Chapter – V

MATERIALS AND METHODS

5. MATERIALS AND METHODS

The proposed work will be conducted on collected sample of plant materials, variation of different agro-soil zones, agro-climatic data, processed tea samples, season wise collected tea samples of different tea gardens situated at Dooars, Terai, and Darjeeling Hills of North Bengal.

5.1 Collection of materials

5.1.1 Plant materials

At first, the agro-climatic regions are classified as Darjeeling Himalaya, Terai and Dooars Regions. A pilot survey has been conducted from the data sources of the tea gardens to document the distribution of tea plant variety and their age (20 to 50 years). The plant specimens [different selected varieties of *Camellia sinensis* (L.) O. Kuntze], *i.e.* fresh tea leaves were collected from the different cultivated section of the tea garden of different agro-climatic areas of North Bengal for the preparation of fresh, handmade tea, intermediately processed tea; mulching materials were also collected from the same plot. An industrial grade of tea was also collected in the same tea garden factory.

5.1.2 Soil Samples

Soil samples were collected as per the methods described by Ranganathan & Natesan (1987) from different agro-climatic areas of North Bengal and samples also were collected from adjacent barren land, multi crops system to document the soil degradation pattern due to tea monoculture. Soil (top and sub soil with 0-22.5 cm and 22.5-45 cm depth respectively) and composite soil were prepared as per the method of Misra *et al*., 2009. The samples were processed for physicochemical analysis viz. pH, electrical conductivity, moisture contents, organic carbon, an available form of nitrogen, potash as K_2O, phosphorus as P_2O_5 and sulphur as SO_4^{-2} (Jackson, 1973).

5.1.3 Agro- climatic and geographical distribution data

The Climatic data like aerial temperature, atmospheric humidity, precipitation, altitudes, and longitudinal, latitudinal positions were collected from the record of CTRI at Coochbehar, DTRC at Darjeeling, and Meteorological station at Gungaram TG.

5.1.4 Records & collections of plant varieties (other than tea)

The Plant variety like shade trees, weeds etc. were recorded from the data sources of the gardens and collected by survey method. Then the samples were identified by Researchers and Dr. A.P. Das, Ex-Professor of Taxonomy and Environmental Biology Laboratory, Department of Botany, North Bengal University. Finally identified voucher specimens were deposited in the 'NBU Herbarium' of Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal.

5.1.5 Collection of mulching materials

The mulching materials *i.e.* pruning litters, grass sp., shaded leaf litter were collected from the tea gardens of different agro-climatic areas of North Bengal.

5.2 METHODOLOGY

5.2.1 Preparation of methanolic plant extracts

Different fresh tea leaves were crushed with mortar and pestle with methanol. The methanol was completely removed by vacuum rotary evaporator at 50°C. One part of these crude extracts used for experiments and other parts were freeze-dried. The powder was stored at 4°C and used for further investigation. The extractive value of the plant materials was calculated on a dry weight basis from the formula given below:

$$\text{Percent extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \times 100$$

Handmade and Industrial grade, processing intermediate tea extracts also used by the same process.

5.2.2 DPPH (2, 2-diphenyl-1-picryl hydrazyl) assay

Radical scavenging activity of plant extracts against stable DPPH (2, 2-diphenyl-1-picrylhydrazyl) was determined spectrophotometrically. The changes in color of DPPH free-radical (from deep-violet to light-yellow) were measured at 517 nm wavelength in presence of antioxidants. Radical scavenging activity of extracts was measured by the standard method proposed by Blois (1958). Two microliters of each sample, prepared at various concentrations were added to 2 ml of 0.2 mM DPPH solution. The mixture was shaken and allowed to stand for 30 min at 20ºC in dark condition and then the absorbance was measured at 517 nm with UV-VIS spectrophotometer (Systronics, 2201). The percentage inhibition activity was calculated by the following equation:

DPPH scavenging effect (%) = $[(A_{control} - A_{sample}) / A_{control} \times 100]$

Where, $A_{control}$ is the initial concentration of the stable DPPH radical without the test compound and A_{sample} is the absorbance of the remaining concentration of DPPH in the presence of methanol. IC_{50} values (mg/ml) were determined from a plotted graph of scavenging activity against the concentrations of the standard extracts, where IC_{50} is defined as the total amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

5.2.3 Superoxide assay

Scavenging capacities of crude and handmade, industrial, processing intermediate tea extracts on superoxide anion would be determined according to the method of Mccord & Fridowish (1969); Nishikimi *et al*. (1972). The superoxide anions generated by phenazine methosulphate (PMS) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), were detected by the reaction with 2, 2´-di-*p*-nitrophenyl-5, 5´-diphenyl-(3, 3´-dimethoxy-4,4´-diphenylene) di-tetrazolium chloride, nitro-blue tetrazolium (NBT). The reaction mixture contained 1 ml samples (different concentration), 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH-7.4) and 1 ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4). Finally, the reaction was accelerated by adding 100 µl PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the

mixture. The reaction mixture was incubated at 25^{o} C for 5 min and the absorbance at 560 nm was measured against methanol as a control. Percentage inhibition and IC_{50} value were calculated using the same formula mentioned above.

5.2.4 Nitric oxide assay

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci *et al*., 1994; Sreejayan & Rao, 1997). 320µl methanol extract, 360µl (5mM) sodium nitroprusside-PBS solution, 216µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride) was mixed and incubated at 25^{o}C for one hour. Lastly, 2 ml water was added and absorbance was taken at 546 nm. The IC_{50} value was calculated by the same procedure mentioned above.

5.2.5 Hydroxyl radical assay

Hydroxyl radical scavenging potency of tea extracts would be assayed according to the method of Jung *et al*., 2008; Jainu & Devi, 2005. Hydroxyl radical scavenging activity was determined by using the 2-deoxyribose oxidation assay (Jung *et al*., 2008; Jainu & Devi, 2005). A solution (0.2 ml) of 10 mM $FeSO_4$, $7H_2O$ and 10 mM ethylenediamine tetraacetic acid was prepared in a screw capped test tube. Then, 0.2 ml of 10 mM 2-deoxyribose solution, 0.5 ml of each sample (different concentration) and 0.1M sodium phosphate buffer (pH 7.4) were added to give a total volume of 1.8 ml. Finally, 200 µl of 10 mM H_2O_2 solution was added to this reaction mixture and incubated at 37º C for 120 min. After incubation, 1 ml each of 2.8% trichloroacetic acid and 1.0% thiobarbituric acid were added to the reaction mixture. The sample was boiled at 100º C for 10 min, cooled in ice, and then its absorbance was measured with a spectrophotometer at 515nm. The IC_{50} value was calculated by the same process mentioned above.

5.2.6 Antilipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the sample extracts was determined by the standard method followed by slight modification with the goat liver homogenate (Bauchet and Barrier, 1998; Ohkawa *et al*., 1979; Sreejayan *et al*., 1994). About 2.8 ml of 10% goat liver homogenate, 0.1ml of 50 mM hydrated ferrous sulfate and 0.1 ml extract was mixed.

This mixture was incubated for 30 minutes at 37^{o}C. 1 ml of the reaction mixture was taken with 2 ml 10% trichloroacetic acid (TCA) -0.67% thiobarbituric acid (TBA) in acetic acid (50%) for blocking the reaction. Then the mixture was boiled for 1hour at 100^{o}C and centrifuged at 10,000 rpm for 5 min. The supernatant was taken for absorbance at 535 nm. ALP% was calculated by using the following formula:

$$\text{ALP percent} = \frac{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{Abs. of sample}}{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{Abs. of control}} \times 100.$$

5.2.7 Determination of ABTS radical scavenging assay

The free radical-scavenging activity was determined by 2,2′ azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ABTS radical cation decolorization assay described by Re *et al.* (1999). ABTS was dissolved in water to a 7 μM concentration. ABTS radical cation ($ABTS^{\cdot +}$) was produced by reacting ABTS stock solution with 2.45 μM potassium persulfate (final concentration) and kept in the dark at room temperature for 12–16h before use. The radical was stable in this form for more than two days when stored in the dark at room temperature . For the study of infusion , the samples containing the $ABTS^{\cdot +}$ solution were diluted with redistilled water to an absorbance of 0.700 (±0.02) at 734 nm and equilibrated at 30°C. A reagent blank reading was taken . After addition of 3.0 ml of diluted $ABTS^{\cdot +}$ solution (734 nm) to 30 μl of plant extracts, the absorbance reading was taken exactly 6 min after initial mixing. The IC_{50} value was calculated by the same procedure mentioned above.

5.2.8 Metal chelating activity

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al.* (1994) with slight modification. To 0.4 ml of methanol extract, 1.6 ml of methanol was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30s, 0.8 ml ferrozine (5 mM) was added. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated using the same formula mentioned above.

5.2.9 Estimation of total polyphenols:

Total phenolic compounds of sample extracts were determined by Folin-Ciocalteu reagent method (Folin and Ciocalteu, 1927; Bray & Thrope, 1954; Malick and Singh, 1980). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocalteu reagent (10 times diluted) and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1hr. at 20ºC and the calibration curve was drawn. 1 ml methanolic leaf extracts (50 mg/ml FWT) was mixed with the same reagent and the mixture was incubated for one hour at room temperature. After 1 hour the absorbance was measured at 765nm.

5.2.10 Estimation of flavonols

Spectrophotometric aluminum chloride method was used for flavonoids determination (Sultana *et al*., 2009; Swain *et al*., 1959; Mahadevan *et al*., 1986). Each methanolic leaf extracts (0.5 ml of 100mg/ml) were separately diluted with 4 ml double distilled water. Then the diluted leaf extracts were mixed with 5% (0.3 ml) $NaNO_2$ and 10% aluminum chloride were then added to reaction mixture. After 6 min 2ml (1.0 M) NaOH and 2.4 ml double distilled water was added and mixed well. There after absorbance was measured at 510 nm in a spectrophotometer. Standard solution of quercetin (0-500 mg L^{-1}) was used as a calibration curve.

5.2.11 Estimation of theaflavin & thearubigins

The theaflavin (TF), thearubigin (TR), high polymerized substances (HPS) and total liquor color (TLC) of the tea samples were determined by the process shown in Figure 5.1. The tea samples obtained from different clone/jat were analyzed for various chemical constituents by standard procedure Masoud *et al*. (2006).

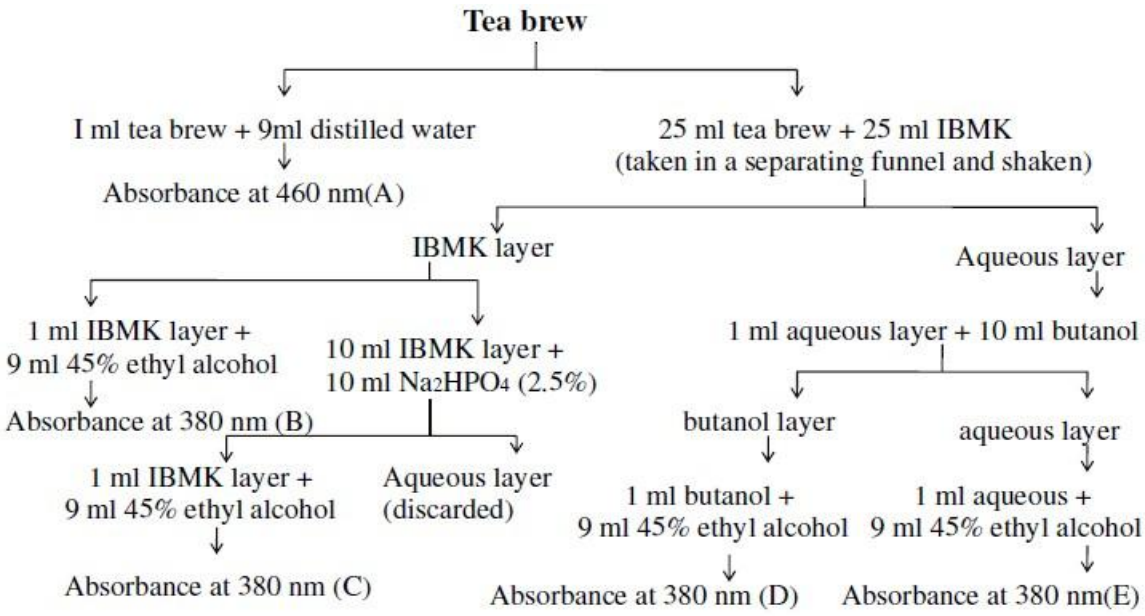

Figure 5.1 Method for determination of TF, TR, HPS and TLC.

TF (%)	=	4.313 x C	TR (%)	=	13.643 x (B+D-C)
HPS (%)	=	13.643 x E	TLC	=	10 x A
TF	=	Theaflavin	TR	=	thearubigin
HPS	=	High polymerized substances	TLC	=	total liquor color
IBMK	=	Iso-butyl methyl ketone			
Na_2HPO_4	=	Di-sodium hydrogen phosphate			

5.2.12 Determination of quinone

Quinone was determined (spectrophotometrically) according to the method described by Mahadevan *et al*. (1986). Accurately weighed 20.00g of the sample was transferred to a mortar and pestle with 100ml chilled phosphate buffer. The samples were centrifuged at 2,000 rpm for 30 min at 4 ºC and supernatants were collected in a 250mL volumetric flask and used as enzyme extract. 3ml of buffer was pipetted out along with 3ml catechol standard and 1.5ml of enzyme extract in a test tube, gently shaken and incubated in a water bath. At different time interval 2ml of samples were withdrawn in duplicate and 4ml of TCA reagent (without ascorbic acid) was added to one sample and another corresponding sample was added with 4ml of TCA reagent containing ascorbic acid. The samples were filtered off for removing the precipitates and recorded at absorbance 400nm. The samples were calculated with the amount of quinone produced by the enzyme extract from ortho-dihydric phenols.

5.2.13 Determination of tannins

Determination of Tannins was done by the method of Sadasivam *et al.*(1992). Accurately weighed 0.5g of the powdered sample was transferred to a 250mL conical flask in 75mL water. The samples were heated in the flask gently and boiled for 30 min. and centrifuged at 2,000 rpm for 20 min and the supernatant was collected in a 100mL volumetric flask and made the volume as required. 1mL of the sample extract was transferred to a 100mL volumetric flask containing 75mL water. Then 5mL of Folin-Denis reagent was added with 10mL of sodium carbonate solution and diluted to 100mL with water and shaken well. The absorbance was read at 700nm after 30 min. Tannins were calculated from the standard curve. An aliquot of the sample extract containing not more than 0.5mg of tannic acid was used and the percentage of tannin was determined.

5.2.14 Estimation of caffeine

Caffeine was determined spectrophotometrically (UV-VIS) as per the standard method of Rogers *et al.* (1998). Briefly, 2gm of instant coffee and 3.2gm of dried tea leaf were taken in a 250ml conical flask, added 200ml of boiling purified water into it and stirred for 30sec, then allowed to cool at room temperature. Simultaneously 1000 ppm of stock standard caffeine was prepared using 198.2mg of caffeine into 200ml boiling purified water. Working standard solution was prepared using suitable aliquots. 50ml of both working and standard solution were poured into separating funnel and 25ml of Dichloromethane was added and caffeine was extracted by inverting funnel by three times and venting the funnel after each inversion. Dichloromethane layer was removed and the procedure was repeated twice more times. The absorbance (λ=272nm) values of the six working standard solutions were measured and calculated the concentration of caffeine in sample using the equation

$$\text{Caffeine content (mg)} = \text{Conc. (ppm)} \times \frac{(\text{Total Sample Vol.})^{2}}{(\text{Measured Sample Vol.}) \times 1000}$$

5.2.15 Estimation ortho-dihydric phenols

Ortho-dihydric phenol was determined by the method of Mahadevan *et al.,* 1986. Accurately weighed 0.5gm of the powdered sample was transferred to a 250mL conical flask in 75mL water. The flask was gently heated and boiled for 30 min, centrifuged at 2,000 rpm for 20 min and the supernatant was collected in a 100mL volumetric flask and make up the volume. Then 1mL of the sample extract was transferred to a 100 mL volumetric flask containing 75mL water and added 1mL of 0.05(N) HCl, Arnow's reagent, 10mL of water, 2ml 1(N) NaOH respectively and shaken well. Absorbance was recorded at 515 nm after 5 min. Ortho-dihydric phenol was calculated from standard curve; catechol was used as a standard.

5.3 ANALYSIS OF SOIL AND MULCHING MATERIALS

Analysis of the soil and mulching materials were analyzed as per prescribe methods of Jackson (1973), Vogel (1962) according to International Society of Soil Science (1929) and Baruah *et al*. (1997).

5.3.1 pH and electrical conductivity

pH and Electrical conductivity of the sample suspension (Soil: Water:: 1:2.5) were analyzed according to the methods described by the Jackson, (1973) using Systronics make calibrated pH and conductivity meter.

5.3.2 Determination of percentage of Organic carbon

Organic carbon (%) of the air dried, sieved (80 mesh) soil sample was determined by wet digestion, volumetric methods of Walkley & Black (1934).

5.3.3 Estimation of available Phosphorus

Available forms of phosphorus were estimated by the colorimetric method of Bray & Kuntz (1945) and Dickman and Bray (1940).

5.3.4 Determination of available Potassium

An available form of potassium was determined by the flame photometric method described by Stanford & English (1949).

5.3.5 Determination of available form of Sulphur

An available form of sulfur was determined by the colorimetric method of Palaskar *et al*., (1981).

5.3.6 Determination of Chloride

The chloride content of the sample was determined by the volumetric methods of Baruah *et al*., (1997).

5.3.7 Moisture Content

The moisture content of the sample was estimated by the method of Baruah *et al.,* (1997).

5.3.8 Determination of particle size distribution of Soil

The Particle size distributions of the soil were evaluated according to the hydrometric method described by Bouyoucos (1927) and Day (1965).

5.4 THIN LAYER CHROMATOGRAPHY FINGER PRINTING

TLC finger printing profiles of ethanol extract of different grades of handmade tea obtained from selected variety tea leaf were carried out using ethanol extract solution. Sample extracts were spotted on a pre-coated silica-gel 60 F_{254} and plate scanned by UV scanner and R_f values of resolved band were recorded according to the method described by Wagner and Bladt (1996).

5.5 TEA PROCESSING CONTROLS

Antioxidants properties were determined, controlling the important parameters like withering, fermentation time, temperature, moisture etc. during processing of tea using the methods described above.

5.6 ESTIMATION OF POLYPHENOL OXIDASE (PPO)

The activity of PPO will be determined during processing of tea on antioxidants properties according to the method described by Augustine *et al*. (1985).

5.7 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The analytical determinations of EGCG and ECG were carried out using reverse phase-high-performance liquid chromatography in isocratic mode. The Waters HPLC system equipped with automated gradient controller, 510 pumps, U6K injector, 481 detectors, 746 data module and Waters µ-bondapak C18 column (3.9 x 300 mm), was used for the analysis. Elution was carried out at an ambient temperature between 24 to 28°C using water: methanol: acetic acid (70: 30: 0.5) as a mobile phase at a flow rate 1.0 mL/min. All extracts were prepared in triplicate and each extract was analyzed in triplicate. The UV detection was carried out at 280 nm.

5.7.1 Standard solution

A standard solution of EGCG was prepared by dissolving 4.72 mg of EGCG in 50 ml methanol. The standard plot for HPLC analysis was prepared by injecting in triplicate a constant volume of 5 µl of serially diluted concentrations containing 9.85, 19.70, 39.40, 78.80, 157.60 and 315.20 ng/5 µ l of EGCG and noting AUC corresponding to each concentration. The standard solution of ECG was prepared by dissolving 3.02 mg of ECG in 10 mL methanol. The stock solution was diluted to make 9.40, 18.80, 37.60, 75.20, 150.40 and 300.8 ng/5 µl dilutions of ECG. A constant volume of 5 µl of each concentration was injected in triplicate. The standard plot was prepared as described for EGCG and same conditions of analysis were used for the two catechins.

5.7.2 Linearity of HPLC systems and its sensitivity

Linear regression was obtained by plotting the peak area versus concentration of a series of dilutions for each phenolic compound. The regression lines, expressed as correlation coefficients, were linear (r^2 =1 and 0.9998 for EGCG and ECG respectively) in the experimental range. Sensitivity (defined as the lowest measurable concentration of a compound in the sample) was determined as that concentration which generated a peak at

least three times higher than the baseline noise range.

5.8 DETERMINATION OF MINERAL CONTENTS IN TEA

The tissues sampled of handmade tea were oven-dried at 68ºC for 48 h and ground. The Kjeldahl method and a Rapid Kjeldahl Distillation Unit were used to determine total N. Macro (P, K, Ca, Mg), and micro elements (Na, Fe, Mn, Zn and Cu) were determined after wet digestion of dried and ground sub-samples in a H_2SO_4-Se-salicylic acid mixture as suggested by Bremner (1996). In the diluted digests, P was measured spectrophotometrically by the indophenol blue method after its reaction with ascorbic acid by UV/VIS spectrophotometer. K, Ca, Na were analyzed by flame photometer and Mg, Fe, Mn, and Zn, the analysis was determined by atomic absorption spectrometry (ECIL made AAS).

5.9 STATISTICAL ANALYSIS

The data were pooled in triplicate and subjected to analysis of correlation coefficient matrix using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) for drawing the relation between different types of antioxidant attributes and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antioxidant attributes of different tea samples. Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $p < 0.05$ was considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE). In order to examine and visualize relationships between different phytochemicals and antioxidant traits, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).