3: MATERIAL AND METHODS:

When going to start the material and methods firstly collected all requirements which are required for this study. The all plants leaves of *Caesalpinia bonduc* L., *Mangifera indica* L. & *Ricinus communis* L. these selected plants leaves were collected from my native place of Ramling Mudgad, which is located in the Latur district of Maharashtra state. After collection of leaves there identification of plants were confirmed by ICMR, Belgaum, India. After confirmation of identity of the selected plants then evaluated the all parameters are following -

3.1: PHARMACOGNOSTIC INVESTIGATION:

The pharmacognostic investigations are important terms for the detection of pure drug because now days near about 80 % human being used herbal drug for various ailments. Therefore, there is need to identified the identity and determination or check there quality and purity.

In industry, in these days there is facing most of the problems by user for because there is an herbal drug, raw materials, semi finished products and finished products are not available in pure quality. Quality assurance is a technique used to the determination of crude drug profile or to cheque the quality of bioactive compound. It is the new analytical tools and most usefull technology. Therefore, it is suggested for practical purpose. Therefore it is most essential to maintain the quality and purity of raw materials, finished products in the commercial market. Therefore, shown that the crude drug having adultrated drug authenticate as compared with standard drugs. Even, people having problem regarding desbesment of drug. Adulteration is a mixing or substituting with original crude drug partially or other spurious substances but the later is eithr free from or inferior in chemical and theralpeutic properties. Adulteration is a simple term is desbasment of of an article. The process of adulteration is done by forcefully, but it can be occur accidentally in few cases. Adulteration is also known as sophistication, deterioration, admixture, spoilage, substitution and inferiority.

The drug evaluation is also important parameter for the pharmacognostic study. The evaluation of crude drug means the determination and confirmation of its identity and checks their quality and purity and observed the nature of adulteration. The determinations of evaluation of a crude drug are importn because of three main resons-

a. Chemical and Biochemical changes of the drug
b. Decomposition or spoilage of drug due to storage

c. Displacement and drug mixing

Generally, the crude drugs were identified with the help of comparison of standard drug value with the crude drug which are using. The organoleptic and microscopical analysis plays important role for identification of organized drug and unorganized drug. According to the advent techniques and instrumentation analysis, which can possible to perform physical evaluation of a crude drug which usefull for the estimation of the qualitative and quantitative investigation.

The different parameter are included in standardization of crude drugs are as follows.

3.1: *Caesalpinia bonduc* L.

3.1.1: Collection of plant and Authentication of plant:

The selected plant leaves of *Caesalpinia bonduc* L. was collected locally from the place of Ramling Mudgad is located in the Latur district and state of Maharashtra. After collection of plant then, authentication was confirmed by ICMR, Belgaum, state of Karnataka, in India.

After, authentication of leaves which are applied for the observation of macroscopical characteristics.

3.1.2: Organoleptic / Macroscopic/Morphological evaluation

The morphological or organoleptic means to the study of external appearance crude drug which are useful for the identification. In this system the crude drug are evaluated by their specific characters like as color, odour, taste, size, shape and also important the special like touch, texture etc. the morphological evaluation means conclusion drawn from impressions on organs of senses [51].

For the present study i.e morphological evaluation of crude drug used the leaves of *Caesalpinia bonduc* L. The observation of organoleptic characteristics which are shown in table-3.1.

<table>
<thead>
<tr>
<th><strong>Caesalpinia bonduc</strong> L.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Pale green</td>
</tr>
<tr>
<td>Odour</td>
<td>Characteristic</td>
</tr>
<tr>
<td>Taste</td>
<td>Bitter</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Size &amp; Shape</td>
<td>2-3.8 by 1.3-2.2 (each leaflet)</td>
</tr>
<tr>
<td>Surface</td>
<td>Elliptic- oblong</td>
</tr>
</tbody>
</table>

Table 3.1: Macroscopic characteristics of *Caesalpinia bonduc* L. Leaves

### 3.1.3: Microscopic Characteristics

The microscopical evaluation is one of the important method for determination or identification of a crude drug and it may used to identified the organized drugs by histological parameters. This method used for the determination of confirmation and check their quality of organized crude drugs and powders forms.

Microscope, having a good method for the quality to magnify, the minute objects under study to be magnified to confirm the histological details of the drug from plant origin. If want to better and effective results, used several coloring reagents for identified the tissue and cellular internal structure.

The Microscopical evaluations also importance study of the chemical constituents by using of chemical method to low quantities of drug in powdered form or to histological section of the drug. A drop of phoroglucinol and concentrated hydrochloric acide give red stain with lignin. Mucilage is stained pink with ruthenium red and also, when treated with corallin soda and few drops of sodium carbonate solution, cellulose swells and dissolved in cuoxam, while N/50 iodine solution stain blue starch and hemicelluloses.

Other important histological aspect is the quantitative microscopy and linear measurements. The various parameter studies here are stomata number and index, palisade ration, vein-islet number, size of starch grain etc [51].

The observation of microscopic characteristics which were shown in Table no. 3.2.

**The Powder Characteristics:**

For the powder characteristic take a the dried plant *Caesalpinia bonduc* L. leaves was prepared into fine powder then, transfer the fine powder in watch glass separately. After that the fine powder are applied for the investigation of microscopic characteristic observation.

**Procedure:**
The fine or pulverized powder of leaves is transferred in watch glass in small quantity and boiled with cleaning reagent such as chloral hydrate solution. Then separate out the powder in three watch glass respectively and add staining reagent after that add one to two drops of phloroglucinol and concentrated hydrochloric acid.

Take the treated powder are mounted a small quantity of the powder on slide and add dilute glycerine then covered the cover slip on treated powdre. Then, the mounted slide is keep on microscope stage and at low power magnification.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Caesalpinia bonduc L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Covering trichomes, covering trichomes attached to epidermic</td>
</tr>
<tr>
<td></td>
<td>Diacytic stomata</td>
</tr>
<tr>
<td></td>
<td>Lignified Xylem</td>
</tr>
<tr>
<td></td>
<td>Prism Calcium oxalate crystals</td>
</tr>
</tbody>
</table>

**Table 3.2: Microscopic character of Leaves Caesalpinia bonduc L.**

3.1.5: **Physical Standardization of Caesalpinia bonduc L. Leaves.**

The physical standareds are to be determined for durgs, wherever possible. In physical evaluation there are few constant present or not present for crude drugs, this evaluation method may help in evaluation, generally with reference to moisture content, specific gravity, foreign matter, determination of physical constant like aqueous extractive value, alcoholic extractive value, LOD, and estimation of drug fluorescence.

After physical evaluation, leaves were dried then powdered. The uniform powder was subjected to standardization for different parameters [39,58].

*Estimation of foreign matter:*
The Foreign matter which is present in raw material or crude drugs due to the attacked of micro-organism, also involved mineral etc. The foreign matters are present in herbal plant like dust, soil, stones etc. The observations of foreign matter which are present specific quantity in drug which are find out the drugs which are spread on a paper. After spreading the foreign matter find out with the help of observation by visual using various type of magnified lens, after visualised the foreign matters which are remove and recorded the percentage of yield.

**The Determination of physical constants**

- **LOD**

  The determination of Loss on drying which are based on the loss of material after drying and determined by percent w/w. Estimation of aqueous and volatile extracts or matter in crude drug with the help of determination of loss on drying method. If the moisture is present in crude drugs then, which are not observed by visually. Therefore, determination of loss on drying of crude drug is required to used the LOD techniques and removed the moisture as soon as possible.

  Take 5 gm of drug weight accurately quantity of coarse crude drug was transfer in a china or porcelain dish. After taking the weight of drug the china dish is kept in hot air oven for 2 hrs. During these 2 hrs maintain the temperature at 110°C. Then, porcelain dish kept for cooling in desiccator at 30-35°C temperature, after cooling the powder take a weighed. Then, calculated the percentage of Loss on drying with the help of following formula.

\[
\text{% Loss on drying} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample}} \times 100
\]

- **Estimation of Ash values**

  The determination of ash values which are crucial parameter for the determination of quality and purity of a crude drug. In generally the crude drugs which are available in form of the powder. The main motto of the organized drugs of ash in which to removed the all traces of inorganic as well as organic compounds, the matters which can be interfering in an estimation of drug. After burning of crude drugs for example bark and leaves parts which contains inorganic components such as phosphates, carbonates, and potassium, silicates of sodium, Ca and Mg. During the preparation ash of ash such as water soluble ash, total ash and acid insoluble ash of
crude drug should take a precaution. When the calcium oxalate present in greater volume there may be acid-insoluble ash is present in crude drug.

- **The Determination Total ash value**

  For the determination of total ash to take accurately weight of crude drug powder are 2-3 gm and which are transferred in silica crucible. During the burning time to maintain the temperature should not exceeding at 450°C for 4 hrs. Otherwise free from carbon, after cooling take weight of ash and calculated the percentages of total ash value as per the reference of the air-dried crude drug with the help of following formula,

  \[
  \% \text{ Total ash value} = \frac{\text{Wt. of total ash}}{\text{Wt. of crude drug taken}} \times 100
  \]

- **The Determination Water soluble ash value**

  For the determination of water soluble ash value to take ash and ash is boile with 25 ml of water. After cooling the ash are filtered through the filter paper and insoluble residue which are collected on ashless filter paper are collecte and wash with hot water and after washing the ash is transfer in crucible. Then, againe ignite the 4 hrs. during ignition time the temperature should not exceeding 450°C. After burning the ash are keep in a desiccator for cooling after cooling recorded the weighed. The weight of total ash from that remove the weight of insoluble ash matter. The water soluble ash is represented by the weight difference of ash. After that noted values are put up in given formula and calculated the % of water soluble ash value.

  \[
  \% \text{ Water soluble ash value} = \frac{\text{Wt. of total ash} - \text{Wt. of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100
  \]

- **Determination Acid insoluble ash value**

  To take the ash and boiled with 25 ml of 2 Mole of hydrochloric acid for 5 min. After coplision of boiling the ash then, filtre through the ashless filter paper and ash are collected in dish. Then, collected ash transfer in a tared crucible and ash is ignited for 4 hrs. during specified time of burning or ignite the temperature should not exceed 450°C. Then, the after coplision of burning the crucible is keep in desicater for cooling after cooling remove the dish and recorded the weight of ash and calcute the percentages of acid insoluble ash value. For the calculation of percentage of yeild of acid insoluble ash values have a specific formula these are following-
**Determination of Extractive Value:**

**The Alcoholic extractive value**-

To take 5 gm of accurately weight of coarsed powde of crude drug transferred in conical flask macerated with the 100 ml of alcohol and the flask is macerated for 24 hrs. During the 24 hrs first five hours shaking frequently and last 1 hr the flask kept a side for settle down. Then, filter the marc through filter paper during filtration to taking a precaution of loss of alcohol. Then, out of them to take 25 ml of alcoholic extracts in tared evaporating dish, the dish is kept on water bath up to dryness. Then, note the weight and calculated the percentage of yield w/w which are based on the air-dried drug.

**Calculations:**

- 25 ml of alcohol extract gives = X g of residue
- 100 ml of alcohol extract gives = 4X g of residue
- 5 g of air dried drug gives = 4X g of alcohol (90%) soluble residue.
- 100 g of air dried drug gives = 80 X g of alcohol (90%) soluble residue.

**Water soluble extractive value**

To take 5 gm of accurately weight of coarsed powde of crude drug and macerated with 100 ml of water and 10 % of alcohol in closed flask and macerated for 24 hrs. During the 24 hrs first five hours shaking frequently and allowing standing for 19 hours. The completion specified time of maceration. Then, filter the marc during filtration have to take precautions against loss of ethanol. Out of 100 ml take 25 ml filtrate and transfer in evaporating dish and kept on water bath for evaporation of water, the after evaporation of water and dry at 105\(^0\) C, then dish is keep in dessicator for cooling after cooling recorded the weigh of dried extract. Calculate yield of ethanol soluble extractive value.

**Calculations:**

- 25 ml of water extract gives = X g of residue
100 ml of water extract gives = 4X g of residue
5 g of air dried drug gives = 4X g of water soluble residue.
100 g of air dried drug gives = 80 X g of water soluble residue.

**Analysis of Fluorescence of drug**

Several or number of crude drugs having fluorescence, if the sample is observed under UV light then, the sample which are shown fluorescence colour. The confirmation of identity and quality of crude drug checked on the based with the reference of fluorescence. The observation of fluorences is not much used in day-light, because they have a low fluorescent effect. During the installation the Fluorescence lamps used the suitable filters, which help to the removal radiation which are produced from the lamp as well as UV transmitted radiation of at the specific wavelength. The several extracts having the specific characters which are shown as fluorescence for there confirmation of identity [69].

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Physico-chemical parameter</th>
<th><em>Caesalpinia bonduc</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Foreign matter</td>
<td>Nil</td>
</tr>
<tr>
<td>2.</td>
<td><strong>Ash values:</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total ash</td>
<td>3.95 %w/w</td>
</tr>
<tr>
<td></td>
<td>Acid insoluble ash</td>
<td>2.40 %w/w</td>
</tr>
<tr>
<td></td>
<td>The Water soluble ash</td>
<td>1.65 %w/w</td>
</tr>
<tr>
<td>3.</td>
<td><strong>The Extractive values:</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>The Alcohol soluble extractive</td>
<td>25.21 % w/w</td>
</tr>
<tr>
<td></td>
<td>Water soluble extractive</td>
<td>36.76 % w/w</td>
</tr>
<tr>
<td>4.</td>
<td>Loss on Drying</td>
<td>2.84 % w/w</td>
</tr>
<tr>
<td>5.</td>
<td>Fluorescence colour</td>
<td>Fluorescence</td>
</tr>
</tbody>
</table>

Tab 3.3: Standardization of *Caesalpinia bonduc* L. Leaves.
3.2: *Mangifera indica* L.

3.2.1: The Collection and Authentication of Plant:

The selected plant leaves of *Mangifera indica* L. was collected locally from the place of Ramling Mudgad is located in the Latur district and state of Maharashtra. After collection of plant then, authentication was confirmed by ICMR, Belgaum, state of Karnataka, in India.

After, authentication of leaves which are applied for the observation of macroscopical characteristics.

3.1.2: Organoleptic / Macroscopic/Morphological evaluation

The morphological or organoleptic means to the study of external appearance crude drug which are useful for the identification. In this system the crude drug are evaluated by their specific characters like as color, odour, taste, size, shape and also important the special like touch, texture etc. the morphological evaluation means conclusion drawn from impressions on organs of senses [51].

For the present study i.e morphological evaluation of crude drug used the leaves of *Mangifera indica* L. The observation of organoleptic characteristics which are shown in table-3.4.
### Table 3.4: Macroscopic character of Leaves of *Mangifera indica* L.

<table>
<thead>
<tr>
<th>Colour</th>
<th>Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odour</td>
<td>Characteristic</td>
</tr>
<tr>
<td>Taste</td>
<td>Acrid</td>
</tr>
<tr>
<td>Size &amp; Shape</td>
<td>25 cm long and 8-cm wide</td>
</tr>
<tr>
<td>Surface</td>
<td>lanceolate-elliptical, pointed at both ends</td>
</tr>
</tbody>
</table>

3.2.3: **Microscopic Characteristics**

The microscopical evaluation is one of the important method for determination or identification of a crude drug and it may used to identified the organized drugs by histological parameters. This method used for the determination of confirmation and check their quality of organized crude drugs and powdered forms.

Microscope, having a good method for the quality to magnify, the minute objects understudy to be magnified to confirm the histological details of the drug from plant origin. If want to better and effective results, used several coloring reagents for identified the tissue and cellular internal structure.

The Microscopical evaluations also importance study of the chemical constituents by using of chemical method to low quantities of drug in powdered form or to histological section of the drug. A drop of phoroglucinol and concentrated hydrochloric acide give red stain with lignin. Mucilage is stained pink with ruthenium red and also, when treated with corallin soda and few drops of sodium carbonate solution, cellulose swells and dissolved in cuoxam, while N/50 iodine solution stain blue starch and hemicelluloses.

Other important histological aspect is the quantitative microscopy and linear measurements. The various parameter studies here are stomata number and index, palisade ration, vein-islet number, size of starch grain etc [51].

The observation of microscopic characteristics which were shown in Table no. 3.5.

**The Powder Characteristics:**
For the estimation of powder characteristic to take a dried powder of *Mangifera indica* L. leaves was prepared into fine powder then, transfer the fine powder in watch glass separately. After that the fine powder are applied for the investigation of microscopic characteristic observation.

**Procedure:**

The fine or pulverized powder of leaves is transferred in watch glass in small quantity and boiled with cleaning reagent such as chloral hydrate solution. Then separate out the powder in three watch glass respectively and add staining reagent after that add one to two drops of phloroglucinol and concentrated hydrochloric acid.

Take the treated powder is mounted a small quantity of the powder on slide and add dilute glycerine then covered the cover slip on treated powdre. Then, the mounted slide is keep on microscope stage and at low power magnification.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th><em>Mangifera indica</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Covering trichomes</td>
</tr>
<tr>
<td></td>
<td>Paracytic stomata</td>
</tr>
<tr>
<td></td>
<td>Lignified bordered xylem vessels</td>
</tr>
<tr>
<td></td>
<td>Prism Calcium oxalate crystals</td>
</tr>
</tbody>
</table>

Table 3.5: Microscopic character of Leaves *Mangifera indica* L.

**3.2.5: Standardization of *Mangifera indica* L., Leaves.**

The physical standareds are to be determined for drugs, wherever possible. In physical evaluation there are few constant present or not present for crude drugs, this evaluation method may help in evaluation, generally with reference to moisture content, specific gravity, foreign matter, determination of physical constant like aqueous extractive value, alcoholic extractive value, LOD, and estimation of drug fluorescence.
After physical evaluation, leaves were dried then powdered. The uniform powder was subjected to standardization for different parameters [39,58].

*Estimation of foreign matter:*

The Foreign matter which is present in raw material or crude drugs due to the attacked of micro-organism, also involved mineral etc. The foreign matters are present in herbal plant like dust, soil, stones etc. The observations of foreign matter which are present specific quantity in drug which are find out the drugs which are spread on a paper. After spreading the foreign matter find out with the help of observation by visual using various type of magnified lens, after visualised the foreign matters which are remove and recorded the percentage of yield.

*The Determination of physical constants*

- **LOD**

The determination of Loss on drying which are based on the loss of material after drining and determined by percent w/w. Estimation of aqueous and volatile extracts or matter in crude drug with the help of determination of loss on drying method. If the moisture is present in crude drugs then, which are not observed by visually. Therefore, determination of loss on drying of crude drug is required to used the LOD techniques and removed the moisture as soon as possible.

Take 5 gm of drug weight accurately quantity of coarse crude drug was transfer in a china or porcelain dish. After taking the weight of drug the china dish is kept in hot air oven for 2 hrs. During these 2 hrs maintaine the temperature at 110°C. Then, porcelain dish kept for cooling in desiccator at 30-35 °C temperature, after cooling the powder take a weighed. Then, calculated the percentage of Loss on drying with the help of following formula.

\[
\text{% Loss on drying} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample}} \times 100
\]

- **Estimation of Ash values**

The determination of ash values which are crucial parameter for the determination of quality and purity of a crude drug. In generally the crude drugs which are available in form of the powdere. The main motto of the organized drugs of ash in which to removed the all traces of inorganic as well as organic compounds, the matters which can be interfering in an estimation of drug. After burning of crude drugs for example bark and leaves parts which contains inorganic
components such as phosphates, carbonates, and potassium, silicates of sodium, Ca and Mg.
During the preparation ash of ash such as water soluble ash, total ash and acid insoluble ash of crude drug should take a precaution. When the calcium oxalate present in greater volume there may be acid-insoluble ash is present in crude drug.

- **The Determination Total ash value**

  For the determination of total ash to take accurately weight of crude drug powder are 2-3 gm and which are transferred in silica crucible. During the burning time to maintain the temperature should not exceeding at 450°C for 4 hrs. Otherwise free from carbon, after cooling take weight of ash and calculated the percentages of total ash value as per the reference of the air-dried crude drug with the help of following formula,

  \[
  \% \text{ Total ash value} = \frac{\text{Wt. of total ash}}{\text{Wt. of crude drug taken}} \times 100
  \]

- **The Determination Water soluble ash value**

  For the determination of water soluble ash value to take ash and ash is boile with 25 ml of water. After cooling the ash are filtered through the filter paper and insoluble residue which are collected on ashless filter paper are collecte and wash with hot water and after washing the ash is transfer in crucible. Then, againe ignite the 4 hrs. during ignition time the temperature should not exceeding 450°C. After burning the ash are keep in a desiccator for cooling after cooling recorded the weighed. The weight of total ash from that remove the weight of insoluble ash matter. The water soluble ash is represented by the weight difference of ash. After that noted values are put up in given formula and calculated the % of water soluble ash value.

  \[
  \% \text{ Water soluble ash value} = \frac{\text{Wt. of total ash} - \text{Wt. of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100
  \]

- **Determination Acid insoluble ash value**

  To take the ash and boiled with 25 ml of 2 Mole of hydrochloric acid for 5 min. After coplision of boiling the ash then, filtere through the ashless filter paper and ash are collected in dish. Then, collected ash transfer in a tared crucible and ash is ignited for 4 hrs. during specified
time of burning or ignite the temperature should not exceed $450^0$C. Then, the after completion of burning the crucible is keep in desicater for cooling after cooling remove the dish and recorded the weight of ash and calculate the percentages of acid insoluble ash value. For the calculation of percentage of yield of acid insoluble ash values have a specific formula these are following:

\[
\% \text{ Acid insoluble ash value} = \frac{\text{Wt. of acid insoluble ash}}{\text{Wt. of crude drug taken}} \times 100
\]

- **Determination of Extractive Value:**

**The Alcoholic extractive value**-

To take 5 gm of accurately weight of coarsed powde of crude drug transferred in conical flask macerated with the 100 ml of alcohol and the flask is macerated for 24 hrs. During the 24 hrs first five hours shaking frequently and last 1 hr the flask kept a side for settle down. Then, filter the marc through filter paper during filtration to taking a precaution of loss of alcohol. Then, out of them to take 25 ml of alcoholic extracts in tared evaporating dish, the dish is kept on water bath up to dryness. Then, note the weight and calculated the percentage of yield w/w which are based on the air-dried drug.

**Calculations:**

- 25 ml of alcohol extract gives $= X$ g of residue
- 100 ml of alcohol extract gives $= 4X$ g of residue
- 5 g of air dried drug gives $= 4X$ g of alcohol (90%) soluble residue.
- 100 g of air dried drug gives $= 80 X$ g of alcohol (90%) soluble residue.

**Water soluble extractive value**

To take 5 gm of accurately weight of coarsed powde of crude drug and macerated with 100 ml of water and 10% of alcohol in closed flask and macerated for 24 hrs. During the 24 hrs first five hours shaking frequently and allowing standing for 19 hours. The completion specified time of maceration. Then, filter the marc during filtration have to take precautions against loss of ethanol. Out of 100 ml take 25 ml filtrate and transfer in evaporating dish and kept on water bath for evaporation of water, the after evaporation of water and dry at $105^0$C, then dish is keep in dessicator for cooling after cooling recorded the weigh of dried extract. Calculate yield of ethanol soluble extractive value.
Calculations:

25 ml of water extract gives  =  X g of residue
100 ml of water extract gives  =  4X g of residue
5 g of air dried drug gives  =  4X g of water soluble residue.
100 g of air dried drug gives  =  80 X g of water soluble residue.

➢ Analysis of Fluorescence of drug

Several or number of crude drugs having fluorescence, if the sample is observed under UV light then, the sample which are shown fluorescence colour. The confirmation of identity and quality of crude drug checked on the based with the reference of fluorescence. The observation of fluorences is not much used in day-light, because they have a low fluorescent effect. During the installation the Fluorescence lamps used the suitable filters, which help to the removal radiation which are produced from the lamp as well as UV transmited of radiation of at the specific wavelength. The several extracts having the specific characters which are shown as fluorescence for there confirmation of identity [69].

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Physico-chemical parameter</th>
<th>Mangifera indica L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Foreign matter</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>The Ash Values:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total ash</td>
<td>1.2 %</td>
</tr>
<tr>
<td></td>
<td>Acid insoluble ash</td>
<td>3.0 %</td>
</tr>
<tr>
<td></td>
<td>The Water-soluble ash</td>
<td>2.80 %</td>
</tr>
<tr>
<td>3</td>
<td>The Extractive values:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcohol soluble extractive</td>
<td>12.10 %w/w</td>
</tr>
<tr>
<td></td>
<td>Water soluble extractive</td>
<td>8.62 %w/w</td>
</tr>
<tr>
<td>4</td>
<td>LOD</td>
<td>6.72 %w/w</td>
</tr>
<tr>
<td>5</td>
<td>The Fluorescence</td>
<td>No fluorescence</td>
</tr>
</tbody>
</table>

Table- 3.6: Standardization of *Mangifera indica* L.
3.3: *Ricinus communis* L.

3.3.1: Collection and Authentication.

The selected plant leaves of *Ricinus communis* L. was collected locally from the place of Ramling Mudgad is located in the Latur district and state of Maharashtra. After collection of plant then, authentication was confirmed by ICMR, Belgaum, state of Karnataka, in India.

After, authentication of leaves which are applied for the observation of macroscopical characteristics.

3.1.2: Organoleptic / Macroscopic/Morphological evaluation

The morphological or organoleptic means to the study of external appearance crude drug which are useful for the identification. In this system the crude drug are evaluated by their specific characters like as color, odour, taste, size, shape and also important the special like touch, texture etc. The morphological evaluation means conclusion drawn from impressions on organs of senses [51].

For the present study i.e morphological evaluation of crude drug used the leaves of *Ricinus communis* L. The observation of organoleptic characteristics which are shown in table - 3.7.

<table>
<thead>
<tr>
<th></th>
<th><em>Ricinus communis</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Green</td>
</tr>
<tr>
<td>Odour</td>
<td>Mild</td>
</tr>
<tr>
<td>Taste</td>
<td>Astringent / Salty</td>
</tr>
<tr>
<td>Size &amp; Shape</td>
<td>Alternative,longpetiolare, stipulate, palmately lobed, lobes 7 or more.</td>
</tr>
</tbody>
</table>

(Table 3.7: Macroscopic character of Leaves of *Ricinus communis* L.)
3.3.3: Microscopic Characteristics

The microscopical evaluation is one of the important method for determination or identification of a crude drug and it may used to identified the organized drugs by histological parameters. This method used for the determination of confirmation and check their quality of organized crude drugs and powderd forms.

Microscope, having a good method for the quality to magnify, the minute objects understudy to be magnified to confirm the histological details of the drug from plant origin. If want to better and effective results, used several coloring reagents for identified the tissue and cellular internal structure.

The Microscopical evaluations also importance study of the chemical constituents by using of chemical method to low quantities of drug in powdered form or to histological section of the drug. A drop of phoroglucinol and concentrated hydrochloric acide give red stain with lignin. Mucilage is stained pink with ruthenium red and also, when treated with corallin soda and few drops of sodium carbonate solution, cellulose swells and dissolved in cuoxam, while N/50 iodine solution stain blue starch and hemicelluloses.

Other important histological aspect is the quantitative microscopy and linear measurements. The various parameter studies here are stomata number and index, palisade ration, vein-islet number, size of starch grain etc [51].

The observation of microscopic characteristics which were shown in Table no. 3.8.

The Powder Characteristics:

For the estimation of powder characteristic to take a dried powder of *Ricinus communis* L. leaves was prepared into fine powder then, transfer the fine powder in watch glass separately. After that the fine powder are applied for the investigation of microscopic characteristic observation.

**Procedure:**

The fine or pulverized powder of leaves is transferred in watch glass in small quantity and boiled with cleaning reagent such as chloral hydrate solution. Then separate out the powder in three watch glass respectively and add staining reagent after that add one to two drops of phloroglucinol and concentrated hydrochloric acid.
Take the treated powder is mounted a small quantity of the powder on slide and add dilute glycerine then covered the cover slip on treated powdre. Then, the mounted slide is keep on microscope stage and at low power magnification.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Ricinus communis L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observation</td>
<td></td>
</tr>
<tr>
<td>Covering trichomes</td>
<td></td>
</tr>
<tr>
<td>Paracytic stomata with covering trichomes</td>
<td></td>
</tr>
<tr>
<td>Lignified scalariform Xylem vessels</td>
<td></td>
</tr>
<tr>
<td>Non-lignified fibers</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.8: Microscopic character of Leaves *Ricinus communis* L.**

**3.3.5: Physical Standardization of *Ricinus communis* L. Leaves**

The physical standareds are to be determined for durgs, wherever possible. In physical evaluation there are few constant present or not present for crude drugs, this evaluation method may help in evaluation, generally with reference to moisture content, specific gravity, foreign matter, determination of physical constant like aqueous extractive value, alcoholic extractive value, LOD, and estimation of drug fluorescence.

After physical evaluation, leaves were dried then powdered. The uniform powder was subjected to standardization for different parameters [39,58].

*Estimation of foreign matter:*
The Foreign matter which is present in raw material or crude drugs due to the attacked of micro-organism, also involved mineral etc. The foreign matters are present in herbal plant like dust, soil, stones etc. The observations of foreign matter which are present specific quantity in drug which are find out the drugs which are spread on a paper. After spreading the foreign matter find out with the help of observation by visual using various type of magnified lens, after visualised the foreign matters which are remove and recorded the percentage of yield.

The Determination of physical constants

➢ LOD

The determination of Loss on drying which are based on the loss of material after drining and determined by percent w/w. Estimation of aqueous and volatile extracts or matter in crude drug with the help of determination of loss on drying method. If the moisture is present in crude drugs then, which are not observed by visually. Therefore, determination of loss on drying of crude drug is required to used the LOD techniques and removed the moisture as soon as possible.

Take 5 gm of drug weight accurately quantity of coarse crude drug was transfer in a china or porcelain dish. After taking the weight of drug the china dish is kept in hot air oven for 2 hrs. During these 2 hrs maintain the temperature at 110°C. Then, porcelain dish kept for cooling in desiccator at 30-35 °C temperature, after cooling the powder take a weighed. Then, calculated the percentage of Loss on drying with the help of following formula.

\[
\% \text{ Loss on drying} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample}} \times 100
\]

➢ Estimation of Ash values

The determination of ash values which are crucial parameter for the determination of quality and purity of a crude drug. In generally the crude drugs which are available in form of the powdere. The main motto of the organized drugs of ash in which to removed the all traces of inorganic as well as organic compounds, the matters which can be interfering in an estimation of drug. After burning of crude drugs for example bark and leaves parts which contains inorganic components such as phosphates, carbonates, and potassium, silicates of sodium, Ca and Mg. During the preparation ash of ash such as water soluble ash, total ash and acid insoluble ash of
crude drug should take a precaution. When the calcium oxalate present in greater volume there may be acid-insoluble ash is present in crude drug.

- **The Determination Total ash value**

  For the determination of total ash to take accurately weight of crude drug powder are 2-3 gm and which are transferred in silica crucible. During the burning time to maintain the temperature should not exceeding at $450^0$C for 4 hrs. Otherwise free from carbon, after cooling take weight of ash and calculated the percentages of total ash value as per the reference of the air-dried crude drug with the help of following formula,

  $$\% \text{ Total ash value} = \frac{\text{Wt. of total ash}}{\text{Wt. of crude drug taken}} \times 100$$

- **The Determination Water soluble ash value**

  For the determination of water soluble ash value to take ash and ash is boile with 25 ml of water. After cooling the ash are filtered through the filter paper and insoluble residue which are collected on ashless filter paper are collecte and wash with hot water and after washing the ash is transfer in crucible. Then, againe ignite the 4 hrs. during ignition time the temperature should not exceeding $450^0$C. After burning the ash are keep in a desiccator for cooling after cooling recorded the weighed. The weight of total ash from that remove the weight of insoluble ash matter. The water soluble ash is represented by the weight difference of ash. After that noted values are put up in given formula and calculated the % of water soluble ash value.

  $$\% \text{ Water soluble ash value} = \frac{\text{Wt. of total ash} - \text{Wt. of water insoluble ash}}{\text{Wt. of crude drug taken}} \times 100$$

- **Determination Acid insoluble ash value**

  To take the ash and boiled with 25 ml of 2 Mole of hydrochloric acid for 5 min. After coplosion of boiling the ash then, filtre through the ashless filter paper and ash are collected in dish. Then, collected ash transfer in a tared crucible and ash is ignited for 4 hrs. during specified time of burning or ignite the temperature should not exceed $450^0$C. Then, the after complosion of burning the crucible is keep in desicater for cooling after cooling remove the dish and recorded the weight of ash and calcute the percentages of acid insoluble ash value. For the calculation of percentage of yeild of acid insoluble ash values have a specific formula these are following-
Determination of Extractive Value:

The Alcoholic extractive value-

To take 5 gm of accurately weight of coarsed powde of crude drug transferred in conical flask macerated with the 100 ml of alcohol and the flask is macerated for 24 hrs. During the 24 hrs first five hours shaking frequently and last 1 hr the flask kept a side for settle down. Then, filter the marc through filter paper during filtration to taking a precaution of loss of alcohol. Then, out of them to take 25 ml of alcoholic extracts in tared evaporating dish, the dish is kept on water bath up to dryness. Then, note the weight and calculated the percentage of yield w/w which are based on the air-dried drug.

Calculations:

25 ml of alcohol extract gives = X g of residue
100 ml of alcohol extract gives = 4X g of residue
5 g of air dried drug gives = 4X g of alcohol (90%) soluble residue.
100 g of air dried drug gives = 80 X g of alcohol (90%) soluble residue.

Water soluble extractive value

To take 5 gm of accurately weight of coarsed powde of crude drug and macerated with 100 ml of water and 10 % of alcohol in closed flask and macerated for 24 hrs. During the 24 hrs first five hours shaking frequently and allowing standing for 19 hours. The completion specified time of maceration. Then, filter the marc during filtration have to take precautions against loss of ethanol. Out of 100 ml take 25 ml filtrate and transfer in evaporating dish and kept on water bath for evaporation of water, the after evaporation of water and dry at 1050 C, then dish is keep in dessicatator for cooling after cooling recorded the weigh of dried extract. Calculate yield of ethanol soluble extractive value.

Calculations:

25 ml of water extract gives = X g of residue
100 ml of water extract gives = 4X g of residue
5 g of air dried drug gives = 4X g of water soluble residue.
100 g of air dried drug gives = 80 X g of water soluble residue.

➤ Analysis of Fluorescence of drug

Several or number of crude drugs having fluorescence, if the sample is observed under UV light then, the sample which are shown fluorescence colour. The confirmation of identity and quality of crude drug checked on the based with the reference of fluorescence. The observation of fluorences is not much used in day-light, because they have a low fluorescent effect. During the installation the Fluorescence lamps used the suitable filters, which help to the removal radiation which are produced from the lamp as well as UV light transmited of radiation of at the specific wavelength. The several extracts having the specific characters which are shown as fluorescence for there confirmation of identity [69].

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Physico-chemical parameter</th>
<th>Ricinus communis L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Foreign matter</td>
<td>Nil</td>
</tr>
<tr>
<td>2.</td>
<td>Ash Values:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total ash</td>
<td>1.10 %w/w</td>
</tr>
<tr>
<td></td>
<td>Acid insoluble ash</td>
<td>2.1 %w/w</td>
</tr>
<tr>
<td></td>
<td>The Water-soluble ash</td>
<td>1.5 %w/w</td>
</tr>
<tr>
<td>3.</td>
<td>The Extractive values:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alcohol soluble extractive</td>
<td>28.00% w/w</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>Water soluble extractive</td>
<td>54.11% w/w</td>
</tr>
<tr>
<td>4. LOD</td>
<td></td>
<td>7.90% w/w</td>
</tr>
<tr>
<td>5. The Fluorescence</td>
<td>At 254 nm</td>
<td>No fluorescence</td>
</tr>
<tr>
<td></td>
<td>At 366 nm</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.9: Standardization of *Ricinus communis* L. Leaves.**

3.2 : EXTRACTION :

The extraction means the separation of chemical constituents from plants sources. The plant and plant derived products are gaining world wide popularity due to numerous advantages including easy availability sources of lead molecules, synergism with modern drugs abd their significance patent opportunities. WHO and other regulatory bodies have emphasized research, especially in extraction and isolation of phytomedicines from the herbs utilized in Complementary and Alternative Medicines (CAM) which can be useful for affirming scientific evidence along with setting good quality control parameters. Here I shall discuss firstly the basic concept of extraction method which is the very first step in any herbal research.

A number of extraction techniques are utilized by researchers. But due to the drawbacks of traditional extraction systems like poor solubility in solvent, time and energy consumption along with thermal degradation led to the invention and introduction of various modern alternative extraction methods which are rapid, solvent free and compatible with thermolabile substances. These modern methods are more promising and found to be fruitful but are very costly. Some of the traditional (e.g. maceration, decoction, percolation, soxhlet extraction) as well as recent (e.g. Super critical fluid) extraction method have been discussed as possible effective methods as per desired phytoconstituents [52,40,59,50].

In present study used maceration and hot continuous extraction method. These are discuss follows-

*Maceration Extraction:*
Maceration means to soften the parts of the plant. It is an extraction process of crude drug from crushed or pressed plant or animal parts which are soaked in a suitable solvent for seven days with occasional stirring at room temperature. The solvent used in the process is called as menstruum. After several days of soaking the crude drug menstruum is strained off and kept separately, the residue left behind is called as marc which is pressed to obtain the remaining menstruum. Compared to other method the intensity of movement is so slow that it can be called extraction at stationary conditions.

3.2.1: EXTRACTION CAESALPINIA BONDUC L.

In the present study, the shade dried leaves of *Caesalpinia bonduc* L. was prepared to coarse powder. After preparation of powder about 100 gm of powder was taken for extraction. The powder was loaded in soxhlet to successive hot continuous extraction. Another batch of powdered drug was macerated with chloroform-water. After the effective extraction, the solvent were concentrating on water bath or usined rotary flash evaporator and remove the water from obtained extraction. Then, recorded the weight of extract and calculated extract value. Its percentage of yield obtained was shown in Table No.3.10.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plant Name</th>
<th>Extracts</th>
<th>Nature of Extract</th>
<th>Colour of extract</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Caesalpinia bonduc</em> L</td>
<td>Alcohol</td>
<td>Semi-solid</td>
<td>Red-brownish</td>
<td>36.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqueous</td>
<td>Semi-solid</td>
<td>Brownish</td>
<td>25.21</td>
</tr>
</tbody>
</table>

Table 3.10: The percentage yield of the aqueous and alcoholic leaves extracts of the *Caesalpinia bonduc* Linn.
The obtained all extracts were used for chemical investigation.

3.2.2: QUALITATIVE CHEMICAL INVESTIGATION:
The alcohol (95%) and aqueous extracts were subjected to qualitative chemical investigation [52,40,59,50,37,24].

PHYTOCHEMICAL INVESTIGATION:
The Preliminary qualitative tests

The determination of phytochemicals investigation having several tests and chemical reagents. For determination of chemical test firstly prepared freshed all reagent. Then, the extracts were used for the all these tests and observations given in below are reported in Table- 3.11.

1) Tests for Carbohydrates:

Test solution:
The extracts were dissolved in water and these solution used as test solution. After that it was hydrolyzed with equal volume of 2 N hydrochloric acid are utilized for the following tests.

a) Molish's test or General test for carbohydrates:

To take 3 to 4 ml of water extracts then, added few drops of molish reagent and shakes well. After that add few drops of concentrated H$_2$SO$_4$ added through the sides of the test tube without shaking. The violet ring is appeared at the junction between two liquids.

b) Fehling’s test:

To take 1 ml of Fehling-A and Fehling-B solution in a test tube and boiled for two minutes. Then, add 2 ml of tests solution which is prepared from extract. Then, solution is heated on water bath for 5 min, the precipitate appears yellow, brick red, or orange colour.

c) Benedict's test:

To take the 2 ml of test solution and add 2 ml of Benedict's reagent in a test tube and mix properly. Then, heated on water bath for 5 mins. The precipitated observed red, green or yellow colour. If red, green and yellow colour are appeared which shows presence of reducing sugars.

d) The Barfoed's test:

To take the test solution then, add equal volume of Barfoed's reagent solution. Then, the solution is heated in water bath for 1 to 2 min. After the cooling the solution appeared red precipitate.

e) Cobalt-chloride test:
Take 3 ml of test solution was mixed with 2 ml cobalt chloride, after that boiled and cooled. Then added FeCl₃ drops on NaOH solution. Solution was shown greenish blue the glucose is present and the solution was observed purplish color the Fructose is present in solution. When the lower layer appeared purplish and the upper layer appeared blue coloured indicated the presence of glucose and fructose.

f) Test of Non-Reducing Sugars:
   To take test solution and treated with Fehling's and Benedict's reagent test solution does not give any response.

g) Tannic acid test to detect starch:
   Take test solution in test tube add 20% tannic acid, precipitate observed.

2) Tests for Proteins:

   Preparation of Test Solution:
   To take the drug extracts and dissolve in water and prepared test sample for following test.

   a) Biuret test (General test):
      To take the 3 ml test solution then add 4% sodium hydroxide solution and few drops of 1% copper sulphate solution, the solution appears purple or violet colour. If shows the purple and violet colour of solution the presence proteins in drug.

   b) Million's test (for proteins):
      For the detection of the of proteins to take the 3 ml of test solution mix with the 5 ml of Million’s reagent, which appeared white precipitate. When the Precipitate get warmed it turns white to brick red colour.

   c) Test of Xanthoprotein:
      To take the 3ml of Test Solution treated with 1 ml concentrated sulphuric acid, the solution shows white precipitate.

   d) Test of Precipitation:
      The test solution is treated with following reagent shows white colloidal ppt. such as alcohol, 5% HgCl₂ solution, 5% CUSO₄ solution, 5% lead acetate and 5% ammonium sulphate.

3) Tests for Steroids:
**Preparation of extract solution for test:**

For the detection of steroids, the saponification of extract is done by refluxing the extract with potassium hydroxide. Then add water to the saponified extract for dilution of the extracts and the unsaponified matter was extracted with diethyl ether. Then, the extract was evaporate on water bath and the remaining residue was used for the following test.

a) **Reaction of Salkowski:**

To take 2 ml of extract mix with 2 ml chloroform and treated with 2 ml conc. H$_2$SO$_4$. Then, the solutions are shaking properly. If the chloroform layer shows red and acid layer observed fluorescence colour.

b) **Libermann-Burchard test:**

To take 2 ml of extract are mixed with chloroform solvent. Then, added 1-2 ml of acetic anhydride and two drops of H$_2$SO$_4$ transferred through the side of test tube then observed test tube layer first layer shown red, second layer blue and finally green colour.

c) **Libermann's test:**

To take the 3 ml extract is treated with 3 ml of acetic anhydride. Then, test tube is heated on flame and cooled. After cooling added few drops conc. H$_2$SO$_4$ the solution appears blue colour.

4) **Tests of Amino Acids:**

a) **Test of Ninhydrin (General test):**

To take 3 m of test solution of crude drug and add 2-3 drops of 5% Ninhydrin reagent then, heated on water bath for 10 min. If the solution appears purple or violet colour the there is presence of amino acid in crude extract drug.

b) **Test for Tyrosine:**

Take 3 ml of test solution and added 3 drops Million's reagent then heat. After heating the solution observed dark red colour.

c) **Test for tryptophan:**

For this test take 3 ml test solution then, added few drops of glycoxallic acid and conc. H$_2$SO$_4$ the solution shows reddish violet ring at junction of the two layers.

5. **Tests for Glycosides:**

*The Preparation of test solution:*
The test solutions were prepared from the crude drug extract is dissolved in alcohol solution. The prepared test solution are used for following test.

Detection tests for Cardiac Glycosides:

a) Baljet’s test:
   
   For the detection of cardiac glycoside take test solution added with sodium picrate. Then, solution is appeared yellow colour change to orange colour.

b) Bromine water test:
   
   To take a test solution dissolved in bromine water shown yellow precipitate

c) Legal's test (Cardenoloids):
   
   To take test solution which are prepared from water or alcohol. Then, added 1 ml of pyridine and 1 ml of sodium nitroprusside the solution observed for pink to red colour.

d) Test for deoxysugars (Killar Krillani test):
   
   To take 2 ml of extract (test solution) and add 1 ml of glacial acetic acid and one drop of 5% ferric chloride and concentrate sulphuric acid when addition the sulphuric acid the solution appeared brown colour at the junction of the two liquid and bluish green colour appeared at upper layers of solution.

e) Libermann's test (For bufadenolids):
   
   To take 3 ml of extract solution mixed with the 3 ml acetic anhydride and heated on water bath and cooled. After cooling the solution added few drops concentrated H₂SO₄ then, the solutions are appeared blue colour.

Test for anthraquinone glycosides:

a) Modified Borntrager’s test:
   
   The C-glycosides of anthraquinones require more specific conditions for doing the hydrolysis. During the hydrolysis of the produced drug take the 5 ml of dilute hydrochloric acid and 5 ml of 5% solution of ferric chloride. For hydrolysis of extract have procedure is described the following test i.e Borntrager’s test.

b) Borntrager’s test:
   
   For the borntrager’s test take powdered drug and boiled with 5 ml of the 10 % sulphuric acid for 5 min. After boiling filtered the solution immediately. Then, cooled and the obtained filtrate are mix with benzene equal volume as filtrate. The benzene layer was separate and the
separated layer was taken in tared dish and adds 10% of ammonia solution. The solution is keeping a side for separating. The ammonical layer appeared pink colour due to the presence of anthraquinones.

Saponin Glycosides Tests:

a) **Test of Foam:**

To take crude drug extract powder and mixed with water then, continuously shake. The solution appeared foam form.

b) **Foaming index:**

To take accurately weight 1 gm of powdered crude drug and transfer in 500 ml of flask which contains 100 ml of boiling water. The temperature is maintained and the drug boils continuously for 30 minutes. When the solution is cool then the solution was filter it into a volumetric flask and make a volume addition of water up to 100 ml.

Take the above solution into ten graduated test-tubes and each test tube containing 1 ml, 2 ml, 3 ml up to 10 ml respectively. Then, adjusted the volume up to 10 ml in each test tube. The test tube are cover and shake for 15 second. After shaking the test tubes are keep a side for 15 minutes and measure the foam height.

The results assed as follows:

i. The each and every test tube having the foaming index is less than 100 when the foam height is one cm.

ii. If the height of the foam is measure 1 cm of any one test tube of the plants material. Then, there is required for the determination of the foaming index. To prepared the dilution to observed better or accurate result.

iii. When the foam height observed above one centimeter in each and every test tube. Then, there is need to determination of the 1000 foaming index. Take one or more tests by using the different decoction dilutions and fid out the result.

\[
\text{Foaming index} = \frac{100}{a}
\]

Where \( a \) = volume of the decoction in 100 ml. which are preparing from the dilutions in the test tube where height of foaming is observed.

Haemolytic test:
To take test solutions add 1-2 drops of the blood and mounted on a glass slide. The slide is observed are Haemolytic zone.

**Tests for Coumarin Glycosides:**

Test solution having pH 7 or alkaline, shown blue or green fluorescence.

6. **Tests for Alkaloids:**

a) **Dragendorff's test:**

Take test solution 2-3 ml which is prepared from crude drug and filter. Then, take a filtrate and added few drops Dragendorff's reagent when added the reagent the solution appeared brown precipitate.

b) **Mayer's test:**

To take 2-3 ml of filtrate or test solution in the test tube and add few drops Mayer's reagent. when addition of Mayer’s reagent the solution is observed precipitate.

c) **Hager's test:**

To take 2-3 ml test solution filtrates and treated with Hagers reagent. when the addition of Hager’s reagent the solution are observed yellow precipitate.

d) **Wagner's test:**

For the detection of alkaloids to take 2-3 ml filtrate and test solution is treated with Wagner's reagent when the addition of reagent the solution are appeaed reddish brown precipitate.

7. **Tests for Flavonoids:**

The structurally all flavonoids are derived from the origin substance are known as flavone. The flavones are found either in the free form or bound to sugars like as a glycosides. To this reason, when determination of flavonoids it is generally better to estimated the flavonoids of plant extracts.

**Preparation of test solution:**

i. Test solution of extract is preapared by addition of a small quantity of extract and the equal volume of hydrochloric acidr 30-40 min. during boiling the temp. should not exceed 100°C.

ii. The prepared extract was filtered with the help of filter paper. Take a filreted extracte and treated with ethyl acetate.
Then, the extract was concentrated on water bath upto dryness. Then, dried extracts was used for the following test -

a) **Shinoda test:**

To take test solution (extract) in test tube then add 5 ml 95 % ethanol in test tube and few drops concentrated hydrochloric acid and finally add 0.5 g magnesium turnings. When addition of magnesium turning appeare pink colour to solution.

b) **Lead acetate test:**

To take small quantity of (extract) or test solution in test tube treated with lead acetate solution. When the addition of lead acetate solution the solution are appeared yellow coloured ppt. If addition of large amount of sodium hydroxide the solution appeared yellow coloured, the yellow coloration was decolourised when addition of any type of acid.

c) **Ferric chloride test:**

To take a test solution and treated with few drops of ferric chloride solution. When addition of FeCl$_3$ in test solution, the solution shown green colour.

8. **Test for Vitamins:**

a) **For Vitamin A:**

Take 10-15 units quantity dissolved in 1 ml chloroform then added 5ml of antimony trichloride solution, immediately the solution appeared blue colour.

b) **Test for vitamin C (Ascorbic acid):**

To take 1 ml of test sample dissolved in 5 ml of water mixed with 2 ml dil. NaOH solution. Then, added 1 drop of solution of sodium nitroprusside. After that added 0.6 ml of hydrochloric acid dropwise and shake the solution color changed yellow to blue.

c) **Test for Vitamin D:**

For the test of vitamin take about 100 units of test sample dissolved a equal volume of chloroform and then, added 10 ml of antimony tricohloride solution, the solution appeared pinkish-red colour which indicated that vitamin D is present.

9. **Tests for Saponins:**

The preparation of test sample:

Take extracts and extracts was dissolved in water these solution used for following tests.

a) **Foam test:**
When test solution stir or shaken then kept a side for 15 min for stable. After stabled solution showed the formation of foam.

b) **Haemolysis test:**

The two tast tube was taken and added 2 ml of 18% sodium chloride. In one test tube added distilled water and second test tube 2 ml test solution. Then, added 1-2 drops of blood which are added in both the test tubes. All test tube were mixed properaly and the haemolysis observed under microscope.

c) **Test for steroidal saponins:**

The extract was hydrolyzed by dilute sulphuric acid and which are extracted using chloroform. Then, tested sterols using chloroform layer.

d) **Test for triterpenoid saponins:**

For the confirmation of triterpenoid saponins extract was hydrolyzed with dilute sulphuric acid after that extracted with chloroform. Then, separated the chloroform layer and layer was tested for triterpenoids.

**10. Tests for Tannins & phenolic compounds:**

To take 2-3 ml of test solution which is prepared from alcoholic or aqueous extracts and treated with few drops of reagents which are follows:

a) **5% ferric chloride solution:** when the solution is treated with ferric chloride solution the solution appeared deep blue-black colour.

b) **Lead acetate solution:** when the addition of lead acetate solution in test solution which appeared white ppt.

c) **Bromine water:** When added bromine water the bromine water is discoloration.

d) **Acetic acid solution:** when the acetic acid is added in test solution which appeared red colour for solution.

e) **Dilute iodine solution:** the addition of the dil. Iodine solution the solution observed red colour.

One drop NH₄OH, excess of 10% AgNO₃ solution. The solution is heated for 20 min in water bath. After boiling the solution is observed white ppt. then, dark silver mirror deposited on side of test tube.
<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Name of Chemical Test</th>
<th>CB</th>
<th>ALE</th>
<th>AQE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Test for Sterols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. Salkowaski test</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. Liebermann-Burchardt</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Test for Triterpenoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. Libermann Burchardt’s</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. Salkowski Test</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Test for glycosides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. Keller – Killaini Test</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. Baljet’s Test</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d. Libermann’s test</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td></td>
<td>Test Cardiac glycosides</td>
<td></td>
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<tr>
<td></td>
<td>Baljets test</td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td></td>
<td>Legals test</td>
<td>+</td>
<td>-</td>
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<td>4.</td>
<td>Test for carbohydrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. Benedict’s test</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. Fehling’s test</td>
<td>+</td>
<td>+</td>
<td></td>
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<td></td>
<td>c. Molisch’s test</td>
<td>+</td>
<td>-</td>
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<tr>
<td>d. Barfoeds test</td>
<td></td>
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<tr>
<td><strong>5. Test for alkaloids</strong></td>
<td></td>
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<tr>
<td>a. Mayer’s test</td>
<td></td>
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<tr>
<td>b. Dragendorff’s test</td>
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<td></td>
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<tr>
<td>c. Hager’s test</td>
<td></td>
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<tr>
<td>d. Wagner’s test</td>
<td></td>
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<tr>
<td><strong>6. Test for flavonoids</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>a. Shinoda test</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Alkaline reagent test</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. Lead acetate test</td>
<td>+</td>
<td>-</td>
<td></td>
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<td><strong>7. Test for tannins</strong></td>
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<tr>
<td>a. Gelatin test</td>
<td></td>
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<tr>
<td>b. Ferric chloride test</td>
<td></td>
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<td></td>
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<tr>
<td>c. Lead acetate test</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>d. Dil HNO₃ test</td>
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<td><strong>8. Tests for proteins</strong></td>
<td></td>
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<tr>
<td>a. Xanthoprotein test</td>
<td></td>
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<td></td>
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<tr>
<td>b. Millon’s test</td>
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<tr>
<td>c. Biuret test</td>
<td></td>
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<tr>
<td>d. Ninhydrin test</td>
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<tr>
<td><strong>9. Test for amino acids</strong></td>
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<td></td>
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<tr>
<td>Ninhydrin test</td>
<td></td>
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<tr>
<td><strong>10. Test for fats</strong></td>
<td></td>
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<tr>
<td>Solubility test</td>
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<tr>
<td>Filter paper test</td>
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<tr>
<td><strong>11. Test for Volatile oils</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Filter paper test</td>
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<td></td>
</tr>
<tr>
<td>solubility test</td>
<td></td>
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</tbody>
</table>
3.2.3: THE STUDY OF CHROMATOGRAPHIC:

For this study the chromatography plays important role. These are classified different methods such as TLC, HPTLC, HPLC, GC etc. out of that the thin Layer Chromatography is the best techniques for evaluation of phytoconstituents. TLC is a play important role for separation of constituent from plant extracts. In thin layer chromatography the extract or sample is given as a small spot at the base line on a glass / plastic / metal plate. In thin layer chromatography method used the mobile phase which is transfer through by capillary action. The mobile and stationary phases are separated the solutes due to their differential partition coefficient and adsorption. The separated component has same transfer time but different transfer distance.

The mobile phase which are prepared with the a single solvent as well as mixture of solvents. Although, a number of sorbents such as cellulose, silica gel, polyamide, chemically modified silica gel, Silica gel F-60 etc. The methods or techniques like pouring, spraying, dipping etc. which are used to prepare the Handmade plates TLC. Presently, precoated plates used for thin layer chromatography which are available in market. After selection of plates, the plates which are required to need activated. The plates should be activated at 110 °C for 15 min. Because presence of water or moisture is removes loosely bound to silica gel surface.

The Retardation Factor (Rf) is calculated according to following formula,

\[ R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}} \]
Qualitative TLC of *Caesalpinia bonducella* L.:

For the investigation of preliminary phytochemical constituents which are obtained from plant extracts like saponins, alkaloids, glycosides, carbohydrates, triterpenoids and flavones etc.

So, for the investigation of active constituents which are present in *Caesalpinia bonducella* L. ethanolic extract of leaves. This extract was subjected to thin layer chromatography to detect the various constituents present in it. The ethanolic extract showed the presence of flavonoids [16,98].

The Details of TLC are -

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Silica gel GF 254 (activated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate Thickness</td>
<td>0.4 mm</td>
</tr>
<tr>
<td>Size of Plate</td>
<td>10 x 20 cm</td>
</tr>
<tr>
<td>Activation of Temp.</td>
<td>110 °C for 15 min.</td>
</tr>
<tr>
<td>Volume of Spot</td>
<td>20 µl</td>
</tr>
<tr>
<td>Solvent system</td>
<td>Ethyl acetate: Glacial acetic acid: Formic acid: Methanol : Water (7: 0.35: 0.35: 2.15: 0.35)</td>
</tr>
</tbody>
</table>

The spots were observed in UV chamber (Light green spot).

Isolation of the Flavonoids:

Preparative TLC analysis

The alcoholic extract of drug shown significant hepatoprotective activity; hence, it was selected for the analysis of detailed phytoconstituents using TLC. The preparative Thin Layer Chromatography plates having 0.4 mm thickness layer were prepared using paste or slurry of the Silica Gel through pouring technique. The plate was kept in hot air oven at 110 °C for 15 mins. for activation of plate. The Sample of alcoholic extract was prepared in distilled water and the sample application was done by capillary tube as a spot on the base line of TLC plate.
During the application of if the sample is applied overload on the plate the tailing effect shown on plate. So, take minute quantity of test sample for applying the band or spot which was avoid the tailing effect on plates. Then, the plates were dried in air or kept for 10 min at 40-50°C in oven. Before keeping the TLC plate in chamber, require to predevelope the chamber with solvent system which are used for qualitative thin layer chromatography. The compound was separated in definite bands. The bands shown light green color just below the solvent front. The separated band was carefully removed with the help of spatula and collected each band separately in container. The scrap of band was dissolved in alcohol and filter with the help of Whatmann filter paper, and washed for two times with alcohol. The filtrate was subjected for TLC to identify flavonoids. Each spot indicated presence of single spot on TLC and Rf values of scraped is calculated and recorded [98,87,102]. They were as follows:

Scrap No.1:  Rf = 0.72

**Isolation and characterization of compound:**

From the separated bands, the substance of interest was scrapped from the plate & it was dissolved in 95% ethanol. Filtered the mixture and evaporated the filtrate to dryness. The isolated compound was subjected to qualitative HPTLC analysis. Spectra are given in annexure 1.3.

**UV spectrum**

The UV absorption spectrum of isolated compound was recorded on JASCO UV 530 spectrophotometer [102]. Spectra are given in annexure 1.4.

**IR spectrum**

The IR absorption spectrum was recorded on THERMO NICOLET IR-200 spectrophotometer using liquid sampling cell [102]. Spectra are given in annexure 1.5.

**High Performance Thin Layer Chromatography**

In this study the alcoholic and aqueous extract of leaves of *Caesalpinia bonducella* L. and isolated compounds were subjected for the investigation of active constituents which are present in extract using the chromatographic techniques such as HPTLC.

The details of high performance thin layer chromatography were as follows:
HPTLC Plate: Precoated with Aluminium plate Silica gel GF$_{254}$

Thickness of Plate: 0.2 mm

Size of Plate: 10 x 10 cms

Volume of Sample: 10 µl

Solvent system: Ethyl acetate: Glacial acetic acid: Formic acid: Ethanol: Water

(7: 0.35: 0.35: 2.15: 0.35)

Wavelength: U.V. are 200, 254 and 366 nm.

Instrument: CAMAG WINCAT

The CAMAG TLC Scanner3 and LINOMAT-V is evaluation system using the help of software of WINCAT for estimation active constituents using thin layer chromatogram which are reflectance or transmission mode by fluorescence with 254 nm.

The fingerprint of HPTLC profile of all extracts and isolated compound were taken by using computer. Rf values of various samples were evaluated by using following formula -

$$R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}$$

HPTLC profile of various extracts of leaves of *Caesalpinia bonducella* L. given in Table No. 3.12.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Tracks</th>
<th>Peak Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Max % End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>1</th>
<th>Peak</th>
<th>Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Max % End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
</tr>
</thead>
</table>


### Table 3.12: HPTLC profile of *Caesalpinia bonducella* L.

<table>
<thead>
<tr>
<th>Track</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Solvent</th>
<th>Peak Area (mm²)</th>
<th>Rf</th>
<th>%Vol</th>
<th>Dens. (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.69</td>
<td>58.0</td>
<td>0.71</td>
<td>101.5</td>
<td>89.58</td>
<td>.75</td>
<td>0.1</td>
<td>1769.3</td>
<td>92.78</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0.77</td>
<td>0.2</td>
<td>0.79</td>
<td>11.8</td>
<td>10.42</td>
<td>.81</td>
<td>0.4</td>
<td>137.6</td>
<td>7.22</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0.69</td>
<td>63.2</td>
<td>0.71</td>
<td>103.7</td>
<td>90.83</td>
<td>.74</td>
<td>1.6</td>
<td>1589.4</td>
<td>94.05</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0.78</td>
<td>0.1</td>
<td>0.80</td>
<td>10.5</td>
<td>9.17</td>
<td>.82</td>
<td>0.8</td>
<td>100.6</td>
<td>5.95</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1</td>
<td>0.69</td>
<td>1.4</td>
<td>0.72</td>
<td>40.3</td>
<td>100.00</td>
<td>.77</td>
<td>0.4</td>
<td>895.5</td>
<td>100.00</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1</td>
<td>0.69</td>
<td>1.9</td>
<td>0.75</td>
<td>62.3</td>
<td>100.00</td>
<td>.79</td>
<td>3.7</td>
<td>1726.2</td>
<td>100.00</td>
</tr>
</tbody>
</table>
A – Isolated Compound
B – ethanolic Extract
Rf Value- 0.71
Fig. No. 3.1: Comparative TLC Profile of ethanolic extracts and Isolated Compound of *Caesalpinia bonducella* L.

Fig. No.3.2: Comparative HPTLC Profile of ethanolic extracts and Isolated Compound of *Caesalpinia bonducella* L.

3.2.2: EXTRACTION *MANGIFERA INDICA* L:
For the present study, the dried leaves of *Mangifera indica* L. was prepared to coarse powder. After preparation of course powder to take about 100 gm of powder was taken for extraction. The powder was loaded in soxhlet to successive hot continuous extraction. Another batch of extraction for that used the powdered drug was macerated with chloroform-water. After the effective extraction, the solvent were concentrating on water bath or usined rotary flash evaporator and remove the water from obtained extraction. Then, recorded the weight of extract and calculated extractive value [52,40,50]. Its percentage of yield obtained was shown in Table No.3.13.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plant Name</th>
<th>Extracts</th>
<th>Nature of Extract</th>
<th>Colour of extract</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Mangifera indica</em> L</td>
<td>Alcohol</td>
<td>Semi-solid</td>
<td>Brownish</td>
<td>12.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqueous</td>
<td>Semi-solid</td>
<td>Brownish</td>
<td>8.62</td>
</tr>
</tbody>
</table>

Table No. 3.13: The percentage yield of alcoholic and aqueous extracts of *Mangifera indica* L.

The obtained all extracts were used for chemical investigation.

3.2.2: QUALITATIVE CHEMICAL INVESTIGATION:

The alcohol (95%) and aqueous extracts were subjected to qualitative chemical investigation [52,40,59,50,37].

The Preliminary qualitative tests

The Preliminary qualitative tests

The determination of phytochemicals investigation having several tests and chemical reagents. For determination of chemical test firstly prepared freshed all reagent. Then, the extracts were used for the all these tests and observations given in below are reported in Table- 3.11.

1) **Tests for Carbohydrates:**

**Test solution:**

The extracts were dissolved in water and these solution used as test solution. After that it was hydrolyzed with equal volume of 2 N hydrochloric acid are utilized for the following tests.
b) **Molish's test or General test for carbohydrates:**

To take 3 to 4 ml of water extracts then, added few drops of molish reagent and shakes well. After that add few drops of concentrated H$_2$SO$_4$ added through the sides of the test tube without shaking. The violet ring is appeared at the junction between two liquids.

b) **Fehling's test:**

To take 1 ml of Fehling-A and Fehling-B solution in a test tube and boiled for two minutes. Then, add 2 ml of tests solution which is prepared from extract. Then, solution is heated on water bath for 5 min, the precipitate appears yellow, brick red, or orange colour.

h) **Benedict's test:**

To take the 2 ml of test solution and add 2 ml of Benedict's reagent in a test tube and mix properly. Then, heated on water bath for 5 mins. The precipitated observed red, green or yellow colour. If red, green and yellow colour are appeared which shows presence of reducing sugars.

i) **The Barfoed's test:**

To take the test solution then, add equal volume of Barfoed's reagent solution. Then, the solution is heated in water bath for 1 to 2 min. After the cooling the solution appeared red precipitate.

j) **Cobalt-chloride test:**

Take 3 ml of test solution was mixed with 2 ml cobalt chloride, after that boiled and cooled. Then added FeCl$_3$ drops on NaOH solution. Solution was shown greenish blue the glucose is present and the solution was observed purplish color the Fructose is present in solution. When the lower layer appeared purplish and the upper layer appeared blue coloured indicated the presence of glucose and fructose.

k) **Test of Non-Reducing Sugarst:**

To take test solution and treated with Fehling's and Benedict's reagent test solution does not give any response.

l) **Tannic acid test to detect starch:**

Take test solution in test tube add 20% tannic acid, precipitate observed.

2) **Tests for Proteins:**

*Preparation of Test Solution:*

To take the drug extracts and dissolve in water and prepared test sample for following test.
e) **Biuret test (General test):**

To take the 3 ml test solution then add 4% sodium hydroxide solution and few drops of 1% copper sulphate solution, the solution appears purple or violet colour. If shows the purple and violet colour of solution the presence proteins in drug.

f) **Million’s test (for proteins):**

For the detection of the proteins to take the 3 ml of test solution mix with the 5 ml of Million’s reagent, which appeared white precipitate. When the Precipitate get warmed it turns white to brick red colour.

g) **Test of Xanthoprotein:**

To take the 3ml of Test Solution treated with 1 ml concentrated sulphuric acid, the solution shows white precipitate.

h) **Test of Precipitation:**

The test solution is treated with following reagent shows white colloidal ppt. such as alcohol, 5% HgCl₂ solution, 5% CUSO₄ solution, 5% lead acetate and 5% ammonium sulphate.

3) **Tests for Steroids:**

**Preparation of extract solution for test:**

For the detection of steroids, the saponification of extract is done by refluxing the extract with potassium hydroxide. Then add water to the saponified the extract for dilution of the extracts and the unsaponified matter was extracted with the diethyl ether. Then, the extract was evaporate on water bath and the remaining residue was used for the following test.

d) **Reaction of Salkowski:**

To take 2 ml of extract mix with 2 ml chloroform and treated with 2 ml conc. H₂SO₄. Then, the solutions are shaking properly. If the chloroform layer shows red and acid layer observed fluorescence colour.

e) **Libermann-Burchard test:**

To take 2 ml of extract are mixed with chloroform solvent. Then, added 1-2 ml of acetic anhydride and two drops of H₂SO₄ transferred through the side of test tube then observed test tube layer first layer shown red, second layer blue and finally green colour.

f) **Libermann's test:**
To take the 3 ml extract is treated with 3 ml of acetic anhydride. Then, test tube is heated on flame and cooled. After cooling added few drops conc. H$_2$SO$_4$ the solution appears blue colour.

4) **Tests of Amino Acids:**

d) **Test of Ninhydrin (General test):**

To take 3 m of test solution of crude drug and add 2-3 drops of 5% Ninhydrin reagent then, heated on water bath for 10 min. If the solution appears purple or violet colour the there is presence of amino acid in crude extract drug.

e) **Test for Tyrosine:**

Take 3 ml of test solution and added 3 drops Million's reagent then heat. After heating the solution observed dark red colour.

f) **Test for tryptophan:**

For this test take 3 ml test solution then, added few drops of glyoxalic acid and conc. H$_2$SO$_4$ te solution shows reddish violet ring at junction of the two layers.

5. **Tests for Glycosides:**

*The Preparation of test solution:*

The test solutions were prepared from the crude drug extract is dissolved in alcohol solution. The prepared test solution are used for following test.

**Detection tests for Cardiac Glycosides:**

f) **Baljet's test:**

For the detection of cardiac glycoside take test solution added with sodium picrate. Then, solution is appeared yellow colour change to orange colour.

g) **Bromine water test:**

To take a test solution dissolved in bromine water shown yellow precipitate

h) **Legal's test (Cardenoloids):**

To take test solution which are prepared from water or alcohol. Then, added 1 ml of pyridine and 1 ml of sodium nitroprusside the solution observed for pink to red colour.

i) **Test for deoxysugars (Killar Krillani test):**

To take 2 ml of extract (test solution) and add 1 ml of glacial acetic acid and one drop of 5% ferric chloride and concentrate sulphuric acid when addition the sulphuric acid the
solution appeared brown colour at the junction of the two liquid and bluish green colour appeared at upper layers of solution.

j) Libermann's test (For bufadenolids):

To take 3 ml of extract solution mixed with the 3 ml acetic anhydride and heated on water bath and cooled. After cooling the solution added few drops concentrated H$_2$SO$_4$ then, the solutions are appeared blue colour.

Test for anthraquinone glycosides:

a) Modified Borntrager’s test:

The C-glycosides of anthraquinones require more specific conditions for doing the hydrolysis. During the hydrolysis of the produced drug take the 5 ml of dilute hydrochloric acid and 5 ml of 5% solution of ferric chloride. For hydrolysis of extract have procedure is described the following test i.e Borntrager’s test.

b) Borntrager’s test:

For the borntrager’s test take powdered drug and boiled with 5 ml of the 10 % sulphuric acid for 5 min. After boiling filtered the solution immediately. Then, cooled and the obtained filtrate are mix with benzene equal volume as filtrate. The benzene layer was separate and the separated layer was takes in tared dish and adds 10 % of ammonia solution. The solution is keeping a side for separating. The ammonical layer appeared pink colour due to the presence of anthraquinones.

Saponin Glycosides Tests:

c) Test of Foam:

To take crude drug extract powder and mixed with water then, continuously shake. The solution appeared foam form.

d) Foaming index:

To take accurately weight 1 gm of powdered crude drug and transfer in 500 ml of flask which contains 100 ml of boiling water. The temperature is maintained and the drug boils contineously for 30 minutes. When the solution is cool then the solution was filter it into a volumetric flask and make a volume addition of water up to 100 ml.

Take the above solution into ten graduated test-tubes and each test tube containing 1ml, 2 ml, 3 ml up to 10 ml respectively. Then, adjusted the volume up to 10 ml in each test tube. The
test tube are cover and shake for 15 second. After shaking the test tubes are keep a side for 15 minitus and measure the foam height.

The results assed as follows:

i. The each and every test tube having the foaming index is less than 100 when the foam height is one cm.

ii. If the height of the foam is measure 1 cm of any one test tube of the plants material. Then, there is required for the determination of the foaming index. To prepared the dilution to observed better or accurate result.

iii. When the foam height observed above one centimeter in each and every test tube. Then, there is need to determination of the 1000 foaming index. Take one or more tests by using the different decoction dilutions and fid out the result.

\[
\text{Foaming index} = 100 \frac{1}{a}
\]

Where a = volume of the decoction in 100 ml. which are preparing from the dilutions in the test tube where height of foaming is observed.

**Haemolytic test:**

To take test solutions add 1-2 drops of the blood and mounted on a glass slide. The slide is observed are Haemolytic zone.

**Tests for Coumarin Glycosides:**

Test solution having pH 7 or alkaline, shown blue or green fluorescence.

6. **Tests for Alkaloids:**

e) **Dragendorff's test:**

Take test solution 2-3 ml which is prepared from crude drug and filter. Then, take a filtrate and added few drops Dragendorff’s reagent when added the reagent the solution appeared brown precipitate.

f) **Mayer's test:**

To take 2-3 ml of filtrate or test solution in the test tube and add few drops Mayer's reagent. when addition of Mayer’s reagent the solution is observed precipitate.

g) **Hager's test:**

To take 2-3 ml test solution filtrates and treated with Hagers reagent. when the addition of Hager’s reagent the solution are observed yellow precipitate.
h) **Wagner's test:**
   For the detection of alkaloids to take 2-3 ml filtrate and test solution is treated with Wagner's reagent when the addition of reagent the solution are appeared reddish brown precipitate.

7. **Tests for Flavonoids:**
   The structurally all flavonoids are derived from the origin substance are known as flavone. The flavones are found either in the free form or bound to sugars like as a glycosides. To this reason, when determination of flavonoids it is generally better to estimated the flavonoids of plant extracts.

**Preparation of test solution:**
iv. Test solution of extract is prepaired by addition of a small quantity of extract and the equal volume of hydrochloric acidr 30-40 min. during boiling the temp. should not exceed 100°C.

v. The prepared extract was filtered with the help of filter paper. Take a filtered extracte and treated with ethyl acetate.

vi. Then, the extract was concentrated on water bath upto drtness. Then, dried extracts was used for the following test -

d) **Shinoda test:**
   To take test solution (extract) in test tube then add 5 ml 95 % ethanol in test tube and few drops concentrated hydrochloric acid and finally add 0.5 g magnesium turnings. When addition of magnesium turning appeare pink colour to solution.

e) **Lead acetate test:**
   To take small quantity of (extractr) or test solution in test tube treated with lead acetate solution. When the addition of lead acetate solution the solution are appeared yellow coloured ppt. If addition of large amount of sodium hydroxide the solution appeared yellow coloured, the yellow coloration was decolourised when addition of any type of acid.

f) **Ferric chloride test:**
   To take a test solution and treated with few drops of ferric chloride solution. When addition of FeCl₃ in test solution, the solution shown green colour.

8. **Test for Vitamins:**
   d) **For Vitamín A:**
Take 10-15 units quantity dissolved in 1 ml chloroform then added 5ml of antimony trichloride solution, immediately the solution appeared blue colour.

e) **Test for vitamin C (Ascorbic acid):**

To take 1 ml of test sample dissolved in 5 ml of water mixed with 2 ml dil. NaOH solution. Then, added 1 drop of solution of sodium nitroprusside. After that added 0.6 ml of hydrochloric acid dropwise and shake the solution color changed yellow to blue.

f) **Test for Vitamin D:**

For the test of vitamin take about 100 units of test sample dissolved a equal volume of chloroform and then, added 10 ml of antimony tricloride solution, the solution appeared pinkish-red colour which indicated that vitamin D is present.

**9. Tests for Saponins:**

**The preparation of test sample:**
Take extracts and extracts was dissolved in water these solution used for folloing tests.

e) **Foam test:**

When test solution stir or shaken then kept a side for 15 min for stable. After stabled solution showed the formation of foam.

f) **Haemolysis test:**

The two tast tube was taken and added 2 ml of 18% sodium chloride. In one test tube added distilled water and second test tube 2 ml test solution. Then, added 1-2 drops of blood which are added in both the test tubes. All test tube were mixed properaly and the haemolysis observed under microscope.

g) **Test for steroidal saponins:**

The extract was hydrolyzed by dilute sulphuric acid and which are extracted using chloroform. Then, tested sterols using chloroform layer.

h) **Test for triterpenoid saponins:**

For the confirmation of triterpenoid saponins extract was hydrolyzed with dilute sulphuric acid afer that extracted with chloroform. Then, sepreted the chloroform layer and layer was tested for triterpenoids.

**10. Tests for Tannins & phenolic compounds:**

To take 2-3 ml of test solution which is prepared from alcoholic or aqueous extracts and treated with few drops of reagents which are follows:
f) **5% ferric chloride solution:** when the solution is treated with ferric chloride solution the solution appeared deep blue-black colour.

g) **Lead acetate solution:** when the addition of lead acetate solution in test solution which appeared white ppt.

h) **Bromine water:** When added bromine water the bromine water is discoloration.

i) **Acetic acid solution:** when the acetic acid is added in test solution which appeared red colour for solution.

j) **Dilute iodine solution:** The addition of the dil. Iodine solution the solution observed red colour.

One drop NH₄OH, excess of 10% AgNO₃ solution. The solution is heated for 20 min in water bath. After boiling the solution is observed white ppt. then, dark silver mirror deposited on side of test tube.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Name of the Test</th>
<th>MI ALE</th>
<th>AQE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Test for sterols</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liebermann-Burchardt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Salkowaski test</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Test for Triterpenoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Libermann Burchardt’s</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Salkowaski Test</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Test for glycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Keller – Killaini Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Baljet’s Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Libermann’s test</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>Cardiac glycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baljets test</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Legals test</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test</th>
<th>Saponins glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Foam test</td>
<td>+</td>
</tr>
</tbody>
</table>

4. **Test for carbohydrates**

<table>
<thead>
<tr>
<th>Test</th>
<th>Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Benedict’s test</td>
<td>+</td>
</tr>
<tr>
<td>Fehling’s test</td>
<td>+</td>
</tr>
<tr>
<td>Molisch’s test</td>
<td>+</td>
</tr>
<tr>
<td>Barfoeds test</td>
<td>-</td>
</tr>
</tbody>
</table>

5. **Test for alkaloids**

<table>
<thead>
<tr>
<th>Test</th>
<th>Alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Dragendroff’s test</td>
<td>+</td>
</tr>
<tr>
<td>Mayer’s test</td>
<td>+</td>
</tr>
<tr>
<td>Hager’s test</td>
<td>-</td>
</tr>
<tr>
<td>Wagner’s test</td>
<td>-</td>
</tr>
</tbody>
</table>

6. **Test for flavonoids**

<table>
<thead>
<tr>
<th>Test</th>
<th>Flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Shinoda test</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline reagent test</td>
<td>+</td>
</tr>
<tr>
<td>Lead acetate test</td>
<td>-</td>
</tr>
</tbody>
</table>

7. **Test for tannins**

<table>
<thead>
<tr>
<th>Test</th>
<th>Tannins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin test</td>
<td>-</td>
</tr>
<tr>
<td>Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td>Lead acetate test</td>
<td>+</td>
</tr>
<tr>
<td>Dil HNO₃ test</td>
<td>-</td>
</tr>
</tbody>
</table>

8. **Tests for proteins**

<table>
<thead>
<tr>
<th>Test</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthoprotein test</td>
<td>-</td>
</tr>
<tr>
<td>Millon’s test</td>
<td>-</td>
</tr>
<tr>
<td>Biuret test</td>
<td>-</td>
</tr>
<tr>
<td>Ninhydrin test</td>
<td>-</td>
</tr>
</tbody>
</table>

9. **Test for amino acids**
### Table No.3.14: List of Phytochemical Investigation various extracts of *Mangifera indica* L.(MI).

<table>
<thead>
<tr>
<th>Test Type</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ninhydrin test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10. Test for fats</td>
<td>Solubility test</td>
<td>-</td>
</tr>
<tr>
<td>Solubility test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Filter paper test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11. Test for Volatile oils</td>
<td>Filter paper test</td>
<td>-</td>
</tr>
<tr>
<td>solubility test</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 3.2.2: THE STUDY OF CHROMATOGRAPHIC:

For this study the chromatography plays important role. These are classified different methods such as TLC, HPTLC, HPLC, GC etc. out of that the thin Layer Chromatography is the best techniques for evaluation of phytoconstituents. TLC is a play important role for separation of constituent from plant extracts. In thin layer chromatography the extract or sample is given as a small spot at the base line on a glass / plastic / metal plate. In thin layer chromatography method used the mobile phase which is transfer through by capillary action. The mobile and stationary phases are separated the solutes due to the their differential partition coefficient and adsorption. The separated component has same transfer time but different transfer distance.

The mobile phase which are prepared with the a single solvent as well as mixture of solvents. Although, a number of sorbents such as cellulose, silica gel, polyamide, chemically modified silica gel, Silica gel F-60 etc. The methods or techniques like pouring, spraying,
dipping etc. which are used to prepare the Handmade plates TLC. Presently, precoated plates used for thin layer chromatography which are available in market. After selection of plates, the plates which are required to need activated. The plates should be activated at 110$^\circ$C for 15 min. Because presence of water or moisture is removes loosely bound to silica gel surface.

The Retardation Factor (Rf) is calculated according to following formula,

\[ R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}} \]

**Qualitative TLC of Mangifera indica L.:**

For the investigation of preliminary phytochemical constituents which are obtained from plant extracts like saponins, alkaloids, glycosides, carbohydrates, triterpenoids and flavones etc.

So, for the investigation of active constituents which are present in Mangifera indica L. ethanolic extract of leaves. This extract was subjected to thin layer chromatography to detect the various constituents present in it. The ethanolic extract showed the presence of flavonoids [16,98].

**The Details of TLC are –**

<table>
<thead>
<tr>
<th>Details</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorbent of TLC</td>
<td>Silica gel GF $^{254}$</td>
</tr>
<tr>
<td>Thickness of TLC plate</td>
<td>0.4 mm.</td>
</tr>
<tr>
<td>Size of TLC Plate</td>
<td>10 x 20 cm.</td>
</tr>
<tr>
<td>TLC Activation Temp.</td>
<td>110$^\circ$C for 1 hr</td>
</tr>
<tr>
<td>Volume of spot on TLC Plate</td>
<td>20 $\mu$l</td>
</tr>
<tr>
<td>Solvent system</td>
<td>Ethyl acetate : glacial acetic acid: formic acid: water</td>
</tr>
<tr>
<td></td>
<td>100 : 11 : 11 : 26</td>
</tr>
</tbody>
</table>

The under UV chamber (Light green spot) spots were observed.

**Isolation of the Flavonoids:**
Preparative TLC analysis

The alcoholic extract of drug shown significant hepatoprotective activity; hence, it was selected for the analysis of detailed phytoconstituents using TLC. The preparative Thin Layer Chromatography plates having 0.4 mm thickness layer were prepared using paste or slurry of the Silica Gel through pouring technique. The plate was kept in hot air oven at 110°C for 15 mins. for activation of plate. The Sample of alcoholic extract was prepared in distilled water and the sample application was done by cappilaty tube as a spot on the base line of TLC plate. During the application of if the sample is applied overload on the plate the tailing effect shown on plate. So, take minute quantity of test sample for applying the band or spot which was aovid the tailing effect on plates. Then, the plates were dried in air or kept for 10 min at 40-50°C in oven. Before keeping the TLC plate in chamber, require to predevelope the chamber with solvent system which are used for qualitative thin layer chromatography. The compound was separated in definite bands. The bands shown light green color just below the solvent front. The separated band was carefully removed with the help of spatula and collected each band separately in container. The scrap of band was dissolved in alcohol and filter with the help of Whatmann filter paper, and washed for two times with alcohol. The filtrate was subjected for TLC to identify flavonoids. Each spot indicated presence of single spot on TLC and Rf values of scraped is calculated and recorded [98,87,102]. They were as follows:

Scrap No.1:  Rf = 0.40

Isolation and characterization of compound:

From the separated bands, the substance of interest was scrapped from the plate & it was dissolved in 95% ethanol. Filtered the mixture and evaporated the filtrate to dryness. The isolated compound was subjected to qualitative HPTLC analysis. Spectra are given in annexure 1.6.

UV spectrum

The UV absorption spectrum of isolated compound was recorded on JASCO UV 530 spectrophotometer [102]. Spectra are given in annexure 1.7.

IR spectrum

The IR absorption spectrum was recorded on THERMO NICOLET IR-200 spectrophotometer using liquid sampling cell [102]. Spectra are given in annexure 1.8.

High Performance Thin Layer Chromatography (HPTLC)
Ethanolic and aqueous extract of leaves of *Mangifera indica* Linn. and isolated compounds were subjected to HPTLC. The details of high performance thin layer chromatography were as follows:

- **Plate**: Precoated plate with Silica Gel GF60\textsubscript{254}
- **Plate Thickness**: 0.2 mm
- **Plate size**: 10 X 10 cm
- **Sample application**: 10 µl
- **Solvent system**: Ethyl acetate : glacial acetic acid : formic acid : water
  
  \[
  \begin{align*}
  &100 : 11 : 11 : 26
  \end{align*}
  \]
- **Detection**: 254 nm
- **Instrument**: CAMAG TLC Scanner3 and LINOMAT-V

The CAMAG TLC Scanner3 and LINOMAT-V is evaluation system using the help of software of WINCAT for estimation active constituents using thin layer chromatogram which are reflectance or transmission mode by fluorescence with 254 nm.

The fingerprint of HPTLC profile of all extracts and isolated compound were taken by using computer. Rf values of various samples were evaluated by using the following formula -

\[
R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}
\]

HPTLC profile of various extracts of leaves of *Mangifera indica* Linn. given in table no. 3.15.
<table>
<thead>
<tr>
<th>Track</th>
<th>Peak</th>
<th>Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Max %</th>
<th>End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.27</td>
<td>0.6</td>
<td>0.34</td>
<td>532.4</td>
<td>100.00</td>
<td>.40</td>
<td>4.8</td>
<td>16994.1</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.27</td>
<td>3.9</td>
<td>0.34</td>
<td>602.7</td>
<td>100.00</td>
<td>.40</td>
<td>3.5</td>
<td>20509.9</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.27</td>
<td>5.9</td>
<td>0.33</td>
<td>631.3</td>
<td>100.00</td>
<td>.39</td>
<td>0.4</td>
<td>24737.2</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.27</td>
<td>10.6</td>
<td>0.33</td>
<td>634.2</td>
<td>100.00</td>
<td>.39</td>
<td>0.2</td>
<td>26390.3</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.27</td>
<td>5.1</td>
<td>0.33</td>
<td>478.2</td>
<td>100.00</td>
<td>.39</td>
<td>0.0</td>
<td>6918.7</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.28</td>
<td>12.2</td>
<td>0.33</td>
<td>271.9</td>
<td>100.00</td>
<td>.38</td>
<td>0.7</td>
<td>7157.1</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>0.28</td>
<td>0.2</td>
<td>0.33</td>
<td>107.4</td>
<td>100.00</td>
<td>.39</td>
<td>0.2</td>
<td>2857.7</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.28</td>
<td>0.4</td>
<td>0.33</td>
<td>131.0</td>
<td>100.00</td>
<td>.37</td>
<td>0.4</td>
<td>3102.0</td>
<td>100.00</td>
<td>Isolated comp.</td>
</tr>
</tbody>
</table>

Table 3.15: HPTLC profile of the extracts of the *Mangifera indica* Linn.
A – alcoholic Extract; B – Isolated Compound;

Rf Value- 0.40

Fig 3.3: Comparative TLC Profile of ALE *Mangifera indica* Linn. and Isolated Compound
**3.2.3: EXTRACTION RICINUS COMMUNIS L.:**

In this study, the dried leaves of *Ricinus communis* L. are used. Then, prepared to coarse powder from leaves of plant. After preparation of powder take about 100 gm of powder was taken for extraction. The course powder was loaded in soxhlet to successive hot continuous extraction. Side by side started the batch extraction in this extraction the powdered drug was macerated with water-chloroform. After completion of extraction, the solvent were concentrating on water bath or using rotary flash evaporator and remove the water from obtained extraction. Then, recorded the weight of extract and calculated extractive value [52,40,50]. Its percentage of yield obtained was shown in Table No.3.16.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plant Name</th>
<th>Extracts</th>
<th>Nature of Extract</th>
<th>Colour of extract</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alcohol</td>
<td>Semi-solid</td>
<td>Greenish-black</td>
<td>28.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ricinus communis</em> L.</td>
<td>Aqueous</td>
<td>Semi-solid</td>
<td>Brown</td>
<td>54.11</td>
</tr>
<tr>
<td>---</td>
<td>----------------------</td>
<td>---------</td>
<td>------------</td>
<td>-------</td>
<td>-------</td>
</tr>
</tbody>
</table>

Table 3.16: The Percentage yield of *Ricinus communis* L. extracts.

The obtained all extracts were used for chemical investigation.

**3.2.3. QUALITATIVE CHEMICAL INVESTIGATION:**

The alcohol (95%) and aqueous extracts were subjected to qualitative chemical investigation [52, 40, 59, 50, 37, 24].

The Preliminary qualitative tests

The determination of phytochemicals investigation having several tests and chemical reagents. For determination of chemical test firstly prepared freshed all reagent. Then, the extracts were used for the all these tests and observations given in below are reported in Table- 3.11.

1) **Tests for Carbohydrates:**

   **Test solution:**

   The extracts were dissolved in water and these solution used as test solution. After that it was hydrolyzed with equal volume of 2 N hydrochloric acid are utilized for the following tests.

   c) **Molish's test or General test for carbohydrates:**

      To take 3 to 4 ml of water extracts then, added few drops of molish reagent and shakes well. After that add few drops of concentrated H₂SO₄ added through the sides of the test tube without shaking. The violet ring is appeared at the junction between two liquids.

   b) **Fehling's test :**

      To take 1 ml of Fehling-A and Fehling-B solution in a test tube and boiled for two minutes. Then, add 2 ml of tests solution which is prepared from extract. Then, solution is heated on water bath for 5 min, the precipitate appears yellow, brick red, or orange colour.

   m) **Benedict's test:**

      To take the 2 ml of test solution and add 2 ml of Benedict's reagent in a test tube and mix properly. Then, heated on water bath for 5 mins. The precipitated observed red, green or yellow colour. If red, green and yellow colour are appeared which shows presence of reducing sugars.

   n) **The Barfoed's test:**
To take the test solution then, add equal volume of Barfoed's reagent solution. Then, the solution is heated in water bath for 1 to 2 min. After the cooling the solution appeared red precipitate.

o) Cobalt-chloride test:

Take 3 ml of test solution was mixed with 2 ml cobalt chloride, after that boiled and cooled. Then added FeCl$_3$ drops on NaOH solution. Solution was shown greenish blue the glucose is present and the solution was observed purplish color the Fructose is present in solution. When the lower layer appeared purplish and the upper layer appeared blue coloured indicated the presence of glucose and fructose.

p) Test of Non-Reducing Sugarst:

To take test solution and treated with Fehling's and Benedict's reagent test solution does not give any response.

q) Tannic acid test to detect starch:

Take test solution in test tube add 20% tannic acid, precipitate observed.

2) Tests for Proteins:

Preparation of Test Solution:

To take the drug extracts and dissolve in water and prepared test sample for following test.

i) Biuret test (General test):

To take the 3 ml test solution then add 4% sodium hydroxide solution and few drops of 1% copper sulphate solution, the solution appears purple or violet colour. If shows the purple and violet colour of solution the presence proteins in drug.

j) Million's test (for proteins):

For the detection of the of proteins to take the 3 ml of test solution mix with the 5 ml of Million’s reagent, which appeared white precipitate. When the Precipitate get warmed it turns white to brick red colour.

k) Test of Xanthoprotein:

To take the 3ml of Test Solution treated with 1 ml concentrated sulphuric acid, the solution shows white precipitate.

l) Test of Precipitation:
The test solution is treated with following reagent shows white colloidal ppt. such as alcohol, 5% HgCl₂ solution, 5% CUSO₄ solution, 5% lead acetate and 5% ammonium sulphate.

3) Tests for Steroids:

*Preparation of extract solution for test:*

For the detection of steroids, the saponification of extract is done by refluxing the extract with potassium hydroxide. Then add water to the saponified the extract for dilution of the extracts and the unsaponified matter was extracted with the diethyl ether. Then, the extract was evaporate on water bath and the remaining residue was used for the following test.

g) **Reaction of Salkowski:**

To take 2 ml of extract mix with 2 ml chloroform and treated with 2 ml conc. H₂SO₄. Then, the solutions are shaking properly. If the chloroform layer shows red and acid layer observed fluorescence colour.

h) **Libermann-Burchard test:**

To take 2 ml of extract are mixed with chloroform solvent. Then, added 1-2 ml of acetic anhydride and two drops of H₂SO₄ transferred through the side of test tube then observed test tube layer first layer shown red, second layer blue and finally green colour.

i) **Libermann's test:**

To take the 3 ml extract is treated with 3 ml of acetic anhydride. Then, test tube is heated on flame and cooled. After cooling added few drops conc. H₂SO₄ the solution appears blue colour.

4) Tests of Amino Acids:

g) **Test of Ninhydrin (General test):**

To take 3 m of test solution of crude drug and add 2-3 drops of 5% Ninhydrin reagent then, heated on water bath for 10 min. If the solution appears purple or violet colour the there is presence of amino acid in crude extract drug.

h) **Test for Tyrosine:**

Take 3 ml of test solution and added 3 drops Million's reagent then heat. After heating the solution observed dark red colour.

i) **Test for tryptophan:**

For this test take 3 ml test solution then, added few drops of glycoxalic acid and conc. H₂SO₄ te solution shows reddish violet ring at junction of the two layers.
5. Tests for Glycosides:

The Preparation of test solution:

The test solutions were prepared from the crude drug extract is dissolved in alcohol solution. The prepared test solution are used for following test.

Detection tests for Cardiac Glycosides:

k) Baljet's test:

For the detection of cardiac glycoside take test solution added with sodium picrate. Then, solution is appeared yellow colour change to orange colour.

l) Bromine water test:

To take a test solution dissolved in bromine water shown yellow precipitate

m) Legal's test (Cardenoloids):

To take test solution which are prepared from water or alcohol. Then, added 1 ml of pyridine and 1 ml of sodium nitroprusside the solution observed for pink to red colour.

n) Test for deoxysugars (Killar Krillani test):

To take 2 ml of extract (test solution) and add 1 ml of glacial acetic acid and one drop of 5% ferric chloride and concentrate sulphuric acid when addition the sulphuric acid the solution appeared brown colour at the junction of the two liquid and bluish green colour appeared at upper layers of solution.

o) Libermann's test (For bufadenolids):

To take 3 ml of extract solution mixed with the 3 ml acetic anhydride and heated on water bath and cooled. After cooling the solution added few drops concentrated H$_2$SO$_4$ then, the solutions are appeared blue colour.

Test for anthraquinone glycosides:

a) Modified Borntrager’s test:

The C-glycosides of anthraquinones require more specific conditions for doing the hydrolysis. During the hydrolysis of the produced drug take the 5 ml of dilute hydrochloric acid and 5 ml of 5% solution of ferric chloride. For hydrolysis of extract have procedure is described the following test i.e Borntrager’s test.

b) Borntrager’s test:
For the borntrager’s test take powdered drug and boiled with 5 ml of the 10 % sulphuric acid for 5 min. After boiling filtered the solution immediately. Then, cooled and the obtained filtrate are mix with benzene equal volume as filtrate. The benzene layer was separate and the separated layer was takes in tared dish and adds 10 % of ammonia solution. The solution is keeping a side for separating. The ammonical layer appeared pink colour due to the presence of anthraquinones.

**Saponin Glycosides Tests:**

e) **Test of Foam:**

To take crude drug extract powder and mixed with water then, continuously shake. The solution appeared foam form.

f) **Foaming index:**

To take accurately weight 1 gm of powdered crude drug and transfer in 500 ml of flask which contains 100 ml of boiling water. The temperature is maintained and the drug boils contineusly for 30 minutes. When the solution is cool then the solution was filter it into a volumetric flask and make a volume addition of water up to 100 ml.

Take the above solution into ten graduated test-tubes and each test tube containing 1ml, 2 ml, 3 ml up to 10 ml respectively. Then, adjusted the volume up to 10 ml in each test tube. The test tube are cover and shake for 15 second. After shaking the test tubes are keep a side for 15 minitus and measure the foam height.

The results assed as follows:

i. The each and every test tube having the foaming index is less than 100 when the foam height is one cm.

ii. If the height of the foam is measure 1 cm of any one test tube of the plants material. Then, there is required for the determination of the foaming index. To prepared the dilution to observed better or accurate result.

iii. When the foam height observed above one centimeter in each and every test tube. Then, there is need to determination of the 1000 foaming index. Take one or more tests by using the different decoction dilutions and fid out the result.
Foaming index = \frac{100}{a}

Where \( a \) = volume of the decoction in 100 ml. which are preparing from the dilutions in the test tube where height of foaming is observed.

**Haemolytic test:**
To take test solutions add 1-2 drops of the blood and mounted on a glass slide. The slide is observed are Haemolytic zone.

**Tests for Coumarin Glycosides:**
Test solution having pH 7 or alkaline, shown blue or green fluorescence.

**6. Tests for Alkaloids:**

i) **Dragendorff's test:**
Take test solution 2-3 ml which is prepared from crude drug and filter. Then, take a filtrate and added few drops Dragendorff's reagent when added the reagent the solution appeared brown precipitate.

j) **Mayer's test:**
To take 2-3 ml of filtrate or test solution in the test tube and add few drops Mayer's reagent. when addition of Mayer’s reagent the solution is observed precipitate.

k) **Hager's test:**
To take 2-3 ml test solution filtrates and treated with Hagers reagent. when the addition of Hager’s reagent the solution are observed yellow precipitate.

l) **Wagner's test:**
For the detection of alkaloids to take 2-3 ml filtrate and test solution is treated with Wagner's reagent when the addition of reagent the solution are appeaed reddish brown precipitate.

**7. Tests for Flavonoids:**
The structurally all flavonoids are derived from the origin substance are known as flavone. The flavones are found either in the free form or bound to sugars like as a glycosides. To this reason, when determination of flavonoids it is generally better to estimated the flavonoids of plant extracts.

**Preparation of test solution:**
vii. Test solution of extract is prepared by addition of a small quantity of extract and the equal volume of hydrochloric acid 30-40 min. during boiling the temp. should not exceed 100°C.

viii. The prepared extract was filtered with the help of filter paper. Take a filtered extract and treated with ethyl acetate.

ix. Then, the extract was concentrated on water bath up to dryness. Then, dried extracts was used for the following test –

a) Shinoda test:

To take test solution (extract) in test tube then add 5 ml 95% ethanol in test tube and few drops concentrated hydrochloric acid and finally add 0.5 g magnesium turnings. When addition of magnesium turning appears pink colour to solution.

b) Lead acetate test:

To take small quantity of (extract) or test solution in test tube treated with lead acetate solution. When the addition of lead acetate solution the solution are appeared yellow coloured ppt. If addition of large amount of sodium hydroxide the solution appeared yellow coloured, the yellow coloration was decolourised when addition of any type of acid.

c) Ferric chloride test:

To take a test solution and treated with few drops of ferric chloride solution. When addition of FeCl₃ in test solution, the solution shown green colour.

8. Test for Vitamins:

a) For Vitamin A:

Take 10-15 units quantity dissolved in 1 ml chloroform then added 5ml of antimony trichloride solution, immediately the solution appeared blue colour.

b) Test for vitamin C (Ascorbic acid):

To take 1 ml of test sample dissolved in 5 ml of water mixed with 2 ml dil. NaOH solution. Then, added 1 drop of solution of sodium nitroprusside. After that added 0.6 ml of hydrochloric acid dropwise and shake the solution color changed yellow to blue.

c) Test for Vitamin D:
For the test of vitamin take about 100 units of test sample dissolved a equal volume of chloroform and then, added 10 ml of antimony tricohloride solution, the solution appeared pinkish-red colour which indicated that vitamin D is present.

9. **Tests for Saponins:**

**The preparation of test sample:**

Take extracts and extracts was dissolved in water these solution used for folloing tests.

i) **Foam test:**

When test solution stir or shaken then kept a side for 15 min for stable. After stabled solution showed the formation of foam.

j) **Haemolysis test:**

The two tast tube was taken and added 2 ml of 18% sodium chloride. In one test tube added distilled water and second test tube 2 ml test solution. Then, added 1-2 drops of blood which are added in both the test tubes. All test tube were mixed properaly and the haemolysis observed under microscope.

k) **Test for steroidal saponins:**

The extract was hydrolyzed by dilute sulphuric acid and which are extracted using chloroform. Then, tested sterols using chloroform layer.

l) **Test for triterpenoid saponins:**

For the confirmation of triterpenoid saponins extract was hydrolyzed with dilute sulphuric acid afer that extracted with chloroform. Then, sepreted the chloroform layer and layer was tested for triterpenoids.

10. **Tests for Tannins & phenolic compounds:**

To take 2-3 ml of test solution which is prepared from alcoholic or aqueous extracts and treated with few drops of reagents which are follows :

k) **5% ferric chloride solution:** when the solutionis treated with ferric chloride solution the solution appeared deep blue-black colour.

l) **Lead acetate solution:** when the addition of lead acetate solution in test solution which appeared white ppt.

m) **Bromine water:** When added bromine water the bromine water is discoloration.
n) **Acetic acid solution:** when the acetic acid is added in test solution which appeared red colour for solution.

o) **Dilute iodine solution:** the addition of the dil. Iodine solution the solution observed red colour.

One drop NH₄OH, excess of 10% AgNO₃ solution. The solution is heated for 20 min in water bath. After boiling the solution is observed white ppt. then, dark silver mirror deposited on side of test tube.

<table>
<thead>
<tr>
<th>Sr.N o</th>
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<th>RC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>1.</td>
<td><strong>Test for sterols</strong></td>
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<tr>
<td>a. Liebermann-Burchard</td>
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<tr>
<td>b. Salkowaski test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td><strong>Test for Triterpenoids</strong></td>
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</tr>
<tr>
<td>a. Libermann Burchardt’s</td>
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<td>-</td>
</tr>
<tr>
<td>b. Salkowaski Test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td><strong>Test for glycosides</strong></td>
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</tr>
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</tr>
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<td>b. Baljet’s Test</td>
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<td>d. Libermann’s test</td>
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<td>-</td>
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<tr>
<td><strong>Test Cardiac glycosides</strong></td>
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</tr>
<tr>
<td>Baljets test</td>
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<td><strong>Test saponins glycosides</strong></td>
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<td>4.</td>
<td><strong>Test for carbohydrates</strong></td>
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<td>b. Benedict’s test</td>
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<td>c. Molisch’s test</td>
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<tr>
<td>d.Barfoeds test</td>
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<td><strong>5. Test for alkaloids</strong></td>
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<td>b. Hager’s test</td>
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<td>c. Dragendorff’s test</td>
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<tr>
<td>d. Wagner’s test</td>
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<td><strong>6. Test for flavonoids</strong></td>
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<td>a. Lead acetate test</td>
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<td>b. Alkaline reagent test</td>
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<td>+</td>
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<tr>
<td>c. Shinoda test</td>
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<td><strong>7. Test for tannins</strong></td>
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<td>b. Ferric chloride test</td>
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<td>+</td>
</tr>
<tr>
<td>c. Lead acetate test</td>
<td>-</td>
<td>+</td>
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<td>d. Dil HNO₃ test</td>
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<td><strong>8. Tests for proteins</strong></td>
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<td>a. Millon’s test</td>
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<td>b. Biuret test</td>
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<td>-</td>
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<td>c. Xanthoprotein test</td>
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<td>-</td>
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<td>d. Ninhydrin test</td>
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<td>-</td>
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<td><strong>9. Test for amino acids</strong></td>
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<td>Ninhydrin test</td>
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<td><strong>10. Test for fats</strong></td>
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<td>Filter paper test</td>
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<td><strong>11. Test for Volatile oils</strong></td>
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<tr>
<td>solubility test</td>
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</table>
3.2.3: THE STUDY OF CHROMATOGRAPHIC:

For this study the chromatography plays important role. These are classified different methods such as TLC, HPTLC, HPLC, GC etc. out of that the thin Layer Chromatography is the best techniques for evaluation of phytoconstituents. TLC is a play important role for separation of constituent from plant extracts. In thin layer chromatography the extract or sample is given as a small spot at the base line on a glass / plastic / metal plate. In thin layer chromatography method used the mobile phase which is transfer through by capillary action. The mobile and stationary phases are separated the solutes due to the their differential partition coefficient and adsorption. The separated component has same transfer time but different transfer distance.

The mobile phase which are prepared with the a single solvent as well as mixture of solvents. Although, a number of sorbents such as cellulose, silica gel, polyamide, chemically modified silica gel, Silica gel F-60 etc. The methods or techniques like pouring, spraying, dipping etc. which are used to prepare the Handmade plates TLC. Presently, precoated plates used for thin layer chromatography which are available in market. After selection of plates, the plates which are required to need activated. The plates should be activated at 110 ⁰C for 15 min. Because presence of water or moisture is removes loosely bound to silica gel surface. The Retardation Factor (Rf) is calculated according to following formula,
Qualitative TLC of *Ricinus communis* L.:

For the investigation of preliminary phytochemical constituents which are obtained from plant extracts like saponins, alkaloids, glycosides, carbohydrates, triterpenoids and flavones etc.

So, for the investigation of active constituents which are present in *Ricinus communis* L. ethanolic extract of leaves. This extract was subjected to thin layer chromatography to detect the various constituents present in it. The ethanolic extract showed the presence of flavonoids [16,98].

The Details of TLC are as-

<table>
<thead>
<tr>
<th>Adsorbent used</th>
<th>Silica gel GF 254 (activated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of plate</td>
<td>0.4 mm</td>
</tr>
<tr>
<td>Size of the Plate</td>
<td>10 x 20 cm</td>
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<tr>
<td>TLC plate Activation Temp.</td>
<td>110°C for 1 hr</td>
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<tr>
<td>Application Volume</td>
<td>20 μl</td>
</tr>
<tr>
<td>Solvent system used</td>
<td>Ethyl acetate : glacial acetic : formic acid : water</td>
</tr>
<tr>
<td></td>
<td>80 : 5 : 5 : 10</td>
</tr>
</tbody>
</table>

Isolation of the Flavonoids:

Preparative TLC analysis

The alcoholic extract of drug shown significant hepatoprotective activity; hence, it was selected for the analysis of detailed phytoconstituents using TLC. The preparative Thin Layer Chromatography plates having 0.4 mm thickness layer were prepared using paste or slurry of the Silica Gel through pouring technique. The plate was kept in hot air oven at 110°C for 15 mins. for activation of plate. The Sample of alcoholic extract was prepared in distilled water and the sample application was done by capillaty tube as a spot on the base line of TLC plate.
During the application of if the sample is applied overload on the plate the tailing effect shown on plate. So, take minute quantity of test sample for applying the band or spot which was avoid the tailing effect on plates. Then, the plates were dried in air or kept for 10 min at 40-50\(^\circ\)C in oven. Before keeping the TLC plate in chamber, require to predevelope the chamber with solvent system which are used for qualitative thin layer chromatography. The compound was separated in definite bands. The bands shown light green colore just below the solvent front. The separated band was carefully removed with the help of spatula and collected each band separately in container. The scrap of band was dissolved in alcohol and filter with the help of Whatmann filter paper, and washed for two times with alcohol. The filtrate was subjected for TLC to identify flavonoids. Each spot indicated presence of single spot on TLC and Rf values of scraped is calculated and recorded [98,87,102]. They were as follows:

Scrap No.1: \( Rf = 0.12 \)

**Isolation and characterization of compound:**

From the separated bands, the substance of interest was scrapped from the plate & it was dissolved in 95% ethanol. Filtered the mixture and evaporated the filtrate to dryness. The isolated compound was subjected to qualitative HPTLC analysis. Spectra are given in annexure 1.9.

**UV spectrum**

The UV absorption spectrum of isolated compound was recorded on JASCO UV 530 spectrophotometer [102]. Spectra are given in annexure 2.0.

**IR spectrum**

The IR absorption spectrum was recorded on THERMO NICOLET IR-200 spectrophotometer using liquid sampling cell [102]. Spectra are given in annexure 2.1.

**High Performance Thin Layer Chromatography**

For the estimation of active constituents from the alcoholic and aqueous extract of leaves of *Ricinus communis* L. and isolated compounds were subjected to HPTLC.

The details of high performance thin layer chromatography were as fallows-

<table>
<thead>
<tr>
<th>Plate</th>
<th>Alluminium plate precoated with Silica Gel GF60254</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness</td>
<td>0.2 mm</td>
</tr>
<tr>
<td>Plate size</td>
<td>10 X 10 cm</td>
</tr>
</tbody>
</table>
Sample application: 10 µl
Solvent: Acetate : Formic acid : Glacial acetic acid : Water (80:5:5:10)
Detection: 254 nm
Instrument: CAMAG TLC Scanner3 and LINOMAT-V

The CAMAG TLC Scanner3 and LINOMAT-V is an evaluation system using the help of software of Wincat for estimation active constituents using thin layer chromatogram which are reflectance or transmission mode by fluorescence with 254 nm.

The fingerprint of HPTLC profile of all extracts and isolated compound were taken by using computer. Rf values of various samples were evaluated by using following formula -

\[
R_f = \frac{\text{Distance travelled by solute from the origin}}{\text{Distance travelled by solvent from the origin}}
\]

HPTLC profile of various extracts of leaves of *Ricinus communis* L. given in table no. 3.18.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Trak</th>
<th>Peak</th>
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<th>Max Rf</th>
<th>Max Height</th>
<th>Max %</th>
<th>End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
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<tbody>
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<td>1</td>
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<td>0.01</td>
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<td>0.05</td>
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<td>6</td>
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<td>38.4</td>
<td>0.19</td>
<td>2.7</td>
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Table 3.18: HPTLC profile of the extracts of the *Ricinus communis* L.
Fig 3.5: Comparative TLC Profile of ALE of *Ricinus communis* L. and Isolated Compound

A – Alcoholic Extract; B – Isolated Compound; 

Rf Value- 0.12

Fig 3.6: Comparative HPTLC Profile of ALE of *Ricinus communis* L. and Isolated Compound

A– ALE; B – AQE; C- Isolated Compound; D- Isolated Compound; 

Rf Value - 0.12
3.4: Preparation of Herbal Formulation:

For the preparation of Herbal Formulation, there are several formulations like tablet, capsules, syrup, emulsion, suspension and parenteral dosage form. But selected tablet formulation. Therefore, tablets are the solid dosage form containing medicaments, generally tablet shapes such as circular in shape, flat or biconvex. For the tablet preparation used compression.

Merits of Tablets:

1. It is easy to be administered.
2. The tablets are easily dispensed.
3. This dosage form is more stable.
4. It maintained the accuracy.
5. If the smell are bitter and nauseous can be given easily.
6. The tablets are lightest and the most compact of all dosage forms.
7. The tablets are easiest and the cheapest and easy for transport.
8. As compared with any other oral dosage form the tablets are the best suitable to large scale production.
9. The tablets are an economical dosage form.

Demerits of Tablets:

1. Some drugs, because of amorphous nature or low density character, resist compression of tablet
2. The manufacture of tablets cost may be increases due the bitter taste and objectionable odour, atmospheric moisture.
3. If the drugs having poor wetting and slow dissolution rate are difficult to preparation tablets in good form and not provide better drug bioavailability.

Manufacturing of Compressed Tablets:

The following steps are involved during the manufacturing of compressed tablets:

1. Preparation of granules for compression:
   a) Weighing the ingredients.
b) Mixing the powdered ingredients and excipients.
c) Preparation of granules.

2. Compression of granules into tablets.
3. Coating of tablets.
4. Quality control of tablets.

3.4.1: Material & Methods:

Materials:

For this study the all selected plant leaves of Caesalpinia bonduc L., Mangifera indica L., & Ricinus communis L. were collected from my native place of Ramling Mudgad, which is located in Latur district (Maharashtra) and the botanical identification of all plats were confirmed by ICMR, Belgaum, (Karnataka), India and other requirement were purchased from locally and some of the other chemicals used and which are Analytical Grade.

Methods:

The preparation of formulation (tablet) using alcoholic extract of Caesalpinia bonduc L., Mangifera indica L., & Ricinus communis L. prepared two dose variation formulation (ALF-1, ALF-2) and also prepared two possible forms of tablets using aqueous extract of Caesalpinia bonduc L., Mangifera indica L., & Ricinus communis L. (AQF-1 and AQF-2). The four dosage forms (tablet) are prepared from the combination, of Caesalpinia bonduc L., Mangifera indica L., & Ricinus communis L.

The General Procedure for preparation of tablet:

To take all extracts and make a mixture of all extracts powder of Caesalpinia bonduc L., Mangifera indica L., & Ricinus communis L. were used for the preparation of tablet. For the preparation of tablets are having various methods but for this study used the wet granulation method, and also used the 10 % w/v of starch mucilage as a binding agent as well as disintegrating agents. For the preparation of tablet required lubricating agent. Therefore, talc is used as a lubricating agents for preparing tablets [12,30].

The following steps which are involved for the preparation of powder of different extract and preparation of granulation then, mixing with all excipients. After that prepared the binding solution and added the binder solution with powder mixture after addition of binding agent then get to form a wet mass, then, the wet mass passes through with the help of sieves that used the
sieves size like six to twelve mesh for drying the granules and the drying the granules with the help of 14 to 20 mesh used for the purpose of screen as well as mixing of drying of granules with lubricating agents. After drying the granulation finally tablet are compressed. Prepared four different forms of tablet formulations for example ALF-1, ALF-2, AQF-1 and AQF-2 were prepared with the help of single punch tablet compressed machine. At the time of the preparation of suitable types of tablet during the preparation of tablet were prepared only one time. Hence, which is to be avoid bias of the second batch. The preparation of formulation of used the different concentration of binding agent with the drug which are shown in Table 3.19.

After manufactured of tablets are checked for efficiency and quality of tablets having various standared preliminary tests or determination for its quality control parameters these are following.

**Tablet Evaluation**

After manufactured of formulation there are required to check thire standared with the help of standared evaluation parameter. So, evaluated for general appearance, hardness test, friability test, disintegration test, dissolution test of four forms of Tablets i.e ALF-1, ALF-2, AQF-1 and AQF-2. The evaluated result of tablets which are shown in Table No.5.2.

The prepared tablets having the spherical shape and brown colour with smooth surface. The all manufactured of tablets were having best quality with standared hardness and friability. So, all form of formulation which are prepared with the help of 10 % w/v starch paste used as disintegrating agent as well as binder. The disintegrating agent which was shown disintegration time is 50 min. [76,41].

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Ingredients</th>
<th>Formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALF-1</td>
</tr>
<tr>
<td>1</td>
<td><em>Caesalpinia bonduc</em> L.,</td>
<td>200.00 mg</td>
</tr>
</tbody>
</table>
Table 3.19: The List of Ingredients preparation of Tablet.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Ingredient</th>
<th>200.00 mg</th>
<th>300.00 mg</th>
<th>200.00 mg</th>
<th>300.00 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td><em>Mangifera indica</em> L.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Ricinus communis</em> L.</td>
<td>200.00 mg</td>
<td>300.00 mg</td>
<td>200.00 mg</td>
<td>300.00 mg</td>
</tr>
<tr>
<td>4</td>
<td>Starch of Paste</td>
<td>10% w/v</td>
<td>10% w/v</td>
<td>10% w/v</td>
<td>10% w/v</td>
</tr>
<tr>
<td>5</td>
<td>Talc</td>
<td>5 mg</td>
<td>5 mg</td>
<td>5 mg</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

Table 3.20: Evaluation parameter of Tablet.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Evaluation Parameters</th>
<th>Observed Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ALF-1</td>
</tr>
<tr>
<td>1</td>
<td>Disintegration</td>
<td>49 min</td>
</tr>
<tr>
<td>2</td>
<td>Dissolution Time</td>
<td>88 min</td>
</tr>
<tr>
<td>3</td>
<td>Hardness</td>
<td>3.5 g/cm</td>
</tr>
<tr>
<td>4</td>
<td>Friability</td>
<td>0.83 %</td>
</tr>
</tbody>
</table>

3.5.1: STUDY OF HEPATOTOXICITY: [56]

*Damage liver cell changes*

Hydropic change of liver cell is a more described word which applies to the liver cell with slightly faintly, water containing cytoplasm and a nucleus. Most of the varietiess of conditions develops the colouring or staining of cytoplasm which is relatively less. The eosinophils are increased in the blood may be find with compound related hydroplasia of the endoplasmic reticulum (smooth) i.e ribosimes are not attached to the E.R. Active regeneraration
of liver cells after liver damage occurs in hepatitis occurs due to viruses or recovery phases of fatty liver created a widespread damaged liver cells changes as well as a cobblestone typed of the liver cords. Due to the drug induced hepatotoxicity or due to the alcohol induced liver damaged shown are the damage and indicated the viral hepatitis and changes of the or recovered the hepatoprotective drug.

**Histopathological basis:**

Hepatocellular fat accumulation may be either large cytoplasmic body composed of minute fine foamy vesicles of fat within a hepatocyte. The liver of Fatty which occurs because of has some reason these are

- The increase intensive in movement of fat from the periphery in the liver,
- Lack of protein is essential for hepatocellular fat release,
- Increase hepatocellular fat formation by metabolic changes and
- Decrease hepatocellular fat degradation. Fatty, liver is common in alcohol ingestion, parental nutrition, tuberculosis, starvation, and certain drugs.

**Hepatocellular Necrosis:**

The hepatocellular necrosis has classified in various types, these are introducing at particular part (Periportal, pervenular and so on), mechanism (lytic, coagulative), amount (submassive versus focal), cellular type (Lymphocytotoxic verses hyline necrosis) and various pattern are composition having different causes diseases. When the acute hepatic injury the common pattern shown like zonal necrosis. The actually coagulation of liver necrosis are having certain typical of acetaminophen injury and can be discussed by various oxygenation and also having the activity of enzyme drug metabolizing. The primary liver necrosis is not generally and did not indicated coma and convulsions during pregnancy. The Midzonal damage is having for yellow fever.

The damaged liver or necrosis of hepatocellular are the lytic type, joined with the small activity that are generally dead hepatocytes are rarely noted; such type of liver necrosis are common shown in widely spread viral hepatitis in the liver, the hepatotoxic reaction due to the given of alcoholic. The composition of necrosis in the liver is having different conditions. This typed parameter are shown by coloring hepatocytes that removal of some staining of the cytoplasm due the inflammatory action the cells being shrunken and the cells are slowly disappear because of the inflammatory action.
**Regeneration:**

The liver cells are having a important capacity for regeneration of cell and it has been recovery the submassive hepatic cell which are indicated death of tissues by enzyme degradation. In liver of adult rat, the liver cell is having capacity of turnover of one mitosis per year and occure weight doubles within 48 hours. The liver cells weights are normally recovered within 3 to 6 days. The human livers have capacity regeneration also having increases and also regeneration in interstitial inflammation of liver. When major hepatic(an agent that acts on the functions of liver) resection completes for tumor or malignancy, the normal volume of cells are regenerated within 3 to 6 months and after surgery the liver function has nirmaly work.

**The liver Fibrosis:**

The Hepatic formation of fibrous tissues (fibrosis) are plays crucial role when the chronic liver cells disease and increases to the cirrhosis and liver function are changes which are produced various clinical manifestation of fatal liver disease.

Types of fibrosis: Simple hepatocellular necrosis does not result in collagen formation, but in severe hepatic necrosis a collapsed stroma may form a framework for other retention of collagen. The continuous or regular necrosis occurs with fibrosis and the most striking fibrosis within the lobule is noted in chronic alcoholic disorders of liver. The formation of fibrous tissues occurs even with a very less response of inflammation. Another same pattern of formation of fibrous tissue occurs in the portal areas in chronic active hepatitis which tends to be more confirmed to the portal tracts and does not extend into the lobule as much as portal fibrosis does in alcoholic.

**Liver Cirrhosis:**

The presentally liver cirrhosis stated that cirrhosis are needed the term to be applied of the liver which are diffuse liver fibrosis (that is entire liver and not focal) and which contains regenerative of nodules which are lobes of hepatocytes decreases the normal blood flow due to the lack of terminal hepatic celss. The cirrhosis are divided in different class as micro or small nodular having the three millimeters in diameter and out of that some are less than 1.5 millimeter in diameter.

The macronodular cirrhosis which are applied when nodules are greater than three millimeters in diameter and these are available in two different forms. The nodules divided by thin septa that are incompleted and have linking an entrance tract.
3.5.2: ENZYME THAT DETECT HEPATOCELLULAR NECROSIS: [85]

Nearby thousands of enzymes are present in liver, but some of them are in very low concentration which are present in serum. The enzymes which are present in serum have no more functions or also having other function and behave like other proteins. The hepatocellular necrosis are distributed in plasma as well as intestinal fluid and they are have specific character are no appearance, they generally measures in days. When the increased rate of liver cells damaged in serum then increases the enzyme activity in serum. The Serum enzymes tests can be grouped into two categories these are –

1) If the enzymes whose increases in damage to hepatocytes and

2) If the enzymes which are elevation in reflects of cholestasis in serum.

The Aminotransferases

The enzyme transaminases like serum aminotransferase are more sensitive indicator of liver necrosis, and useful in identifying acute hepatatic disorders such as hepatitis. Alanine aminotransferases (ALT), serum glutamic-pyruvic transaminases (SGPT)) and Asparate aminotransferases (AST), serum glutamic-oxaloacetic transaminases (SGOT)), these are the important biochemical parameters which are measured activities in serum of liver.

All enzymes are protein in nature so the enzymes derived from the amino acids such as aspartic acid, alanine etc. catalyze the reactions in krebs cycle, glycolysis. The ketoglutarate forms the pyruvate and oxaloacetic acid. The number of methods used for measurement of Alanine aminotransferases and Aspartate aminotransferases activity or mechanism in serum, in that the most significant and effective method which are helps to the synthesis of oxaloacetate and pyruvate. The enzymatic reduction to lactic acid and malic acid. Reduced form of the coenzyme such as nicotinamide adenine dinucleotide (NADH) which is derived from vitamin B , the cofactor in this reduction NADH get aerobically converted to nicotinamide adenine dehydrogenase (NAD). Which the light is absorbed at 340 nanometer and that events can occurs as follows.

By the Spectrophotometrically the low absorptivity of 340 nm, and the both the aminotransferases enzymes are generally present in serum at low concentrations, and they are having less than 30 to 40 IU/L.

The Asparate aminotransferases which is shown in the liver, as muscles like skeletal, cardiac, the brain, kidneys, the lungs, the pancreas, which are in decreasing order of the
concentration, where alanine amino transferase is present at largest concentration in the liver. If the ALT and AST serum values are increase then damage of tissue they changed the permeability of cell membrane. Hence, the ALT and AST are allowed to the leak into serum.

Aminotransferases typically are elevated in all liver disorders. This includes the cirrhosis, hepatitis such as chronic, acute hepatitis, and infectious mononucleosis, alcohol liver disease etc. these diseases raises the limit than normal limit nearby more eight times. Again these diseases are non-specific and they may occur in the form of any one or more diseases of then liver. The highest increases occur in diseases associated hepatic cell injury extensively. The hepatic injuries occurs due to viruses, exposure of hepatotoxins such as carbon tetrachloride and paracetamol etc. ALT is somewhat higher than AST. The increases the aminotransferases can be diminished under certain circumstances. The drugs like increased aminotransferases values if older tests are used for colorimetric estimation. Low concentration of aspartate amino transferase may present in uremia. These values increase after dialysis.

3.5.3: DRUG-INDUCED HEPATOTOXICITY:

The main function performed by liver is metabolism of drugs and compounds which are given orally. The compounds which are metabolized by the liver are mostly soluble in fats and lipids and again they passes through intestinal cell membrane and after passing through the cell membrane and they go through the process of excretion by converting into water-soluble compounds and excreted through the urine.

All the metabolic processes of drug or compounds occurred in the liver by the system of microsomal fraction. Smooth endoplasmic reticulum is the microsomal fraction of the liver. The metabolism of drugs or compounds in liver is by the enzymes like cytochrome P450 enzymes & cytochrome C reductase enzyme and they are catalysed in the presence of a co-factor such as Reduced NADPH which increases the activity of these enzymes. The drug polarity is increased by hydroxylation (in which addition of – OH group) or oxidation (addition of O2). And this polarity is further raised by conjugation or combination of several substances such as glucuronic acid, sulphuric acid etc.

By the administration of drugs into the liver, there is enlargement of liver is occurred which relates to enzyme induction. Para-amino salicylic acid is the inhibitor of the enzyme system. Several active drugs which may be compete for the binding site of enzyme which takes
the more time or slow process for catalyzing the reactions or the drugs which take more time to metabolise.[85].

**Mechanism of drug hepatotoxicity:**

The Drugs which are shown damage of liver by a directly react on the liver cell. The drugs are having two other mechanisms are usually involved. The first one is combined with the proteins cells and which are indicated related to the metabolite substances and second mechanism is that which is modified the immunological reaction are inceases to the drug concentration of hepatocytes. Sometimes the liver damage or the injury of liver shown when the hepatotoxic drugs are taken such as paracetamol. The immunological reactions are sometimes predominant when the drugs like anaesthetics such as for halothane.

**Direct action (metabolite – related):**

The hepatic enzymes that takes important part in the metabolism of compounds or drugs and these enzymes make the drug chemically active and stable to the drugs and convert them into more potent agents like acylating agents, arylating agents, alkylating agents etc. For the life of liver cells and liver necrosis are essential to the combination of covalently to the liver of the macromolecules. The liver generally included the substances, like glutathione, which has a capability of more preferentially together with a toxic metabolite. If storage of these components can be removed the necrosis of the liver.

**Immunological reaction for the drug:**

Generally small or some part of liver is affected i.e about nearby 1%. and those drugs causes liver injury. Skin rashes, fever are the symptoms of immunological reactions. But generally children are not affected for that.

The diagnosis may be only being confirmed with the help of drug challenges. The diagnosis is ethically or un-justified when the the original reaction has been a serious one.

**Toxicity of Individual Drugs:**

**Carbon tetrachloride**

It may be administered into the body accidentally or it may be taken for suicidal condition. It is very toxic to the body. During filling of fire extinguisher or for dry cleaning when it is used or when it mixed into the drinks it may be inhaled into the body. Because of the toxic metabolism of microsomal which combined with the covalently with the proteins cell are induced the liver injury and liver necrosis. The after reduction of protein which are decreases the
drug metabolism of enzymes and induced effect are enhanced by alcohol, barbiturates etc. These types of effect which are shown under-developed countries and the hepatotoxic effects due to the carbon tetrachloride.

**Tetracyclines**

Chlortetracycline and oxytetracycline interfere with protein synthesis. In 1963, to cure the pyelonephritis the larger dose of tetracycline is given i.e 3-6 g per day intravenously to the six women during pregnancy in the third trimester. When failure of hepato-renal system, death occurs. Tetracycline may be inhibitory to protein synthesis particularly affecting the transport lipoproteins which remove triglyceride from the liver. Hepatotoxicity occurs when large doses of tetracyclins are intravenously administered during pregnancy.

**Paracetamol**

For hepatic necrosis paracetamol is a best agent i.e the dose near about 10-12 g is necessary. But the administration of paracetamol is very difficult because it gives immediate unreliable histories and vomiting. Most preferred drug for paracetamol is Glutathione is always combine with the electrophonic metabolite of paracetamol. Glutathione when causes hepatic necrosis the metabolites of paracetamol arylates essential nucleophilic macromolecules.

**Methotrexate**

It is first metabolized to 6-mercaptopurine. The Hepato-toxicity includes hepatic fibrosis and hepatic cirrhosis occurs when a toxic metabolite of microsomal origin affects on the liver.

**Isoniazid**

The hydrazine is a inhibitor associated with hepatotoxicity which is a weak amino-oxidase inhibitor. This amino oxidase inhibitor used individually in asymptomatic person which has a tuberculin skin tests. During the starting six months of starting isoniazid, in Washington there are a lot of government employees which suffered from liver diseases like fibrosis, cirrhosis. All of that total thirteen persons were suffered from jaundice and two were died. Hydrazine forms when acylation of isoniazid occurs. This formed hydrazine is converted to acylating agent which is more potent by drug metabolizing enzymes, which gives necrosis.

\[
\text{Isoniazid} \rightarrow \text{Acetyl isoniazid} \rightarrow \text{Acetyl hydrazine} \rightarrow \text{acylating agent}
\]

Liver cell necrosis \hspace{1cm} \text{CytochromeP}_{450}
**Halothane**

Convincing evidence has come from challenge experiments such as that in an anaesthetist who developed clinical, biochemical and hepatic histological deterioration when rechallenged with halothane. Two controlled prospective clinical trials have been reported. The first, from Oxford, reported liver function tests in women receiving multiple anaesthetics for the treatment of cancer of the uterine cervix. The second, from Southampton in addition studied men receiving multiple anaesthetics during the treatment of cancer of the bladder. In both there was an increased incidence raised transaminases values in those receiving halothane.

When metabolism of Halothane is occurs then there is a formation of halogens like chloride, bromide and also trifluoroacetic acid. The formation of the first product of reductive metabolism is unstable and toxic and again they covalently bound to the metallic enzymes of liver which gives the injury to the liver directly.

3.5.4: LIVER FUNCTION TESTS: [89,37].

**Test for formation and excretion of Bile**

1. **Bilirubin:**

   Bilirubin pigments can be detected in serum, faeces and urine. Serum bilirubin estimation is based on van den Bergh diazo reaction by spectrophotometric method. Diazo reagents consist of diazotized sulphanic acid. Water soluble conjugated bilirubin is determined by van berg reaction with diazo reagent, whereas alcohol soluble unconjugated bilirubin by indirect van den Bergh reaction. In faeces, excretion of bilirubin is assessed by inspection of stools, clay-coloured stools due to absence of faecal excretion of the pigments indicates obstructive jaundice. In urine, conjugated bilirubin can be detected by commercially available dipsticks, Fouchet’s test, Foam test or Ictotest tablet method.

2. **Urobilinogen:**

   It is normally excreted in the urine. Its semiquantitative estimation in the urine can be done by preparing dilutions with Ehrlich’s aldehyde reagent or by dipsticks method. An increase in uribilinogen in the urine is found in hepatocellular dysfunctions such as in alcoholic liver disease, cirrhosis and malignancy of the liver.

3. **Bile Acids (Bile salts):**
In the hepatic cells or liver cells, the cholesterol synthesizes two acids namely cholic acid and cheno-deoxy cholic acid which are termed as the primary bile acids. The secondary bile acids synthesized when these bile acids like cholic and deoxy-cholic acids on secretion into the gut come in contact with colonic bacteria and undergo deconjugation. When there is an increase in concentration or level of bile acids of the serum, the hepatobiliary diseases are associated with cholestasis and these diseases produces itching. In the hepatobiliary diseases like cholestasis, the excretion of bile acids are through the urine by the mechanism of passive diffusion and active transport and these bile acids are confirmed by simple methods as hay’s test and dipsticks.

1. Serum Enzyme Assays:

The estimation of several serum enzymes are most commonly used in various types of liver injuries, whether hepatocellular or cholestatic, as well as in quantifying liver damage. A combination of serum transaminases and alkaline phosphatase estimation adequate to diagnose of liver injury.

2. Alkaline phosphatase:

The enzyme like Serum alkaline phosphatase is synthesized from number of organs and tissues like intestine, liver, specially from bone, and placenta. Elevation of enzyme activity may be indicated in various diseases of liver, bone, and also associated in pregnancy.

3. Glutamyl transpeptidase:

The primary source of the enzyme, in serum is the liver. Its serum level parallels serum alkaline phosphatase and these enzyme detect that there is an increased level of an enzyme serum alkaline phosphatase is from the hepatobiliary origin. If the increased level of Glutamyl transpeptidase in hepatobiliary diseases like in cholestasis and hepatocellular disease, it is present in high concentration in those patients which are with alcohol abuse but there is no liver damage.

4. Transaminase (Aminotransaminase):

The Serum Aminotransaminase which are plays important role for estimation of the hepatocellular injury and it is most commonly used in detection of the acute hepatocellular disease like as a hepatitis. These enzymes catalyzes the transfer of the keto group of the
ketoglutaric acid and amino group of the amino acid which forms respectively the oxaloacetate and pyruvate from by SGPT and SGOT.

### 3.5.5: THE ROLE OF CYTOCHROME P<sub>450</sub> IN DRUG-INDUCED LIVER DISORDERS: [27,86].

The chemical-induced liver which are generally not shown the result from the effects of the original compounds. The conversion of the original compounds to toxic metabolites within the liver. This statement applies not just to drugs, but also to environmental chemicals such as aflatoxins, bromobenzene and carbon tetrachloride. The major families of liver enzyme which are produced they are responsible for the generating potentially toxic metabolites which are from the cytochromes P<sub>450</sub>.

**The role of cytochrome P<sub>450s</sub>:**

The most of drugs are capable of producing liver toxicity appears through the generation of toxic metabolites which are present in the liver. The important role of metabolites in chemical-induced injury probably reflects the fact that in order to be absorbed into the body and reached the liver, chemicals must generally be lipophilic and metabolically stable. Metabolism of drugs which are generally produces metabolites that are safely removed from body. However, under the certain circumstances P<sub>450s</sub> can be generated most potential and reactive toxic metabolites. The major enzymes responsible for the production of hepatotoxic metabolites are the P450s. Species differences in P<sub>450</sub> catalytic activity and regulation probably contributes to the imperfect animal studies to identify hepatotoxins.

For Example-Acetoaminophen is the compound which believed to cause the toxicity in the liver occurs the production of the substance like n-acetyl benzoquinone amine metabolite (NAPQI). The Studies include with recombinant enzymes of human liver suggested that this reactive and potential metabolite could be produced by several cytochrome.

**Carbon tetrachloride (CCl<sub>4</sub>) induced hepatotoxicity** [82,90,83].

The Carbon tetrachloride (CCl<sub>4</sub>) is considered as a very toxic and powerful agent to the liver and this is is used for the induced or damaged the liver which is useful for the observation of liver necrosis as well as liver steatosis in the rat. These free radicals are produced cytochromes
P-450 mediated which are reductive process. During the process the carbon tetrachloride is converted into (CCl₃) and (CCl₃O₂). These radicals which are reactive and their time of action is very short carbon tetrachloride created necrosis is most severe in centrilobular hepatocytes during this process the concentration is high of cytochrome P-450. As per the above observation the free radicals which are important for the produce lipid peroxidation of biological membranes, protein binding, covalently to lipids and nucleic acids. Others are considered that the trichloromethyl radical which are important for the lipid peroxidation and covalent bonds.

3.6: MATERIALS AND METHODS
3.6.1: ANIMAL SELECTION:

For this study selected the Female albino wistar rats (150-200 gm) breed. These selected animals which are used for assessing hepatoprotective activity and acute toxicity study respectively. The animals which are used for the study allow free access to water and food. The rats were housed in a group of six in clean cages at 25 °C±5 and 12 hours dark, 12 hours light. Take ethical clearance from (IAEC) Institutional Animal Ethics Committee before conducting the experiment.

3.6.2: DRUGS AND CHEMICALS:

The drugs and chemicals used are as follow-

- Carbon tetrachloride (scientific sells, Latur)
- Liv52 (Himalaya drug; Bangalore)
- Olive oil (Seven ship brand, Nirmal chemical, Bangalore)
- Estimation kit (SGPT, SGOT, SALP and Serum billirubin)
  - **SGOT** - (Crest Biosystem Ltd. Santacruz).
  - **SGPT** - (Crest Biosystem Ltd. Santacruz).
  - **SALP** - (Crest Biosystem Ltd. Santacruz).
  - **Serum Billirubin** - (Crest Biosystem Ltd. Santacruz).

All other reagents were of analytical grade.

3.6.3. HERBAL FORMULATIONS USED:

Herbal Formulation:

- Alcoholic formulation (ALF)
- Aqueous formulation (AQF)

3.6.4. TOXICITY STUDIES:

The Acute Oral Toxicity:

For this study the oral toxicity is required. The toxicity study was carried out as per the guidelines which was prepared by Organization of Economic Co-operation and Development (OECD) and received draft guidelines 423, these guidelines which was prepared by committee for the control and observation of experiments on Animals (CPCSEA), by Government of India [69].
3.6.5 : PRINCIPLE OF TEST:

The principle which is based on step by step methods for the use of animals per steps in that necessary information is obtained to enable its classification. The fixed doses of drugs are given through the orally for the group animals which are used for experimental study. The substances are tested on animals according stepwise procedure and the used 3 animals of a single sex (normally males) for each step. Due to the presence or absence of a compound related with mortality of the animal’s doses will determines the next step.

- Administration of dosing to the three additional animals with the same dose.
- Administration of dosing to three additional animals at the next lower or the next higher dose levels.
- No further testing is required.

The method which was enables to estimate or judgment with respect to divided to the test substances to one of the sequence of toxicity which are stated according to cut off values of LD₅₀.

3.6.6 : METHOD OF DESCRIPTION :

The Selection of animal species:

The healthy young female albino rats of weighing between 150-200 gms and 8-10 weeks old which were used for acute toxicity study to determine LD₅₀. for determination used the Herbal Formulation and make three groups, each groups consist of three animals.

The Housing and feeding conditions:

For the storage of animals the temperature should be maintain in the experimental room was around 25 °c ± 5. The Lightening was artificial and the sequence being 12 hrs. dark and 12 hrs. light. The conventional laboratory diet was fed and the drinking water which is supplied unlimited.

The Preparation of Animals:
The animals were selected randomly and make a indication mark for animal to allow identification after identification mark all animals are kept in their respective cages for 5 days. The laboratory condition to maintained before 5 days giving the dose.

**Dose Preparation:**

Each tablet such as ALF-1, ALF-2, AQF-1 and AQF-2 contains 200 mg and 300 mg of active ingredients. The tablet is converted in powdered was suspended in 0.5 % Carboxy Methyl Cellulose in distilled water and then the dose was given to animals with the help of rat through oral gavaging needles.

**Dose Administration:**

The test substances were given in a single unit dose through gavage using intragastric tube. The animals were found prior to dosing, following period fasting the animals were weighed and test substance was administered. When given the dosing, the food was avoided for a further 3-4 hrs in rats.

**Number of animals and dose levels:**

In this study the each steps three animals were used. Since there was information on the substances to be tested (i.e. Herbal Formulation), starting dose was selected to be 1000 - 4000 mg/kg body weight no mortality which is considered as the end point was observed. The procedure of dose selection and finalizing LD$_{50}$ cut off values are shown in the Table No.3.21.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of Herbal Formulation</th>
<th>LD$_{50}$ cut off mg/kg b.w.</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ALF</td>
<td>3500</td>
<td>Water</td>
</tr>
<tr>
<td>2</td>
<td>AQF</td>
<td>4000</td>
<td>Water</td>
</tr>
</tbody>
</table>

**Table 3.21: Dose Selection and LD$_{50}$ cutoff values of Herbal Formulation.**

So, according to the 1/10$^{th}$ of this lethal dose was taken as effective dose (therapeutic dose) for subsequent Hepatoprotective activity.

**Observations:**

Animals were observed initially then; the dose was given after administration of dosing observed at least one during 30 minutes interval up to the first 24 hours. In all cases death was
observed within first 24 hours. The additional observations such as the skin and eyes and mucous membranes colour are has been changed. Also changes the central nervous systems, a motor activity and behavior pattern and also respiratory, circulatory. So, Attention was also given to observation of tremors and convulsions.

3.7. HEPATOPROTective ACTIVITY:

3.7.1. Method of screening:

In this study for the screening of hepatoprotective activity the method are used according to Handa S.S and Anupam Sharma [84,60].

The prepared Herbal Formulations were administered in each group of six female albino Wistar rats which were weighing about 150-200 gm. All animals were administered with carbon tetrachloride dose of 2 ml/kg i.p. for induce hepatotoxicity. Then, the increases the serum level of SGPT, SGOT, SGPT, SALP and Serum Bilirubin was taken as indication of hepatotoxicity [20, 75, 2, 45].

The animals were divided in seven groups as-

- **Group A** – The group A served as control and received single daily dose of 1 ml/kg i.p of sucrose solution for 4 days along with 1 ml/kg s.c of olive oil on 2\textsuperscript{nd} and 3\textsuperscript{rd} days.
- **Group B** – The group B is received single dose daily of 1 ml/kg i.p of sucrose solution for 4 days along with 2 ml/kg of Carbon tetrachloride by subcutaneous route dissolved in an equal volume of olive oil on 2\textsuperscript{nd} and 3\textsuperscript{rd} days.
- **Group C** – The group C is received standard drug Liv-52 as a single dose daily of 5 ml/kg oral route for 4 days along with 2 ml/kg of Carbon tetrachloride by subcutaneous route dissolved in an equal volume of olive oil on 2\textsuperscript{nd} and 3\textsuperscript{rd} days.
- **Group D and E** – These two groups are received single dose daily of ALF-1 (200 mg/kg) and ALF-2 (300 mg /kg) respectively, the does were given by oral route for 4 days along with 2 ml/kg of Carbon tetrachloride by subcutaneous route on 2\textsuperscript{nd} and 3\textsuperscript{rd} days.
- **Group F and G** – The group F & G are received single dose daily of AQF-1 (200 mg/kg) and AQF-2 (300 mg/kg) respectively, the does were given by oral route for 4 days along with 2 ml/kg of Carbon tetrachloride by subcutaneous route on 2\textsuperscript{nd} and 3\textsuperscript{rd} days.
The rats in all groups are sacrificed on 5th day by giving anesthesia like light ether and withdrawn the blood through the retro orbital plexus. Withdrawn blood was allowed to coagulated for 30 min and in that serum was separated by centrifugation through at a speed of 2500 rpm for 10 minutes. Again the serum was used to estimation of Alanine phosphatase (ALP), serum bilirubin, Serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT).

**The Histopathological studies:**

For this observation take one animal from each group such as treated groups, hepatotoxin, positive control, and control groups were utilized for this purpose. The animals are sacrificed on day 5th and cut the abdomen and remove the liver. After removed the liver. Then, separated the about 5 mm thick pieces of the liver kept in 10 % formalin solution for 12 hrs and then embedded in paraffin, using suitable method and cut into the 5 m thick sections and stained by using the staining agent such as haematoxylin-eosin dye and lastaly mounted in diphenylxylene.

### Table 3.22: Schedule for Carbon tetrachloride Model.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>GROUP</th>
<th>DOSE</th>
<th>DAYS</th>
<th>Animal were sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>A- control</td>
<td>1 ml/kg</td>
<td>SS</td>
<td>SS,OO</td>
</tr>
<tr>
<td>2</td>
<td>B - CCl4</td>
<td>2 ml/kg</td>
<td>SS</td>
<td>SS,CCl4</td>
</tr>
<tr>
<td>3</td>
<td>C - Liv-52</td>
<td>5 ml/kg</td>
<td>TS</td>
<td>SD</td>
</tr>
<tr>
<td>4</td>
<td>D -ALF-1</td>
<td>200 mg/kg</td>
<td>TS</td>
<td>TS, CCl4</td>
</tr>
<tr>
<td>5</td>
<td>E -ALF-2</td>
<td>300 mg/kg</td>
<td>TS</td>
<td>TS, CCl4</td>
</tr>
<tr>
<td></td>
<td>F -AQF-1</td>
<td>200 mg/kg</td>
<td>TS</td>
<td>TS,CCl4</td>
</tr>
<tr>
<td></td>
<td>G -AQF-2</td>
<td>300 mg/kg</td>
<td>TS</td>
<td>TS, CCl4</td>
</tr>
</tbody>
</table>
After that the sections was observed under the microscope for the study of histopathological changes which occurs in liver architecture and taken the photomicrographs.

3.7.2. Evaluation of Hepatoprotective Activity:

The Evaluation of biochemical parameter for hepatoprotective activity has been selected according to the study of G.S. achliya et al. method. Following parameters are evaluated for hepatoprotective activity.

- SGPT (Serum glutamate pyruvate transaminase).
- SGOT (Serum glutamate oxaloacetate transaminase).
- Serum Alanine phosphatase (SALP).
- Serum Bilirubin.

Serum glutamate pyruvate transaminase activity as an index of hepatic damage has been widely used. Human serum contains several transaminase of which aspartate transaminase (AST) also called as glutamate oxaloacetate transaminase as (GOT) and alanine transaminase (GPT) are of diagnostic significance. One more enzyme occurred in serum i.e. alkaline phosphatase (ALP) is also having significant presence in liver injury. Evaluated levels of all these enzymes have been observed in hepatic injury. Diagnosis of these enzyme level can be assess in viral hepatitis, liver damage due to drugs or chemicals and for the differentiation of these condition from obstructive jaundice.

So the efficacy of the chloroform extract and alcoholic extract to reduce serum transaminase and alkaline phosphatase levels in CCl₄ induced hepatic failure model has been considered as hepatoprotective activity of respective extract.

Biochemical Investigation:

The Estimation of SGPT and SGOT:

The Serum glutamate pyruvate transaminase activity as an index of hepatic damage has been widely used. Human serum contains several transaminases of which aspartate transaminase (AST) also known as glutamate oxaloacetate transaminase (GOT) and alanine transaminase (ALT) also known as glutamate pyruvate transaminase (GPT) are of diagnostic significance. Elevated levels of serum transaminase have been observed in hepatic injury and are widely used both clinically and in experimental model to reveal the extent of hepatic injury. They are useful for diagnosis of acute viral hepatitis, liver damage due to drugs or chemicals and for the
differentiation of these conditions from obstructive jaundice. Thus the capacity of different extracts to reduce the elevated serum transaminase levels in carbon tetrachloride induced acute hepatic failure model has been used as a biochemical parameter to expose its hepatoprotective capacity.

**Estimation of Serum Glutamate Pyruvate Transaminase:**

The Serum Glutamate Pyruvate Transaminase or Alanine amino trasaminase is located in the cytosol of the liver cell. When inflammation of liver cell occurs, which are released in the blood circulation because of raised cell membrane permeability which are break down of liver cells. There, the determination of SGPT as index of the extent of liver damage.

For the estimation of SGPT USED the Diagnostic reagent kit and SGPT also known as "Alanine amino transaminase" (ALT) activity by method of Reitman and Frankel.

**Principle:**

The SGPT (ALT) Catalyses having the following reaction:

\[
\alpha\text{-Ketoglutarate} + L\text{-Alanine} \rightleftharpoons L\text{-glutamate} + \text{pyruvate}
\]

Pyruvate formed is coupled with 2,4-Dinitro phenyl hydrazine (2, 4 DNPH) to give the corresponding hydrazone, which gives brown color in alkaline medium and this can be measured by colorimetrically.

**Reagents:**

- Reagent 1: Buffered alanine $\alpha$-KG substrate, pH 7.4.
- Reagent 2: DNPH Colour Reagent.
- Regent 3: Sodium hydroxide, 4N.
- Reagent 4: Working pyruvate standard, 2 mM.
Reagent Preparation:

- **Solution I**: To take 1 ml of Regent and dissolved with 3 to 10 ml of purified water.

Precautions:

- Serum samples must be completely free from haemolysis, since RBCs are very rich in this enzyme. This may result in erroneous results.
- The use of detergents was avoided for the cleaning of glasswares.
- Cleaned and dry glasswares were used.
- Reagent 3 is corrosive, contact with skin was avoided.

Advantages:

- Highly popular and simpler method.
- This method gives reproducible results.
- Substrate and standard are specially stabilized.
- Very economical.

Stability:

- 2-8°C till expiry date

Wavelength:

- On spectrophotometer at 505 nm, on photocolorimeter using green filter.

Cuvettes:

- 1 cm light path.

Standardization of Kit:

To avoid variations due to inter-laboratory assay conditions, standardisation of kit was done before assay and standard curve was developed as shown in graph no.1.
**Assay:**

For this assay all solutions were pipette out into clean dry test tube and the test tube was labeled as Test (T) as shown below.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent 1:</strong> The buffered alanine having the pH 7.4 – it is incubated at 37°C for 5 minutes.</td>
<td>0.25 ml</td>
</tr>
<tr>
<td><strong>Serum:</strong> the serum was mixed properly and incubated at 37°C for 30 minutes.</td>
<td>0.05 ml (50 µl)</td>
</tr>
<tr>
<td><strong>Reagent 2:</strong> DNPH Reagent – the reagent is mixed properly and allowed to stand for 20 mins at room temperature.</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

**Solution 1:**

To take the solution and mixed properly then, allows to stand for about 10 min. at normal temperature and the absorbance are recorded at 505 nm of wavelength.

**The Estimation of SGOT:**

The serum glutamate oxaloacetate transaminase is located on the cytosol of liver cell. It is also found in the mitochondria. It is also found in many tissue such as heart, liver, skeletal muscle and kidney which rich source of SGOT in that order, liver are being the second richest source of SGOT the importance of SGOT levels in hepatic damage of hepatic cells to increased levels of serum glutamate oxaloacetate transaminase in blood serum.

**Methodology:**

SGOT Kit is based according to the Reitman and Frankel's method. The SGOT catalyzes the transfer of the amino group of L-aspartate (ASP) to α-ketoglutarate of the (α-KG) resulting in the formation of oxaloacetate (OAA) and L-glutamate (L-Glu).
The oxaloacetate is allowed to react with 2, 4-DNPH to form 2, 4 dinitrophenyl hydrazone derivative, which is brown coloured in alkaline medium. The hydrazone derivative of oxaloacetate similar to pyruvate is considerably more chromogenic than that of α-KG. The final colour developed does not obey Beer's law.

**Principle:**

![Reaction diagram](image.png)

**Reagents:**
- Reagent 1: Buffered substrate........:2 x 12.5 ml
- Reagent 2: DNPH colour regent......:2 x 12.5 ml
- Reagent 3: Sodium hydroxide........:1 x 25.0 ml
- Regent 4: Pyruvate standard 2mM...:1 x 3.0 ml

**Reagent Preparation:**
- Dilute sodium hydroxide 1: 10 with distilled water before use.

**Precautions:**
- SGOT (AST) kit is for in-vitro diagnostic use only.
- Clean and dry glassware’s were used; presence of impurities or detergent interferes with enzyme activity.
Reagents were stored as 2-8°C, it is necessary that working reagents reaches the temperature of measurement before adding the sample. If to serum cold reagents were added, it would reduce enzyme activity.

Haemolysis should be avoided as red cells contain GOT.

Stability:

- 0-4°C for 1-3 days.
- 20°C for more than 3-4 days

Wavelength:

- On spectrophotometer at 505 nm
- On photo colorimeter using green filter.

Cuvettes:

- 1 cm light path.

Standardization of Kit:

To avoid variations due to inter-laboratory assay conditions, standardisation of kit was done before assay and standard curve was developed as shown in graph no.2.

Assay:

All solutions were pipette out into two clean and dry test tubes with labeled blank (B) and Test (T) as shown below:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>(B)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffered substrate (1) ml</strong> : the substances was incubated at 37°C for 3 minutes.</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Serum (ml): the is mixed properly and incubated at 37°C for 60 minutes.

<table>
<thead>
<tr>
<th>Serum (ml)</th>
<th>0.1</th>
</tr>
</thead>
</table>

DNPH Color Reagent (2) ml: The reagent is mixed properly and allowed to stand for 20 min..

<table>
<thead>
<tr>
<th>DNPH Color Reagent (2) ml</th>
<th>0.5</th>
<th>0.5</th>
</tr>
</thead>
</table>

Distilled water (ml):

<table>
<thead>
<tr>
<th>Distilled water (ml)</th>
<th>0.1</th>
<th>--</th>
</tr>
</thead>
</table>

Working NaOH (ml):

<table>
<thead>
<tr>
<th>Working NaOH (ml)</th>
<th>5.0</th>
<th>5.0</th>
</tr>
</thead>
</table>

All test tubes were shaked and allowed to stand for 10 minutes at room temperature and measure the absorbance at 505 nm of Test (T) and blank (B) was measured on spectrophotometer.

On calibration curve: X-axis – Enzyme activity

Y-axis – Absorbance

Linearity:

If the enzyme activity of the specimen was above 190 units/ml, the test was repeated using dilute serum with normal saline and multiplied the final results with appropriate dilution factor.

Estimation of Alkaline Phosphatase (ALP):

Serum alkaline phosphatase was estimated using ERBA test kit of transasia Biomedical Ltd; Daman.

Principle -

\[
\text{P-nitro phenol phosphatase + water } \xrightarrow{\text{ALP } \text{mg}^{++}} \text{P-nitro phenol + phosphate.}
\]

Reaction temperature: 37°C
Wavelength: 405nm

*Blank:* carried out with distilled water.

*Units:* IU/l

The absorbances is converting into international units (IU) of activity having the following formula –

\[
\text{IU/l} = \frac{(\text{delta} \ A/\text{min}) \times T.V \times 106}{S.V \times \text{absorptivity} \times \text{XP}}
\]

Where,

- T.V means = Total reaction volume in ul
- S.V means = Sample Volume in ul.

Absorptivity = Millimolar Absorptivity of p-nitro phenol at 405nm

\[= 18.8 \times 103\]

= cuvette light path (cm) = 1cm

Activity of ALP at 37°C (IU/L) = (delta A405/min) X factor 2713.

### 3.7.3. Statistical Analysis:

The results of biochemical estimation were reported according to the analysis of Mean ± S.E.M for estimation was significant intergroup difference between each parameter was analysed separately by one-way analysis of variance and biostastistical calculated with the using the ANOVA Tukey test [45,84].

### 3.7.4. Plotting the calibration graph

To obtain the standard curves of SGOT and SGPT, solutions were prepared of the concentration mentioned in the standard procedure. Optical density was observed on spectrophotometer and plotted against respective concentration (U/ml).

<table>
<thead>
<tr>
<th>Units /ml</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.294</td>
</tr>
<tr>
<td>24</td>
<td>0.351</td>
</tr>
<tr>
<td>Value</td>
<td>SGOT</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>61</td>
<td>0.431</td>
</tr>
<tr>
<td>114</td>
<td>0.517</td>
</tr>
<tr>
<td>190</td>
<td>0.592</td>
</tr>
</tbody>
</table>

Standard curve of SGOT
Graph 3.1: Standardization cure of SGOT

Standard curve of SGPT

<table>
<thead>
<tr>
<th>Units /ml</th>
<th>Optical Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.302</td>
</tr>
<tr>
<td>28</td>
<td>0.414</td>
</tr>
<tr>
<td>57</td>
<td>0.483</td>
</tr>
<tr>
<td>97</td>
<td>0.545</td>
</tr>
<tr>
<td>150</td>
<td>0.601</td>
</tr>
</tbody>
</table>
Graph 3.2 : Standardization of curve of SGPT