3. MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

- The following chemicals, reagents and kits used for the present study were procured from Sigma-Aldrich Mumbai, Merck and Sd-Fine Chemicals, Chennai; 5-(hydroxymethyl) furfural, oxalic acid, fructose, 1,1,3,3-tetramethoxy propane, disodium hydrogen phosphate, 5,5'-dithiobis(2-nitrobenzoic acid), tricholoacetic acid, citric acid, L-glutathione reduced, trichloroacetic acid, n-butanol, pyridine, dipotassium hydrogen phosphate, hydrogen peroxide, sucrose, sodium pyrophosphate, phenazine methosulfate, nitroblue tetrazolium, nicotinamide adenine diphosphate, sodium salt monohydrate (NADH), dinitrophenyl hydrazine (Brady’s reagent), ascorbic acid, sodium acetate, thio urea, etc. Kits include triglyceride, total cholesterol, HDL cholesterol direct, creatinine, protein, albumin, urea etc.

- Vitamin C tablets 500 mg were purchased from the local market and resveratrol capsules 250 mg were obtained as gift sample from Biotivia Bioceuticals International, USA.

3.1.2 Instruments used

- Manual weigh machine
- Sphygmomanometer and stethoscope
- Blood Glucose Monitor and strips (Dr. Morepen Gluco-One blood glucose monitor system)
- HbA\textsubscript{1C} Monitor (A1C Now\textsuperscript{+} Bayer Healthcare LLC, USA)
- UV Spectrophotometer (Shimadzu; UV-1700)
- Auto analyser (Merck Microlab 200; CA-536)
Materials and Methods

- Centrifuge (Remi; R8C)
- Digital pH meter (Sartorius; R200D&172)

3.1.3 Outcome Measures

Health related quality of life (HRQoL) instrument and Diabetes treatment satisfaction questionnaire (DTSQ) were obtained from Health Psychology Research Ltd., Orchard Building, Royal Holloway, University of London, Egham, Surrey. These instruments were obtained after signing a user agreement between JSS College of Pharmacy and the concerned organization. Some of the other patient reported outcome measures, namely, patient adherence, and patient knowledge and attitude care were collected from the University of Wisconsin-Madison, USA, and MCOPS, Manipal, respectively.

3.1.3.1 Audit of Diabetes-Dependent Quality of Life (ADDQoL)

The ADDQoL has been designed for measuring the impact of diabetes on the quality of life for adult and older adolescents with type 1 or type 2 diabetes mellitus (Bradley et al., 1999). The instrument is a 19 items scale based on a modification of existing ADDQoL 18 instruments, which in turn was modified from the ADDQoL 13. It consists of two overview items for audit purposes, namely, generic “present QoL” and diabetes specific “impact of diabetes on QoL”. It also includes 19 items concerning the impact of diabetes on specific aspect of life which includes social, physical and emotional functioning. The ADDQoL has some important advantages over other questionnaire based instruments, allowing patients to indicate which aspect of life apply to them and which are not, impact of diabetes on that aspect of life and whether the impact is positive or negative and the perceived importance of each aspect of life for their QoL.

It can be administered by email or in the clinic, for a range of purposes including an assessment tool for individual, for groups, for a broad cross section survey, a routine part of clinical audit cycles and an outcome measure for clinical research trials evaluating new
treatment. The questionnaire includes 19 life domain specific items to be scored between -9 to +3 depending upon the impact of the disease on the quality of life. Each item of the questionnaire consists of two sets (part a and part b), namely, impact of diabetes on specific domain and importance of those domain for their quality of life.

The instrument has been translated into more than 23 languages including Hindi and English. In the present study Audit of Diabetes Dependent Quality of Life (ADDQoL19) English for S. Asians (India & UK) 7.11.04 (from standard UK English rev. 13.10.03C) and Audit of Diabetes Dependent Quality of Life (ADDQoL19) Hindi for India & UK (from English for S. Asians rev. 7.11.04) were used.

### 3.1.3.2 Diabetes Treatment Satisfaction Questionnaire (DTSQ)

The diabetes Treatment Satisfaction Questionnaire (DTSQ) is an 8 item scale designed to measure patient satisfaction with treatment and individual glycaemic control perception (Bradley, 1994; Bradley & Lewis, 1990). Here each item is rated on a seven point Likert scale (0-6). Six items (items 1 and 4-8) are summed to produce a measure of treatment satisfaction with scores ranging from 0 (very dissatisfied) to 36 (very satisfied). The remaining two items are treated individually. Item 2 measures perceived frequency of hyperglycemia and item 3 measures perceived frequency of hypoglycemia. The both items score ranging from 0 (none of the time) to 6 (most of the time). The instrument is translated into more than seventy two languages. In the present study Diabetes Treatment Satisfaction Questionnaire (status) (DTSQs) English for India 10.4.06 (from standard UK English rev. 7/94) and Diabetes Treatment Satisfaction Questionnaire (status) (DTSQs) Hindi for India & UK 8.11.04 (from standard UK English rev. 7/94) were used.

### 3.1.3.3 Brief Medication Questionnaire (BMQ)

BMQ is a 4-part medication adherence tool (Svarstad et al., 1999), namely, regimen screen, belief screen, recall screen and access screen. Regimen screen consists of 7 items checking
how and whether the patients took the medication according to what was prescribed. Belief Screen consists of 2 items that asks about the drug effects and bothersome features. Recall Screen consists of 2 items about potential difficulties remembering and 2 items in access screening that ask about difficulty in paying and refilling medication. The adherence risk scale (ARS) measures the number of adherence risk factors present and is constructed by adding the subtotals listed above (Subtotal A + Subtotal B + Subtotal C + Subtotal D= ARS). The ARS score ranges from 0 to 4, with “0” indicating no self-reported nonadherence or barriers to adherence and “4” indicating the presence of self-reported nonadherence and three types of barriers (belief or motivational barrier, recall barrier, and access barrier).

3.1.3.4 Knowledge, Attitude and Practice (KAP)

The Knowledge, Attitude, and Practice (KAP) Questionnaire was used to assess the patient knowledge, attitude and practice. The questionnaire was developed by the Department of Medicine, Kasturba Hospital, Manipal. The original questionnaire was prepared in three languages: Kannada, Malayalam, and English. It consists of 25 questions: seven attitude/practice questions (numbers 8, 11, 13, 16, 17, 23, and 24) and 18 knowledge-related questions. For the knowledge related questions, each question was scored as one (1) for a correct answer and zero (0) for an incorrect answer. For the practice questions, adhering to the guidelines for disease management or instructions from the patient’s healthcare providers merits a score of 1 and non-adherence a score of 0. The English version of this questionnaire was used for this study.

3.1.3.5 Framingham risk score

An individual’s risk for future cardiovascular events is modifiable, by life style changes and preventive medical treatment. It is important to be able to predict the risk of an individual patient, in order to decide when to initiate life style modification and preventive medical treatment or to evaluate the effectiveness of life style modification and treatment. The
Framingham risk score is used to estimate the 10-year cardiovascular risk of an individual. The Framingham Risk Score is based on data obtained from the Framingham heart study (D'Agostino et al., 2008). The Framingham risk score is one of a number of scoring systems used to determine an individual's chances of developing cardiovascular disease. The Framingham Risk score gives a good indication of the likely benefits of prevention. They are useful for both the individual patient and for the clinician in helping to decide whether lifestyle modification and preventive medical treatment and for patient education, by identifying men and women at increased risk for future cardiovascular events. The CHD risk at 10 years in percent can be calculated with the help of the Framingham risk score.

3.2 Methods

3.2.1 Study design and site

The study was designed as a prospective, open label and randomized control trial to assess the health outcome measures and vascular risk factors in patients with T2DM receiving vitamin C and resveratrol as supplementation. The study was carried out at the Outpatients Department of Government Headquarters Hospital, Ootacamund, The Nilgiris, Tamilnadu, India.

3.2.2 Study duration

Each patient was followed up for a period of 6 months.

3.2.3 Study period

2 Years

3.2.4 Sample size

90 Eligible patients, 30 patients in each group control, group receiving vitamin C and group receiving resveratrol.

3.2.5 Primary outcome measures

Glycaemic Control (12 weeks and 24 weeks)
3.2.6 Secondary outcome

a) Blood pressure, Lipid Profile, Oxidative Stress, Body Weight (12 week and 24 week of supplementation),

b) Health outcome measures include Health related quality of life (HRQoL), Diabetes treatment satisfaction, medication adherence, knowledge attitude and practice and economic outcome.

3.2.7 Ethical consideration

The study protocol was approved by the Institutional Human Ethics Committee of J.S.S. College of Pharmacy, Ootacamund, Tamil Nadu, India. The trial was also registered with Clinical Trial Registry of India (Registration No. CTRI/2011/05/001731).

3.2.8 Intervention and comparator agents

Vitamin C tablet 500 mg/OD for 6 months.

Resveratrol 250 capsule mg/OD for 6 months.

Both the supplements are available in the market in oral dosage form.

3.2.9 Inclusion and exclusion criteria

Participants for this study were selected from volunteers from a population of south India with T2DM residing in Ootacamund, who receive care at the Government Head Quarter Hospital Ootacamund. Selection of participants was based on the inclusion and exclusion criteria.

*Inclusion criteria* Male /female subjects between the ages group of 30 to 70 years, clinically diagnosed with T2DM were selected. To maximize the accuracy of classification of subjects as having diabetes, at least one of the following criteria was made sure to be true: ICD-9-CM hospital discharge diagnosis of diabetes or a prescription for an oral hypoglycemic drug or the participants who answered “yes” to the baseline question about a physician diagnosis of diabetes and having HbA1c ≥ 6.7 % recorded in the medical history record. Duration of
Materials and Methods

disease should not be less than 3 years and patients were on at least 6 months of ongoing oral antidiabetic treatment like glibenclamide and/or metformin. Patients should have the ability to comply with the procedure and able to give consent for the study participation. The patient or his/her care taker must be capable of speaking English or Hindi.

Exclusion criteria  Pregnant or lactating women, paediatric population T1DM. patients with any other co-morbidity and/or contraindication that may interfere with the study result and patients with significant hepatic, renal dysfunction and blood disorder.

3.3 Procedure

3.3.1 Selection of subjects and baseline data collection

Patients were first identified. The objectives of the study were explained to them at the screening visit after verifying the inclusion criteria. The subjects who met the inclusion criteria were enrolled after getting their consent. The standard demographic data collection form and case report form were constructed to obtain data on key variables including age, sex, education, occupation, income, marital status, living status, social habit and food habits. The medical information includes body weight, height, blood pressure, duration of disease, pharmacological treatment, co-morbidity, co medication, family history of diabetes, direct and indirect cost, etc. Health related quality of life (HRQoL), diabetes treatment satisfaction (DTSQ), and medication adherence were also assessed.

3.3.2 Patient randomization

The enrolled patients were then randomized into three groups, namely, the control group and the two intervention groups by using computer assisted method of randomization. After randomization, a coloured identity card was issued to each patient for identification. On the initial visit patients were asked to come on fasting for blood sample collection. Knowledge, practice and attitude (KPA) and patient satisfaction were also assessed.
3.3.3 Supplementation with antioxidant

Patients in the control group received only oral antidiabetic agents, whereas patients in the intervention group received vitamin C supplementation (500 mg/day) or resveratrol supplementation (250 mg/OD) along with their antidiabetic treatment.

3.3.4 Follow up and assessment

Patients were asked to visit every month until six months and prognosis was assessed by health outcome measures and biochemical parameters. During the follow up, drugs refilling and general counseling were also done. The follow up plan used is presented in Table 3.

Table 3: Follow up plan

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
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<td></td>
<td>Control</td>
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<tr>
<td></td>
<td>Control</td>
<td>√</td>
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</tr>
</tbody>
</table>

3.3.5 Bicochemical analysis

Fasting (12h) veinular blood sample (5mL) was collected from the patients for biochemical estimation at the baseline, after 3 months and at the end of 6 months. The plasma/serum was separated by centrifugation and analyzed. HbA1C was measured by using A1C Now⁺ (A1C Now⁺ Bayer Healthcare LLC, USA) monitor and fasting blood sugar was monitored by using
Materials and Methods

Dr. Morepen Gluco-One blood glucose monitor system. Lipid profile and urea, creatinine, total protein, albumin were analyzed in the centre diagnostic laboratory of Government Headquarter Hospital, Ootacamund. Oxidative stress parameters were measured by using colorimetric methods.

3.3.5.1 Glycated hemoglobin (HbA1C)

Bayer A1C Now+ consists of microelectronic, optic and dry reagent chemistry strips within a reusable, self contained, integrated hand held monitor and single use test cartridge. When unmeasured whole blood mixture is directly applied to the sample port, the results are displayed in numeric form on the monitor’s liquid crystal display after 5 minutes. Having no switches or buttons the monitor self activates upon insertion of the cartridge. The A1C Now+ monitor utilizes both immunoassay and chemistry technology to measure A1C and total hemoglobin, respectively. Upon the addition of diluted blood sample, blue microparticles conjugated to anti-A1C antibody migrate along the reagent strips. The amount of blue micro particles captured on the strips reflects the amount of A1C in the sample.

3.3.5.2 Fasting blood glucose

Dr. morepen blood Gluco-One blood glucose monitor is an instrument which enables to test the blood sugar very simply and with confidence. It requires Dr. morepen blood Gluco-One blood glucose monitor, test strips and Lancing system for testing blood sugar. On each test strip there is a test area (pad) containing sensitive chemicals. When blood is applied to this area, a chemical reaction takes place causing the color of the test pad change. The Dr. morepen blood Gluco-One blood glucose monitor registers this color change and, with the aid of the lot-specific information held in the coding cheap, converts the signal obtained into a displayed reading. Determination of glucose in fresh capillary blood was done by reflectance photometry.
3.3.5.3 Triglycerides (TGL)

Triglycerides were measured by colorimetric/ enzymatic method with glycerophosphate oxidase. This is based on the method of Wako modified by Mc Gowan et al., and Fossati et al. Triglycerides catalyze the following reactions;

\[
\text{Triglyceride} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty Acid},
\]
\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-Phosphate} + \text{ADP},
\]
\[
\text{Glycerol-3-Phosphate} + \text{O}_2 \xrightarrow{\text{GPO}} \text{Dihydroxy acetone phosphate} + \text{H}_2\text{O}_2,
\]
\[
2\text{H}_2\text{O}_2 + \text{Aminoantipyrine} + 4\text{-Chlorophenol} \xrightarrow{\text{POD}} \text{Chinonimine} + 4\text{H}_2\text{O}
\]

Where, GK is glycerokinase, GPO is glycerol-3-phosphate oxidase and POD is peroxidase. The absorbance of the sample and standard were measured against the blank at 505 nm (500-540). The intensity of chromogen formed is proportional to the triglyceride in the sample. The triglyceride levels were expressed as mg/dL.

\[
\text{Triglycerides [mg/dL]} = (A \text{ Sample} / A \text{ Standard}) \times \text{Concentration of standard}
\]

3.3.5.4 Total Cholesterol (TC)

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyzes the esters. In the subsequent enzymatic oxidation by cholesterol oxidase, $\text{H}_2\text{O}_2$ is liberated. This is converted into colored quinoneimine with 4-aminoantipyrine and phenol catalyzed by peroxidase (Jacobso, 1996) as per the following reaction;

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{Fatty Acid}
\]
\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{CHO}} \text{Cholesterol-3-one} + \text{H}_2\text{O}_2
\]
\[
2\text{H}_2\text{O}_2 + \text{Amino antipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\text{H}_2\text{O}_2
\]
Where, CHE is cholesterol esterase, CHO is cholesterol oxidase and POD is peroxidase. The absorbance of the sample and standard was measured against the reagent blank at 546 nm. Cholesterol level was expressed as mg/dL.

\[
\text{Cholesterol [mg/dL]} = \left( \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \right) \times \text{Concentration of standard}
\]

### 3.3.5.5 HDL-Cholesterol

Anti-human β-lipoprotein antibody binds to lipoproteins (LDL, VLDL and Chylomicrons) other than HDL. The antigen-antibody complexes formed block enzyme reaction. Cholesterol esterase and cholesterol oxidase react only with HDL-C. Hydrogen peroxide produced by the enzyme reactions with HDL-C yield a blue color complex upon oxidative condensation of F-DAOS [N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,3-dimethoxy-4-fluroaniline, sodium salt] and 4-aminoantipyrine in the presence of peroxidase. By measuring the absorbance of the blue color complex produced, at the optimum wavelength of 593 nm, the HDL-C concentration in the sample was calculated and compared with the absorbance of the HDL-C calibrator (Rifai and Warnick, 1994).

\[
\text{HDL concentration [mg/dl]} = \frac{(\text{OD}_2-\text{OD}_1)_{\text{sample}}}{(\text{OD}_2-\text{OD}_1)_{\text{Calibrator}}} \times \text{Calibrator concentration}
\]
3.3.5.6 LDL Cholesterol and VLDL Cholesterol

The LDL and VLDL were calculated by using Friedewald equation (1972).

\[
LDL = \text{Total cholesterol} - \text{HDL} - (\text{Triglycerides} \div 5)
\]

\[
VLDL = \text{Triglycerides} \div 5
\]

3.3.5.7 Total Protein (TP)

Peptide bonds of protein react with cupric ions in an alkaline solution. This reaction, called as the Biuret reaction, is particularly easy to carry out that gives reproducible results, which are in good agreement with Kjeldahl method. The absorbance of the colored complex is directly proportional to the protein concentration in sample materials. The absorbance of the sample and standard total protein was measured against the Biuret reagent and the absorbance of the blank against distilled water at 578 nm (Koller, 1984). Total protein level is expressed as g/dL.

\[
\text{Total Protein [g/dL]} = \left(\frac{A_{\text{Sample}}}{A_{\text{Standard}}}\right) \times 6.5
\]

3.3.5.8 Albumin

Albumin act as a cation at pH 3.68 and binds to the anionic dye bromocresol green (BCG) forming a green color complex (Gendler, 1984). The absorbance of color was measured at 630nm. The color intensity of the complex is proportional to albumin concentration in the sample.

\[
\text{Albumin [g/dl]} = \left(\frac{\text{Absorbance of test}}{\text{Absorbance of standard}}\right) \times 4
\]

3.3.5.9 Globulin The globulin was calculated by using the following formula;

Globulin = Total Protein –Albumin

3.3.5.10 Creatinine

Creatinine reacts with alkaline picrate to produce an orange-yellow color (the Jaffe reaction). Specificity of the assay has been improved by introduction of an initial rate method. The
absorbance of the orange-yellow color formed is directly proportional to creatinine concentration and was measured photometrically at 500-520 nm (Bower, 1980).

\[
\text{Creatinine [mg/dl]} = \frac{\Delta A \text{ of test}}{\Delta A \text{ of standard}} \times \text{Concentration of standard (mg/dl)}
\]

Where \( \Delta A = A_2 - A_1 \)

### 3.3.5.11 Urea

Urea hydrolyses in presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, ammonia so formed reacts with hypochlorite and phenolic chromogen to form colored indophenols, which was measured at 578 nm (570-600nm). Sodium nitroprusside acts as a catalyst. Intensity of color is proportional to the concentration of urea in the sample (Murray, 1984).

\[
\text{Urea concentration [mg/dl]} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 50
\]

### 3.3.5.12 Superoxide Dismutase (SOD)

The activity of SOD was estimated by measuring of the inhibition of the formation of the blue coloured formozan from nitro blue tetrazolium (NBT) in the presence of phenazine methosulphate (PMS) and reduced nicotinamide adenine dinucleotide (NADH). One unit of activity of SOD is defined as the amount of enzyme that inhibits the rate of reaction by 50% under special conditions. The incubation mixture consisted of 40 \( \mu \)L of plasma, sodium pyrophosphate buffer (pH 8.3, 0.052 M, 1.2 mL), phenazine methosulphate (186 \( \mu \)M), nitroblue tetrazolium (300 \( \mu \)M) and NADH (780 \( \mu \)M, 0.2 mL). The reaction was initiated by
the addition of NADH; following incubation was done for 90 seconds at 37° C. The reaction was terminated by the addition of glacial acetic acid (1 mL). n-Butanol (4 mL) was added, shaken vigorously, centrifuged at 4000 rpm for 1 min. and the upper butanol layer was read at λ- 560 nm against butanol blank (Kakkar et al., 1984).

Reagents

Phenazine metho sulphate (PMS) (186 µM): 5.698 mg of phenazine methosuphate was dissolved in 100 mL of distilled water giving a concentration of 186 µM.

Nicotinamide Adenine Dineucleotide Sodium Salt Monohydrate (NADH) solution (780 µM): 55.3 mg of NADH was dissolved in 100 mL of distilled water giving a concentration of 780 µM.

Nitro Blue Tetrazolium (NBT) (300 µM): 24.5 mg of nitro blue Tetrazolium was dissolved in 100 mL of distilled water giving a concentration of 300µM.

Sodium Pyrophosphate Buffer (0.052 M, ph 8.3): 1.3826 gms of sodium pyrophosphate was dissolved in 100 mL of distilled water giving the concentration of 0.052 M. The pH was adjusted to 8.3 with 1 N sodium hydroxide solution (NaOH) or 0.1 N Hydrochloric acid solutions (HCl).

3.3.5.13 Catalase (CAT)

CAT measurement was done based on the ability of CAT to inhibit oxidation of hydrogen peroxide (H₂O₂). Potassium phosphate buffer (2.25 mL 65 mM, pH 7.8) and 100 µL of the serum (0.32 M) were incubated at 25°C for 30 minutes. H₂O₂ (7.5 mM; 650 µl) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3 min. The dy/dx for every min for each assay was calculated and the results were expressed at CAT units of protein (Beers et al., 1952).
Reagents

Potassium Phosphate Buffer (65mM, pH 7.8): 2.2116 gm of dipotassium hydrogen phosphate was dissolved in 250 mL of distilled water (Sol. A) and 11.323 gm of potassium di hydrogen phosphate was dissolved in 1 L of distilled water (Sol. B). Sol A and B were mixed and the pH was adjusted to 7.8 with potassium di hydrogen phosphate.

Sodium chloride 0.154 M containing 0.1 mM EDTA: 9 gm of sodium chloride and 29.22 mg of EDTA were dissolved in 1 L of distilled water.

Hydrogen peroxide 7.5 mM: 1.043 mL original (30 % w/w, 0.08821M) was made up to 100 mL with sodium chloride in EDTA.

Sucrose 0.3199 M: 10.95 gm of sucrose was dissolved in 100 mL of distilled water.

3.3.5.14 Thiobarbituric Acid Reactive Substances (TBARs)

The quantitative measurement of thiobarbituric acid reactive substances (TBARS) is an index for lipid peroxidation. The incubation mixture was made up to 5.0 mL with double distilled water and then heated in boiling water bath for 30 min. After cooling, the red chromogen was extracted into 5 mL of a mixture of n-butanol and pyridine (15.1v/v) and centrifuged at 4000 rpm for 10 min. The organic layer was taken and its absorbance at 532 nm was measured. 1, 1, 3, 3 Tetra ethoxypropane (TEP) was used as an external standard and the levels of lipid peroxides were expressed as nmole/ml protein. (Okhawa et al., 1979).

Reagents

Sodium dodecyl sulphate (SDS): 8 g of SDS was dissolved in 100 mL of distilled water.

Acetic acid solution (20%): 20 mL of glacial acetic acid solution was taken and the volume was made up to 100 mL. The pH was adjusted to 3.5 with 1 N sodium hydroxide solution (NaOH) or 0.1 N Hydrochloric acid solutions (Hcl).

Thiobarbituric acid solution: 0.9 g of the thiobarbituric acid was weighed and dissolved in 100 mL of distilled water by means of ultrasound sonicator for 20-35 minutes until a clear
solution was obtained. The pH was adjusted to 7.4 with 1 N of Sodium hydroxide or 0.1 N Hydrochloric acid.

Extraction mixture: 15 mL of n-butanol and 1 mL of pyridine was mixed to form two immiscible layers in the ratio of 15: 1 v/v.

1,1,3,3 Tetraethoxy propene: 0.82 mL of the standard 1, 1, 3, 3-tetramethoxy propane was diluted to 5 mL with distilled water to obtain 1 M solution. 1 mL of this solution was further diluted to 10 mL with distilled water and this dilution process was further repeated for seven times to get 10 nM 1, 1, 3, 3-tetramethoxy propane.

3.3.5.15 Reduced Glutathione (GSH)

The supernatant of homogenate was mixed with trichloroacetic acid (10% w/v) in 1:1 ratio. The tubes were centrifuged at 1000 g for 10 min at 4 °C. The supernatant obtained (0.5 mL) was mixed with 2 mL of 0.3 M disodium hydrogen phosphate. Then 0.25 mL of 0.001 M freshly prepared DTNB [5, 5`-dithiobis (2-nitrobenzoic acid) dissolved in 1% w/v citric acid] was added and the absorbance was noted spectrophotometrically at 412 nm. A standard curve was plotted using 5-50 µM of the reduced form of glutathione and results were expressed as micromoles of reduced glutathione per mg of protein (Beutler et al., 1963).

Reagents

Preparation of 10% trichloroacetic acid: 10 g of trichloroacetic acid was dissolved in 100 mL of distilled water.

Preparation of 0.3 M disodium hydrogen phosphate: 4.26 g of anhydrous disodium hydrogen phosphate was dissolved in 100 mL distilled water.

Preparation of 5, 5`-dithiobis (2-nitrobenzoic acid) in 1% citric acid: 7.92 mg of 5, 5`-dithiobis (2-nitrobenzoic acid) was dissolved in 20 mL of 1% citric acid.

Preparation of 50 µM of reduced glutathione: 6.14 mg of reduced glutathione was dissolved in 400 mL distilled water.
3.3.5.16 Ascorbic Acid

Serum (250 µl) with trichloroacetic acid was centrifuged at 4000 rpm for 10 min. To the supernatant, 62 µl of DPNH reagent was added and the mixture was heated at 85°C for 30 minutes. Sulphuric acid (430 µl) was slowly added through the sides of the test tube under cooling ice water. After complete mixing of the tubes the substance was allowed to stand for 30 minutes at room temperature. The absorbance was taken at 540 nm. The standard ascorbic acid was prepared in sodium acetate buffer (1mM) and the calibration curve was prepared through which the absorbance was correlated and the levels or concentration of the ascorbic acid was found out (Natelson et al., 1963).

Reagents

Preparation of 4 % Trichloro acetic acid: 4 mL of trichloroacetic acid was diluted 100 mL in a standard volumetric flask.

Dinitrophenyl hydrazine (DNPH) reagent: 5 mL of 5 % thiourea and 5 mL of 0.6 % copper sulphate (CuSO₄) were mixed in 90 mL of DNPH. DNPH was prepared by dissolving 2.2 g of DNPH in 100 mL of ION H₂SO₄.

Sulphuric acid solution (65%): Sulphuric acid (65 ml) was diluted to 100 ml with water.

Sodium acetate buffer (1mM): Anhydrous sodium acetate (0.08203g) dissolved and diluted to 1000 mL of water giving a concentration of one millimole (1 mM). Standard ascorbic acid solution (1 mg/mL) was prepared in sodium acetate buffer (1mM). The pH of the solution was adjusted to 4.5 by ortho-phosphoric acid or sodium hydroxide solution (1N).
3.4 Statistical analysis and interpretation of data

The data obtained from the study was subjected to statistical analysis using graphPad InStat and interpreted. All data are expressed as mean ± SD. Descriptive statistics was performed to obtain mean and standard deviations for parametric data. The probability value less than 0.05 are considered for statistical significance. Repeated measure ANOVA with post hoc tests was used for interpreting the data.