3.1 Protocol for clinical trials
3.1.1 Patient information sheet

What is the study about?

Ayurveda has laid emphasis on maintenance of positive health and prevention of diseases, besides management/treatment of diseases. Medhya Rasyana drugs of Ayurveda besides acting on mental disorders and stress related conditions also provide strength to the body. In the present study selected Medhya Rasyana drug will be evaluated for above conditions. You are invited to participate in this study where you will be provided Ayurvedic drug in capsule one capsule is to be taken daily at bed time.

What will you have to do?

- Your doctor will explain clearly what you have to do.
- It is important that you follow the instructions scrupulously.
- The study will take approximately 12 months to complete
- During this period, you are expected to visit us 13 times.
- Before you start treatment, during the first visit to the clinic, you will undergo a complete physical examination,
- Blood will be taken.
- This is to make sure that you are eligible for the study.
- If you are found eligible, you would be put on trial treatment for 12 months
- The daily dosage will be 300 mg.
- At each visit, you will be supplied with sufficient quantities of drugs to last until your next visit.
3.1.2 Consent Form (Certificate by Investigator)

I certify that I have disclosed all details about the study in the terms easily understood by the patient.

Signature______________________ Name_________________________
Date :__________________________

CONSENT BY SUBJECT

I have been informed to my satisfaction, by the attending physician, the purpose of the clinical trial and the nature of drug treatment and follow-up, including the laboratory investigations to be performed to monitor and safeguard my body functions

I am also aware of my right to opt out of the trial at any time during the course of the trial without having to give the reasons for doing so.

I, exercising my free power of choice, hereby, agree to be a subject for clinical trial on Ayurvedic drug as per dose schedule and also agree to undergo various biochemical and other required tests at specified times.

Signature or Thumb impression……………………………….
Name . . . . . . . . . . . . . Date……………………………….
Date of birth…………  Age (years)……………………….
Signature or Thumb impression of witness:………………..
Name: . . . . . . . . . . . . . . . . . Relationship ..............………............
Date……………………………Signature of Investigator…………………………….

3.1.3 Form I - Screening Performa

- Code No. (Of clinical trial)………………..
- Center……………………………………
- Name of the person…………………………
- Gender  Male….  Female………………
- Date of birth………..  Age in years………
- Address……………………………………
Confidential

The contents of Form IA – history sheet will be kept confidential and no portion, in whole or part will be revealed.

3.1.4 FORM IA – History Sheet

- Code No. (Of clinical trial)……………………
- Centre ………………………………………
- Sl. No. of Subject: …………………………
- Name of the Subject………. ………………..
- Gender Male…… Female…..
- Date of Birth ………… Age (In Yrs)…..
- Educational status…………………………
  Illiterate…. ……………………
  Read & Write…… …………………
  Educational qualifications …………
- Occupation:
  Office work ……………………………
  Field work……………………………
  Work with physical labor………………
  Indicate nature of work………………
  Hours of work per day…………………
- Type of living arrangement;
  Living alone ………………….
  Living with spouse …………..
  Living with family ……………
  Others (specify)…………………
- Income / month…………………
- Chief Complaints with duration (if Any) in days
  Loss of appetite Yes . .  No…
  Duration . . . . . . . …………………

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constipation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vague Pain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weakness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forgetfulness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Recurrent frequent attacks of fever:**
  - Yes... No...
  - If yes indicate frequency of attack / month ....

- **Rhinitis/upper respiratory tract infection:**
  - Yes... No...
  - If yes indicate frequency of attack / month ....

- **Urinary tract infection:**
  - Yes... No...
  - If yes indicate frequency of attack / month ....

- **Other complaints (Specify):** .................

- **History of Past illness:**
  - Tuberculosis: Yes... No......
  - Rheumatic fever: Yes... No......

- **Major hospitalization/surgery during past three years:**
  - Yes... No......
  - If yes, specify...............  

- **Personal History:**
  - **Diet:** Only Veg ...... Only Non-Veg... Both....
Sleep:  
A. Normal …… Duration in hours ….  
B. Abnormal… Duration in hours ….  
C. Freshness in the morning:  
   Yes…. No…

Presence of anxiety:  
Yes…. No…

Addiction  
Smoking:  
   Yes…. No…

If yes specify:  
   (a) No of Cigarettes . . . ........
   (b) Total duration (years).............  

Tobacco:  
   Yes… No…

If yes specify  
   (a) Quantity .....  
   (b) Total duration (years) ....

Alcohol  
   Yes…. No…

If yes specify:  
   (a) Quantity (ml)......................  
   (b) Total duration (years).............

Are you happy with your work –place  
   Yes…. No…

Do you find difficult to adjust with your colleagues  
   Yes…. No…

Any other (specify)  ................................

Physical Examination  
Height (cm).…………………………………….
Weight (kg).…………………………………….
BMI……………………………………………
Pulse (per min).…………………………….
Body temperature……………………………
Respiration rate (per min)……………………
Anemia  
   yes…. No…

Systemic examination  
   CVS:  
      Normal …… Abnormal ……..
   If abnormal, details……………………
- **CNS:**
  - Normal
  - Abnormal
  - If abnormal, details

- **Respiratory system:**
  - Normal
  - Abnormal
  - If abnormal, details

- **Digestive system:**
  - Normal
  - Abnormal
  - If abnormal, details

- **Urogenital system:**
  - Normal
  - Abnormal
  - If abnormal, details

- **Vision:**
  - Normal
  - Abnormal
  - If abnormal, details

- **Hearing:**
  - Normal
  - Abnormal
  - If abnormal, details

- **Locomotor’s system:**
  - Normal
  - Abnormal
  - If abnormal, specify

**Date:** 

**Signature of physician:** 

**Signature of Investigator:**
3.1.5 FORM II – CLINICAL ASSESSMENT

- Code No. (of clinical trial)……………………
- Name ..............................................
- Gender Male…. Female …… ....
- Date of Birth…… Age (In yrs)………
- Date of Assessment ..............................
- Month of Assessment ............................

Clinical Symptoms: Visual Analogue Scale:

- Dizzy spells/Giddiness 0………………10
- Breathlessness on exertion 0………………10
- Constipation 0………………10
- Urgency to micturate 0………………10
- Aching muscles or joints 0………………10
- Low back or shoulder pain 0………………10
- Numbness 0………………10
- Tremors 0………………10
- Sleep Abnormality 0………………10
- Loss of Appetite 0………………10
- Any other non-specific symptoms……
  Yes……  No……  If yes, specify………………
- Adverse reaction: Yes …… No….  If yes, details………………
- Overall clinical assessment by the Physician:
  Improved …… No change ……. Deteriorated…
- Overall impression of well-being by the Subject:
  Improved…… No change…. Deteriorated…

Status of the patient:

Under treatment…

Drop out: Give reason:………………

Death: Give cause:………………

Date: ……. Signature of Physician……….
### 3.1.6 FORM-III LABORATORY INVESTIGATIONS AND PHYSIOLOGICAL PARAMETERS

#### Table-2 Hematological parameters

<table>
<thead>
<tr>
<th>Test Name</th>
<th>Value</th>
<th>Reference Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (Hb)</td>
<td>12.5-18.00</td>
<td></td>
</tr>
<tr>
<td>TLC (Total Leucocyte Count)</td>
<td>4000-11000</td>
<td></td>
</tr>
<tr>
<td>Differential Leucocyte Count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>45-75</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>20-45</td>
<td></td>
</tr>
<tr>
<td>Eosinophil</td>
<td>01-06</td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>01-10</td>
<td></td>
</tr>
</tbody>
</table>

#### Biochemistry

<table>
<thead>
<tr>
<th>Test Name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose random</td>
<td>70-150</td>
</tr>
<tr>
<td>Blood urea</td>
<td>15-45</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0.6-1.4</td>
</tr>
<tr>
<td>Serum uric acid</td>
<td>2.5-7.0</td>
</tr>
<tr>
<td>Serum cholesterol</td>
<td>150-220</td>
</tr>
<tr>
<td>Serum triglycerides</td>
<td>50-160</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>30-70</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>90-210</td>
</tr>
<tr>
<td>VLDL</td>
<td>13-34</td>
</tr>
<tr>
<td>Serum bilirubin</td>
<td>0.2-1.0</td>
</tr>
<tr>
<td>Unconjugated (I.D.Bilirubin)</td>
<td>0.2-0.8</td>
</tr>
<tr>
<td>Conjugated (D.Bilirubin)</td>
<td>0.00-0.2</td>
</tr>
<tr>
<td>SGOT/AST</td>
<td>10-55</td>
</tr>
<tr>
<td>SGPT/ALT</td>
<td>10-50</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>60-170</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>3.2-4.6</td>
</tr>
<tr>
<td>Total protein</td>
<td>6.5-8.5</td>
</tr>
</tbody>
</table>
PHYSIOLOGICAL PARAMETERS

Table-3 Physiological parameters

<table>
<thead>
<tr>
<th>Month of Assessment</th>
<th>value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm of Hg)</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mm of Hg)</td>
<td></td>
</tr>
<tr>
<td>Upper mid arm circumferences (cm)</td>
<td></td>
</tr>
<tr>
<td>Chest circumference (cm)</td>
<td></td>
</tr>
<tr>
<td>Hand grip</td>
<td></td>
</tr>
<tr>
<td>Left Hand (kg)</td>
<td></td>
</tr>
<tr>
<td>Right Hand (kg)</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
</tr>
<tr>
<td>SPO2</td>
<td></td>
</tr>
<tr>
<td>Pulmonary Function FEV1</td>
<td></td>
</tr>
</tbody>
</table>

Date..........................................................

Signature of Physician...................... Signature of Investigator.......................

3.2 Methodology

3.2.1 Placebo: Placebos are inert substances without any action but people who take them believe that they were real (Jadad 1998), placebos are used to make attitudes in both treatment groups as similar as possible (same schedule same looking medication)

3.2.2 Randomization: Participants receive the interventions in random order to ensure and unbiased assignment to treatment by removing any difference between the groups. Similarity of characteristics at the start of the comparison can be achieved through a variety of procedures. Individual groups and the order in which measurements are obtained can all be randomized. (Altman, 1991).
3.2.3 **Random Sample**: Random sample is a set of items that have been drawn from a population in such a way that each time an item is selected; every item in the population has an equal opportunity to appear in the sample. (Hoffman, 2000)

3.2.4 **Controlled Study**: In this study, the patients would be randomized into two groups; one group would be treated with the Brahmi, while the other group would be given a placebo having similar shape, size, color and consistency. The aim of such study is to exclude the psychological effect of the patient, as the patients do not know whether they are treated with the Brahmi or placebo.

3.2.5 **Blinding**: Masking or blinding means that subject are unaware of treatment they were receiving. It represent any attempt made by investigator to keep one or more of the people involved of the trials (that is, the participants or the investigator) unaware of the interventions that is being given and evaluated. The purpose of blinding is to reduce the risk of ascertainment or observation bias. This bias is present when the assessment of the outcome of an intervention is influenced systematically by knowledge of which intervention a participant is receiving. Blinding can be implemented at least six different levels in a randomized clinical trial. These include the participants, the investigator or clinicians who administer the interventions or clinicians who take care of the participants during the trial, the investigator who assess the outcome of the intervention, the data analysis, and the investigators who write the result of the trial (Jadad, 1996, 1998a) therefore, depending on the extent of blinding, randomized controlled trials can be classified as open, single blind, double blind, triple blind, and quadruple blind.

3.2.6 **Double-Blind Study**: This study is planned in such a way that neither the clinician nor the patient is aware of which patient is administered with the Brahmi or with placebo. This study excludes the psychological factors of both the clinician and the patient.

3.2.7 **Cross-Over Study**: This is done to evaluate whether the effect observed is due to the formulation or some psychological factor. This study helps the variations in
particular patients. A patient treated with formulation is shifted to placebo in due course of time.

3.2.8 Place of Work

A randomized, control, double blind, cross-over study was designed and carried out in the pharmacology laboratory of College of pharmacy, IFTM, Moradabad, from July 2008 to August 2009. All the healthy human volunteers of either sex were accessed for eligibility of inclusion/exclusion criteria.

3.2.9 Plant Description

![Bacopa monniera](image)

*Figure-9. The herb of *Bacopa monniera*. 
Latin Nomenclature: Bacopa monniera (Linn.) Wettst
Synonyms: Herpestis monniera (Linn.) HB&K
Grahola monniera Linn.
Moniera cunefolia Michx
Vernacular Names: Hindi: Brahmi, Jalneem, Kapodvanka, Somwalli, Sarsvati
Bengali: Brahmi shak; Udhavni
Telagu: Shambrani; Chettu
Tamil: Nirabrahmi
English: Bacopa
Family: Scrophulariaceae

Bacopa monniera is a small, creeping herb with numerous branches, small oblong leaves, and light purple flowers. In India and the tropics it grows naturally in wet soil, shallow water, and marshes. It is also found in Nepal, Srilanka, China, Taiwan, Vietnam, Florida and Southern states of USA (Parrott, 2001; Russo et al., 2005).

The herb can be found up to an altitudes of 4,400 AMSL, and can be easily cultivated if adequate water is available. Flowers and fruit appear in summer and the entire plant is used medicinally (Chopra, 1958; Bone, et al., 1996).

The genus Bacopa includes over 100 species of aquatic herbs distributed through out the warmer regions of the world. In the United States the herbs are recognized as weeds in rice fields and found abundantly growing in marshes and wetlands of warmer regions (Barrett and Strother, 1978).

Brahmi is frequently mentioned in the religious, social and medical treatises of India since the period of Verdic civilization antiquity and can be traced to the time of Ather ved (800 BC) where Brahmi finds a mention in the very first richa of the third chapter.
Although, it has been frequently mentioned in the religious, social and medical treatises of India since the time of Athar-Ved (C.800 BC), The first clear reference to its memory augmenting property is to be found in Charak Samhita (C.100BC), where brahmi is prescribed as a cure for mental disorder (retardation) leading to insanity (10:62). The etiology of the mental disorder according to Charak is a combination of anxiety, weak intellect and lack of concentration. Another authentic Ayurveda treatise, i.e., Susruta Samhita has described Brahmi as efficacious in loss of intellect and memory.

The Bhavaprakasha Nighantu (Indian Materia Medica of Bhavnishra c.1500-1600 AD) has described Brahmi as bitter in taste, produces coolness and increases vigor. It acts as a bitter, laxative and astringent. It is light in digestion, acts as a brain tonic and promotes longevity. It increases memory, heals whitlow and cures anemia. It is useful in renal disorder, blood disease, cough and poisoning. It is also antipyretic and anti-inflammatory.

It is not clear why this plant was called Brahmi. But, because of its unique properties may eminent ideologists have postulated that the origin of the word Brahmi is from lord “Brahma”, the mythical creator in the Hindu pantheon. Because the brain is the center for creative activity, any compound that improves the brain health is called Brahmi.

3.2.9.1 Medicinal Properties and Uses

The whole plant is the source of the Ayurvedic drug Brahmi, an important ingredient of several Ayurvedic preparations such as Brahmi, Brahmirasayana, Sarasvatarista, Brahmitaila and Misrakasneha used for treatment of various mental disorders specially memory loss. (Parotta, 2001)

Brahmi has been used by Ayurvedic medical practitioners in India for almost 3000 years and is classified as a medhya rasayana, a drug used to improve memory and intellect (medhya). It has been used to treat conditions such as bronchitis, coughs,
Experimental Work

asthma, hoarseness, arthritis, allergies, rheumatism, inflammatory conditions, constipation, boils, ulcers, fever, digestive problems, epileptic fits, depression including post natal depression, diarrhoea, irritable bowel syndrome, frigidity, irregular menstruation, mental and physical fatigue, exhaustion, restlessness, insomnia and over active mind, mental deterioration of the elderly, forgetfulness, confused and cloudy thoughts, anxiety, stress, nervous breakdown, insanity and to improve circulation, strengthen capillaries and stimulate hair, skin and nail growth.(Bone, 1996)

The juice of the leaves is used in the treatment of bronchitis and diarrhoea in children. A paste of the leaves is applied to treat arthritis (Bone, 1996). Tribal inhabitants of Maharashtra believed that eating 5 leaves per day for a month improve the speech of stutterers. Bhil women in Rajasthan apply the boiled leaves to the abdomen to relieve postnatal pains, yet other Bhils use the warmed leaves as a poultice to relieve swellings from beatings (Parotta, 2001).

3.2.9.2 Usual Dosage

Traditional daily doses of Brahmi are 5-10 g of non-standardized powder, 8-16 ml of infusion, and 30 ml of syrup. Dosages of a 1:2 fluid extract are 5-12 ml per day for adults and 2.5-6 ml per day for children age group of 6-12. For Bacopa extracts standardized to 20% bacosides A and B the dosage is 200-400 mg daily in divided doses for adults and for children 100-200 mg daily in divided doses.(Monograph, Bacopa Monniera. 2004)

This plant is mentioned in three standard Ayurvedic treatises, Charak Samhita and Sushruta Samhita and Ashtanghridya. All these treatises are recognized as authoritative and are included in the First Schedule of the Drugs and Cosmetics Act 1940. The fact that the Brahmi does not find a place in Schedule E1 (which contains the name of poisonous drug of Ayurveda) bears testimony to its safety profile.
3.2.10 Dosege Forms with its standard specification

Since the memory enhancing effect of Bacopa was localized in bacosides, a standardized extract of Bacopa containing an optimum 55 ± 5% of Bacosides with an optimum concentration of Bacogenines (especially A3), were obtained from the Lumen Research Foundation, Chennai, the sole license of CDRI patented process of Bacopa extract used in the study.

3.3 Materials

Lumen Research Foundation, chennai, funded Rs. Two Lakh for the purpose of further development of its product called “Memory Sure” in India, Memory gold in south Asia markets and Keen Mind in other parts of World. The firm provided 10,000 capsules of standardised extract of Brahmi (Bacopa monniera) along with 10,000 capsules of placebo and 1800 capsules of Revital (Ranbaxy) was purchased from the market. One Pulse Oximeter (BPL: Model-Cleo) and one Semi-auto analyzer (Transasia: Model-Erba chem 5 Plus V2) were purchased from the grant provided by the Lumen Research Foundation.

3.3.1 Human Ethical Committee

According to ICMR guidelines for Biomedical Research on Human Participants constitution of Human Ethical Committee is a mandatory requirement and hence a committee was constituted, which included chairman, two clinicians, two basic medical scientists, one social scientist, one lawyer, one philosopher, one layman and one member secretary. As per the requirement a meeting of all members was held on 29th May 2008 in which the committee approved the research methodology after evaluation and discussion.

3.3.2 Selection of Volunteers

95 volunteers of different age groups, gender and health, were selected for clinical trials of drug and were divided into three groups. The total 79 volunteers have
completed the study. Volunteers were of 20 to 75 years of age group from both genders who were healthy and were not suffering from any critical illness/disease.

3.3.2.1 Inclusion Criteria:

Subjects were included in the study who fulfilled all the following criteria:
1. Subjects who were willing to provide written consent.
2. Healthy, age between 20-75 years
3. Ambulatory and cooperative.
4. Patients having hypertension and diabetes were also included.

3.3.2.2 Exclusion Criteria:

Following subjects were not included in the study
1. Suffering from Cancer, AIDS, kidney related disorder and liver dysfunction.
2. Undergoing treatment for serious chronic illness.
3. Who had undergone major hospitalization or any surgery during past 3 years.

3.3.3 Instruction to Volunteers

Before starting the clinical trial, instruction was given to volunteers who showed their interest to be the part of the research as volunteers. They were provided comprehensive description of the research, its objectives, benefits, and limitations and about the role to be played by the volunteers. The doubts of some volunteers were cleared and comprehensive consent form carrying volunteer information and health history was also documented. All patients provided written consent to participate after a full explanation of the study.

3.3.4 Dose Schedule

For minimizing error of the large data, the volunteers were distributed in Randomized Double Blind and divided in three groups,

**Group I: Brahmi treated group** - The volunteers received bacosides enriched standardized *Brahmi* extract at a dose of 300 mg per day for 180 days in a capsule form.
**Group II: Placebo treated group**- The volunteers received placebo capsule (lactose) at a dose of 300 mg per day for 180 days. The placebo capsules were identical to the Brahmi capsule in color and shape.

**Group III: Control Group**- The volunteers received prescribed dose of standard reference drug (Ranbaxy’s Revital) per day for 180 days.

### 3.3.5 Distribution of Drugs

The manufacturers supplied the drugs (Standardized Brahmi and Revital) to the External supervisor, who after coding it provided to the investigator for clinical trial. Since the study was double blind, after each month testing of volunteers, fixed numbers of capsules were given to them into a dark colored coded container. In addition to the trial regime (one bottle for 4 weeks), additional capsules ranging in number from 1-10 (randomly allocated) were also placed in the bottles so that compliance could be accurately examined. After the completion of 4 weeks, participants were asked to bring their bottles and the remaining capsules were counted. Participants were excluded if greater than 10% of the total number of capsules required were not consumed by the end of the 4 weeks. At each study visit including the initial visit all volunteers were subjected to the same estimation and testing as for the base line.

### 3.3.6 Study Design

A clinical assessment of each eligible volunteer was carried out at a base line (0 month) and scheduled clinical visits at 1, 2, 3….up to 12 months.

Among the 79 volunteers, 56 volunteers were given bacoside enriched standardized extract of Brahmi (*Bacopa monniera*), 10 volunteer were given standard drug, Revital (Ranbaxy) and 13 volunteers received placebo up to 180 days.

After the break of fifteen days the volunteers were cross-over. The 41 volunteers previously receiving Brahmi were given placebo and the volunteers of placebo group were given capsule of standardized extract of Brahmi and the group receiving standard reference drug (Revital) were given placebo up to 180 days.
volunteers who were taking revital, their next six months data were not included in the crossover study.

The remaining 15 volunteers of group one; (Brahmi group) continued the drug for another 180 days. The progress of trial and compliance of instruction were weekly monitored by personal visit or telephonically. (Figure-10)

During the course of the trial treatment serious condition, if developed, which requires urgent treatment such subjects, were withdrawn from the trial.

![Design of Study](image-url)
3.4 Laboratory Investigations

3.4.1 Assessment of Various Biochemical, Hematological and Physical Parameters

As per the research plan, before administration of drug to volunteers, the samples of blood were taken for analysis and the data recorded in a file of each volunteer. Peak respiratory flow, weight, height, blood pressure and other parameter as per protocol were also recorded. All the volunteers were given monthly schedule for coming for blood analysis and other tests.

3.4.1.1 Method to Measure HDL (Warnick et al. 2001):

The serum was reacted with the polyethylene glycol contained in the precipitating reagent; all the VLDL and LDL were precipitated. The serum was centrifuged at 2500-3000 rpm. HDL remained in the supernatant. The supernatant was then mixed well with Cholesterol reagent, incubated at 37°C for 5 min. The absorbance was taken at 505 nm by semi-autoanalyzer.

The amount of HDL was determined by following formula -

\[
\text{HDL Cholesterol in mg/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 25 \times 2
\]

(Where 2 is the dilution factor due to the deproteinization step)

3.4.1.2 Method to Measure Triglycerides (Trinder et al. 1969):

Triglyceride in the serum was measured by DES-GPO method. After separating the serum by centrifuge, the triglyceride reagent was added. The absorbance of triglyceride was taken at 505nm by semi-autoanalyzer.

The amount of triglyceride was determine by the following formula -

\[
\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}
\]
3.4.1.3 **Method to Measure Cholesterol (Roeschlau’s et al. 1974)**:

The lipid profile was measured by CHOD-PAP method using specific kits. After separating the serum by centrifuge, the cholesterol reagent was added. The absorbance was noted at 505nm by semi-autoanalyzer.

The amount of Cholesterol was determined by following formula-

\[
\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}
\]

**Determination of LDL-Cholesterol (Friedewald’s equation)**

\[
\text{LDL Cholesterol} = \frac{\text{Triglycerides}}{5} - \text{HDL} + \text{Total cholesterol}
\]

**VLDL was calculated as Triglycerides/5**

3.4.1.4 **Methods to Measure Alkaline Phosphatase (Wilkinson et al. 1969)**:

The ALP was measured by TRIS CARBONATE BUFFER method using specific kits. In this method serum was taken and ALP reagent was added. The absorbance was taken at 405 nm by semi-autoanalyzer.

The amount of ALP was determined by the following formula-

\[
\text{IU/L} = \frac{(\Delta A/\text{min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorptivity} \times \text{P}}
\]

Where:

- \((\Delta A/\text{min})\) = The mean absorbance change
- T.V. = Total reaction volume in µl
- S.V. = Sample volume in µl
- Absorptivity = Millimolar absorptivity of p-nitrophenyl Phosphate at 405 nm = 18.8
- P = Cuvette lightpath (cm) = 1cm.
Activity of ALP
At 37º C (IU/L) = (∆A<sub>405</sub>/min) x Factor (2713)

3.4.1.5 Method for Measurement of Albumin (Gustafsson et al. 1977) :

The albumin level was measured by BCG Dye Method using specific kits and semiautoanalyzer. The serum was separated by centrifugation. The albumin reagent was added in the serum and the absorbance was read at 630 nm on a semi-autoanalyzer.

The amount of albumin was determined by the following formula-

\[
\text{Albumin (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}
\]

3.4.1.6 Method for Calculation of Bilirubin (Pearlman et al. 1974) :

The amount of bilirubin was measured by DIAZO (BIT & BID) method using specific kits and semiautoanalyzer. The unhaemolysed serum was added in the serum and the absorbance was taken for total bilirubin and direct bilirubin at 546 nm on semi-autoanalyzer.

\[
\text{Total Bilirubin (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}
\]

\[
\text{Direct Bilirubin (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)}
\]

\[
\text{Total Bilirubin} = \text{Indirect Bilirubin} + \text{Direct Bilirubin}
\]

3.4.1.7 Method for Measurement of S.G.P.T. (IFCC 1986) :

The SGPT was measured by IFCC Method using specific kits. In this method serum was taken and SGPT reagent was added. The absorbance was taken at 340 nm by semi-autoanalyzer.

The amount of SGPT was determined by following formula-
Units (IU) of activity are:

\[
\text{IU/L} = \frac{(\Delta A/\text{min}) \times T.V. \times 10^3}{S.V. \times \text{Absorptivity} \times P}
\]

\((\Delta A/\text{min})\) = The mean absorbance change

Where:  
- **T.V.** = Total reaction volume (µl)  
- **S.V.** = Sample volume (µl)  
- **Absorptivity** = Milimolar absorptivity of NADH at 340 nm  
  = 6.22  
- **P** = Cuvette light path (cm)  
  = 1 cm  

Activity of AST = \(\Delta \text{Abs/\text{min}} \times 1768\)

### 3.4.1.8 Method for Measurement of S.G.O.T. (IFCC 1986):

The SGOT was measured by IFCC Method using specific kits. In this method serum was taken and SGOT reagent was added. The absorbance was taken at 340 nm by semi-autoanalyser.

\[
\text{IU/L} = \frac{(\Delta A/\text{min}) \times T.V. \times 10^3}{S.V. \times \text{Absorptivity} \times P}
\]

Where:

- \((\Delta A/\text{min})\) = The mean absorbance change

Where:  
- **T.V.** = Total reaction volume (µl)  
- **S.V.** = Sample volume (µl)

- **Absorptivity** = Milimolar absorptivity of NADH at 340 nm  
  = 6.22  
- **P** = Cuvette light path (cm)  
  = 1 cm  

Activity of AST = \(\Delta \text{Abs/\text{min}} \times 1768\)
3.4.1.9 **Method for Measurement of Total protein (Goodwin et al. 1965):**

The total protein level was measured by BIURET method using specific kits and semiautoanalyzer. The serum was separated by centrifugation, the protein reagent was added in the serum and the absorbance was read at 546nm a semi-autoanalyzer.

The amount of total protein was determined by the following formula-

\[
\text{Total protein (g/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (g/dl)}
\]

3.4.1.10 **Method for Measurement of Creatinine (Bowers et al. 1980):**

The creatinine level was measured by JAFFE’S method using specific kits. In this method serum was taken and creatinine reagent was added. The absorbance was taken at 500-520 nm by semi-autoanalyser.

The absorbance change (\(\Delta A\)) was determined by using formula - \(\Delta A = A_1 - A_2\)

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

3.4.1.11 **Method to Measure Urea (BUN) (Young et al. 1990):**

The urea was measured by GLDH-UREASE method, using specific kits. In this method unhaemolysed serum was taken and urea reagent was added. The absorbance was taken at 340 nm on a semi-autoanalyser.

The amount of urea was determined by the following formula-

The absorbance change (\(\Delta A\)) was determined by using formula - \(\Delta A = A_1 - A_2\)

\[
\text{Urea (mg/dl)} = \frac{\Delta A \text{ of test}}{\Delta A \text{ of Standard}} \times \text{Concentration of standard (mg/dl)}
\]
3.4.1.12 **Method to Measure the Uric Acid (Trivedi et al. 1976)**:

The uric acid was measured by Des Triender method using specific kits. In this method serum was taken and uric acid reagent was added. The absorbance was taken at 505 nm by semi-autoanalyzer. The amount of uric acid was determined by the following formula:

\[ \text{Uric Acid (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)} \]

3.4.1.13 **Method to Measure Glucose (Trinder et al. 1969)**:

The glucose level was measured by GOD-POD method using specific kits and semi-autoanalyzer. The serum was separated by centrifugation. The glucose reagent was added in the serum and the absorbance was read at 505nm on a semi-autoanalyzer.

The amount of glucose was determined by the following formula:

\[ \text{Glucose (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{Concentration of standard (mg/dl)} \]

3.4.1.14 **Estimation of Haemoglobin (Ghai 2005)**:

Hemoglobin was measured by Sahli’s method, 0.1N Hcl was taken in the haemoglobin tube, filling up to 20 per cent, the finger was pricked and the first one or two drops of blood with a cotton swab were wiped. When a good-sized drop was formed at the puncture site, then the pipette tip was held horizontally and blood was drawn to the 20 cmm mark. Immediately the blood was expelled into the Sahli tube containing the 0.1N Hcl. Then contents were mixed quickly by gently shaking the tube. The solution was put back to comparator. It was kept for 10 minutes. The tube was taken out from comparator, a few drops of distilled water were added and the content was stirred with the glass rod. The dilution was continued till its colour matched with that of the standard.
3.4.1.15 **Determination of Total Leucocyte Count (TLC) (Ghai 2005):**

The counting chamber was placed with its ‘centred’ cover slip near the microscope and turk’s fluid was taken on a watch glass. Finger was pricked and blood was sucked by WBC pipette exactly to the mark 0.5, followed by Turk’s fluid to the marks 11. After thorough mixing of the blood and the fluid, the first 2-3 drops of the diluted blood was discarded and the counting chamber was charged. The ‘charged’ haemocytometer was placed on the stage of the microscope and allowed the cells to settle for 2 minutes. Under low power objective, the distribution of WBC was identified and checked in the four corner WBC squares. The numbers of WBCs in each WBC square preferably, under low power objective, were counted as per rules observed for counting the cell (Jain, 2000).

**Observation:** The volume of one small square is 1/160 cmm (side=1/4mm, area=1/4X1/4=1/16sq mm, depth=1/10 mm, volume=1/4X1/10=160 cubic millimetre).

\[
N \times \text{Dilution factor} = \frac{N \times 50 \text{ mm}^3}{0.4}
\]

\(N=\) no of cells

3.4.1.16 **The Differential Leucocyte Count (DLC) (Ghai 2005):**

The slides of blood sample were prepared using the method described by Ghai (2005). the best slides was than stained using the standard method and DLC was counted using an optical microscope.

3.4.1.17 **Measurement of Pulmonary Function (Ghai 2005):**

The Pulmonary Function Tests (PFTs) was done for measuring the lung function, specifically the measurement of the amount (volume) and/or speed (flow) of air that can be exhaled by the volunteers. The volunters was asked to take the deepest breath that they can, and then exhale into the sensor as hard as possible, for as long as possible, and was normally repeated at least three times to ensure reproducibility. The spirometer was used.
3.4.1.18 Measurement of Oxygen Caring Capacity (SPO2) and Pulse:

The oxygen caring capacity and pulse was measured by using the pulseoxymeter, the probe was positioned on the finger, and the digital reading was recorded after giving the proper rest to the patient.

3.4.1.19 Measurement of Blood Pressure (Korotkoff, 1905.):

The subjects were sit or lie supine and allow 5 minutes for mental and physical relaxation. The cuff was place around the upper arm, the cloth was wrapped, covered around the arm so as to cover the rubber bag completely, and was preventing it bulging out from under the wrapping on the inflation. The point of the arterial pulsation was marked with a sketch pen. The chest – piece of the stethoscope were placed on this point and keep it in position with our fingers and thumb of the left hand. The cuff was inflated rapidly, by compressing and releasing the air pump alternately, raised the pressure to 40 to 50 mmHg above the systolic level. The pressure was lower gradually until a clear sharp, tapping sound is heard. Continued to lower the pressure and noted a change in the character of the sound. The point where the sound is disappearing was the diastolic level.

3.4.1.20 Measurement of Hand Grip (Nurgul, 2002):

Grip strengths were measured using a standard adjustable handle Jamar dynamometer at standing position with shoulder adducted and neutrally rotated and elbow in full extension. Results were recorded as kilograms. For standardization, the dynamometer was set at the second or third handle position of which the participant claims to be more suitable. One-minute rests were given between each attempt and hands were alternated to minimize fatigue affects. No verbal encouragements were performed.

3.4.1.21 Measurement of Chest (Clements 1954):

The chest girth was measure by the following technique: The volunteer was stand erect during the measurement of the chest, with his feet together and his arms rose above his head. The tape was then so adjusted that it’s upper border touches the lower angles of the scapulae behind and its lower border the nipples in front. The
arms were then lowered slowly to the sides, the tape being retained in position, and the girth was noted after both extreme expiration and extreme inspiration. The mean of the measurement of chest girth recorded at extreme inspiration and expiration was taken as the measurement of chest girth throughout this analysis.

### 3.4.1.22 Measurement of Mid Arm Circumference:

Measure the arm circumference with the subject standing upright, shoulders relaxed, and the right arm hanging loosely. It is important to be certain that the muscle of the arm is not flexed or tightened, which could yield a larger and inaccurate reading. Place the measuring tape around the upper arm at the crossed point (+), perpendicular to the long axis of the upper arm. Hold the measuring tape gently on the skin’s surface. Pull the two ends of the overlapping tape together so that the zero end is held below the measurement value and the measurement is taken on the lateral aspect of the arm. National Health and Nutrition Examination Survey, (2004)

The test like constipation, forgetfulness, low back pain, sleep abnormality, stress, appetite, well being self and by researcher, hypertension, anxiety, walking effect, dizziness were measured by the visual analogue scale. The VAS scale was based on a 10 cm line from 0 (no improvement) to 10 (improvement). (Victorian quality council 2007).

### 3.5 Statistical analysis:

Statistical analysis for cross over study was done by using cross-over analysis of variance of area under the curve data. For comparative study the normal data were taken, statistical analysis was done by two way analysis of variance, followed by Newman-Keuls test for Treatments Comparisons and Time Comparisons. The AUC data obtained from one year study was subjected to statistical analysis by student t test. The significance level was observed at (p<0.05), (p<0.01) and (p<0.001).
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