# CHAPTER-4

# MATERIALS & METHODS

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## 4.1 Phytochemical analysis of herbal extracts

### 4.1.1 Procurement of herbal extracts:

**Table 1 Herbal extract used in the tablet formulation**

| Extracts | Type of extract | Provider Company for Gift Sample |
|---|---|---|
| Aghedo extract | Hydro alcoholic | Vasu Research centre |
| Garcinia extract | Hydro alcoholic | Sun Pure |
| CurryLeaves Extract | Hydro alcoholic | Tulsi Amrit |
| Guggul extract | Ethyl acetate | Sun Pure |

### 4.1.2 Organoleptic analysis:

In this analysis all herbal extract are analyse by its colour, odour and compared with standard monographs.

### 4.1.3 Proximate Investigation of Herbal Extracts:

All herbal extracts are exposed to different chemical test to determine presence of different plant metabolites like alkaloids, tannin, glycoside, proteins, amino acid etc.

The pharmacological action of crude drugs is determined by the nature of its constituents. Plant species may therefore be considered as a biosynthetic laboratory not only for the chemical constituents that are utilized as food, but also for a multitude of compounds including alkaloids, terpenoids, flavonoids, glycosides, etc. which exert definite physiological effects. The phytochemistry of herbal drugs embraces a thorough consideration of these chemical entities that are termed as constituents. As the plant drugs contain so many phytoconstituents, it is essential to separate and screen them to find out which constituent is responsible for the physiological effect. For the identification of different phytoconstituents chemical tests are performed. The tests that were performed are given below:

**Table 4.2Chemical test used for phytochemical screening**

| Metabolites | Test | Procedure |
|---|---|---|
| **Carbohydrates** | Molisch's Test | To 2-3 ml of aq. extract add few drops of alpha naphthol solution in alcohol, shake and add conc. $H_2SO_4$ from sides of the test tube. A violet ring formed at the junction of two liquids indicates presence of carbohydrates. |
| **Protein** | Biuret Test | To 3 ml of aq. extract add 4% NaOH solution and few drops of 1% $CuSO_4$ solution. Appearance of violet or pink color indicates presence of protein. |
| | Precipitation Test | If protein is present, the test solution gives white colloidal precipitates with: 5% $HgCl_2$ solution, 5% $CuSO_4$ solution, 5% ammonium sulphate solution. |
| **Amino Acids** | Ninhydrin Test | To 3 ml of test solution add 3 drops of 5% ninhydrin solution and heat in a boiling water bath for 10 min. appearance of purple or bluish colour shows presence of amino acids. |
| **Saponins** | Foam Test | Shake the extract vigorously with water. Persistent foam indicates presence of saponins. |
| **Coumarins** | Fluorescence Test | The extract when made alkaline shows blue or green fluorescence if Coumarins are present. |
| **Tannins and Phenolics** | $FeCl_3$ Test | Add 5% $FeCl_3$ solution to the extract. Development of deep blue-black colour shows presence of tannins and phenolics. |
| **Alkaloids** | Dragendorff's Test | To 2 ml of extract add few drops of Dragendorff's reagent. Alkaloids give orange brown precipitates. |

### 4.1.4 TLC Profiling of Extracts:

- ✓ Traditionally, analytical TLC has found application in the detection and monitoring of compounds through a separation process. In the case of known natural products, qualitative and quantitative information can be gathered concerning the presence or

absence of a metabolite or breakdown product. Qualitative initial screening of extracts can be routinely performed to ascertain the presence of specific components with the help of TLC. All the extracts were subjected to screening for different class of compounds using the following mobile phase and detection:

| Extracts | Mobile Phase | Detection |
|---|---|---|
| *Achyranthes aspera* extract | Toluene:EthylAcetate:Methanol: Acetone(14:4:1:1) | Spray with Methanolic $H_2SO_4$ UV scan @ 3666 nm |
| *Garcenia indica* extract | n butanol: Formicacid: water (4:2:4) | UV scan @t 273 nm |
| *Murraya koenigii* Extract | Pet.Ether:Chloroform(7:3) | UV scan @ 254 nm |
| *Commiphora mukul* extract | Toluene: Acetone(9:1) | UV scan @240 nm |

**Table 4.3Thin Layer Chromatography system**

### 4.1.5 Microbial Analysis:

Raw materials of animal, plant and even mineral origin are frequently carriers of numerous, possibly pathogenic, microorganisms which can be passed on to subsequent processing stages. All starting material must therefore be stored in such a way that multiplication of any bacteria originally present is impossible. Normally a large number of bacteria and moulds are present in medicinal plant materials because of the contamination from the soil and environment. The practices of harvesting, handling and production may cause further contamination. Hence it is important to check and bring the microbial state to limits that are acceptable for consumption.

### 4.1.6 Loss on Drying (LOD):

It determines the amount of water and other volatile content in a substance. Moisture is an inevitable component of crude drugs which must be eliminated as far as possible. Excess moisture suggests that drug has been incorrectly prepared or incorrectly stored. It can lead to

breakdown of important constituents by enzymatic activity and may also encourage growth of yeast and fungi during storage.

Procedure:

- 10 g of the drug, accurately weighed, was taken in an evaporating dish.
- It was placed in an oven at 105 ºC for 5 hours and weighed.
- The drying was continued until constant weight was obtained. (Constant weight is reached when two consecutive weighing after drying for 30 minutes and cooling for 30 minutes in a desiccator show no more than 0.01 g difference.)

### 4.1.7 Heavy metal Analysis:

The medicinal plant materials can be contaminated with heavy metals which can be attributed to many causes including environmental pollution and pesticide residues. As these components even in trace amounts are dangerous, they have to be monitored. The heavy metals which are usually found and are tested in crude materials are: Lead, Arsenic, Cadmium and Mercury. The permissible limits for each are given below:

**Table 4.4Limit of Heavy metal**

| Heavy Metal | Permissible Limits (ppm) |
|---|---|
| Lead | 10 |
| Arsenic | 3 |
| Cadmium | 0.3 |
| Mercury | 1 |

## 4.2 Determination of Secondary metabolites:

### 4.2.1 Estimation of Total Phenolics content:

Total phenolics were determined in all the plant extracts using spectrophotometric method. Folin-Ciocalteu Assay method was used for the determination and gallic acid was taken as the reference standard.

Procedure:

- In a 25 ml volumetric flask, 1 ml of extract and 9 ml of distilled water were taken.

- 1 ml of Folin-Ciocalteu phenol reagent was added to the mixture and shaken well.
- After 5 minutes, 10 ml of 7% sodium carbonate solution was added and the volume was made upto 25 ml with distilled water.
- A set of standard solutions of gallic acid (20, 40, 60, 80, 100 ppm) were prepared in the same manner.
- The solutions were incubated at room temperature for 90 minutes.
- The absorbance was determined against reagent blank at 550 nm with a uv-visible spectrophotometer.
- Total phenol content was expressed as %w/w and was calculated from the regression equation obtained from the calibration curve of gallic acid.

### 4.2.2 Estimation of Total tannins content:

The reactivity of condensed tannins with molecules of biological significance such as proteins and polysaccharides has important physiological consequences, and hence its determination in plant materials is important. Total Tannins were estimated in the water extracts of all plants by AOAC Method. It is based on a redox titration using aq. solution of potassium permanganate as the titrant.

Procedure:

- 25 ml of the test solution was taken in a conical flask, then, 25 ml of indigo solution and 750 ml of distilled water were added to it.
- 0.1 N aq. solution of $KMnO_4$ was used for titration until blue coloured solution changed to green colour.
- Then few drops at time were added until solution became golden yellow.
- The readings were taken in triplicate.
- Blank reading was also taken.
- Standard solution of indigo carmine was prepared as following: 6 g indigo carmine was dissolved in 500 ml of distilled water by heating. After cooling 50 ml of conc. $H_2SO_4$ was added and the solution was diluted upto 1 litre and then filtered.

Calculation: The % tannin content was calculated by the following formula:

$$T(\%) = \frac{(V - V_0)\ x\ 0.004157\ x\ 250\ x\ 100}{g\ x\ 25}$$

Where, V=volume of 0.1 N aq. solution of $KMnO_4$ required for titration of sample; $V_0$=volume of 0.1 N aq. solution of $KMnO_4$ required for blank; g=mass of sample taken for analysis.

### 4.2.3 Estimation of Total flavonoids:

Flavonoids were determined in all the plant extracts using spectrophotometric method. Aluminium chloride colorimetric assay was employed for the estimation and quercetin was taken as the reference standard.

Procedure:

- In a 10 ml volumetric flask, 1 ml of extract and 4 ml of distilled water were taken.
- To it, 0.3 ml of 5% sodium nitrite solution was added and after 5 minutes 0.3 ml of 10% aluminium chloride was added and mixed well.
- After 5 minutes, 2 ml of 1 M sodium hydroxide solution was added and the volume was made upto 10 ml with distilled water.
- A set of standard solutions of quercetin (20, 40, 60, 80, 100 ppm) were prepared in the same manner.
- The absorbance was determined against reagent blank at 510 nm with a uv-visible spectrophotometer.
- Flavonoid content was expressed as %w/w and was calculated from the regression equation obtained from the calibration curve of quercetin.

## 4.3 Formulation and Development:

Excipients used for tablet formulation: Avicel PH 102, Talc, Magnesium Stearate, SSG, Cross Carmellose sodium, Syloid silica

Equipment used: Compression machine, digital weighing balance etc.

### 4.3.1 Dose Selection:

Dose of herbal extract is selected on the basis of different literature and available marketed formulation

**Table 4.5**

| Sr.No. | Plant Extract | Dose |
|---|---|---|
| **1** | *Commiphora mukul* | 80-200mg |
| **2** | *Murraya koenigii* | 75-250 mg |
| **3** | *Garcinia Indica* | 80 -500 mg |
| **4** | *Achyranthus aspera* | 70 -300 mg |

### 4.3.2 Optimization of excipients is done by D optimal Factorial design software

#### 4.3.2.1 Variables used to check

**Table 4.6 variables**

| Sr. No. | Variables | Lower limit | Upper limit | Unit |
|---|---|---|---|---|
| **1.** | Avicel PH102 | 10 | 20 | gram |
| **2.** | SSG(Sodium starch Glycolate) | 0.5 | 10 | gram |
| **3.** | Cross Carmellose sodium | 0.5 | 5 | gram |
| **4** | Syloid silica | 0.25 | 5 | gram |

#### 4.3.2.2 The obtained design matrix after

**Table 4.7 Design Matrix**

| Sr. No. | Avicel PH 102 | Sodium starch glycolate | Cross carmellose sodium | Syloid slilica |
|---|---|---|---|---|
| **1.** | 12 | 0.5 | 0.5 | 4.98 |
| **2.** | 13 | 6.68 | 0.57 | 0.25 |
| **3.** | 10 | 8.86 | 0.5 | 0.25 |
| **4.** | 18 | 10 | 0.5 | 4.68 |

| | | | | |
|---|---|---|---|---|
| **5.** | 10 | 5.0 | 0.54 | 3.0 |
| **6.** | 20 | 2.86 | 5.05 | 0.25 |
| **7.** | 14 | 2.22 | 2.98 | 2.56 |
| **8.** | 10 | 0.54 | 0.5 | 0.25 |
| **9.** | 18 | 5.58 | 7.5 | 0.00 |
| **10.** | 20 | 0.5 | 9.5 | 0.25 |
| **11.** | 15 | 5.0 | 5 | 0.25 |
| **12.** | 20 | 0.5 | 0.5 | 0.50 |
| **13.** | 11 | 4.32 | 0.5 | 3.0 |
| **14.** | 10 | 5.0 | 10 | 0.50 |
| **15.** | 10 | 4.95 | 9.8 | 0.5 |
| **16.** | 18 | 5.0 | 10 | 2.88 |
| **17.** | 16 | 5.0 | 7.5 | 3.00 |

### 4.3.2.3 Desired values of variables:

**Table 4.8 Received desired responses**

| Sr. No. | Variable | Goals |
|---|---|---|
| 1. | Avicel PH102 | Target |
| 2. | Sodium Starch Glycocollate | Maximize |
| 3. | Cross Carmellose sodium | Maximize |
| 4 | Syloid silica | Maximize |
| 5 | Disintegration time(Minute) | Minimize |
| 6 | Hardness | Maximize |

### 4.3.2.4 Results of Run in software:

| | Std | Run | Block | Factor 1 A:Avicel PH10 gm | Factor 2 B:S.S.G gm | Factor 3 C:C.C.S. gm | Factor 4 D:Syloid gm | Response 1 Disintegration t Minutes | Response 2 Hardness Kg/cm2 |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 1 | Block 1 | 10.00 | 0.50 | 0.50 | 5.00 | 16 | 2.9 |
| | 17 | 2 | Block 1 | 14.10 | 6.64 | 0.57 | 0.25 | 15 | 3.5 |
| | 14 | 3 | Block 1 | 10.00 | 8.93 | 0.50 | 0.25 | 11 | 2.1 |
| | 3 | 4 | Block 1 | 20.00 | 10.00 | 0.50 | 5.00 | 15 | 4.9 |
| | 1 | 5 | Block 1 | 10.00 | 0.50 | 5.00 | 3.00 | 14.5 | 2.6 |
| | 10 | 6 | Block 1 | 20.00 | 5.03 | 2.86 | 0.25 | 19.5 | 5.6 |
| | 16 | 7 | Block 1 | 14.05 | 2.91 | 2.19 | 2.00 | 17 | 3.9 |
| | 5 | 8 | Block 1 | 10.00 | 0.50 | 0.50 | 0.25 | 17 | 3.1 |
| | 8 | 9 | Block 1 | 16.00 | 7.50 | 5.00 | 0.00 | 13 | 3.5 |
| | 13 | 10 | Block 1 | 20.00 | 10.00 | 0.50 | 0.25 | 17 | 4.2 |
| | 7 | 11 | Block 1 | 16.00 | 5.01 | 5.00 | 0.25 | 15.5 | 3.8 |
| | 9 | 12 | Block 1 | 20.00 | 0.50 | 0.50 | 0.50 | 25 | 6.2 |
| | 11 | 13 | Block 1 | 11.00 | 0.50 | 4.32 | 3.00 | 16 | 5.2 |
| | 6 | 14 | Block 1 | 10.00 | 10.00 | 5.00 | 0.50 | 9 | 2 |
| | 4 | 15 | Block 1 | 10.00 | 10.00 | 5.00 | 0.50 | 6.5 | 1.8 |
| | 12 | 16 | Block 1 | 20.00 | 10.00 | 5.00 | 2.00 | 13 | 3.4 |
| | 15 | 17 | Block 1 | 16.00 | 7.50 | 5.00 | 3.00 | 10.5 | 4 |

**Figure 4.1 Rsult of run with desired value**

### 4.3.2.5 Statistical analysis for Disintegration time:

To determine the parameters ANOVA is performed.

**Response 1 Disintegration time**

**ANOVA for Response Surface Linear Model**

**Analysis of variance table [Partial sum of squares - Type III]**

| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|
| Model | 276.56 | 4 | 69.14 | 87.37 | < 0.0001 | significant |
| *A-Avicel PH102* | *110.48* | *1* | *110.48* | *139.61* | *< 0.0001* | |
| *B-S.S.G* | *149.53* | *1* | *149.53* | *188.97* | *< 0.0001* | |
| *C-C.C.S.* | *25.43* | *1* | *25.43* | *32.14* | *0.0001* | |
| *D-Syloid* | *7.31* | *1* | *7.31* | *9.24* | *0.0103* | |
| Residual | 9.50 | 12 | 0.79 | | | |
| *Lack of Fit* | *6.37* | *11* | *0.58* | *0.19* | *0.9596* | *not significant* |
| *Pure Error* | *3.13* | *1* | *3.13* | | | |
| Cor Total | 286.06 | 16 | | | | |

**Figure 4.2 Statistical analysis using ANOVA**

From the above analysis f value of 87.37 implies the model is important. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

**Statistical analysis for Disintegration time**

| | | | |
|---|---|---|---|
| Std. Dev. | 0.89 | R-Squared | 0.9668 |
| Mean | 14.74 | Adj R-Squared | 0.9557 |
| C.V. % | 6.04 | Pred R-Squared | 0.9355 |
| PRESS | 18.44 | Adeq Precision | 35.772 |

**Figure 4.3 Statistical analysis using ANOVA**

This above result shows that Pred R-Squared of 0.9355 is in realistic agreement with the Adj R-Squared of 0.9557. Adeq Precision shows noise to signal ratio.

$$\text{Disintegration time} = +14.97 + 3.27 * A - 3.99 * B - 1.37 * C - 0.97 * D$$

Form above analysis suggests that all excipients affect the DT.

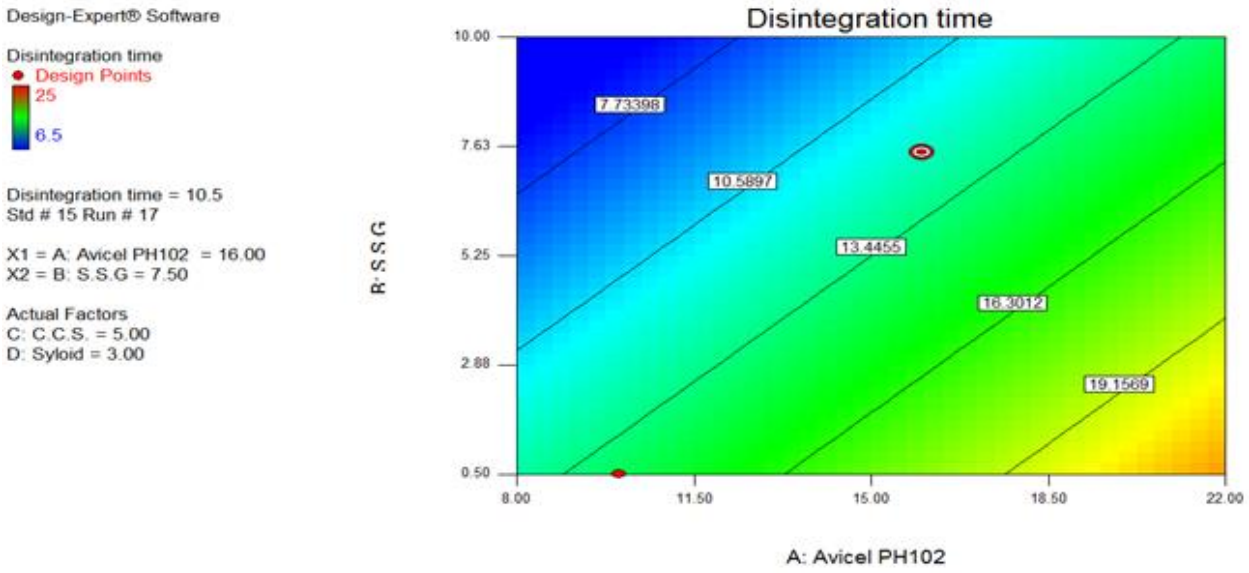



**Figure 4.4 Plot contour for Disintegration time**

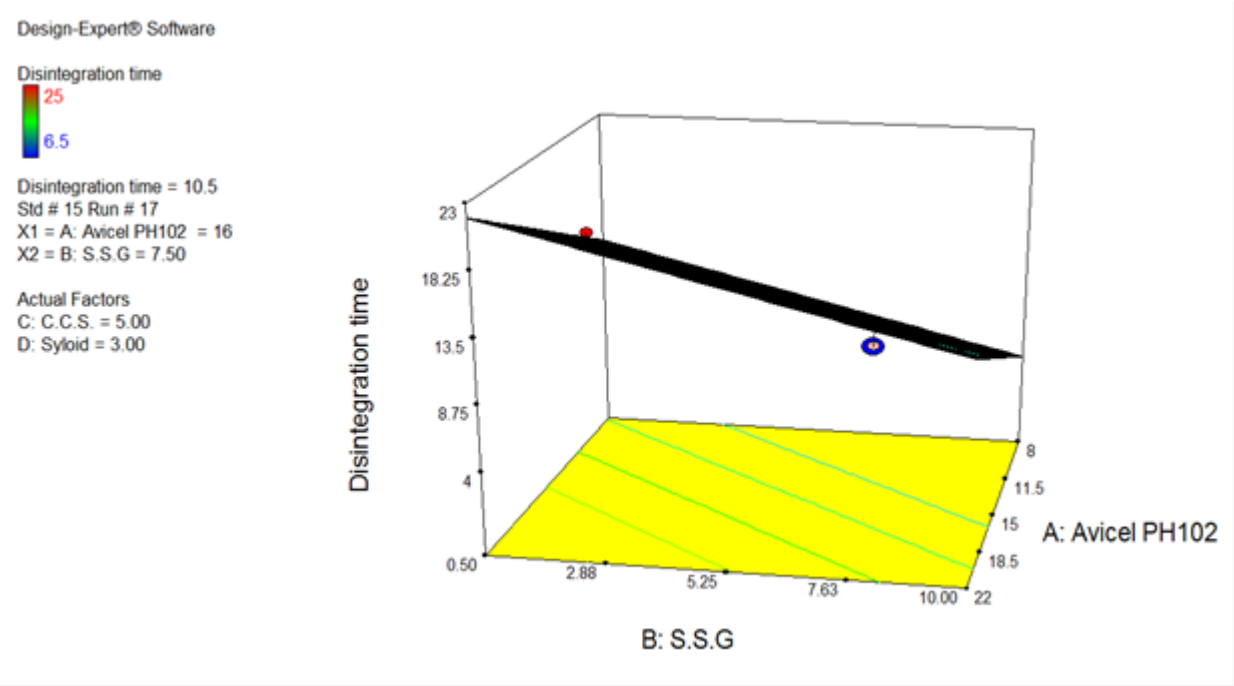



**Figure 4.5 3D surface graph for DT**

### 4.3.2.6 Statistical analysis for Hardness:

To find the important parameters and their interaction and analysis of variance is performed for each parameter.

**Response 2 Hardness**

**ANOVA for Response Surface Linear Model**

**Analysis of variance table [Partial sum of squares - Type III]**

| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|
| Model | 20.29 | 4 | 5.07 | 11.97 | 0.0004 | significant |
| *A-Avicel PH102* | *16.95* | *1* | *16.95* | *39.98* | *< 0.0001* | |
| *B-S.S.G* | *6.20* | *1* | *6.20* | *14.63* | *0.0024* | |
| *C-C.C.S.* | *0.038* | *1* | *0.038* | *0.091* | *0.7684* | |
| *D-Syloid* | *0.028* | *1* | *0.028* | *0.067* | *0.8006* | |
| Residual | 5.09 | 12 | 0.42 | | | |
| *Lack of Fit* | *5.07* | *11* | *0.46* | *23.03* | *0.1613* | *not significant* |
| *Pure Error* | *0.020* | *1* | *0.020* | | | |
| Cor Total | 25.38 | 16 | | | | |

**Figure 4.6 Statistical analysis for Hardness**

Above result shows F-value of 11.97 suggested that model is significant.

**Statistical analysis study Results for Hardness:**

| | | | |
|---|---|---|---|
| Std. Dev. | 0.65 | R-Squared | 0.7996 |
| Mean | 3.69 | Adj R-Squared | 0.7328 |
| C.V. % | 17.65 | Pred R-Squared | 0.5706 |
| PRESS | 10.90 | Adeq Precision | 12.160 |

The above result of ANNOVA suggested that this model is use the design space.

**Calculation in Terms of Coded Factors:**

$$\text{Hardness} = +3.90 + 1.28 * A - 0.81 * B - 0.053 * C + 0.060 * D$$

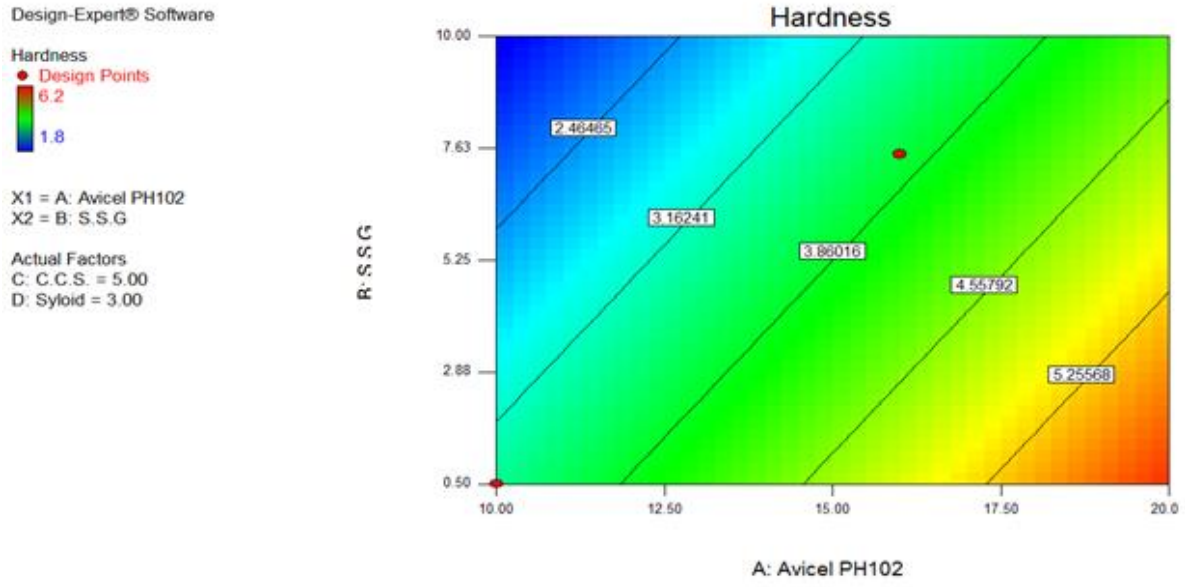



**Figure 4.7 Contour plot for Hardness**

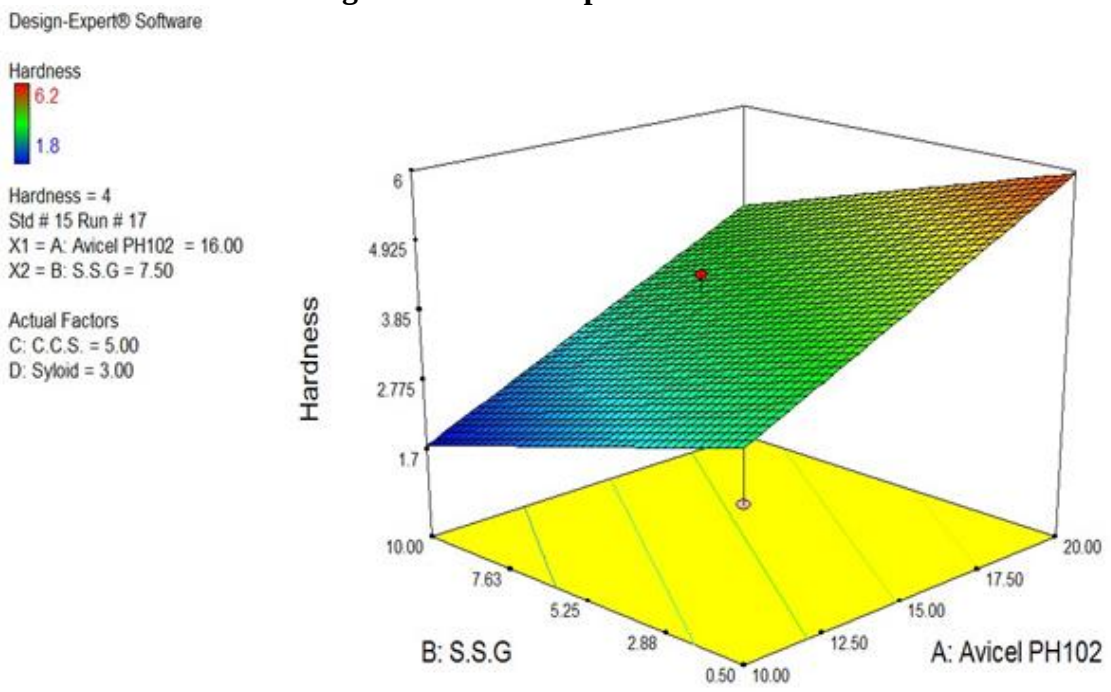


**Figure 4.8 3D surface plot for Hardness**

### 4.3.3 Finalize the formula by Desirability Curve

For Finalize the formula desirability curve is used. Based on the upper and lower limit

| Name | Goal | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|---|---|---|---|---|---|---|
| Avicel PH | is target = 16.00 | 10 | 16 | 1 | 1 | 3 |
| S.S.G | maximize | 0.5 | 7.5 | 1 | 1 | 3 |
| C.C.S. | maximize | 0.5 | 5 | 1 | 1 | 3 |
| Syloid | maximize | 1 | 3 | 1 | 1 | 3 |
| Disintegra | minimize | 6.5 | 15 | 1 | 1 | 3 |
| Hardness | maximize | 1.8 | 6.2 | 1 | 1 | 3 |

**Final formula has maximum desirability**

| Number | Avicel PH102 | S.S.G | C.C.S. | Syloid | Disintegration | Hardness | Desirability |
|---|---|---|---|---|---|---|---|
| 1 | 16.00 | 7.50 | 5.00 | 3.00 | 12.2111 | 3.73134 | 0.724 |
| 2 | 15.89 | 7.50 | 5.00 | 3.00 | 12.1418 | 3.7043 | 0.723 |
| 3 | 16.00 | 7.44 | 5.00 | 3.00 | 12.2625 | 3.74219 | 0.721 |
| 4 | 16.00 | 7.50 | 4.95 | 3.00 | 12.2434 | 3.73299 | 0.721 |
| 5 | 15.90 | 7.50 | 5.00 | 2.93 | 12.1721 | 3.70415 | 0.718 |
| 6 | 16.00 | 7.35 | 5.00 | 3.00 | 12.3405 | 3.75764 | 0.717 |
| 7 | 15.37 | 7.50 | 5.00 | 3.00 | 11.7996 | 3.57065 | 0.717 |
| 8 | 15.15 | 7.50 | 5.00 | 2.99 | 11.6576 | 3.51371 | 0.713 |
| 9 | 16.00 | 7.50 | 5.00 | 2.85 | 12.2724 | 3.72796 | 0.712 |
| 10 | 16.00 | 7.50 | 4.74 | 3.00 | 12.3683 | 3.73783 | 0.710 |
| 11 | 14.87 | 7.50 | 5.00 | 2.98 | 11.4772 | 3.44156 | 0.707 |
| 12 | 14.80 | 7.49 | 5.00 | 3.00 | 11.4356 | 3.42681 | 0.706 |
| 13 | 15.42 | 7.19 | 5.00 | 3.00 | 12.0899 | 3.636 | 0.706 |
| 14 | 16.00 | 7.50 | 4.59 | 3.00 | 12.4621 | 3.74144 | 0.702 |
| 15 | 14.83 | 7.50 | 5.00 | 2.88 | 11.4912 | 3.42821 | 0.698 |
| 16 | 16.00 | 7.50 | 5.00 | 2.68 | 12.3417 | 3.72355 | 0.697 |
| 17 | 15.77 | 7.26 | 5.00 | 2.75 | 12.3637 | 3.70789 | 0.692 |
| 18 | 14.17 | 7.50 | 5.00 | 3.00 | 11.0141 | 3.26283 | 0.690 |
| 19 | 14.90 | 7.50 | 5.00 | 2.69 | 11.6201 | 3.44273 | 0.684 |
| 20 | 13.73 | 7.50 | 5.00 | 3.00 | 10.7274 | 3.15069 | 0.677 |
| 21 | 14.98 | 6.53 | 5.00 | 3.00 | 12.36 | 3.63709 | 0.673 |
| 22 | 13.26 | 7.50 | 5.00 | 3.00 | 10.4184 | 3.02967 | 0.659 |
| 23 | 14.46 | 7.50 | 5.00 | 2.41 | 11.441 | 3.32088 | 0.650 |
| 24 | 16.00 | 7.50 | 3.98 | 2.45 | 13.0562 | 3.74211 | 0.620 |
| 25 | 15.61 | 6.47 | 5.00 | 2.31 | 13.1086 | 3.79225 | 0.612 |

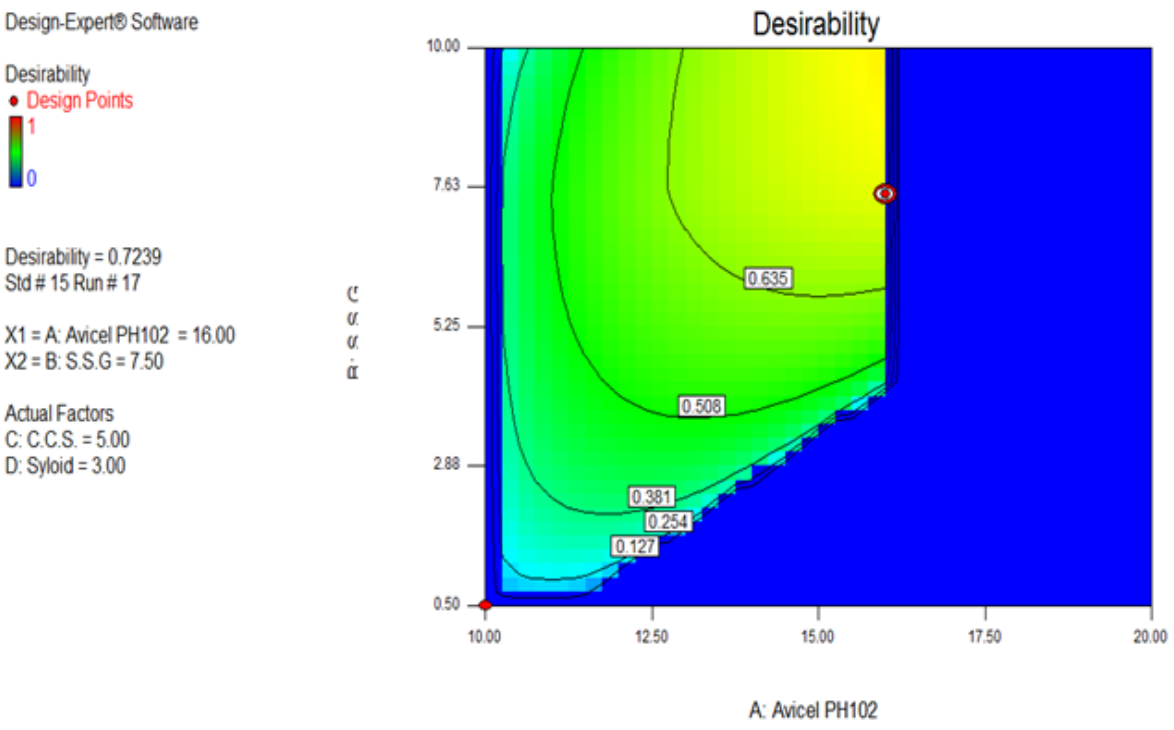



**Figure 4.9 Contour plot or final batch**

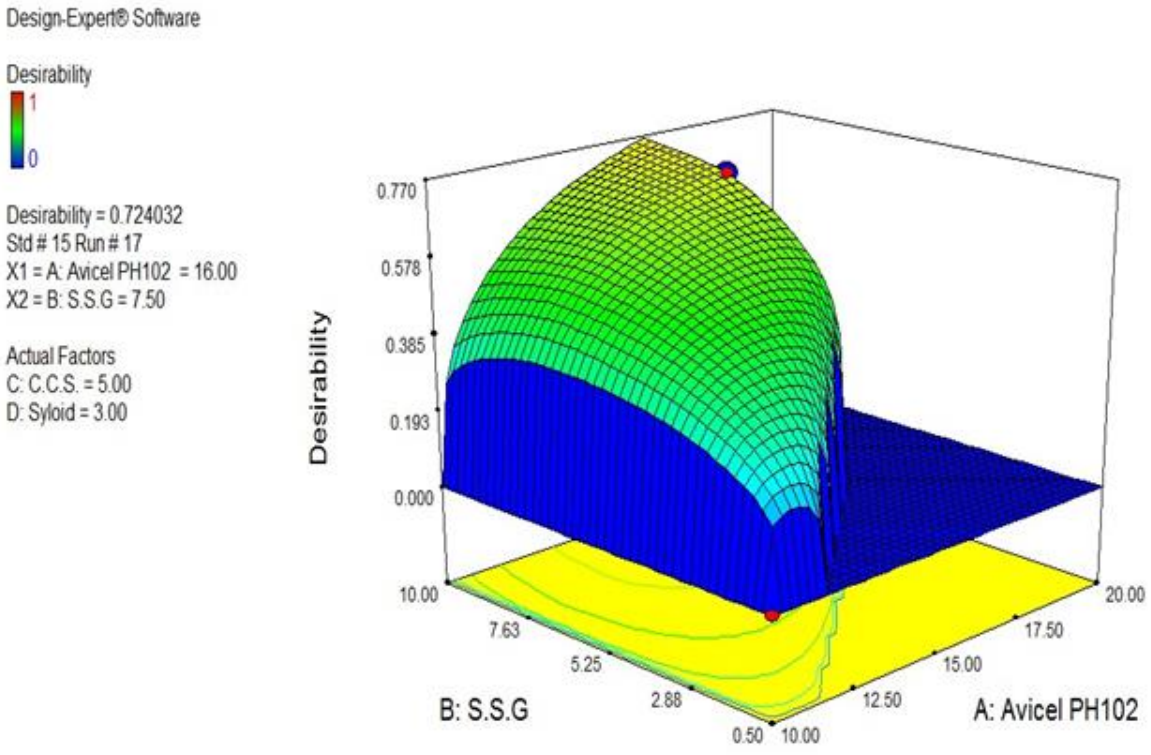



**Figure 10 D surface) for final batch**

## Overlay Plot

Overlay plot is used for making a design space. Design space is formed in such a mode that the response would be between a specific limit.

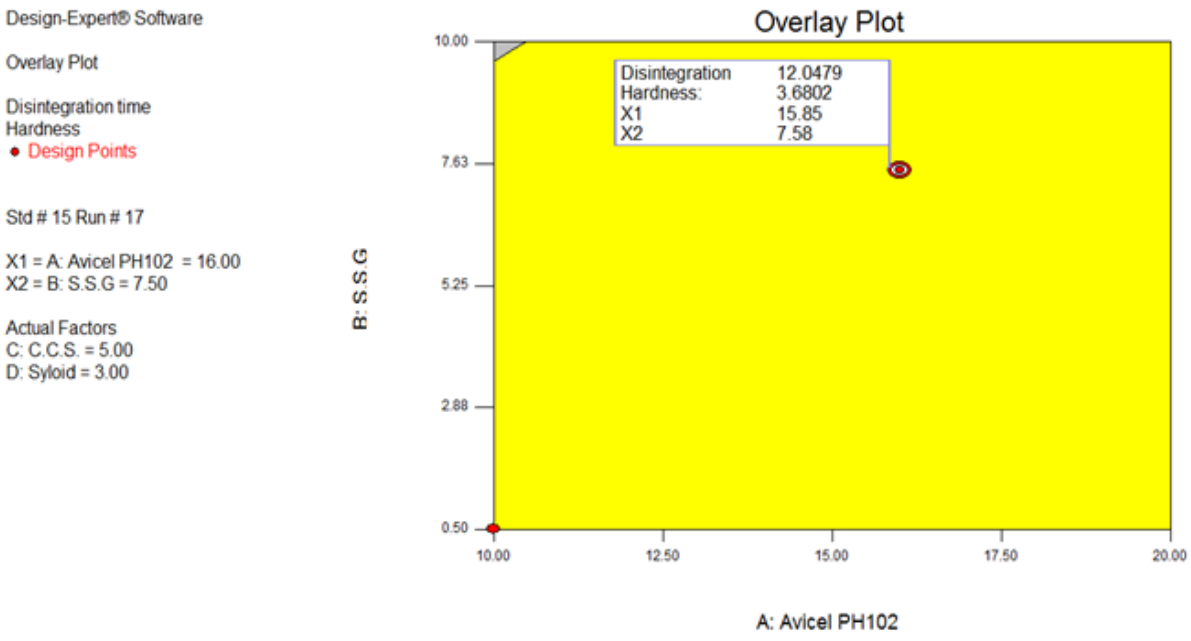



**Figure 11Overlay plot**

### 4.3.1 Batch Analysis by check point:

**Table 4.9T-Test Analysis**

| Sr. No. | DT (Minutes) | | Hardness(Kg/cm2) | |
|---|---|---|---|---|
| | Projected value | Practical value | Projected value | Practical value |
| 1. | 10.50 | 9.50 | 3.50 | 3.80 |
| 2. | 10.50 | 10 | 3.50 | 3.50 |
| 3. | 10.50 | 9.80 | 3.50 | 3.40 |
| T-TEST value | 0.2276 | | 0.6347 | |

The result of T-Test analysis, we observed complies within the standard value showed in the above table. Hence we can predict that model ss validated.

**Point Prediction and Confirmation:**

| Factor | Name | Level | Low Level | High Level | Std. Dev. | Coding |
|---|---|---|---|---|---|---|
| A | Avicel PH102 | 15.00 | 10.00 | 20.00 | 0.000 | Actual |
| B | S.S.G | 5.25 | 0.50 | 10.00 | 0.000 | Actual |
| C | C.C.S. | 2.75 | 0.50 | 5.00 | 0.000 | Actual |
| D | Syloid | 2.63 | 0.25 | 5.00 | 0.000 | Actual |

| Response | Prediction | SE Mean | 95% CI low | 95% CI high | SE Pred | 95% PI low | 95% PI high |
|---|---|---|---|---|---|---|---|
| Disintegration t | 14.9683 | 0.26 | 14.40 | 15.54 | 0.93 | 12.95 | 16.99 |
| Hardness | 3.90396 | 0.19 | 3.49 | 4.32 | 0.68 | 2.43 | 5.38 |

From above table we can predict that we can achieve the disintegration in the range of 12.95 - 16.99 minutes and hardness 2.43 - 5.38 kg/cm$^2$.

### 4.3.2 Final concentration of excipients for tablet formulation.

**Table 4.10 Final concentration of excipients for tablet formulation**

| Excipients | Range | Final selected concentration |
|---|---|---|
| **Avicel PH 102** | 10-20% | 16.66% |
| **Syloid silica** | 0.25-5% | 2.66% |
| **Sodium starch glycolate** | 0.5-10% | 7.5% |
| **Cross carmellose sodium** | 0.5-5% | 5% |
| **Talc** | 1% | 1% |
| **Methyl paraben** | 1% | 1% |

### 4.3.3 Stability Study:

Stability study is used for the prediction of the shelf life of the herbal formulation. It is more important for the herbal formulation because it is very sensitive to moisture and microbial contamination. Tablet formulation was kept in sealed container and put in desiccators for 3 months. At the end of three month, the tablets were analysed.

## 4.4 Analytical method development using HPLC:

### 4.4.1 Analytical method development:

**Chemical and reagents:**

Oleanolic acid is purchased from Sigma Aldrich. Gallic acid is purchased from Sulab. Other reagents and chemical are purchased from Rankem and all are HPLC grade.

**Instrument Specification:**

| | |
|---|---|
| **Equipment** | HPLC SHIMADZU LC-20AD |
| **Column** | Hyperchrom 5µ C18 |
| **Detector** | SHIMADZU SPD-20A with UV/VIS Detector |
| **Injector** | Rheodyne 7725 injector |
| **Software** | LC solution |

#### 4.4.1.1 Standard solution preparation.

**Standard solution Preparation (Gallic Acid)**

Take 10 mg Gallic acid and dissolve in 10 ml of methanol. (1000 µg/ml = 1ppm) form this prepared stock solution 1 ml of taken and diluted to 10ml using methanol.

**Standard solution Preparation (Oleanolic acid)**

Take 10 mg Oleanolic acid and dissolve in 10 ml of methanol. (1000 µg/ml = 1ppm) form this prepared stock solution 1 ml of taken and diluted to 10ml using methanol.

#### 4.4.1.2 Teat sample Preparation:

About 10 prepared herbal tablets are crushed and dissolve in 50 ml of methanol and then solution is filtered. From this solution 1 ml is taken and diluted up to 10 ml with methanol.

**Lambda Max Selection for UV scanner**

Both the selected markers are subjected to full UV scan at between 200-400nm. Form the scan data intercept is selected as a final scan wavelength for the detection.

**Mobile Phase optimisation:**

Mobile phase is optimizing by traditional trial and error techniques. Different solvents, like Methanol, Water, Acetonitrile and Orthophosphoric acid in different ratio. To finalize the mobile phase different parameter are observed like retention time, shape of the peak and tailing.

### 4.4.2 Method Validation parameters:

Developed Method is validated according to the ICH Guidelines and data complying with the standards were obtained. All validation parameters were check for developed method. Validation parameter includes Accuracy, Precision, Specificity, Selectivity, Linearity, LOD, LOQ, and Robustness.

**Linearity:**

Physical mixture with known concentration is prepared and injected in increasing concentration. From the above run the peak area is recorded. Graph is plotted peak area Vs concentration of markers.

**Table 4.11Conc. of Gallic acid and Oleanolic acid**

| Sr. No | Conc. Of Gallic acid in µg/ml | Conc. Of oleanolic acid in µg/ml |
|---|---|---|
| 1. | 1 | 50 |
| 2. | 2 | 60 |
| 3. | 3 | 70 |
| 4. | 4 | 80 |
| 5. | 5 | 90 |
| 6. | 6 | 100 |

**Precision**

The precision is determined by replicate analysis. For intraday precision, each Marker is analyzed at three time intervals in a single day for three different concentrations. For interday precision, each Marker is analyzed at three consecutive

days for three different concentrations. The variation in the data is represented as %RSD.

**Robustness**

In this parameter the method is explored under different conditions like changes in the composition ratio of mobile phase, temperature, and flow rate of mobile phase.

**Limit of Detection and Limit of Quantification:**

The LOD and LOQ represent sensitivity of the method and were determined using the following equation:

$LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$

Where, $\sigma$= average standard deviation; S= slope of the response

**Accuracy:**

The accuracy of the method was determined by the Standard Addition Method. Known amount of Markers were added to the sample at three different levels (80, 100 and 120 %) and the mixture was analysed. Percent Recovery was calculated as the mean of three determinations at each standard addition level.

**4.5 Analytical method development using HPTLC:**

**Chemical and reagents:**

Oleanolic acid and E-Guggulsterone is purchased from Sigma Aldrich. Gallic acid is purchased from Sulab. Other reagents and chemical are purchased from Rankem and all are HPLC grade.

**Instrumental Specification:**

HPTLC aluminium plates pre-coated with silica gel 60 F254 were used as the stationary phase in this study. Sample application was done using Camag Linomat V Sample Applicator. The linear ascending development was carried out in a Camag twin trough glass chamber saturated with mobile phase. Scanning was done using Camag Linomat Scanner 3. Data acquisition and integration was performed using winCATS software version 1.2.3

**4.5.1.1Standard solution preparation**

**Standard solution Preparation (Gallic Acid)**

Take 10 mg Gallic acid and dissolve in 10 ml of methanol. (1000 µg/ml = 1ppm) form this prepared stock solution 1 ml of taken and diluted to 10ml using methanol.

**Standard solution Preparation (Oleanolic acid)**

Take 10 mg Oleanolic acid and dissolve in 10 ml of methanol. (1000 µg/ml = 1ppm) form this prepared stock solution 1 ml of taken and diluted to 10ml using methanol.

**Standard solution Preparation (**E**-Guggulsterone)**

Take 10 mg E-Guggulsterone dissolved in 10 ml Methanol to prepare 1000 µg/ml solution. Dilute 2 ml of this solution up to 10 ml methanol to prepare 200µg/ml

**4.5.1.2 Teat sample Preparation:**

About 10 prepared herbal tablets are crushed and dissolve in 50 ml of methanol and then solution is filtered. From this solution 1 ml is taken and diluted up to 10 ml with methanol. Sample application is done by using Linomat applicator.

**4.5.2. Mobile Phase optimisation**

Mobile phase is optimizing by traditional trial and error techniques. Different solvents, like Methanol, Water, Toluene and ethyl acetate in different ratio. Final mobile phase is selected based on the good resolution and separation of spot and retention factor is considered.

### 4.5.2.1 Method Validation parameter:

Developed Method is validated according to the ICH Guidelines and data complying with the standards were obtained. All validation parameters were check for developed method. Validation parameter includes Accuracy, Precision, Specificity, Selectivity, Linearity, LOD, LOQ, and Robustness. .

**Linearity:**

Linearity of the method is performed by analysing three markers in increasing concentration spotted on plate and then scan and area is calculated. The calibration curve is plotted by using the peak area v/s concentration of compound.

**Table 4.12HPTLC Linearity table**

| Sr.No | Injection volume (µl) |
|---|---|
| **1.** | 5 |
| **2.** | 10 |
| **3.** | 15 |
| **4.** | 20 |
| **5.** | 25 |
| **6.** | 30 |

**Precision**

The precision is determined by replicate analysis. For intraday precision, each Marker is analyzed at three time intervals in a single day for three different concentrations. For interday precision, each Marker is analyzed at three consecutive days for three different concentrations. The variation in the data is represented as %RSD.

**Robustness**

In this parameter the method is explored under different conditions like changes in the composition ratio of mobile phase, temperature, and flow rate of mobile phase.

**Limit of Detection and Limit of Quantification:**

The LOD and LOQ represent sensitivity of the method and were determined using the following equation:

LOD= 3.3σ/S and LOQ= 10σ/S

Where, σ= average standard deviation; S= slope of the response

**Accuracy:**

The accuracy of the method was determined by the Standard Addition Method. Known amount of Markers were added to the sample at three different levels (80, 100 and 120 %) and the mixture was analysed. Percent Recovery was calculated as the mean of three determinations at each standard addition level.

**HPTLC method is used for developed formulation:**

Above Method is used for the quantification of marker compounds in the developed herbal tablets. Finally quantification is done by following quation.

$$\% \text{ Assay} = \frac{Area\ of\ sample\ x\ Std\ wt\ taken\ x\ Sample\ dilution}{Area\ of\ std\ x\ Std\ dilution\ x\ Sample\ wt\ taken} \text{ x } 100$$

**HPTLC of Mahanimbine:**

**Sample preparation:**

Standard stock solution (1µg/ml) was prepared in HPLC grade methanol. Sample solution was also prepared in HPLC grade methanol.

**Sample:**

5µL of the extract was applied.

Mobile phase: n-hexane: ethyl acetate (9:1)

Stationary phase: The stationary phase was precoated plate, Aluminum oxide 150 F254, neutral

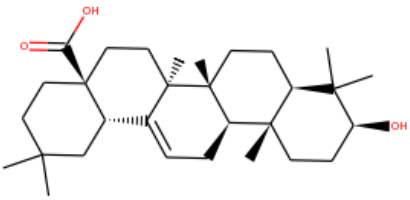
**Detection:**

The plate was scanned at 254 nm. The derivatization can also be done with 5 or 10 % alcoholic H2SO4

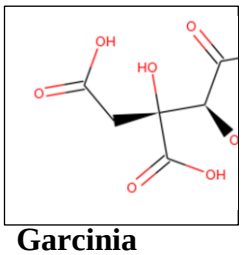
## 4.6. Anti-obesity evaluation of markers present in selected herbs using In Silico Approach.

### 4.6.1 Markers and selected class of drugs for evaluation.

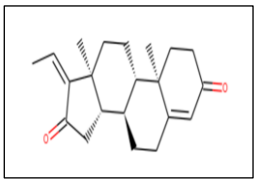
**A**



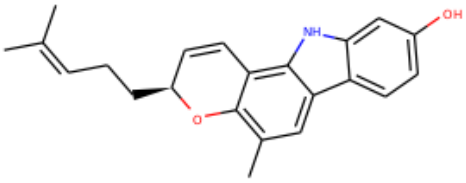
**Oleanolic acid**



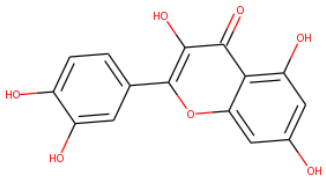
**Garcinia**



**Guggulsterone**

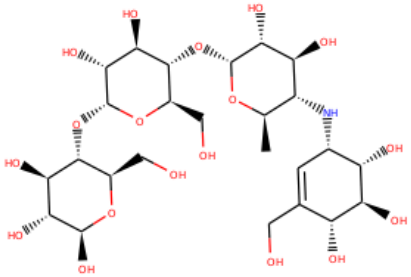


**Mahanine**

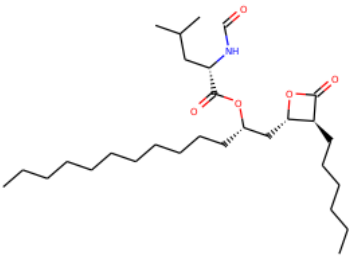


**Quercetin**

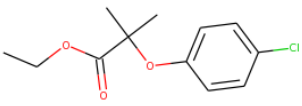
**B**



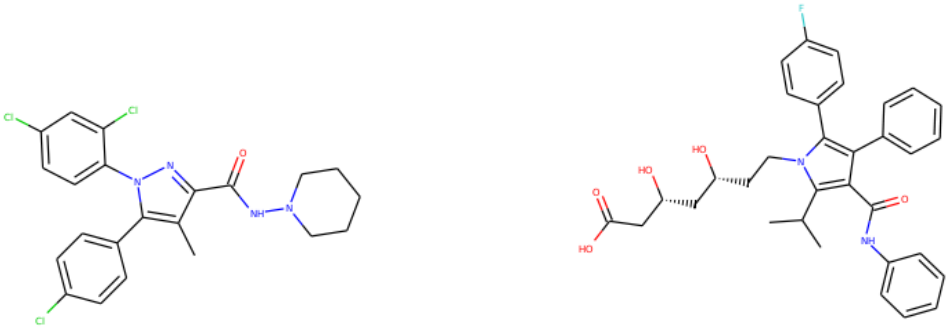
**Acarbose**



**Orlistat**



**Clofibrate**



**Rimonabant** **Atorvastatin**

**Figure 4.12Structures of (A) Natural Products NPs and (B) Drug molecules collected from ZINC database**

### 4.6.2 Pancreatic α-Amylase (Inhibitory activity):

#### 4.6.2.1 Molecular Docking Study:

**Identification of Active site of α-Amylase:**

The PDB structure (6GXV) of the AliC GH13 alpha-amylase from *Alicyclobacillus* sp. from www.rcsb.org has been selected for the molecular docking activity. Co Crystallized structure of alpha amylase is available in complex with alpha-glucosidase inhibitors Acarbose. A good resolution (2.07 A) X-Ray diffraction protein structure showed interactions with D-glucopyranose. Based on the interactions of glucopyranose observed with the protein structures, active site of the protein structure has been identified. The identified active site has been again confirmed with the online server 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html).

**Molecular Docking using Autodock Vina:**

To obtain details about the binding interaction as well as the relative orientation of the natural products (NPs), molecular docking was used. To obtain the comparative results, the standard drugs are also considered for the molecular docking. The protein structure of alpha-amylase was obtained from the protein data bank (PDB id: 6GXV, resolution: 2.07 Å). Autodock MGL Tool was used to modify the protein (alpha-amylase) and ligands (NPs and Drug molecules) structures. The protein (alpha-amylase) was prepared by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. After which Grid box

was prepared according to the identified active site. Center Grid box was selected using x: 66.817, y: 24.937, z: -6.517, and the number of points in all dimensions x,y,z were considered 40x40x40 Å , and the grid spacing was selected as 0. 50 Å.

The structures of NPs and the standard drugs were downloaded from the ZINC database. Addition of Gasteiger charges, detecting root, and choosing torsions was done from the Autodock Tools panel's torsion tree for the preparation of ligands. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking of natural products as well as drugs was carried out using AutoDock vina on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.

### 4.6.3 Pancreatic Lipase:

#### 4.6.3.1Molecular Docking Study

**Identification of Active site of human gastric lipase**

The PDB structure (1HLG) of recombinant human gastric lipase with the resolution of 3.0-Å was selected which is described under mammalian acid lipase family. The Crystallized structure of human gastric lipase is available in complex with 2-acetamido-2-deoxy-beta-D-glucopyranose. Based on the interactions of glucopyranose observed with the protein structures, active site of the protein structure has been identified. The identified active site has been again confirmed with the online server 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html).

**Molecular Docking using Autodock Vina**

To obtain details about the binding interaction as well as the relative orientation of the natural products (NPs), molecular docking was used. To obtain the comparative results, the standard drugs are also considered for the molecular docking. The protein structure of human gastric lipase was obtained from the protein data bank (PDB id: 1HLG). Autodock MGL Tool was used to modify the protein (human gastric lipase) and ligands (NPs and Drug molecules) structures. The protein (human gastric lipase) was prepared by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. After which Grid box was prepared according to the identified active site. Center Grid box was selected using x: 51.910, y: 70.449, z: 82.856, and the number of points in all dimensions x,y,z were considered 40x40x40 Å , and the grid spacing was selected as 0. 50 Å.

The structures of NPs and the standard drugs were downloaded from the ZINC database. Addition of Gasteiger charges, detecting root, and choosing torsions was done from the Autodock Tools panel's torsion tree for the preparation of ligands. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking of natural products as well as drugs was carried out using AutoDock vina on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.

### 4.6.4 PPARs (peroxisome proliferator activated receptor) (PPARalpha):

#### 4.6.4.1 Molecular Docking Study:

**Identification of Active site of Human Peroxisome proliferator-activated receptors (PPARs) alpha**

The PDB structure (3VI8) of Human Peroxisome proliferator-activated receptors (PPARs) alpha from www.rcsb.org has been selected for the molecular docking activity. The protein is Co Crystallized structure with a synthetic agonist APHM13. A good resolution (1.75 Å) X-Ray diffraction protein structure showed interactions with agonist APHM13. Based on the interactions of agonist APHM13 with the protein structures, active site of the protein structure has been identified. The identified active site has been again confirmed with the online server 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html).

**Molecular Docking using Autodock Vina**

To obtain details about the binding interaction as well as the relative orientation of the natural products (NPs), molecular docking was used. To obtain the comparative results, the standard drugs are also considered for the molecular docking. The protein structure of Human PPAR alpha was obtained from the protein data bank (PDB id: 3VI8 ). Autodock MGL Tool was used to modify the protein (alpha-amylase) and ligands (NPs and Drug molecules) structures. The protein (alpha-amylase) was prepared by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. After which Grid box was prepared according to the identified active site. Center Grid box was selected using x: 12.325, y: 5.943, z: -4.419, and the numberof points in all dimensions x,y,z were considered 40x40x40 Å , and the grid spacing was selected as 0. 50 Å.

The structures of NPs and the standard drugs were downloaded from the ZINC database. Addition of Gasteiger charges, detecting root, and choosing torsions was done from the Autodock Tools panel's torsion tree for the preparation of ligands. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking of natural products as well as drugs was carried out using AutoDock vina on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.

### 4.6.5 Leptin ( LEP-R, LEP-Rb):

#### 4.6.5.1Molecular Docking Study:
#### Identification of Active site of human obesity protein, leptin

The PDB structure (1AX8 and 3V6O) of human obesity protein, leptin and leptin receptor-antibody complex from www.rcsb.org has been selected for the molecular docking activity. The aim was to consider Leptin receptor and its antibody complex using cocrystallized structure. The selected good resolution (2.40 Å and 1.95 Å) X-Ray diffraction protein structure showed interactions with monoclonal antibody 9F8 fab fragment Heavy chain and 2-acetamido-2-deoxy-beta-D-glucopyranose. Based on the interactions, active site of the protein structure has been identified and the identified active site has been again confirmed with the online server 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html).

**Molecular Docking using Autodock Vina**

To obtain details about the binding interaction as well as the relative orientation of the natural products (NPs), molecular docking was used. To obtain the comparative results, the standard drugs are also considered for the molecular docking. The protein structure of human obesity protein, leptin and leptin receptor-antibody complex were obtained from the protein data bank (PDB id: 1AX8 and 3V6O). Autodock MGL Tool was used to modify the protein (human obesity protein, leptin and leptin receptor-antibody complex) and ligands (NPs and Drug molecules) structures. The proteins were prepared by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. After which Grid box was prepared according to the identified active site. Center Grid box was selected using x: 57.983, y: -31.125, z: 4.499 for 1AX8 & x: 38.665, y: 34.386, z: 15.606 for 3V6O and the number of

points in all dimensions x,y,z were considered 40x40x40 Å, and the grid spacing was selected as 0. 50 Å.

The structures of NPs and the standard drugs were downloaded from the ZINC database. Addition of Gasteiger charges, detecting root, and choosing torsions was done from the Autodock Tools panel's torsion tree for the preparation of ligands. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking of natural products as well as drugs was carried out using AutoDock vina on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.

### 4.6.6 Cannabinoid receptor type 1(CB1):

#### 4.6.6.1 Molecular Docking Study
#### Identification of Active site of Cannabinoid receptor type 1(CB1)

The PDB structure (7V3Z) of Cannabinoid receptor type 1(CB1) from www.rcsb.org has been selected for the molecular docking activity. The structure of cannabinoid receptor type 1 is available in complex with small molecules like flavin mononucleotide, cholesterol and 2-[(1r,2r,5r)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol. Based on the interactions of small molecules with the protein structure, active site of the protein structure has been identified. The identified active site has been again confirmed with the online server 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html).

#### Molecular Docking using Autodock Vina

To obtain details about the binding interaction as well as the relative orientation of the natural products (NPs) and selected drug molecules, molecular docking was used. To obtain the comparative results, the standard drugs are also considered for the molecular docking. The protein structure of cannabinoid receptor type 1 was obtained from the protein data bank (PDB id: 7V3Z). Autodock MGL Tool was used to modify the protein (cannabinoid receptor type 1) and ligands (NPs and Drug molecules) structures. The protein (cannabinoid receptor type 1) was prepared by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. After which Grid box was prepared according to the identified active site. Center Grid box was selected using x: -40.487, y: -111.762, z: 247.410, and the number of

points in all dimensions x,y,z were considered 40x40x40 Å , and the grid spacing was selected as 0. 50 Å.

The structures of NPs and the standard drugs were downloaded from the ZINC database. Addition of Gasteiger charges, detecting root, and choosing torsions was done from the Autodock Tools panel's torsion tree for the preparation of ligands. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking of natural products as well as drugs was carried out using AutoDock vina on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.

### 4.6.7 HMG CoA Reductase:

#### 4.6.7.1 Molecular Docking Study:

**Identification of Active site of human HMG-CoA reductase**

The PDB structure (1DQA) of the human HMG-CoA reductase from www.rcsb.org has been selected for the molecular docking activity. Co Crystallized structure of human HMG-CoA reductase is available in complex with HMG, CoA, and NADP+. A good resolution (2.00 Å) X-Ray diffraction protein structure showed interactions with HMG, CoA, and NADP+. Based on the interactions observed with the protein structures, active site of the protein structure has been identified. The identified active site has been again confirmed with the online server 3D Ligand Site (https://www.wass-michaelislab.org/3dlig/index.html).

**Molecular Docking using Autodock Vina**

To obtain details about the binding interaction as well as the relative orientation of the natural products (NPs), molecular docking was used. To obtain the comparative results, the standard drugs are also considered for the molecular docking. The protein structure of human HMG-CoA reductase was obtained from the protein data bank (PDB id: 1DQA). Autodock MGL Tool was used to modify the protein (human HMG-CoA reductase) and ligands (NPs and Drug molecules) structures. The protein (human HMG-CoA reductase) was prepared by removing water, unwanted chain and a bound ligand. Missing amino acids have been checked, and the protein structure has been added with polar hydrogen and Kollman charges. After which Grid box was prepared according to the identified active site. Center Grid box was selected using x: -17.851, y: 15.494, z: 25.000, and the numberof points in all dimensions x,y,z were considered 60x60x60 Å , and the grid spacing was selected as 0. 50 Å.

The structures of NPs and the standard drugs were downloaded from the ZINC database. Addition of Gasteiger charges, detecting root, and choosing torsions was done from the Autodock Tools panel's torsion tree for the preparation of ligands. The docking procedure was carried out using the Lamarckian genetic algorithm. Docking of natural products as well as drugs was carried out using AutoDock vina on windows platforms with machine configuration of 8 GB RAM and Intel i5 processor.