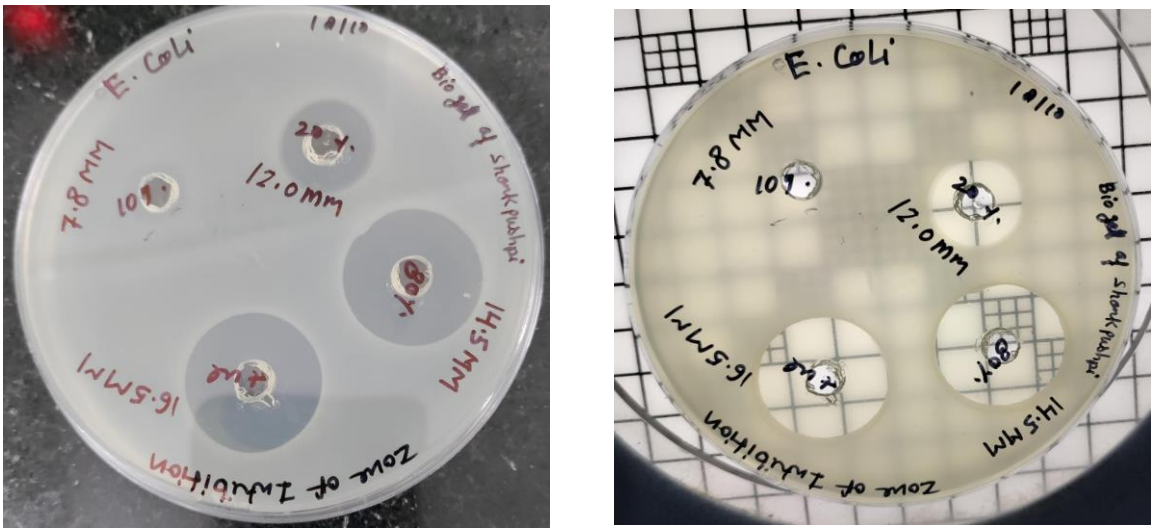


Figure 7.6: Inhibitory effect of *E. coli* at different concentration of biogel (dia. mm)

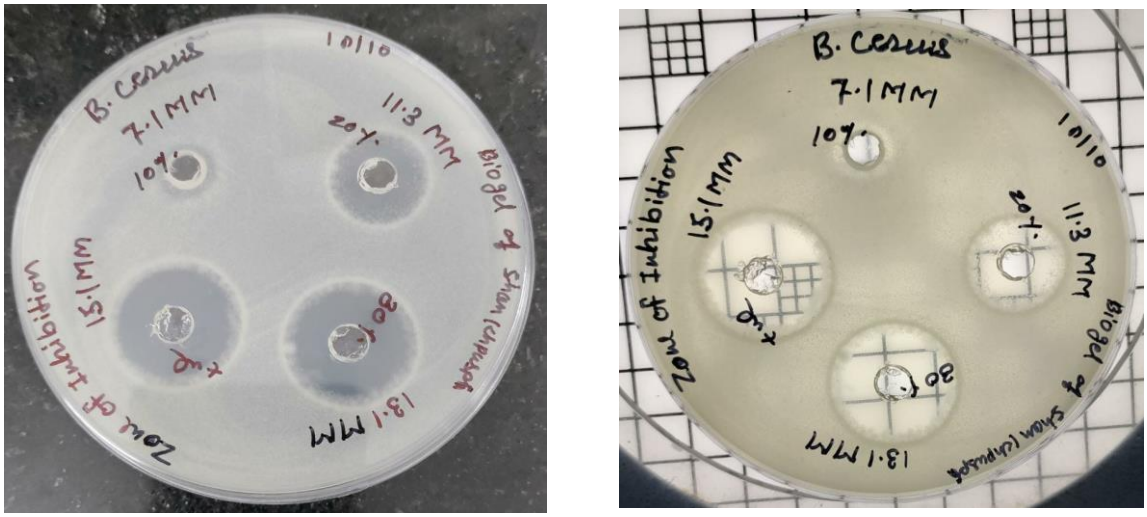


Figure 7.7: Inhibitory effect of *B. cereus* at different concentration of biogel

The antifungal activity of CT biogel was observed from the Table 7.6 and Figure 7. 8 showed that higher inhibition zone against positive control obtained from *Candida albicans* (13.3 mm/14.8 mm) at 30% concentration of biogel as compared to concentration of 10% and 20% i.e.7.5mm and 11.5 mm respectively. It was also observed that effect of biogel was shown at 48 hours for anti-bacterial activity whereas it was found 72 hours for anti-fungal activity. From Table 7.6 it is observed that after increase of concentration of biogel, the anti-bacterial and anti-fungal activity were increased.

Figure 7.8: Inhibitory effect of *C. albicans* at different concentration of Biogel

7.7. Toxicity test of *Clitoria ternatea*'s Biogel

Method: The biogel was analysed through the Instrument ICP-MS (Inductively Coupled Plasma Mass Spectrometry) for heavy metal toxicity. Through ICP-MS heavy metal is measured at trace level at milligram to nanogram level in biological fluids, food, plant etc (Figure 7.9). The 0.25g/ml sample was used in the liquid form and inserted by syringe in the instrument of nebulizer. The Nebulizer of Instrument convert liquid into gas as fine mist. The instrument's detector detects the particles of the sample with its mass.

Result: For the determination of toxicity biogel of C. ternatea, the formulated biogel was analyzed through ICP-MS instrument for the detection of heavy metals of Arsenic and Lead. The result was observed that negligible presence of Arsenic(As) and Lead (Pb) in the biogel i.e. 0.01 mg/kg for each. It indicated that biogel was free from heavy toxic metals and could be used as medicated gel. As per World Health Organisation (WHO), the maximum permissible limit is fixed 0.01 mg/kg for Arsenic and 0.05 mg/kg for lead for the cosmetics. The formulated biogel of C. ternatea is within the permissible limit of As and Pb. Hence, it is safe for use.

Figure 7.9: Inductively Coupled Plasma Mass Spectrometry(ICP-MS)

7.8.Discusion

Findings of biogel was showed above important benefits which have natural properties in opposing different diseases in the human body. Natural remedies are more acceptable in the belief that they are safer with rarer side effects than the synthetic ones. The biogel was prepared in two formulations i.e. 100 mg and 200mg dried extract of blue flower of CT. According to results of physical parameters, biogel was observed that 200 mg dry extract's formulation was better spreadability and viscosity. The results observed that the both formulated biogel were exhibited deep blue colour, transparent, superior stability, standard P^H (6.9-7.04), good viscosity (2100-2200 cps) and good spreadability (range 18-25gm.cm/sec) at against control. Additionally, the biogel was tested on human skin up to 7 days used twice in a day. The result on skin observed with non-irritant and non-allergic. In fact, regular use of the biogel resulted in smoother and more radiant skin texture. Furthermore, *Clitoria ternatea* could be improve the quality of blood by potentially enhancing its ability to efficiently carry oxygen and nutrients to the body's cells.

The antimicrobial activity of biogel, the highest inhibition zone of *Escherichia coli* (14.5 mm/16.5 mm) followed by *Staphylococcus aureus* (13.2 mm/16.0 mm) and *Bacillus cereus* (13.1mm/15.1 mm) at 30% concentration of biogel in 42 hours whereas antifungal activity (*Candida albicans*) was observed (13.3 mm/14.8 mm) at

30% concentration of biogel in 72 hours were found. The toxicity of biogel was tested for the detection of Arsenic and Lead through ICP-MS and obtained the result of toxic elements was detected in very minute quantity i.e. 0.01 mg/kg which could be ignored.

The observation exhibits remarkable properties like antioxidants, nontoxic and antimicrobial in CT biogel which also contains many phytochemicals like flavonoids, phenols and antioxidant enzymes. The overall it can say that biogel of *Clitoria ternatea* could be used safely for medicated purposes and applicable for industrial production as herbal medicines.

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CHAPTER- VIII

SUMMARY AND CONCLUSION

CHAPTER- VIII
SUMMARY AND CONCLUSION

8.1. Summary and Conclusion:

Belonging to the fabaceae family, *Clitoria ternitea L.* is a perennial herbaceous plant having numerous medicinal qualities. It is often referred to as butterfly pea or blue pea plant. Its plant parts have various photo-chemicals and are utilized in numerous kinds of pharmacological activities. They are scientifically validated and found wide range of enzymatic antioxidant, anti-diabetic, remembrance enhancer, anti-depressant properties, tranquilizing antistress as well as sedative agents. Due to its many therapeutic benefits, it has a long history of usage in traditional Chinese and Indian Ayurvedic medicine despite of knowing its phytoconstituents. Previous research done by various reserchers and scientist were studied in the Chapter II- Review of Literature. It was observed that the research is lacking for comparartive study phytochemicals, enzymatic, non-enzymatic activities and therapeutic values of different parts of plant of *Clitoria ternatea*. The medicinal properties of *C. ternatea* are attributed to its various range of bioactive compound including total carbohydrates, amino acids, total phenols, saponin, flavonols, terpenoids, flavonoids, quinones, tannins, oxalates, resins and anthocyanins. Hence, this research was performed.

The aim of the study was based on selected parameters of all objectives (as mentioned at Para 2.14 of Chapter II) which were done to compare the antioxidant activities of four different plant tissues (roots, seeds, leaves and flower parts) of blue flowered genotypes of *Clitoria ternatea*.

Chapter III*:*

Qualitative tests of any compound revealed that secondary metabolites are strongly and moderately present or absent in the extracted sample based on the indication of dark to light colour. For the analysis, the extractions of the all phytochemicals were performed in 60% ethanol. The qualitative analysis of different bioactive phytochemicals from ethanol extracts of different plant tissue (roots, seeds, leaves, and flowers) of *Clitoria ternatea* blue flower genotypes was performed. The

qualitative test of various phytochemical constituents' presence and absence of plant parts are done.

The qualitative test of these secondary metabolites revealed that the carbohydrates, present in all tissue of CT plant. The result showed that the seeds are the main source of terpenoids, sterol, carbohydrates, tannins, amino acids, and proteins. However, resins, phenols, flavonoids, glycosides, as well as alkaloids are present in moderates, while quinines in less in seeds. Other secondary metabolites like oxalates and saponins were absent in seeds of *Clitoria ternetea*.

As per outcome of the study, flower extract contain more present of alkaloids, flavonoids, phenols, tannins, terpenoids, quinones, and moderate present of resins while less present of oxalates, sterols, amino acids & proteins, glycosides. However, the flower extract had no saponin. Alkaloids, glycosides, phenols, tannins, sterols, quinones, qxalates, and resins are missing from the root extract of Clitoria, whereas flavonoids, amino acids, proteins, and terpenoids are less prevalent in it.

The leaves are very important parts of plant because the most of primary and secondary metabolites biosynthesis during the time of photosynthesis and most of translocated and present in leaves parts of the plant. The qualitative test showed that phenols were highly present in the leaf extract whereas flavonoids, tannins, sterols, quinones, were moderately present. While resins, terpenoids, amino acids & proteins, glycosides were showed in less and alkaloids, saponin and oxalates were absent in CT leaves. The study revealed that *Clitoria ternatea* plant parts have presence of various secondary metabolites.

Based on the aforementioned qualitative investigation, it can be inferred that the *Clitoria ternatea* plant is a significant producer of secondary metabolites with significant therapeutic potential because of the natural chemicals it contains. Furthermore, the results showed that every part of the high medicinal value plant *Clitoria ternatea*, including its roots, leaves, flowers, and seeds, may be used to make herbal medicines and potentially treat severe human illnesses.

Chapter IV:

In comparison to qualitative analysis, quantitative analysis is a more thorough and practical approach because the findings of the studies can be applied to drug discovery, herbal drug standardisation, plant medicinal potential explanation, and plant toxicity level determination. To assess the quality of the plant and identify any possible medicinal uses, precise quantification of its bioactive components is necessary.

Under this chapter, the quantitative analysis was done adopting various research methods in laboratory. Observations were recorded of the total carbohydrates, total phenols, flavonoids, tannins and anthocyanin in different parts of extract (seeds, flower, leaves and roots) of *Clitoria ternetea*. The total carbohydrate was quantified through ethanol extract of plant tissue of all parts. The biochemical studies revealed that the total carbohydrates i.e. 89.01 mg Glc/g Fresh wt. content was found maximum in leaves tissues, and its average mean value was obtained 76.75 mg Glc/g Fresh wt. Similarly, the other parameters such as total phenolic content (93.00 mg Glc/g Fresh wt.), total flavonoids (469.34 Catechine mg/g Fresh wt.), total tannins (72.00 mg TAE/g Fresh Wt.), and total anthocyanins (178.33 mg CGE/g Fresh wt.) were found highest in flowers extract and its mean value were 70.50, 273.75, 41.00 and 87.75 respectively.

This study revealed that *C. ternatea* plant has highest saturation of secondary metabolites, or maximum amounts of carbohydrates, in its leaves, while its flowers are rich in polyphenols such anthocyanin, tannin, flavonoids, and phenols. The findings indicate that the *C. ternatea* plant, particularly its flower, has great significance in the Ayurvedic and pharmaceutical industries. *Clitoria ternetea* holds great promise as natural source of bioactive compounds with different health benefits. These compound contribute to its medicinal properties and have the important health implication.

Chapter V*:*

In this chapter, study of quantitative estimation of antioxidants was undertaken through various assays in CT plant. The DPPH (diphenyl-picryl hydrazine), FRAP, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and superoxide radical

scavenging tests were among these assays which helps in assessment of *Clitoria ternatea's* antioxidant activity. The observation recorded maximum activities of FRAP in leaves (399.73). Whereas, DPPH, ABTs and superoxide scavenging were maximum in flower 92.43, 89.36 and 89.33 respectively. From results, the highest percentage inhibition of DPPH and ABTS activities was observed in 88.43 mg (ascorbic acid)/ g FW and 96.70 (GAE) mg/g FW in flowers of the *C. ternatea* over the mean 85.81 and 81.92 respectively. Whereas, the inhibition percentage of DPPH and ABT activities in roots (48.8 and 68.73), leaves (79.73 and 85.40), flower (88.43 and 96.70) and seeds (48.20 And 90.23) were observed the hightest at 1.0 mg/ml concentration. The results showed in general, maximum inhibition activities of FRAP, ABTs, DPPH and superoxide were recorded maximum at concentration of 1.0 mg/ml over 0.2, 0.4, 0.6, 0.8 and 1.0 ml concentration against control of different part extract of CT. The comparative study showed that superoxide inhibition activity was found the highest in all parts of CT plant in comparison to other selected antioxidants.

Evaluating the antioxidant activity of natural products, food additives, and foods is an essential part of research in the field of functional factors. Antioxidants trapped free radicals during stress condition. Oxidative stress, affected by an imbalance between reactive oxygen species (ROS) production and the body's ability to neutralize them, has been implicated in various diseases including cancer, asthma, cardiovascular diseases, and neurodegenerative disorders. The antioxidant potentials in butterfly pea plant are also useful to treat many disease, and good source of memory enhancer.

Chapter VI:

In this chapter, oxidative stress enzymes like peroxidase, Guaiacol peroxidase, superoxide peroxidase and polyphenol oxidase were studied. The results showed that peroxidase activity was highest in flower 391.20 unit/ml followed by leaves (376.33) and lowest value was recorded in seed (289.14 unit/ml) whereas mean value was recorded at 340.23. Superoxide dismutase activity was also recorded highest in flower (772.33 Unit/ml). Guaiacol peroxidise and Polyphenol oxidase were observed highest in leaves i.e. 552.90 Unit/ml and 870 Unit/ml respectively in compared to other plant parts. The study concluded that maximum activity of antioxidant enzymes was found in flowers than leaves and other parts of CT plant. Maximum antioxidants

potentials showed the maximum power of trapping free radicals of the cells. These antioxidants molecules protect the cell organelles from oxidative damage, and they are used as a defence mechanism in removing or repairing damaged molecules. Scavenging of these toxic reactive speciesisperformed by both antioxidant metabolites, such as glutathione (GSH) and ascorbate (AsA) as well as others antioxidant enzymes. The functions of these enzymes like peroxidase and superoxide dismutase (SOD) are catalyze the conversion of O_2^- radical into O_2 and H_2O_2. The peroxidases enzymes are critical constituents of cellular detoxification system that maintained intracellular level of Hydrogen peroxide (H_2O_2). Phenolic and flavonoids compound are protecting the cells from damages of the diseases.`

Statstiscally, all data of the results Chapter IV, V, & VI) were observed highly significant at 5%.

Chapter VII:

In this chapter, the biogel was prepared from different chemical ingredients of CT blue flower extract and showed its effect of antimicrobial activity as well as toxicity. The studies revealed that the two biogel formulations (0.1% and 0.2%) consisting *Clitoria ternetea* blue flower extract comparatively better (0.2%) than other formulations. Studies of observation of biogel formulations were found clear, homogenized, with blue color, transparent, good viscosity, excellent stability at different temperature and humadity alongwith favorable pH 6.9-7.04. The process of screening biogel was also undertaken to examine and analyse the biogel's safety profile before using it to treat different types of skin.

The biogel of the blue flower of CT was tested by its antibacterial and antifungal activities for its use in medicinal purposes and in the pharmaceutical industries at large. The test was performed by zone of inhibition at three different biogel concentrations i.e. 10%, 20% and 30% w/v. Three bacterial culture i.e. *Staphylococcus aureus* (NCIM-2079), *Escherichia coli* (NCIM-2065) and *Bacillus cereus* (MTCC-430) were selected. Also, for the antifungal activity, one microorganism was selected as *Candida albicans* (NCIM-3100) for the present study. The observation revealed that the highest inhibition zone against positive control obtained from *Escherichia coli* (14.5 mm/16.5 mm) followed by *Staphylococcus*

aureus (13.2 mm/16.0 mm) *and Bacillus cereus* (13.1mm/15.1 mm) whereas antifungal activity of *Candida albicanes* observed 13.3 mm/14.8 mm at 30% concentration of biogel.

The presence of heavy or toxic metals like Arsenic and Lead in formulated biogel of *Clitoria ternatea* was also analyzed through the ICP-MS and noticed negligible presence of Arsenic (As) and Lead (Pb) in the biogel i.e. 0.01 mg/kg for each. It indicates that biogel is free from heavy or toxic metals and could be used as a safe medicated gel. It is a best effort has made to establish the medicinal plant extract biogel containing *Clitoria ternatea* blue flowers extract. *Clitoria ternatea* flower bio-gel has shown important benefits and natural properties in opposing different diseases in the human body.

Herbal formulations have growing demand in the world market. Natural remedies are more acceptable in the belief that they are safer with rarer side effects than the synthetic ones. The medicinal plant *Clitoria ternatea* can be growing and cultivated as a crop globally, gaining acceptance from medical specialists and the pharmaceutical industry due to its natural properties and effectiveness in treating several well-known diseases.

Overall, it can say that biogel of blue flower of *Clitoria ternatea* could be used safely for herbal medicine as well as pharmaceutical industry. Finally, it can be said that the study has achieved the result of its objectives.

8.2. The Future Prospect of Present Research:

The present study on the butterfly pea plant found some gaps that might be improved and included some new research work for future plans:

- Need to investigate more phytochemicals and enzymes which can be used in different applications in the field of pharmaceuticals for the treatment of various diseases.
- Isolation of pure form of bioactive compounds extracted from *Clitoria ternatea* for formation of herbal medicine.
- Due to Anti-microbial properties of *C. ternatea*, toothpaste (dental cream), face cream, body lotion. Soap may be manufactured.

- ❖ Preparation of value added product of each part of plant like herbal health drinks, biscuits, candy and chocolates.

Therefore, future studies ought to concentrate on delving deeper into the possible uses and health advantages of the bioactive substances found in *Clitoria ternatea*. As a naturally occurring source of bioactive substances with a range of health advantages, *Clitoria ternatea* has a lot of potential. New therapeutic agents and the advancement of herbal medicine will both benefit from more research and development in this area.

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APPENDIX-I

List of Chemicals used for Analytical Study

S.No.	Chemicals	Company Name /Make
1.	1α naphthol	Oxford Lab Fine Chem LLP
2.	ABTs solution	Innovative Lab
3.	Acetic acid	The Pioneer Chemical Company
4.	Acetic anhydride	The Pioneer Chemical Company
5.	Acetone	Oxford Lab Fine Chem LLP
6.	acid n-butanol	Oxford Lab Fine Chem LLP
7.	Antrhone	Oxford Lab Fine Chem LLP
8.	Ascorbic acid	Advance Inorganic
9.	BHT- Butylated Hydroxy Toluene	Innovative Lab
10.	Carbopol 940	Prime Chem
11.	Catechine	Merck Life Science Pvt. Ltd
12.	Chloroform	Merck International
13.	Citric acid	Pioneer Chemical Company
14.	Cyanidin-3-glucoside equivalents	A B enterprises
15.	Disodium EDTA	Mrinal chemtech Pvt. Ltd.
16.	Distilled Water	DSS Chem Enterprises LLP
17.	DPPH	Innovative Lab
18.	Ethanol	Shri Sai Lab & Pharma Co.
19.	Ethylene diamine tetra acetic acid (EDTA)	R.S. Dye Chem
20.	Ferric chloride	Gurdial Mal Chhoga Lal Jain Co.
21.	Folin-Ciocalteu reagent	Vital Science Industry
22.	Gallic acid	Marc Flavours
23.	Glacial acetic	Hindustan Organic Corporation
24.	Glucose	Innovative Lab
25.	Glycerine	Charu Chem & La services
26.	Guaiacol Solution	Jigs chemical Ltd.
27.	Hydrochloric acid	Sunder Chemicals
28.	Hydrogen Peroxide	ARK Chemicals

29.	Hydroxylamine hydrochloride	Sigma Inc.
30.	Iodine	Oxford Lab Fine Chem LLP
31.	Methanol	P S Polychem
32.	Methyl paraben	Goyal Chem Associates
33.	NADH(Nicotinamide adenine nucleotide)	Innovative Lab
34.	NBT (Nitroblue tetrazolium)	Innovative Lab
35.	Ninhydrin solution	Innovative Lab
36.	Phenazine Methosulphate	R P Chemicals
37.	Phosphomolybdic acid	Oxford Lab Fine Chem LLP
38.	Phosphoric acid	**Colorchem Industries Limited**
39.	Potassium Ferricyanide	Cynor Laboratories
40.	Potassium iodide	R P Chemicals
41.	Potassium Persulphate	Advance Chemicals Sales Corp.
42.	Potassium Phosphate buffer	Oxford Lab Fine Chem LLP
43.	Propylene glycol	Acuro Organics Ltd.
44.	Sodium acetate	Oxford Lab Fine Chem LLP
45.	Sodium Carbonate	Oxford Lab Fine Chem LLP
46.	Sodium hydroxide	Vardhman Impex
47.	Sodium Nitrate	Oxford Lab Fine Chem LLP
48.	Sodium pyrophosphate buffer	Tricon Speciality Chemicals Pvt Ltd
49.	sodium tungstate	Koncept solutios
50.	Sorbitol	Advance Inorganics
51.	Sulfuric acid	Ess Dee Chemicals
52.	Sulphosalicylic acid	Polychemtech India
53.	Tannic acid	Rajvi Enterprise
54.	Triethanolamine	Oxford Lab Fine Chem LLP
55.	Tris-HCL buffer	Oxford Lab Fine Chem LLP

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