4.3 BIOCHEMICAL ANALYSIS

Biochemical analysis was performed to identify and compare the presence of key phytochemicals such as alkaloids, flavonoids, phenols, proteins, saponins, steroids, and carbohydrates in both plasma-treated and control seed samples. This analysis helped evaluate the effect of plasma on enhancing the phytochemical profile of the seeds.

4.3.1 Extraction Process for Fenugreek Seeds (Soxhlet Method)

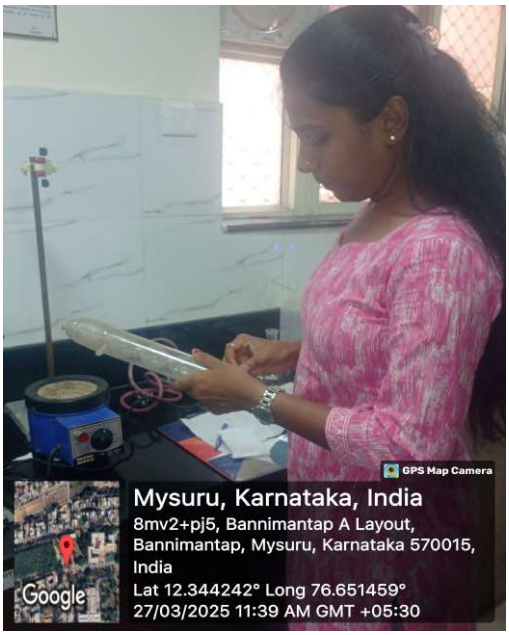
For fenugreek seeds, the **Soxhlet extraction method** was employed due to its efficiency in extracting heat-stable phytochemicals in large quantities. The process involved the following steps:

Seed Preparation:

Cleaned fenugreek seeds were shade-dried and ground into fine powder using mortar and pestle.

Soxhlet Extraction:

About 10g of fenugreek seed powder was packed into a thimble and placed in the Soxhlet apparatus. **Ethanol** was used as the solvent. The solvent was heated, vaporized, and condensed repeatedly to allow continuous extraction over several hours (typically 6–8 hours) until the solvent in the siphon tube appeared clear.



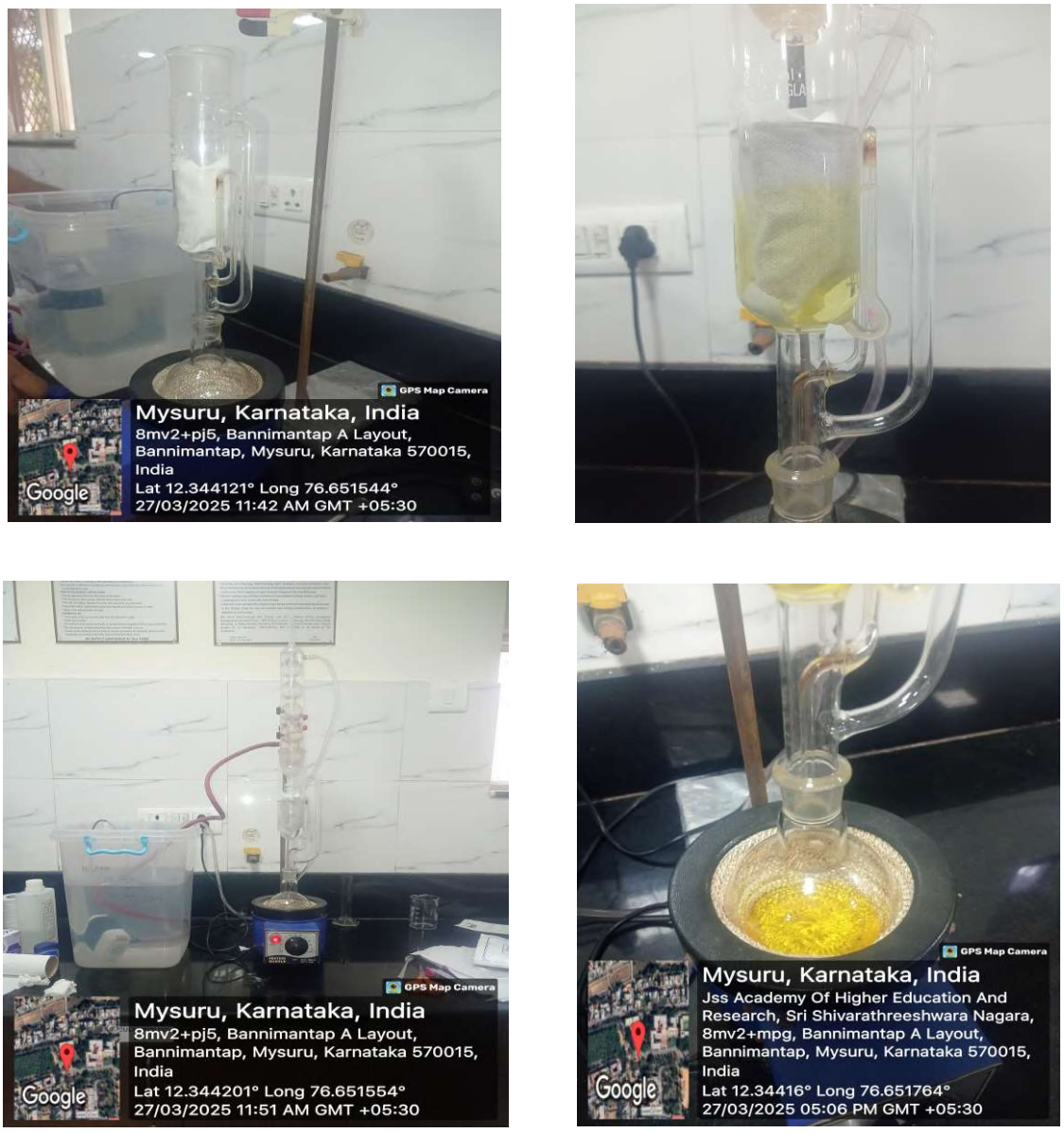


Fig 4.3.1 : Extraction process done for fenugreek seed with the help of the guide at JSS campus(Life Science Laboratory) by Soxhlet Extration Method

Post-extraction Procedure:

The extracted solution was collected and concentrated using a water bath to evaporate excess solvent. The thick residue was then scraped, collected, and stored in **sterile microtubes (MTC tubes)** under refrigerated conditions for further biochemical testing.This method ensured a thorough extraction of active compounds like flavonoids, alkaloids, tannins, and saponins from the fenugreek seeds.

4.3.2 Phytochemical Test Results for Fenugreek and Other Selected Seeds

Qualitative phytochemical screening was performed on ethanolic and methanolic extracts of various seeds (both control and plasma-treated) including **fenugreek, spinach, radish, beans, hyacinth, and lady's finger**. The tests were conducted using standard reagents to detect the presence of:

1. **Alkaloids** (Wagner's test)
2. **Flavonoids** (Alkaline reagent test)
3. **Tannins and Phenolic Compounds** (Ferric chloride test)
4. **Steroids** (Salkowski test)
5. **Saponins** (Froth test)
6. **Carbohydrates** (Fehling's test)
7. **Proteins** (Ninhydrin test)

Observations : In **fenugreek seeds (ethanolic extract)**, both control and treated samples showed strong presence (+++) of **alkaloids, flavonoids, tannins, saponins, carbohydrates, and proteins**.

Phytochemical Analysis	Ethanol Fenugreek (control)
Alkaloids	+++
Flavonoids	+++
Tannins and Phenolic Compounds	+++
Steroids	+++
Saponins	+++
Carbohydrates	+++
Proteins	+++

Table 07 :Phythochemical Test Results of Control(untreated) fenugreek seed

Phytochemical Analysis	Ethanol Fenugreek (control)
Alkaloids	+++
Flavonoids	-
Tannins and Phenolic Compounds	++
Steroids	+++
Saponins	+++
Carbohydrates	+++
Proteins	+++

Table 08:Phythochemical Test Results of Plasma Exposed(treated) fenugreek seed

Where **(+++)** indicates the phytochemical is Strongly present ,**(++)** indicates the phytochemical is Moderately present & **(-)** indicates the phytochemical is Absent.

germination rate, and signs of infection. In the context of seed germination and early plant growth, morphological traits provide essential information regarding the quality and performance of seeds under given environmental conditions. This analysis helps in identifying the most vigorous plants and assessing the overall success of the germination process.

In the laboratory, a 14-day morphological study was conducted where daily observations were recorded for various parameters. Seeds were first placed in a moist medium under controlled environmental conditions to initiate germination. Each day, the longest and shortest shoot and root lengths were measured using a scale, and the number of germinated and non-germinated seeds was noted. In addition to this, any seeds showing visible signs of fungal infection were counted to track health and contamination levels during the observation period.

The daily measurements allowed for tracking the progress of seedling development, identifying variations among samples, and analyzing patterns in growth behavior. The data collected was used to calculate the average growth rate and the percentage of successful germinations. The comparison of shoot and root lengths gave insights into which seeds developed more vigorously and consistently, while the fungal infection data highlighted any negative influence of environmental conditions on seed health. Such analysis not only assists in understanding plant behavior but also in improving cultivation techniques and selecting high-quality seed batches.

2.7 BIOCHEMICAL ANALYSIS

Biochemical analysis is the systematic investigation of the chemical composition and biochemical processes occurring in living organisms. It plays a critical role in understanding how organisms function at the molecular level by studying metabolites, enzymes, and biomolecules.

In plant science, biochemical analysis focuses on identifying, detecting, and quantifying **phytochemicals**—naturally occurring bioactive compounds that contribute to the plant's defense mechanisms, flavor, color, growth, and potential medicinal properties. These include:

Alkaloids – nitrogen-containing compounds with therapeutic effects.

Flavonoids – potent antioxidants responsible for color and UV filtration.

Phenols – compounds with antioxidant, antimicrobial, and anti-inflammatory activity.

Saponins, tannins, steroids, proteins, and more.

The analysis of these compounds in plant parts such as seeds, roots, or leaves allows researchers to evaluate the nutritional and pharmacological potential of the plant, especially when assessing the impact of treatments like **cold plasma**, priming, or storage conditions.

Figure 2.10: Initial discussions made for biochemical tests

Figure 2.11: Discussions made at JSS AHER

2.7.1 Importance of performing Biochemical Analysis on Plasma-Treated Seeds

Plasma treatment is an emerging non-thermal technology that modifies the surface properties and internal metabolism of seeds through the interaction of reactive oxygen and nitrogen species. It enhances seed germination rates, boosts stress tolerance, and influences overall seed vigor.

Biochemical analysis of plasma-treated seeds provides a deeper insight into:

- **Comparative evaluation** of treated vs. untreated (control) seeds to assess any enhancement in bioactive content.
- **Validation of physiological responses** at a molecular level, correlating external growth improvements with internal phytochemical changes.
- **Detection of alterations in secondary metabolite synthesis**, such as increases in phenolics, flavonoids, and antioxidants that may be triggered by plasma-induced oxidative stress.
- **Supporting the application of plasma in agriculture** by providing measurable biochemical evidence of its benefits, ensuring reproducibility and scientific credibility.

Thus, biochemical analysis acts as a critical tool for validating the effectiveness and safety of plasma treatment on seeds for both academic research and practical application in sustainable agriculture.

2.7.2. Role of Biochemical Tests in Differentiating Treated vs. Control Seeds

Biochemical tests serve as a crucial tool in evaluating the physiological and molecular differences between plasma-treated seeds and untreated control seeds. These tests provide measurable indicators of how plasma exposure influences the internal composition of seeds, especially in terms of phytochemical content and antioxidant potential.

Through these tests, researchers can:

- **Identify variations in the concentration of key phytochemicals** such as phenolics, flavonoids, alkaloids, and saponins, which may be altered due to oxidative stress induced by plasma treatment.
- **Detect the emergence or suppression of specific bioactive compounds** that are either enhanced or degraded as a result of the treatment.
- **Evaluate the enrichment in antioxidants and other beneficial metabolites**, which are essential for improving seed performance, stress tolerance, and potential therapeutic value.
- **Establish a biochemical fingerprint** that distinguishes treated seeds from untreated ones, enabling researchers to understand the functional impacts of plasma at the cellular and molecular level.

This comparative biochemical profiling is essential for validating plasma treatment as a viable, non-chemical method to improve seed quality and promote sustainable agricultural practices.

2.7.3. Step-by-Step Experimental Workflow

Step 1: Morphological Screening

- Initial visual and germination-based screening of seeds was conducted to assess plasma treatment impact.
- Seeds treated with various **combinations of voltage and exposure time** were observed.

- The combination that yielded the **best morphological traits and germination efficiency** was selected for further biochemical analysis.

Step 2: Literature Survey

- A comprehensive **literature review** was conducted to identify common **phytochemicals** reported in the selected seed types.
- The review guided the selection of suitable **solvents** and **extraction methods** based on phytochemical polarity and extraction efficiency.
- Decision made to analyze **phenolics, flavonoids, alkaloids, steroids, and proteins**.

Step 3: Sample Preparation

- Seeds were **cleaned to remove debris** and **shade-dried at controlled temperature** (not exceeding 50°C) to retain bioactive compounds.
- Once dried, seeds were ground into **fine powder using mortar and pestle** to increase surface area for extraction.
- For seeds that had undergone **priming**, the external layer was gently peeled off and seeds were re-dried before grinding.

Figure 2.13: Seeds kept inside Oven

Figure 2.12: Oven used for seed drying

Figure 2.14: Powdering of dried seeds

Step 4: Extraction of Phytochemicals

Two extraction methods were used based on compound stability and solvent compatibility:

- **Soxhlet Extraction**:

Used for **exhaustive and continuous hot extraction**. It is best suited for **heat-stable, non-volatile compounds**. It also enables repeated solvent cycling for better yield.

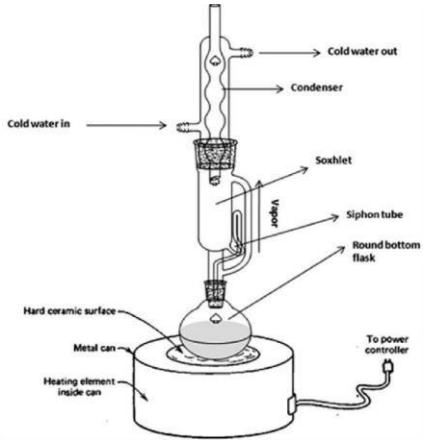


Figure 2.15: Diagrammatic representation of Soxhlet Apparatus

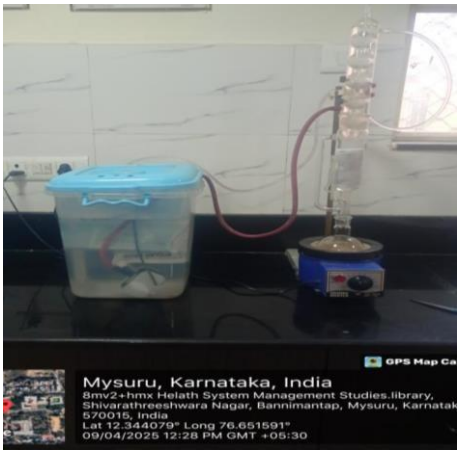


Figure 2.16: Practical Implementation of extraction

- **Maceration Extraction**:

A **cold, passive method** used for **thermolabile or delicate phytochemicals**. It involves soaking in solvent for extended periods with occasional agitation.

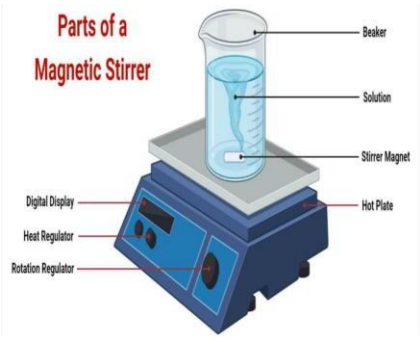


Figure 2.17: Diagrammatic representation of Maceration Apparatus

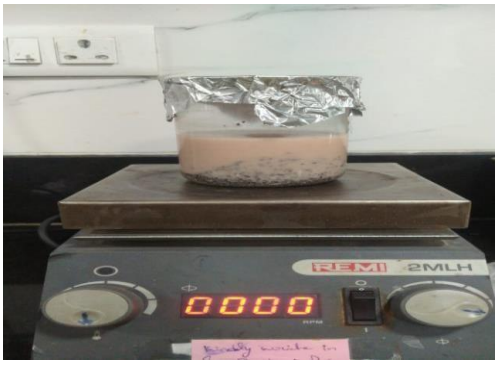


Figure 2.18: Practical Implementation of extraction

- **Solvent Selection Criteria:**

Polar solvents (e.g., methanol, ethanol, water): Extracts polar compounds like phenols, flavonoids, proteins.

Non-polar solvents (e.g., hexane, chloroform): Extracts lipophilic compounds like steroids, lipids, and terpenoids.

The choice was guided by the **solubility of the target compounds** and preservation of their bioactivity.

Step 5: Post-Extraction Handling

- After extraction, samples were **filtered using Whatman filter paper**.
- The filtrate was then **concentrated by evaporation** using a **water bath or rotary evaporator**.
- Dried residues were gently scraped from beakers and **transferred into sterile MTC (Microcentrifuge) tubes**.
- Samples were labeled and stored at **4°C** for further qualitative and quantitative biochemical analysis.
- Extracts were **filtered, dried using water bath/evaporator**, and scraped.
- Final samples were **stored in sterile MTC tubes** and labeled accordingly.

Figure 2.19: Filteration Of extract

Figure 2.20: Sample is kept in petridish for drying

Figure 2.21: Sample kept in oven

Figure 2.22: Samples kept for storage

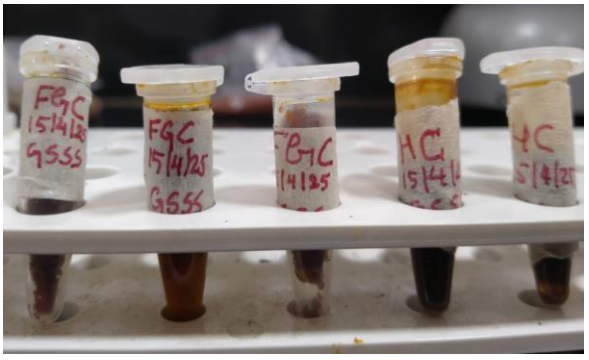


Figure 2.23: Samples stored in MTC tubes

2.7.4. Phytochemical Screening Tests Performed

Phytochemical screening involves a series of **qualitative tests** that help detect the presence of key classes of bioactive compounds in seed extracts. These tests rely on **chemical reactions between specific reagents and phytochemicals**, often producing color changes or precipitates that indicate the presence of the compound.

These qualitative observations serve as a preliminary confirmation of the biochemical diversity within both **plasma-treated and control seeds**. The tests form the basis for further **quantitative assays** (such as TPC and TFC) and **instrumental analysis (GC-MS)**.

SL NO	TEST	PROCEDURE	INFERENCE
1	Wagner's Test	Wagner's Reagent	Reddish Brown Precipitate
2	Flavonoids	1mL extract + 2mL water + 5mL 20% NaOH	yellow coloration
3	Ferric Chloride Test(Tannins & Phenols)	1mL extract + 1% FeCL3	green color to blue black
4	Froth Test(saponins)	1mL extract + 5mL water in testube & shake it vigourously	Persistent froth formed
5	Salkowski Test(steroids)	1mL extract+ 10ml chloroform + equal volume of conc. H2SO4 was added by the sides of test tube	upper layer turned red abd sulpuric acid layer formed flurescent green with yellow
6	Carbohydrates	he extracts were treated with 5mL of fehling's solution & kept in boiling water bath	Yellow or Red colour precipitate shows the presence of reducing sugar
7	Nonhydrin Test(Protein)	Two drops of 0.2 freshly pepared Ninhydrin Solution added to 1ml of extract	Production of purple colour shows the presence of protein

Figure 2.23: Table Phytochemical screening

2.7.5. Quantitative Biochemical Tests

Quantitative biochemical analysis provides precise estimations of specific phytochemical groups. These tests are essential to assess the concentration of antioxidants and other health- promoting compounds present in seed extracts.

2.7.5.1 DPPH Radical Scavenging Assay

To evaluate the antioxidant activity of spinach (Spinacia oleracea) extracts by measuring their ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals.

Materials Required:

1. DPPH (2,2-diphenyl-1-picrylhydrazyl)
2. Methanol (analytical grade)
3. Microplate (96-well, flat-bottom, clear)
4. Spinach leaf extracts (control and plasma-treated)
5. Standard: Ascorbic acid (or Trolox)
6. Micropipettes + sterile tips

7. Aluminum foil
8. Multichannel pipette (optional)
9. Plate shaker (optional)
10. Microplate reader (capable of reading at **517 nm)**

Solution Preparation:

The DPPH working solution (0.1 mM) was freshly prepared by dissolving DPPH powder in methanol and protected from light to maintain stability. Spinach leaf extracts, both control and plasma-treated, were prepared by methanolic extraction and subsequently diluted to desired concentrations for the assay. A standard antioxidant solution (ascorbic acid) was also prepared in methanol and serially diluted to generate a calibration curve for comparative analysis.

Microplate Reader Analysis:

The antioxidant activity was assessed using a 96-well microplate format where equal volumes of sample or standard and DPPH solution were mixed. The reaction mixtures were incubated in the dark at room temperature for 30 minutes to allow for complete radical scavenging. Absorbance was then measured at 517 nm using a microplate reader, with methanol serving as a blank. The percentage inhibition of DPPH radicals was calculated from absorbance values to evaluate antioxidant capacity.

2.7.5.2 Total Phenolic Content (TPC) Estimation Procedure Using 96-Well Plate Materials and Reagents

1. Spinach extracts (control and plasma-treated), prepared in suitable solvent (e.g., methanol or ethanol)
2. Folin–Ciocalteu reagent (commercially available)
3. Sodium carbonate solution (7.5% w/v)
4. Gallic acid standard (for calibration curve)
5. Distilled water
6. 96-well microplate

7. Microplate reader capable of measuring absorbance at 765 nm

Total Phenolic Content (TPC) of spinach extracts (control and plasma-treated) was determined using the Folin–Ciocalteu colorimetric method adapted for a 96-well microplate. First, a standard calibration curve was prepared by diluting gallic acid stock solution to obtain a range of concentrations (0, 20, 40, 60, 80, and 100 µg/mL). Twenty microliters of each standard and appropriately diluted spinach extract samples were pipetted into the wells of the microplate in triplicates. To each well, 100 µL of 10% (v/v) Folin–Ciocalteu reagent was added, and the plate was gently mixed to ensure homogeneity. The mixture was allowed to incubate at room temperature for 5 minutes, after which 80 µL of 7.5% sodium carbonate solution was added to each well. The plate was then incubated in the dark at room temperature for 30 minutes to allow full color development. Absorbance was measured at 765 nm using a microplate reader. The phenolic content of the spinach samples was quantified by interpolating their absorbance values from the gallic acid standard curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight of spinach. Throughout the assay, all measurements were conducted in triplicates, and blank wells containing distilled water instead of sample were used for baseline correction. This method provides a reliable and efficient means to evaluate the total phenolic content in spinach extracts using minimal sample volume and reagent consumption.

TPC = (mg GAE/g sample) = C×V/M

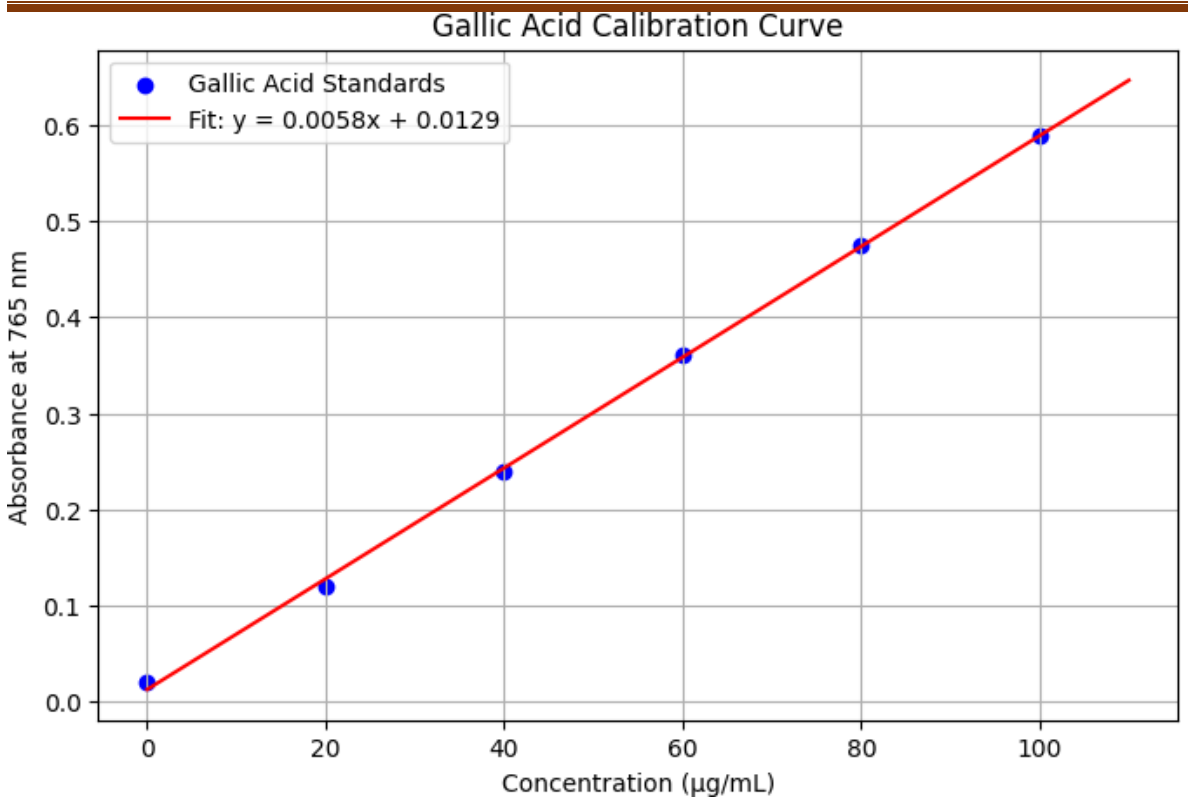
where:

1. C = concentration of gallic acid obtained from the calibration curve (mg/mL or µg/mL converted to mg/mL)
2. V = volume of extract used for the assay (mL)
3. M = mass of the dry sample used for extraction (g)

Sample Type	Concentration (µg/mL)	Absorbance Rep 1	Absorbance Rep 2	Absorbance Rep 3
Gallic Acid Std 0	0	0.019	0.021	0.020
Gallic Acid Std 20	20	0.118	0.121	0.122
Gallic Acid Std 40	40	0.238	0.242	0.241
Gallic Acid Std 60	60	0.359	0.361	0.360
Gallic Acid Std 80	80	0.472	0.478	0.475
Gallic Acid Std 100	100	0.588	0.591	0.590

Table 01: Triplicate Absorbance Values of Gallic Acid Standard Solutions at Various Concentrations (µg/mL)

The table 01 displays the absorbance values for a series of gallic acid standard solutions at different concentrations (0, 20, 40, 60, 80, and 100 µg/mL), measured in triplicate. At the lowest concentration (0 µg/mL), the absorbance values were minimal, ranging between 0.019 and 0.021, indicating no phenolic content. As the concentration increased, the absorbance values showed a clear upward trend, reflecting a proportional relationship between gallic acid concentration and absorbance. At 20 µg/mL, the mean absorbance was 0.118 to 0.122, and it continued to rise with higher concentrations, reaching 0.238 to 0.241 at 40 µg/mL, 0.359 to 0.361 at 60 µg/mL, 0.472 to 0.478 at 80 µg/mL, and 0.588 to 0.591 at 100 µg/mL. These absorbance values can be used to construct a calibration curve for Total Phenolic Content (TPC), which will help in determining the phenolic content of spinach or other sample extracts. By correlating the absorbance of unknown samples to the calibration curve, the phenolic content can be quantified in terms of gallic acid equivalents (GAE).



Graph 1: Calibration Curve Parameters for Gallic Acid Standard

Parameter	Value
Slope	0.0058
Intercept	0.0129
R-squared	0.9995

Table 02: Calibration Curve Parameters for Gallic Acid Standard: Slope, Intercept, and Coefficient of Determination (R^2)

The table 02 provides the key parameters for the calibration curve of gallic acid, which is used to quantify the Total Phenolic Content (TPC). The slope of the curve is 0.0058, which indicates the rate at which absorbance increases with the concentration of gallic acid in the solution. The intercept is 0.0129, which represents the absorbance at zero concentration (the baseline absorbance when no gallic acid is present). The R-squared value of 0.9995 is extremely close to 1, which indicates an almost perfect linear relationship between absorbance and concentration. This high R-squared value suggests that the calibration curve is highly reliable

and can be used with confidence to determine the phenolic content in unknown samples by comparing their absorbance to the standard curve.

2.7.5.3 Total Flavonoid Content (TFC) Determination Using 96-Well Plate

Sample Preparation:

Prepare spinach extracts (control and plasma-treated) by appropriate solvent extraction (usually methanol or ethanol). Dilute samples if necessary to fit within the linear range of the assay.

Reagents Needed:

1. 10% Aluminum chloride ($AlCl_3$) solution
2. 1 M Potassium acetate (CH_3COOK) solution
3. Distilled water
4. Quercetin standard solutions (for calibration curve)

Procedure:

To determine the total flavonoid content (TFC) in spinach samples, a quercetin standard curve is first prepared. This involves creating a series of quercetin solutions with known concentrations, typically ranging from 0 to 100 µg/mL, by diluting a stock quercetin solution accordingly.

For the assay, a 96-well microplate is used. Each well receives 100 µL of either the spinach extract sample or one of the quercetin standards, all prepared in triplicate to ensure accuracy and reproducibility. To each well, 100 µL of 10% aluminum chloride ($AlCl_3$) solution is added, followed by 100 µL of 1 M potassium acetate. Then, 400 µL of distilled water is added to bring the final volume in each well to 700 µL.

The reaction mixtures are then incubated at room temperature in the dark for 30 minutes, allowing the color to develop fully. Following incubation, the absorbance of each well is measured at 415 nm using a microplate reader.

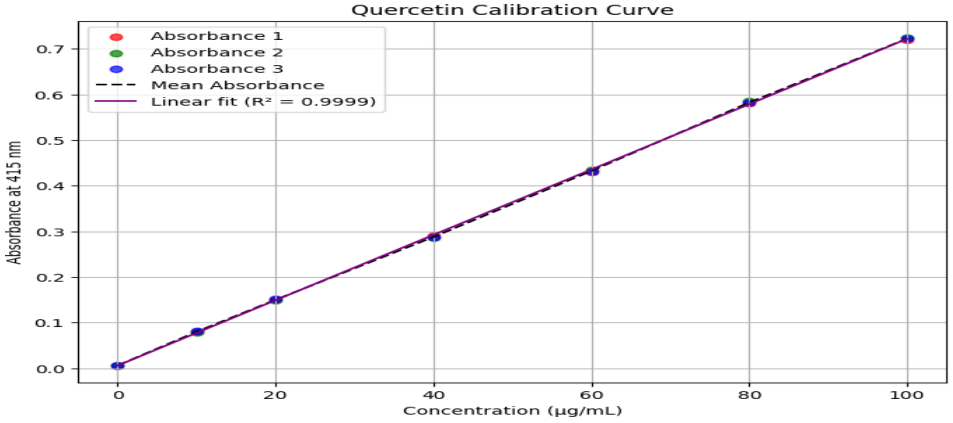
Using the absorbance data from the quercetin standards, a calibration curve is constructed by plotting absorbance against concentration. The flavonoid content of the spinach samples is determined by interpolating their absorbance values onto this standard curve. Finally, the flavonoid content is expressed as micrograms of quercetin equivalents (QE) per milliliter of the

sample, providing a quantitative measure of the total flavonoid content present.

Quercetin Concentration (µg/mL)	Absorbance 1	Absorbance 2	Absorbance 3
0	0.005	0.007	0.006
10	0.080	0.078	0.082
20	0.150	0.148	0.152
40	0.290	0.285	0.288
60	0.430	0.435	0.432
80	0.580	0.585	0.582
100	0.720	0.725	0.722

Table 03: Triplicate Absorbance Values of Quercetin Standard Solutions at Various Concentrations (µg/mL)

This table 3 effectively demonstrates the relationship between **Quercetin Concentration** and corresponding **Absorbance** values, vital for creating a standard curve. Across all tested concentrations, from 0 to 100 µg/mL, there's a clear and consistent increase in absorbance as the quercetin concentration rises, indicating a strong positive correlation. The data also highlights remarkable precision, with the three replicate absorbance readings for each concentration showing minimal variation, reinforcing the reliability of the measurements. Furthermore, the very low absorbance values recorded at 0 µg/mL confirm negligible background interference, ensuring the method's specificity. Ultimately, this well-structured data set provides a robust foundation for accurately quantifying quercetin in unknown samples through spectrophotometry.



Graph 2: Quercetin Calibration Curve