MATERIALS AND METHODS

Chapter III

Objective 3.1: Selection and segregation of appropriate germplasm from different bio-geographical zones of Gujarat.

T. cuneifolia were collected from three different biogeographical zones of Gujarat and the plant material was identified in the field itself. The plants were collected in the flowering and fruiting stages. The plants were reconfirmed in the laboratory with the available local flora (Shah, 1978). During the field studies habit, habitat, height of plant, colour of flower, associations and other ecological parameters were also recorded. For the purpose of making herbarium sheets, the plants were taken in their flower and fruiting stages and kept in good condition. The plants' germplasm from three distinct locations was sent to the National Bureau of Plant Genetic Resources (ARIS Cell), Pusa Campus, New Delhi. In order to compare the phytoconstituents of *T. cuneifolia*, the roots of *Glycyrrhiza glabra* were purchased from the local market of Vadodara, Gujarat. While visiting the sites various ecological parameters were studied along with the organoleptic properties.

Based on the sugar content of the plant samples collected from three different zones, Bagodara was finalized for further analysis of soil. The soil samples for the macro and micronutrient analysis were collected from the Bagodara location. An experimental pit of 1 x 1 x 1 ft. was dug, and soil samples were taken from the top, middle, and bottom layers. The physical and chemical parameters of the obtained soil were studied. The physical properties of studies include colour, texture, and ability to retain water.

The following are the soil chemical properties studied.

- **Carbon (Chromic acid Method) (Perur *et al.*, 1973)**

Rating of organic carbon: Low < 0.50%, Medium 0.50-0.75%, High > 0.75%

- **Available Phosphorous in soil: (Ghosh *et al*., 1983)**

Available Phosphorous is determined by Olsen’s extractant.

Observations: Rating of available P (Kg/ha) Low < 11, Medium 11-25, High > 25

- **Available Potassium in soil (Perur *et al.*, 1973)**

Instruments: Electric shaker, flame photometer

Rating of available K_2O Low < 120 kg/ha, Medium 120-280 kg/ha, High > 280 kg/ha. 1.

- **Soil pH: (Perur *et al.*, 1973)**

Instrument: pH meter

Soil pH was measured by a pH meter after preparing soil paste with distilled water (1:5 ratio).

3.1.1 Electrical conductivity of soil: (Perur *et al.*, 1973)

Instrument: Conductivity meter and a conductivity cell with known cell constant, suction machine.

- **Available Sulphur in soil: (Chesnin & Yein, 1950)**
  Apparatus: Kett Summerson, water bath, etc
- **Available micronutrients in soil: (Ghosh *et al.*, 1983)**

The term 'micronutrients' refers to important nutrients that are needed in minute amounts for proper plant growth and development. Included in this group are zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), boron (B), molybdenum (Mo), and chlorine (Cl). Micronutrients serve several intricate functions in plant nutrition, but the majority of them are necessary for the function of a variety of enzyme systems. However, the particular activities of the different micronutrients in plant and microbial development processes vary considerably.

3.1.2 Apparatus: Atomic absorption spectrophotometer (AAS), Electrical shaker.

Reagents: Diethylene-triamine penta-acetic acid (DTPA) 0.005M solution, Tri-ethanolamine (TEA) 0.1M, $CaCl_2.2H_2O$ 0.01M solution, Zinc metal, Dilute HCL (1:5) with double distilled water. The extracting reagent is prepared by taking 1.967g of DTPA and 1.470g $CaCl_2.2H_2O$ in a beaker. To this 20-25ml distilled water is added and 13.3ml of TEA followed by 100ml distilled water. This is transferred to a 1 litre volumetric flask, washed 3 to 4 times, and the volume is brought up to the mark with water. With diluted HCl, the pH of the solution is adjusted to 7.3. Establishing standard curves:

Zinc: 1 gramme of pure zinc metal is entirely dissolved in the smallest quantity of weak hydrochloric acid, brought to 1 litre with distilled water, and then transferred to a plastic container. This solution includes 1000g Zn per millilitre. In six 100ml volumetric flasks, the necessary aliquots are taken, standards of 0, 0.5, 1.0, 1.5, 2.0, and 2.5 ppm zinc solution are prepared, and a standard curve is plotted against the atomic absorption spectrophotometer readings.

Copper: A stock solution of 1000ppm Cu is prepared by dissolving 1g of Cu metal in 50ml of diluted (1:1) nitric acid, and then diluting the solution with 100ml of distilled water. In a volumetric flask, 0.25, 0.5, 1.5, 2.0, 2.5, and 3ppm working solutions are made.

Iron: A stock solution of 1000ppm Fe is made by dissolving 1g of Fe metal in 50ml of diluted (1:1) nitric acid, and then diluting the solution with 1 litre of distillate water. In 100ml volumetric flasks, the working solutions (1, 2, 4, 6, 8, 10 ppm) are prepared.

Manganese: For a 1000 ppm Mn stock solution, dissolve 1g of pure Mn metal in 50ml of weak nitric acid and make it up to 1 litre. Concentrations of 1, 2, 4, 6, 8, and 10 ppm Mn are taken in a 100 ml volumetric flask.

Procedure: A sample of 10 gms of soil is mixed with 20 millilitres of DTPA reagent in a 100 millilitre conical flask and then agitated for two hours. Filtering the extract before AAS analysis for micronutrient content.

Rating of available micronutrients:

Zinc: Low <0.5ppm, Medium <0.5-1.0ppm, High >1.0ppm

Iron and Manganese: Low <5ppm, Medium <5-10ppm, High >10ppm

Copper: Low <0.2ppm, Medium <0.2-0.4ppm, High >0.4ppm

Objective 3.2: Standardization and validation of sugars (sweeteners) and amino acids

Plant material was procured as stated in Section 3.1. The collected *Taverniera cuneifolia* root material was cleaned, shade-dried for two weeks, and then put in an oven preheated to 37°C for two days to remove any moisture. Each of the dried plant components was pulverised separately using an electric mixture-grinder and sieved through 80-mesh BSS (Jayant Scientific India, Mumbai) to facilitate the extraction procedure (Benbelaid *et al*., 2013). Powdered material has been depicted in Figure 2.10 and 2.11, respectively.

The *T. cuneifolia* powder was then kept in airtight containers at room temperature, away direct sunlight, and in an environment that was water-, fire-, and rodent-proof. The containers were properly labelled with pertinent information, including the name of the plant, the day, month, and year of harvest, as well as the time necessary for shade and oven drying.

3.2.1 Preparation of Plant Extracts

Powdered plant samples of *T. cuneifolia* and *G. glabra* were extracted using several solvents i.e., in distill water, methanol, Hexane, and ethyl acetate, by the soxhlet and reflux extraction techniques for 6 to 8 hours. The obtained extracts were then subjected to rota-evaporation to remove the excess solvent and were totally evaporated to yield powdered extracts for further analysis. In accordance with the Indian pharmacopoeia 2010, the solvent extractive values were also calculated using following formula:

Extractive value (%) = (W1-W2)/ W3 X 100

Where,

W1 = Weight of beaker with extract (g)
W2 = Weight of empty beaker (g)
W3 = Weight of sample taken (g)

3.2.2 Phytochemical analysis

Phytochemicals are chemical compounds synthesized during the various metabolic processes. Various phytochemicals are known to play an important role to identify the bioactivity of medicinal plants (Patil *et al*., 2015). The preliminary work on qualitative phytochemical analysis of *T. cuneifolia* was done by Manglorkar, 2014.

Fingerprinting is a means for establishing a characteristic chemical pattern for the plant material (Shirolkar *et al*., 2013). Chemical fingerprints obtained by chromatographic techniques present a relatively good integral representation of various phytochemicals and provide the means for a convenient identity check of plant materials (Kamboj, 2012). Thus, chromatographic fingerprinting is the most recommended and globally accepted tool for the quality evaluation of herbals (Shirolkar *et al*., 2013). Various analytical techniques like HPTLC, HPLC, GC-MS, etc., aid in the development and establishment of characteristic fingerprints for plants.

3.2.3 Chromatographic fingerprinting of plant powder

Fingerprinting analysis was conducted to evaluate the quality of the phytochemicals found in Glycyrrhiza glabra in comparison with *T. cuneifolia* prior to the standardization and validation of sugar and amino acid. *Taverniera abyssanica*, a closely related species, was also procured during the fingerprinting investigation to serve as a reference material. The *T. cuneifolia* root bark and pith were separated during the method development of fingerprinting analysis, with

the goal of determining which part of the root has the greatest concentration of phytochemicals and hence the greatest number of detectable bands. The sample preparation and instrumental conditions are discussed below.

3.2.4 Preparation of sample

Plant powder (0.5 g) for HPTLC extracted in methanol (10.0 mL) and hydroalcholic (ethanol : water ; 7:3), vortexed for 5mins, sonicated for 20 mins and filtered using Whatman filter paper no. 1 followed by filtration through nylon filter paper. The filtrate was used for development of chromatographic fingerprints.

3.2.5 Chromatographic conditions for HPTLC

For the analysis, a CAMAG HPTLC system with a Linomat V Automatic Sample Spotter (CAMAG, Muttenz, Switzerland) and a CAMAG TLC Scanner IV with winCATS planar chromatography manager software was used. Using the Linomat-V sample applicator equipped with a 100 L syringe, 10 l of samples were transferred to the plate. On TLC plates pre-coated with silica gel 60 F254 (Merck, Darmstadt, Germany) of 0.2 mm thickness with aluminium sheet support in 8 mm bands at 10 mm from the bottom, 15 mm from the sides, and 8 mm gap between bands, chromatographic separation was obtained. The plates were generated in a twin trough chamber (CAMAG, Muttenz, Switzerland) lined on one of the inner walls with Whatman filter paper no. 1. Mobile phase of ethyl acetate: Formic acid: Glacial acetic acid: water (15: 1: 1: 2, v/v/v/v) was poured on the filter paper to saturate the filter paper thoroughly. The chamber was then tilted 45 degrees to disperse the mobile phase evenly in both troughs. For 20 minutes, the chamber was saturated with mobile phase. The plate was developed ascendingly in the pre-saturated chamber up to a height of 85 mm from the base of the plate. The separated bands were quantified by densitometric scanning in absorbance mode at 254 nm and 366 nm prior to derivatization under the following conditions: slit width- 6 mm x 0.45 mm, scanning speed- 20 mm/s, and data resolution- 100 m/step. The Reprostar 3 system (CAMAG, Muttenz, Switzerland) was used for photodocumentation at 254 nm, 366 nm, and 550 nm. All measurements were conducted at a temperature of 22 1°C. The plates were derivatized with 10% Methanolic Sulphuric acid and Anisaldehyde-sulphuric acid reagent and then dried at 120°C for 20 minutes in a hot air oven.

3.2.6 Standardisation and validation of Sugars

The chromatographic separation was performed on silica gel 60 F 254 HPTLC plates (glass plates 20 ×10 cm) in a saturated (33 % relative humidity) automatic developing chamber (ADC 2, CAMAG) at ambient temperature. The plates were pre-saturated with the mobile phase for

60 min, automatically developed to a distance of 85 mm at room temperature and dried for 5 min. The obtained chromatographic results were documented using an HPTLC imaging device (TLC Visualizer 2, CAMAG) under white light. The chromatographic images were digitally processed and analysed using a specialised HPTLC software vision CATS 3.0 (Server LABSERVER, version 3.0.20196.1) all the instrumentation modules were controlled with vision CATs 3.0 software.

3.2.7 Reagents and chemicals

All solvents were of analytical grade and were obtained from Merck (Darmstadt, Germany). The standard compounds (Arabinose, glucose, fructose, Maltose, Manitol, Galactose, Inositol, Lactose, Mannose, Rhamnose, Ribose, Sorbitol, Sucrose and Xylose) and other chemicals such as 1-Butanol, Boric acid, Diphenylamine, Aniline, Methanol and Phosphoric acid were purchased from Merck (Darmstadt, Germany). Silica gel 60 F254 HPTLC glass plates (20 cm×10 cm) were purchased from Merck (Darmstadt, Germany).

3.2.8 Standard and reagent preparation

Standard Arabinose, glucose, fructose, Maltose, Manitol, Galactose, Inositol, Lactose, Mannose, Rhamnose, Ribose, Sorbitol, Sucrose and Xylose solutions (250 µg/mL) were prepared by dissolving 2.5 mg each in 10 mL of 50% aqueous methanol with sonication. A solution of boric acid (5 mg/mL) was prepared by dissolving 50 mg of boric acid in 10 mL deionised water. A mixture of 1-butanol-2-propanol–boric acid in water (5 mg/mL) (30:50:10, V/V/V) was used as the mobile phase. The derivatizing reagent as a dipping solution was prepared as follows: 2gm of diphenylamine and 2mL of aniline were dissolved in 80 mL of methanol, 10mL of Phosphoric acid (85%) were added and then the solution was diluted to 100 mL with methanol.

3.2.9 Preparation of Plant extract solution

The roots were extracted with methanol by using soxhlet apparatus. The plant materials were shade dried and coarsely powdered before soxhlet apparatus application. 10 g of each dried and powdered roots were applied for the methanolic extraction independently in soxhlet apparatus. The extracts were concentrated using rota-evaporator and then lyophilized. Powdered extracts were weighed and used for further analysis. The working sample solutions (1 mg/mL) were prepared in methanol after sonication for 30 mins.

3.2.10 Sample application

The standard solutions were applied as 8 mm bands at 8 mm from the lower edge of the HPTLC plate. 5 µL of sample extracts solution was spotted on the HPTLC Silca gel plate as 8 mm band

length, using a Camag ATS 4 automatic TLC sampler. To prepare the glucose, fructose and sucrose standard curves, 1 µL, 2 µL, 3 µL, 4 µL, 5 µL, 6 µL and 7 µL of the respective standard solutions were applied.

3.2.11 Sample development

The chromatographic separation was performed on silica gel 60 F254 HPTLC plates (glass plates 20 × 10 cm) in a saturated (33 % relative humidity) automatic developing chamber (ADC 2, CAMAG) at ambient temperature. The plates were pre-saturated with the mobile phase for 60 min, automatically developed to a distance of 85 mm at room temperature and dried for 5 min. The obtained chromatographic results were documented using an HPTLC imaging device (TLC Visualizer 2, CAMAG) under white light. The chromatographic images were digitally processed and analysed using a specialised HPTLC software vision CATS 3.0 (Server LABSERVER, version 3.0.20196.1) all the instrumentation modules were controlled with vision CATs 3.0 software.

3.2.12 Plate derivatisation

After initial documentation of the chromatographic results, each plate was derivatised by using Camag immersion device III, developed plate was automatically dipped in immersion device III chamber 20x10 filled with 180-200 ml of 2 mL of aniline–diphenylamine–phosphoric acid reagent. The derivatised plate was heated for 3 min at 110 °C using a CAMAG TLC Plate Heater III. Afterwards, the plate was cooled to room temperature and analysed with the HPTLC imaging device Visulizer 2 under R white and R 366nm. Three sets of images, remission white (R white), transmission white (T white) and remission-transmission white (RT white), were taken for analysis.

3.2.13 Method validation

The developed method for the determination of glucose, fructose and sucrose in root samples of T. cuneifolia was validated as the resolution passed in International Conference on Harmonisation (ICH) guidelines (Borman & Elder, 2017)

3.2.13.1 Specificity

The specificity of a method for the determination of a compound is ascertained by comparing the compound with a reference standard. This is also done to demonstrate that an analytical procedure is specific for a particular analyte as well as able to discriminate between compounds of closely related structures which are likely to be present in the analytical sample of interest. Peak purity was assessed by comparing the peak start, peak apex and peak end position. The

bands for standards were also confirmed as being present in roots by comparing the corresponding Rf values and peak purity spectra with the respective standards.

3.2.13.2 Linearity

The linearity of the analytical method was evaluated by analysing standards at different volumes. Three replicate measurements were conducted using the standards. Each standard solution was used to produce Five-point linear calibration curves, and the obtained peak area and peak height versus the corresponding concentration of standards were evaluated by linear regression analysis. The coefficient of determination (r^2), slope (m), y-intercept (c) and standard deviation (SD) of the calibration curves were determined to assess the linearity of the method.

3.2.13.3 Sensitivity (limits of detection and limit of quantification)

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value under the experimental conditions. The limit of quantification (LOQ) is the lowest amount of analyte that can be detected and quantified with suitable precision, accuracy and reproducibility. The LOD and LOQ were calculated based on the standard deviation of the regression lines and slope of the calibration curves (n=3) using the formula described in the ICH guidelines:

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

where σ is the standard deviation of the regression line and S is the slope of the calibration curve. The sensitivity of the method (LOD and LOQ) was determined for glucose, fructose and sucrose standards from their corresponding calibration curves**.**

3.2.13.4 Precision

Precision is the closeness of results between a series of measurements obtained from multiple analyses of samples under the same conditions but at different times, on different instruments or by different analysts. Precision is considered at three levels, repeatability (same operating conditions over a short interval of time), intermediate precision (within-laboratory variations: different days, different analysts, different equipment) and reproducibility (between laboratories). The precision of the current method was validated for intra-day and inter-day precision. Intra-day precision was determined in triplicate using the same method for the same concentrations for sugars and amino acid standard solutions and expressing the obtained peak

area or peak height as percent relative standard deviation (%RSD). The precision of the method for the quantitative analysis of sugars and amino acid were determined in the same way.

3.2.13.5 Accuracy as recovery

The accuracy of the method was established through a standard spiking or addition method. Sample bands were over-spotted with known quantities of each reference standard in three different ranges. Each experiment was performed in triplicate, and the accuracy was calculated as the % recovery and %RSD of standards.

3.2.13.6 Repeatability (system precision)

Repeatability as system precision is determined by repeatedly analysing a particular sample concentration. In this case, repeatability was studied by analysing six times bands containing standard compounds respectively. The findings were expressed as %RSD of standards.

3.2.13.7 Robustness

Robustness of an analytical method is evaluated by studying the influence of small, but deliberate changes to the method parameters in order to provide an indication of its reliability during normal usage. Robustness was examined in this study by making small changes to the mobile phase composition, volume, and chamber saturation time during the triplicate analysis of the satndards. The results were examined in terms of % recovery and Rf values of individual compounds.

3.2.14 Standardisation and validation of Amino acids

Refer section 3.2.6.

3.2.15 Reagents and chemicals

All solvents such as 2- Propanol, Glacial acetic acid, Acetic acid, 1-Butanol, Ninhydrin, Methanol were of analytical grade and were obtained from Merck (Darmstadt, Germany). The standard compounds (Alanine, Aspargine, Arginine, Aspartic acid, Cystine, Glycine, Glutamic acid, Glutamine, Histidine, Lysine, Leucine, Isoleucine, Metheonine, L- Phenylalanine, Proline, Serine, DL-Serine, Threonine, Tyrosine, Trypthophan, L-Valine, DL Valine) were purchased from Merck (Darmstadt, Germany). Silica gel 60 F254 HPTLC glass plates (20 cm×20 cm) were purchased from Merck (Darmstadt, Germany).

3.2.16 Standard and reagent preparation

Standard Alanine, Aspargine, Arginine, Aspartic acid, Cystine, Glycine, Glutamic acid, Glutamine, Histidine, Lysine, Leucine, Isoleucine, Methionine, L- Phenylalanine, Proline,

Serine, DL-Serine, Threonine, Tyrosine, Trypthophan, L-Valine and DL Valine were prepared by dissolving 0.5mg/ml in Water with sonication.

3.2.17 Sample Preparation

Refer section 3.2.9.

T. cuneifolia extract in water (TAV water), T. cuneifolia extract in methanol (TAV MEOH) and G. glabra extract in methanol (GG MEOH), 20mg each dissolved in 1 ml of water (double distill water has been used throughout the process).

Mobile Phase: n-Butanol: acetic acid: water (3: 1: 1 V/V/V)

Derivatization reagent: Ninhydrin reagent

Reagent preparation: Weight and transfer 0.6gram of ninhydrin in 190 ml of 2-Propanol and 10 ml of glacial acetic acid.

3.2.17.1 Sample application

The standard solutions were applied in 8 mm bands eight millimetres from the plate's lowest edge. Using a Camag ATS 4 automated TLC sampler, 5 µL of sample extracts solution was spotted as 8 mm bands on an HPTLC Silica gel plate.

3.2.17.2 Sample development

Refer section 3.2.11.

3.2.17.3 Sample derivatisation

Refer 3.2.12.

3.2.18 Method validation

Refer 3.2.13.

Objective 3.3: Standardization and validation of active phyto-constituents of *T. cuneifolia*:

The intricacy of analysis techniques is one of the most difficult parts of method development in quantitative analysis. The optimal procedure is the simplest one that can be executed by a variety of operators in a variety of laboratories (Hajimehdipoor *et al*., 2012).

A review of the literature indicated the availability of several separation conditions and mobile phases for the determination of phytoconstituents, both individually and synergistically in the presence of other markers, from different plants and their extracts by HPLC (Tables 5.1 and 5.2, respectively, provide a synopsis of numerous documented techniques for the quantification of Liquiritigenin, Quercetin, Naringenin, Genistein, Kaempferol, Apigenin, Glycyrrhizin,

Glabridin, *18 α-Glycyrrhetinic acid*, *18 β-glycyrrhetinic* acid, Stigmasterol and β-Sitosterol from plant extracts using HPLC.

Separation and quantification of these 12 phytoconstituents from *T. cuneifolia* root extracts in contrast to *G. glabra* root extracts have not yet been reported. Consequently, our endeavour to separate and quantify phytoconstituents from these plant extracts is the first of its type.

3.3.1 Standardization and validation of Glycyrrhizin

3.3.1.1 Reagents, standards, and solutions

HPLC-grade solvents (butanol, water, acetonitrile, methanol) and chemicals (acetic acid and ammonium acetate) acquired from Merck Specialties India Pvt. Ltd., India. Sigma Aldrich chemie supplied 95% glycyrrhizic acid mono-ammonium salt (steinheim Germ any). Deionized Milli-Q water was used throughout the analysis.

3.3.1.2 TLC analysis

The plant material was sonicated for 15 minutes with 1 mL of methanol before being filtered. The extract was placed in bands on a silica gel 60 F254 TLC sheet (Merck). As standards, the first and second bands were used. The mobile phase consisted of butanol : acetic acid : water (6:1:3 v/v). The plates were developed, dried, and then treated with an anisaldehyde sulphuric acid reagent. After heating the plate, it was examined using ultraviolet light.

3.3.2 LC-MS/MS analysis

3.3.2.1 Preparation of LC samples

1 gm of powder was combined with 5 mL of a 1:1 mixture of acetonitrile and water and sonicated. The combination was centrifuged at 5000 RPM (at room temperature) for 10 minutes, after which the TC (T. cuneifolia) and GG (G. glabra) supernatants were diluted to 0.1 mg mL^{-1} and 0.01 mg mL^{-1} respectively for LC-MS/MS analysis.

3.3.2.2 Standard solution preparation for LC-MS/MS

Glycyrrhizin standard calibration curve in water: acetonitrile (60:40) v/v was generated from the stock solution (10000 ng mL^{-1}) of glycyrrhizin standard in concentrations of 5 ng mL^{-1}, 10 ng mL^{-1}, 50 ng mL^{-1}, 100 ng mL^{-1}, 200 ng mL^{-1} and 500 ng mL^{-1}.

3.3.2.3 Liquid chromatography-mass spectrometry condition

Chromatographic development was performed using a Shimadzu NEXERA-X2 UHPLC (Ultra High Performance Liquid Chromatograph) system with LC-30AD pumps, SIL-30A autosamplers, and CTO-20AC as a column oven. LabSolutions was the software used by the data processor. On the Shimadzu LCMS-8040, MRM transitions for glycyrrhizin have been

optimised (Triple Quadrupole Mass Spectrometer). For the analysis, a Shimadzu shim-pack XR-ODS, C18 column (L 75 mm x 3.0 mm x 2.2 m) was used. Mobile phase-A was 20 mM ammonium acetate in water, while mobile phase-B was acetonitrile. The volume of injection was 5 L, and the flow rate was maintained at 0.3 mL/min. Glycyrrhizin was isolated from the matrix using gradient chromatography. The gradient programme consisted of 0.05 min - 25% B, 1.0 min - 60% B, 3.0 min - 90% B, 5.0 min - 90% B, and 8.0 min - 25% B. The column oven was set at 40 degrees Celsius.

Using the APCI (Atmospheric Pressure Chemical Ionization) interface in positive mode with a capillary voltage of 4V, analysis was conducted. The following MS parameters were used in the analysis: Nebulizing Gas flow: 2L min^{-1}, Drying Gas flow: 15L min^{-1}, Interface temperature: 350°C, DL (Desolvation Line), temperature: 200°C, and Heating Block: 400°C.

3.3.2.4 Method Validation

The proposed analytical technique has been verified to demonstrate its suitability for its intended use. The technique has been verified in accordance with the ICH recommendations for specificity, linearity, range, accuracy, precision, and sensitivity as validation parameters.

3.3.2.4.1 Linearity

A stock solution with 10000 ng mL^{-1} concentration of glycyrrhizin standard was prepared in water and acetonitrile. Different aliquots were made to acquire six different desired concentrations ranging from 5 ng mL^{-1} to 500 ng mL^{-1} which were injected (5µL each) by autosampler and chromatographed according to the previously mentioned protocol. To avoid degradation due to light exposure, the stock solution was stored in the dark. The experiment was performed in triplicate, and the average was taken in the calculations. Peak area was plotted against analyte concentrations to generate the calibration graph and data were statistically analysed using correlation and least square linear regression.

3.3.2.4.2 Specificity

The method's specificity was established by comparing the standard and the sample. The peak for Glycyrrhizin was confirmed by comparing the retention time and spectra of the sample to those of the standard. For Glycyrrhizin determination in methanolic extracts of Taverniera cuneifolia and Glycyrrhiza glabra root, the HPLC-developed assay was used. This was accomplished by injecting 5 µL of sample working solution and measuring and quantifying the area against the calibration curve.

3.3.2.4.3 Accuracy as Recovery

Three different concentrations of standard were injected into the previously tested samples. i.e., 50 ng mL^{-1}, 100 ng mL^{-1}, and 200 ng mL^{-1}, and then re-analyzed using the suggested method. The recovery experiment was performed three times, and the recovery percentage was determined. The procedure was carried out in triplicate. The GG root extract was diluted 20,000 times using the solvent system described above, whereas the TC root extract was diluted 2,000 times.

3.3.2.4.4 Precision

Repeatability and intermediate precision experiments were conducted to assess the method's accuracy. To assess inter- and intra-day precisions, three different concentrations of standard were made and applied on the same day and on three successive days. Each analyses' assay was computed, and %RSD was determined.

3.3.2.4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the procedure was determined by measuring the limit of detection (LOD) and limit of quantification (LOQ). The sample concentration yielding a signal-to-noise ratio of three was labelled as the LOD, while the sample concentration yielding a signal-to-noise ratio of ten was marked as the LOQ.

3.3.3 Simultaneous method development and validation of phytoconstituents using HPLC

T. cuneifolia methanolic extracts were compared to G. glabra methanolic extracts for their concentrations of ten different phytoconstituents: liquiritigenin, quercetin, naringenin, genistein, kaempferol, apigenin, glycyrrhizin, glabridin, and *18 α-glycyrrhetinic acid*, *18 β-glycyrrhetinic* acid. For the analysis of mid-polar components, sonicated T. cuneifolia chloroform extract has also been extracted. Additionally, a unified strategy for the detection and verification of both Stigmasterol and *β*-Sitosterol in these samples has been developed. The table below contains all the necessary instrumental and methodological information.

Table 3.1: Instrument parameters for Method Validation

Parameters	**HPLC (other standards)**	**HPLC (For *β*-sitosterol and stigmasterol)**
Stationary phase	Shim-pack GIST (Shimadzu) C18 (2) 250X 4.6 mm, 5.0µm	Kinetex C18 (2) 250X 4.6 mm, 5.0µm
Column temperature	35°C	35°C
Column Oven	CTO-20 AC	CTO-20 AC
Instrument	Shimadzu LC 20AD	Shimadzu LC 20AD
Mode of separation	**Gradient**	**Isocratic**
Mobile phase	A. 0.1 % H_3PO_4 acid in water B. Methanol C. ACN	A. Methanol (90) B. ACN (10)
Flow rate	0.8 mL/min	1.0 mL/min
Pump	Quarternary LC-20AD	Quarternary LC-20AD
Injector	Autosampler (SIL-20 AC HT)	Autosampler (SIL-20 AC HT)
Spotting/injection volume	10.0µL	20.0µL
Scanner/Detector	PDA (SPD-M20A)	PDA (SPD-M20A)
Run Time	50min	40 min
Wavelength	254 nm	205 nm
Software		

The gradient method applied for the separation of Liquiritigenin, quercetin, naringenin, genistein, kaempferol, apigenin, and glabridin 18 *α*- and *β*- glycyrrhetinic acid is given in table 3.3.

Table 3.2: Gradient Mobile Phase

	MPA	**MPB**	**MPC**
0.01	60	30	10
1	85	0	15
25	75	25	0
25.50	100	0	0
30	40	0	60
40	40	0	60
41	60	30	10
50	Controller	Stop	Stop

For the validation purpose, the HPLC method was developed to achieve a reproducible method by optimizing mobile-phase composition, column oven temperature, wavelength, flow rate, and change columns. A gradient program was used to separate the active constituents in a single run. The detection wavelengths were set according to the ultraviolet (UV) absorption maxima (λ*max*) of compounds.

3.3.3.1 System suitability

In the suitability of the system, the following parameters were considered to define the optimal performance of the method. (i) Injection of mix standard preparation in duplicate; (ii) peak resolution between **2** and **3** which was found to be >2.0; (iii) the tailing factor for each analyte, found as <1.5. The system suitability tests presented CV values less than 2% for the tested parameters, thus suggesting that the chromatographic system is adequate for the analysis and meets the expectations of the analyst.

3.3.3.2 Linearity, LOD and LOQ

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of the analyte in the sample. A regression line is drawn using appropriate statistical methods and the correlation coefficient, y-intercept and slope are derived from the regression line. After performing linear regression, the linearity was assessed by determining goodness-of-fit (coefficient of the correlation) at seven different levels to address the wide range of the applicability. The residuals (SSR) over these concentration ranges were determined with AUC (peak area/concentration) at each concentration level. The % CV for area of the samples was found to be below 2 and the % nominal was within the acceptance range.

3.3.3.3 Accuracy as recovery

Accuracy is typically assessed by spiking known amounts of the analyte substance into a sample and compared to the amount measured in the samples upon analysis (Dowell et al., 2015). Standard was introduced into the pre-analyzed samples at three different concentration levels and the mixtures were then re-analyzed with the proposed technique. The recovery experiment was repeated three times, and the percentage recovery of the results was calculated.

3.3.3.4 Precision

Precision is the measure of repeatability of an analytical method under normal conditions (Bansal & DeStefano, 2007). It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the

prescribed conditions (ICH, 2005). Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

3.3.3.5 Robustness

Robustness assesses the effect of minor changes to instrumental conditions on the analyte measurement and is a measure of capacity of method to remain unaffected by small, but deliberate variations in its parameters. It provides an indication of reliability of the method during normal usage (Farooqui *et al*., 2015; ICH, 2005). In this research work, robustness was aimed to examine the sources potentially subject to variations through evaluation of one or a set of responses inherent to the method. In this sense, effect on change in analyst, batch of HPLC column, increase or decrease in spotting volume, flow rate and mobile phase composition was studied on the response of quality control samples of markers using HPLC techniques. It was observed that the chromatographic separation of markers was not affected deliberately making small changes. Nonetheless, the % CV and % mean difference values were found to be within the acceptable limits. The findings of the study thus suggest that the method is robust and suitable for use.

3.3.4 Assay of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, stigmasterol and β-sitosterol from plant extracts:

Assay represents a quantitative measurement of the analyte present in a given sample. It provides an exact result which allows an accurate statement on the content or potency of the analyte in a sample (ICH, 2005). In the present study, the proposed validated HPLC methods for estimation of liquiritigenin, apigenin, genistein, glabridin and glycyrrhizin were subjected to determine and quantify from the TAC (*T. cuneifolia* chloroform extracts) and methanolic extracts of TAV MEOH (*T. cuneifolia* methanolic extracts) and GG MEOH (*G. glabra* methanolic extracts). The content of liquiritigenin, apigenin, genistein, glabridin, glycyrrhizin, Stigmasterol and β-Sitosterol from the extracts of plants was determined by linear regression equations.

3.3.6 LC-MS/MS-Q-TOF analysis of Targeted metabolites

3.3.6.1 Sample Preparation

The root of *T. cuneifolia* (5gm) was soaked in hexane for two hours, then sonicated in ethyl acetyl acetate. After letting the solution sit for two hours, a rota-evaporator was used to remove the solvent and filter the remaining solution. Then, it was all put together in a vile and given the name HXEATAV. *T. cuneifolia* methanolic extract (named as MEOHTAV) was made in a

similar manner. *T. cuneifolia* root extract (CHTAV) and G. glabra root extract (CHGG) were extracted in chloroform for the purpose of comparison. The analysis was done by firstly dissolving 1 mg of the plant extract in 1 mL of HPLC grade methanol followed by sonicating for 10 minutes, and finally filtering through 0.22 µm polyvinylidene fluoride (PVDF) membrane syringe filters into a 1 mL LC auto-sampler vial. The aforementioned focused metabolomic examination was then performed on a total of four samples using LCMS/MS-QTOF analysis.

3.3.6.2 LC-MS/MS-Q-TOF instrument parameters

Agilent 6520 Accurate-Mass Q-TOF was applied in a positive mode. Zorbax eclipse Plus, C18 (1.8 µm, 3X100 mm) column was used for the separation of targeted compounds, and the flow rate was set at 0.4ml/min. An aliquot of 1 µL from each extract was diluted 50 times and injected with gradient method**.** MassHunter Workstation Software (LC/MS Data Acquisition for 6200 series TOF/6500 series Q-TOF) was used for extraction and identification of Liquiritigenin, Naringenin, Kaempferol, Apigenin, Glycyrrhizin, Glabridin, *Glycyrrhetinic acid* and Stigmasterol in the *T. cuneifolia* root extract. The instrument parameters and methodology used are as follows:

Table 3.3: LC data acquisition parameters

Parameter	**LC (6200 series TOF/6500 series Q-TOF)**		
Mobile Phase A:	0.1% Formic Acid in water		
Mobile Phase B:	0.1% Formic Acid in ACN		
Column	Zorbax eclipse Plus C18 (1.8 µm, 3X100mm) Max Pressure:600Bar		
Gradient	**Time (Min)** **0** **1** **4** **6** **8** **10**	**A%** 70 70 45 25 25 70	**B%** 30 30 55 75 75 30
Run Time	15 Min		
Post Time	0 Min		
Column Temp.	30 °C		
Flow Rate	0.4ml/Min		
Injection Volume	1 µl		
DAD Wavelength	210, 225, 258, 254, 270 nm		

Table 3.4: MS data acquisition parameters

Parameter	MS-MS
Ion Mode	Positive
Drying Gas Temp	320 °C
Drying Gas Flow	8 L/Min
Sheath Gas Temp.	350 °C
Sheath Gas Flow	11 L/Min.
Nebulizer Pressure	35 psi
Capillary Voltage	3000V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
Data Acquired	2GHz Extended Dynamic Range
Instrument Status	1700m/z

Objective 3.4: Purification of the active fractions by Column chromatography.

3.4.1 Extraction of sample for column chromatography

The root sample of *T. cuneifolia* were extracted in methanol by using Soxhlet apparatus as mentioned in first objective. The 2 gm of dry extract were mixed with 0.5 gm of silica gel 60 to remove its stickiness completely.

3.4.2 Column Chromatography

TLC and column chromatography are two most widely used methods for the isolation and purification of bioactive compounds from the sample matrix. Column chromatography was carried out to fractionate the active or pure compound from a complex sample matrix. Briefly, silica gel (60-200 mesh, Merck) was dried in an oven for one hour at 100°C and subsequently 30 g of silica gel was wet packed with Hexane (Analytical grade, Merck) on to a glass column (60 x 3 cm, Borosil, India) fitted with a sintered disc inside. 2.5 gm of the Soxhlet methanolic root extract of *T. cuneifolia* residue was loaded on to the column and sample was covered with a layer of cotton. The sample extract was then fractionated by passing solvents of varying polarity through a column at a constant pace under gravity. The elution was carried out using a gradient of Hexane-Ethyl Acetate-Methanol (100:0, 50:50, 0:100). Total 120 fractions were collected. Each fraction was collected individually in a test tube and sequentially numbered for thin layer chromatography examination. Each fraction is applied to activated TLC plates at a 1/2-inch distance from the lower edge of the plate using a capillary tube, and the plates are kept in a developing chamber containing a suitable solvent system for a specific period of time until the developing solvent reaches the upper edge of the plate. Plate is removed from the

developing chamber, allowed to dry, and the solvent front is marked with a lead pencil. Compound bands/spots seen on a TLC chromate plate were visualised in an iodine chamber or under UV light (254 nm). The fractions obtained from Hexane-Ethyl acetate (100:0-0:100) were further analysed by using GCMS for the non-polar compound identification. Similarly, the fractions obtained from Ethyl acetate-Methanol (100:0-0:100) were analysed by TLC and similar fractions were put together and further subjected for further identification.

3.4.3 LC-MS-Q-ToF Analysis for untargeted plant metabolite

Metabolite profiling has not yet been reported in any investigations on *T. cuneifolia* root extract. For the metabolic profile of *T. cuneifolia*, the crude methanolic Soxhlet extract and the fifth fraction from column chromatography (ethyl acetate: methanol, 80:20) were selected as this fraction were giving different TLC profile.

Liquid chromatography linked to quadrupole time-of-flight mass spectrometry (LC-QToF-MS) was used to profile metabolites. Using an Agilent 6540 LC-QToF-MS system comprised of an Agilent 1290 LC and a 6540 UHD accurate-mass QToF mass spectrometer, metabolite analysis of *T. cuneifolia* root methanolic extract and fraction generated via column chromatography was performed. A 0.1% solution of formic acid in water (phase A) and acetonitrile made up the mobile phase (phase B). The whole duration of the performance was 60 minutes.

The flow rate applied was 0.6 mL min^{-1}, and the injection volume was 10.0 µl. MS analysis was performed using a 6540 Agilent Ultra-High-Definition Accurate-Mass QToF-MS linked to the LC and equipped with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) interface in positive ionisation mode under the following conditions mentioned in table (3.5, 3.6). Mass Hunter (Agilent Technologies, Santa Clara, California, United States)74 was used for data integration and refinement. Agilent Technologies has furnished the METLIN Personal Compound Database with precise mass MS/MS Library (PCDL). The METLIN, PCDL contains every chemical as well as precise mass Q-TOF MS/MS library reference spectra.

Table 3.5: Parameters for Liquid Chromatography analysis

LC Condition	
Column	ZORBAX 300SB C-18,
	4.6 x 100 mm 3.5-Micron
Column Temperature	25 °C
Injection Volume	10 µl
Mobile phase	A = 0.1 % Formic acid in Water
	B =Methanol 0.1 % Formic acid
Flow	0.6 ml / min
Gradient	B= 5% at 0 min
	B = 5 % at 3 min
	B = 100 % at 55 min
	B = 5 % at 56 min
	B = 5 % at 60 min
Run time	60 min

Table 3.6: Parameters for QToF instrument

QToF condition	
Ion Mode	Positive, ESI ionization mode (MS mode)
Drying Gas Temperature	350 °C
Drying Gas flow	10 L/min
Vaporize / sheath gas Temperature	300 °C
Sheath Gas Flow	12 L/min
Capillary voltage	4000 V
Capillary	0.078 uA
Fragmentor	150 V
Skimmer	65 V
Mass Range	80 to 1500 m/z
Aqusition rate	5 spectra/Sec
Time	200 ms/spectrum
extended dynamic range	2 GHz

3.4.3.1 LC-MS/MS-Q-TOF analysis of Targeted metabolites from column chromatography

3.4.3.2 Sample Preparation

In addition to the untargeted metabolomic analysis of crude and fractionated samples, the two fractions, i.e., the 4th fraction of ethyl acetate: methanol (5:5) (ColTAVEAMEOH) and the 2nd fraction of the 100% methanol (ColMEOHTAV), were subjected to the previously

mentioned targeted metabolomic analysis of Liquiritigenin, Naringenin, Kaempferol, Apigenin, Glycyrrhizin, Glabridin, Glycyrrhetinic acid and Stigmasterol.

3.4.3.3 LC-MS/MS-Q-TOF instrument parameters

All the information of this instrument for targeted metabolomic analysis is given in 3.3.6 section, table 3.3.

3.4.3.4 GCMS Analysis

3.4.3.4.1 GCMS Analysis of Column fraction

Sample preparation

The root extract of *T. cuneifolia* was fractionated by non-polar fraction using column chromatography (discussed in detail in section 3.4.2) and then analysed using gas chromatography-mass spectrometry. Those giving same TLC pattern mixed together for further separation. Selected fractions were mixed, and gas chromatography-mass spectrometry (GC-MS) were performed. The final sample were dissolve in 9:1 hexane: ethyl acetate.

3.4.3.4.2 GCMS Analysis of *T. cuneifolia* leaves and seed extract

Sample preparation

The seed and the leaves were taken apart, dried, and then ground up in a mixer. 112 gm of seed powder and 40 gm of leaves were put into a thimble and mixed with petroleum ether for 24 hours at 45 ℃ to carry out Soxhlet extraction. The extra solvent were evaporated using rota-evaporator and the extract was put in a vile and weighed.

Specification for GCMS is as follows

Perkin Elmer Clarus 680/SQ8C Gas Chromatography and Mass Spectrometry (With Quadruple detector). The Chromatographic separation was achieved using gradient programming on Rxi-5ms; 30m x 0.25mm ID x 0.25 µm dt., phase 5% diphenyl and 95% dimethyl polysiloxane is used as a stationary. The separated peaks were matched with the National Institute of standard and Technology (NIST) library.

3.4.3.5 Acquisition Parameters

Oven: Initial temp 70°C for 0 min, ramp 10°C/min to 200°C, hold 1 min, ramp 4°C/min to 250°C, hold 5 min, Injection volume=0 µL, Split=50:1, Carrier gas=Helium, Solvent delay=3.50 min, Transfer temperature=250°C, Source temperature=250°C, Scan: 50 to 600Da, Column 30.0m x 250µm**.**