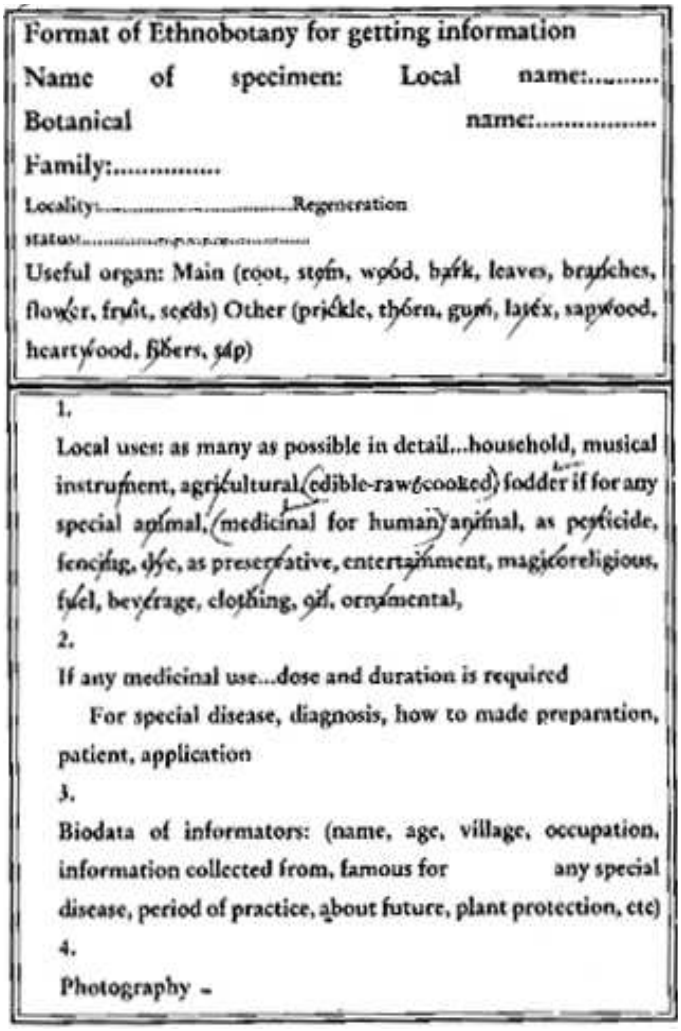
3. Materials and Methods

3.1 Ethnomedicinal Survey

Field explorations were well organized covering all seasons, but with more frequency during rainy seasons so as to cover all the ephemeral floral components for identification of species. During collection trips plants were collected in different developmental stages and exhaustive field notes were taken. Many of the plants were identified in the field with the help of Gujarat Flora vol. I & II (Shah, 1978) and Flora of Bombay Presidency vol. I, II & III (Cook, 1958). Different characters such as, habit, habitat, abundance etc., were noted on the spot. During exploration trips representative samples were collected in different developmental stages. Preliminary identifications done in the field were confirmed in the laboratory by dissecting the floral parts and confirmed with local floras. Different forest views, photographs of interesting plants were taken during various field trips.



Format of Ethnobotany for getting information

Name of specimen: Local name:.....

Botanical name:.....

Family:.....

Locality:.....Regeneration status:.....

Useful organ: Main (root, stem, wood, bark, leaves, branches, flower, fruit, seeds) Other (prickle, thorn, gum, latex, sapwood, heartwood, fibers, sap)

1.

Local uses: as many as possible in detail...household, musical instrument, agricultural (edible-raw/cooked) fodder if for any special animal, (medicinal for human/animal, as pesticide, fencing, dye, as preservative, entertainment, magico-religious, fuel, beverage, clothing, oil, ornamental,

2.

If any medicinal use...dose and duration is required

For special disease, diagnosis, how to made preparation, patient, application

3.

Biodata of informators: (name, age, village, occupation, information collected from, famous for any special disease, period of practice, about future, plant protection, etc)

4.

Photography –

The specimens collected during the field explorations were taken to the local tribals (*vaidya*, *bhuva*, *maharaj*, local medicine men) who know much about the utility of plants. The correct locals were obtained by putting quaternaries about the

characters, when the person is very much sure about its name. If the person is well aware of name of the plant, quarries regarding which part is being used, mode of collection, processing, preservation of medicine, administration of drug, dosages etc. Frequent visits were made to confirm such practices in various areas with various medicine men.

Not only the Ethnobotanical information was collected from the tribal of the area but also the details regarding the usages method, form of preparation, duration of use; is provided along with the ethnobotanical uses of some wild plants. For verification and authentification of data at any stage in later by any user getting the benefit of the ethnobotanical information by the user directly from the source of information (Tribal inhabitants).

In various seasons, various ethnomedicinal useful plants parts like; root, stem, bark, leaves, flowers and fruits were collected fresh/dry for laboratory work.

Selected wild medicinal plant species were identified with dissecting floral parts (in the field/laboratory) and with the help of regional floras for scientific justification.

Five medicinal plant species were selected for the *in vitro* antibacterial screening. The scientific names of these plants, local names, parts used and traditional uses, status, chemical compounds and properties are presented in **Table 1**.

All the specimens were critically examined in the laboratory under light microscope and identified with the help of the state and/or the regional floras and other authentic literature on taxonomy available in the College / University library. The identification was finally confirmed by matching with the help of authentic herbarium specimens available at S. P. University Herbarium, Vallabh Vidyanagar (Gujarat) and Saxton & Sedgwick's collection available at Gujarat College, Ahmedabad.

3.3 Extraction of Plant Materials

Young branches with leaves and inflorescence of *Anisomeles indica* (Lamiaceae), The gum exuded from stem of *Boswellia serrata* (Burseraceae), Root

tubers of *Chlorophytum borivilianum* (Liliaceae), Young branches with leaves and inflorescence of *Euphorbia hirta* (Euphorbiaceae) and Roots, young branches with leaves and flowers of *Evolvulus alsinoides* (Convolvulaceae) were washed thoroughly under running tap water and dried on paper towel, then kept in room temperature for proper drying and finally powdered.

10 gm of air dried plant powder (leaves, stem, fruits, etc.) is successively with 100 ml of aqueous and organic solvents petroleum ether, acetone and methanol separately in 250 ml sterile conical flask and covered with cotton wool. It was then plugged and wrapped with aluminum foil and shaken vigorously for 24 hrs. at room temperature. The mixture was then filtered using a Whatman No. 1 filter paper. The filtrate was evaporated at 50-55ºC on a water bath in reflux condition to obtain crude extract. Extraction procedure was done further twice for complete extraction. Then the crude extracts were resuspended in the respective solvents to prepare various concentrations of 100, 50, 25, 12.5µg/µl before testing it for antibacterial activity. The same procedure was followed for all solvent extraction. (Omogbai *et al.,* 2011; Kokate *et al.,* 2007; Akharaiyi *et al.,* 2010; Sundaram *et al.,* 2011)

3.4 Bacterial Cultures

The following bacteria were employed in the screening: Gram-positive: *Staphylococcus albus, Staphylococcus aureus and Staphylococcus citreus.* Gram-negative: *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris,* and *Klebsiella pneumoniae* were isolated from hospitalized patients from the A. M. C. M. E. T College, L. G Hospital Compound, Ahmadabad (North Gujarat) and its identify confirmed by biochemical tests. The stock cultures were maintained at 4°C on slopes of Nutrient agar and sub cultured for 24 hrs before use. (*Ghosh et al.,* 2008; Sundaram *et al.,* 2011).

3.5 Antibiotics

Hi-media antibiotics used in the study were Amoxicillin (30 mcg), Bacitracin (10 units), Ciprofloxacin (5 mcg), Gentamicin (10 mcg), Nalidixic Acid (30 mcg), Penicillin-G (10 units), Streptomycin (10 mcg), Tetracycline (30 mcg).

3.6 Preparation of inoculum

Direct colony suspension method of choice for UTI organisms, e.g. *Escherichia coli, Staphylococcus albus, Staphylococcus aureus, Staphylococcus citreus, Pseudomonas aeruginosa, Proteus vulgaris,* and *Klebsiella pneumoniae* colonies are taken directly from the plate into distilled water. The suspension should match or exceed the density of the 0.5 McFarland standard. (Andrews, 2001) These suspensions should be used within 30 min of preparation.

3.7 Preparation of the McFarland standard

Add 0.5ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) to 99.5 ml of 0.18 M H_2SO_4 (1% v/v) along with continual mixing. Disperse the standard are hand out with the help of the same dimensions and volume screw cap tubes those utilized in expanding the particular broth cultures. Then close screw cap tube for the stop damage by evaporation. Retailer protected against gentle at room temperature. Energetically agitate the turbidity standard over a vortex mixing machine just before use. Standards might be stored for up to 6 months after which period they must be dumped. (Andrews, 2001)

3.8 Adjustment to the density of organism suspension of the 0.5 McFarland standard

Add sterile distilled water for adjust density of bacterial suspension equal to the 0.5 McFarland standard compare with white background with contrast black line. (Andrews, 2001; Wayne, 1997)

3.9 Antibacterial Sensitivity Testing

3.9.1 Kirby-Bauer Disc Diffusion Method

Antibacterial activity of the aqueous, petroleum ether, acetone and methanol extracts were determined using the agar disc diffusion by Kirby-Bauer method. (Baur *et al.,* 1966; Mohamed *et al.,* 2010). Kirby-Bauer method is recommended by the National Committee for Clinical Laboratory Standards (1993) and the World Health Organization (WHO). Sterile Petri plates containing Mueller-Hinton agar (Hi-media) used for the assays and 100 µl standardized inoculum (which has been adjusted to 0.5

McFarland standard), was spread using a sterile glass spreader by spread plate method. 6mm diameter of sterile Whatman® No.1 filter paper discs was prepared. Negative control was prepared using respective solvent, while the standard Hi-media antibiotic discs served as a positive control were aseptically placed over sterile Mueller-Hinton agar plates seeded with respective test organisms. 100 µg/µl of crude extract were aseptically transferred to these discs. The plate incubate for 5 min at 37˚C for the diffusion of compound. The plates were incubated inverted position at 37˚C or 24 hrs and each extract was tested on three repeat plates. At the end of incubation inhibition zone formed around the disc were measured in mm (millimeter) and the results were recorded.

3.9.2 Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibition Concentrations (MIC's) was determined using Inhibitory Concentrations in Diffusion (ICD) method (Guerin-Faublee *et al.*, 1996; Zaidan *et al.*, 2005). The MIC method was applied on extracts that proved their high efficacy against m.o. The highest dilution of a plant extract that still retain an inhibitory effect against the growth of an m.o. is known as MIC. Minimum Inhibitory Concentration (MIC) was carried out with Whatman® No.1 filter paper was impregnated with various concentration (100, 50, 25, 12.5 µg/µl) of extracts prepared using respective solvent were placed on Mueller-Hinton agar plates. 100 µl standardized inoculum 10^7-10^8 cfu/ml 0.5 McFarland standards was spread using a sterile glass spreader by spread plate method. All the plates were incubated at 37˚C for 24 hrs. After 24 hrs, the zone of inhibition appearing around the discs was measured in each concentration and recorded in millimeter diameter. The lowest concentration of the extracts which inhibits the growth of tested bacteria is observed.

3.10 Literature search

Survey and literature collection for various databases were consulted for this task including Science Direct, PubMed (NCBI), PubMed Central, Biomed Central, Scirus (Pitchai *et al.*, 2010) details from the publications were noted.

3.11 Ligand library creation

Various ligands were selected from literature survey and 3D structure of chemical compound were downloaded *.sdf file format from PubChem Compound database (Wang *et al.,* 2009) for creation of ligand library. 3D structure of the compounds were created *.mol (MDL Molfile) format with Accelry Discovery studio visualizer 2.0 software and ACD/ChemSketch version 11.2 software for details like molecular formula, IUPAC name and SMILES notation, Molar refractivity which were also recorded.

3.12 First stage QSAR study for Lipinski's Rule of Five

Entire library compounds were analyzed for the Lipinski`s Rule of Five (Lipinski *et al.,* 2001; Oprea *et al.,* 2001; Ghose *et al.,* 1999) by ACD/ChemSketch version 11.2 software and online cheminformatics software tools Molinspiration property engine v2011.04 (Rajasekaran *et al.,* 2010). The Lipinski's Rule of Five requirements with no more than one violation. All the compounds were found to have no more than one violation in terms of hydrogen bond donors (<5 hydrogen bond donor) and hydrogen bond accepter (<10 hydrogen bond acceptor), Molecular weight was in the range of 160 to 500, Partition coefficient log *P* in -0.4 to +5.6 ranges, Molar refractivity from 40 to 130, Topological Polar Surface Area (TPSA) no greater than 140 $Å^2$ (Ghose *et al.,* 1999)

3.13 Second stage QSAR study for Toxicity, Carcinogenicity and Mutagenicity

Entire library compounds were analyzed Batch predictions for Developmental Toxicity and Mutagenicity by Consensus method (by Benigni/Bossa rule) using T.E.S.T. version 4.0 software (Toxicity Estimation Software Tool) (Piparo and Worth *et al.,* 2010; Cassano *et al.,* 2010; Zhu *et al.,* 2008). Further analyzed for verhaar model of fish base-line toxicity, daphnia base-line toxicity and algae base-line toxicity by using DRAGON version 5.4 software (Cassano *et al.,* 2010; Verhaar *et al.,* 1992). Furthermore, potential *S. typhimurium* TA100 strain mutagen based on QSAR, structural alert for non genotoxic carcinogenicity, structural alert for genotoxic carcinogenicity, potential carcinogen based on QSAR, unlikely to be carcinogen based on QSAR and unlikely to be a *S. typhimurium* TA100 strain mutagen based on

QSAR predicted were examined by Toxtree version 2.1.0 software with default parameters (Franke *et al.,* 2001; Benigni *et al.,* 2007).

3.14 Selection of the drug like compound

Selection of the drug like compound was done by selecting the compound confirming no more than one violation in Lipinski's Rule of Five, non-mutagen, non-carcinogen and non toxicant.

3.15 Calculation of bioactivity of drug like compounds

The bioactivity for GPCR ligand, Ion channel modulator, Kinase inhibitor, Nuclear receptor, Protease inhibitor and Enzyme inhibitor of filtered drug-like compounds were predicted applying web based chemoinformatics software Molinspiration bioactivity score v2011.06 (Rajasekaran *et al.,* 2010) and also by online PASS (Prediction of Activity Spectra for Substances) software (Lagunin *et al.,* 2005; Da Silva *et al.,* 2010; Pospieszny *et al.,* 2010).

3.16 Selection of target

Various successful and research target of Cancer, TB, HIV, Malaria, Bacterial Infection, Infertility female and Infertility male were selected from literature survey and TTD (Therapeutic Target Database) (Zhu *et al.,* 2012; Chen *et al.,* 2002; Oprea *et al.,* 2006). 3D structure of protein was downloaded from the Protein Data Bank (PDB) (Abola *et al.,* 1996; Kouranov *et al.,* 2006). Then target was further analyzed for active site details and hydrogen was added by using Raswin and Accelry Discovery studio visualizer 2.0 software (Abola *et al.,* 1996).

3.17 Molecular Docking studies

The compounds having drug like properties were selected as ligands to carry out molecular docking studies in GOLD 3.2 (Genetic Optimization for Ligand Docking) software (Verdonk *et al.,* 2003; Bharatham *et al.,* 2007) against the receptors, which were obtained from PDB (Protein Data Bank) (Abola *et al.,* 1996; Kouranov *et al.,* 2006). Scoring was done by Gold score (Jones *et al.,* 1997; Jacobsson, 2008) and Chem score method (Eldridge *et al.,* 1997; Jacobsson, 2008) using GoldMine v1.0.1 software. Then further analyzed for Consensus scoring (Clark

et al., 2002; Klon *et al.,* 2004; Jacobsson, 2008) was done by collative score from Gold docking program.

3.18 Hydrogen bond interaction

Best ligand was selected from the Consensus score and further analyzed by SILVER 1.1.1 software for the hydrogen bond interaction study (Sperandio *et al.,* 2006).

3.19 ADME studies

ADME properties like Absorption, Bioavailability, Aqueous Solubility, Distribution, Ionization, Absolv equations, Physicochemical properties, Maximum Recommended Daily Dose (MRDD) of selected ligand were predicted by using ADME Boxes 3.5 (Bharath *et al.,* 2011; Dearden *et al.,* 2007) and Cytochrome P450-Mediated Drug Metabolism was predicted by using Toxtree version 2.1.0 software (Franke *et al.,* 2001; Benigni *et al.,* 2007).

3.19.1 Absorption

Prediction of Human Intestinal Absorption (HIA) after oral administration and a classification of absorption level. The method is based on calculations of logP, pKa (Acid), pKa (Base) and Permeability of Human jejunum scale (pH=6.5), caco-2 scale (pH=7.4, 500 rpm) and Absorption rate Ka.

3.19.2 Bioavailability

Predicts the oral Bioavailability of Human less than 30% probability that compound has %F (oral) >30% and %F (oral) >70%. Predict the solubility, stability (pH<2), passive absorption, P-gp efflux and active transport, first pass metabolism.

3.19.3 Aqueous Solubility

Predicts aqueous solubility in water. The Aqueous solubility of compound in pure water at >10mg/ml, >1mg/ml, >0.1mg/ml, Sw (mg/ml) were predict the value. The value of LogS based on pH dependent aqueous solubility of compound in buffer at different pH like pH 1.7 (stomach), pH 4.6 (Duodenum), pH 6.5 (Jejunum & Ileum), pH 7.4 (Blood), pH 8.0 (colon).

3.19.4 Distribution

Predicts the value of Drug Binding to Plasma Proteins and Volume of Distribution in Human.

3.19.5 Ionization

Predicts the value of ionization based on LogD at pH dependent distribution coefficient. The predicted value of LogD at different pH like 1.7 (stomach), 4.6 (Duodenum), 6.5 (Jejunum & Ileum), 7.4 (Blood), 8.0 (colon) respectively the value of all different pH.

3.19.6 Absolv equations

Predicts the Blood-brain distribution, water-skin permeation, water-human serum albumin, water-skin partition of a molecule, defined as the ratio of the concentrations of compound. Gas-solvent partitions for Urine, Blood, Plasma, Liver, Muscle, Lung, Kidney, Heart, Brain as the ratio of the concentrations of compound.

3.19.7 Physicochemical properties

Predicts the value of Physicochemical properties like molecular weight, no. of hydrogen bond donors, no. of hydrogen bond acceptors, TPSA (Polar surface area), no. of rotatable bonds and LogP.

3.19.8 Maximum Recommended Daily Dose (MRDD)

Predicts the value of Maximum Recommended Daily Dose (MRDD). Then probability value (MRDD<3 mg/kg/day) and to predict Acute toxicity in mouse at LD 50 oral and LD 50 Intravenous. The effect on health is predicted respectively the probability in gastrointestinal, lungs, cardiovascular, liver, blood, kidney.

3.19.9 Metabolism

Predict the Cytochrome P450-Mediated Drug Metabolism based on SMARTCyp sites of metabolism (Rydberg *et al.*, 2010)

3.20 *In silico* workflow diagram