3.1. COLLECTION

Authentic samples: Various market samples of selected plants were procured from various tribes living in tribal pockets of Mt. Abu, arid zone of Rajasthan, in the month of Feb, 2010. These plants were used by these tribes in their daily lives to cure various ailments.

3.2. IDENTIFICATION

All the samples were authenticated and were given identification numbers. The identification was as follows:

- Gymnema sylvestre R.Br. (gudmar) - Whole plant
- Adiantum lunulatum Burm (hasraj) - Whole plant
- Bryonia laciniosa Linn. (shivlingi) - Fruit and stem
- Tectona grandis Linn. (sagwan) - Stem and leaf
- Viola odorata Linn. (banpasha) - Whole plant
- Dashmool
- Solanum xanthocarpum SCHRAC&WENDLE (pasarkateli) – Whole plant
- Withania coagulans (Stocks) Dunal (paneerphal) - Fruit

These samples were authenticated and submitted to Ethno medicinal Herbarium, Centre of Excellence (funded by DST), Department of Biotechnology, MGIAS, JECRC, Jaipur (Rajasthan).

3.3. PROCESSING OF PLANT MATERIALS

During the course of the study, each sample was screened for its foreign matter and milled, before use.

3.4. EXPERIMENTAL DETAILS

The following studies were performed on selected plants-

1. Experimental details
   a) Phytochemistry
   b) TLC
   c) HPLC
2. **Specific objectives**
   a) **Selection of plants.**
   b) **Phytochemistry characterization of active extracts.**
   c) **Extraction and isolation of active extract/fraction/bioactive as antimicrobial agents.**
   d) **TLC for characterization of active extracts.**
   e) **HPLC (qualitative and quantitative studies)**
   f) **NMR spectrum of isolation pure compounds.**
   g) **Antimicrobial assay against selected bacteria.**
   h) **MIC (Minimum Inhibitory Concentration)**

3.5. **PHYTO-CHEMICAL TESTS**

3.5.1. **Extraction procedure**

For phytochemical profile of selected species, each of the dried and powdered (g) test samples were soxhelt extracted in petroleum ether, benzene, chloroform, ethyl acetate, ethanol and distilled water for 6 hours. These extracts were filtered, evaporated to dryness and weighed. Each extract was used for phytochemical screening of plant active compound.

The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. After then, there will no longer be a mass transfer of the active principle from plant material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhance the mass transfer. Moreover, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is changed. This gives rise to different types of extractions: cold percolation, hot percolation and concentration.

Increasing the temperature of the solvent increases the solubility of the active principle, which increases the concentration gradient and therefore enhances the mass transfer of active principle from solid material to the solvent, provided that active principle is not
heat-sensitive. The other type of instrument for extraction of medicinal ingredients from plant material is the soxhlet apparatus, which consists of an extractor, a distillation unit, a tubular condenser for the distillation unit, a tubular condenser for the recovery of solvent from the marc, a receiver for collecting the condensate from the condenser, and a solvent storage tank. The plant material is fed into the extractor, and solvent is added until it reaches the siphon point of the extractor. Then, the extract is siphoned out into the distillation unit, which is heated with steam. The solvent vapours go to the distillation condenser, get condensed and return to the extractor. The level of the solvent in the extractor again rises to the siphon point and the extract is siphoned out into the distillation still. In this way, fresh solvent comes in contact with the plant material a number of times, until the plant material is completely extracted. The final extract in the distillation unit, which is rich in active principle, is concentrated and the solvent is recovered.

The enriched extract from percolators or extractors, known as miscella, is fed into a wiped film evaporator where it is concentrated under vacuum to produce a thick concentrated extract. The concentrated extract is further fed into a vacuum chamber dryer to produce a solid mass free from solvent. The solvent recovered from the wiped film evaporator and vacuum chamber dryer is recycled back to the percolator or extractor for the next batch of plant material. The solid mass thus obtained is pulverized and used directly for the desired pharmaceutical formulations or further processed for isolation of its phyto-constituents.

In a conventional Soxhlet system, plant material is placed in a thimble-holder, which is filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. Solute is left in the flask and fresh solvent passes back into the plant solid bed. The operation is repeated until complete extraction is achieved.
Fig. 3.1. Soxhlet apparatus (a and b)
Phytochemical screening was performed using standard procedure:

3.5.2.  **Test for Reducing Sugars (Fehlings test)**

The aqueous petroleum ether, benzene, chloroform, ethyl acetate, methanol and distilled water extract (0.5 g in 5 ml of water) was added to boiling Fehling’s solution (A and B) in a test tube. The solution was observed for a colour reaction.

3.5.3.  **Test for Terpenoides (Salkowski test)**

To 0.5 g of each of the extract was added 2 ml of chloroform. Con. Sulphuric acid (3 ml) was carefully added to form a layer. Reddish brown coloration of the interface indicates the presence of terpenoides.

3.5.4.  **Test for Flavonoides**

4 ml of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of Concentrated Hydrochloric acid was added and red colour was observed for flavonoids and orange colour for flavones.

3.5.5.  **Test for Tannins**

About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish green or a blue-black coloration.

3.5.6.  **Test for Saponins**

To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

3.6.  **CHEMICAL FINGERPRINTING**

3.6.1.  **Thin layer chromatography (TLC) for fingerprinting**

3.6.1.1. **Extraction procedure**

For TLC profile of selected species, each of the dried and powdered (100 g) test samples were soxhelt extracted in petroleum ether for 6 hours. These extracts were filtered, evaporated to dryness and weighed. Each extract (10 mg) was dissolved in 10 ml to make a concentration of 1mg/ml used for further studies.
3.6.1.2. TLC plates

Each extract was applied on silica gel G Thin Layer Chromatography (TLC) coated plates (Merck: 20x20 cm; with thickness 0.2-0.3mm) which were activated at 100°C for 30 minutes and brought to room temperature, just before use. Each extract of various species was applied 1cm above the edge of the chromatographic plates along with the reference compounds and developed in air-tight chamber already saturated with 200 ml of various solvent systems (Harborne, 1973).

3.6.1.3. TLC solvent system

Various extracts of test samples were subjected to different solvent systems for identification of significant bio molecules. After having used different solvent systems, on the basis of better resolution of spots for generating “Thin Layer Chromatography (TLC) fingerprints” for chemical libraries of the test drugs following solvent system were used in the present study- Acetone : Hexane (1:3) for petroleum ether extracts.

3.6.1.4. TLC spraying reagents

During these studies, different visualizing reagents i.e. 10% sulphuric acid (10 ml conc. Sulphuric acid dissolved in 100 ml absolute alcohol), I_2 vapour (Saturated iodine chamber) and Drangen-droff reagent were used.

3.6.1.5. Qualitative TLC

Thin glass plates were coated (0.2-0.3 mm) with silica gel G (30 g/60 ml distilled water) and dried at room temperature. The coated plates were activated in an oven at 100°C for 30 minutes and cooled. The plates were then placed in developing tanks having 150 ml of an organic solvent mixture of Acetone: Hexane (1:3) for petroleum ether extracts. The lid of the developing tanks was sealed with vacuum grease. The plates were removed after making the solvent front and were air-dried. The dried plates were sprayed with 10 % sulphuric acid (10 ml concentrated Sulphuric acid dissolved in 100 ml absolute alcohol) and further addition of 28 g (KI) and alkaloid positive spot (R_f value) was calculated.
3.6.1.6. Preparative TLC

Silica gel G thick layer plates were activated at 100 °C for 30 min. The petroleum ether extract and the reference compound β- sitosterol (Heftmann, 1974), Stigmasterol and Lupeol were applied separately as a streak 1 cm above the edges of the plates and developed in an organic solvent mixture of Acetone: Hexane (1:3). A portion of the plate containing the applied standard reference and the extract was visualized under 10 % sulphuric acids (10 ml conc. Sulphuric acid was dissolved in 100 ml absolute alcohol) and also exposed to I₂ vapour for 10 min. The spots coinciding to the reference compounds were marked and compared with that of standard reference compound.

3.6.2. High pressure liquid chromatography (HPLC) chromatograms

HPLC was developed in the mid 1970s and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. This technique was commonly used for the separation of chemical compounds, and later techniques improved for the separation, identification, purification and quantification of the compounds far away from the previous one, as computers and automation have been added. Improvements in type of columns, as micro-column and affinity column had further increased reproducibility and faster analysis. In the present work, various Indian medicinal plants were subjected onto the HPLC analysis using Shimadzu Model LC2010 AHT Auto Sampler (UV – VIS Detector).

Height/ Area method: This method utilizes the fact that the area of a peak is a function of its height and standard deviation. To determine efficiency, values for peak height and area are used in a different formula:

\[ N = \frac{2\pi (ht)^2}{A^2} \]

(Where h=peak height; t_r-retention time; N-number of theoretical plates; A-area). A computer is usually necessary to use this method in order to calculate area and height.
3.6.2.1. Columns

Out of various columns such as guard, derivatizing, capillary, fast and a preparatory column with Hypersil, BDS C18 column was used. This column has a large column diameter which is designed to facilitate large volume of injection as compared to analytical ones. The back pressure regulator is placed immediately posterior to the HPLC detector which generates constant pressure to the detector outlet and prevents the formation of air bubbles within the system to protect the column from damage. Thus the baseline is enhanced.

Packing for columns are diverse, since there are many modes of HPLC. They are available in different sizes, diameter and pore sizes. Column size (250 × 4.6 mm) and 100 Å pore size, 5 µm particle diameters was used to performed various analysis.

3.6.2.2. Mobile Phase

The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected onto the mobile phase of an assay through the injected port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample and thus, will have variable retention times. There are several types of mobile phases, these include: isocratic gradient, and polytypic. In the present study 15V ethanol: 85V Acetonitrile were used as mobile phase to evaluate better resolution of chromatograms and their co-comparison.

3.6.2.3. Stationary Phase

The stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. Column containing various types of stationary phase is commercially available. Some of the more common stationary phase includes liquid-liquid, liquid-solid (adsorption), size exclusion, normal phase, reverse phase, ion exchange and affinity.

The reverse phase operates on the basis of hydrophilicity and lithophilicity. The stationary phase consists of silica based packing with an alkyl chain covalently bound. For example C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more
hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus, hydrophilic compounds elute more quickly than hydrophobic compounds.

### 3.6.2.4. Injectors for HPLC

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection value and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a micro-syringe (20 µl) and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. In the present work 20 µl loops were used to carry out the studies. Injection was given by a specific syringe with a blunt tip.

### 3.6.2.5. HPLC Pumps

There are several types of pumps available for use with HPLC analysis viz: reciprocating piston pumps, syringe type pumps and constant pressure pumps.

Syringe type pump are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500 ml. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor and in the present study LC-solution (Shimadzu) liquid chromatography pumps were used.

### 3.6.2.6. Detectors and Detection limits

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and in most cases the detection and sensitivity parameters may also be controlled. There are many types of detectors that can be used with HPLC. Some of the more common detectors include: refractive index (RI), ultra-violet (UV), fluorescent, radiochemical, etc.
Ultra-violet (UV) detectors measure the ability of a sample to absorb light. This can be accomplished at several wavelengths: UV detectors have sensitivity to approximately $10^{-8}$ or $10^{-9}$ g/ml. It can have fixed wavelength measures at one wavelength, usually 254nm, variable wavelength measures at one wavelength at a time, but can detect over a wide range and/or diode array measures a spectrum of wavelengths simultaneously. In the present work, the UV detector was used for chromatogram analysis of various species.

3.6.2.7. Flow Rate

The speed of solvent by which it moves in the column varies according to different modes where as in the present studies; attempts have been made to analyze the chromatograms with 1ml/min flow rate. The wavelength which was used is 254 nm and the column temperature was ambient.

3.6.2.8. Sample preparation for HPLC chromatograms

HPLC is used as marker in identification of antibiotic compound which are present in various medicinal plants. Dried powdered plants of selected species were extracted (6 hours) in petroleum ether, filtered and concentrated to dryness. For HPLC analysis, 1 mg extract of each drug were dissolved in 10 ml methanol and used for fingerprinting analysis. 20 µl of each sample was subjected onto Shimadzu HPLC system in an analytical mode. Various peaks were observed at different retention time (rt) and each retention time reflects a compound.

3.6.2.9. Standard Preparation

1 mg of standard compound that is lupeol, stigmasterol, β- sitosterol, was isolated from TLC fingerprinting of various plants and authenticated by spectral analysis; then it was dissolved in methanol the volume of which was raised to 1ml and used.

3.6.2.10. Composite standard curve

The area of corresponding extracts peak and conc. in various medicinal plants were plotted as composite standard curve.

3.6.3. Nuclear magnetic resonance (NMR) analysis.

Each of the compound(s) was subjected to NMR analysis (model Brukur-DPX-300 MHz, using CDCL$_3$ and DMSO- d$_6$ as an internal reference) along with the standard reference compound.
3.7. BIOEFFICACIES

3.7.1. Antimicrobial efficacies

3.7.1.1. Sources of test organisms


3.7.1.2. Culture of test microbes

Microorganisms were maintained on Nutrient broth media. Culture of test microbes:

For the cultivation of bacteria, Nutrient Agar Medium (NAM) was prepared by using 20 g Agar, 5 g Peptone, 3 g beef extract and 3 g NaCl in 1 L distilled water and sterilized at 15 lbs pressure and 121°C temperature for 25-30 minute. Agar test plates were prepared pouring approximately 15 ml of NAM into the Petri dishes (10 mm) under aseptic conditions. A saline solution was prepared (by mixing 0.8% NaCl) in distilled water, followed by autoclaving and the bacterial cultures were maintained on this medium by regular sub-culturing and incubation at 37°C for 24-48 hour.

To prepare the test plates, in bacteria, 10-15 ml of the respective medium was poured into the Petri plates and used for screening. For assessing the bactericidal efficacy, a fresh suspension of the test bacteria was prepared in saline solution from a freshly grown agar slant, while for fungicidal efficacy; a uniform spread of the test fungi was made using sterile swab.

3.7.1.3. Preparation of test extracts

Crushed powders of species were successively soxhlet extracted. Later, each of the homogenates was filtered and the residue was re-extracted twice for complete exhaustion, the extracts were cooled individually. Each filtrate was concentrated to dryness in vitro and re-dissolved in respected solvent, until screened for antimicrobial activity.

In this method, the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus (Figure 3.1). The extracting solvent in flask A is heated, and its vapors condense in condenser D. The
condensed extract drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

3.7.1.4. Bactericidal and fungicidal assay

For both, bactericidal in vitro Disc diffusion method was adopted (Gould and Bowie, 1952), because of reproducibility and precision. The different test organisms were proceeded separately using a sterile swab over previously sterilized culture medium plates and the zone of inhibition were measured around sterilized dried discs of Whatman No. 1 paper (6 mm in diameter), which were contained five different concentration (A1-50 mg/20 discs, A2-100 mg/20 discs, A3-150 mg/20 discs, A4-200 mg/20 discs, A4-250 mg/20 disc), its control (of the respective solvent) and tetracycline as reference drugs (standard disk) separately. Such treated discs were air-dried at room temperature to remove any residual solvent, which might interfere with the determination, sterilized and inoculated. These plates were initially placed at low temperature for 1 hour so as to allow the maximum diffusion of the compounds from the test disc into the agar plate and later, incubated at 37°C for 24 hours in case of bacteria, after which the zones of inhibition could be easily observed. Five replicates of each test extract were examined and the mean values were then referred.

The Inhibition Zones (IZ) in each case were recorded and the Activity Index (AI) was calculated and compared with those of their respective standard reference drugs (AI = Inhibition Zone of test sample/Inhibition zone of standard).

A1 = 50 mg of test extract/20disc
A2 = 100 mg of test extract/20disc
A3 = 150 mg of test extract/20disc
A4 = 200 mg of test extract/20disc
A5 = 250 mg of test extract/20disc

3.8. STATISTICAL ANALYSIS
Statistical analysis is defined as a collection of methods which is used to process a large amount of data and report overall trends. It is particularly useful when dealing with noisy data. All tests and analyses were run in triplicate.

3.8.1. Retardation factor (R_f)

The retardation factor (R_f) is defined as the ratio of the distance travelled by the center of a spot to the distance travelled by the solvent front. Thus R_f value is calculated by the formula:

\[ R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \]

3.8.2. Mean of Zone of inhibition

Diameter of bacterial and fungal growth was measured and expressed as means of percentage growth inhibition of three replicates. Significant differences within the means of treatments and controls were calculated using the LSD statistical test.

\[ \text{Mean (μ)} = \frac{\Sigma x_i}{N} \]

Here Σ is the summation (addition) sign, x_i is each individual number, and N is the no of readings.

3.8.3. Linear equation

The linear equation represents the straight line function, \( y=mx+c \) which discusses ‘m’ and ‘c’, and shows how to draw graph and some different forms of this function. Where function: \( y=f(x) \) that graphs as a straight line, has an equation of this form

\[ y = mx+c \]

Here, x and y are the coordinates of the points that satisfy the function and so lie on the straight line graph.

\[ m = \text{gradient (number that represents the steepness of the straight line)} \]

\[ c = \text{y intercept (y coordinates of where the straight line cut the y-axis)} \]

3.8.4. R^2 (coefficient of determination)

The coefficient of determination, R^2 is used in the statistical models. Its main purpose is the prediction of future outcomes on the basis of other related information. It is the
proportion of variability in a data set that is accounted for by the statistical model. The most general definition of the coefficient of determination is given in the formula:

\[ R^2 = 1 - \frac{SS_{err}}{SS_{tot}} \]

Where, \( SS_{err} \) = the sum of square of residuals
\( SS_{tot} \) = the total sum of squares

3.8.5. MIC (Minimum Inhibitory Concentration)

Antimicrobial activity against namely \( P. \ aeruginosa, S. \ aureus, K. \ pneumoniae, S. \ typhi, S. \ flexneri, P. \ vulgaris, E. \ aerogenes \) and fungi \( C. \ albicans, A. \ niger, T. \ rubrum \) microbes was inferred through replicate disc diffusion assays; and it was observed and statistically predicted that MIC values can be determined through replicate serial dilution assays. The MIC is defined as the lowest concentration of antibiotic or extract at which there is no visible growth. The agar plates without extract or standard antibiotic (the negative control) and the plates containing (2.5 μg/ml) of a standard antibiotic, tetracycline (the positive control) were also streaked with the micro organisms. The agar plates were incubated at 37°C for 24 hour (for the bacteria) and at 25°C for 48 hour (for the fungus). The inhibition zone diameter, the measure of activity, was consequently determined by plotting the square of the inhibition zone diameter (IZD2) against the log concentration of the extract and the MIC calculated from the intercept on the log concentration axis.

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test microorganisms. At the end of incubation, the plates were collected and zones of inhibition that developed were measured. The average of the zones of inhibition was calculated. The minimum inhibitory concentration (MIC) was calculated by plotting the natural logarithm of the concentration of extract against the square of zones of inhibition. A regression line was drawn through the points. The antilogarithm of the intercept on the logarithm of concentration axis gave the MIC values (Esimone, et al., 1998, Osadebe and Ukwueze, 2004).

3.8.6. Standard error

The term "standard error" is used to refer to the standard deviation of various sample statistics such as the mean or median. Standard error is a statistical term that measures the accuracy with which a sample represents an activity. The standard error of a statistic is the standard deviation of the sampling distribution of that statistic. Standard errors are important
because they reflect how much sampling fluctuation a statistic sample will show. The interefential statistics involved in the construction of confidence intervals and significance testing are based on standard errors. The standard error of a statistic depends on the sample size. In general, the larger the samples size the smaller the standard error. The standard error of a statistic is usually designated by the Greek letter sigma (σ) with a subscript indicating the statistic. For instance, the standard error of the mean is indicated by the symbol: σₘ.

$$\text{Standard error} = \frac{\sigma}{\sqrt{n}}$$

The standard error is the standard deviation of the sampling distribution of a statistic. The term may also be used to refer to an estimate of that standard deviation, derived from a particular sample used to compute the estimate.