MATERIALS

AND

METHODS
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(1) Location, sample size and criteria for registration of the subjects:

The study was conducted in the department of Biochemistry, GSVM medical college, Kanpur, India. Healthy human (n=120), non-insulin dependent diabetic (Type-II) (n=90), ischaemic heart disease patients (n=90) and non-insulin dependent diabetic + ischaemic heart disease patients (n=90) of either sex and of matched age group subjects who were attending OPD, diabetic clinic and indoor patients of Department of Medicine and LPS Institute of Cardiology, GSVM Medical College, Kanpur were registered for the study. All participants were provided a specific written information about the aims of the study before written consent is obtained, in accordance with the declaration of Helsinki. All these individuals were registered at different times and at different locations, because they were not available at one time and lived in the same urban area (Kanpur City and Kanpur Dehat i.e. Districts of Kanpur).

(2) Selection of the Subjects and their groupings:

1. Normals: Comparised of volunteered normal healthy subjects of different age groups and either sex (age group 30-60 years and above with male to female ratio of 3:1) were selected from college campus residents and staff members of GSVM Medical College, Kanpur and its departmental staff, their friends and relatives residing in the same afore-said locations. Prior consent of the subjects were taken before including them in the study. Subjects were advised not to take Cinnamon / its kinds of preparations of the diet from the last 15 days before the study and during the trial period. Further, subjects were asked to take their usual daily diet and to stick it during the trial period so that they were isocaloric during the study period. Only those subjects were taken under the study who were free from any illness and have not taken any other kind of drug during the trial period. Normal subjects were divided into three sub groups as 1A, 1B, 1C (each minimum N=30) depending upon taking three corresponding drug preparations designated A, B and C.

2. Diabetics: Poor glycemic controlled or glycemic controlled and established (not less than 5 years) non-insulin dependent diabetic subjects (Type-2 diabetes) in age group of
30-60 years and above of either sex in a ratio of male to female 3:1 were selected from indoor and outdoor of LLR Hospital, associated Hospitals and Diabetic Clinics, Department of Medicine of GSVM Medical College, Kanpur. Taking into account the inclusion and exclusion criteria, only those were included in study, who have no other disease (CVD/CHD, hepatic, renal, neurological etc.) except diabetes mellitus. As per declaration of Helsinki, their written consent were obtained and made them understand the trial. They were advised to take their prescribed doses of drug preparation under the guidance of the physician. Further they were suggested and called for periodical checkup and followed up treatment by the treating physicians.

Diabetic subjects were divided into three subgroups as 2A, 2B and 2C (each minimum N=30) depending upon taking three corresponding, two cinnamon drug preparations and a placebo designated A, B and C of the study respectively.

3. **Heart Patients (IHD)**: Established non-diabetic Ischeamic Heart Diseased patients, admitted, indoor, outdoor of Cardiology department of LPS Institute of Cardiology, formed the group-3 of Heart patients. These patients included in the one group suffered from different types of heart related diseases (CVD/CAD/CHD/IHD, unstable angina, its acute or chronic conditions established by definite EKG changes and other related biochemical parameters in blood). Each patients followed the drug regimen as prescribed by the treating physician for the particular illness. Subjects were divided into 3 subgroups as 3A, 3B and 3C (each minimum N=30) depending upon taking two corresponding drug preparations and a placebo designated A, B and C of the drug trial respectively.

4. **Diabetics with IHD (DM+HD)**: Type – II diabetic subjects having one or more IHD episodes of various kinds as described for group-3 patients and receiving antidiabetic drugs along with drug regimen for IHD etc as prescribed to them by the physicians, were included in this group.

Subjects were divided into 3 subgroups as A, B and C correspondingly received two Cinnamon preparations and placebo during the trial period (minimum in each group N=30 or otherwise specified).
(3) **Cinnamom drugs preparation**:  
Cinnamomum zeylanicum (Dalchini bark) bark of one lot was purchased in bulk from the reputed shop of the local market and was finely grounded to powder in waring blender at room temperature (20-30°C) in the month of November 2006 and then in November 2007. Cinnamon powder was stored and kept in tightly capped wide mouth brown/Amber color glass bottles and kept in the shaded cupboard at ambient temperature of room. 3g packets of cinnamon in aluminium foil were prepared.

(4) **Selection of Effective dose / dose prescribed in the study**:  
Cinnamon in a dose of 1, 3 and 6 g have been reported to possess significant hypoglycemic and hypolipidemic effects (93). Appropriately 9 g cinnamon is GRAS (Generally recognized as safe) by the American Herbal Product Association (AHPA) reported in Hand Book of Botanical Safety; however AHPA suggests pregnant women & other severely diseased patients should not exceed dosage. Hence, optimum 3g dose / day had been selected for the present study.

(5) **Cinnamom drug preparation and its mode of Administration**:  
Two types of drug preparation and a placebo was prepared and administered to all subjects of respective subgroups (A,BC) of each major four (1,2,3 and 4) groups designated as follows:

A- 3g Cinnamon powder was taken by the subjects during breakfast with/along 150 ml tea liquor (without milk) prepared by boiling ½ teaspoonful tea without sugar for 5 minutes.

B- Subgroup B subjects were assigned to drink 150 ml of tea liquor without milk and sugar prepared by boiling 3g Cinnamon powder + ½ teaspoonful tea for five minutes in that much of water which after boiling remained to about 150 ± 10 ml.

C- Placebo of 150 ml tea liquor alone prepared as described above without Cinnamon powder was administered to C subgroup of all the subjects of four major groups 1 to 4 (1-Normals, 2- Diabetic, 3- Heart (IHD etc.) and 4- Diabetic with IHD etc.)
(6) **Duration of the Cinnamom drug preparation trial**:

Separately, above two drug preparations and placebo were administered to 1- normals, 2- Diabetics, 3- Heart patients (IHD) and 4- Diabetics with Heart disease (IHD etc. as described previously) continuously for 30 days. After 30th day, each preparations (A, B and C) administration were withdrawn. At the 30th day each subjects of all four major groups were advised to take the three preparations (Cinnamon preparations A and B and placebo of 150 ml Tea) in the early morning without breakfast. Morning blood samples of each subjects of different subgroups were collected at 0 day i.e. before administration of drugs (for initial or basal values) and at 30th day of drug trial and at 45th day i.e. after 15 days of drug withdrawal.

(7) **Collection of blood samples, preparation of haemolysate & biochemical analysis**:

Approximately 5 ml 10-12 hours fasting heparinised blood samples from median vein of arm were taken from each subjects of the subgroups of 4 major groups on day 0, 30th and 45th of drug trial. About 1.0 ml blood sample were collected in the fluoride vial and the rest transferred to sterilized centrifuge tubes. The whole blood was centrifuged for 10 minutes in a refrigerated centrifuge machine at 15000 rpm for plasma separation and RBC collection. Plasma samples were collected / stored in refrigerator at 4-5°C for later analysis. For different experiments, blood samples were processed accordingly for analysis.
I Preparation of haemolysate:

Reagents:-

1) Stock solution of Sodium Citrate: 3.8 g of Sodium Citrate + 100 ml distil water.

2) Normal saline (0.9%): -0.9 gm of NaCl + 100 ml distil water.

<table>
<thead>
<tr>
<th>S. N.</th>
<th>Preparation of haemolysate: (All operations were carried out at 0-5°C or otherwise stated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>0.4 ml of stock solution + 1.6 ml of blood + mix + centrifuge at 6000 rpm for 10 minutes = separate plasma.</td>
</tr>
<tr>
<td>2)</td>
<td>wash with normal saline (3 times) + 1.0 ml distil water (refrigerated for 10 minutes) + vigorously shake (for 2 minutes) + 0.5 ml of chloroform + centrifuge at 3000 rpm for 20 minutes = 3 separate layers.</td>
</tr>
<tr>
<td>3)</td>
<td>pipett off uppermost layer + filtered through two layers of filter paper + store into clear dry test tube.</td>
</tr>
</tbody>
</table>

Note: - Haemolysate can be preserved for 7 days at 4°C.

METHODOLOGY AND ESTIMATION PROTOCOL OF PARAMETERS

(1) Plasma Glucose Levels: Plasma glucose level is as determined by the Enzymatic Kit method of GOD/POD (Trinder, 1969).

Reagents Required / Prepared:

2. Reagent – II: Glucose diluent (Phenol preservative).
3. Working Reagents III: One vial reagent – 1 and .50 ml reagent – 2
4. Glucose Standard: Dextrose with preservative (100 mg/dl).

Procedure:

Preparation of 1) Blank: Working reagent 1500 µl
2) Standard: Working reagent 1500 ml + Glucose standard 20 µl
3) Test: Working reagent 1500 µl + plasma 20 µl

Each tubes were mixed well and incubated at 37°C for 10 minutes or at room temperature (15-30 C) for 30 minutes or longer and OD recorded at 505 nm.

Calculation: Plasma glucose (mg/dl) = \( \frac{OD_{of\ T}}{OD_{of\ S}} \)
(2) **Glycated hemoglobin (HbA1C)** estimation done by modified colorimetric method of Fluckiger R, Winterhalter KH (1976)\(^{112}\).

**Preparation of Reagents:**

1) 0.3% oxalic acid w/v  
2) 40% TCA w/v  
3) 0.05 M Thiobarbituric acid (TBA)

**Test for glycated haemoglobin (Procedure):**

To 2 ml of haemolysate, 1 ml of 0.3% oxalic acid was added and heated in a boiling water bath for 60 minutes. After cooling 1 ml of 40% TCA was added and shaken well and then centrifuged. To the 2 ml supernatant, 0.5 ml of 0.05 M TBA was added and incubated at 37 C for 30 minutes and absorption read on 443 nm.

Calculation: HbA1c was calculated on assumption that HbA1c corresponds to an absorption of 0.029 at 443 nm.

(3) **Plasma Malonaldehyde (MDA) level.** by the method as described by Satho K, (1978)\(^{113}\).

**Reagents will be required:**

1. TCA (tri-chloro acetic acid) 15% w/v  
2. TBA (thiobarbituric acid) 0.375% w/v  
3. 0.25N HCl  
4. 1N NaOH

The TCA-TBA-HCl solution will be freshly prepared by mixing equal volume of 15% TCA 0.375% TBA and 0.25 N HCl.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Test Procedure</th>
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</thead>
<tbody>
<tr>
<td>1)</td>
<td>0.8ml of plasma + 1.2ml of TCA-TBA-HCl reagent.</td>
</tr>
<tr>
<td>2)</td>
<td>mixed immediately + kept in a boiling water bath (for 10 minutes)</td>
</tr>
<tr>
<td>3)</td>
<td>cooled + 2ml of 1N NaOH (freshly prepared) to eliminate centrifugation.</td>
</tr>
<tr>
<td>4)</td>
<td>O.D.at 535nm against blank which contained normal saline in place of serum/plasma.</td>
</tr>
</tbody>
</table>
Calculations:

The content of MDA was calculated using molar extinction coefficient \((1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1})\) and expressed as µmole of MDA per 100 ml of plasma.

4) Total antioxidant power (TAP) (FRAP value) by the method as described by Benzie and Strain (1999)

Preparation of Reagents:

1) FRAP reagent:- 1.62 ml conc. HCl + 100 ml acetone.
   i) 10.0 ml of 22.78 mM sodium acetate buffer, pH 3.6,
   ii) 1.0 ml of 20 mM ferric chloride
   iii) 1.0 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine solution prepared in 40 mM HCl.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Test Procedure:</th>
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<tbody>
<tr>
<td>1)</td>
<td>0.1 ml of plasma + 900 µl of freshly prepared FRAP reagent. (both FRAP reagent and plasma samples were preincubated for 5 min at 30° C before starting the reaction)</td>
</tr>
<tr>
<td>2)</td>
<td>incubated for 5 min at 30°C.</td>
</tr>
<tr>
<td>3)</td>
<td>Read the O.D. at 593nm.</td>
</tr>
</tbody>
</table>

Blank Procedure: Instead of serum, procedure is same as for test.

Calculations:

The “total antioxidant power” (FRAP value) was calculated by utilizing a standard ferrous sulfate (µmole/l) by plotting standard calibration curve.

(5) Glutathione Peroxidase (GPx) by Hafeman D.G. method (1974)

Preparation of Reagents:

1) Metaphosphoric acid precipitating solution: 1.67 gm of Metaphosphoric acid + 0.2 gm EDTA + 30 gm of NaCl per 100 ml Triple distil water

2) DTNB Reagent:- Tri-sodium Citrate Solution 40 mg of DTNB (di-thiobis nitrobenzoic acid) + 100 ml 1% w/v

3) Sodium Phosphate buffer : 0.4M, pH = 7.0:- Prepared by mixing followings in appropriate ratio.
i) \( \text{NaH}_2\text{P0}_4 \cdot 2 \text{H}_2\text{O} = 28.39 \text{gm} / 500 \text{ ml. D.W.} \)
ii) \( \text{Na}_2\text{HPO}_4 = 35.58 \text{gm} / 500 \text{ ml. D.W.} \)

4) Sodium Azide (\( \text{NaN}_3 \)) : 0.01M
5) Hydrogen peroxide : 1.25mM
6) GSH : 2mM

**Procedure:**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Tubes</th>
<th>Test Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Test</td>
<td>0.3 ml of haemolysate + 1 ml of Sodium Phosphate buffer + 0.5 ml of NaN(_3) + 1.0 ml of GSH.</td>
</tr>
<tr>
<td>2)</td>
<td>Standard</td>
<td>1 ml of Sodium Phosphate buffer + 0.5 ml of NaN(_3) + 1.0 ml of GSH</td>
</tr>
<tr>
<td>3)</td>
<td>Blank</td>
<td>1 ml of water or 0.15 M KCl + 1 ml of Sodium Phosphate buffer + 0.5 ml of NaN(_3).</td>
</tr>
</tbody>
</table>

**COMMON STEPS:**

a. made up all the three tubes upto 4 ml with distilled water.
b. kept for 5 minutes.
c. added 1 ml of \( \text{H}_2\text{O}_2 \) (pre-warmed to 370°C) to each of the three tubes.
d. after 3 minutes, taken 1 ml of aliquot from all three test tubes.
e. added 4 ml of metaphosphoric precipitating solution in each test tube.
f. centrifuged and supernatant collected.
g. taken 2 ml of supernatant from each test tube.
h. added 2 ml of Sodium phosphate solution and 1 ml of DTNB to each test tube.
i. read at 412 nm within 2 minutes.

**Calculations:**

Specific activity of enzyme = \( \frac{\text{O.D. of Test}}{\text{O.D. of standard}} \times \frac{\text{Units of enzyme per ml}}{\text{Conc. Of Hb in gm percent}} \)
Preparation of Reagents:-

1. **Phosphate buffer (0.1M; pH 7.5)**: It comprises of following ratio mixed in appropriate.
   a. \( \text{Na}_2\text{HPO}_4 = 0.7098 \text{ gm} / 500 \text{ ml distilled water} \)
   b. \( \text{KH}_2\text{PO}_4 = 0.68045 \text{ gm} / 500 \text{ ml distilled water (D.W.)} \)

2. **GSSG (oxidized glutathione); 0.2mM**: 1.22 mg + 10 ml D.W.

3. **NADPH (0.12mM)**: 1mg/10 ml D.W.

Procedure:

Take 0.1ml RBC hemolysate and added 0.9ml chilled glass triple distilled water for 1:10 dilution.

<table>
<thead>
<tr>
<th>S.N.</th>
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<tbody>
<tr>
<td>1)</td>
<td>2100µl of Phosphate buffer + 300µl of GSSG + 300µl of Haemolysate (diluted) + 300µl of NADPH.</td>
</tr>
<tr>
<td>2)</td>
<td>O.D. at 340nm, 7 times in decreasing order after 30 sec</td>
</tr>
</tbody>
</table>

Calculations:

Specific activity of GR = \( \frac{\text{O.D. change per minute}}{6.3 \times 10^3} \times \frac{\text{ml of reaction mixture}}{\text{ml of sample volume}} \times 10^6 \)

Or Specific activity of GR = \( \frac{\text{O.D. change per minute}}{6.3} \times \frac{\text{ml of reaction mixture}}{\text{ml of sample volume/Hb gm dl}^{-1}} \)

(7) **CATLASE**: colorimetric assay of catalase by Ashok K. Sinha (1971)

Preparation of Reagents:

1) **Dichromate acetic acid reagent**: 2.5 gm of potassium dichromate in 50 ml distilled water +150 ml glacial acetic acid. (98-100%)
2) Hydrogen peroxide (0.2M)
3) Phosphate buffer (0.01M) pH=7: contains two chemicals, solution A and B mixed in required proportions.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Test Procedure:</th>
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<tbody>
<tr>
<td>1)</td>
<td><strong>Reaction mixture:</strong> 4ml of H₂O₂ in small beaker + 5 ml of phosphate Buffer + 1 ml of diluted sample (haemolysate 1:10) added rapidly + mixed gently by swirling motion</td>
</tr>
<tr>
<td>2)</td>
<td>Taken 2ml of dichromate acetic acid reagent in another test tubes labeled 1,2,3,4. + 1ml of <strong>reaction mixture</strong> added to the each test tube at an interval of 60 second.</td>
</tr>
<tr>
<td>3)</td>
<td>Read the O.D. at 570nm of all the four tubes 1,2,3,4 containing H₂O₂ 40,80,120 and 160 µmoles</td>
</tr>
</tbody>
</table>

**Procedure of Blank:** - 2ml of dichromate acetic acid + 1ml of distilled water.

**Calculations:**

The activity of catalase was expressed as µmoles of H₂O₂ consumed/min/g Hb or units/gmHb and calculated by calibration curve.

(8) **Superoxide Dimutase (SOD)** in plasma/ RBC hemolysate both by Mishra H.P. & Fridovich (1972) Later partially modified by Polidoro et al, 1984

**Preparation of Reagents:**

1) **Epinephrine or adrenaline (1.8mM)**: - 0.059 gm of adrenaline bitartarate dissolved in + 100 ml. of D.W. (distilled water)

2) **Sodium carbonate buffer (0.3M); pH=10.2** : - It comprises of following .

   a) **Solution A (Na₂CO₃)** = 7.95 gm / 250 ml D.W.
   b) **Solution B (NaHCO₃)** = 6.3 gm / 250 ml D.W.
For making 25 ml solution of Sodium carbonate buffer :- Solution A + Solution B in ratio 8:17

3) EDTA (0.6mM):- 0.0175 gm / 100 ml D.W.

Procedure :-

<table>
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<tr>
<th>S.N.</th>
<th>Test Procedure</th>
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<tbody>
<tr>
<td>1)</td>
<td>0.5ml serum + 0.5ml sodium carbonate buffer + 0.5ml EDTA + 1.0ml glass distilled water + 0.5ml epinephrine</td>
</tr>
<tr>
<td>2)</td>
<td>Measured the increase in absorbance at 480nm. (every 30 second till 2.5 minutes.)</td>
</tr>
</tbody>
</table>

Blank Procedure :- Instead of serum, Normal saline was added rest the procedure is same as for test.

Calculations:-

Specific activity of enzyme (SOD) = Units per ml enzyme
Protein in mg per ml or Hb g/dl

Unit per ml enzyme = 50% inhibition of reaction by SOD/0.1 x 50

Percentage inhibition (%) = \( x \times \frac{100}{A} \); 50% inhibition =100% inhibition /2

Where,
\( x \) = O.D. change in experimental reaction – O.D. change in control(blank reaction)

\( A \) = O.D. change in experimental reaction.

(9) Estimation of Total-cholesterol and HDL-cholesterol (T-C, HDLC) : was estimated by the CHOD/POD – phosphotungustate Method of Allain. 1974119.

Cholesterol ester → Cholesterol + Free Fally acid

\[ \text{CHE} \]

Cholesterol → Cholest-4-ene + \( \text{H}_2\text{O}_2 \)

\[ \text{CHOD} \]

\( \text{H}_2\text{O}_2 + \text{Phenol} + 4\text{AAP} \rightarrow \text{Red Quinoneimine} + \text{H}_2\text{O}_2 \)

\[ \text{POD} \]
On addition of the precipitating reagent to the serum, followed by centrifugation, HDL fractions remain in the supernatant while the other lipoprotein precipitate out.

Reagents:
1) Enzyme reagent (CHE, CHOD and POD)
2) Buffer Solution
3) Precipitating Reagents (Phosphotungustic acid and magnesium ions)
4) Cholesterol standard 200 mg/dl
5) Working reagent: 10 ml reagent – 2 added in reagent-1 (each vial) mixed gradually to constitute working reagent.

**Procedure Protocol**

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Test Procedure</th>
</tr>
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<tbody>
<tr>
<td>1)</td>
<td>0.2 ml plasma/ serum + 0.3 ml precipitating reagent in centrifuge tube mixed well by swirling and allowed to stand minimum for 5 minutes</td>
</tr>
<tr>
<td>2)</td>
<td>Above centrifuged to get clear supernatant</td>
</tr>
<tr>
<td>3)</td>
<td>Four tubes prepared as follows</td>
</tr>
<tr>
<td>a)</td>
<td>Working reagents 1.0 ml 1.0 ml 1.0 ml 1.0 ml</td>
</tr>
<tr>
<td>b)</td>
<td>Cholesterol Standard - - - 0.1 ml</td>
</tr>
<tr>
<td>c)</td>
<td>Plasma - 0.01 ml - -</td>
</tr>
<tr>
<td>d)</td>
<td>Supernatant - - 0.1 ml -</td>
</tr>
<tr>
<td>e)</td>
<td>Water 0.1 ml 0.1 ml - -</td>
</tr>
</tbody>
</table>

Each tube mixed well and incubated for 5 minutes at 37°C and O.D. read at 505 nm against blank

Calculation:

\[
\text{T-C (mg/dl)} = \frac{\text{Absorbance of T-C}}{\text{Absorbance of S}} \times 200
\]

\[
\text{HDL-C (mg/dl)} = \frac{\text{Absorbance of HDL-C}}{\text{Absorbance of S}} \times 5
\]

(10) Estimation of LDL-C: LDL-C was estimated in the sample by using Friedward’s and Fredrichsen’s (1972) formula

\[
\text{LDL-C} = \frac{\text{T-C} - \left(\frac{\text{HDL-C} + \text{TG or VLDL}}{5}\right)}{5} \times 100
\]

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(11) **Estimation of VLDL – Cholesterol**: VLDL-C was estimated in the samples by using Friedwand’s and Fredrichsen’s Formula (1972) \(^{120}\)

\[
VLDL-C = \frac{TG}{5} \text{ (mg/dl)}
\]

(12) **Estimation of Triglycerides (TG)**: By the method of GPO/POD method with ESPAS (Bueoloetal 1973) \(^{121}\)

Lipase

Principle: \(TG \rightarrow \text{Glycerol} - 3-P + H_2O_2\)

ATP, glycerol

Kinase

\(H_2O_2 + POD + 4 - \text{aminoantipyrine} \rightarrow \) Color complex + H\(_2\)O

\[\text{Absorbance read at 540 nm}\]

Reagent: All the reagents were supplied with the Kit.

Reagent – 1 - Enzyme reagent (Lipase, peroxidase, 4-amino antipyrine and ESPAS)

Reagent – 2 – TG Standard (200 mg/dl)

**Procedure**: Three Test tubes were taken and labeled as Blank (B), Standard (S) and Test sample (T). In each tubes 1 ml of enzyme reagent-1 were added. Then 0.01 ml TG Standard was added into S tubes and 0.01 ml sample was added in the T tube. Contents were mixed and incubated for 10 minutes at 37°C. Absorbance of the color developed was read at 540 nM.

**Calculation**:

\[
TG = \frac{\text{Absorbance of } T \times 200}{\text{Absorbance of } S} \text{ (mg/dl)}
\]

**STATISTICAL ANALYSIS**: For analysis of the data the mean value was calculated in each of the groups along with the standard deviations for the different parameters at different days of the study. The student’s ‘t’ test was employed for finding out the statistical significance (p-value) of the results between different groups (sub groups) and at days of study period. Paired ‘t’ test was done for matched groups and unpaired ‘t’ test was done for unmatched groups to analyse the effect of drug. According to the statistical analysis the
“Statistically significant” is not the same as “Scientifically important”. To interpret the results in a scientific context, both ends of the confidence intervals were considered and seen whether they represent a difference between means that would be scientifically important or scientifically trivial (Harvey Motulsky, 1999).