4. Experimental Work

4.1. Chemicals and instruments

4.1.1 Solvents and chemicals

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemical/Reagents</th>
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</tr>
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<tr>
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<tr>
<td>2</td>
<td>Ethanol</td>
<td>Qualigens</td>
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<tr>
<td>3</td>
<td>Hexane</td>
<td>Qualigens</td>
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<tr>
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<tr>
<td>7</td>
<td>Butanol</td>
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<tr>
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<td>10</td>
<td>Dioxane</td>
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<tr>
<td>13</td>
<td>Sodium sulphate</td>
<td>Qualigens</td>
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<tr>
<td>14</td>
<td>Folin- Ciocalteu Reagent</td>
<td>Sisco Research Laboratories.Pvt.LTD.</td>
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<td>15</td>
<td>DPPH</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>16</td>
<td>Ascorbic acid</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

4.1.2 Chromatographic instruments:

CAMAG (Switzerland), Model: Applicator- Linomat IV automated TLC applicator ATS 4, Densitometer- CAMAG Model–3 TLC scanner equipped with CAMAG CAT S 4 software. Desega Applicator, Densitometer- Model–4 TLC scanner
### 4.1.3 Instruments for physical and structural identification:

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruker Ultrashield spectrometer 400 MHz</td>
<td>Bruker</td>
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<tr>
<td>QTRAP LCMS-3200 with ESI probe</td>
<td>Appliebiosystem</td>
</tr>
<tr>
<td>Melting point apparatus</td>
<td>Buchi</td>
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<tr>
<td>UV Spectrophotometer</td>
<td>Perkin-Elmer</td>
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<tr>
<td>FTIR Spectrum RXI</td>
<td>Perkin-Elmer</td>
</tr>
<tr>
<td>GC FID detector Clarus 500</td>
<td>Perkin-Elmer</td>
</tr>
</tbody>
</table>

### 4.1.4 Other instruments:

- Rotary evaporator                                   | Equitron       |
- Ultrasonic bath                                      | Elma           |
- Leica DMLS microscope                               | leica          |

All solvents and reagents used were of analytical reagent grade and procured from commercial sources.
4.2 Experimental of *Cassia absus*

4.2.1 Standardization of *Cassia absus*

4.2.1.1 Pharmacognostical Study

**Collection, Identification & Authentication**

The seeds of Chaksu-*Cassia absus* was purchased from Piyush Ayurvedic Bhandar Mumbai, India. The identification and authentication of plant material was done by Dr. Vinayak Naik, Senior Research Scientist, Piramal Life Sciences Ltd, Mumbai and it was submitted to the herbarium of Natural Product Botany Department, Piramal Life Sciences Ltd Mumbai.

**Powder analysis**

Dried powder of seeds of *Cassia absus* was examined for its macroscopic characters. The powder was passed through sieve # 60 and observed under the microscope for microscopical characters.

Powder was boiled with chloral hydrate to remove coloring matter and viewed under microscope after mounting it on a glass slide using glycerin and covering with a cover slip. Then the powder was stained with Phloroglucinol in presence of hydrochloric acid to observe the lignified structures like vessels, tracheids, stone cells etc. and again viewed under microscope as described earlier. Iodine water was used to locate starch.

The photomicrography of the sections at different magnifications as demanded by the anatomical details were taken using Leica DMLS microscope, attached with Leitz MPS 32 camera.
4.2.1.2 Physicochemical parameters

**Foreign organic matter**

**Procedure:**

About 100 - 500 g or the quantity specified in the individual monograph, of the original sample was weighed accurately and spread out in a thin layer. The sample was inspected with the unaided eye or with the use of a 6X lens and the foreign organic matter was manually as completely as possible. The percentage of foreign organic matter was weighed and determined with reference to the weight of the drug taken.

**Determination of moisture content or Loss on drying**

**Procedure:**

About 1 g of drug was taken and powdered. A glass-stoppered bottle was dried for 30 minutes under the same conditions to be employed in the determination and the weight of the bottle was taken. The sample was transferred into the bottle and weight of the bottle with the contents was noted. The sample was distributed evenly and was placed in the drying chamber (Oven). The stopper was removed and left in the chamber. The drying was carried out by heating to 100-105°C. Then, the bottle was removed from the oven and the bottle was closed promptly. The bottle was allowed to cool to room temperature and weighed. The experiment was repeated till constant value was obtained.

**Ash values**

**Total ash**

2 gm of accurately weighed seed powder was taken in tarred silica crucible. The drug material was spreaded in fine even layer at bottom of the silica crucible. The silica crucible was kept in muffle furnace at 450°C. The powder was incinerated, until it becomes free from carbon. Crucible was then taken out from furnace, cooled in dessicator and weighed. The percentage of total ash was calculated with reference to air-dried drug.

**Acid insoluble ash**

The ash obtained was boiled with 25 ml of 2 N hydrochloric acid for 5 min. Insoluble matter was collected on ashless filter paper and washed with hot water. The material was further ignited and weighed. The percentage of acid insoluble ash was calculated with reference to air-dried material.
Water soluble ash
The ash obtained was boiled with 25 ml water for 5 min. All insoluble matter was collected on ash less filter paper, washed with hot water and ignited for 15 min at the temperature not exceeding 450°C. The weight of insoluble matter was subtracted from total ash to get water-soluble ash. The percentage of water soluble ash was calculated with reference to air-dried substance.

Sulphated ash
A silica crucible was heated to redness for 10 minutes, allowed to cool in a desiccator and weighed. 1 gm of powder was transferred to crucible. The crucible and the contents were weighed accurately. Then the crucible was ignited, gently at first, until the substance is thoroughly charred. The crucible was cooled and the residue was moistened with 1 ml of sulphuric acid. It was heated gently until white fumes were on longer evolved. Then contents were ignited at 800°C until all black particles have disappeared. The crucible was allowed to cool and few drops of sulphuric acid were added to the residue. The contents were ignited, cooled and weighed. The operation was repeated until two successive weighing do not differ by more than 0.5mg.

Loss on drying
2 gm of air dried drug reduced to powder was placed in a tarred silica crucible. The powder was spread in a thin uniform layer. The crucible was then placed in the oven at 105°C for 4 hours. After specified time crucible was taken out from the oven, cooled in desiccators to room temperature and weighed.

Elemental analysis of ash $^{89, 90}$
The seed powder was taken in a platinum crucible and incinerated in muffle furnace to obtain ash. The ash was treated with 50% hydrochloric acid for 30 minutes and filtered. The filtrate was used for the detection of elements by specific tests.
Elemental analysis of ash

Presence of Aluminum
a) To the test solution, dilute ammonia solution was added. If gelatinous precipitate, soluble in hydrochloric acid, acetic acid and sodium hydroxide solution but nearly insoluble in dilute ammonia solution is formed it indicates presence of aluminum.
b) To the test solution, ammonium sulphide solution was added. If gelatinous precipitate, soluble in hydrochloric acid, acetic acid and sodium hydroxide solution but nearly insoluble in dilute ammonia solution is formed it indicates presence of aluminum.

Presence of Chlorides
a) To the test solution, manganese dioxide and sulphuric acid were added. Odour of chlorine indicates presence of chlorides.
b) To the test solution, solution of potassium iodide was added. Blue colour indicates presence of chlorides.
c) To the test solution, solution of silver nitrate was added. A white, curdy precipitate soluble in dilute ammonia solution but insoluble in nitric acid indicates presence of chlorides.

d) To the test solution dilute ammonia solution was added. Greenish blue precipitate indicates presence of copper.

Presence of Copper
a) To the test solution, hydrogen sulphide was added. Brownish-black precipitate indicates presence of copper.
b) To the test solution a solution of sodium hydroxide was added. Light blue precipitate indicates presence of copper.
c) To the test solution a solution of ammonium thiocyanate was added. Black precipitate indicates presence of copper.
d) To the test solution dilute ammonia solution was added. Greenish blue precipitate indicates presence of copper.

Presence of Calcium
a) To the test solution ammonium carbonate solution was added. A white precipitate which after boiling and cooling is insoluble in solution of ammonium sulphide forms then it indicates presence of calcium.
b) To the test solution ammonium oxalate solution was added. A white precipitate, soluble in hydrochloric acid but insoluble in acetic acid forms then it indicates presence of calcium.

c) To the test solution potassium chromate solution was added. A yellow, crystalline precipitate indicates presence of calcium.

d) To the test solution potassium ferrocyanide solution was added. Immediate precipitate, but on addition of excess of reagent in presence of excess of ammonium chloride, yields a white precipitate then it indicates presence of calcium.

**Presence of Carbonates and Bicarbonates**

a) Dilute hydrochloric acid was added to the test solution, effervescence due to liberation of carbon dioxide gas indicates presence of Carbonates and Bicarbonates.

b) Mercuric chloride was added to the test solution, Brownish-red precipitate indicates presence of Carbonates and White precipitate indicates presence of Bicarbonates.

c) To the test solution silver nitrate solution was added. A white precipitate, which does not, becomes yellow on addition of excess of reagent indicates presence of Carbonate.

**Presence of Iron**

a) To the test solution dilute hydrochloric acid and solution of potassium permanganate were added. Faint pink colour indicates presence of Iron.

b) To the test solution dilute hydrochloric acid and solution of ammonium thiocyanate were added. Blood red colour indicates presence of Iron.

c) To the test solution potassium ferrocyanide was added. White precipitate indicates presence of Iron.

d) To the test solution sodium hydroxide solution was added. Dull green precipitate indicates presence of Iron.

**Presence of Magnesium**

a) To the test solution ammonium carbonate solution was added and boiled. White precipitate indicates presence of Magnesium.
b) To the test solution dilute ammonia solution and solution of sodium phosphate were added. White crystalline precipitate indicates presence of Magnesium.

c) To the test solution sodium hydroxide solution was added. White precipitate indicates presence of Magnesium.

Presence of Phosphates

a) To the test solution silver ammonio-nitrate solution was added. A light yellow precipitate, readily soluble in dilute ammonia solution and in cold nitric acid indicates presence of Phosphates.

b) To the test solution magnesium ammonio-sulphate solution was added. A white crystalline precipitate indicates presence of Phosphates.

c) To the test solution ammonium molybdate and nitric acid solution was added. A yellow precipitate indicates presence of Phosphates.

Presence of Potassium

a) To the test solution perchloric acid was added. A white crystalline precipitate indicates presence of Potassium.

b) To the test solution sodium cobalt nitrite and acetic acid solution was added. A yellow precipitate indicates presence of Potassium.

c) Sample was moistened with hydrochloric acid and introduced on platinum wire into the flame of Bunsen burner violet colour to the flame indicates presence of Potassium.

Presence of Sodium

a) To the test solution uranyl zinc acetate solution was added. A yellow crystalline precipitate indicates presence of Sodium.

b) Sample was moistened with hydrochloric acid and introduced on platinum wire into the flame of Bunsen burner yellow colour to the flame indicates presence of Sodium.

Presence of Sulphates
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Experimental Work

a) To the test solution barium chloride solution was added. A white crystalline precipitate insoluble in hydrochloric acid indicates presence of Sulphates.

b) To the test solution lead acetate solution was added. A white precipitate soluble in solution of sodium hydroxide indicates presence of Sulphates.

Presence of Zinc

a) To the test solution ammonium sulphide solution and solution of sodium hydroxide were added. A white precipitate soluble in hydrochloric acid indicates presence of Zinc.

b) To the test solution potassium ferrocyanide solution was added. A White precipitate insoluble in dilute hydrochloric acid indicates presence of Zinc.

Extractive values determination:

Five gram of previously weighed air-dried drug was taken in a stoppered flask and 100 ml of 95% pet ether was added to it. It was shaken continuously for 4 hr on a magnetic stirrer. Then it was filtered rapidly taking precautions against loss of the solvent. Twenty five ml of filtrate was evaporated to dryness in a tared flat-bottomed petri dish, dried at 105°C and weighed. The percentage of petroleum ether soluble extractive was calculated with reference to air-dried drug.

The marc obtained from the process was used for the determination of extractive values viz chloroform extractive value, ethyl acetate extractive value, ethanol extractive value, hydroalcoholic extractive value & aqueous extractive value using above mentioned process.

Foaming index

Procedure

One gram of the plant material was reduced to a coarse powder (sieve size 125), weighed accurately and transferred into 500 ml conical flask containing 100 ml of boiling water. It was maintained at moderate boiling for 30 min, cooled and filtered into a 100 ml volumetric flask. Sufficient water was added through the filter and the volume made upto 100 ml.

The above decoction was placed into 10 stoppered test tubes, in a series of successive portions of 1, 2, 3, up to 10 ml and the volume of the liquid adjusted in each tube with water to 10 ml. The tubes were stoppered and shaken in a lengthwise motion for15 sec at two frequencies per second, allowed to stand for 15 min and the height of foam measured.
1. When the height of the foam in every tube was less than 1cm the foaming index is less than 100.

2. When in any tube a height of foam of 1cm was measured, the dilution of the plant material in this tube (a) was the index sought.

3. When the height of the foam was more than 1 cm in every tube the foaming index was over 1000. In this case, the determination had to be made on a new series of dilutions of the decoction in order to obtain a result.

   **Foaming Index = 1000/a**

   Where a, is the volume in ml of the decoction used for preparing the dilution in the tube where foaming was observed.

**Determination of swelling index**

**Procedure**

One gram of the drug was transferred into a 100 ml stoppered cylinder containing 90 ml of water. After shaking well for 30 seconds, it was allowed to stand for 24 hours, shaking gently on three occasions during this period. Sufficient water was added to produce 100 ml. It was mixed gently for 30 seconds and allowed to stand for 5 hours. The final volume was measured. The average of 3 readings was taken.

**Mucilage content**

**Procedure**

Five gram of the powdered drug was taken and 100 ml of water added to obtain the aqueous extract of the powder. It was filtered through cotton and the mucilaginous filtrate collected. 10 ml of this mucilage solution was mixed with 25 ml of absolute alcohol to precipitate the mucilage. This mucilage precipitate was collected by filtration through a tared filter paper. The filter paper was dried along with the mucilage. After drying, the filter paper was carefully weighed and the mucilage content was calculated from the weight.

**Fluorescence analysis**

Fluorescence analysis of dried powder of *Cassia absus* was carried out. Dried powder sample of *Cassia absus* was placed on a slide, treated with several drops of specified reagents and observed immediately under UV lamp. Then the dried specimen was mounted in nitrocellulose, allowed to dry and observed under UV lamp. The colour emitted by the dried powder of Cassia absus were designated in
terms of the three primary colours (red, yellow, blue), three secondary colours (orange, green, purple) and brown, which is a mixture of three primary colours.

Heavy Metals

The limit for heavy metals is indicated in the individual monographs in terms of ppm, i.e., the parts of lead, Pb, per million parts (by weight) of the substance under examination.

- Standard solution: Into a 50-ml Nessler cylinder 1.0 ml of lead standard solution (20 ppm Pb) was added and diluted with water to 25 ml. With dilute acetic acid/dilute ammonia solution to a pH between 3.0 and 4.0 was adjusted, diluted with 35 ml of water.
- Test solution. 1 gm of powder was dissolved in 25 ml of water. With dilute acetic acid/dilute ammonia solution a pH was adjusted between 3.0 and 4.0, diluted with 35 ml of water.
- Procedure. To each of the cylinders containing the standard solution and test solution respectively, 10 ml of freshly prepared hydrogen sulphide solution was added.
  - The solution was mixed and was diluted to 50 ml with water,
  - It was allowed to stand for 5 minutes and viewed downwards over a white surface
  - The colour produced with the test solution was compared.

Limit test for Arsenic

Preparation of reagent:
- Stannated hydrochloric acid: Stannous chloride solution 1 ml was diluted with 100 ml of hydrochloric acid.
- Dilute Arsenic solution: Strong arsenic solution 1 ml was diluted with 100 ml of water

Procedure:
- Gutzeit test apparatus was used for limit test of Arsenic

Preparation of sample:
- 1 gm of ethyl acetate extract of Cassia absus was placed in the wide mouth bottle of Gutzeit apparatus to this 1 gm of potassium iodide, 10 gm of zinc was added.
• Gutzeit apparatus was closed and was allowed to stand for 40 minutes.
• A yellow stain was produced on the mercuric chloride paper and was compared with stain produced by standard arsenic solution

**Preparation of standard stain:**

- 50 ml of water, 10 ml of stannated hydrochloric acid, and quantities of dilute arsenic solution varying from 0.2 ml to 1 ml was placed in the wide mouth bottle of Gutzeit apparatus to this 1 gm of potassium iodide, 10 gm of zinc was added.
- Gutzeit apparatus was closed and was allowed to stand for 40 minutes.
- A yellow stain was produced on the mercuric chloride paper and was compared with stain produced by standard arsenic solution.

**4.2.1.3 Phytochemical study**

**Extraction Methodology for Cassia absus**

The extraction of whole plant powder was done by following method

1. **Extraction by successive cold maceration:**

   5 kg of Cassia absus (Seed) powder was macerated with petroleum ether, chloroform, ethyl acetate, ethanol, hydro-alcoholic and water each for seven days, with frequent shaking. It was filtered and concentrated with the help of rotary vacuum evaporator. The extracts were stored in refrigerator.

Characterization of extract by chemical tests: The extracts were tested for various chemical constituents with the help of different chemical tests.

1. **Alkaloids:**

   a) **Mayer’s test:** - To the extract Mayer’s reagent was added, cream colored precipitate indicates presence of alkaloids.

   b) **Wagner’s test:** - To the extract Wagner’s reagent was added, reddish brown precipitate indicates presence of alkaloids.

   c) **Hager’s test:** - To the extract Hager’s reagent was added, yellow precipitate indicates presence of alkaloids.

2. **Amino acids:**

   a) **Millon’s test:** - To the extract, 2ml of Millions reagent was added, white precipitate indicates presence of amino acids.
b) Ninhydrine test: - To the extract Ninhydrine solution was added, boil, violet color indicates presence of amino acid.

3. Carbohydrates:
   a) Molisch’s test: -To the extract few drops of alcoholic α-naphthol, few drops of concentrated sulphuric acid through sides of test tube was added, purple to violet color ring appears at the junction.
   b) Barfoed’s test: -1ml of extract was heated with 1ml of Barfoed’s reagent, if red cupric oxide is formed, monosaccharide is present. Disaccharides on prolong heating (about 10 min.) may also cause reduction, owing to partial hydrolysis to monosaccharides.
   c) Selivanoff’s test (Test for ketones): -To the extract crystals of resorcinol and equal volume of concentrated hydrochloric acid was added and heated on a water bath, if rose color is produced. (E.g. Fructose, honey)
   d) Test for pentose: -To the extract equal volume of hydrochloric acid containing a small amount of phloroglucinol was added, and heated, if red color is produced indicates positive

4. Flavonoids:
   a) Shinoda test: - To the extract few magnesium turnings and concentrated hydrochloric acid drop wise was added, pink scarlet, crimson red or occasionally green to blue color appears after few minutes.
   b) Alkaline reagent test: - To the extract few drops of sodium hydroxide solution was added, if intense yellow color is formed which turns to colorless on addition of few drops of dilute acid indicate presence of flavonoids indicates positive.
   c) Zinc hydrochloride test: - To the extract add a mixture of zinc dust and conc. Hydrochloric acid was added, it gives red color after few minutes if positive

5. Glycosides:
   I. General test: -
   Test A: Extract 200mg with 5ml of dilute sulphuric acid by warming on a water bath. Filter it. Then neutralize the acid extract with 5% solution of sodium hydroxide. 0.1ml of Fehling’s solution A and B was added, until it becomes alkaline (test with pH paper) and heated on a water bath for 2 minute. Note the quantity of red precipitate formed and compare with that of formed in Test B.
**Test B:** Extract 200mg using 5ml of water instead of sulphuric acid. After boiling add equal amount of water as used for sodium hydroxide in the above test. 0.1ml Fehling’s A and B until alkaline (test with pH paper) was added, and heated on water bath for 2 minute. Note the quantity of red precipitate formed. Compare the quantity of precipitate formed in Test B with that of formed in Test A. If the precipitate in Test A is greater than in Test B then Glycoside may be present. Since Test B represents the amount of free reducing sugar already present in the crude drug, whereas Test A represents free reducing sugar plus those related on acid hydrolysis of any glycoside in the crude drug.

6. Steroids and Triterpenoids:
   a) **Libermann-Burchard test:** - Treat the extract with few drops of acetic anhydride, boil and cool. Then add concentrated sulphuric acid from the side of the test tube, if brown ring is formed at the junction two layers and upper layer turns green which shows presence of steroids and formation of deep red colors indicates presence of triterpenoids.
   b) **Salkowski test:** - Treat the extract with few drops of concentrated sulphuric acid red colors at lower layer indicates presence of steroids and formation of yellow colored lower layer indicates presence of triterpenoids.

7. Phenolic compounds (Tannins):
   a) **Ferric chloride test:** - Treat the extract with ferric chloride solution, blue color appears if hydrolysable tannins are present and green color appears if condensed tannins are present.
   b) **Gelatin test:** - To the extract add 1% gelatin solution containing 10% sodium chloride. Precipitate is formed.

8. Anthraquinone:
   a) Borntrager’s test: To the extract dil. H₂SO₄ was added. Boiled and filtered. To the cold filtrate, equal volume of chloroform was added. Separated the organic solvent. Ammonia was added. Ammonical layer turns pink or red.
   b) Modified Borntrager’s test: To the extract dil. H₂SO₄, FeCl₃ and dil. HCl were added. Heat for 5 min in boiling water bath. Cool and add any organic solvent. Shake well. The organic layer was separated. Equal volume of dilute ammonia was added. Ammonical layer shows pinkish red colour.
4.2.1.4 Estimation of Total Polyphenolic Content

Flavonoids attracted growing global interest during the last decade and, as a result of this upsurge in research, the number of known Flavonoids has increased dramatically: About 800 different Flavonoids were known. At the start of the 1990s, the number of reported flavonoid structures had increased to and currently almost 6500 different Flavonoids are known.

During the 1990s, Flavonoids were shown to possess several biological effects, some of which were also related to human health. Phenolic compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom.

Flavonoids have certain health effects, with considerable amount of research has been directed towards their activity as antioxidants Radical scavengers, antimicrobial as well as their anti-mutagenic and anti-carcinogenic properties. However, the most interest has been paid to their potential in the prevention of coronary heart disease. Both tannins, which are large Phenolic molecules, and the more simple phenols and Phenolic acids are antimicrobial mechanisms thought to be responsible for Phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulphhydryl groups or through more non-specific interactions with the proteins.

Preparation of Reagent:
Sodium carbonate (350 gm) was dissolved at 70-80°C in and volume was made with distilled water (up to 1 lit). It was filtered through glass wool after allowing it to stand overnight.

Total polyphenolic content of the extracts was estimated by the method described.

Standard solution: Standard stock solution; an accurately weighed quantity of Gallic acid (100 mg) was dissolved in methanol and volume was made up to 100 ml with methanol to make concentration 100 µg/ml.

Standard working solution: The aliquot portions of standard stock solution of Gallic acid were diluted appropriately with methanol to obtain a concentration range of 10-100 µg/ml.
**Preparation of calibration curve**

1ml of Gallic acid working solution was taken into 25 ml volumetric flask to this 10 ml of water and 1.5 ml of Folin- Ciocalteu reagent was added. The mixture was kept for 5 min and then 4 ml with double distilled water and then 4 ml of 20 % sodium carbonate solution was added it.

The mixture was kept for 30 min and the absorbance blue colored complex was measured at 765 nm. The graph was plotted as absorbance verses concentration. All the determinations were performed at triplicate. The solutions were mixed thoroughly and absorbance was measured at 765nm against a reagent blank after an hour.

**4.2.1.5 Determination of Total Tannin Content**

Tannins and tannin like substances are widespread in nature and are probably present in all plant materials. Those are polyphenolic compounds divided into two main groups-hydrolysable and condensed. Hydrolysable tannins contain a polyhydric alcohol usually, if not always, glucose esterified with Gallic acid or with hexahydroxydiphenic acid. Condensed tannins are mostly flavan-3-ol (Catechin) and these cannot be hydrolyzed to simple components. The total tannins in the *Cassia absus* were estimated by Folin-Denis method.

**Principle:**

Tannin like compounds reduces phosphotungustomolybdic acid in alkaline solution to produce a highly colored blue solution, the intensity of which is proportional to the amount of tannins. The intensity is measured in a spectrophotometer at 760nm.

1. Folin Denis Reagent: To 750ml of water, 100g of sodium tungstate (Na$_2$WO$_4$.2H$_2$O), 20g of phosphomolybdic acid and 50ml of 85%phosphoric acid (H$_3$PO$_4$) was added. Mixture was refluxed for 2 hours and cooled to 25°C and diluted to 1000ml with water.

2. 35% Sodium Carbonate Solution: To 100ml of water, 35g of anhydrous sodium carbonate was added. Dissolved at 70-80°C and cooled to room temperature and allowed to stand overnight. Solution was filtered through Whatman filter paper No.1 before use.

3. Tannic Acid Standard Solution: 100mg of tannic acid was dissolved in 1litre of water and fresh solution was prepared for each determination (1ml=0.1mg of tannic acid).
Preparation of Working Standard

0.1g of Tannic Acid powder was weighed in a dry 100mL volumetric flask. It was dissolved in distilled water and volume was made up to the mark. Appropriate dilutions were prepared from this stock for color development. i.e. 10mL → 100mL → 5mL for color development. 75mL of distilled water, 5mL Folin-Denis reagent and 10mL Na₂CO₃ solution was added into the volumetric flasks and volume was made up to 100mL with distilled water. Solution was mixed well and color was measured after 30 mins. At 760 nm against experimental blank (i.e. 75mL of distilled water, 5mL Folin-Denis reagent and 10mL Na₂CO₃ solution diluted to 100mL with distilled water) and adjusted to zero absorbance.

Preparation of Sample

1 gram of the powder sample was weighed accurately in a 50 mL of water and was well shaken. It was then kept on water bath for about 1 hour with occasional shaking. Mixture was then cooled to room temperature and diluted up to 100mL with distilled water. It was kept overnight with occasional shaking. Next day filtrate was filtered through Whatman’s filter paper No.1 and further 2ml of the filtrate was diluted to 50mL with the same distilled water and shaken well. 2ml of this solution was taken out and color was developed by adding 5mL Folin-Denis reagent and 10mL Na₂CO₃ solution. Color was allowed to develop for about 30 minutes and finally diluted up to the mark of 100mL with distilled water. The absorbance was recorded at 760nm.

4.2.1.6: Determination of residual solvents:

Instruments and Materials: Gas Chromatograph perkin-elmer Clarus 500 was used. Gas chromatograph was equipped with standard oven option for temperature ramping, split/splitless injection ports and flame ionisation detector. BP 624 column (30m X 0.53mm i.d. X 0.25µm coating thickness, 4% cyanoethyl phenyl and 94% dimethyl poysiloxane stationary phase), with nitrogen as carrier gas in the split mode by direct injection method was used. Analytical grade solvents methanol, ethanol, isopropyl alcohol and dimethyl sulfoxide (DMSO) were purchased from Qualigens, Mumbai, India.

Preparation of standard: Dimethyl sulfoxide (DMSO) was selected as the standard and sample diluent, based on its ability to dissolve wide variety of substances. Also DMSO is a solvent with high boiling point that does not interfere
with more volatile solvents tested by GC for the method involving analysis of high boiling point solvents.

Standard stock of ethyl acetate was prepared by diluting with DMSO in 10 mL volumetric flask to get concentration of 1000 µLmL⁻¹. From these stocks 8 serial working standard solutions were prepared to obtain concentrations ranging from 10-400 µLmL⁻¹ for ethyl acetate respectively, volume was made with DMSO. 1 µL of working standards were injected in to gas chromatograph and standard calibration curves was obtained.

**Preparation of Sample:** Accurately weighed 1 g of ethyl acetate extract of Cassia absus dissolved and sonicated with DMSO, filtered through whatman filter paper No 1 and volume made up to 10 mL with DMSO, in separate 10 mL volumetric flask. From these samples 1 µL samples was injected and concentrations of ethyl acetate in sample was calculated by interpolating standard calibration curve.

**Gas chromatographic conditions:** The experimental conditions were used; 1 µL volume of either standard or sample solutions was injected in GC injection port. The injection port maintained at temperature 35⁰C with a split ratio 1:10. Nitrogen used as a carrier gas with pressure 16 kpa for an expected flow of 3.5 mL min⁻¹. Temperature of the detector was set at 250⁰C.
4.2.2. *In vitro* antioxidant assay

4.2.2.1. DPPH Free Radical Scavenging Assay

**Chemicals and Reagents:** were procured from Sigma-Aldrich and Qualigens

**Preparation of Sample solution:**

10 mg of extract was weighed in an ephendorff and dissolved in methanol with sonication for 1 minute. The mixture was then transferred in a 100mL volumetric flask and the volume was made up to the mark with methanol to obtain a 100µg/mL stock solution.

From this stock solution 0.4 mL, 0.8 mL, 1.2 mL, 1.4 mL & 1.8 mL were taken in 10 mL volumetric flasks & by serial dilution with same solvent, final volume were made up to 10 mL whose concentrations were then 4µg/mL, 8µg/mL, 12µg/mL, 16µg/mL & 20µg/mL respectively

**Preparation of Standard Solution:**

100µg/ml stock solution was prepared by dissolving 10mg of ascorbic acid in 100 mL of methanol. From this 4µg/mL, 8µg/mL, 12µg/mL, 16µg/mL & 20µg/mL respectively ascorbic acid solutions were prepared.

**Preparation of DPPH Reagent:**

It was prepared by dissolving 10mg of DPPH in 100 mL of analytical grade Methanol and kept in dark amber colored bottle to protect from sunlight.

**Procedure:**

1) 1ml of different concentrations of extract solutions and standard solutions were taken in different amber colored volumetric flasks and 3 mL of Methanol was added.

2) To this solution of 1 mL of DPPH solution was added, shaken well and mixture was incubated at 37°C for 10 mins.

3) The absorbance was measured against Methanol as blank at 517nm. All the tests were performed in duplicate and the graph was plotted with the mean values.

4) Percent radical scavenging activity was calculated by using formula.
\[
\text{% Radical Scavenging activity} = \frac{\text{Abs}_{\text{Con}} - \text{Abs}_{\text{Sam}}}{\text{Abs}_{\text{Con}}} \times 100
\]

Where,

\(\text{Abs}_{\text{Con}}\) is absorbance of Control,
\(\text{Abs}_{\text{Sam}}\) is absorbance of Sample solution.

4.2.2.2 Reducing Power Assay: \(^{103}\)

Chemicals and Reagents: were procured from Sigma-Aldrich and Qualigens

Preparation of Reagents:

0.2 M sodium hydroxide solution: 0.8 gram of sodium hydroxide was weighed and diluted to 100 mL with water

0.2 M Phosphate Buffer (pH 6.6)

2.722 g of monobasic potassium phosphate (KH\(_2\)PO\(_4\)) was weighed and diluted to 100 mL with water. 62.5 mL of above solution was taken in 250 mL volumetric flask to which 20.5 mL of 0.2 M sodium hydroxide solution was added and volume was made up to 250 mL with water.

Potassium ferricyanide \([K_3Fe(CN)_6]\) (1% w/v) solution

0.5 g of Potassium ferricyanide was weighed and diluted to 50 mL with water.

Trichloroacetic acid (10% w/v)

5 g of Trichloroacetic acid was weighed and diluted to 50 mL with water.

Ferric Chloride (0.1%)

0.1 g of Ferric Chloride was weighed and diluted to 50 mL with water.

Preparation of Standard Solution:

100µg/mL stock solution was prepared by dissolving 10mg of Ascorbic acid in 100 mL of methanol. From this 2, 4, 6, 8 and 10µg/mL Ascorbic acid solutions were prepared.

Preparation of Sample Solution:

100µg/mL stock solution was prepared by dissolving 10mg of Ethyl acetate extract in 100 mL of water. From this 2, 4, 6, 8 and 10µg/mL concentration sample solutions were prepared.
Procedure:
1. 1ml of different concentrations of the ethyl acetate extract (2-10 µg/mL) in deionised water were mixed with 2.5 mL of 0.2 M (pH 6.6) phosphate buffer and 2.5 ml of 1% potassium ferricyanide.
2. The resulting mixture was incubated at 50°C for 20 min
3. After incubation, aliquots of trichloroacetic acid (10% w/v), (2.5 mL) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min.
4. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of ferrous chloride (0.1%, w/v). All the tests were performed in duplicate and the graph was plotted with the mean values.
5. The absorbance was measured at 700 nm against a blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.
6. Various concentrations of the extracts (30 to 60 µg/mL) in 1.0 ml of deionized water were mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide.
7. The absorbance was measured at 700 nm. A blank was prepared without adding extract.
8. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

4.2.2.3. Nitric Oxide Scavenging activity: 104

Preparation of Reagents:
Chemicals and Reagents: were procured from Sigma-Aldrich and Qualigens

0.2 M sodium hydroxide solution: 0.8 gram of sodium hydroxide was weighed and diluted to 100 mL with water.

0.2 M Phosphate Buffer (pH 7.4): 2.722 gram of monobasic potassium phosphate (KH₂PO₄) was weighed and diluted to 100 mL with water. 62.5 mL of above solution was taken in 250 mL volumetric flask to which 48.9 mL of 0.2 M sodium hydroxide solution was added and volume was made upto 250 mL with water.

Sodium Nitroprusside solution (10mM)
Accurately weighed 0.148 g of Sodium nitroprusside in 100mL volumetric flask it was dissolved in standard phosphate buffer solution and volume was adjusted with phosphate buffer.

**20% Glacial Acetic acid:** 20ml of glacial acetic acid was pipette out and diluted upto 100mL with water.

**0.33 % Sulphanilic Acid Reagent:** 0.33 g of sulphanilic acid was weighed and the volume was made upto 100 mL in 20% glacial acetic acid.

**0.1% w/v 1-naphthylamine:** 0.1 g of 1-naphthylamine was weighed and dissolved in 100mL methanol.

**Procedure:**

1. The reaction mixture (3 mL), containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and sample extract at various concentrations (4-20 µg/mL) or standard solution (0.5 mL) was incubated at 25ºC for 150 min.
2. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipetted out and 1mL of sulphanilic acid reagent (0.33%) was added & mixed.
3. Above mixture was allowed to stand for 5 mins for completing diazotization.
4. 1ml of naphthylamine (1%) was added to the mixture.
5. The mixture was incubated at room temperature for 30 min, followed by the measurement of absorbance at 540 nm. All the tests were performed in duplicate and the graph was plotted with the mean values.

The amount of nitric oxide radical inhibited by the extract was calculated using the following equation:

\[
\text{NO radical scavenging activity} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100
\]

Where; \( \text{Abs}_{\text{control}} \) is the absorbance control reaction; \( \text{Abs}_{\text{sample}} \) is the absorbance of NO radical + sample extract.
4.2.2.4. ABTS assay\textsuperscript{105,106}

**Sample preparation:**
25mg of sample was dissolved in 1ml of methanol and made up to 10ml with 0.01M phosphate buffered saline (pH 7.4).

**Procedure:**
The ABTS radical cation was prepared by the following method.

- ABTS 2mM (0.0548g in 50 ml) was prepared in distilled water.
- Potassium per sulphate 70mM (0.0189g in 1ml) was prepared in distilled water. 200µl of ammonium per sulphate and 50 ml of ABTS were mixed and used after 2 hrs. This solution is called as ABTS radical cation, which was used for the assay.
- To the 0.5 ml of various concentration of extract, 0.3 ml of ABTS radical cation and 1.7 ml of Phosphate buffer, pH 7.4 was added. For control, instead of extract methanol was taken. The absorbance was measured at 734 nm. The experiment was performed in triplicate.

\[
\text{Control - Test} \times 100
\]

\[
\% \text{ Scavenging} = \frac{\text{Control}}{\text{Test}} \times 100
\]

4.2.2.5. ORAC assay\textsuperscript{107}

**Sample preparation:**
10mg of each sample was dissolved in 250µl of methanol and made up to 5ml with 75mM phosphate buffer (pH 7.4). Further dilutions were made as required.

**Procedure:**

- A pre-incubation mixture of 140µl contained 20µl of 75mM sodium phosphate buffer, pH 7.4 / test solution / TROLOX of various concentrations and 120µl of sodium fluorescein (117nM), mixed and incubated at 37°C for 10mins.
- Following pre-incubation, 60µl of AAPH (40mM) was added and mixed for 15 seconds. The reaction was carried out for 90 minutes at 37°C. The fluorescence measurements were taken at 485 nm excitation and 520nm emission filter with the following settings:
  1. Mode: Fluorescence intensity
  2. Filters: Excitation 485nm, emission 520nm
3. Number of cycles: 90
4. Number of flashes: 10
5. Cycle time: 60 seconds
6. Plate used: Costar 96, Code 3792 from Corning

Data reduction and ORAC value calculation was done as per Davalos et al., 2004 with some changes.

4.2.3. Invivo antioxidant assay\(^{108,109}\)

Experimental animals

Wistar albino rats (male) were purchased from Haffkine Institute, Mumbai, India and used in the present study. All animals were maintained in an air-conditioned room at 23\(^0\)C ± 2\(^0\)C, with a relative humidity of 75% ± 5%, a 12-h light/dark cycle. A basal diet (Amrut feeds, Maharashtra, India) and tap water were provided \textit{ad libitum}.

Male rats were assigned to each dose group by stratified random sampling based on body weight. The animals were kept under laboratory conditions for an acclimatization period of 7 days before carrying out the experiments.

4.5.1. Preparation of Streptozotocin solution

Preparation of 0.1 M citrate buffer solution pH 4.5:

- Accurately weighed quantity of trisodium citrate (14.9g) was dissolved in sufficient distilled water to produce 1000 ml and the necessary pH (4.5) was adjusted with Con. HCl.
- A solution of STZ was prepared by dissolving the weighed quantity of streptozotocin in 0.1 M freshly prepared ice cold citrate buffer (pH 4.5) solution. The solution of STZ so prepared was administered in the volume of 0.5-1ml.
- The selected male animals of 90 days old, weighing between 250 - 300 g were fasted overnight were administered with Nicotinamide 120 mg/kg i.p route and after 15 minutes Streptozotocin 60 mg/kg IP.
- Fasting blood sugar levels were determined on 14\(^{th}\) day after administering STZ/NIC to confirm stable hyperglycemia.
• The diabetic rats after confirmation of stable hyperglycemia, were divided into different groups of 6 rats each. That day was considered as the 0\textsuperscript{th} day. Drug and doses were administered as mentioned as below daily for 14 days.

1. Group I (Control 1) - water.
2. Group II (Control 2) - received dose of 300 mg/kg of ethyl acetate extract
3. Group III (Control 3 Diabetic) - water
4. Group IV (Diabetic) - received dose of 300 mg/kg of ethyl acetate extract
5. Group V (Diabetic) - received dose of 0.6 mg/kg of glibenclamide.

4.5.1 Preparation of liver post mitochondrial supernatant \textsuperscript{110}
(Liver-PMS)
At the end of the study (after four week of dosing) animals were decapitated and cut open to excise the liver. The excised livers immediately and thoroughly washed with ice-cold physiological saline. The tissue of 100mg was homogenized in 1ml of 0.1M cold tris-HCl buffer (pH7.4) in a potter-Elvehjem homogenizer fitted with a Teflon plunger at 600rpm for 30 min. The homogenate was centrifuged at 10,000g for 20 min at 40\degree C and the supernatant with firmly packed pellets were resuspended by homogenization in 100mM Tri-HCl buffer containing 20\%w/v glycerol and 0.1ml of 10mM EDTA, pH 7.4. The post mitochondrial supernatant was used to assay LPO, reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) activity.

Estimation of lipid peroxidation (LPO) from liver PMS \textsuperscript{111}
In 1ml of the reaction muddle, 0.58ml phosphate buffer (0.1 M, pH 7.4), 0.2ml of hepatic PMS (10\%w/v), 0.2 ml ascorbic acid (100mM) and 0.02 ml ferric chloride (100mM) and was incubated at 37\degree C in a shaking water bath for 1hr. The reaction was clogged by the addition of 1ml TCA (10\%, w/v), subsequently 1ml TBA (0.67\%w/v) was added and all the tubes were kept in a boiling water bath for 20 min. The tubes were shifted to ice-bath and centrifuged at 2500\times g for 10 min. The amount of malondialdehyde (MDA) formed in each of samples was assessed by measuring the optical density of the supernatant at 535nm allied with reagent blank without tissue homogenate. The molar extinction coefficient for MDA was taken to be 1.56\times 10\textsuperscript{5}M\textsuperscript{−1}cm\textsuperscript{−1}.
Calculation = $3 \times \text{absorbence of sample}/50.156 \times (\text{mg of tissue taken}) = \mu M/\text{mg tissue}$.

**Estimation of Reduced glutathione (GSH) from liver PMS**

Glutathione was assayed by the method of Jollow *et al.*, 1974. An aliquot of 1ml of hepatic PMS(10%w/v) was mixed with 1ml of sulphosalicylic acid (4%w/v) and centrifuged at 1200g for 5 min and filtered. From the filtrate, 0.1ml filtered aliquot, 2.7ml phosphate buffer (0.1M, pH 7.4) and 0.2ml DTNB (40mg/10ml of phosphate buffer 0.1M, pH7.4) in a total volume of 3.0ml. The yellow color developed was comprehended at 412 nm on a spectrophotometer.

**Estimation of Superoxide dismutase (SOD) from liver PMS**

The reaction mixture consisted of 0.5ml of hepatic PMS, 1ml 50mM sodium carbonate, 0.4ml of 25µM NBT (Nitro blue tetrazolium) and 0.2ml, 0.1mM EDTA. The reaction was initiated by addition of 0.4ml of 1mM hydroxylamine-hydrochloride. The change in absorbance was recorded at 560nm. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required inhibiting the reduction of NBT by 50%.

**Estimation of catalase (CAT) from liver PMS**

The assay mixture consisted of 1.95ml phosphate buffer (0.05M, pH 7), 1ml $\text{H}_2\text{O}_2$ (0.019M),0.05ml of hepatic PMS (10%w/v). Changes in absorbance were recorded at 240nm for 2min with 60seconds interval.
4.2.4 TLC & HPTLC fingerprinting of *Cassia absus*

**A) TLC**

Plate dimensions: 10 x 20cm.
Stationary phase: Silica gel G for TLC.
Mobile phase: Butanol:Water:Acetic acid (4:1:1)
Spraying agent: Dragendorff’s reagent.

**B) HPTLC**

**Sample preparation**

For development of HPTLC fingerprints the extracts of *Cassia absus* was prepared by taking 5 gm of powder in 250 ml of conical flask and 100 ml of corresponding solvents petroleum ether, chloroform, ethyl acetate, butanol, methanol was added and heated on water bath for one hour. The extract was filtered and evaporated to dryness. 20 mg/ml samples were prepared by reconstituting with same solvent. The solution was filtered through a 0.45-µm membrane and solvent systems were developed for their separation by thin layer chromatography.

**Chromatographic conditions**


**Stationary Phase**: Silica gel 60 F254 TLC plates

**Chamber saturation time**: 20 minutes.

**Spotting dimension**: 8 mm

**Distance between two spots**: 12.0 mm

**Wavelength**: 200- 400 nm

**Mobile Phase**: hexane– ethyl acetate–formic acid 8.0:1.0:0.5 (v/v)

**Development of chromatogram and Detection of spots**

After the application of spots, the chromatogram was developed in Twin trough glass chamber 20x 10 cm saturated with solvents developed for each extracts before putting the plates for 20 mins. The air-dried plates were viewed in ultraviolet radiation to mid day light. The chromatograms were scanned by densitometer at 360 nm. The Rf values and fingerprint data were recorded by WIN CATS software.
4.2.5 Procedures for extraction and isolation of phytoconstituents

Extraction:
The powdered drug (1.5 kg) was successively extracted using the solvents of increasing polarity i.e., petroleum ether (40-60°C), chloroform, ethyl acetate and ethanol respectively. After the removal of solvents in vacuo at 40-50°C, petroleum extract (25 gm), chloroform (34 gm), ethyl acetate (72 gm), ethanol (150 gm) were obtained. Invivo and invitro antioxidant assay were carried out for selection of extract for isolation of bioactive constituent (antioxidant & antidiabetic). Extract showing prominent antioxidant activity were selected for isolation.

The fractionation of the ethyl acetate extract

The ethyl acetate extract (15.5 gm) was fractionated using silica G-60 column chromatography. Silica G-60 900 gm was packed using petroleum ether in to a glass column (14 cm, i.d. x 90 cm, length), which was eluted with petroleum ether-ethyl acetate gradiently (100:0 to 90:10 to 80:20 to 70:30 to 60:40 to 50:50 to 40:60 to 20:80 to 10:90 to 0:100). Fractions of 45 ml were collected and combined. After removal of the solvents in vacuo, the fraction A (70:30) (Fr. 25-42 6.0 gm), fraction B (40:60) (Fr. 142-172 3.0 gm), fraction C (10:90) (Fr. 352-418 2.0 gm). The scheme of the fractionation is presented in Figure 4.1.

Fraction A, fraction B and fraction C revealed highest concentration of phytoconstituents, so these three fractions were chosen for the isolation and characterization of the marker compounds.

Isolation:
The isolation of the marker compounds from three fractions was achieved by using column chromatography with Silica G-60.

Isolation of CA-01

Fraction A (6.0 gm) was chromatographed on silica gel quick column (200 gm, 10 cm, i.d. x 15 cm, length), eluted with chloroform: ethyl acetate, gradiently (100:0 to 90:10 to 80:20 to 70:30 to 60:40 to 50:50 to 40:60 to 20:80 to 10:90 to 0:100). Fractions of 45ml were collected and combined. The enriched fraction A4 was continuously isolated. This fraction was re-chromatographed on silica gel column (480g, 10 cm, i.d. x 9 cm, length), eluted with chloroform: methanol, 80:20. Fractions of 300 ml were collected and combined.
The combined fractions were evaporated under reduced pressure on rotary evaporator. The fraction A4 was affording 20 mg of compound CA-01 (Figure 4.2)

Isolation of CA-02
Fraction B (3.0 gm) was chromatographed on silica gel quick column (200 gm, 10 cm, i.d. x 15 cm, length), eluted with chloroform: ethyl acetate, gradiently ((100:0 to 90:10 to 80:20 to 70:30 to 60:40 to 50:50 to 40:60 to 20:80 to 10:90 to 0:100). Fractions of 20ml were collected and combined. The enriched fraction was continuously isolated. This fraction was re-chromatographed on silica gel column (480g, 10 cm, i.d. x 9 cm, length), eluted with chloroform: methanol, 80:20. Fractions of 200 ml were collected and combined.
The combined fractions were evaporated under reduced pressure on rotary evaporator. The fraction B4 were affording 10 mg of compound CA-02. (Figure 4.3)

Isolation of CA-03
Fraction C (2.0 gm) was chromatographed on silica gel quick column (159 gm, 10 cm, i.d. x 9 cm, length), eluted with chloroform: ethyl acetate, gradiently ((100:0 to 90:10 to 80:20 to 70:30 to 60:40 to 50:50 to 40:60 to 20:80 to 10:90 to 0:100)..
Fractions of 20ml were collected and combined. The enriched fraction continuously isolated. This fraction was re-chromatographed on silica gel column (350g, 10 cm, i.d. x 9 cm, length), eluted with chloroform: methanol, 80:20. Fractions of 150 ml were collected and combined. The combined fractions were evaporated under reduced pressure on rotary evaporator. The fraction C3 were affording 10 mg of compound CA-03. (Figure 4.4)
Figure 4.1: Column chromatography of ethyl acetate extract

\[ \text{Cassia absus ethyl acetate extract 15.5 gm} \]

Column chromatography silica gel. Petroleum ether: chloroform: ethyl acetate, gradient elution

- A (6.0 gm)
- B (3.0 gm)
- C (2.0 gm)

Fig. 4.2
Fig. 4.3
Fig. 4.4

Figure 4.1: Column chromatography of ethyl acetate extract
Figure 4.2: Isolation of compound CA-01 from fraction A
Figure 4.3: Isolation of compound CA-02 from fraction B
Figure 4.4: Isolation of compound CA-03 from fraction C
Identification:

Melting points
The melting points of the isolated compounds were determined by the Electrothermal 9100 (ENG., LTD.)

Ultraviolet spectra
The ultraviolet spectra of the isolated marker compounds were recorded with a UV spectrometer (Perkin elmer).

Infrared spectra
The IR spectra in KBR disc of the isolated compounds were recorded with a FT-IR spectrometer (Perkin elmer)

Nuclear magnetic resonance spectra
The $^1$H-NMR was measured on NMR spectrometer (Bruker) at the Piramal Life Science Limited

Mass spectra
The ESI-MS were determined at Piramal Life Science Limited,

4.2.6 Analytical Method Development and Validation by HTPLC

Material, Reagents
The seeds of Chaksu- Cassia absus was purchased from Piyush Ayurvedic Bhandar Mumbai, India. The identification and authentication of plant material was done by Dr. Vinayak Naik, Senior Research Scientist, Piramal Life Sciences Ltd, Mumbai and it was submitted to the herbarium of Natural Product Botany Department, Piramal Life Sciences Ltd. The powder was passed through an 85-mesh sieve and the sieved powder was used for this research.

Analytical grade ethyl acetate and methanol were obtained from Qualigens Fine Chemicals, Mumbai, India. Distilled water used for the mobile phase, was obtained from a Millipore (USA) Milli Q water-purification system.

Preparation of Stock and Working Standard Solutions
A stock solution (1000 µg mL$^{-1}$) of Rhein was prepared by mixing 10 mg Rhein standard in a 10 mL volumetric flask and sonicating for 10 min. A stock solution (2000.0 µg mL$^{-1}$) of Emodin was prepared by mixing 40.0 mg Emodin standard in a 20-mL volumetric flask and sonicating for 10 min. A stock solution (1000.0µg mL$^{-1}$) of Chrysophanol was prepared by mixing 25.0 mg Chrysophanol standard in a 25-
mL volumetric flask and sonicking for 10 min. The solutions were cooled to room temperature (28 ± 2°C) and the contents of the flask were then diluted to volume with methanol.

Working standard solutions were prepared by diluting stock solution to 10.0 mL, with methanol, in volumetric flasks, to furnish concentrations in the range 25-75 µg mL⁻¹ for emodin, 100–300 µg mL⁻¹ for rhein and 150–450 µg mL⁻¹ for chrysophanol.

Sample Preparation
*Cassia absus* seed powder (approx. 1000 mg) was accurately weighed in a stoppered test tube and 10.0 mL warm (40 ± 2°C) methanol was added. The mixture was sonicated for 10 min and cooled to room temperature (28 ± 2°C). The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India) and the filtrate was used as the sample solution for assay.

Chromatography
HPTLC was performed on 100 mm × 100 mm aluminum backed plates coated with 0.2 mm layers of silica gel 60 F₂₅₄ (Merck, Mumbai, India). Standard solutions of Rhein, Emodin and Chrysophanol and sample solutions were applied to the plates as bands 8.0 mm wide, 7.0 mm apart, and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100-µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28 ± 2°C), with hexane–ethyl acetate–formic acid 8.0:1.0:0.5 (v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapor for 30 min. After development, the plates were dried in air and then scanned at 287 nm with a Camag TLC Scanner with CATS3 software, using the deuterium lamp. The method was validated according to the ICH guidelines.

Validation of the Method
The method was validated for linearity, precision and accuracy. Standard solutions of, Emodin concentration 25-75 µg mL⁻¹, Rhein concentration of 100–300 µg /mL⁻¹ and Chrysophanol of concentration 150–450 µg /mL⁻¹ were applied as bands to the same TLC plate. The procedure was repeated three times. The densitograms were recorded and mean Chrysophanol, Emodin, Rhein peak areas (Y axis) were plotted against the corresponding concentrations (X axis).
The limits of detection (LOD) and (LOQ) were determined as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively.

A system suitability test was conducted to determine whether the method gave accurate results. System suitability were determined by applying freshly prepared standard solutions of Rhein (200 µg mL\(^{-1}\)), Emodin (50 µg mL\(^{-1}\)) and Chrysophanol (300 µg mL\(^{-1}\)), five times to the same chromatographic plate. The plate was developed under the optimized chromatographic conditions then scanned and the densitograms were recorded.

The measured peak areas for all standards and their retention factors were noted and values of the mean peak area, the standard deviation (SD), and the relative standard deviation (RSD,\%) were calculated.

Instrumental precision, intra-assay precision, and intermediate precision of the method were determined. Instrumental precision were measured by replicate (\(n = 10\)) application of the same Rhein, Emodin and Chrysophanol standard solutions (25–75 µg /mL\(^{-1}\) for Emodin, 100–300 µg /mL\(^{-1}\) for Rhein and 150–450 µg /mL\(^{-1}\) for Chrysophanol). Intraassay precision was evaluated by analysis of six replicate applications of freshly prepared sample solutions of same concentration, on the same day. Intermediate precision was evaluated by analysis of six replicate applications of sample solutions of same concentration on three different days.

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of *Cassia absus* samples prior to extraction. The resultant samples were then extracted and analyzed with the described method. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. The recovery of the method was in the range of 99.1–99.8%. Considering the results, the method was deemed to be accurate.

**Use of the Validated Method for Quantification of Rhein, Emodin and Chrysophanol in *Cassia absus* seeds.**

The extract of *Cassia absus* (10.00 µL) was applied seven times to the same plate and the plate was developed, using the optimized chromatographic conditions, and scanned, as described above. Rhein, Emodin and Chrysophanol peak areas were recorded for each band and the amount of all standards were calculated by use of the
calibration plot. The procedure was repeated seven times, with a new sample of plant powder each time.

4.2.7 Safety Studies
4.2.7.1 Acute toxicity study
It was carried out by using OECD guidelines (No. 423) with slight modifications.

Experimental animals
Male Swiss albino mice were used in the present investigation. The animals were maintained in polypropylene cages in the Departmental Animal House Facility. The temperature in the experimental animal room was maintained at 23 ºC ± 2ºC. The relative humidity was maintained at 75% ± 5%. Lighting was artificial with the sequence of 12 hours light, 12 hours dark. For feeding, a conventional laboratory diet (Amrut feeds, Maharashtra) was used with an unlimited supply of drinking water.

The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatisation to the laboratory conditions.

Preparation of doses
Ethyl acetate extract was prepared in double distilled water. The maximum volume administered to the animals was 2 ml/100 g body weight. Doses were prepared shortly prior to administration.

Experimental design
Three animals are used for each step. As there was no information on extracts to be tested, for animal welfare reasons, 300 mg/kg body weight was selected as the starting dose for the study. The next two doses selected for studies were 1000 mg/kg and 2000 mg/kg. The test substances were administered in a single dose by using oral feeding needle.

Animals were fasted for 3-4 hours prior to dosing. Following the period of fasting, the animals were weighed and the test substance was administered. After administration of the substance the food was withheld for 1-2 hours.
Observations
Animals were observed individually after dosing once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days.

Cage Side Observations
Observations included changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Special attention was directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

Body Weight, Food and Water Intake
Body weight, food and water intake were recorded at two days intervals.

4.2.7.2.Repeated dose toxicity study
It was carried out by using OECD guidelines (No. 407) with slight modifications.

Experimental animals
Wistar albino rats of either sex were purchased from Haffkine Institute, Mumbai, India and used in the present study. All animals were maintained in an air-conditioned room at 23°C ± 2°C, with a relative humidity of 75% ± 5%, a 12-h light/dark cycle. A basal diet (Amrut feeds, Maharashtra, India) and tap water were provided *ad libitum*.

Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The animals were kept under laboratory conditions for an acclimatization period of 7 days before carrying out the experiments.

Experimental design
Repeated toxicity studies were conducted on four groups of rats (Control, 300 mg/kg low dose, 1000 mg/kg medium dose and 2000 mg/kg high dose), each containing five males and five females. Whilst the extract was orally administered using gavage to test groups, distilled water was administered to control group for 28 days. All animals were supplied with standard food and water *ad libitum* during the testing periods.

Preparation of serum and isolation of organs:
Animals were sacrificed on the 28th day by cervical dislocation, the jugular vein was cut and blood flowed freely. About 1.5 ml of blood was collected into vials.
containing 2.5 mcg of (EDTA) as an anticoagulant and 3 ml of the blood was collected into tubes without anticoagulant. The blood without anticoagulant was allowed to clot before centrifugation (1600 at 4°C for 10 min) to obtain serum which was collected and stored at -20 °C. After collecting blood, animals were dissected and the organs were freed of fat and connective tissues and organ weight was noted down.

**Histopathology**

Liver, kidney, stomach, intestine, spleen, pancreas, adrenal, lungs, heart, brain and gonads were fixed immediately in 10% formalin for routine histopathological examination. The tissues were embedded in paraffin, and then sectioned, stained with haematoxylin and eosin and were examined under light microscope. Histopathological evaluations were performed by pathologist. Photomicrographs of the microscopical sections were taken with the help of Motic photomicroscope (Canada) provided with Motic Images Plus 2.0 software.

**Observations**

General clinical observations were made once a day. All animals were observed for morbidity and mortality, twice daily. Different Signs noted include, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern).

Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. selfmutilation, walking backwards) were also recorded.

**Body Weight, Food and Water Intake**

Body weight, water and food intake were measured once a week.

**Hematology**

Hematological analysis was performed using an automatic hematological analyzer (Sysmex, Japan). Hemoglobin, hematocrit, total red blood corpuscles (RBC), total white blood corpuscles (WBC), platelets and red cell indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of blood samples were recorded.

**Clinical Biochemistry**
Cholesterol, high density lipoproteins (HDL), triglycerides (TGL), bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, albumin, blood urea nitrogen (BUN), creatinine, glucose were recorded, using an autoanalyzer (Erba Chem 7, Germany).

Pathological Examination

Gross Necropsy

All animals in the study were subjected to a full, detailed gross necropsy which included careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, lungs, kidneys, adrenals, gonads, spleen, heart and brain of all animals were removed and their wet weights were taken immediately after dissection to avoid drying.

4.2.8. Antidiabetic activity of ethyl acetate extract of Cassia absus in streptozotocin-induced diabetic rats

4.2.8.1 Induction of diabetes

Diabetes was induced in rats by the intraperitoneal (i.p.) injection of streptozotocin (STZ) at a dose of 55 mg/kg (i.p. dissolved in ice cold sodium citrate buffer, 0.01 M, pH 4.4). 48 hours after the injection, the plasma glucose levels were measured. Each animal with a plasma glucose level above 250 mg/dl was considered to be diabetic. The animals were divided into four groups- Group I diabetic control (n = 6), Group II and III, the ethyl acetate extract treated (250 and 500 mg/kg/day) (n=6) and Group IV glibenclamide (0.6 mg/kg) along with these groups, there was one age matched control group (n = 6) also. To overcome the hypoglycemia which occurred during the first 24 h following the STZ administration, diabetic rats were orally given 5% glucose solution. In all experiments, rats were fasted for 16 h prior to STZ injection.

4.2.8.2. Acute effects

The test samples (ethyl acetate extract and glibenclamide) were administered orally by using a oral feeding needle. Plasma glucose levels were determined at 30, 60, 120, 240 and 360 min after the administration of the test samples.

4.2.8.3. Subacute effects

The test samples (ethyl acetate extract and glibenclamide) were administered consecutively. Plasma glucose levels were determined on 7th, 14th, 21st and 28th days after the administration of test samples.
4.2.9 Docking studies: 116, 117

- To study the nature of interactions, binding mode and selectivity of insulin receptor protein with isolated compounds, docking was carried out with, Autodock 4.0 and Arguslab 4.0.1.

- **Protein**: The structure of Insulin receptor protein complexed with peptide substrate (PDB Code: 1IR3) was obtained from PDB.

- **Ligands**: Mol Inspiration, an online tool was used to identify suitable biological targets. Based on Lipinski’s Rule of Five probable ligands were selected.

- **Active Site Analysis**: Q-site Finder, an online tool which uses hydrophobic probes was used to predict possible binding sites. Energetically favorable probes sites were clustered and then ranked according to the sum of interaction energies. Ligand explorer of PDB was also used to study the interactions.

- **DOCKING SOFTWARES**

  - **Autodock 4.0**: Autodock 4.0 predicts the interaction of small molecules with macromolecular targets. Autodock performs the docking of the ligand to a set of grids (pre-calculated by Autogrid) describing the target protein. The energy grid was built within a cubic box of dimensions 40X16X50 Å with a spacing of 1.0 Å. The docking was performed based on Lamarckian Genetic Algorithm.

  - **Arguslab 4.0.1**: Arguslab 4.0.1 is Molecular modeling and Drug Docking software. It is very flexible and can reproduce crystallographic binding orientations. Arguslab, provides a user friendly graphical interface and uses Shape Dock algorithm, to carry out docking studies.