Chapter - III
MATERIALS AND METHODS
MAP - 1. Karnataka Map Showing Study Area *Viz.*, Hassan, Mysore and Chamarajanagar Districts.
MATERIALS AND METHODS

The prominent rivers of these districts are Kaveri, Lakshmanateertha, Kapila, Hemavathi, Yagachi and Nugu-hole.

SOILS

It is clearly observed in the district of Mysore the soils of Mysore district as classified by large physical studies and by the survey of the National Commission on Agriculture (State Soil Survey, Bangalore) that these soils have been classified into the five broad categories.

1. The Red loam soil
2. The Clay loam soil
3. The Laterite type of soil
4. The Black soil, and
5. Forest soil

Soils are natural bodies which help the sustainable plant growth of largely agriculture oriented it becomes the major source of livelihood. Soil is the surface and adjoining horizons of parent materials, which have undergone more or less natural change under the influence of water, air and various species of organisms living or dead. This change is reflected to a certain degree in the composition, structure and colour of the products of weathering. Soils mainly being the resultant are matter of textures, colour and contents.

1) TOPOGRAPHY AND CLIMATIC CONDITION OF THE STUDY AREA

A. HASSAN

Location and Extent

Hassan is the headquarters’ and town of the district, and the district is also called by the same name. But the original name of the place, according to
2. HASSAN DISTRICT MAP SHOWING PLACE OF SAMPLE COLLECTION CENTRES AND TALUK HEAD QUARTER
the certain inscription of the veeragal dated in the year 1140 A.D. occurs the
word Hassan after the goddess Hasan-amma or Hasanamba the presiding deity
of the local Hasanamba temple, which means smiling mother or goddess.

a) Physiographic Structure

Hassan belongs to a group of districts partly in southern maidan
(plains) and partly in Malnad and is situated in the eastern part of Karnataka.
The district situated between 12° 13' and 13° 33' north latitude and between
75° 83' and 76° 38' east latitude.

It is bounded on the north by Chikkamagalore district, on the east by
Tumkur and Mandya districts, on the south by Mysore and kodagu districts
and on the west by Dakshina kannada district. There are 8 taluks in the
district viz., Alur, Arkalgud, Arsikere, Belur, Channarayapatna, Hassan,
Holenarasipura, Sakleshpur (Map 2).

Hassan district may be divided into three regions on the basis of
physical aspects, climate, rainfall and so on.

1. Southern malnad
2. Semi malnad
3. Southern maidan

The western and north-eastern portions of the Belur taluk, western and
central part of Alur taluk and the whole of the Sakaleshpur taluk constitute
the southern malnad region the central part of the Arakalgud taluk, the
western portion of the Hassan taluk, the eastern portion of the Alur taluk, the
central & eastern parts of the Belur taluk & western part of the Arsikere taluk
form the semi-malnad region.
The southern maidan region includes the whole of the Holenarasipur and Channarayapatna taluks, eastern parts of the Arasikere & Hassan taluks and the southeastern portions of the Arkalgud taluk.

The southern malnad is a forest - clad hilly region with a heavy rainfall. On the western peripheral are the picturesque ghats extending from the pass at Bisle Ghat to the Jenkal-betta, with some lofty peaks in them, perhaps there is no scenery in India more beautiful than the southern part of this track hills covered with finest grass or equally verdant crops of dry grain adorned and crowned with clumps of noble forest trees. The peaks like Pushpagiri (1,715 metres). Devarabetta (1,282 mts). The low ranges of granite hills running along the northern limits of the district through the Belur, Hassan and Arsikere taluks. The hills in Hassan taluk are Seegegudda (1286 mts.). Mukundur-betta (1,049 mts) and karle Kaval Hippli-betta (1,063 mts.) and Mallappana-betta (1064) in arakalgud & Holenarasipur taluk.

Three important rivers viz., the Cauvery, Hemavathi and Yagachi flow through the Hassan district. While Hemavathi is a tributary of the river Cauvery.

b) Rainfall

The average annual rainfall in the district is 1,040.7 mm. The western part of the district in the vicinity of the Western Ghats gets heavy rainfall. The rainfall decreases rapidly from the west to the east. Rainfall is mostly confined to the period from May to October, July being the rainiest month.

c) Climate

The climate of this district has an agreeable climate. The year may be divided into four seasons;

1. The dry season, with clear bright weather, is from December to February.
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c) Climate
The climate of this district has an agreeable climate. The year may be divided into four seasons;

1. The dry season, with clear bright weather, is from December to February.
2. The period from March to May constitutes the hot season.
3. The southwest monsoon season is from June to September.
4. October and November may be termed the post-monsoon season.

d) **Seasons**

There is a meteorological observatory in the district at Hassan. The period from March to April is one of the continuous raise in temperatures. April is usually hottest month. Maximum temperatures may sometimes reach $37.8^\circ C$ with an advance of the southwest monsoon over the district in June, the temperature drops appreciably and through out the monsoon season, the weather is pleasant. There is a slight increase in day temperature, and a secondary. After October, temperature decreases steadily and weather remains cool till February. December is generally the coolest month of the year. The daily minimum temperature in the cold season some times goes down $12.3^\circ C$ to $11.4^\circ C$.

The relative humidity is high during the southwest monsoon period and the post monsoon seasons. February and March are the driest months of the year during that times the relative humidity in the afternoons are less than 35 percent.

e) **Forest wealth**

The total area under forests in the district is a little over 510 sq. Kilometers. The Hassan district has a rich and varied flora. The major contributing factors to this variety are the difference in rainfall and topography within the district, a rapid transition from scrub to the monsoon forests as one move from east to west. The intermediately stages of dry deciduous to wet deciduous and semi evergreen form a continuous pattern as rainfall increases and the plateau breaks up into the lofty peaks and deep valleys of the western ghats.
The scrub can be found in Ramanahalli and Belavathalli state reserve and along with the lower slopes leading to Nagpuri in Arasikere taluk. The gravelly soil is sparsely covered by a few prostrate herbs with well developed root stocks. As climatic and soil conditions improve, a number of deciduous trees establish themselves. The maidan is dotted with numerous irrigation tanks often supporting on interesting aquatic flora. Rolling green hills with narrow paddies in their shallow valley are typical of areas around Sakleshpur and Hanur. Where the soil is rich and humidity high, wet deciduous trees form compact associations especially along streams and on steep slopes. These wooded patches have often been cleared and replanted with coffee and the accompanying shade trees.

In the large valleys opening on to the plains that of flora of the district attains its real splendour the Kabbinala, Kempuhole, Kenchenkumri, Kenchenkumri, Kagengiri and Bisle forests, situated in these valleys. The canopy trees may be over 40 mt tall and festooned with innumerable climbers and epiphytes. The soft woods are represented by Artocarpus hirsutus, Michalia champaka, and Dipterocarpus indicus the hard woods are represented by Dalbargia latifolia, Lagerstroemia microcarpa. Natural forest glades are usually dotted with Bombax ceiba and Emblica officinalis.

The herbaceous monsoon flora of these forests is rich and interesting. The innumerable flowering plants thrive during the wet season. Hassan district with over 1,500 species of vascular plants affords a good cross section of the flora of the entire Karnataka state.

B. MYSORE

Mysore is one of the twenty seventh districts in the state of Karnataka and it is situated in the southern part of Deccan Peninsula and it forms the southern most district of Karnataka State. Mysore is the name by which Karnataka state was known prior to 1973. It is known as one of the heritage
3. MYSORE DISTRICT MAP SHOWING PLACE OF SAMPLE COLLECTION CENTRES AND TALUK HEAD QUARTER
city of India, and also known throughout the world for the pomp and gaiety of its traditional Dasara festival. In the days of Haider and Tippu, it came in limelight internationally. The district is situated in the south of Karnataka State at 11° 60' to 12° 17' north latitude and 75° 19' to 77° 77' east longitude. It is 610 metres above sea level. It has Chamarajanagar district to east and south, Mandya and Hassan districts to the North, and Kodagu district, along with the state of Kerala to the West. The district covers an area of 6269 sq. kms and consists of seven taluks, namely, Heggadadevanakote, Hunsur, Krishnarajanagar, Mysore, Nanjangud and T. Narasipura.

f) Physiographic Structure

Physiographically, Mysore district comprises of maidan. It is described as an undulating tableland, fertile and well watered by perennial rivers whose waters dammed by anicuts enrich their banks by means of canals.

The Mysore district has a variety of topographical situations from plains in the angle where the Eastern and Western Ghat ranges converge into a group of hills. Lofty mountain ranges covered with vast forests. The principal ranges of hills are the Bettadapura hills in the northeast and the Chamundi hills near Mysore. The Chamundi betta rises to a height of 1,074 m above MSL. Bettadapura hill is an isolated conical hill, 1,338.6m above MSL and on the hill is a celebrated temple of Mallikarjuna.

g) Rainfall

The district receives the major portion of its rainfall from the southwest monsoon. The normal annual rainfall is spread over a period of about seven calendar months from the later half of April to October. The average annual rainfall in the district is 761.9mm.
The rainfall in the district is scanty and erratic with uneven distribution during monsoon. Larger variation in rainfall is noticed from year to year and amongst the taluks it varies to a large extent.

h) Climate

The district enjoys an agreeable climate during March to May. There will be continuous rise in temperature resulting in experiencing the highest temperature (34°C) during the month of May in the year, with mean temperature of 33.4°C at Mysore which can be taken as representative of the district in general.

With and advancement of southwest monsoon in early June, the temperature decreases, thought the monsoon season, the weather remains pleasant. At the end of southwest monsoon and the beginning of cold season i.e. November day and night temperature begins to reduce, with mean daily temperature of 25.8°C in the month of December.

i) Seasons

On the basis of climate the district enjoys four seasons in a year. They are;
1. Dry Season: with clear bright weather from December to February.
2. Hot Season: From March to May.
3. South West Monsoon Season: From June to end of October, and
The retreating season begins in November.

C. CHAMARAJANAGAR

The birthplace of Jayachamaraja Wodeyar, father of Mummadi Krishna Raja Wodeyar, named after him in 1818.

Chamarajanagar district, which is at the southern most tip of the state of Karnataka, has achieved a unique place for itself. It is one of the seven
CHAMARAJANAGAR DISTRICT MAP SHOWING SAMPLE COLLECTION CENTRES AND TALUK HEAD QUARTER.
district formed a district out of Mysore on 15th August established in 1997. It is known for silk, sandalwood and forest produce. The tributaries of river Cauvery, Suvarnavati and Chikkahole, flow in this district. It is situated between 11°15' and 12°18' north latitude and between 76°26' and 77°47' east latitude.

This district is bounded in the east by Salem and Coimbatore district of Tamil Nadu, in the north by Mandya and Bangalore districts, in the west by Mysore district and in the south by the Nilgiri district of Tamil Nadu. It is enclosed by a range of hills on the southeast.

The district has an area of 5,101 sq km having 4 taluks viz., Chamarajnagar, Gundlupet, Kollegal and Yelandur.

j) Physiographic Structure

Physiographically, Chamarajnagar district comprises of maidan areas. A range of hills on the southeast encloses the district.

The general elevation of the district is more than 800 mt above MSL. Lofty mountain ranges covered with vast forests. The principal ranges of the hills are the Biligiri Rangana Betta in Yelandur taluk and the Mahadeswara Betta in the Kollegal taluk. A part from these two, there are several other isolated hills such as the Gopal Swamy Betta in the south near Gundlupet.

The Biligiri Rangana Betta forms a Hilly terrain with lofty mountains raising to 1,687 metres above MSL. The hill run north to south for nearly 16 km. on the highest point is the temple of Biligiri Ranganatha Swamy from which the hills take their name. The Mahadeswara hills from a hill range of about 976 mt above MSL contain 77 hills such as the Anemale, Kadumale, Jenumale, etc. A Gopalaswamy hill is a lofty hill extremely picturesque in appearance rising to a height of 1,468m above MSL. It is generally enveloped in clouds and mist, hence the name Himavad Gopalaswamy Betta.
The extreme south of the district forms a terrain of dense forests and a major portions of the land here is uniformly covered by red loamy soil. The main forest areas are located in the southern and southwestern taluks of Kollegal, Yelandur, Chamarajanagar and Gundlupet.

k) **Rain fall**

The district receives the major portion of its rainfall from the southwest monsoon. The average normal rainfall of the district was 704 mm. the eastern taluks of Kollegal receive more rainfall (775mm). Most of the rainfall in the district is confined heaviest rainfall is 24 hours recorded at any station in the district was 205.5mm at Chamarajanagar on 17th October 1916.

l) **Climate**

The Climate of the district is moderate throughout the year.

m) **Seasons**

On the basis of climate the district enjoys four seasons in a year. They are

1. Dry season: With clear bright walker from December to February.
2. Hot season: From March to May, and
3. Southwest monsoon season: From June to end of October.

n) **Forest wealth**

Mysore and Chamarajanagar is essentially forest rich and flora is luxuriant and highly diversified. The slopes of Western Ghats are clothed with dense vegetation containing valuable timber species. The heavy rainfall in this area stimulates the growth of forest. Originally, the forest in the district was largely evergreen but now different types of forests ranging from the deciduous to the scrubs type will be seen in these districts. Cultivation being confined mainly to the plains and the bottom of the valleys, amongst the laterite hills and plateau jungles varying from moderate forest to scrub.
Further, cultivation is also predominant through the large areas of waste land.

Practically all types of forest can be met with evergreen in the Ghat belt, Semievergreen in the foot hills, deciduous in the outer ridges and in the areas bearing secondary growth and scrub type in exposed lateritic flat topped table land.

In the dry deciduous areas bamboo is common. The principal timbers in this area are Matti (Terminalia tomentosa), Maruvu (Terminalia sp.) and Jack (Artocarpus sp.). In moist deciduous type covering the protected valleys and some of the localities, which have escaped the ravages of man, the principal species are Acacia sp., Anogeissus latifolia, Terminalia sp., Pongamia pinnata, etc. It is difficult to demarcate areas where the semi evergreen type dominates, because this type of forest is more or less a transitory phase between the deciduous and the evergreen type.

2) **BOTANICAL SURVEY AND COLLECTION**

a) **Centres identified for melittopalynological investigation**

Production of honey depends upon the availability of nectar/pollen to the honey bees, plants flowering period, density of nectar/pollen producing plants in study area. Hence, in consultation with apiculture department of Hassan, Mysore and Chamarajnagar districts of Karnataka. General surveys of all beekeeping areas were conducted and following places were identified as potential beekeeping centres. In Hassan district three centres which were identified are Sakleshpur, Belur and Alur. In Mysore district four places which were identified are Mysore, Piriyapatna, Heggadadevanakote and Hunsur. In Chamarajnagar district four places were identified are Gundlupet, Chamarajnagar, Biligirirangana hills and Hanur.
b) Botanical Survey and plant collection

Botanical survey is prerequisite to Melittopalynological studies. A thorough knowledge of the area under investigation is very much essential. A systematic field botanical survey was undertaken in Hassan, Mysore and Chamarajanagar districts with special emphasis on beekeeping field stations. Field trips were undertaken to record botanical observation on habit, flowering period and mode of pollination of plants with reference to bee forage. Fresh flowers were collected; reference slides were prepared and preserved for reference. In order to establish a permanent herbarium of bee forage plants of the districts, plant specimens have been collected and identified by referring Flora of Presidency of Madras (Gamble, 1979), Manual of cultivated plants (Bailey, 1948), Flora of Bangalore District (Ramaswamy and Ravi, 1973), Flora of Hassan District (Saldanha & Nicolson, 1976), Flora of Karnataka (Saldonha, 1984), Flora of Presidency of Bombay (Cooke, 1967), Flora of Coorg (Keshavamurthy and Yoganarasimhan.).

c) Pollen herbarium

An extensive collection of plants growing in and around the study area and other parts of Hassan, Mysore and Chamarajanagar districts were made and pollen reference slides were prepared. The mature flower buds were stored in 70% alcohol for preparation of pollen reference slides. Wodehouse (1935) and Erdtman acetolysis (1960) techniques were employed for preparing reference slides. These slides are deposited at Palynology and Paleobotany Laboratory Department of Botany, Bangalore University, Bangalore.

3) Sample Collection

Eleven centres were selected for the present investigation. Honey samples were collected with the assistance of apiary staff or bee keepers. About 50-75 grams of honey samples were collected and stored in clean, dry, coloured glass bottles and labeled indicating the date, place of collection and colour.
The present investigation embodies one hundred honey samples collected from taluks of Hassan, Mysore & Chamarajanagar. Illustrated Maps 2 to 4. Out of 100 samples investigated, 45 samples were collected from *Apis dorsata* combs. 42 samples were collected from *Apis cerana* combs from apiary and wild, 13 samples were collected from *Apis mellifera* combs. Information such as place, date of collection, colour of the honey are presented in Table 1. Of the 42 samples collected from *Apis cerana* (both from apiary and wild) 30 samples were from squeezed honey and the rest 12 from extracted honey. All the 13 samples collected from AMH combs represent extracted honeys. All the 45 samples collected from *Apis dorsata* were squeezed honeys.

The squeezing of honey from the comb was preformed under special care. For the removal of honey, only the honey storage portion of the comb was subjected to squeezing, but in case of *Apis dorsata* comb, there was a possibility of slight mixing up with one or two pollen loads from the pollen stores.

The reason for selecting 15 specific honey samples for chemical analysis was based on the nature of samples, namely unifloral and multifloral. This is to find out the nutritional value endowed by the presence of pollen grains qualitatively and quantitatively.

4) **Method used for preparation of reference slides**

In the present Melittopalynological investigation, pollen grains deposited in honey, cells of the comb and pollen were identified by comparing them with the reference slide collection maintain in the paleobotany and palynology laboratory. This palynarium is enriched with slides prepared from polliniferous material collected from the centres selected for present
Preserved pollen materials were used for the preparation of reference slides by employing standard Wodehouse (1935) and Erdtman's (1952) acetolysis techniques.

Preparation of mounting medium. Glycerine jelly was used as a mounting medium for all the reference slides and honey pollen slides. It was prepared using 350 ml distilled water, 300 ml glycerin, 100 g gelatin and 7 g phenol crystals.

Gelatin, Glycerin and distilled water were mixed in a beaker and heated on water bath for 3 hours. When the mixer is cooled, phenol crystals were added to prevent fungal infection and filtered into a clean beaker. The solidified glycerin jelly thus prepared was melted at the time of use by keeping hot water bath.

5) Melittopalynological methods used for the analysis of honey sample

The pollen grain present in the honey samples is the only basis of identification of the plant source. The microscopic analysis of the honey samples reveals their botanical origin. The method proposed by International Commission for Bee Botany (ICBB, 1962; Louveaux, Anna Maurizio and Vorwohl, 1970) was followed.

a) Acetolysis method - Erdtman (1952)

Acetolysis method is used in the study of pollen morphology. The pollen grains subjected to acetolysis became transparent revealing the structural pattern for correct identification.

Procedure

Fresh flower buds which are about to bloom were selected and the anthers were removed with the help of forceps. The anthers were taken into a
centrifuge tube containing 70% alcohol. The material was crushed with the help of glass rod. The pollen grains released from the anthers were filtered through a sieve. The pollen grains free from the fats and oils were collected by placing a centrifuge tube beneath the sieve. The solution was centrifuged and the supernatant portion was decanted. To the sediment, glacial acetic acid was added and centrifuged. The supernatant was discarded and to the sediment 5 cc of freshly prepared acetolysis mixture (9:1 acetic anhydride and concentrated sulphuric acid) was added. The centrifuge tube with the content was placed in a water bath, when the colour of the solution changes to golden brown, the centrifuge tube was removed from the water bath, cooled and centrifuged. The supernatant was decanted into a separate bottle and to the sediment, glacial acetic acid was added, centrifuged and the supernatant was decanted. After washing the sediment several time with water (by centrifuging the solution), the sediment at the bottom of the centrifuge tube was taken on a pellet of glycerine jelly using a clean needle and placed on a glass slide. The slide was gently heated over the flames of the spirit lamp. The molten jelly was spread evenly using a clean needle and a cover slip was placed over it. The sides of the coverslip were sealed using wax to avoid contamination. The slide was number and labelled.

b) **Wodehouse Technique (1935)**

The anther of the flower bud which is about to bloom was placed on a clean glass slide. The pollen grains were released by teasing out the anther wall. After removing the debris, the pollen material was treated with a drop of 70% alcohol to remove fats and oil present in the pollen grains. After a few seconds a rig was formed around the pollen grains. The area around the pollen grains was cleaned using a cotton bud. A drop of melted glycerine jelly was added to the fat free pollen grain and a clean cover slip was placed over it. The edges of the coverslip were sealed with paraffin wax.
For staining pollen grains, a small piece of glycerine jelly prestained with saffarin was placed over the fat free pollen material. The slide was gently heated over the flames of a spirit lamp to melt the jelly and a clean cover glass was placed over the material. The edges of the cover glass were the material. The edges of the cover glass were sealed with wax. The permanent slides thus prepared were labelled, numbered and deposited in the Pollen Herbarium of the Paleobotany and Palynology Laboratory, Bangalore University, Bangalore.

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6) Microscopic Examination

Slides prepared from honey sediments were subjected to critical microscopic examination to identify constituents such as types of pollen grains present in the sample. The sediments help to determine the botanical origin of honey and an attempt was made to identify the pollen up to species level.

The pollen grains counted by scanning the whole slide or slides prepared are expressed in percentage frequencies represented in Table 2 (pollen spectrum of honey sample).

"Predominant pollen" means constituting pollen count of more than..... 45%
Secondary pollen ................................................................. 16-45%
Important minor pollen ....................................................... 3-15%
Minor pollen ................................................................. < 3%

49
The honey sample is classified as unifloral honey, if the honey contains pollen of one plant species, in other words, if the pollen of that species predominant i.e., above 45%. In case of multifloral honey, the individual pollen count is less than 45%.

7) Methods used for Qualitative and Quantitative Analysis of Honey Samples

All genuine honeys derived from floral nectaries contain pollen grains which can be studied microscopically to infer their botanical origin, geographical origin, method of extraction, etc. Further calculation of percentage frequency of pollen grains helps in characterising honey as unifloral or multifloral.

In the present investigation the method proposed by International Commission for Bee Botany (ICBB 1962, Louveaux J, Maurizio A, and Vorwohl G 1970) is adapted.

a) Qualitative Analysis

10 grams of honey was weighed and dissolved in 20ml of hot water (not above 40°C). The solution was centrifuged for 10-15 minutes. The supernatant liquid was decanted. For the better removal of the honey sugars the sediment was dispersed again in 10ml of distilled water and the solution was centrifuged for 10 to 15 minutes. The supernatant liquid was decanted and the sediment was taken on to a clean glass slide with the help of a piece of glycerin jelly. The jelly was cut into a small pieces and placed on the glass slide, warmed, spread cover with coverslip and then it was sealed with wax. The slides were numbered and preserved for microscopic examination.

b) Quantitative Analysis

One gram honey was weighed and dissolved in 10 ml of distilled water and the solution was centrifuged for 10-15 min at 3,000 rpm. The supernatant
was decanted for better removal of the honey sugars. Later the sediment was redissolved in 1 ml distilled water.

A drop of solution was placed in 1 mm square of the Haemocytometer using 0.1 ml pipette and cover with cover slip. The pollen types and their number present in the central squares were counted. A minimum of 10 reading was taken for each sample and the average was calculated by multiplying the average by 1,000 for 10g of honey. The absolute pollen count of the honey sample and the amount of sediment determined from the graduated centrifuge to provide information about method of honey extracted.

8) Chemical analysis of honey

a) Determination of sugars

(i) Preparation of honey solution

One gram of honey sample was dissolved in 250 ml of volumetric flask using distilled water and the solutions were made up to 250 ml and kept aside in a stopper container for analysis.

(ii) Total reducing sugars

In order to determine the percentage of total reducing sugars, samples were chemically analysed using the method recommended in “ISI Hand Book of Food Analysis” (1984).

(iii) Standardisation of copper sulphate solution

5ml of 0.05 N copper sulphate solution and potassium sodium tartrate were pipetted out into 250 ml conical flask and boiled on asbestos gauze after adding 48 ml standard invert sugar solution and 1 ml (0.2%) methylene blue indicator. The samples were titrated after boiling to a red end point with in 3min.
From the volume of invert sugar used, the strength of copper sulphate was calculated by multiplying the titre value by 0.001 (mg/ml of the standard invert sugar solution). The value so obtained is the amount of standard invert sugar solution required to reduce the copper present in 5 ml copper sulphate solution.

b) Estimation of total reducing sugars

5 ml of copper sulphate and potassium sodium tartrate solution were added into a conical flask and kept for boiling over asbestos gauze. The mixture was titrated against honey solution using 1 ml 0.2% methylene blue indicator until the blue colour changes to red.

\[
\text{Total reducing sugar} = \frac{250 \times 100 \times S}{H \times M}
\]

Where, S is the strength of copper sulphate solution, H is the volume of honey solution required for titration in ml and M is the mass in g of honey.

c) Estimation of Sucrose

An aliquot of 100 ml stock honey solution in a conical flask was mixed with 1 ml concentrated HCl and heated to near boiling. The solution was kept aside overnight and subsequently neutralized with sodium carbonate. The total reducing sugar was determined as described above.

Calculation

Sucrose, percent by mass = |(reducing sugars after inversion, percent by mass) - (reducing sugars before inversion, percent by mass)| x 0.95

d) Estimation of Fructose - Glucose ratio

A mixture of 40 ml Iodine (0.05N), 25 ml 0.1 N NaOH and 50 ml honey samples in 250 ml stopper flask were kept in dark for 20 min. The mixture was
acidified with 5 ml concentrated $\text{H}_2\text{SO}_4$ and titrated excess of iodine quickly against standard sodium thiosulphate (0.05N) solution till decolorisation take place. For blank, 50 ml distilled water was similar by titrated in lieu of honey sample.

\[
\text{Glucose} \% = \frac{(B - S) \times 0.004502 \times 100}{A}
\]

Where, B is the volume of sodium thiosulphate solution required for the blank, S is the volume of sodium thiosulphate solution required for the sample, and A is the mass of honey taken for test.

**Fructose (Levulose)**

Calculated by subtracting percentage from the percentage of total reducing sugars. L/D (Fructose to Glucose) ratio was then determined.

\[
\text{Fructose} \% = \frac{\text{Total reducing sugars} - \text{Glucose} \%}{0.925}
\]

\[
\text{F-G ratio} = \frac{\text{Fructose}}{\text{Glucose}}
\]

e) **Determination of moisture**

The moisture content of fresh samples was estimated by using refractometer. The refractometer reading of the honey sample was recorded at $20^\circ\text{C}$ and the moisture contents were noted from standard refractive index and moisture content relative table.

f) **Determination of pH**

The pH values of crude honey samples were recorded using Digisun electronics pH meter – DI 707.
g) **Determination of ash**

Five grams of honey samples and few drops of pure Olive oil in silica crucible were heated carefully over a low flame until swelling ceases. Further ignition of the sample was carried out in a muffle furnace at 600 ± 20°C for 5 hr till the white ash is obtained. The ash was cooled in a desiccator and weight was determined.

\[
\text{Ash (\% W)} = \frac{100 (M_2 - M_1)}{M_1 - M}
\]

Where, \(M\) is the mass of empty silica crucible in g, \(M_1\) is the mass of the crucible + material taken for test in g and \(M_2\) is the mass of the crucible + ash in g.

h) **Determination of acidity**

10 grams of honey sample was dissolved in 75 ml carbon dioxide free water and mixed thoroughly. The same was titrated against standard 0.05 N sodium hydroxide solution using 4-6 drops of carefully neutralized phenolphthalein solution to pink end point, which persist at least 10s. The blank was determined by titrating the distilled water and indicator. The titre value of blank was subtracted from that of the aliquot.

\[
\text{Acidity (Formic acid)} = \frac{0.23 \times V}{M}
\]

Where, \(V\) is the corrected volume of 0.05 N sodium hydroxide solution required for titration, \(M\) is the mass in g of the sample taken for the test.

9) **Determination of optical density of honey**

Two grams of honey samples were dissolved in distilled water and the volume was adjusted to 10 ml in a measuring cylinder. The colorimeter was set
to 100 percent transmittance using distilled water in a cuvet at 660 nm. The honey solution was taken in a cuvet and optical density was recorded (Table - 1).

10) **Photography**

   For the photography, FM-2, Nikon camera with Konica 100ASA film was used for outdoor as well as photomicrography.