CHAPTER-8

8. Estimation of Biochemical test associated with resistance to wilt and blight disease

8.1 Introduction

People have been investigating plant species since the beginning of time in an effort to discover new medicines, which has contributed to the widespread use of medicinal plants that have the ability to cure a wide range of diseases. In developing countries, the value of therapeutic plants is becoming more and more apparent. According to estimates, 80% of the population in India uses plants for self-care, with 60% of that population regularly using medicinal plants to treat various diseases and approximately 40% of people using such plants in the pharmaceutical industries (Hiren, Manisha D, Sheju et al. 2013). Numerous plants include substances that were initially proven to possess antiviral, anti-ulcer, antifungal, antiplasmodial, antiseptic, and antipyretic properties.

Research on phytochemicals and extracts from plants has long been done to develop pesticide alternatives with less detrimental impacts on the ecosystem and human health. According to reports, plant-derived pesticides have the power to alter the ratio of different biochemical components (carbohydrates, lipids, proteins, etc.) in an insect's body. This disruption of the insect's internal metabolism results in the insect's reduced activity or death.

Since the leaves of this plant have strong meditative properties, this study aims to link their antifungal action and biochemical properties. This biochemical test was conducted to extract, estimate and compare the proteins, total sugar, phenol, and carbohydrate from the selected leaves.

8.1.1 Protein

The growth, maintenance, and repair of the plant depend heavily on protein, one of the essential macronutrients. Along with carbohydrates and lipids, proteins are considered the source of energy (Aregheore et al. 2012 Young V.R. et al.1992). Additionally,

proteins perform a variety of other functions in the cell, including transporting nutrients, participating in enzymatic activity, and other biochemical reactions (Young V.R. et al 1991). It is necessary to provide the plant with sufficient protein through any treatment in order to sustain these vital processes. There are two main sources of protein for plant extract and the other is soil (Furst ;1989 Reddy Narendra et al. 1999). In various regions of India and Gujarat, these plant leaves have been ingested for a very long time. To our knowledge, these leaves have never been linked to any negative side effects. In addition to proteins, the majority of these chosen plants include significant amounts of eco-friendly biochemicals, like flavonoids, alkaloid substances, steroids, and phenolic compounds that help in the prevention of plant diseases caused by microorganisms. (Krishnaiah D et al. 2008). These secondary biomolecules, when present with basic biomolecules like carbohydrates, proteins, and lipids, increase the capacity of the immune system to absorb nutrients and fight against disease. This type of study may help in the evaluation of better crops, and the creation of nutrient-rich products (Kris-Etherton PM et al. 2002).

8.1.2 Phenol

One of the most significant substances identified in plant secondary metabolites is phenolic derivatives. Reviewing different techniques of extracting these substances from plants has attracted more interest as a result of its numerous applications in the agricultural, culinary, chemical, and pharmaceutical industries. In this chapter, an overview of the extraction and estimation of phenol from the leave have been discussed.

The bioactive phenolics found in plants are abundant and useful resources. They can be used as antioxidants, antibacterial agents, anti-inflammatory agents, anticancer agents, antiviral agents, analgesic agents, and antipyretic agents. As a result, they have attracted the interest of several organizations in the field of plant science for their antifungal properties (Gaytán V.G. et al 2013).

8.1.3 Total sugar

One of the main components of plants is carbohydrates. They make up the majority of organic substances and are produced by plants' photosynthetic processes. According to Bernier (1986), carbohydrates in plants serve a variety of physiological purposes. For

example, cellulose serves as a structural component, starch functions as a reserve energy source, acts protectively to prevent tissue desiccation, and so on (see Smith and Montgomery 1959). The reserve carbohydrates are broken down during plant development and respiration, which occurs in different regions of the plant. Plants utilize it for both growth and recovery. (Jeanes and Hodge 1985).

In this biochemical analysis total sugar test was performed by the phenol sulphuric acid method. The total number of carbohydrates in the sample was determined rapidly with colorimetry. The function of sulfuric acid is to change disaccharides, oligosaccharides, and polysaccharides into simple sugars.

8.1.4 Chlorophyll

Several aspects of crop production and research efforts, such as crop breeding, insect control, water management, analysis of environmental stress, and nutritional analysis, have made use of the measurement of leaf chlorophyll content (Wood et al. (1992); Rodriguez Miller (2000); Ravier (2018) Yuan et al. (2016). As a result, it may be possible to use the measurement of leaf chlorophyll concentration as a phenotyping method and to examine its relationship to leaf protein content. The most accurate way to quantify the amount of leaf chlorophyll is to measure it in vitro using leaf extracts and a spectrophotometer.

8.2 Methods

8.2.1 Collection of Samples

The collection of selected plant samples for a biochemical experiment from different parts of Rajkot City was done between January and March 2023. The leaves were cleansed with tap water and then twice-distilled water after collecting (Figure 8.1) to remove all the dust. Extracts of the selected plant were prepared at the biochemistry lab at Atmiya University using a mortar and pestle. The extract was placed in a glass container after filtered with filter paper for future study (Cheynier V. et al., 2011; Salinas-Moreno,2019).

Figure:8.1 plants used for Biochemical test

8.2.2 Extraction and estimation of protein

For the estimation of protein from the leaves sample, many techniques are used. The Folin-Lowry method was used to estimate protein in this research. No device is 100 percent sensitive. The extract can be quantified by breaking the protein and calculating the amino acid. The Lowry method is widely used because it is sensitive enough to provide a reasonably constant value. In this method, BSA is used as a stock solution.

8.2.2.1 Principle

In a solution containing alkaline media, a polypeptide chain's core integrates with copper sulfate to produce a blue-colored complex. Additionally, the phosphomolybdate and phosphotungstate constituents of the Folin reagent are reduced by the protein residues that contain tryptophan and tyrosine to generate a blue color complex which helps to increase the effectiveness of this approach. The blue color complex is measured by a colorimeter at 660 nm.

8.2.2.2 Preparation of a pH 7.0 buffer with phosphate (0.1 M)

80ml of double-distilled water was mixed with 0.02 g of potassium chloride, 0.8 g containing sodium chloride, 0.1gm of sodium dihydrogen phosphate, and 3mg of dihydrogen phosphate to prepare 100ml of phosphate buffer saline (pH 7.4). The pH was then adjusted using a pH meter after 20 ml of double-distilled water had been added to 100 ml.

8.2.2.3 Extraction of protein

The buffers were used for the process of extraction (Figure 8.2). Using a mortar and pestle, 1gm of leaf tissue was measured and thoroughly ground in a solution of phosphate buffer having a pH of 7.0. The extract was centrifuged, and its protein concentration was calculated.

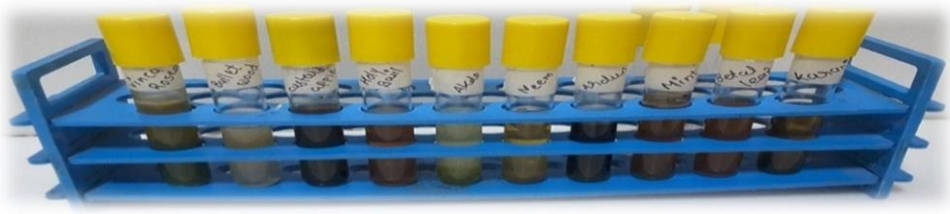

Figure 8.2: Extraction of protein from selected botanicals

8.2.2.4 Quantification of Total Protein Content

In the present study, normal values of the total amount of protein in the range of 0.1 to 1.0 ml were taken in different test tubes with labeling. One ml of water was added to each tube. The blank was a tube containing 1 ml of distilled water. Then, C reagent (5ml) was added to all the tubes of the experiment including Blank as well as Unknown. These tubes' contents were mixed by shaking/vertexing them before keeping them remain still for a period of ten minutes. 500µl of solution D has added right away and fully mixed, then allowed to settle in the dark room for thirty minutes. At a wavelength of 660 nm, a blank space was used to measure absorbance. The protein concentration was plotted along the X-axis (Concentration) and Y- axis (Absorbance) at 650nm to create the standard curve (Figure 8.3). The amount of protein in the sample was calculated by using the standard curve. The sample, which is unknown, contains...g protein/ml.

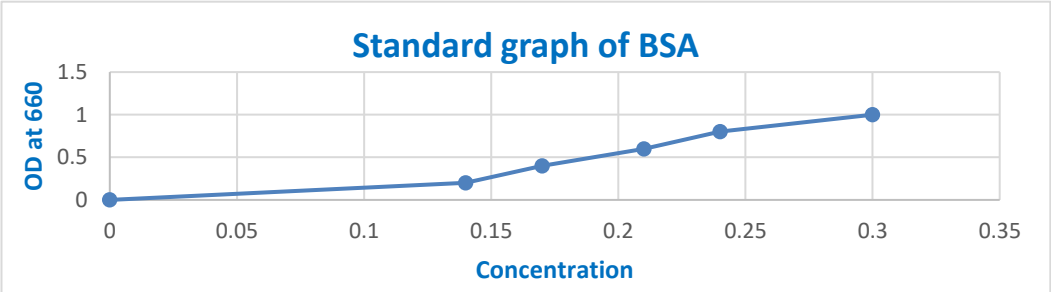

Figure 8.3: Standard graph of BSA for protein estimation.

8.2.2.5 Calculation formula for protein Estimation

A reference applied to determine the amount of protein was the Bovine Serum Albumin (BSA). The amount of protein in the material being studied was calculated and converted to mg per gram using the equation below.

$$Protien\left(\frac{mg}{g}\right) = Graph\ factor \times \frac{Sample\ reading}{Weight\ of\ sample\ (g)} \times \frac{Total\ volume}{Taken\ volume\ (ml)}$$

8.2.3 Estimation and extraction of phenol

The Folin reagent was used to estimate the overall phenol content.

8.2.3.1. Principle

Folin reagent reacts with the phenol present in the sample and produces the phosphomolybdic acid in order to create a blue-colored complex which is measured by using a colorimeter.

8.2.3.2 Extraction of phenol

The leaf tissue (0.5 g) was homogenized in either rectified spirit in water or 80% methanol before being made up to 10 ml with the same solvent. Centrifugation of the homogenate was carried out at 8,000 rpm for 10 mins. The upper layer of the tubes known as the supernatant was collected and re-extraction of residues was done two times then the supernatant was pooled out. The compound phenol content of the extracts was calculated. (Figure 7.4)

Figure 8.4: Extraction of phenol from selected plant extract

8.2.3.3 Calculating the concentration of total phenol

Several methods are used to determine the total amount of phenol in the sample. The total amount of phenolic compounds was determined in this experiment by the Folin Ciocalteu procedure. Normal Catechol (10,20,40,60,80, or 100 g/ml) in samples of 0.2 to 2 ml was added to the experiment tubes, and then 500 µl distilled water and 0.5ml Folin reagent was added and mixed properly. After that 20% Sodium carbonate and 10 ml distilled water were added after a period of five minutes. For two hours, it was incubated at room temperature. A deep blue color appeared. O.D of all samples were taken at 660 nm by using a UV/ Visible spectrophotometer. A standard curve was created utilizing various catechol concentrations (Figure 8.5). The extracts were duplicated three times. A solution containing a blank was used to make comparisons with the other tubes. Normal Catechol has been added to the calibration curve in order to plot results.

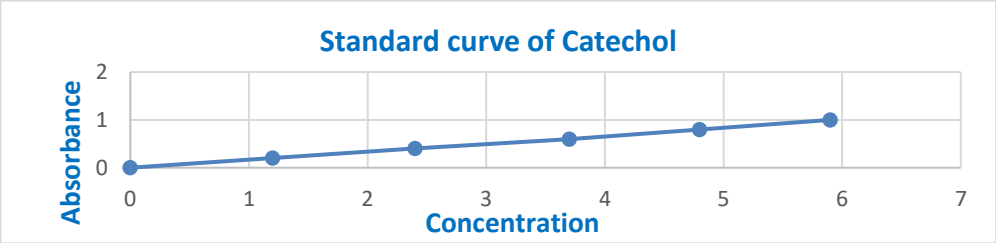

Figure 8.5: Standard graph of Catechol for phenol estimation.

8.2.3.4 Calculation formula

The amount of phenol in the selected plant was calculated by the following formula

$$\text{Phenol } \left(\frac{mg}{g}\right) = Graph\ factor \times \frac{Sample\ reading}{Weight\ of\ sample\ (g)} \times \frac{Total\ volume}{Taken\ volume\ (ml)}$$

8.2.4. Total sugar estimation and extraction

The method of measuring the total carbohydrates using phenol-sulfuric acid is described below.

8.2.4.1 Principle

Dehydration of glucose produces hydroxymethyl furfural. When combined with phenol, this produces a bluish-green compound with maximum absorption at 490 nm.

8.2.4.2 Extraction of total sugar

The leaf material (500 mg) was homogenized in either 80% rectified spirit in water or 80% methanol after being made up to 10 ml using the same solvent. The content of the tube was refluxed in a boiling water bath for one hour at 70 °C. A residue was twice extracted from filtered material. All supernatants were mixed, and a volume of 25 ml of methanol at an 80% concentration was created. In order to measure total soluble sugar, the extract (Figure 8.6) was used.

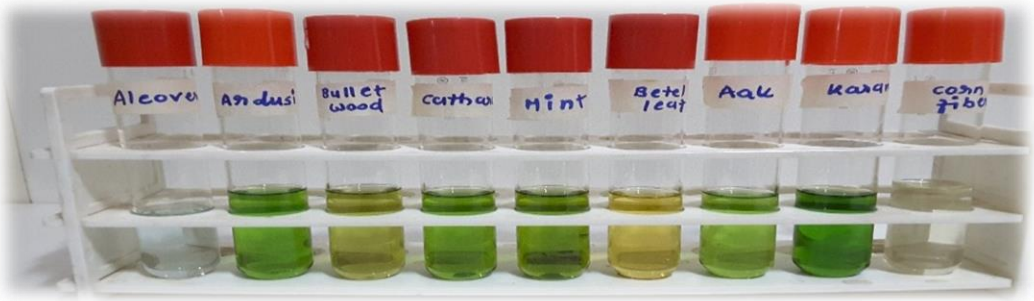

Figure 8.6: Extraction of total sugar from selected plants

8.2.4.3 Phenol sulphuric acid technique for calculating total sugar

In this experiment, the different concentration (0.2 to 1.0 ml) of the sample was taken in different tubes. Then water was added to dilute the solution up to 1ml .5ml phenol solution was added to each tube. The content of the experimental tubes was vigorously shaken well after 10 minutes and then soaked in a water bath heated to 25 to 30 °C for twenty minutes. Observe the color at 490 nm. Using the standard graph, the quantity of the unknown sample was calculated. By using the common graph, determine the sample solution's total carbohydrate content. The glucose solution was used as the starting point for the graph. As a basis for comparison, a glucose solution was used (Figure 8.7).

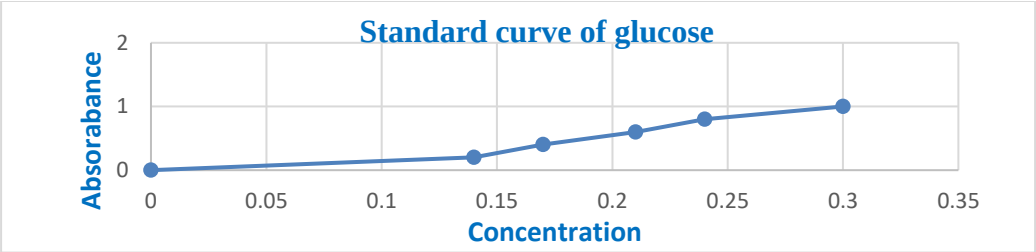

Figure 8.7: Standard graph of glucose for estimation of total sugar

8.2.4.4 Calculation formula

The sugar in the selected plant sample was calculated by the following formula.

$$\text{Total sugar } \left(\frac{mg}{g}\right) = Graph\ factor \times \frac{Sample\ reading}{Weight\ of\ sample\ (g)} \times \frac{Total\ volume}{Taken\ volume\ (ml)}$$

8.2.5 Extraction and Estimation of Chlorophyll Lenz and Zeitzchlen Method

The technique described by Arnon (1949) was implemented to calculate the quantified quantities of chlorophyll a, chlorophyll b, and the total quantity of chlorophyll.

AV= Total volume of chlorophyll the extract in 80% acetone

The fresh plant materials were crushed into chilled 80% acetone. Weighed 0.1 gm, and then suspended in test tubes containing 2 ml of dimethyl sulphoxide. The filter paper Whatman No. 1 was utilized to filter the entire paste. The contents of the experiment tubes had been incubated for a period of ten minutes at 80° C in a water bath. The remaining sample collected a further three ml of DMSO, and the entire mixture was kept for incubation at 70 ºC for 10 minutes after the remaining solution was previously decanted. The supernatant, the upper layer, has been collected and the volume was raised to 10 ml by the addition of DMSO. Using an instrument called a spectrophotometer, the optical density of a chlorophyll extract solution was measured at 645 and 663 nm compared to a blank (DMSO) solution. The following formula was used for the estimation of chlorophyll content from the leaves.

$$Chlorophyll\ a\ mg/g\ fresh\ wt. = \frac{(13.8\ x\ O.D.at\ 665 - 2.68x\ O.D.at\ 650)}{1000xWxA} XV$$

$$Chlorophyll\ b \frac{mg}{g} fresh\ wt. = \frac{(21.9\ x\ O.D.at\ 650 - 4.68\ x\ O.D.at\ 665)}{1000xWxA} XV$$

$$\text{Total clorophyll mg/g fresh wt.} = \frac{(20.2\ x\ O.D.at\ 650 + 8.02\ x\ O.D.at\ 665)}{1000xWxA} XV$$

Where O.D. 663 = O.D (Absorbance) at 663 nm

O.D. 645= O.D (Absorbance) at 645nm

V = ml of the extract's volume

W= Initial weight of the sample in grams

A= Path length (Usually 10 cm)

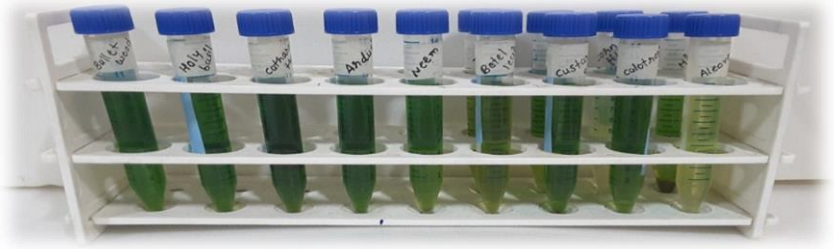

Figure 8.8: Extraction of chlorophyll in acetone from selected plant

8.2.6 Statistical Analysis

The averages and the standard deviations from each experiment were calculated in three separate experiments. Using the MS Excel 2010 programmed, the significance of the means, standard errors, standard deviations and were computed.

8.3 Result

Name of Sample	OD of 0.5ml sample			Mean OD	Conc. in mg of 0.5ml sample			Mean Conc. mg/g
	R1	R2	R3		R1	R2	R3	
Karanj	0.28	0.29	0. 35	0.30	83.5	85.5	86.5	85.83±1.52
Bullet wood	0.28	0.34	0.22	0.28	80	85	80	66.60±2.88
Aak	0.058	0.052	0.045	0.22	14.25	13	11.25	12.83±1.50
Holy basil	0.081	0.052	0.065	0.06	14.85	16	16.25	15.66±0.80
Neem	0.19	0.25	0.22	0.22	54.5	55.5	55	85.00±0.5
Piper betle	0.052	0.039	0.044	0.04	12	10.85	11	11.25±0.66
Mint	0.066	0.059	0.08	0.06	16.5	14.85	18.5	16.25±1.39
Adulsa	0.96	0.88	0.91	0.91	230	230	228	229±1.83
Vinca rosea	0.44	0.39	0.4	0.41	104	102.5	101	102.5±1.5
custard apple	0.61	0.58	0.65	0.61	152	155	152	153±1.83
Aloe vera	0.38	0.41	0.38	0.39	98	98	98	98.33±0.58

Table 8.1 Estimation of protein by Folin-lowry method

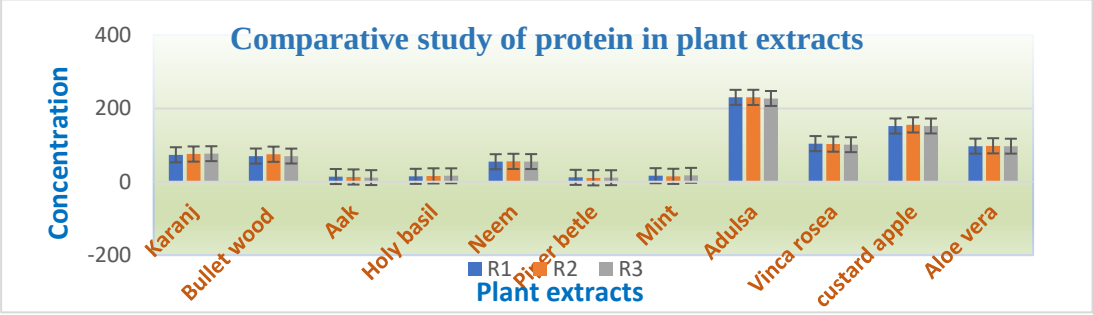

Figure 8.9: Graph of protein concentration from selected plants

By performing the protein estimation of selected leaves by the Folin-Lowry method it was found that a higher amount of protein has been found in Adulsa 229.0 mg/g. In the Custard apple, the amount of protein was 153.5 mg/g and stood in the second position.

Vinca rosea was in the third position 102.5 mg/g. According to the Data (Table 8.1) in the range of 66.60 to 98.33 mg/g protein was found by Karanj, Bullet wood, Neem, and Aloe Vera. A minimum level of protein in the leaves was found in Mint, Piper betle, Aak, and Holly basil.

8.3.2 Estimation of phenol by Folin-Ciocalteu method

Name of Sample	OD of 0.5ml sample			Mean OD	Conc. in mg of 0.5ml sample			Mean Conc. mg/g±SD
	R1	R2	R3		R1	R2	R3	
Karanj	0.14	0.14	0.14	0.28	33.6	33.6	33.6	33.6±0.00
Borsali	0.32	0.38	0.35	0.69	80.8	84.8	84	83.2±2.11
Aak	0.28	0.22	0.3	0.52	64.8	62.8	62	63.2±1.44
Holy basil	0.23	0.18	0.15	0.38	45.2	43.2	46	44.8±1.44
Neem	1.00	0.92	0.96	1.92	230	229.8	230.4	230.4±0.30
Piper betle	0.89	0.83	0.83	1.6	196.6	195.2	198.2	196±1.02
Mint	0.32	0.38	0.35	0.69	81.8	83.8	84	83.2±1.21
Adulsa	0.35	0.35	0.34	0.69	84	84	81.6	83.2±1.38
Vinca rosea	0.19	0.25	0.26	0.46	55.6	55	58.4	56±1.84
custard apple	0.22	0.22	0.21	0.43	52.8	52.8	50.4	52±1.38
Aloe vera	0.14	0.09	0.11	0.22	28.6	29.6	26.4	28.2±1.61

Table 8.2: Estimation of phenol from selected plants

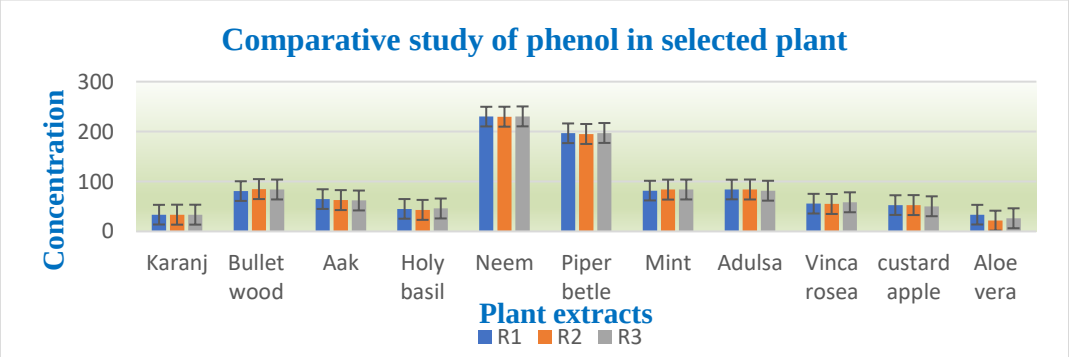

Figure 8.10 Graph of phenol concentration from plant extract

In this study, it was found that all plant extract contains a good level of total phenols, exhibits higher antioxidant and antifungal activity, and is a potential candidate for the treatment of fungus-associated diseases. Phenolic compound of the plant extract was measured by the Folin method, and the results are presented in the Data (Table 8.2). The phenol concentration in the tested extracts was in ranged of 28.2 to 230 mg/g. The higher amount of protein was found in Neem (230.4mg/g). The data table also revealed that the 196mg/g phenol was detected in Piper betle. In the range of 83.2mg/g to 96mg/g phenol was found in plant extract of Adulsa, Mint and Neem.

8.3.3 Estimation of total sugar

Name of Sample	OD of 0.5ml sample			Mean OD	Conc. of mg of 0.5ml sample			Mean Conc.mg/g ±SD
	R1	R2	R3		R1	R2	R3	
Karanj	1.09	1.15	1.45	2.46	315	312	318	344±2.51
Borsali	1.5	1.63	1.6	3.1	445	446	443	441±1.52
Aak	1.05	0.81	1.06	1.94	294	290	296	282±3.05
Holy basil	2.14	2.14	1.86	4.09	588	589	586	583±1.52
Neem	1.05	1.18	1.93	2.8	344	348	345	388±1.52
Piper betle	1.23	1.53	1.61	2.91	426	428	430	408±3.05
Mint	0.91	0.91	1.28	2.06	254	254	255	288±0.58
Adulsa	0.95	1.04	1.28	2.18	262	260	258	305±2.00
Vinca rosea	1.35	1.21	1.38	2.62	388	384	386	368±2.00
custard apple	1.33	1.35	1.53	2.80	386	388	385	392±1.58
Aloe vera	1.08	1.02	1	2.06	283	285	280	289±2.51

Table 8.3 Estimation of total sugar from selected plant extract

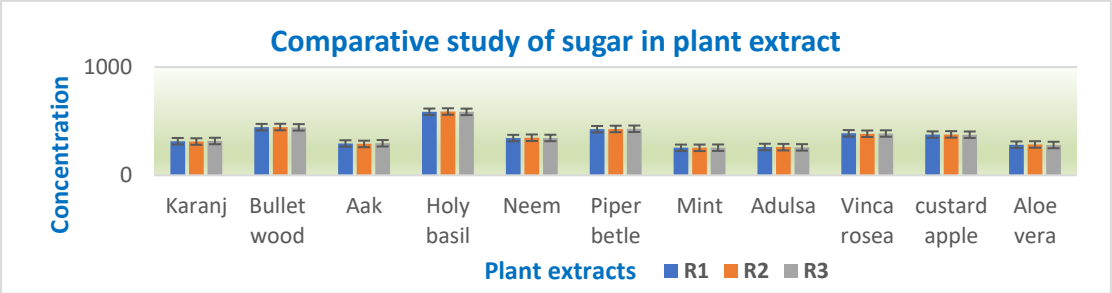

Figure 8.11 Graph of sugar concentration from plant extract

The total sugar in the plant extract was calculated in this experiment using the phenol-sulphuric acid method. The plants had a good quantity of total sugar. Glucose was used as the standard for measuring total sugar. The data (table 8.3) showed that the plant extract's total sugar content ranged from 282 to 583 mg/g. Holy basil plant extract (583 mg/g) had the greatest amount of total sugar, followed by bullet wood (441 mg/g) and piper betel (408 mg/g). The total sugar content of Karanj, Neem, Vinca rosea, Adulsa, and custard apple ranged from 305 mg/g to 392 mg/g.

Treatment	A665	A649	Amount of chlorophyll in 500mg of leaves extract (Mean)±SD		
			Chlorophyll a	Chlorophyll b	Total Chlorophyll
Karanj	2.0	1.63	19.36±0.65	31.88±0.34	41.88±0.54
Borsali	2.12	1.68	20.66±1.23	33.98±1.68	43.26±1.53
Aak	1.93	1.11	19.89±0.28	33.06±0.93	32.11±0.86
Holy basil	2.14	1.81	20.56±2.88	33.65±0.18	45.88±1.84
Neem	1.93	1.83	18.31±1.98	29.85±1.39	43.10±0.32
Piper betle	0.98	0.58	10.01±1.38	16.80±1.43	16.59±0.88
Mint	1.15	0.54	12.08±0.68	20.32±2.45	16.98±1.56
Adulsa	2.11	1.81	20.45±0.39	33.58±0.86	43.91±0.68
Vinca rosea	2.12	1.85	20.48±1.28	33.56±1.39	44.68±0.34
custard apple	2.02	1.28	20.45±0.34	34.04±0.29	35.53±0.56
Aloe vera	0.24	0.13	2.48±2.45	4.15±0.12	5.85±0.38

Table 8.4 Estimation of chlorophyll from plant extracts

8.3.4 Estimation of chlorophyll

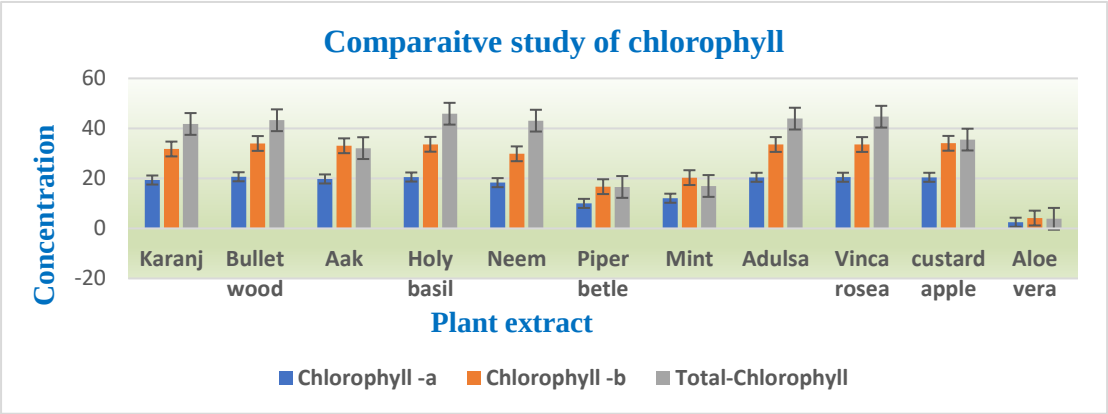

Figure 8.12: Graph of chlorophyll concentration from plant extracts

Data in Table 8.4 was the mean reading of three replicates. All most all the plants have a similar value of total chlorophyll, chlorophyll-a, and chlorophyll-b. The amount of chlorophyll in the range of 18.31 mg/g to 20.66mg/g was detected in the plant extract of Karanj, Bullet wood, Aak, holy basil, Adulsa, Vinca rosea, and Custard apple.12.08mg/g and 10.01mg/g Chlorophyll-a was detected in plant extract of Mint and Piper betle respectively. A very low amount of chlorophyll-a was detected in the plant extract of Aloe vera 2.48mg/g.

In the case of chlorophyll b, 29.84 mg/g to 34.4 mg/g was recorded in the plant extract of Karanj, Bullet wood, Aak, holy basil, Adulsa, Vinca rosea, and Custard apple. 16.80mg/g and 20.32mg/g Chlorophyll was detected by Mint and Piper betle respectively. Lower amount of chlorophyll-b was recorded in the plant extract of Aloe vera.

Total chlorophyll in the range 32.11mg/g to 44.68 mg/g was detected in the plant extract of Karanj, Bullet wood, Aak, holy basil, Adulsa, Vinca rosea and Custard apple.16.58 mg/g and 16.98 mg/g Chlorophyll was recorded by the plant extract of Mint and Piper betle respectively. Very low value of total chlorophyll was detected in the extract of Aloe vera 5.85mg/g.

By comparing this all the data (Figure 8.12) it was found that the Holy basil has a higher amount of all chlorophyll and followed by the Vinca rosea. Lower value of chlorophyll was found in the extract of Aloe vera.

Figure 8.13: Estimation of protein by Folin-Lowry method

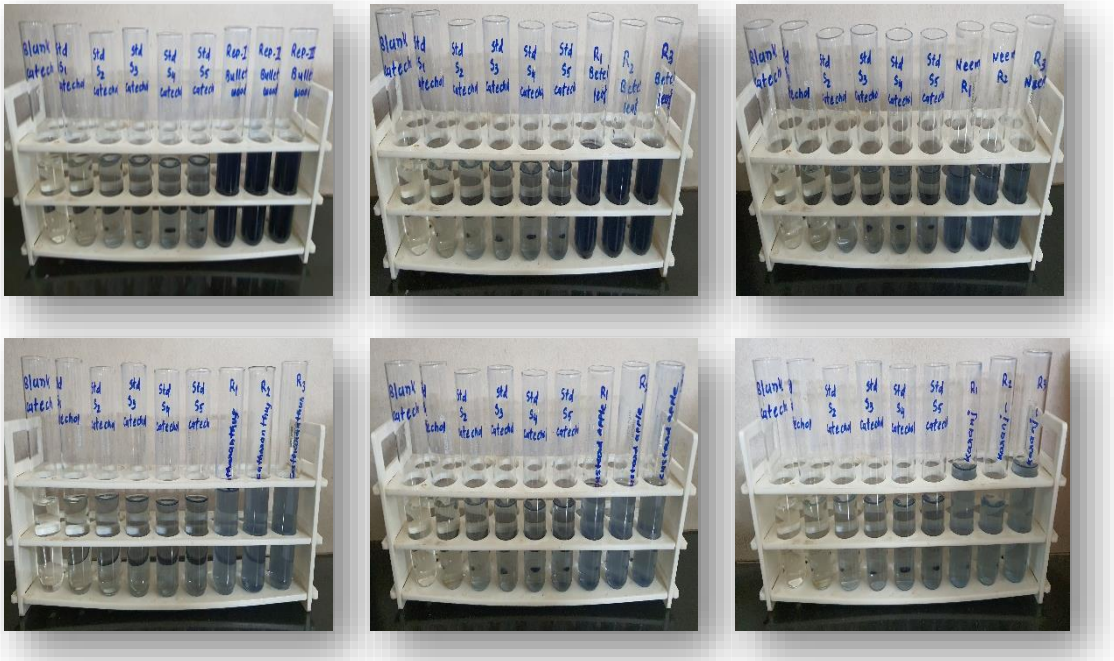

Figure 8.14 Estimation of phenol by Folin-Ciocalteu method

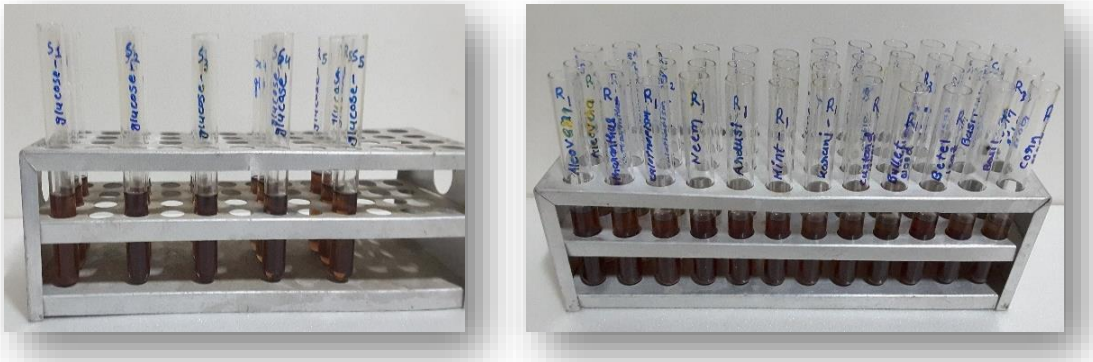

Figure 8.15: Total sugar estimation using the phenol-sulfuric acid test.