The present research was carried out to investigate the role of honey as a nutraceutical ingredient in functional foods. The research work was conducted in the Ph.D Laboratory of Foods and Nutrition of P.G. Department of Home Science, Sardar Patel University, Vallabhb Vidyanagar during 2007-2011. To fulfill the objectives six experiments were conducted. Materials and Methods used in all the six experiments are described in this chapter.

Experiment - I
The aim of the experiment was to study the non nutritive antioxidants, total antioxidant capacity as well as moisture, total sugar and colour of few commercial brands of honey as well as honey procured from four different regions/ forest of Gujarat.

Procuring honey samples:
Four honey samples were procured from the beekeepers of four different forest of Gujarat viz., Dang (Sample A), Banaskantha (Sample B), Panchmahal (Sample C) and Saurashtra (Sample D). Commonly consumed five brands of the marketed honey samples were also purchased from the local market. Both forest and marketed honey samples were stored in a refrigerator until analysed.

Estimation of biochemical and physical parameters of honey samples:
Each sample was diluted with distilled water in the ratio of 1:25(w/v) and the total phenolic contents and flavonoids as well as the percentage antioxidant activity were analysed by the following methods-

- Total phenols-by Singleton et al (1999)
- Flavonoids-by Singleton et al (1965)
- Total Antioxidant Capacity by three different methods-
  - Ability to scavenge 1, 1, diphenyl-2, picrylhydrazy radicals (DPPH) described by Brand-Williams et al (1995).
  - 2, 2′- Azino-bis (3-ethyl benzothiazoline-6-sulfonate-diammonium salt) radical discoloration assay (ABTS) by Rice-Evans et al (1996).
- Moisture (by AOAC, 1990)
- Total sugar content (Sadasivam, 1996)
Materials and Methods

- Colour (CIE L*a*b* method)

**Sample preparation**
1gm of honey was taken and dissolved in 25 ml of distilled water.

**Estimation of total phenols:**
The colorimetric assay based on the reaction of Folin-Ciocalteu reagent (FCR) is a method widely used for the determination of total phenols in honey. The method consists of calibration with a pure phenolic compound, extraction of phenols from the sample and the measurement of absorbance after the colour reaction. A typical protocol using the Folin-Ciocalteu method was outlined by Singleton et al (1999). The results are expressed as total phenols mg Equivalent to Gallic Acid /100gms of honey (mg GAE/100gm).

*Sample:* 0.05 ml from diluted sample was taken as an aliquot. To this 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1) was added. The content was cyclomixed for 4 min and 10 ml sodium carbonate was added. Content was made upto 12 ml with distilled water and allowed to incubate for 1 hour at room temperature. The colour intensity was measured at 750 nm on a UV spectrophotometer.

*Blank:* 0.5 ml of Folin Ciocalteu reagent (1:1) was taken. The content was cyclomixed for 3 minutes followed by addition of 10 ml of sodium carbonate and 1.5 ml of distilled water, then treated same as sample.

*Standard:* Standard series of known concentration of gallic acid (5-20µg) were prepared and To this 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1) was added. and there after treated in same way as sample.

**Calculation:**

\[
mg \text{ GAE/100gm} = \frac{Standard \ Concentration}{O.D \ of \ Standard} \times \frac{O.D \ of \ Sample}{Aliquot \ Taken} \times \frac{Total \ Volume \ made}{Sample \ Taken \ (gm)} \times \frac{100}{1000}
\]

**Estimation of Flavonoids:**
The total flavonoids content was measured by using colorimetric assay, used by Singleton et al, 1965). The results are expressed as flavonoids mg Equivalent to Rutin /100gms of honey (mg RE/100gm).
0.1ml of diluted honey sample was taken and volume was made up to 5 ml with distilled water. At 0 minute, 0.3 ml of sodium nitrite, at 5 minutes 0.6 ml of 10% aluminium chloride and at 6 minutes 2 ml of 1N sodium hydroxide were added to the mixture. This was followed by the addition of 2.1 ml of distilled water to it. The solution was mixed well and the intensity of pink colour was measured at 510 nm in a UV visible spectrophotometer (Hitachi 220s Japan) against blank.

**Blank:** 5 ml of distilled water was taken and treated same way as sample.

**Standard:** Standard series of known concentration of Rutin (20-80 μg) was prepared and final volume was made up to 5 ml with distilled water and there after treated in same way as sample.

\[
mg RE/100gm = \frac{Standard\ Concentration}{O.D\ of\ Standard\ Aliquot\ Taken} \times \frac{O.D\ of\ Sample\ Taken\ (gm)}{Total\ Volume\ made} \times \frac{100}{1000}
\]

**Total Antioxidant Capacity by three different methods**

a) **Ferric Reducing Antioxidant Power (FRAP)**:

The procedure described by Benzie and strain (1996), was used to evaluate the TAC of honey. The principle of this method is based on the reduction of a ferric - 2,4,6-tripyridyl-s-triazine complex (Fe³⁺- TPTZ) to its ferrous colored form (Fe²⁺ -TPTZ) in the presence of antioxidants. The results are expressed as total phenols mg Equivalent to Trolox, Gallic Acid, Ascorbic Acid /100gms of honey (mg TE, GAE, AAE/100gm).

**Sample:** 100µl of diluted honey sample was taken and volume was made up to 300µl with distilled water. 1.8 ml of FRAP reagent was added and allowed to incubate at 37°C for 10 minutes. The coloured complex was measured at 593nm using double beam U.V. spectrophotometer (Hitachi # 220s, Japan).

**Blank:** To 300µl of distilled water, 1.8 ml of FRAP reagent was added and mixed well.

**Standard:** Standard series of known concentration of trolox (1-4µg), gallic acid (0.2-0.8 μg) ascorbic acid (1-4µg) were taken and the volume was made up to 300µl with distilled water. There after all three tubes were treated in the same way as sample.

**Calculation:** TAC was expressed as mg TE/GAE/AAE/ 100gms of honey.
Materials and Methods

\[ TAC = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot Taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times \frac{100}{1000} \]

b) **Determination of antioxidant activity in the reaction with DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical:**

The antioxidant activity was determined by the ability of extract to scavenge DPPH radicals. This method was described by Brand-Williams et al (1995). The assessment of antioxidant activity is a free radical colorimetry that relies on the reaction of specific antioxidant with a stable free radical DPPH dissolved in methanol. As a result of reduction of DPPH by antioxidant, the optical absorbance at 517 nm of this purple colored solution of DPPH in methanol decreases. This change is detected by UV spectrophotometer.

0.1 ml of diluted honey sample was taken and volume was made up to 1 ml with methanol. 3 ml of DPPH reagent was added followed by vigorous shaking. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. To 1 ml of methanol 3 ml DPPH was added and used as control.

**Blank:** Methanol was used as blank.

**Standard:** Standard series of known concentrations of trolox (10-40 µg), gallic acid (1-4 µg) ascorbic acid (10-40 µg) were taken and volume was made up to 1 ml with methanol, there after all tubes were treated in the same way as sample.

**Calculation:** TAC was expressed as mg TE/GAE/AAE/ 100 gms of honey.

\[ TAC = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot Taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times \frac{100}{1000} \]

c) **ABTS (2, 2-Azimobis 3-ethyl benzo thiazolin-6-sulfonic acid diammonium salt) radical discoloration assay:**

The radical scavenging capacity (antioxidant activity) of yoghurt samples were measured by the modified ABTS (2, 2-azinobis, 3-ethylbenzothiazelen-6 sulfuric acid diammonium salt) radical discolourization assay by Rice Evans et al (1996).

**Sample:** 0.05 ml of sample aliquot was taken. Ethanol was added to make up volume to 1 ml. 3 ml of ABTS reagent was added. The discoloration caused reduction of the
cation by antioxidants from the sample which was measured at 734 nm on a U.V. visible spectrophotometer (Hitachi 220S, Japan). For control 1 ml of ethanol plus 3 ml of ABTS was used.

**Blank:** 50 µl of distilled water was taken and treated same as sample.

**Standard:** Standard series of known concentrations of trolox (10-40µg), gallic acid (1-4µg) ascorbic acid (10-40µg) were taken and volume was made up to 1ml with methanol, there after all tubes were treated same as sample.

**Calculation:** TAC was expressed as mg TE/GAE/AAE/ 100gms of honey.

\[
TAC = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot Taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times \frac{100}{1000}
\]

**Total moisture content:**

Moisture content was determined by the method given in AOAC. 2gm of sample was taken in a preweighed petri plate and kept in a well ventilated oven maintained at 60°C, weighed periodically at every hour. This process was repeated till constant weight loss was obtained. The loss in weight due to moisture evaporation was calculated using the following formula.

\[
\text{Moisture (gm %)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100
\]

**Total Sugar content:**

Total sugar was estimated colorimetrically by Sadasivam (1996).

**Sample:** 1gm sample was taken and diluted with 9ml distilled water. From this 0.1ml aliquot was taken, volume was made up to 1ml with distilled water and 1ml Dinitrosalicylic Acid reagent was added. The tube was kept in a water bath for 10 minutes followed by addition of 10 ml distilled water. The colour developed was read at 540 nm.

**Blank:** Blank consisted of water instead of sample and treated in the same way as sample.
**Standard:** Standard series of known concentration of standard glucose were taken. The volume was made up to 1 ml with distilled water and treated in the same way as sample.

**Calculation:**

\[
\text{Total Sugar (gm%) = } \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot taken}} \times \frac{\text{Total Volume made}}{\frac{\text{Sample Taken (gm)}}{1000}}
\]

**Color measurement –**

Colour parameters (L*, a* and b*) were established in the CIELAB system by reflection method (measuring geometry d/8, illuminant D65, range 400-700 nm, observer 10) using a CM 3500D spectrophotometer (Konica–Minolta). Measurements of colour parameters were carried out in triplicate.

**Experiment II**

The experiment was planned to study the effect of storage on non nutritive antioxidants. Samples were aliquoted into 2-ounce clear glass bottles and stored at room temperature under natural laboratory lighting. 3 samples were stored per honey for a period of six months. Analytical determinations were performed at least in triplicate for each honey sample. Each honey sample was analyzed for total antioxidant capacity (by FRAP, DPPH & ABTS), total phenols and flavonoids, the materials and methods for which is described earlier in this chapter under the head experiment I.

**Experiment III**

The aim of this experiment was to find optimal concentrations and combination ratio of antioxidant mixture (vitamin E, β-carotene, and vitamin C) in a model honey system that produces a synergistic antioxidant effect. In the present experiment, The “Design-Expert” Version 7.0, State-Ease Inc., Minneapolis, USA was used for experimental design (Central Composite Design, CCD), as well as regression and graphical analysis of data obtained. Three independent variables namely vitamin E, β-carotene and vitamin C were studied at five different levels. The dependent variables were total antioxidant capacity measured by three different methods namely FRAP,
DPPH and ABTS. The materials and methods used in this experiment is discussed in results and discussion chapter (Page 139 to146).

**Experiment IV**

The present investigation was planned with the principle objective of standardizing and developing biscuits, by replacing sugar with honey at different levels. Oxidative stability of control and honey fortified biscuits was measured after one month of storage. The study was carried out into six phases.

**Phase–1:** Procurement of raw materials.

**Phase–2:** Preparation of control and experimental biscuits (honey fortified biscuits).

**Phase – 3:** Sensory evaluation of the control and experimental biscuits.

**Phase – 4**  
A. Determination of rheological properties of dough prepared for control and experimental biscuits  
B. Determination of physical and textural properties of control and experimental biscuits

**Phase – 5:** Estimation of total antioxidant and total phenolic compounds from control and experimental biscuits.

**Phase–6:** Determination of peroxide value from stored control and experimental biscuits.

**Phase–1:** This phase deals with the procurement of raw materials from appropriate sources (Table 4).

**Phase–2:** This phase deals with the preparation of control and experimental biscuits (honey fortified). **Composition of control and experimental biscuits is presented in Table 5.**

- **Preparation of control biscuits:** Control biscuits were prepared by the method given in AACC (American Association of cereal Chemists, 1995). Numbers of trials were conducted to prepare the control biscuits.
### Table: 4 Treatment, storage and use of raw ingredients purchased

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Ingredients</th>
<th>Brand Name &amp; Company</th>
<th>Treatment, Storage &amp; use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Refined wheat flour (maida)</td>
<td>“Uttam” Shree bhagwati flour &amp; foods pvt. Ltd.</td>
<td>• Sieved</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Placed in airtight food grade plastic container</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Stored at ambient room temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Again sieved before use</td>
</tr>
<tr>
<td>2.</td>
<td>Sugar powder</td>
<td>“J.M.Patel” V.V.Nagar</td>
<td>• Cleaned, ground to fine powder in pin mill at J.M.Patel</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Followed rest of the procedure similar to Refined wheat flour</td>
</tr>
<tr>
<td>3.</td>
<td>Vanaspati Ghee</td>
<td>“Rasada” Ashwin vanaspti India pvt. Ltd.</td>
<td>• stored at ambient temperature plastic/tin pack containers as available during purchase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Used as such</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Allowed to acquire ambient temperature before use.</td>
</tr>
<tr>
<td>5.</td>
<td>Salt</td>
<td>“TATA” Tata chemicals Ltd., Mumbai</td>
<td>• Placed in air tight food grade plastic container</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Stored at ambient temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Used as such</td>
</tr>
<tr>
<td>6.</td>
<td>Distilled water</td>
<td>Prepared in laboratory using double distillation electric system</td>
<td>• Stored at ambient room temperature in glass bottle.</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th></th>
<th>Ingredient</th>
<th>Supplier</th>
<th>Storage Details</th>
</tr>
</thead>
</table>
| 7 | Sodium Bicarbonate                  | “Helios” Helios food Additives Pvt. Ltd., Mumbai | - Placed in air tight food grade plastic container  
|   | Ammonium Bicarbonate                |                                               | - Stored at refrigeration temperature                                              |
|   |                                     |                                               | - Allowed to acquire ambient temperature before use                               |
| 8 | Essence (Vanilla)                   | “Bush” Bush Boake Allen (India) Ltd., Chennai | - Stored at ambient temperature in the glass bottle in which purchased.           |
|   |                                     |                                               | - Used as such                                                                   |
| 9 | Honey                               | “Dabur” Dabur India Ltd., New Delhi           | - Stored at ambient temperature in the glass bottle in which purchased.           |
|   |                                     |                                               | - Used as such                                                                   |
| 10| Glucose powder                      | “Glucon –D” Heniz (India) Pvt. Ltd., Mumbai   | - Placed in air tight food grade plastic container.  
|   |                                     |                                               | - Used as such                                                                   |
Table 5: Composition of control and experimental biscuits

<table>
<thead>
<tr>
<th>Ingredients</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Refined wheat flour (gm)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sugar powder (gm)</td>
<td>30</td>
<td>25</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Fat (gm)</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Essence (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Skimmed milk powder (gm)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Glucose (gm)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Salt (gm)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium bicarbonate (gm)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium bicarbonate (gm)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>water (ml)</td>
<td>20</td>
<td>20</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>Honey (gm)</td>
<td>-</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

A – Control biscuits
B – 5 gm honey fortification
C – 10 gm honey fortification
D – 15 gm honey fortification
Method:

The biscuits were prepared basically using the rubbing method. The refined wheat flour, glucose powder and skimmed milk powder were mixed and sieved twice to ensure uniform homogenous blending as ‘Dry mix’. The vanaspati ghee was creamed till light and fluffy. To this sugar powder was gradually added, continuing the creaming process till the mixture became light. Salt, sodium bicarbonate and ammonium bicarbonate were dissolved in small quantity of water and mixed into ghee-sugar mixture, followed by addition of essence. This mixture is termed as ‘paste’. The ‘Dry mix’ was added to ‘paste’ gradually, mixed gently and kneaded into smooth dough. The dough was sheeted on the platform to a thickness of 0.5 cm using a wooden rolling pin. The dough-sheet was cut into circular shape using a metallic cutter of 5 cm diameter. Each raw biscuits was transferred and systematically arranged on a baking sheet about 10 mm apart from each other. The tray was transferred, as quickly as possible, to a preheated baking oven (Bajaj Ltd) at 180º c and baked for 7 minutes. The biscuits were immediately transferred on a container having holes on the surface for cooling. The cooled biscuits were packed in aluminum foil.

Preparation of Experimental biscuits:

Experimental biscuits were prepared by incorporating honey at 5, 10, and 15% levels.

Method:

For the preparation of honey fortified biscuits similar procedure as for the control biscuits were followed except that honey, at different levels, was mixed together in the ‘paste’.

Phase–3 :

This phase deals with the sensory evaluation of the control and experimental biscuits. Sensory evaluation was carried out by a team of six panel member using composite scoring test (Shrilaxmi, 2001). The control and experimental biscuits were evaluated for various visual and sensory attributes like color, surface character, crumb color, crumb texture, taste, mouth feel and overall quality. The score card is presented in Appendix-I (A).
**Phase – 4:**

This phase deals with studying the rheological properties of dough of control and experimental biscuits as well as evaluating the physical and textural properties of the control and experimental biscuits.

**A. Rheological Properties of dough:**

The rheological analyses of dough of control and experimental biscuits (Appendix I(B) a and b) were performed by using a food texture analysis System “Lloyd LF plus and Nexygen Software” (Lloyd Instruments Ltd., U.K.) model TA500. The dough was sheeted between two polyethylene films with a rolling pin over a platform, to give a sheet 0.5 cm thick. Compression was exerted with a cylindrical probe at a displacement speed of 0.5 mm S\(^{-1}\). The force \(F_0\) (Newtons) measured at 20% compression was determined. This compression was maintained for 2 minutes, and the force \(F_{20}\) exerted on the probe as measured. Elasticity was calculated as \(F_{20}/F_0\).

The measurements of dough firmness, consistency, adhesiveness and cohesiveness were performed on discs of dough of 5 cm diameter, with two compression cycles at 50% compression, according to Damasio and Fiszman (2000). Dough adhesiveness was calculated as the negative force area obtained after the first compression cycle, representing the work necessary to pull the compressing plunger away from the sample. Dough firmness was defined as the force (Newton) exerted during the first compression cycle. Dough cohesiveness was calculated as the ratio of the positive force area during the second compression with that during the first compression \((A_2/A_1)\) (Bourne, 1978 and Yamul and Lupaño, 2003).

**B. Physical and textural properties of the control and experimental biscuits:**

**Physical properties -**

1. **Biscuit Width:**

The width of biscuits was determined as per AACC (1995) method no. 10-50 D (200). After baking, the biscuits were allowed to cool for 30 minutes, on a mesh like structure. After that, 5 biscuits were placed diameter to diameter on a smooth platform such as the top of a sunmica table. The length of all 5 biscuits together was measured as “width” and recorded in mm. Each biscuit was rotated at 90° angle and width was measured. Average of 5 biscuits was calculated on the both
occasions and the final average was considered as the width of the individual biscuit.

2. **Biscuit Height** :

The height of biscuits was determined as per AACC (1995) method no. 10-50 D (200). After baking, the biscuits were allowed to cool for 30 minutes, on a mesh like structure. After that, 5 biscuits were stacked one on top of another and height of all 5 biscuits together was measured as “thickness” using vernier calipers and recorded in mm. The biscuits were re-stacked in a different order and again measured for thickness. Average of 5 biscuits was considered as height of biscuits.

3. **Spread Ratio** :

The spread ratio of the biscuits was calculated as per the formula of AACC (1995) method no. 10-50 D (200).

\[
\text{Spread ratio} = \frac{\text{Biscuit Width (mm)}}{\text{Biscuit Thickness (mm)}}
\]

**Textural properties**

The hardness of control and experimental biscuits (Appendix I(B) c and d) was measured by following the modified triple beam snap (also called three-point break) technique of Gaines (1991) using a food texture analysis System “Lloyd LF plus and Nexygen Software” (Lloyd Instruments Ltd., U.K.) model TA500. The sample was rested on two supporting beams (equidistant from either beam) spaced at a constant distance of 35 mm. Another identical beam was brought downwards to contact the sample. Then the downward movement of the top beam was continued to cause a degree of deformation of sample before it breaks (snaps). The peak force representing brittleness was read from graph and expressed as hardness. The thickness of biscuits was measured using a vernier caliper, and their fracture properties were studied by three-point bending tests. This test was set at 0.1 mm s⁻¹. The biscuits were placed on supports with their top surface down. Fracture stress of (k N.m⁻²) and fracture strain \(\varepsilon_f\) were calculated. Three series of each type of biscuits prepared in duplicate were analyzed.

The fracture stress \(\sigma\) (N.m⁻²) for a sample of rectangular section is given by:

\[
\sigma = \frac{3FL}{2db^2}
\]
Materials and Methods

Where F is the Force (N), L the distance between supports (m), d the width of the test-piece (m) (Baltsavias et al, 1997).

The fracture strain $\varepsilon$, can be calculated from:

$$\varepsilon = \frac{6by}{L^2}$$

(2)

Where $y$ is the deflection (m) (Balstavias et al, 1997).

Phase–5:

This deals with estimation of total antioxidant capacity and total phenolic content from control and experimental biscuits.

1. Total Antioxidant Capacity:

It was determined using Ferric Reducing Antioxidant Power Assay as per the method described given by Benzie and Strain (1996).

Procedure:

Sample: 1gm of sample was taken and volume was made to 40 ml with distilled water/methanol and mixed well. It was then filtered through whatman no. 1 filter paper. 100 µl of above aliquot was taken in a test tube and volume was made up to 300 µl with distilled water/methanol. 1.8 ml of Ferric Reducing Antioxidant Power (FRAP) working reagent was added and the contents were incubated at 37º C for 10 minutes. The absorbance was read at 593 nm using the double beam UV Spectrophotometer (Hitachi, Japan model 2205).

Blank: To 300µl of distilled water and 1.8 ml of FRAP reagent was added.

Standard: Standard series of known concentration of trolox (1-4µg), gallic acid (0.2-0.8 µg) ascorbic acid (1-4µg) were taken and the volume was made up to 300µl with distilled water. There after all three tubes were treated in the same way as sample.

Calculation: TAC was expressed as mg TE/GAE/AAE/ 100gms of honey.

$$TAC = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot Taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times \frac{100}{1000}$$

2. Total Phenolic compound:
Materials and Methods

The total phenolic compound was analyzed by the method described by Singleton et al (1999).

Sample Preparation: 1 gm powder of control and experimental biscuits were taken in separate beakers. To all of these 25 ml of 0.3 N HCL was added and shaken for about 1 hour followed by centrifugation at 8000 rpm for about 10 minutes. The supernatant obtained was evaporated to dryness in a vacuum dryer at 40° C. The residue was dissolved in hot water in a volumetric flask to a known concentration.

Sample: 0.05 ml from the above sample was taken as an aliquot. To this 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1) was added. The content was cyclomixed for 4 min and 10 ml sodium carbonate was added. Content was made upto 12 ml with distilled water and allowed to incubate for 1 hour at room temperature. The colour intensity was measured at 750 nm on a UV spectrophotometer.

Blank: 0.5 ml of Folin Ciocalteu reagent (1:1) was taken. The content was cyclomixed for 3 minutes followed by addition of 10 ml of sodium carbonate and 1.5 ml of distilled water, then treated same as sample.

Standard: Standard series of known concentration of gallic acid (5-20µg) were prepared and To this 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1) was added, and there after treated in same way as sample.

Calculation:

\[
mg \text{ GAE}/100gm = \frac{\text{Standard Concentration} \times O.D \text{ of Sample} \times \text{Total Volume made}}{\text{O.D of Standard} \times \text{Aliquot taken} \times \text{Sample Taken (gm)} \times 100} / 1000
\]

Phase–6:

This deals with determination of peroxide value from the fat extracted from control and experimental biscuits at 0, 15th, and 30th day of storage. Fat was extracted by gravimetric solvent extraction procedure according to soxhelt method. Rancidity tests were carried out by following method.

Peroxide value: The method adopted for estimation of peroxide value was the one prescribed by Sadasivam (1996).

Procedure: 5 gm oil sample was weighed in a 250ml glass stopper conical flask. 30 ml of acetic acid:chloroform (3:2 by volume) mixture was added followed by addition
of potassium iodide solution. The solution was swirled for exactly one minute and then 30ml distilled water was added. Finally the content of flask was titrated with standard 0.01 N sodium thiosulphate solution with constant and vigorous shaking using freshly prepared starch solution used as an indicator. Titration was continued until the yellow color almost disappeared. Then 2 ml starch solution was added. The titration was again continued by drop wise addition of standard 0.01 N sodium thiosulphate solution till it showed a change in color from blue violet to colorless.

**Blank:** A blank determination was also carried out without sample and the peroxide value was calculated using the following formula:

\[
\text{Calculation:} \quad m\text{Eq.} /\text{Kg} = \frac{(\text{Sample reading} - \text{Blank reading}) \times N\ of\ sodium\ thiosulphate}{\text{Weight of sample}} \times 1000
\]

**Experiment V**

The present study was undertaken to formulate synbiotic yoghurt incorporated with honey. Yoghurt samples were prepared with varying concentrations of honey along with different bacterial cultures. Physico-chemical properties, microbial analysis and sensory profile were compared with the control yoghurt.

**I. Selection, maintenance and activation of bacterial culture:**

**(a) Preparation of starter culture** - Freeze dried pure culture of *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus* and *Lactobacillus casei* were obtained from NDRI, Karnal. The freeze dried cultures were transferred in sterile MRS broth and incubated at 37°C for 24 hours. Then the transfer was given thrice in fresh MRS broth. A known quantity of MRS broth was centrifuged and the supernatant was discarded. The cell palate was washed twice in the sterile distilled water. 0.5 ml of sterilized skim milk was added to the culture, mixed thoroughly and transferred quantitatively to test tubes having 10 to 20ml presterilized cooled skim milk. All test tubes were incubated at temperature of 42-43°C till the firm coagulum was obtained.

**(b) Preparation of batch culture** - Batch culture was prepared by inoculating 3 percent of mother or starter culture into 100 ml of pre sterilized cooled skim milk and
incubated at 41-43°C till firm coagulum was formed, which was stored in refrigerator till further use in the entire investigation.

II. Materials required:

Materials used were stainless steel vessels and borosilicate glasswares. Stainless steel vessels as well as glassware were autoclaved at 121°C, at 15lb pressure for 15 minutes. Standardized milk (AMUL), sugar and skim milk powder (SAGAR) were purchased from the local market.

III. Yoghurt Preparation:

A. Details of manufacture

Control Yoghurt preparation

Standardized Amul milk (4.5 % fat, 8.5 % SNF) was taken in a stainless steel vessel (Sterile). Heat treatment was given at 90°C for 5 minutes. The temperature was monitored constantly by a thermometer followed by cooling to 42-44°C. Activated culture of S. thermophilus, L. bulgaricus, L. acidophilus and L. casei were added at a concentration of 1 %. Each activated culture was weighed in sterile, empty 100 ml beakers, and then the culture was transferred in milk. The contents were mixed properly. The vessel was covered with aluminium foil and incubated in an incubator at 37°C for about 3 hours or till acidity reached to 0.9 %. It was then placed under refrigeration for cooling at 5-7°C.

Experimental yoghurt samples:

Different experimental yoghurt samples were prepared with varying concentration of honey along with different yoghurt cultures. In experimental samples, honey was added at a concentration of 1 % and 2 % before inoculation of respective cultures. The remaining process was same as for the control yoghurt. The formula for control and experimental yoghurt samples are presented in table 6.

Packaging and storage:

Yoghurt samples were packed aseptically in 100 ml U.V. sterilized plastic cups. Control and experimental yoghurt samples were analyzed for the following
Table 6. *Formula for control and experimental yoghurt samples*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Type of Culture</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control sample</td>
<td><em>L. bulgaricus</em> &amp; <em>S. thermophilus</em></td>
<td>Yoghurt Culture with zero level honey</td>
</tr>
<tr>
<td>Experimental Sample I (E.S-I)</td>
<td><em>L. bulgaricus</em> &amp; <em>S. thermophilus</em></td>
<td>Yoghurt culture with 2% honey</td>
</tr>
<tr>
<td>Experimental Sample II (E.S-II)</td>
<td><em>L. bulgaricus</em> &amp; <em>S. thermophilus</em></td>
<td>Yoghurt culture with 4% honey</td>
</tr>
<tr>
<td>Experimental Sample III (E.S-III)</td>
<td><em>L. acidophilus</em></td>
<td><em>L. acidophilus</em> with zero level honey</td>
</tr>
<tr>
<td>Experimental Sample-IV (E.S-IV)</td>
<td><em>L. acidophilus</em></td>
<td><em>L. acidophilus</em> with 2% honey</td>
</tr>
<tr>
<td>Experimental Sample V (E.S-V)</td>
<td><em>L. acidophilus</em></td>
<td><em>L. acidophilus</em> with 4% honey</td>
</tr>
<tr>
<td>Experimental Sample VI (E.S-VI)</td>
<td><em>L. casei</em></td>
<td><em>L. casei</em> with zero level honey</td>
</tr>
<tr>
<td>Experimental Sample VII (E.S-VII)</td>
<td><em>L. casei</em></td>
<td><em>L. casei</em> with 2% honey</td>
</tr>
<tr>
<td>Experimental Sample VIII (E.S-VIII)</td>
<td><em>L. casei</em></td>
<td><em>L. casei</em> with 4% honey</td>
</tr>
</tbody>
</table>
parameters on the day of manufacture as well as on 7\textsuperscript{th} and 14\textsuperscript{th} day of storage at refrigerated temperature (5-7\textdegree C).

**Chemical analysis:**

- The yoghurt samples were analyzed for -
  1. pH
  2. Titratable Acidity
  3. Total Solids
  4. Syneresis
  5. Total Antioxidant Capacity (TAC) by following methods:
    - DPPH (1, 1, diphenyl-2, picrylhydrazyl radicals described by Brand-Williams et al (1995).

6. Sensory evaluation

**Chemical analysis:**

1. **pH:** pH of the control and experimental yoghurt samples was measured by using a digital pH meter (ELICO).

2. **Titratable Acidity:**
   Acidity is one of the most important parameters to check the lactic acid production by cultures in cultured milk products. The procedure used was a titrimetric method (BIS, 1981). 9 gm of yoghurt sample was taken in a 50 ml beaker. 18 ml of distilled water was added followed by addition of 0.5 ml of 1% phenolphthalein. The contents were titrated against 0.1N NaOH until permanent pink color existed for at least 30 seconds to 1 minute (end point was pale pink color).

   Acidity was expressed as % lactic acid (1 ml of 0.1 N NaOH - 0.009 g of lactic acid).

**Calculation -**

\[
\text{% Acidity} = \frac{\text{ml of } \text{NaOH} \times 0.009}{\text{Weight of sample}} \times 100
\]
3. **Total Solids:**

Total Solids were estimated by the procedure given by BIS, 1981. 2 g of sample was weighed in a clean dry preweighed crucible. The crucible was placed on a boiling water bath with support at the base. The crucible was kept horizontal to promote uniform drying.

It was boiled for 30 minutes. After 30 minutes, the crucible was removed, wiped the bottom and kept in an oven at 98 - 100°C. After 3 hours, the crucible was immediately transferred to a dessicator. It was allowed to cool and weighed immediately. The crucible was kept in the oven for one hour. Again, it was transferred to the dessicator, cooled and weighed. The process was repeated till a constant weight was obtained between two successive weighings. Total solid content was calculated from the following formula:

**Calculation -**

\[
\text{Total solids (% by weight)} = \frac{100 \times w}{W}
\]

Where,

- \(w\) = Weight in g of the residue after drying
- \(W\) = Weight in g of the sample taken for the test.

4. **Syneresis determination:**

Syneresis was determined by dispensing 125g of yogurt into a cheesecloth line funnel placed on top of a graduated cylinder. The amount of whey, ‘milk serum’ in ml was measured after manufacture and on 7\(^{th}\) and 14\(^{th}\) of storage at refrigeration temperature (8±1°C). The amount of whey drained off (expressed as milliliters per 125g of sample) was calculated as the syneresis index (Marshal, 1993).

5. **Total Antioxidant Capacity (TAC):**

DPPH and ABTS assays were performed from yoghurt samples.

a) **Determination of antioxidant activity in the reaction with DPPH (1, 1-diphenyl-2- picrylhydrazyl) radical**

The antioxidant activity was determined as the ability of yoghurt sample to scavenge
1, 1, diphenyl-2-picrylhydrazyl (DPPH) radicals. This method was described by McCue et al (2003).

**Procedure:** 0.1 ml of sample aliquot was taken and the total volume was made up to 1 ml with methanol. 3 ml of DPPH reagent was added and the contents were mixed properly. It was then incubated at 37°C for 20 minutes. After incubation, 1 ml of chloroform was added, followed by centrifugation at 3500 rpm for 5 min. The absorbance of the supernatant was measured at 517 nm.

**Blank:** Methanol was used as blank.

**Control:** To 1ml methanol, 3 ml DPPH was added and treated same as sample.

**Calculation:** 
\[
\% \text{ Inhibition} = \left( \frac{\text{Control} - \text{Experimental}}{\text{Control}} \right) \times 100
\]

b) **ABTS (2, 2 Azinobis 3-ethyl benzo thiazolin-6-sulfonic acid diammonium salt) radical discoloration assay**

The radical scavenging capacity (antioxidant activity) of yoghurt samples were measured by the modified ABTS (2, 2-azinobis, 3-ethylbenzothiazelin-6 sulfuric acid diammonium salt) radical discolorization assay by Rice Evans et al (1996).

**Procedure:** 50 μl of the sample was taken. To it, 2 ml of ABTS reagent was added. It was vortexed for 10 seconds followed by addition of 1 ml chloroform. It was then centrifuged at 3500 rpm for 5 min. The clear supernatant was taken directly in the cuvette and the discolorization caused by reduction of the cation by antioxidants from the sample was measured at 734 nm on a U. V. Spectrophotometer. ABTS reagent was used as control.

**Blank:** 50 μl of distilled water was taken and treated same as sample.

**Calculation:** 
\[
\% \text{ Inhibition} = \left( \frac{\text{Control} - \text{Experimental}}{\text{Control}} \right) \times 100
\]

**Sensory evaluation**

For the sensory evaluation of control and experimental yoghurt, the panel consisted
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semi trained six judges, students of the Department of Home Science. Organoleptic qualities studied were flavor, body and texture, appearance, product acidity and container and closure. The score card (Appendix II) for composite scoring test used in the study was developed by Dairy Chemistry department of Dairy Science College, Anand, Gujarat.

Microbial analysis:

Table 7 depicts the details of microbial analysis from control and experimental yoghurt samples.

Lactobacillus MRS Agar, pH 6.5 ± 0.2 was used for microbial analysis. The above mentioned media and the required glasswares were autoclaved at 15 psi for 15 min. The control and experimental yoghurt samples (0.5 ml) were diluted with 4.5 ml sterile D/W. From these samples 10⁻¹, 10⁻² … 10⁻⁶ dilutions were prepared. 0.1 ml of aliquot was taken from 10⁻⁴ and 10⁻⁶ dilutions and spreaded on the respective solidified MRS agar plates with sterile spreader. These plates were incubated at 37 °C for 48 hours.

NOTE: For the growth of L.acidophilus and L. casei, selective media was prepared by adding Salicin and Vencomycin, respectively.

Experiment VI

The present investigation was carried out to study the effect of honey feeding on the plasma antioxidant behavior and lipid profile of young females.

The study was carried out in 2 phases.

Phase 1: It deals with:-

- Enrollment of subjects
- Procuring honey
- Collection of blood samples
- Honey feeding
- Final data

- Enrollment of subject:

25 young females in the age group of 20-24 years were enrolled from P.G. Department of Home science, Sardar Patel University. All the subjects were divided into control (N=10) and experimental (N=15) groups. The subjects were chosen on the basis of the following criteria:
Table 7: **Microbial analysis from control and experimental yoghurt samples**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Samples</th>
<th>Micro-organisms analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Experimental sample I</td>
<td><em>L. bulgaricus</em> and <em>S.thermophilus</em></td>
</tr>
<tr>
<td>3.</td>
<td>Experimental sample II</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Experimental sample III</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Experimental sample IV</td>
<td><em>L.acidophilus</em></td>
</tr>
<tr>
<td>6.</td>
<td>Experimental sample V</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Experimental sample VI</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Experimental sample VII</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Experimental sample VIII</td>
<td></td>
</tr>
</tbody>
</table>

*L. bulgaricus* and *S.thermophilus* are identified as present in samples 1, 2, and 3. *L.acidophilus* is identified in samples 4, 5, and 6. *L. casei* is identified in samples 7, 8, and 9.
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1. Willingness to co-operate.
2. Free from any apparent complications.

- **Procuring Honey:**
  Honey was purchased (brand name “Dhanvantari”) from a local supplier.

- **Collection of blood sample:**
  Approximately 5ml of venous fasting blood sample was drawn from each subject and collected in a clean dry centrifuge tube containing dried Ethylene Diamine Tetra Acetic Acid (EDTA) before and after honey supplementation. The samples were then spun at 3,000 rpm for 10 min. and serum was separated. The serum was stored at -20º C temperature until analyzed.

- **Feeding honey to subjects:**
  25ml of honey was diluted in 50ml of water and fed to the subjects in the morning (9 to 9:30 a.m.) in front of the investigator for 30 days. 5ml of water was fed to the control subjects for a period of one month.

- **Final data:**
  At the end of experimental period all the initial parameters (mentioned in phase 2) were again checked from blood as well as serum.

**PHASE 2:** It deals with the analysis of honey and biochemical parameters.

- **A- Honey analysis**
  Honey was analyzed for the following parameters:
  - Total phenols by Singleton et al (1999)
  - Flavonoids by Singleton et al (1965)
  - Total Antioxidant Capacity was measured by two different methods-
    - Ability to scavenge 1, 1, diphenyl-2, picrylhydrazyl radicals (DPPH) described by Brand-Williams et al (1995)

- **Total phenolic compounds:**
  Total phenolic compounds were estimated according to the method described by Singleton et al (1999).
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**Sample:** 0.05 ml from diluted sample was taken as an aliquot. To this 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1) was added. The content was cyclomixed for 4 min and 10 ml sodium carbonate was added. Content was made upto 12 ml with distilled water and allowed to incubate for 1 hour at room temperature. The colour intensity was measured at 750 nm on a UV spectrophotometer.

**Blank:** 0.5 ml of Folin Ciocalteu reagent (1:1) was taken. The content was cyclomixed for 3 minutes followed by addition of 10 ml of sodium carbonate and 1.5 ml of distilled water, then treated same as sample.

**Standard:** Standard series of known concentration of gallic acid (5-20µg) were prepared and To this 0.5 ml of Folin-Ciocalteu reagent (diluted 1:1) was added and there after treated in same way as sample.

**Calculation:**

\[
mg \text{ GAE/100gm} = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times \frac{100}{1000}
\]

**Total flavanoids:**

The total flavonoids content was measured by using colorimetric assay, used by (Singleton et al, 1965).

**Sample:** 0.1ml of diluted honey sample was taken and volume was made up to 5 ml with distilled water. At 0 minute, 0.3 ml of sodium nitrite, at 5 minutes 0.6 ml of 10% aluminium chloride and at 6 minutes 2 ml of 1N sodium hydroxide were added to the mixture. This was followed by the addition of 2.1 ml of distilled water to it. The solution was mixed well and the intensity of pink colour was measured at 510 nm in a UV visible spectrophotometer (Hitachi 220s Japan) against blank.

**Blank:** 5 ml of distilled water was taken and treated same way as sample.

**Standard:** Standard series of known concentration of Rutin (20-80 μg) was prepared and final volume was made up to 5 ml with distilled water and there after treated in same way as sample.

\[
mg \text{ RE/100gm} = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot Taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times \frac{100}{1000}
\]
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**Total Antioxidant Capacity**

*a) Ferric Reducing Antioxidant Power (FRAP)*

The procedure described by Benzie and Strain (1996), was used to evaluate the TAC of honey. The principle of this method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe³⁺-TPTZ) to its ferrous colored form (Fe²⁺-TPTZ) in the presence of antioxidants.

*Procedure:* 1gm of honey was taken and dissolved in 25 ml of distilled water. 0.1ml of diluted honey sample was taken and volume was made up to 300µl with distilled water. 1.8 ml of FRAP reagent was added and allowed to incubate at 37°C for 10 minutes. The coloured complex was measured at 593nm using double beam U.V. spectrophotometer (Hitachi # 220s, Japan).

*Blank:* To 300µl of distilled water and 1.8 ml of FRAP reagent was added.

*Standard:* Standard series of known concentration of Ascorbic acid (ASA) (1-4µg), trolox (1-4µg), and gallic acid (0.2-0.8 µg), were taken and the volume was made up to 300µl with distilled water. There after all three tubes were treated in the same way as sample.

*Calculation:* TAC was expressed as mg Trolox Equivalent per 100gms of honey.

\[
TAC = \frac{Standard\ Concentration}{OD\ of\ Standard} \times \frac{O.D\ of\ Sample}{Aliquot\ Taken} \times \frac{Total\ Volume\ made}{Sample\ Taken\ (gm)} \times 100
\]

*b) Determination of antioxidant activity in the reaction with DPPH (1,1-diphenyl-2-picrylhydrazyl radical)*

The antioxidant activity was determined by the ability of extract to scavenge DPPH radicals. This method was described by Brand-Williams et al (1995).

*Procedure:*

The assessment of antioxidant activity is a free radical colorimetry that relies on the reaction of specific antioxidant with a stable free radical DPPH dissolved in methanol. As a result of reduction of DPPH by antioxidant, the optical absorbance at 517nm of this purple colored solution of DPPH in methanol decreases. This change is detected by UV spectrophotometer (Brand-Williams et al, 1995).
1gm of honey was taken and dissolved in 25 ml of distilled water. 1ml of diluted honey sample was taken and volume was made up to 1ml with methanol. 3ml of DPPH reagent was added followed by vigorous shaking. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. To 1ml of methanol 3ml DPPH was added and used as control.

**Blank:** Methanol was used as blank.

**Calculation:**

\[
\% \text{ Inhibition} = \frac{O.D \text{ of control} - O.D \text{ of experimental}}{O.D \text{ of control}} \times 100
\]

**B-Biochemical Parameters**

- Whole blood:
  - i) Glutathione by Ellman (1959)

- Serum analysis:
  - i) Total antioxidant capacity by Benzie & Strain (1996)
  - ii) Vitamin E by Desai (1984)
  - iii) Ascorbic acid by Roe & Kuther (1943) & Bessey et al (1947)
  - iv) Total cholesterol
  - v) Triglyceride
  - vi) HDL
  - vii) VLDL
  - viii) LDL

**I. Whole blood:**

**Glutathione (GSH):**

Glutathione was estimated by the method given by Ellman (1959).

**Sample preparation:** 0.4 ml of blood sample was mixed with 0.4 ml of 5% TCA in a plastic vial. The mixture was mixed in a cyclomixer followed by centrifugation at 2000 rpm for 10 minutes to obtain a protein free supernatant. To 0.01ml of supernatant 1.99 ml of phosphate buffer (pH-8) and 0.1 ml of 5,5’-dithiobis-(2-nitrobenzoic acid) solution were added. The contents were mixed in a cyclomixer and allowed to stand for 10 minutes at room temperature. The colour developed was read
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at 412nm in a double beam UV visible spectrophotometer (Hitachi, Japan-model 2205).

**Standard:** The standard series of glutathione ranging from 2 to 10µg were prepared. The volume was made up to 1ml with phosphate buffer (pH-8) and treated in the same way as sample.

**Blank:** The blank was prepared using 1ml of phosphate buffer (pH-8) and treated in the same way as sample.

**Calculation:**

\[ GSH (mg\%) = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{0.4} \times \frac{100}{1000} \]

II **Serum analysis:**

i) **Total Antioxidant capacity (Ferric Reducing Antioxidant Power (FRAP))**

The procedure described by Benzie and Strain (1996), was used to evaluate the TAC of honey. The principle of this method is based on the reduction of a ferric - 2,4,6-tripyridyl-s-triazine complex (Fe³⁺ - TPTZ) to its ferrous colored form (Fe²⁺ - TPTZ) in the presence of antioxidants.

0.02ml of serum was taken in a test tube and volume was made up to 300 µl with distilled water. 1.8ml of FRAP reagent was added and allowed to incubate at 37°C for 10 minutes. The coloured complex was measured at 593nm using double beam U.V. spectrophotometer (Hitachi # 220s, Japan).

**Blank:** 300µl of distilled water and 1.8ml FRAP reagent was added.

**Standard:** Standard series of known concentration of trolox (1-4µg) were taken and the volume was made up to 300µl with distilled water. There after all tubes were treated in the same way as sample.

**Calculation:** TAC was expressed as mg TE/100ml of serum

\[ \frac{mg \ TE}{100ml} = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot taken}} \times \frac{100}{1000} \]

ii) **Vitamin E:-**

Plasma vitamin E was estimated by modified method given by Desai (1984).

**Sample preparation:-**
Exactly 0.5ml of plasma was mixed with 0.5ml of 1% pyrogallol solution in a centrifuge tube, mixed well and incubated at 70°C for 2 minutes in the water bath. 0.1ml of saturated potassium hydroxide was added and incubated for 30 minutes at 70°C in a water bath. Sample was cooled in ice and 1ml of distilled water and 4ml of chilled hexane was added. Mixture was mixed vigorously for 2 minutes in a cyclomixture. This was followed by the centrifugation at 1500 rpm for 5 minutes. The upper layer was carefully taken in a dry test tube and known amount was evaporated to dryness at 40°C in a vacuum dryer. The residue was dissolved in 1ml of ethanol. 2ml of 0.2% of bathophenanthraline reagent was added and mixed thoroughly. To this, 0.2ml of ferric chloride reagent was added and mixed well using a cyclomixture. Exactly after one minute 0.2ml of orthophosphoric acid reagent was added and the color intensity was read at 536nm in a digital spectrophotometer.

**Standard:** The standards were prepared ranging from 2 to 8µg (i.e. 0.1to 0.4ml) from working solution of α – tocopherol acetate. The volume was made up to 1ml with ethanol and treated in the same way as sample.

**Blank:** 1ml of ethanol was treated in the same way as sample.

**Calculation:**

\[
\text{Vitamin E (mg%)} = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot taken}} \times \frac{\text{Total Volume made}}{\text{Sample Taken (gm)}} \times 100
\]

**iii) Ascorbic acid:**

Vitamin C was estimated by the method given by the Roe and Kuther (1943) and Bessey et al (1947).

Exactly 0.4 ml of serum was mixed with 0.4 ml of chilled 10% in a test tube. The content was centrifuged at 5000 rpm for 10 minutes and from this 0.2 ml of supernatant was taken in a plasma tube. Volume was made up to 0.5 ml with 5% Trichloroacetic Acid. 0.1 ml of Dinitrphenylhydrazine – Thiourea - Copper Sulphate solution was added. This mixture was incubated at 37°C for 3 hrs. 0.75 ml of 65% H₂SO₄ was added, mixed and allowed to stand at room temperature for 30 minutes. Absorbance was determined at 520 nm in a spectrophotometer.
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**Standard:** Standards ranging from 2 to 8 µg were prepared. Volume was made up to 0.5 ml with 5% TCA and treated in the same way as sample.

**Blank:** 0.5ml of 5% TCA which was treated in the same way as sample.

**Calculation:**

\[
Vitamin \ C (mg%) = \frac{\text{Standard Concentration}}{\text{O.D of Standard}} \times \frac{\text{O.D of Sample}}{\text{Aliquot taken}} \times \frac{\text{Total Volume made}}{0.4} \times \frac{100}{1000}
\]

iv) **Total cholesterol:**

Serum total cholesterol was estimated by ROESCHCLAU et al method using the cholesterol kit supplied by Eve’s Inn diagnostic, Baroda, India.

2.5 ml working reagent was pipetted in a clean test tube followed by addition of 0.02 ml sample. The contents were mixed properly and incubated for 10 minutes at 37⁰C and absorbance of standard and test was measured at 505 nm or with green filter against reagent blank within 30 minutes.

**Blank:** 2.5ml of working reagent was taken and treated in the same way as same sample.

**Standard:** 2.5ml of working reagent was taken and added 200mg/dl cholesterol standard provided in Kit. Mixed well & treated in the same way as sample.

**Calculation:**

\[
\text{Serum Cholesterol (mg%)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200
\]

v) **Triglyceride:**

Plasma triglyceride was estimated by GPO method using the triglyceride kit supplied by Eve’s Inn diagnostic, Baroda, India.

1ml of working reagent was pipetted into a clean dry test tube, followed by addition of 10µl sample. The contents were mixed well and incubated at 37⁰C for 10 minutes. Absorbance of standard & test was measured against the blank at 520nm within 30 minutes.

**Blank:** 1 ml of working reagent was treated in the same way as the sample.
Materials and Methods

**Standard:** 1ml of working reagent & 10µl triglyceride standard provided in kit was added & content was mixed & treated as for the sample.

**Calculation:**

\[
Serum Triglyceride (mg%) = \frac{Absorbance \ of \ test}{Absorbance \ of \ standard} \times 200
\]

**vi) HDL:**

Serum HDL cholesterol was estimated by “precipitation & enzymatic method” using the HDL cholesterol Kit supplied by Eve’s Inn diagnostic, Baroda, India.

For precipitation of VLDL & LDL 0.1ml precipitating reagent was pipetted into a clean dry centrifuge tube, followed by addition of 0.1ml sample. The contents were incubated at room temperature for 5 minutes & centrifuged at 2000-3000rpm for 10 minutes. To 0.05ml clear supernatant 1ml of working reagent was added and mixed well. It was incubated in a water bath at 37° C for 5 minutes. Absorbance of standard & test sample was measured against the blank at 505nm.

**Blank:** 1ml of working reagent & 0.05ml distilled water were mixed & treated in the same way as the sample.

**Standard:** HDL cholesterol standard was mixed with 1ml working reagent & treated in the same way as the sample.

**Calculation:**

\[
HDL \ Cholesterol (mg%) = \frac{Absorbance \ of \ test}{Absorbance \ of \ standard} \times 100
\]

**vii) Serum VLDL:**

VLDL cholesterol was calculated according to the formula given by Friedewald (1972).

**Calculation:**

\[
VLDL \ Cholesterol (mg%) = \frac{Triglycerides}{5}
\]

**viii) Serum LDL:**
LDL cholesterol was calculated according to formula given by Friedewald (1972).

**Calculation:**

\[
LDL \ Cholesterol (mg\%) = Total \ cholesterol - (VLDL + HDL \ Cholesterol)
\]

**STATISTICAL ANALYSES:**

Appropriate statistical tools (One-way Analysis of variance – ANOVA - post-hoc Duncan. SPSS. Version 10; paired t-test, Pearson correlation, Regression analyses – M S Excel, 2007) was applied to obtain data wherever it was required.
Reagent Preparation

- **Acetate buffer (pH 3.6):**
  
  3.1 gm of sodium acetate and 16 ml acetic acid dissolved in 1 liter of distilled water.

- **Alcohol**: as such

- **Aluminium chloride (10% AlCl₃):**
  
  10ml AlCl₃ .6 H₂O is dissolved in 100 ml distilled water.

- **Ascorbic acid standard:**
  
  Stock: - 100 mg of ascorbic acid was dissolved in 100 ml of 5% trichloroacetic acid.
  
  Working: - 2ml stock was dissolved in 100 ml of 5% trichloroacetic acid.

- **ABTS Reagent (7mM):**
  
  38.4 mg of ABTS was dissolved in 100 ml of D/W. 5ml of this 7mM ABTS and 88µl of 140mM potassium persulphate were mixed, incubated in dark for 16hrs and then diluted with ethanol and O.D was set 0.7 at 734nm.

- **Bathophenanthroline (0.02%):**
  
  20 mg of bathophenanthroline was dissolved in 100 ml of alcohol.

- **CO₂ free water**: Distilled water was boiled for 15 to 20 minutes and cooled.

- **Copper sulfate (CuSO₄):**
  
  5g of copper sulfate was dissolved in water and made up the volume to 100 ml.

- **Dinitrophenyl hydrazine - Thiourea- Copper sulphate solution (DTC solution):**
  
  3 gm DNPH, 0.4 gm Thiourea, 0.05 gm Copper sulphate were dissolved in 100 ml of 9N H₂SO₄.

- **Dinitrosalicylic Acid:**
  
  1 gm of 3.5 of Dinitrosalicylic Acid was dissolved in 20 ml of 2.0N sodium hydroxide. Small quantity of water was added and mixed thoroughly followed by addition of 30gms of sodium potassium tartarate to the mixture.

- **DPPH Reagent:**
  
  20 mg of DPPH was dissolved in 100 ml of methanol and adjusted the absorbance to 1.0 at 517 nm.

- **5, 5, Dithiobis -2- nitrobenzoic acid (0.01%) (DTNB):**
5 mg of DTNB was dissolved in 50 ml of phosphate buffer (pH 8.0).

- **Ferric chloride:**
  For Total Antioxidant Capacity:-
  540.6 mg of FeCl$_3$.6H$_2$O (20mm/liter) was dissolved in 100 ml of D/W.
  For plasma vitamin E:-
  13.5 mg of (0.001 M) FeCl$_3$ was dissolved in 50 ml of alcohol.

- **FRAP:**
  25 ml of acetate buffer and 2.5 ml each of TPTZ and FeCl$_3$ solutions were mixed properly.

- **Folin- Ciocalteu reagent:**
  Folin ciocalteu reagent was diluted with D/W in a ratio of 1:1.

- **Gallic acid standatd (100 mg%):**
  
  **Stock:** - 0.1 gm gallic acid was dissolved in 100 ml D/W.
  
  **Working:** - 1 ml of stock diluted with 200 ml D/W.

- **Glucose Standard:**
  100 mgs of glucose dissolved in 100 ml of distilled water.

- **Glutathione (reduced):**
  
  **Stock:** - 100 mg of reduced glutathione was dissolved in 100 ml of D/W.
  
  **Working:** - 1 ml of stock was diluted to 50 ml of D/W.

- **Hexane:** As such.

- **Hydrochloric acid (HCl):**
  0.1 N HCl: - 0.1 ml HCl diluted with 11.9 ml D/W.
  0.01 N HCl: - 0.01 ml HCl diluted with 11.9 ml D/W.
  40mmol HCl: - 1.016 ml HCl diluted with 100 ml D/W.

- **Methanol:**
  As such

- **Ortho phosphoric acid (0.001 M):**
  
  **Stock:** -0.98 ml of orthophosphoric acid was dissolved in 100 ml of alcohol.
  
  (0.1M)
  
  **Working:** - 1 ml of stock diluted to 100 ml with alcohol. (0.001 M)

- **Oxalic acid (0.1 N):**
  3.1512 g of oxalic acid dissolved in 500 ml of distilled water.

- **Potassium iodide solution (saturated):**
Materials and Methods

Excess of Potassium iodide in was dissolved fresh boiling distilled water.

- **Phosphate buffer.**
  Solution A: - 31.0 gm NaH$_2$PO$_4$.2H$_2$O in was dissolved 1 liter of D/W.
  Solution B: - 28.39 gm Na$_2$HPO$_4$.7H$_2$O in was dissolved 1 liter of D/W.
  pH - 7 39.0 ml of solution-A mixed with 61.0 ml of solution-B.
  pH - 8 5.3 ml of solution-A mixed with 94.7 ml of solution-B.
  Total volume was made to 100 ml and pH was adjusted with 0.1 N HCL or 0.1 N NaOH and volume was made up to 200 ml with D/W.

- **Phenolphthalein (1g %)**
  1 g of phenolphthalein dissolved in 60 ml absolute alcohol and volume made up to 100 ml with distilled water.

- **Potassium Hydroxide (saturated):**

- **Potassium persulphate (140mM):**
  378.4 mg potassium persulphate in 10 ml of D/W.

- **Pyrogallol :**
  1 gm of pyrogallol (1%) dissolved in 100 ml of alcohol.

- **Rutin (10 mg %):**
  10 mg of Rutin was dissolved in 100 ml of methanol.

- **Sodium Hydroxide (NaOH):**
  0.1N NaOH:- 0.4 gm NaOH was dissolved in 100 ml D/W.
  1 N NaOH:- 4 gm NaOH was dissolved in 100 ml of D/W.

- **Sulphuric acid (H$_2$SO$_4$):**
  9N H$_2$SO$_4$: 9 ml of H$_2$SO$_4$ was diluted to 36 ml with D/W.
  65% H$_2$SO$_4$: 65 ml H$_2$SO$_4$ was diluted to 100 ml with D/W.

- **Sodium carbonate (7.5%):**
  7.5 gm of sodium carbonate was dissolved in 100 ml D/W.

- **Sodium Nitrite:**
  5 gm Sodium Nitrite was dissolved in 100 ml D/W.

- **Solvent mixture:**
  3 volume of acetic acid was mixed with 2 volume of chloroform.

- **Starch indicator (1%):**
  1 gm of soluble starch was dissolved in 100 ml of boiled distilled water.
Materials and Methods

- **Trolox (10 mg %):**
  
  For total antioxidant capacity
  
  *Stock:* 10 mg trolox was dissolved in 100 ml D/W.
  
  *Working:* 1 ml of stock was dissolved in 10 ml of D/W.

  For DPPH
  
  10 mg% trolox as such

  For ABTS
  
  *Stock:* 10 mg trolox was dissolved in 100 ml D/W.
  
  *Working:* 1 ml of stock was diluted to 5 ml of D/W.

- **Tocopherol:**
  
  *Stock:* 100 mg α-tocopherol dissolved in 100 ml alcohol.
  
  *Working:* 1 ml of stock was diluted to 10 ml with alcohol.

- **Trichloro Acetic Acid (TCA):**
  
  5 gm of TCA was dissolved in 100 ml of D/W.

- **2, 4, 6 Tripyridyl-S- triazine (TPTZ 10mm):**
  
  313 mg of TPTZ was dissolved in 100 ml of 40 mmol HCl (Sigma chemical company).