Materials And Methods

3.1 MACROMORPHOLOGY

Macromorphological studies were done by studying the standard characters of plants. This includes its habitat, phyllotaxy, floral features and fruit. The plants were collected in flowering and fruiting stage from the field and identified with the help of floras (*Flora of Gujarat State* (Shah, 1978) and *Flora of Saurashtra* (Santapau, 1968; Bole and Pathak, 1988) and monographs before processing for herbarium. The plant specimens were dried using blotting papers and conventional cardboard plant press. Poisoning of dried specimens was done by Mercuric chloride (0.2%w/v) to protect specimen from pest attack. Dried plant specimens were mounted on herbarium sheets by strapping method using masking tape (Gummed linen tape). Standard herbarium sheets of size 29 cm x 41.5 cm were used. (Bridson and Forman, 1992)

Identity of the specimens was confirmed by comparing to the herbarium material lodged in the Herbaria depository of The Maharaja Sayajirao University of Baroda (BARO), Vadodara and Botanical Survey of India, Jodhpur (BSJO). Voucher specimens were deposited in the BARO herbarium, Vadodara.

3.2 MOLECULAR CHARACTERIZATION USING *matK* GENE.

Molecular characterization was used for cladistic understanding of endemic *Tephrosia* species in comparison to other commonly occurring species of this genus. Ribosomal RNA maturase (*matK*), an Open Reading Frame (ORF) in chloroplast DNA, was found embedded in the intron of the *trnK* gene. Due to its high substitution rate, it has been widely employed as powerful tool in evaluating genetic diversity in higher plants. Currently, more and more *matK* genes were used for identification and quality evaluation of medicinal plants. However, the molecular identification of *Tephrosia* species based on the *matK* is not been reported. Furthermore, by detecting the marker nucleotides for selected *Tephrosia* species and analyzing the phylogenetic relationship, the botanical origins of the inspected drugs were determined.

There are three major steps for the *mat*K sequence generation. They are

- Plant material processing and DNA isolation
- PCR amplification and sequencing
- Phylogenetic analysis

PLANT MATERIAL PROCESSING AND DNA ISOLATION

The five *Tephrosia* species (*T. purpurea, T. villosa, T. jamnagarensis, T. strigosa* and *T. collina*) were collected from the field and identification was done. The leaves of these species were washed and dried. It was crushed with liquid nitrogen and homogenised with Cetyl Trimethyl Ammonium Bromide (CTAB) buffer for extracting DNA of each plant samples. This homogenise mix was heated for 30 min at 65˚C over water bath. It was cooled and centrifuge at 13500 rpm for 12 min and separated in to filtrate and supernatant. The supernatant was twice treated with chloroform: isoamylalcohol (24:1) at 5 min at 1000 rpm. Then the supernatant was removed, isopropylanol was added and it left for overnight. After 24 hours it was centrifuge at 13000 rpm for 10 min at 03˚C and DNA is extracted in isopropylanol. The final wash was done with 70% ethanol by centrifuging at 10000 rpm for 5 min at 3˚C. The ethanol is decanted and kept for air drying. The DNA palettes obtained was treated with Tris ETDA buffer. Isolated DNA was subjected to gel electrophoresis to check the purity, shrinkage and contamination of RNA/ protein. The high intensity of DNA is then subjected to the PCR analysis **(Doyle *et al.*, 1987).**

PCR AMPLIFICATION OF THE PARTIAL *matK* GENE

The *matK* gene was amplified from the total genomic DNA using Polymerase chain reaction (PCR). PCR amplification of the partial *matK* gene was performed using 50-100 ng of total DNA as template in 25μL of a reaction mixture. The reaction Master Mix consists of combination of assay buffer, $MgCl_2$, 10mmol of each dNTPs and 1 unit of taq polymerase from Genei^{TM}, Bangalore, India. The *matK* locus was amplified with the primer pair: 3F_KIM f CGTACAGTACTTTTGTGTTTACGAG and 1R_KIM r ACCCAGTCCATCTGGAAATCTTGGTTC (Ki-Joong Kim, School of Life Sciences and Biotechnology, Korea University, Korea, unpublished results). PCR amplification was carried out with the following cycling condition: hot start at 92°C for 2 min denaturation, followed by 40 cycles of 92°C for 1min, 51 .5°C for 1 min for annealing and 72°C for2 min and final extension for 7min at 72°C. PCR product was electrophoresed through 2% agarose gel to determine the size of fragment. Positive products were then purified using the Exo-Sap-IT reagent (USB, Cleveland OH, USA) using the manufacturer's recommended protocol (2 μL reagent per 5μL amplified DNA product). Thereafter the purified samples were subjected to nucleotide sequencing using Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) as per manufacturer's protocol. After purification, the reaction products were analyzed on an ABI PRISM Genetic Analyzer 3130 (Applied Biosystems).

CONSTRUCTION OF PHYLOGENETIC TREE

The partial *mat*K sequence were assembled and aligned by the Bioedit progam. They were deposited in the Genbank nucleotide sequence database. These sequences were utilized for generating phylogenetic tree, which was constructed by Neighbor-joining method using the computer program MEGA 5.05. Branch lengths were calculated by Kimura's two parameter method. Bootstrap analysis (1000 replications) was performed to estimate the confidence of the consensus tree. *Cassia abbreviata* was taken as outgroup for the phylogenetic analysis of the *matK* locus.

3.3 THE DISTRIBUTION PATTERN ECOLOGICAL STUDIES

Distributional pattern was studied based on the available literature and survey of different probable sites from where *T. jamnagarensis* and *T. collina* were reported. Even the surrounding regions were studied. To study the ecological pattern of these plants in their natural habit following method was adopted. The two major parameters are abiotic and biotic parameters of field area. The abiotic parameters like rainfall, temperature, humidity and edaphic factors were studied.The biotic parameters were population size, population density and population growth its Extent of occurrence (EOO) and area of occurrence (AOO) studied over period of time. The EOO and AOO were studied using IUCN Red List Categories and Criteria version 11 and counted using google pro software. Along with it the associated plant species were taken into account.

3.3.1 ABIOTIC PARAMETERS

Under abiotic factors the climatic and edaphic data was collected. The climatic data was collected from the meteorological department. The edaphic factor was studied by collecting soil from the site. For this a pit of 1 x 1 x 1 ft. was made and various strata (upper, middle and lower) were collected and mixed. The soil collected was subjected for the physical and chemical properties analysis. The physical property of soil in studies includes colour and texture. Following are the chemical property studied in soil.

Chemical properties ofthe soils was studied by following method

1) Organic Carbon (Chromic acid Method) (Perur, 1973)

The chromic acid method was used for analysis. The results obtained were rated as:

Low < 0.50%, Medium 0.50-0.75%, High > 0.75%

2) Available Phosphorous (Ghosh *et al.*, 1983)

The olsen's extractant method was used for analysis. The results obtained were rated as: P (Kg/ha)-Low < 11, Medium 11-25, High > 25

3) Available Potassium (Perur, 1973)

Rating of available K_2O was done as below:

Low < 120 kg/ha, Medium 120-280 kg/ha, High > 280 kg/ha.

4) Soil pH: (Perur, 1973)

5) Electrical conductivity (Perur, 1973)

6) Available Sulphur (Chesnin and Yein, 1950)

7) Available micronutrients (Ghosh *et al.*, 1983):

The four micronutrients considered were zinc, copper, iron and manganese and analysed by using Atomic absorption spectrophotometer (AAS). The results obtained were rated as:

Zinc: Low<0.5ppm, Medium0.5-1.0ppm, High>1.0ppm

Iron and Manganese: Low<5ppm, Medium5-10ppm, High>10ppm

Copper: Low<0.2ppm, Medium0.2-0.4ppm, High>0.4ppm

3.3.2. BIOTIC PARAMETERS

The population studies were done by using quadrate method along the distribution belt. The population size were studied by belt transect method. All the plant along the belt of 5 meters was studied. All the plants in the locality were quantitatively studied. The data collected on the number of individual and its occurrence was used for calculating population density, frequency and growth. The status of the plant was studied based on the IUCN criteria by considering the parameters like Extent of occurrence (EOO) and Area of Occupancy (AOO).

Exponential population growth was determined by dividing the change in population size (ΔN) by the time interval (Δt) for a certain population size (N):

$$\frac{\Delta N}{\Delta t} = rN$$

The other associated plant species were identified and collected.

3.4 SEED GERMINATION AND PHENOLOGY

3.4.1 SEED GERMINATION

Seed germination was studied for understanding its dormancy and viability. Seeds of *T. jamnagarensis* and *T. collina* were collected from the wild. The morphological characteristics were studied and photography was done by D M Canon camera under Leica stereozoom microscope. Viability of seeds was tested with Tetrazolium chloride 0.1 % at 25°C and weight of hundred seeds was recorded. Prior to seed germination, surface sterilization of the seeds was done with sodium hypochlorite (AOSA, 1978). Seeds were germinated on 3 sheet of 9 cm diameter filter paper placed in Petri dishes with 10 ml of distilled water. Ten seeds were used for each treatment with three replicates each. The effect of seed treatment was studied by soaking the seeds in hot water, cold water, warm water, concentrated H_2SO_4 for 5 minutes and by treating seed with three different concentration of GA_3 like 250 ppm, 500ppm and 1000 ppm. Effects of treatments were observed for 15 days.

After establishment of the seed treatment protocol, the germinated seeds were taken to experimental site (The arboretum, Department of Botany, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat) for its phenology study.

Life cycle of *T. jamnagarensis* and *T. collina* was understood by germinating seeds in polybags till four leaves stage and at this stage sapling were shifted to the field. The seedlings were grown in a row at the distance of 40 cm apart from each other. Planting holes should be 30 cm deep, filled with soil mixed with leaves, small branches and dung. Prior to shifting of seedling, the mother soil was well aerated and mixed with organic dry matter and sand. Three permanent quadrates (1 x 1 m^2) were marked out before onset of monsoon during April. The deweeding of plot was done prior to seedling transplantation in soil. Then these clean quadrates were left for 2-3 day as it for sunlight to penetrate the soil and kill the harmful organism. Finally, organic fertilizer of dried leaves was applied before sowing of the seeds.

During transplanting, care was taken to avoid bending of the tap root. The soil was pressed firmly around the plant to prevent wilting and to help the plant to recover from the shock of transplanting.

3.4.2 PHENOLOGICAL PARAMETER

The phenology of both these endemic plants was observed at experimental site at regular interval from April 2012 to November 2012. The characters from initiation of seed germination to vegetative stage to flowering stage were studied. During this period morphological variations

were also studied. Even the microclimatic data and soil properties were also studied. Vegetative and generative growth and dormancy periods were determined according to Leon and Bertiller (1982).

3.5 PHARMACOGNOSY

3.5.1 ANATOMY

Anatomical features were studied as per the charactersitc feautes given by Fahn (1997) and Kokate (1994). The free hand sections of all the plant parts were done. Sections of 10-15 µ thickness were selected for further study. These selected sections were stained with safranin (0.5%) in water and the mounted in 50 % glycerine. For permanent slides perparation, fresh plant samples were cut into suitable size of 4-5 cm in length and were immediately fixed in Formalin Acetic Alcohol (FAA) solution (Berlyn and Miksche, 1976) trimmed and locks were sectioned in transverse planes at thickness of 12-15 µm by using Leitz sliding microtone. Sections were stained with safranin: fast green combination (Johansen, 1940) and mounted in Dibutly phthalate xylene (DPX) after passing through the ethanol-xylene series. The slides were then observed under the microscope and the sizes of various cells observed in the tissues were measured using an ocular micrometer. The least count of the micrometer was calculated for this purpose. The sections were photographed under Leica DM 2000 trinocular microscope connected to Digital Canon Camera.

3.5.2 MICROMORPHOLOGY

Fresh plant materials were washed and small fragments of leaves were taken from the middle region of the mature leaf lamina. The epidermal layer was peeled off with the help of pointed needle and blade and was washed in water, stained with safranin (0.5%) and then mounted on slide and viewed under the microscope. Stomatal index, palisade ratio, vein termination number and vein islet number were then calculated using standard procedures (Anonymous, 2002).

3.5.3 POWDER STUDIES

Completely dried plant material was finely powdered and sieved through BSS mesh No. 44. The fine powder obtained was stained using safranin in water. The stained powder was mounted on a slide and observed under a microscope to locate and identify the distinguishing

characters. The characters observed were photographed under Leica DM 200 microscope connected to a Digital Canon camera **(**Wallis, 1957**)**.

3.5.4 HISTOCHEMICAL TESTS

Specific reagents for identification of important classes of compounds were prepared according to procedures prescribed in the WHO guidelines. Sections and powder of root, stem and leaves were treated with reagents and mounted on slides for observations under microscope.

3.6 PHYTOCHEMISTRY

3.6.1 PHYSICOCHEMICAL STANDARDIZATION

Evaluation of crude drugs is of great importance for the pharmaceutical industry. It involves the determination of identify, purity and quality of crude drug. Purity depends upon the absence of foreign matter whether organic or inorganic, while quality refers to the concentration of the crude drug i.e. the plant materials can be gathered by determination of various parameters like solvent extractive values, ash values (total ash, acid insoluble ash as well as water soluble ash) and loss on drying (Mukherjee, 2007).

Raw materials and powders of *T. jamnagarensis* and *T. collina* was subjected to proximate analysis in order to evaluate the content of foreign matter, ash content, extractive values and loss on drying.

1) DETERMINATION OF FOREIGN ORGANIC MATTER

This is one of the parameter essential for the quality of the raw material used in formulation. The plant materials should be entirely free from visible signs of contaminants including animal excreta. It should not have an abnormal odour, discolouration etc. It is not possible to obtain plant materials that are entirely free from any form of foreign matter. Foreign matter is the material consisting of any or all of the following:

- Parts of the organs from which the drug is derived other than the parts named in the definition and description given in the monograph.
- Matter that is not collected from the plant source
- Moulds, insects or other animal contaminants.

PROCEDURE: Raw powdered plant material of aerial part and root of *T. jamnagarensis* and *T. collina* (100-500 g each) were spread separately on a paper to form a thin layer. The samples were examined in daylight with unaided eye using a magnifying lens (6x or 10x). The foreign or suspected particles (if any) was separated and picked out manually. The separated matter was weighed and its percentage was calculated. The experiment was repeated thrice and documented.

Foreign organic matter was calculated as:

$$\text{Foreign organic matter \%} = \frac{(W_1 - W_2)}{W_3} \times 100$$

Where W_1 is weight of dish with foreign matter, W_2 is weight of empty and W_3 is weight of sample taken in grams.

2) LOSS ON DRYING

Loss on drying is used for determining moisture content of crude drugs. Moisture is an inevitable component of crude drugs, which must be eliminated as far as practicable. Insufficient drying favours spoilage by moulds and bacteria and makes possible the enzymatic destruction of active principles. Hence, drying plays a very important role in the quality as well as the purity of the material (Mukherjee, *l.c.*).

PROCEDURE: Place about 10 g accurately weighed up to third decimal place of drug in tarred evaporating dish. The place dish in oven and dries at 105°C for 5 hours and weigh. Continue the drying and weighing at one hour interval until difference between two successive weighing corresponds to not more than 0.25 percentage. Constant weight is reached when two consecutive weighing after drying for 30 minutes and cooling for 30 minutes in a desiccators, show not more than 0.001 g difference.

3) DETERMINATION OF ASH VALUES

The ash of any plant material is composed of their non-volatile inorganic components like metallic salts and silica which is obtained by controlled incineration of crude drugs. Ash values of plant materials vary within fairly wide limits and are therefore a parameter for evaluation of quality of crude drugs. In certain drugs a marked difference in the percentage of ash values indicates the change in its quality. Different contaminations in the plant materials like unwanted plants parts, sand, earth etc can causes an increase in the ash values and thus, these contaminants can be detected. The ash value is determined by measuring the total ash, acid insoluble ash and water soluble ash (Mukherjee, *l.c.*).

TOTAL ASH

The total amount of material produced after complete incineration of the drug at 450°C to remove all carbon compounds is called as total ash. High ash value is indicative of contamination, substitution, adulteration or carelessness in preparation of the crude drug. Total ash consists of carbonates, phosphates, silicates and silica. It includes physiological ash derived from the plant

tissue and non-physiological ash derived from residue of sand, soil etc which adhere to the plant materials (Mukherjee, *l.c.*).

PROCEDURE: Powder of aerial part and root of *T. jamnagarensis* and *T. collina* (2 g each) was weighed separately and transferred to per-crucibles. These crucibles were incinereated at 450°C in muffle furnace until free from carbon. After incineration, the crucibles were cooled in desiccators, weighed and the percentage of total ash was calculated for the respective plant raw materials. The experiment was repeated thrice and documented (India pharmacopeia. 2010; Mukherjee, *l.c.*).

Total ash was calculated as:

$$\text{Total ash (\%)} = \frac{(W_1 - W_2)}{W_3} \times 100$$

Where, W_1 is weight of crucible with ash (g), W_2 is weight of empty crucible (g) and W_3 is weight of powder taken (g).

ACID INSOLUBLE ASH

Acid insoluble ash is the residue obtained after treating the total ash with dilute hydrochloride acid. It indicates contamination with siliceous material e.g. silica and sand (Mukherjee, *l.c.*).

PROCEDURE: Total ash of aerial part and root of *T. jamnagarensis* and *T. collina* was obtained using the above described procedure. 25 ml of 2N HCl was added to the respective ash and the mixtures were kept in boiling water bath for 5 min. The mixtures were filtered through ashless filter paper (Whatman filter paper no. 41) and washed with warm water to remove the traces of acid in it. The respective filter papers (with the insoluble matter) were transferred to preweighed crucibles and ignited in muffle furnace at 450°C till constant weight was obtained. The crucibles were cooled in desiccators, weighed and the percentage of acid insoluble ash in aerial part and root of *T. jamnagarensis* and *T. collina* was calculated. The experiment was repeated thrice and documented (India Pharmacopeia, 2008; Mukerjee, *l.c.*).

Acid insoluble ash was calculated as:

$$\text{Acid insoluble ash (\%)} = \frac{(W_1 - W_2)}{W_3} \times 100$$

Where, W_1 is weight of crucible with ash (g), W_2 is weight of empty crucible (g) and W_3 is weight of powder taken for obtaining total ash (g).

WATER SOLUBLE ASH

The part of total ash content in the raw material that is soluble in water is called as water soluble ash. It is the difference in weight between the total ash and the residue obtained after treatment of total ash with water. It is a good indicator of either previous extraction of the water soluble salts in the drug or incorrect preparation of the raw material (Mukherjee, 2007).

PROCEDURE: Total ash of powdered aerial part and root of *T. jamnagarensis* and *T. collina* was obtained using the procedure described earlier. 25 ml of water was added to the respective ash and the mixtures were boiled in water bath for 5 min. The mixture were filtered through Whatman filter paper no 41. These filter papers (with insoluble matter) were transferred to per-weighed crucibles and ignited at 450°C in muffle furnace till constant weight was obtained. The crucibles were cooled in dessicator and weighed. The weight of the insoluble matter was subtracted from the weight of ash and the difference of weight represented the water soluble ash. The percentage of water soluble ash in aerial part and root of *T. jamnagarensis* and *T. collina* was calculated. The experiment was repeated thrice and documented (Indian Pharmaacopia, 2008; Mukherjee, 2007).

Water insoluble ash was calculated as:

$$\text{Water insoluble ash (\%)} = \frac{(W_1 - W_2)}{W_3} \times 100$$

Where, W_1 is weight of crucible with ash (g), W_2 is weight of empty crucible (g) and W_3 is weight of powder taken for obtaining total ash (g).

Water soluble ash = Total ash – Water insoluble ash

4) DETERMINATION OF SOLVENT EXTRACTIVE VALUES

The amount of phytoconstituents in the given amount of plant material can be determined by extracting it with different solvents of varying polarities. The determination of ethanol soluble and water soluble extractive is used as a means of evaluating the quality of crude drugs (Indian Pharmacoepia, *l.c.*).

ETHANOL SOLUBLE EXTRACTIVE

Powder of aerial part and root of *T. jamnagarensis* and *T. collina* (4 g each) was taken in separate stoppered conical flask and 100 ml of ethanol was added to it. The flasks were shaken well and then allowed it to stand for 1 hour. The flasks were attached to reflux condenser and

boiled gently for 1 hour; cool and weighed. Readjustment original weight with the ethanol was done. Shaken well and then filtered. Transfer 25 ml of the filtrate to a tared flat-bottomed dish and evaporate to dryness on a water-bath. Dry at 105°C for 6 hours, cool in a desiccators for 30 minutes, then weigh without delay. Calculate the content of extractable matter was expressed in mg per g of air-dried material.

Ethanol soluble extractive was calculated as:

$$\text{Ethanol soluble extractive} = \frac{(W_1 - W_2)}{W_3} \times 100 \times 4$$

Where, W_1 is weight of beaker with extract (g), W_2 is weight of empty breaker (g) and W_3 is weight of powder taken (g).

WATER SOLUBLE EXTRACTIVE

PROCEDURE: Powder of aerial part and root of *T. jamnagarensis* and *T. collina* (4 g each) was taken in separate stoppered conical flask and 100 ml of ethanol was added to it. The flasks were shaken well and then allow it to stand for 1 hour. The flasks were attached to reflux condenser and boil gently for 1 hour; cool and weighed. Readjustment of the original total weight with the water was done. Shaken well and then filtered. Transfer 25 ml if the filtrate to a tared flat-bottomed dish and evaporate to dryness on a water-bath. Dry at 105°C for 6 hours, cool in a desiccators for 30 minutes, then weigh without delay. Calculate the content of extractable matter was expressed in mg per g of air-dried material.

$$\text{Water soluble extractive (\%)} = \frac{(W_1 - W_2)}{W_3} \times 100$$

Where, W_1 is weight of breaker with extract (g), W_2 is weight of empty breaker (g) and W_3 is weight of powder taken (g).

5) DETERMINATION OF SWELLING INDEX

Many medicinal plant materials are of specific therapeutic or pharmaceutical utility because of their swelling properties, especially gums and those containing an appreciable amount of mucilage, pectin or hemicelluloses. The swelling index is the volume in ml taken up by the swelling of 1 g of plant material under specified conditions.

PROCEDURE: The accurately weighed (1 g each) fineness powder of aerial part and root of *T. jamnagarensis* and *T. collina* was taken in 25- ml glass-stoppered measuring cylinder. The internal diameter of the cylinder should be about 16 mm, the length of the graduated portion about 125 mm, marked in 0.2 ml divisions from 0 to 25 ml in an upwards direction. Unless otherwise indicated in the test procedure, add 25 ml of water and shake the mixture thoroughly at every 10 minutes of an hour. Allow to stand for 3 hours at room temperature, or as specified. Measure the volume in ml occupied by the plant material, including any sticky mucilage. Calculate the mean value of the individual determinations related to 1 g of plant material (Anonymous, *l.c.*).

6) DETERMINATION OF FOAMING INDEX

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of a foaming index.

PROCEDURE: The accurately weighed (1 g each) coarse powder of aerial and root parts of *T. jamnagarensis* and *T. collina* was taken in 500 ml conical flask containing 100 ml of boiling water. The moderate boiling was done for 30 minutes. Cooled and filtered into a 100 ml volumetric flask by adding sufficient water through the filter to it diluted. This decoction was poured in 10 stoppered test-tubes in successive portions of 1 ml, 2 ml, 3 ml, etc upto 10 ml and the volume of liquid in each tube was adjusted with water to 10 ml. The tubes were stoppered and shaken in a lengthwise motion for 15 seconds, two shakes per second. Allowed it to stand for 15 minutes and measurement of the foam height was done. The results were assessed as per the standard scale (Anonymous, *l.c.*).

7) HEAVY METAL ANALYSIS

Preparation of standard solution:

LEAD (Pb):

10 ppm Pb (Working standard): 0.25 ml standard solution of Pb (1000 ppm) was pipette out accurately in 25 ml volumetric flask and the volume was made up with distilled water. 0.1ppm Pb: 25 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water. 0.5 ppm Pb: 1.25 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water1.0 ppm Pb: 2.5 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water. 2.0ppm Pb: 5.0 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water. 3.0 ppm Pb: 7.5 ml solution from working standard (10 ppm) was pipette out in 25 ml volumetric flask. The volume was made up with distilled water.

CADMIUM (Cd):

10 ppm Cd (Working standard): 0.25 ml standard solution of Cd (1000 ppm) was pipette out accurately in 25 ml volumetric flask and the volume was made up with distilled water. 0.1 ppm Cd: 0.25 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water. 0.2 ppm Cd: 0.50 ml solution from working standard (10 ppm) was pipette out in 25 ml volumetric flask. The volume was made up with distilled water. 0.3 ppm Cd: 0.75 ml solution from working standard (10 ppm) was pipetted out in 25 ml volumetric flask. The volume was made up with distilled water. 0.4 ppm Cd: 1.0 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water. 0.5 ppm Cd: 1.25 ml solution from working standard (10 ppm) was pipette out in a 25 ml volumetric flask. The volume was made up with distilled water

ARSENIC (As):

1000 ppb As: 0.1 ml standard solution of As (1000 ppm) was accurately pipette out in 100 ml volumetric flask and the volume was made up with distilled water. 100 ppb As (working standard): 10 ml solution of Arsenic (1000 ppb) was accurately pipette out in 100 ml volumetric flask and the volume was made up with distilled water.

5 ppb As: 2.5 ml working standard (100 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled water. 10 ppb As: 5.0 ml working standard (100 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled

water. 15 ppb As: 7.5 ml working standard (100 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made with distilled water. 20 ppb As: 10.0 ml working standard (100 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled water.

MERCURY (Hg)

1000 ppb Hg (working standard): 0.1 ml standard solution of Hg (1000 ppm) was accurately pipette out in 100 ml volumetric flask and the volume was made up with distilled water. 20 ppb Hg: 1.0 ml working standard (1000 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled water. 40 ppb Hg: 2.0 ml working standard (1000 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled water. 60 ppb Hg: 3.0 ml working standard (1000 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled water. 80 ppb Hg: 4.0 ml working standard (1000 ppb) was accurately pipette out in 50 ml volumetric flask and the volume was made up with distilled water.

PREPARATION OF SAMPLE: Accurately 0.5-1.0 g weighed of the coarse powder of the samples was examined. It was transfered into casparin/ Erlenmeyer flask. 5-10 ml of the mixture of nitric acid and percholic acid (4:1) was added. A small hopper was added on the flask top. Maceration was done for overnight by heating it on the electric hot plate with occasional shaking. Keep somewhat boiling, if the colour of solution is brownish-black. Add again a quantity of the above acid mixture. Heat continuously till the solutions became transparent. The raise temperatures, heat continuously to thick smoke till white smoke disperse. The slaked solution becomes colourless and transparent or a light- yellow. Cool, transfer it into a 50 ml volumetric flask. Wash the container with two percent nitric acid solution. Add the washing solution to the desired volume and shake well. Prepare synchronously the reagent blank solution according to the procedure above.

INSTRUMENT: - Atomic Absorption spectroscopy (AAS) to detect heavy metals.

Atomic absorption spectroscopy relies on the Beer-Lambert law to determine the concentration of a particular analyte in a sample. The absorption spectrum and molar absorbance of the desired sample element are known, a known amount of energy is passed through the atomized sample and by then measuring the quantity of light it is possible to determine the concentration of the element being measured. Atomic Absorption Spectrometers are available

with flame, graphite furnace and vapour hydride generation atomizers. These atomizers aspirate the sample into the light path where it is illuminated by a hollow-cathode lamp (HCL), which emits light at the wavelength characteristic of the desired elements. A built-in detector measures the light emissions both in presence and absence of sample and the ratio of the absorbances are used to determine the analyte concentration.

3.6.2 PRELIMINARY PHYTOCHEMICAL ANALYSIS

3.6.2.1 QUALITATIVE ANALYSIS

For all the qualitative and quantatitive analysis the methanolic extracts of aerial, root and seed of *T. jamnagarensis* and *T.collina* was taken. The details procedure as described by Evans (2002) was followed.

TEST FOR CARBOHYDRATES

Fehling test: 1 ml of Fehling A added in to 1 ml of Fehling B solution was mixed and few drops of test solution were added. It is allowed to boil for few minutes in boiling water bath. The precipitation of yellow or brown or red colour was observed then reducing sugar present.

Benedict test: To 2 ml of Benedict test reagent added to five drops of the test solution. Boil for five minutes in water bath. Cool the solution. Formation of red yellow, green colour precipitate indicates presence of reducing sugar.

Molisch's test: Two drops of molisch's reagent (5% alpha naphthol in alcohol) + 2 ml test solution mix well and add about 1 ml of concentrated H_2SO_4 acid along side of the tube. A red cum violet ring appears at the junction of the liquid.

TEST FOR PROTEINS

To 1 ml of plant extracts few drops of Millon's reagents was added. If the solution stains red on warming then it indicates presence of protein.

TEST FOR ALKALOIDS

Dragendroff's test: In a test tube containing 1 ml of methanolic extract of samples, few drops of dragendroff's reagent was added and the color change was noticed. Appearance of orange colour indicates the presence of alkaloids.

Mayer test: To 1 ml extract 2 ml of Mayer's reagent was added. A dull white precipitate indicates presence of alkaloids.

TEST FOR IRIDIODES

Trim hill reagent (1 ml) was added to 2 ml of methanolic plant extract. If solution turns blue then iridiods present.

TEST FOR ANTHOCYNANIN

Cut plant sample in sufficient amount to it's add 200 ml of 2 N hydrochloride acid and stirred it. Warm it on water bath and filtered the extract. Filtrated was cooled and shaken with 50 ml of ether to removed flavonoids. The aqueous layer was again shaken with 50 ml amyl/isoamyl alcohol. Anthocyanin move to isoamyl layer and turns deep pink to red colours, mark the presence of anthocyanin and it can be further confirmed by chromatography technique.

TEST FOR FLAVONOIDS

2 ml of plant methanolic extract was taken in test tube. In it sodium hydroxide pallets was dissolve and the dilute hydrochloride was added. The colour change observation from yellow to colourless indicates presence of flavonoids.

TEST FOR LIGNANS

In this test 2% furfuraldheyde was added to 2 ml of methanolic plant extract concentrated. When this was acidified with hydrochorlic acid, the development of red colour was taken as positive for lignans.

TEST FOR TANNIN

To 1 ml of methanolic plant extract, ferric chloride was added. Formation of a dark blue or greenish black colour showed the presence of tannins.

TEST FOR SAPONINS

Foam test: The methanolic plsnt extract was shaken vigorously with water. The formation of persistent foam indicates presence of saponins.

TEST FOR PHENOLS

Fresh plant samples were collected. The sliced tissues was immersed in 2N HCl in a test tube and heated for 30 min on boiling water bath. The extract was cooled extract with ether; the ether extract was pipette out and evaporated to dryness, the residue used for test.

Neutral $FeCl_3$ test- the residue was treated with neutral $FeCl_3$, formation of blue, green red or violet colour indicates presence of phenolics.

TEST FOR TERPENOIDS

To 0.5 ml of plant methanolic extract 2 ml chloroform was added with further addition of 1 ml concentrated sulphuric acid carefully. The observation of brown colour at interface marked the presence of terpenoids.

TEST FOR STEROID

To the 1ml of plant extract 2 ml acetic anhydride was added and with further addition of 2 ml concentrated sulphuric acid. Observations of colour change from violet to blue or green in sample indicate the presence of steroids.

TEST FOR QUINOINES

To 1 ml of the extract 1 ml of concentrated sulphuric acid was added. Formation of red color marked the presence of quinones.

3.6.2.2 QUANTITATIVE ANALYSIS

ESTIMATION OF TOTAL CARBOHYDRATE CONTENT

MATERIALS: 2.5 N HCl, Anthrone reagent: Dissolve 200 mg anthrone in 100 ml of ice-cold 95% H_2SO_4. Prepare fresh before use. Standard glucose: Stock- Dissolve 100 mg in 100 ml water. Working standard—10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene.

PROCEDURE: Weigh 100 mg of the sample into a boiling tube. Hydrolyse by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cool to room temperature.

Neutralise it with solid sodium carbonate until the effervescence ceases. Make up the volume to 100 ml and centrifuge. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard.'0' serves as blank. Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. Then add 4 ml of anthrone reagent. Heat it for eight minutes in a boiling water bath. Cool rapidly and observe the intensity of green at 630 nm in UV-VIS spectrophotmeter. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph calculate the amount of carbohydrate present in the sample tube. All the samples were analyzed in triplicates.

Total carbohydrate was calculated as:

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

Sample preparation for protein, phenolic and Flavonoids content

5 g of plant powders (aerial, root and seed of *T. jamnagarensis* and *T. collina*) was reflux with 100 ml methanol for 1 hour. After 1 hour methanol was fitered in volumentic flask and volume was again make up to 100ml with methanol. In case of Phenolic and Flavonoids further dilution was done.

ESTIMATION OF TOTAL PROTEIN CONTENT

1 ml of plant methanolic solution was taken. To this 5 ml of alkaline solution (solution 3) was added. The solutions were mixed and allowed it to stand at room temperature for 10 minutes. Further 0.5 ml of diluted Folin reagent was added and mixed. After incubation period of 30 minutes the absorbance was read against the appropriate blank at 750 nm. Prepare standard graph and extrapolated the reading of unknown solution on standard graph and calculated the concentration of the unknown solution. All the samples were analyzed in triplicates.

ESTIMATION OF TOTAL PHENOLIC CONTENT

Methanolic solution of the extracts in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 1:10 diluted Folin-Ciocalteu reagent was added. After 4 min, 2 ml of saturated sodium carbonate solution (about 75g/lt) was added. After 2 hour of incubation at room temperature, the absorbance of the reaction mixture was measured at 760 nm. Gallic acid was used as reference standard and

result were expressed as milligram gallic acid equivalent (mg Gallic) dry weight of plant material. All the samples were analyzed in triplicates.

ESTIMATION OF TOTAL FLAVONOID CONTENT

Total flavonoid content was measured by the aluminium chloride colorimetric assay. An aliquot (1 ml) of extracts or standard solution of quercetin (20, 40, 60, 80 and 100 mg/l) was added to 10 ml volumetic flask containing 4 ml of deionised water. To the flask 0.3 ml 5% $NaNO_2$ was added. After 5 min, 0.3 ml 10% aluminium chloride was added. Further, to it at 6^{th} min, 2 ml 1M sodium hydroxide was added and the total volume was made up to 10 ml with deionised water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoids content was expressed as mg quercetin equivalents QE/100 g crude drug. All the samples were analyzed in triplicates.

3.6.3 PHYTOCHEMICAL STANDARDIZATION

All the procedures followed in the present work were separately performed for both the *Tephrosia* species on their aerial and roots parts. The extracts of these parts were focused for polyphenols groups like Flavonoids, Steroids, Phenolic acid and Terpenoids. The seeds extracts of these plants were focused for Rotenoids and Fatty acids analysis. Details are as follow:

FLAVONOIDS

Fifty grams of aerial and root powder were extracted in a Soxhlet's apparatus with methanol for 48 hrs or till the plant material became colourless. The methanolic extract was concentrated to dryness on a water bath. 25-30 ml of water was added to the dry residue and the water soluble phenolic glycosides were filtered out. The filtrate was hydrolysed in waterbath for one hours using 7% HCl.

This hydrolysate was extracted with diethylether/ solvent ether, whereby the aglycones got separated into ether fraction (fraction A). The remaining aqueous fraction was further hydrolysed for another 10 hours to ensure the complete hydrolysis of all the O-glycosides. Aglycones were once again extracted into diethylether (fraction B) and the residual aqueous fraction was neutralized and evaporated for the analysis of glycoflavones.

Ether fraction A and B were combined and analysed for aglycones using standard procedures (Mabry *et al.*, 1970; Harborne, 1984). The combined concentrated extract was banded on Whatmann no. 1 paper. The sample system used was 30% glacial acetic acid, the developed chromatograms were dried in air and the visibly colour compounds were marked out. These chromatograms were observed under ultra-violet light (360 nm) and the brands were marked. Duplicate chromatograms were then sprayed with 10% Na_2CO_3 and 1% $FeCl_3$ and the colour changes were reported. The marked bands of the compounds were cut out from unsprayed chromatograms and were eluted with spectroscopic grade methanol. The UV absorption spectra of these compounds were recorded in methanol using Perkin Elmer Lambda 25 UV/Vis' spectrophotometer.

PHENOLIC ACIDS

Analysis of combined ether extract (A and B) was carried out by two dimensionals ascending paper chromatography. Toluene: acetic acid: water (6:7:3 v/v/v, upper organic layer) in the first direction were used as irrigating solvents. The sprays used to locate the compounds on the

chromatograms were diazotized p-nitroniline or diazotised sulphanilic acid and a 10% Na_2CO_3 overspray (Ibrahim and Towers, 1960).

DIAZOTIZATION:

0.7 g of p-nitraniline/ sulphonic acid was dissolved in 9 ml of HCl and the volume made up to 100 ml. Five ml of 1% $NaNO_2$ was taken in a volumetric flask and kept in ice till the temperature was below 4°C. The diazotized sprays were prepared by adding 4 ml of p-nitraniline/ sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made up to 100 ml with ice cold water; the various phenolic acids present in the extract were identified based on the specific colour reaction they produce with the spray reagents.

THIN LAYER CHROMATOGRAPHY (TLC)

CARBOHYDRATES ANALYSIS

Samples were extracted with methanol and hydrolysed with HCL. Analyses of various sugars were done with various sugar standard procured from Sigma Aldrich. Mobile phase was Ethylacetate: Acetic Acid: Methanol: Water (60:15:15:10). Saturation for 30 mins of chromagraphic chamber was done. Observation was made in visible light. The developing of TLC was done by derivatzing reagent (p Anisaldehyde-sulphuric acid) at 110°C for 10 mins.

AMINO ACID ANALYSIS

Accutately weighed 1 g powder was extracted with 10 ml methanol. The mixture was vortexed for 1 min and kept standing overnight. The mixture was sonicated for 20 mins and the filtered through Whatmann filter paper no 1. The filtrate obtained was concentrated and subjected to TLC with 20 amino acid standards procured from Sigma Aldrich. Mobile phase was n-Butanol: ethylacetate: acetic acid: water (1:1:1:1). Saturation of chromatographic chamber was done for 30 mins. Observation was made in visible light after developing TLC plate with derivatizing reagent (1% Ninhydrin reagent) at 110°C for 5 mins.

HPTLC

Flavonoids, Terpenoids and steroids were analysed with HPTLC. The specification of instrument is as follows:

- HPTLC plates: Merck, aluminium sheet precoated with silica gel F254 procured from Anchrom laboratories, Mumbai, India.

- Applicator: CAMAG linomat 5 sample spotter (Camag Muttenz, Switzerland) equipped with syringe (Hamiliton).
- Developing chamber: CAMAG twin toughchamber.
- Densitiometer scanner: Camag TLC scanner 4 conjugated with winCATS software.
- Photodocumentation : Camag Reprostar 3.

Solvents

Organic solvent of analytical grade were procured from Merck Specialties Private Limited, India.

Standard:

Reference compounds (standard) β-sitosterol (98% purity) and lupeol (95% purity), Quercetin (98% purity) and Kaempferol (98% purity was procured from Sigma Aldrich chemical Pvt. Limited Bangalore, India.

TERPENOIDS AND STEROIDS

Samples Preparation: Accurately weighed powder (0.5 g) was extracted with methanol (10 ml). The mixture was vortex for 1 min and kept standing overnight. The mixture was filtered through Whatmann filter paper no. 41 and filtrate obtained was diluted with methanol in equal proportion (1:1) and subjected to HPTLC analysis. Mobile phase used was Toluene: Methanol (8:1) in a chamber which was saturated for 30 mins. The TLC was observed at 366 nm by derivatizing agent (10% methanolic sulfuric acid) at 110°C for 5 mins.

FLAVONOIDS

Sample preparation: The methanolic extract was hydrolysed with 7% HCL and residue was separated in separating funnel with ethylacetate. The ethylacetate extract residue with help of methanol was loaded to TLC plate. Mobile phase used was Chloroform: Methanol (96:4) in a chamber which was saturated for 30 mins. The TLC plate was observed at 254 nm.

HPLC analysis

HPLC was done for the analysis of **Flavonoids** and **Rotenoids** in the aerial, root and seed of *T. jamnagarensis* and *T. collina.*

Instrumentation:

HPLC analysis was carried out on Shimadzu LC 20AT liquid chromatography, equipped with a photodiode array detector. The analytical column used was Cosmosil C18 (250 x 4.6; 5

μm). Mode of elution was isocratic. The mobile phase used for Flavonoids analysis was Acetonritrile and water with 1% formic acid (45:55) and for rotenoids methanol 80% and water 20% was used. The flow rate in both the case maintained at 1.0 ml per minute for total run time minutes. Detection wavelength for was set at 254 nm wavelength and for rotenoid 294 nm. All samples were filtered through a 0.45 μm acrodisc syringe filter (Pall Corporation, Mumbai, India) before analysis. Reference standards solutions were prepared in methanol. Presences of standard compounds were identified by congruent retention time and UV spectra when compared. All samples were prepared and analyzed in duplicates.

Standards and chemicals:

Reference compounds (standard) Quercetin (98% purity), rutin (98% purity), Naringenin (98% purity) and Rotenone (98% purity was procured from Sigma Aldrich chemical Pvt. Limited Bangalore, India. HPLC grade methanol, HPLC grade water, HPLC grade acetonitrile, formic acid, acetone and ethylacetate was purchased from Loba Chemie, Mumbai, India.

Sample preparation

Flavonoids: 5 g of accurately weighed powder was refluxed with methanol (100 ml) for 2 hour. After 2 hour methanol was distill in rotavapour and residue was hydrolysis with 7% HCl for 1 hour. After this residue wash with ethylacetate and it was taken for analysis.

Rotenoids: Accurately weighed 5 g powder was extracted in 100 ml acetone. The mixture was vortex for 2 min and kept standing overnight. The mixture was filtered through Whatman filter paper no. 41 and filtrate obtained is subjected to HPLC analysis.

FATTY ACIDS

The mature fruit from both the *Tephrosia* Species was collected from the field after monsoon. The mature seed from the mature pods were taken out and powder for further analysis.

Extraction: The oil from the seed was extracted completely with petroleum ether (60-80°C) in a Soxhlet apparatus. The resulting extract was filtered and distilled off in a rotary evaporator leaving behind dark yellowish to brown oil. It was evaluated for its various chemical properties as per AOCS standard methods. The presence of various fatty acids was confirmed by GCMS analysis.

Column chromatography and GC-MS analysis

50 g of aerial part's powder of both *T. jamnagarensis* and *T. collina* was extracted with petroleum ether in soxhlet apparatus for 8 hours. The petroleum ether was removed with rota

vapour and residue was dried and subjected to column chromatography with silica Gel G. The mobile phase of column chromatography was petroleum ether and ethylacetate in range from 9:1, 8:2, 7:3, 6:4 and 5:5. The different fractions were collected and store in the vials, similar fraction were confirmed using TLC with solvent system petroleum: ethylacetate 9:1 with spray reagent 10% sulphuric acid. Each collected fractions were concentrated in vacuum using rota vapour. The pure fractions were analysed using GC-MS.

GC-MS-I

Accurately weighed 5 g powder was extracted in 100 ml acetone. The mixture was vortex for 2 min and kept standing overnight. The mixture was filtered through Whatmann filter paper no. 41 and filterate was derivatized using chlorotrimethylsilane (Procured from Sigma Aldrich). For derivation 1 ml of sample was treated with 0.5 ml of chlorotrimethylsilaneon magnetic stirrer for 30 min. After derivatization, silane was removed and final HPLC grade methanol is added. These samples were filtered through 0.45 μm nylon syringe filters and subjected to GCMS analysis.

GC-MS-II

Accurately weighed 50 g of seed powder of *T. jamnagarensis* and *T. collina* was extracted in petroleum ether and concentrated in vaccum in rota vapour. The oil so obtained was derivatized using chlorotrimethylsilane. After derivatization, silane was removed and final HPLC grade methanol is added. These samples were filtered through 0.45μm nylon syringe filters and subjected to GCMS analysis.

Specification of GCMS:

The instrument was Shimadzu QP 2010 Ultra Gas chromatography and Mass Spectrometry (with Quadruple detector). The chromatographic separation was achieved using gradient programming with 5% diphenyl and 95% dimethyl polysiloxane as stationary phase. The separated peaks were matched with the National Institute of standard and Technology (NIST) library.

Ultra performance liquid chromatography/ tandem mass spectrometry (UPLC/MS/MS)

Liquid chromatography separation was performed on an EXSIGENT UPLC system (AB SCIEX Framingham, USA) with ANALYST software, Hypersil C18 column 250 x 4.6 mm, 5 μm pariticle size (leknokorma, Barcelona, Spain) was used for separation of rotenoids. This UPLC/MS/MS analysis, was performed using binary mobile phase consisting of 80% methanol and 20 % water. The eluting stream from the UPLC was introduced into AB SCIEX 3200 Q

TRAP atmospheric pressure ionization (API) mass spectrometer (ANALYST SOFTWARE, AB SCIEX Crop, Framingham, USA) equipped with electrospray ionization (ESI) multi-mode ionization probe (ESI APCI). All spectra were obtained in positive mode ESI and the scan was set at m/z 300-1000Da. The LC-ESI source operation parameters were as the following: ion spray voltage, -2700V. Nitrogen was curtain and desolvation gas at respective pressure of CUR: 20, GSI: 50, GS2: 50 (arbitrary unit). Block source temperature at 700uC, with the respective voltages ISV: 4500 V, EP:-10V, CXP: 35 V.

NMR

Bruker H_1 NMR at 400 MHz was used for the analysis of the structure of crystal which was obtained in the acetonitrile fraction of the *Tephrosia jamnagarensis* aerial part. The sample was dissolve in DMSO.

3.7 PHARMACOLOGY

3.7.1 *In-vitro*

Sample preparation: Plant powders of endemic two *Tephrosia* species (*i.e.*: TJA and TCA) was done in sterile condition. The 5 g of each dried material were extracted by successive soaking in distilled water for 24 hours. The extracts were filtered using Whatmann filter paper no. 1. They were again filter sterilized with the help of Millipore Syringe filters of 0.2 µm pore size before use. The extracts were stored at 4°C for further use. The three different concentration of the extract was prepared that are 50 µl, 100 µl and 150 µl for both the sample (Rao *et al*., 2014)

Handling and maintenance of cell culture: The details of cell line cluture maintainence is given in table 3.7.1

TABLE 3.7.1 DETAILS OF CELL LINE CHARACTERS

Cell line character	**HepG2**	**Lymphocyte**
Culture type	Monolayer	Adherent Suspension
Growth Medium	DMEM + 10% FBS (supplemented with l- glutamine (3 mm), streptomycin (100 mg/ ml), penicillin (100 IU/ ml), 10% fetal bovine serum (FBS) and buffer)	RPMI-1640 + 10% FBS (supplemented with l-glutamine (3 mm), streptomycin (100 mg/ ml), penicillin (100 IU/ ml), 10% fetal bovine serum (FBS) and buffer)
Subculturing	❖ A culture was maintained by the addition of fresh medium. Alternatively, was established by centrifugation with subsequent resuspension in fresh medium. ❖ An inoculum of 2 x 105 to 3 x 105 cells/ ml was maintained. ❖ Subculture when cell concentration is 2 x 106 cells/ ml.	❖ A culture was maintained by the addition of fresh medium. Alternatively, was established by centrifugation with subsequent resuspension in fresh medium. ❖ An inoculum of 2 x 105 to 3 x 105 cells/ ml was maintained. ❖ Subculture when cell concentration is 2x 106 cells/ ml.

Cell line character	HepG2	Lymphocyte
	❖ Depending on cell density, a fresh medium was added at every 2 to 3 days.	❖ Depending on cell density, a fresh medium was added at every 2 to 3 days.
Culture Conditions	Temperature: 37°C Atmosphere: air, 95%; carbon dioxide (CO_2), 5%	Temperature: 37°C Atmosphere: air, 95%; carbon dioxide (CO_2), 5%

Cell counting protocol

Cell counting was performed with hemocytometer. According to the following way:

1. Hemocytometer and cover slip was cleaned with alcohol.
2. Selected cell culture was harvested and 10 µL was added to the hemocytometer.
3. The chamber was monitored in the inverted microscope under a 10x, 20x and 40x objectives.
4. Cells were counted in the large, central gridded square (1 mm). Cell numbers were multiplied by 104 to estimate the total number of cells per ml.

TABLE 3.7.2 INSTRUMENTAL SPECIFICATION:

Instrument	Make/model
Inverted Microscope	Carl Zeiss-Primovert
Camara	Zeiss ERc5
Biosafety Cabinet	Imset-Class-2, Type-2
CO2 Incubator	Nuaire, NU-5800E
High Speed Centrifuge	Remi- RM-12C-BL
Deep Freeze	Elanproo- EFS 340, -22 degree
Digital Weighing Balance	Wensar
Autoclave	Yorco
Hemocytometer	Rohem-BL 748
pH meter	Systronic-MK-VI

Determination of cell concentration and viability by Trypan blue dye exclusion

Lymphocytes treated with different concentrations of plant extracts for 2 days (48 hrs). At the end of treatment period, the cells were counted with the aid of a hemocytometer and cell viability was determined by trypan blue dye exclusion method (Jin *et al*., 2008). Trypan blue was prepared at a concentration of 0.4% in phosphate buffered saline (PBS)

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5 - diphenyltetrazolium bromide) assay

MTT (3-(4,5 -Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay was performed to assess the cytotoxicity of the plant extracts. (MTT is a yellow dye, which is reduced into purple formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells). HepG2 cells and lymphocytes were cultured in 96-well microtiter plates and were treated with varying concentrations of different plant extracts for 48 hrs. At the end of treatment period, to each well, 20 µl of MTT was added. After addition of MTT, the plates were incubated for 3 h in a dark chamber. Then, 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 540 nm using ELISA reader (Mosmann, 1983).

Statistical Methods

All experiments were carried out in triplicates. The results were calculated as mean ± standard error (SE) values. The significance was calculated using one-way analysis of variance (ANOVA) and Student's t-test. A value of $P<0.05$ was taken as statistically significant.

3.7.2 **Hepatoprotective activity** *In-vivo*

For *In-vivo* hepatoprotective analysis extracts of *T. collina* aerial part was studied. The extracts was subjected to hepatoprotective screening after inducing acute hepatotoxicity using hepatotoxins like carbon tetrachloride, paracetamol and thioacetamide using the following procedural details.

A. Animals

Wistar albino rats weighing 175-225 gm of either sex maintained under standard husbandry conditions (Temp 23± 2°C, relative humidity 55 ± 10% and 12 h light dark cycle) were used for all studies. Animals were allowed to take standard laboratory feed and tap water.The experiments were performed after the experimental protocols approved by the Instituitional Ethics Committee, The M.S.University of Baroda, Vadodara, Gujarat. The Animals were divided into groups consisted of 6 rats.

B. Preparation of solutions for administration:

1. Carbon tetrachloride (CCl_4): 50% v/v solution of carbon tetrachloride was prepared in liquid paraffin.The solutions were administered at the dose of 1.25 ml/kg p.o.
2. Suspensions of test substance: Selective extracts were suspended in 5 % acacia solution and administered at the dose levels of 100, 200 mg/kg for methanol extract and aqueous extract while Silymarin being positive control was suspended in 5 % acacia solution and administered at a dose level of 100 mg/kg.

3.7.2.1 CCl_4 induced hepatotoxicity

Methanol and aqueous extract obtained from aerial parts of *Tephrosia collina* were subjected to the evaluation of hepatoprotective activity *in vivo* against CCl_4 induced toxicity by assessing them through biochemical parameters. Each set of experiment was divided into groups consisting of control, toxicant, standard and test. Groups consisted of 6 rats each unless otherwise mentioned. The protocol followed for CCl_4 induced hepatotoxicity was given in table.

Table 3.7.3 The protocol for *in vivo* hepatoprotective activity

Group	**0 h**	**12 h**	**24 h**	**36 h**
Control	Vehicl e	Vehicle	Vehicle	Withdrawal of Blood
CCl_4	Vehicle + CCl_4	Vehicle	Vehicle	
Silymarin	Silymarin + CCl_4	Silymarin	Silymarin	
Test	Extract + CCl_4	Extract	Extract	

Vehicle: 5 % acacia solution, Test: Extracts prepared in 5 % acacia solution.

The rats of control group received three doses of 5 % *Acacia* solution (1 ml/kg, p.o.) at 12 h intervals (0 h, 12 h and 24 h).The rats of carbon tetrachloride group received three doses of vehicle at 12 h intervals and a single dose of carbon tetrachloride (1.25 ml/kg p.o.) diluted in olive oil (1:1) 30 min after the administration of first dose of vehicle.

The animals in silymarin group received three doses of silymarin (100 mg/kg) at 0h, 12 h and 24 h. CCl_4 (1.25 ml/kg o.p.)was administered 30 min after the first dose of silymarin while the test groups were given first dose of extract in 5 % acacia solution at 0 h which was followed by a dose of CCl_4 (1.25 ml/kg o.p) after 30 min, while at 12 h and 24 h the second and third dose of respective extracts.After 36 h of administration of CCl_4, blood was collected and serum was separated and used for determination of biochemical parameters.

3.7.2.1 Assessment of Liver Function

Blood was collected from all the groups by puncturing the retro-orbital plexus and was

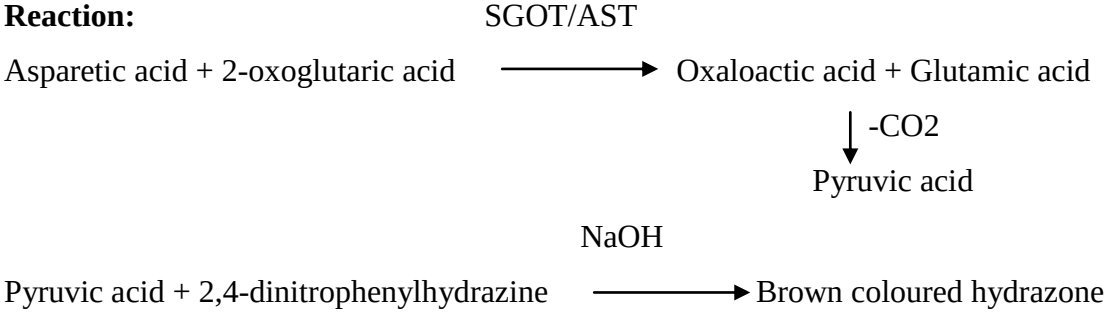
allowed to clot at room temprature and serum was separated by cetrifugation at 2500 rpm for 10 min. the serum was used for estimation of biochemical parameters todetermine the function of the liver. Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT) were estimated by a UV- Kinetic method based on the reference method of international Federation of Clinical Chemistry. Alkaline phosphatase (ALKP) was estimated by PNPP method, while total bilirubin (TBL) by Jendrassik and Grof method. Albumin was estimated by bromo cresol green method.

All the estimations were carried out using standard kits.

(A) Estimation of serum glutamate oxaloacetate transaminase (SGOT)

Principle: SGOT catalyses function is to transfer an amino group from aspartic acid (alpha amino acid) to 2-oxoglutaric acid (alpha keto acid) with the formation of oxaloacetic acid and glutamic acid. Oxaloacetic acid spontaneously converted to pyruvic acid by loss of carbon dioxide (decarboxylation). The pyruvic acid reacts with 2, 4-dinitrophenylhydrazine to form brown coloured hydrazone in alkaline medium. The intensity of brown colour is proportional to the SGOT activity and is measured calorimetrically using green filter or at 505 nm on spectrophotometer.

Reaction:



Reagents:

1. Buffered Aspartate-a-KG substrate, pH 7.4
2. 2.2,4-DNPH colour reagent
3. 3.Sodium Hydroxide,4 N
4. 4.Working pyruvate standard, 8 mM (114 IU/L)

Procedure:

Table 3.7.4 Estimation of SGOT

Pipette into tube marke	**Blank**	**Standard**	**Test**	**Control**
Reagent 1 (ml)	0.25	0.25	0.25	0.25
Serum (ml)	-	-	0.05	-
Standard	-	0.05	-	-
Mix well and incubate at 37°C for 60 minutes				
Reagent 2	0.25	0.25	0.25	0.25
Deionised Water	0.05	-	-	-
Serum	-	-	-	0.05
Mix well and allowed to stand at Room temperature(15-30 °C) for 20 minutes				
Solution I	2.5	2.5	2.5	2.5

Mix well and read the O.D against purified water in a colorimeter using Green filter or on photometer at 505 nm, within 15 minutes.

SGOT was calculated as

$$\text{AST (GOT) activity (in IU/L)} = \frac{\text{Absorbance of test- absorbance of control}}{\text{Absorbance of standard-Absorbance of blank}} \times \text{Conc. Standard}$$

(B) Estimation of serum glutamate pyruvate transaminase (SGPT)

Principle: Transaminase enzyme catylyses the transfer of an amino group from an alpha amino acid to an alpha keto acid. As result different alpha amino acids and alpha keto acids are formed.

SGPT (Alanine transaminase ALT) catalyses the transfer of an amino group from L-alanine (alpha amino acid) to 2-oxoglutaric acid (alpha keto acid) with the formation of L-glutamic acid and pyruvic acid.Formed pyruvic acid couples with 2,4-dinitrophenyl-hydrazine in alkaline medium to form a brown coloured hydrazone.The intensity of brown colour is proportional to the SGPT activity and is measured photometrically using green filter on spectrophotometrically at 505 nm.

Reaction:

$$\text{2-oxoglutaric acid + L-Alanine} \xrightarrow{\text{SGPT/ALT}} \text{L-Glutamic acid + Pyruvic acid}$$

$$\text{Pyruvic acid + 2, 4-dinitrophenylhydrazine} \xrightarrow{\text{NaOH}} \text{Brown coloured hydrazone}$$

Reagents:

1. Buffered alanine-α-KG substrate pH 7.4
2. 2,4-DNPH colour reagent
3. Sodium Hydroxide, 4 N
4. Working pyruvate standard, 8 mM (150 IU/L)

Procedure:

Table 3.7. 5 Estimation of SGPT

Pipette into tube marke	**Blank**	**standard**	**Test**	**control**
Reagent 1 (ml)	0.25	0.25	0.25	0.25
Serum (ml)	-	-	0.05	-
Standard	-	0.05	-	-
Mix well and incubate at 37°C for 60 minutes				
Reagent 2	0.25	0.25	0.25	0.25
Deionised Water	0.05	-	-	-
Serum	-	-	-	0.05
Mix well and allowed to stand at Room temperature (15-30 °C) for 20 minutes				
Solution I	2.5	2.5	2.5	2.5

Mix well and read the O.D against purified water in a colorimeter using Green filter or on photometer at 505 nm, within 15 minutes.

Calculation

$$\text{ALT (GPT) activity (IU/ L)} = \frac{\text{Absorbance of test- absorbance of control} \times \text{Conc. Standard}}{\text{Absorbance of Standard- absorbance of blank}}$$

(C) Estimation of total bilirubin in Plasma (Jendrassik & Grof Method)

Clinical Significance:

Total and direct Bilirubin estimation in serum or plasma is used for the diagnosis , differentiation and follow up of jaundice.

(a) Hemolytic jaundice

Increased hemolysis results in elevation of unconjugated bilirubin level.

(b) Obsructive jaundice

The conjugated bilirubin increases due to regurgitation of bile in to hepatic circulation,

because of blockage of bile passage.

(c) Hepatic jaundice

In this case increase of both conjugated and unconjugated bilirubin in serum is estimated in order to assess the extent of liver damage and subsequent progree or regress.

	Haemolytic	**Hepatic**	**Obstructive**
Direct/ Soluble Bilirubin	Normal	Increased	Increased
Indirect/Soluble Bilirubin	Increased	Increased	Normal

Principle:

Bilirubin reacts with diazotized sulphanilic acid to form an azocompound the colour of which is measured at 546 nm (530-560 nm) and is proportional to the concentration of bilirubin.For total bilirubin the reaction is accelerated by caffeine reagent.The readings for total bilirubin are taken after 5 mins. Incubation and the readings for direct bilirubin are taken after 3 mins incubation.

Test reagent: Reagent 1 consisted of sodium nitrate 10 mmol/l, reagent 2 sulphanilic acid 23 mmol/l and reagent 3 sodium acetate 0.9 mol/l, sodium benzoate 0.5 mol/l and caffeine 0.25 mol/l.

Reaction parameters for total bilirubin:

Monochromatic

Type of Reaction: End Point

Wavelength: 546 nm (530-560 nm)

Flowcell temperature: 30°C

Incubation time: 5 min. at R.T. (25°C+ 5°C)

Factor: 26.312

Sample volume: 50 µl

Reagent volume: 1.1 ml

Zero setting with: Distilled water

Procedure for Total Bilirubin

Solution 1 and solution 2 are mixed in equal volumes to form working solution 1 and working solution 2 consisted of one volume of saline and two volume of reagent 2, where as working solution 3 consisted of equal volumes of reagent 3 and distilled water. To the 50 µl of serum added 100 µl, 100 µl of solution 2 and 1000 µl and solution 3 and incubated for 5 min at

room temperature and read before 10 min.

(A) Monochromatic method:

Table 3.7.6 Estimation of Bilirubin

	Sample Blank	Test
Sample	50 µl	50 µl
Working Solution 1	-	100 µl
Solution 2	100 µ l	-
Solution 3	1.0 ml	1.0 ml

Mix & incubate for 5 mins at R.T. & read the absorbance against sample blank at 546 nm. Read the result before 10 mins.

(D) Estimation of albumin

The method of BCG was used for the estimation of albumin content in the serum.

Principle: Albumin forms blue-green complex with bromocresol green at slightly acidic pH which is measured photometrically at 630 nm.

Test reagents:

Reagent 1 consists of citrate buffer 30 mmol/l, bromocresol green 0.26mmol/l and reagent 2 consisits of albumin standard 5 g/dl.

Procedure: For sample 10 µl of serum was mixed with 1000 µl of reagent 1, for standard 10 µl of reagent 2 and for blank 1000 µl of reagent 1 was added.Then all the solutions were mixed well and incubated at 37°C for 10 min and absorbance was measured at 630 nm.

(E) Estimation of Alkaline phosphtase:

The estimation of alkaline phosphatase was carried out by the method of bessey-lowry-brock modified by bowers and Mc Comb using standard kits.

Principle: p-nitrophenylphosphate undergoes hydrolysis in presence of alkaline phosphatase to yield phosphate and p-nitrophenol which gives a strong yellow color in alkaline solution. Therefore the formation of this product can be monitored directly by measuring the change in absorbance at 405 nm.

Test Reagent:

The reagent and solution includes Diethanolamine HCl buffer pH 9.8 (1mol/l), Magnesium chloride (0.5 mmol/ l) (Reagent 1) and p-nitrophenylphosphate (10 mmol/l) (reagent 2)

Procedure: About 20 µl of the serum was mixed with 1000 µl of reagent solution 1 and 250 µl of reagent solution 2. Mixed well and after one minute the increase in absorbance was measured.

3.7.2.2 Statistical analysis

The mean values ± SEM were calculated for each parameter. Percentage restoration against the hepatotoxin by the test samples was calculated by considering the difference between hepatotoxin treated group and the control group as 100 % restoration.For the determination of significant inter group difference each parameter was analysed separately and one way analysis of Variance (ANOVA) (Gennaro, 1995) was carried out. After that individual comparisions of group mean values were done using Dunnet's test (Dunnet, 1964).