Chapter 3

MATERIALS AND METHODS

1. METHODS

Selection of foods

Certain plant foods / drinks containing both cyanogenic and polyphenols viz. peanut seed coat (Arachis hypogaea), moringa leaves (Moringa oleifera), spinach (Spinacia oleracea) and sugarcane (juice) (Saccharum sp.) have been selected for the present study. Fresh samples of those plants were collected at random from local markets of Kolkata for the measurement of goitrogenic constituents (cyanogenic glucosides, glucosinolates, thiocyanate, and total polyphenols) and also to evaluate their goitrogenic/anti-thyroid potential after feeding those plant foods respectively by replacing a portion of their normal diet for different durations in experimental animals and examining thyroid gland morphology, histology, thyroid functional status by thyroid peroxidase (TPO) activity, thyroidal 5’-deiodinase-1 activity and (Na⁺-K⁺)-ATPase activity along with the assay of thyroid hormone profiles including TSH.

Preparation of plant foods

The plant foods selected for this investigation were prepared by coarsely chopping in a cutter (except sugarcane juice which was prepared by graining in sugarcane juice extractor) and then incubated in an oven at 30-40°C for 4 hours following the method of De Groot et al., (1991) and then mixed with the other ingredients of the standard diet as used in the laboratory for animals feeding.

Measurement of dietary goitrogens in plant foods

Estimation of Cyanogenic glucosides

Principle

Edible parts of each of those fresh plants were hydrolyzed by the enzyme glucosidase and hydrocyanic acid thus liberated was trapped in sodium hydroxide. Cyanide content of trapped hydrocyanic acid was then determined quantitatively. Cyanogenic glycoside was measured following the method of Lambert et al., (1975).
Reagents

i. Standard sodium cyanide (NaCN) solution - 0.2 mM NaCN solution in 0.1 M NaOH was made by diluting 1 ml 10^{-2} NaCN in 1 ml NaOH to 50 ml 0.1M NaOH. For standard curve construction, 100-500 µl solutions were taken and each made up to final volume of 1 ml with 0.1 M NaOH.

ii. 1M acetic acid

iii. Succinimide and N-chloro succinimide - A solution of 2.5 gm succinimide and 0.25 gm N-chloro succinimide in 1L double distilled water was employed in the experiment.

iv. Barbituric acid and pyridine mixture – 24 gm barbituric acid and 120 ml pyridine were taken and made up to 400 ml with double distilled water.

Procedure

1. Foods were rapidly homogenized without adding liquid.
2. An aliquot of the homogenate was weighed and transferred into a 25 ml Erlenmeyer flask containing 5 ml of acetate buffer (0.1 M; pH 5.5) with the enzyme glucosidase and 0.5 ml 1 M NaOH was added in centre well.
3. The flask was closed hermatically and incubated at 37°C for 20 hours with agitation.
4. The hydrocyanic acid liberated by autolysis was trapped in the centre well.
5. Then it was assayed against standards as follows: the standards or the samples from the centre wells were mixed with 0.9 ml 0.1 M NaOH, 0.5 ml 1.0 M acetic acid, 5 ml succinimide/ N-chloro succinimide reagent (succinimide 0.25% and N-chloro succinimde 0.025%) and 1 ml barbituric acid / pyridine reagent (barbituric acid 6% and pyridine-water, 30:70 v/v).
6. After 10 minutes optical density was measured against blank at 580 nm.

Estimation of glucosinolates

Principle

The enzyme myrosinase (thioglucosidase) reacts with thioglucosides present in plant foods to generate thiocyanate. Following this principle glucosinolates (thioglucosides) was measured by the procedure of Gmelin and Virtanen, (1960). Thiocyanate thus formed by the action of myrosinase was estimated following the method of Aldridge, (1945) as modified by Michajlovskij and Langer, (1958).
**Reagents**

i) Methanol

ii) 70% alcohol

iii) 20% Lead acetate solution - 20 gm lead acetate was dissolved in 100 ml of double distilled water.

iv) Hydrogen sulphide

v) Myrosinase

vi) Phosphate buffer (pH 7.0)

vii) 15% Trichloro acetic acid (TCA) - 15 gm of TCA was dissolved in 100 ml double distilled water.

viii) Saturated bromine water - Bromine was added into some amount of double distilled water and mixed. It was ensured that there were some drops of undissolved bromine at bottom of the flask.

ix) 4% Arsenious tri oxide (As$_2$O$_3$) in 2% NaOH - 2 gm of NaOH was dissolved in 100 ml double distilled water. 4 gm of As$_2$O$_3$ was mixed with some amount of 2% NaOH in a hot plate during heating and then the total volume of NaOH was added.

x) Mixture of benzidine hydrochloride and pyridine - benzidine hydrochloride and pyridine were mixed immediately before use 1.6 ml of benzidine hydrochloride and 2 ml of pyridine was mixed for a sample.

xi) Stock thiocyanate solution - 500 mg of potassium thiocyanate (KSCN) was weighed instantly and dissolved in 100 ml double distilled water. To make SCN solution of 100mg/dl strength from the stock solution a calculated amount of KSCN solution was taken into 100 ml volumetric flask and the volume was made 100 ml with double distilled water.

xii) Working thiocyanate solution - stock solution was diluted with double distilled water to make working standards of 0.1 mg/dl, 0.25 mg/dl, 0.5 mg/dl, 1.0 mg/dl and 1.5 mg/dl.
Procedure

1. 10 gm of fresh edible part of respective fresh plant was immersed in adequate amount of absolute methanol.
2. The methanol solution was decanted and the material was thoroughly ground in mortar and was once more extracted boiling in 50 ml of 70% of methanol.
3. Both the extracts were evaporated in vacuo.
4. The residue was taken in double distilled water and the solution was treated with 0.6 ml of 20% lead acetate solution.
5. Excess lead ions in the filtrate were precipitated by hydrogen sulphide.
6. The filtrate was concentrated in vacuo to about 20 ml and was brought to 25 ml.
7. 10 ml of this solution, 1 ml of myrosinase (thoglucosidase obtained from Sigma Chemicals.Co) and 1 ml phosphate buffer of pH 7.0 were incubated for 2 hours at 37°C.
8. 0.5 ml of test solution was mixed with 2 ml of 15% trichloroacetic acid and centrifuged for 10 minutes.
9. Aliquot of the supernatant (1.5 ml) was transferred to a test tube.
10. Standards were prepared by mixing 0.5 ml KSCN solutions (0.1, 0.25, 0.5, 1.0 and 1.25 mg SCN/dl) with 2 ml of 15% trichloroacetic acid.
11. The following reagents were then added subsequently to each of the sample and standard: 0.2 ml saturated bromine water, 0.2 ml 4% arsenous trioxide in 2% NaOH and 3.6 ml bezidine-pyridine mixture (2 ml of pyridine and 1.6 ml of 1% benzidine hydrochloride acidified with HCl).
12. After 30 minutes optical density was measured against blank at 525 nm using spectrophotometer.

Estimation of thiocyanate

Principle

Following the crushing of the plant foods thiocyanate is released and thiocyanate content of the plant foods was then measured by the method of Aldridge, (1945) as modified by Michajlovskij and Langer, (1958).
Reagents

i) 15% Trichloroacetic acid (TCA) - 15 gm of TCA was dissolved in 100 ml double distilled water.

ii) Saturated bromine water - Bromine was added into some amount of double distilled water and mixed. It was ensured that there were some drops of undissolved bromine at the bottom of the flask.

iii) 4% Arseneous tri oxide (As$_2$O$_3$) in 2% sodium hydroxide (NaOH) - 2 gm of NaOH was dissolved in 100 ml double distilled water. 4 gm of As$_2$O$_3$ was mixed with some amount of 2% NaOH in a hot plate during heating and then the total volume of NaOH was added.

iv) Mixture of benzidine hydrochloride and pyridine - benzidine hydrochloride and pyridine were mixed immediately before use; 1.6 ml of benzidine hydrochloride and 2 ml of pyridine was mixed for each sample.

v) Stock thiocyanate solution - 500 mg of potassium thiocyanate (KSCN) was weighed instantly and dissolved in 100 ml double distilled water. To make SCN solution of 100 mg/dl strength from it calculated amount of KSCN solution was taken into 100 ml volumetric flask and volume was made 100 ml with double distilled water.

vi) Working thiocyanate solution - Stock solution was diluted with double distilled water to make working standards of 0.1 mg/dl, 0.25 mg/dl, 0.5 mg/dl, 1.0 mg/dl and 1.5 mg/dl.

Procedure

1) The plant foods (10 gm) were crushed with clean sand and then extracted with about 25 ml double distilled water and refluxed for 20 minutes subsequently in a conical flask.

2) The substance was cooled and filtered.

3) The residue in filter paper was washed repeatedly with double distilled water and volume of the filtrate was made 100 ml.

4) 0.5 ml of this extract containing thiocyanate was treated with trichloroacetic acid followed by saturated bromine water and arsenous trioxide and allowed to react with pyridine-benzidine hydrochloride mixture. The intensity of color thus formed was measured using spectrophotometer. (UV-1240 Shimadzu).
Estimation of total polyphenols

Principle

The method is based on the oxidation of molecule containing –OH groups. The tannin and tannin like compound reduce phosphotungustomolybdic acid in alkaline solution to produce a highly blue colored solution, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds (Matthaus 2002).

Reagents

i) Methanol solution was prepared in 8:2 and 6:4 dilution with double distilled water.
ii) Folin-ciocalteau reagent was prepared in 1:1 dilution with double distilled water.
iii) 2% Sodium carbonate (Na₂CO₃) - 2 gm of Na₂CO₃ was dissolved in 100 ml double distilled water.
iv) Stock gallic acid solution - 100 mg of gallic acid was weighed instantly and dissolved in 100 ml double distilled water.
v) Working gallic acid solution - stock solution was diluted with double distilled water to make working standards of 20µg/µl, 40µg/µl, 60µg/µl, 80µg/µl and 100µg/µl.

Procedure

1) The plant samples were oven-dried (5 days) at 70°C and extracted by using methanol. The 2g sample was extracted in 200 ml using water-methanol (8:2) as the diluting fluid placed in quick fitted conical flask and shaked over night (24) h using a shaker at medium speed. The extract was filtered and kept in the dark at -4°C until tested.
2) 0.2 ml of filtrate was mixed with 0.8 ml double distilled water and made volume up to 1ml.
3) Folin-ciocalteau (diluted 10 folds) - 1 ml of diluted folin ciocalteau reagent was added to each sample, standard and blank, mixed well in vortex.
4) 0.8 ml of 2% Na₂CO₃ was added and the volume made up to 10 ml using water-methanol (4:6) as the diluting fluid.
5) After 30 min, the absorbance was measured at 740 nm using a spectrophotometer.
6) The standard curve was prepared using gallic acid standard solutions of known concentrations, and the results were expressed as mg gallic acid equivalent/g sample.
Body and thyroid weight

At the end of the experimental period, body weights of rats were recorded and the animals were sacrificed following ethical procedure. Just after sacrifice, the rat thyroid glands were dissected and weighed. The relative weight of thyroid gland (mg) was expressed per 100 g body weight.

Morphological and histological study of thyroid gland

After completion of the experimental period thyroid glands of rats in each group were dissected out. The weight of dissected thyroid gland was noted and then fixed in 10% neutral buffered formalin for subsequent histological examination and then embedded in paraffin bath after usual processing. The sections were cut at 5-6 micron thickness and stained with hematoxyline and eosin. The stained sections of each group were then micro photographed and compared thereafter. The diameters of the thyroid follicles selected were measured using camera lucida.

Assay of thyroid peroxidase (TPO) activity

Principle

Peroxidase catalyzes the formation of I$_2$ and periodide formation is then instantaneous when there is an excess iodide substrate. Nearly all off the enzymatically generated I$_2$ is bound as I$_3^-$. Periodide can be spectrophotometrically determined at its absorption peaks at 353 nm and the yield of I$_3^-$ is directly proportional to peroxidase concentration in reaction mixtures containing approximate amounts of hydrogen peroxide (H$_2$O$_2$) and enzyme. Thyroid peroxidase activity was measured following I$_3^-$ from iodine in presence of H$_2$O$_2$ in the assay medium by method of Alexander, (1962). The tissue protein level was determined by the method of Lowry et al., (1951) using bovine serum albumin as standard. The results are expressed as ΔOD/min/mg protein.

Reagents

i) Phosphate buffer (pH 7.2, 100 mM)
ii) Sucrose solution (500 mM)
iii) Acetate buffer (pH 5.2, 50 mM)
iv) Potassium iodide (1.7 mM)
v) Hydrogen peroxide (0.3 mM)
Procedure

1. A 10% homogenate was prepared using thyroid tissues collected from the sacrificed animals; in phosphate buffer (pH 7.2, 100 mM) and sucrose solution (500 mM) at 4°C. Homogenisation was carried out in a glass homogenizer (Potter-Elvehjem) for 45-60 sec at 4400 g and about 15 strokes/min.
2. The homogenate was centrifuged at 1000 g for 10 min.
3. This low speed supernatant was further centrifuged at 10,000 g for 10 min at 4°C to get the mitochondrial fraction.
4. The microsomal fraction containing most of the peroxidase activity was obtained by centrifuged the post mitochondrial supernatant at 1,05,000 g for one hour.
5. Immediately after centrifugation the precipitate was solubilised in phosphate buffer (pH 7.2).
6. Thyroid peroxidase activity was measured in a 1 ml cuvette containing 0.9 ml acetate buffer (pH 5.2, 50 mM), 10 µl potassium iodide (1.7 mM), 20 µl microsomal fraction of thyroid tissue and freshly prepared 20 µl hydrogen peroxide (0.3mM) was added lastly to start the reaction for assaying the TPO activity (ΔOD/min/mg protein) in spectrophotometer (UV-1240 Shimadzu) at 353 nm.

*Thyroidal sodium potassium triphosphate (Na⁺ - K⁺ -ATPase) assay*

**Principle**

The membrane Na⁺ - K⁺ -ATPase activity was determined using Ouabin as an inhibitor of the enzyme activity according to the method of Esmann *et al.*, (1988) modified by Sarkar *et al.*, (2002). The enzyme activity was measured following the rate of formation of inorganic phosphate (Pi), a product of ATP hydrolysis. Pi thus formed, reacts with ammonium molybdate in an acidic solution to form phosphomolybdc acid that gives a blue color. The color production is measured at 850 nm according to the method of Baginski *et al.*, (1967).

**Reagents**

i) Imidazole-HCl buffer (pH 7.4, 30 mM)
ii) Sodium chloride (130 mM)
iii) Potassium chloride (120 mM)
iv) Magnesium chloride (4mM)
v) Ouabin (1mM)
vi) Sodium Dodecyl Sulphate (SDS) (20%)
vii) Tris-ATP (Hydroxymethyl aminoethane hydrochloride adenosine triphosphate)
(4 mM)
viii) Potassium Dihydrogen Phosphate

**Preparation of Tris-ATP solution**

Acidic cation exchanger was used to prepare Tris-ATP from Na$_2$H$_2$ATP. Dowex 50-X8 ($H^+$, 59-100 dry mesh, 1% cross-linkage) was soaked overnight with 0.1 M HCl. It was washed repeatedly with double distilled water to remove excess chloride ions completely. The resin was then taken in a small column and washed with 1 M Tris base to make free the acid ($H^+$) to neutrality. The excess Tris base was removed by washing again the column with double distilled water. A calculated amount of Na$_2$H$_2$ATP (22.044 mg/ml) was next dissolved in double distilled water and placed over the column. It was eluted very slowly with 0.1 M Tris-HCl buffer, pH 7.4 and collected. The pH of the resulting Tris-ATP solution was ultimately adjusted to 7.0 with 1 M Tris base. The total ion-exchange chromatographic procedure was conducted in a cold room at 4°C.

**Inorganic phosphate assay reagent**

I. **Reagent A**

Freshly prepared reagent A was obtained by dissolving 750 mg ascorbic acid in 25 ml 0.5 N HCl. To this medium 1.25 ml of a 10% ammonium molybdate solution was added drop wise with slow stirring under cold condition.

II. **Reagent B**

20 gm each of sodium-meta-arsinite and trisodium citrate-2-hydrate were dissolved in 980 ml double distilled water. To this solution 20 ml concentrated acetic acid was added.

**Procedure**

1. Microsomal fraction of thyroid tissue homogenate was incubated in reaction mixtures of (i) 30 mM imidazole HCl, 130 mM NaCl, 20 mM KCl, 4 mM MgCl$_2$ and (ii) 30 mM imidazole HCl, 4 mM MgCl$_2$ and 1 mM ouabin at pH 7.4.
2. Samples containing 20-50 µg protein were incubated in the medium for 60 min at 0°C for complete binding of ouabain to the active site of the enzyme. The assay medium was equilibrated at 37°C for 50 min.

3. The enzymatic reaction was started by addition of 4 mM Tris-ATP at 37°C and stopped with 0.1 ml of 20% SDS after 10 min.

4. About 0.2 ml (from a total volume of 1.1 ml) of the above reaction mixture was taken with 1.3 ml of H₂O, followed by quick addition of 0.5 ml reagent A. The tubes were kept in ice for 10 min. Then 1 ml reagent B was added.

5. The color developed after 10 min at room temperature was read at 850 nm in a spectrophotometer (Model Shimadzu, UV-mini 1240, Japan) by the method of Baginski et al., 1967.

6. The enzyme activity was expressed as µmols of Pi liberated per hour per mg protein calculated from a standard curve of potassium dihydrogen phosphate. The pooled sample was assayed in duplicate.

**Thyroidal 5’– deiodinase type I (D1) assay**

**Principle**

Iodothyronin 5’– deiodinase type I (5’–D1) activity has been measured according to the method of Kodding et al., (1986) with slight modifications.

**Reagents**

Tris-HCl (Hydroxymethyle aminoethane hydrochloride) buffer (pH 7.4, 0.1 M)

i) Ethelene Diamine Tetra Acetic acid (EDTA) (3mM)

ii) Dithioerythritol (DTE) (150mM)

iii) L-thyroxine (T4) (0.4µM)

iv) Absolute ethanol (800µl)

v) Propythiouracil (PTU)

**Procedure**

1. A substrate solution of 0.1 M Tris-HCl buffer (pH 7.4), 3 mM EDTA and 150 mM DTE containing 0.4µM T4 and 100-150 µg thyroid tissue protein in a final volume of 400 µl was incubated at 37°C for 30 min.
2. The monodeiodination reaction of T4 to T3 was terminated by the addition of 800 µl ice-cold absolute ethanol, followed by shaking for 8 min at 4ºC.

3. The reactants were centrifuged at 10,500 g at 4ºC for 8 min and the ethanol supernatants were collected for measurement of T3 content. For all samples, values for zero time were prepared by adding the tissue to the substrate containing T4 after the addition of alcohol.

4. The concentration of T3 in the ethanolic extract after 0 and 30 min of incubation were estimated by ELISA. The activity of 5’–D1 was calculated as the difference of the 0 and 30 min values and expresses in terms of pmoles T3 formed/mg of protein. The pooled sample was assayed in duplicate.

5. The validity of assay method has been justified by the preincubation of samples with the 5’–D1 inhibitor, propylthiouracil (PTU) the resulted in > 50 % inhibition of the enzyme activity. It needs to be mentioned here that conversion of T4 to rT3 by 5’–D1 cannot proceed under such simulated conditions, as rT3 formation can occur only under a high pH and substrate concentration, unlike T4 to T3 monodeiodination as found in our experimental condition.

**Estimation of tissue protein**

**Principle**

When protein is placed in an alkaline solution containing Cu²⁺, a weak colored complex can form between the peptide bonds in the protein and the copper atom. It is thought that this bonding involves the reduction of Cu²⁺ to Cu⁺. This “Biuret” reaction has been used for several decades to estimate the quantity of protein in samples. The reaction is not very sensitive and large quantities of protein have to be used to get accurate results. Lowry *et al.* (1951) added Folin’s reagent to the biuret assay, greatly improving its sensitivity. The Lowry method combines the biuret reagent with another reagent (the Folin-Ciocateaus phenol reagent) that reacts with tyrosin and tryptophan residues in proteins. This gives a bluish color which can be read somewhere between 500-750 nm, depending on the sensitivity required.

**Reagents**

i) Bovine serum albumin (BSA) - 10 mg BSA in 10 ml 0.9% sodium chloride (NaCl) solution is prepared one day prior to measurement.

ii) NaCl solution 0.9%
iii) Sodium carbonate (Na$_2$CO$_3$) solution was prepared with 10 gm of Na$_2$CO$_3$ dissolved in 100 ml of double distilled water, 50 ml of 0.1 N NaOH was added to solution and then the volume was made up to 500 ml.

iv) 1% Sodium potassium tartrate

v) 0.5 % Copper sulphate

vi) Alkaline copper reagent was prepared freshly by mixing 1% sodium potassium tartrate and 0.5 % copper sulphate in 1:1 proportion and then 1 ml of above solution was mixed with 50 ml of Na$_2$CO$_3$ solution.

vii) Folin ciocalteus reagent was prepared in 1:1 dilution with double distilled water.

**Procedure**

1. From BSA solution 25 µl, 50 µl, 100 µl, 150 µl and 200 µl standards were taken and mixed with 0.975 ml, 0.950 ml, 0.900 ml, 0.850 ml and 0.800 ml of NaCl solution and in blank 1 ml of NaCl solution was taken and for the measurement of tissue protein 25 µl of sample was taken and mixed with 0.975 ml of NaCl solution.

2. 5 ml of freshly prepared alkaline copper reagent was added to each sample, standards and blank, mixed well in vortex and kept for 15 minutes.

3. 0.5 ml of 1:1 freshly prepared Folin ciocalteus reagent was added to each sample, standard and blank, mixed well in vortex and kept for 30 minutes.

4. After 30 minutes optical density was measured at 660 nm in spectrophotometer.

**ELISA of serum total triiodothyronine (T3)**

**Principle**

Serum total triiodothyronine (T3) level was assayed using ELISA total T3 kit obtained from RFCL Limited India. This total ELISA kit is based on the competition principle and the microtiter plate separation. In a competitive EIA, there exists a competitive reaction between native antigen and enzyme antigen conjugate for a limited number of insolubilized binding sites on the antibody coated on the microwell. After the antigen-antibody reaction has taken place, which does not bind to the coated well, i.e., the unbound fraction is then washed away. The enzymatic activity in the antibody bound fraction which is inversely proportional to the native antigen concentration is measured by addition of the substrate.
Reagents
i) **T3 Antibody Coated Microplate** – 1 microtiter plate 12 x 8 wells strips coated with Sheep Anti-T3 Serum.

ii) **T3-Enzyme Conjugate** – 1 vial of T3 – horseradish peroxidase (HRP) conjugate in an albumin-stabilizing matrix.

iii) **T3 Conjugate Buffer** – 1 bottle reagent containing buffer, red dye, preservative and binding protein inhibitors.

iv) **T3 Calibrators** – 6 vials of serum reference for T3 at concentrations of 0, 0.5, 1.0, 2.5, 5.0 and 7.5 ng/ml.

v) **Substrate A** – 1 bottle containing tetramethylbenzidine (TMB) in buffer.

vi) **Substrate B** – 1 bottle containing hydrogen peroxide ($H_2O_2$) in buffer.

vii) **Wash Solution Concentration** – 1 vial containing surfactant in phosphate buffered saline.

viii) **Stop Solution** – 1 bottle of stop solution containing a strong acid (1 N HCl).

Procedure
1. 50 µl of the calibrators and sample were dispensed into appropriate wells.
2. 100 µl of the prepared enzyme T3 reagent added to selected wells.
3. The microplate gently shaken for 20-30 seconds to mix and covered and then incubated for 60 minutes at room temperature.
4. The contents of the wells aspirated and filled them completely (approximately 300µl) with the diluted washing solution. The washing procedure repeated twice.
5. 100 µl of working substrate solution was added to each well, at timed intervals.
6. Incubated for 15 minutes at room temperature (2 1-25°C), protect from light.
7. The enzymatic reaction was stopped by adding 50 µl of stop solution to each at the same intervals as in step 5 and mixed well for 15-20 seconds and then the absorbance of each well was determined at 450 nm in ELISA reader.
8. The sensitivity of the rT3 assay was 0.04 ng/ml.

**ELISA of serum total thyroxin (T4)**

**Principle**

Serum total thyroxin T4 level was assayed using ELISA total T4 kit obtained from RFCL Limited, India. This kit is based on the competition principle and the microtiter plate separation. In a competitive EIA, enzyme linked antigen competes with antigen from the
specimen for a limited number of binding sites on the immobilized antibody coated on the micro wells. Unbound antigen fraction is then washed away. The enzymatic activity in the antibody bound fraction which is inversely proportional to the native antigen concentration is measured by addition of the substrate.

Reagents

i) **T4 Antibody Coated Microplate** – 1 microtiter plate 12 x 8 wells strips coated with Sheep Anti-T4 serum.

ii) **T4-Enzyme Conjugate** - 1 vial of T4-horseradish peroxidase (HRP) conjugate in a bovine albumin-stabilizing matrix.

iii) **T4 Conjugate Buffer** – 1 bottle reagent containing buffer, red dye, preservative and binding protein inhibitors.

iv) **T4 Calibrators** – 6 vials of serum reference for T4 at concentrations of 0, 2.0, 5.0, 10.0, 15.0 and 25.0 µg/dl.

v) **Substrate A** – 1 bottle containing tetramethylbenzidine (TMB) in buffer.

vi) **Substrate B** – 1 bottle containing hydrogen peroxide (H₂O₂) in buffer.

vii) **Wash Solution Concentration** – 1 vial containing surfactant in phosphate buffered saline.

viii) **Stop Solution** – 1 bottle of stop solution containing a strong acid (1 N HCl).

Procedure

1. 25 µl of the calibrators and sample were dispensed into appropriate wells.
2. 100 µl of the prepared enzyme conjugate solution added to selected wells.
3. The microplate gently shaked for 20-30 seconds to mix and covered and then incubated for 60 minutes at room temperature.
4. The contents of the wells was aspirated and filled them completely (approximately 300 µl) with the diluted washing solution. The washing procedure repeated twice.
5. 100 µl of working substrate solution was added to each well, at timed intervals.
6. Incubated for 15 minutes at room temperature (21-25°C), protected from light.
7. The enzymatic reaction was stopped by adding 50 µl of stop solution to each at the same intervals as in step 5 and mixed well for 15-20 seconds and then the absorbance of each well was determined at 450 nm in ELISA reader.
8. The sensitivity of the total T4 assay was 0.4 µg/dl.
**ELISA of serum thyroid stimulating hormone (TSH)**

**Principle**

The microtiter plate provided in this kit has been pre-coated with an antibody specific to TSH. Standards or samples are then added to the appropriate microtiter plate wells with a horseradish peroxidase (HRP) - conjugated antibody preparation specific for TSH and incubated. Then substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of TSH in the samples is then determined by comparing the O.D. of the samples to the standard curve.

**Reagents**

i) **TSH Antibody Coated Microplate** – 12 x 8 (break apart) strips with 96 wells; Wells coated with a monoclonal TSH antibody.

ii) **Rat TSH Calibrator** - 1 vial, lyophilized, in serum/buffer matrix containing highly purified rat TSH. The standard curve concentrations used for the ELISA were 12 µIU/ml, 6 µIU/ml, 3 µIU/ml, 1.5 µIU/ml, 0.6 µIU/ml, 0.3 µIU/ml.

iii) **Enzyme-Labeled anti rat TSH Antibody** - 1 vial, red, ready to use; contains a horseradish peroxidase-labeled polyclonal anti TSH antibody (goat), in a buffered solution with preservative.

iv) **Substrate A** – 1 bottle containing tetramethylbenzidine (TMB) in buffer.

v) **Substrate B** – 1 bottle containing hydrogen peroxide (H₂O₂) in buffer.

vi) **Wash Solution Concentration** – 1 vial containing surfactant in phosphate buffered saline. 15 ml of wash buffer Concentrate was diluted into deionized or distilled water to prepare 300 ml of Wash Buffer.

vii) **Solution** – 1 bottle of stop solution containing a strong acid (1 M H₂SO₄).

**Procedure**

1. A blank well was set without any solution.
2. Add 100µl of standard or sample was added per well. All the standard test were done in duplicate.
3. 50µl of HRP-conjugate was added to each well (not to blank well) and the mixed well and then incubated for 2 hours at 37°C.
4. Each well was aspirated and washed, repeating the process two times for a total of three washes. There were then washed by filling each well with Wash Buffer (200µl).
using a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer, and let it stand for 10 seconds; complete removal of liquid at each step was essential for better performance. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

5. Added 50μl of Substrate A and 50μl of Substrate B to each well, mixed well and incubated for 15 minutes at 37°C; keeping the plate away from drafts and other temperature fluctuations in the dark.

6. Added 50μl of Stop Solution to each well and gently tapped the plate to ensure thorough mixing.

7. The optical density of each well was determined within 10 minutes, using a microplate reader set to 450 nm.

**Analysis of urine**

**Estimation of iodine**

**Principle**

Iodine in urine generally occurs as the iodide ion. Most of the available methods for urinary iodine determination include an initial stage in which the urine is either digested in strong alkali or ashed at high temperature. Following the step, iodine is measured by its catalytic action on the reduction of the ceric ion (Ce⁴⁺) to cerous ion (Ce³⁺) coupled to the oxidation of arsenite As³⁺ to As⁵⁺. This reaction is called Sandal-Kolthof reaction (Sandal and Kolthof, 1937). The ceric ion (Ce⁴⁺) has a yellow color while the cerous ion (Ce³⁺) is colorless. The course of reaction can be followed by disappearance of yellow color as the ceric ion reduced. With the other reactants held stable, the speed of this color disappearance is directly proportional to the amount of iodide catalyzing it. It was then measured by dry ashing following the method of Karmarkar *et al.*, (1986).

**Reagents**

i) Potassium carbonate (K₂CO₃) 2.5 N - 17.5 gm of anhydrous K₂CO₃ was dissolved in small amount of double distilled water and diluted to 100 ml. It was stored in a polyethylene bottle.

ii) Ceric ammonium sulphate [Ce (NH₄)₄ (SO)₄ 2H₂O] - 0.005N - 3.17 gm of ceric ammonium sulphate was dissolved in 500 ml of double distilled water and 57 ml
concentrated H$_2$SO$_4$. It was diluted to 1L and stored in dark bottle away from light at room temperature.

iii) Sodium meta arsenite (NaAsO$_4$) 0.03N - 2.27 gm of sodium meta arsenite was dissolved in 500 ml of double distilled water. 46 ml of concentrated H$_2$SO$_4$ was added to this and the volume was made up to 1L by adding double distilled water.

iv) Stock iodine solution (1mg iodine/ml).

v) Working iodine standard - Stock iodine solution was diluted with double distilled water to make working standards of 5µg /dl, 10µg/dl, 15 µg /dl, 20µg /dl and 25µg /dl and stored in dark bottle.

**Procedure**

1. Urine sample was mixed to evenly suspend any sediment.
2. 100 µl each urine sample or diluted urine sample was taken and 100 µl of double distilled water was taken as blank.
3. 100 µl of each working standards was also taken.
4. 300 µl K$_2$CO$_3$ solutions was added to each tube and mixed.
5. The samples were placed in a test tube rack and kept in the oven for drying at a temperature of 80-100°C overnight.
6. The dried samples were placed in the muffle furnace for ashing at 600°C for two hours.
7. The furnace was switched off, the temperature was allowed to come down and the rack was removed from the furnace, usually when ashing to complete no black residue found.
8. When the tubes were cooled to room temperature, 3 ml of sodium meta arsenite solution was added to each tube. If the ash was not completely soluble, the tubes were centrifuged at 2500g for 10 minutes and then the supernatant was transferred to another sets of tubes.
9. The tubes were incubated with the supernatant for 5 minutes in a water bath at 56°C.
10. 3 ml of ceric ammonium sulphate was added at 30 seconds intervals to each tube and mixed and placed in water bath.
11. Twenty minutes after the addition of ceric ammonium sulphate, its transmittance was read at 420 nm in a colorimeter against water blank. A stopwatch was used to keep a constant interval i.e. 30 seconds, between additions of two successive tubes.
12. Then the concentration of iodine was determined by interpolation from the standard curve using the blank corrected transmittance. The obtained result was expressed as µg/dl and moles/L.

**Estimation of thiocyanate**

**Principle**

The thiocyanate in urine is oxidized by bromine. The oxidized form of thiocyanate then reacts with the chromophore benzidine hydrochloride to form a coloured complex. The intensity of the colour formed is essential at 525 nm. Urinary thiocyanate (SCN) concentration was measured in the same urine samples using the method of Aldridge, (1945) and modified by Michajlovskij and Langer, (1958).

**Reagents** As mentioned earlier.

**Procedure**

Trichloroacetic acid was added to 0.5 ml of urine sample, mixed and centrifuged. To the supernatant saturated bromine water was added and 4% arsenic trioxide (As₂O₃) was then added to oxidize all bromine present in the sample. After that benzidine hydrochloride and pyridine mixture were added and the colour developed gradually. After 30 minutes optical density was measured at the wavelength of 525 nm.

**Statistical analysis**

Results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was first carried out to test for differences across the mean values of all groups. If between-group differences were established, the values of the treated groups were compared with those of the control group using Tukey’s procedure. A value of $p < 0.05$ was interpreted as statistically significant. Statistical analyses were performed using Origin 8 and MS-Office Excel 2007 software packages.

2. MATERIALS

**Animals**

Three-months-old adult male albino rats of the Wistar strain weighing 150 ± 5g were used in the present investigation. The animals were maintained according to national guidelines and protocols, and the study was approved by the Institutional Animal Ethics Committee (IAEC/PROP/AC-1/2010 dated 12.08.2010). The animals were housed in clean polypropylene cages and maintained in a temperature controlled environment at 22°C ± 2°C and relative humidity (40% - 60%) in an animal house with a constant 12 hour light/12 hour
dark schedule. The animals were fed on a standardized diet which consisted of 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined sesame oil, 0.25% shark liver oil, and water ad libitum (Chandra et al., 2007). Control rats were fed with a normal laboratory standardized diet as above whereas experimental rats in each group received a normal laboratory diet with one-third of the diet replaced (Chandra et al., 2006) by selected plant foods (viz. peanut seed coat, moringa leaves, spinach and sugarcane juice) for 30 and 60 days respectively; obtained from a local markets in Kolkata. Feed consumption, corrected for wasted feed, and body weight were measured every seven days. During the last week of the treatment animals in each group were kept in metabolic cages for 24 hr to collect urine over xylene for the analysis of iodine and thiocyanate. At the end of the experimental period the body weights of the rats were recorded and the animals were sacrificed at the end of the 30th and 60th days of the experiment, respectively.

All the animals were sacrificed after 24 hours of the last feeding (i.e. during 9 am to 10 am on the day of experiment to avoid any discrepancy that may arise for diurnal variation) following standard protocols and maintaining ethical procedures. Blood samples were collected and serum was separated for hormone assay.

**Reagents**

DTE, Tris-HCl and Na₂ATP, imidazoles were purchased from Sisco Research Laboratories (SRL), Mumbai, India. Ouabains, PTU, T4, bovine serum albumin, EDTA, MnCl₂ were purchased from Sigma Chemical Company, Steinheim, Germany. SDS was purchased from LOBA Chemie Pvt. Ltd, Mumbai, India, Dowex 50WX8 from Alfa Aesar.