4.0 MATERIALS AND METHODS

4.1 Collection and Authentication of Plant Materials:

The green leaves of plant *Corchorus fascicularis* L. were collected from village Tande of Shirpur tehasil in Dhule district (MS); India in the month of July 2009. The plant was taxonomically identified by Professor Dr. L. K. Kshirsagar, Taxonomist, Department of Botany, S.S.V.P.S's L. K. Dr. Ghogre Science College, Dhule, North Maharashtra University, Jalgaon.

4.2 Physico-chemical analysis[1,2,3,4]:

Physicochemical values such as the percentage of ash values and extractive values were performed according to official methods prescribed in Indian Pharmacopeia 1996 and WHO guidelines on quality control methods for medicinal plant material.

4.2.1 Extractive Values[5]: -

Extractive values of crude drugs are useful for their evaluation, especially when the constituents of a drug cannot be readily estimated by any other means. Further, these values were determined to indicate the nature of the constituents present in a crude drug.

Determination of Alcohol Soluble Extractive Value:-

10 gm. of the air-dried coarse powder of *Corchorus fascicularis* L. leaves were macerated with 100 ml of 90% ethanol in a closed flask for 24 hours shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, it was filtered rapidly. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of ethanol soluble extractive value was calculated with reference to the air-dried drugs.

Determination of Water Soluble Extractive Value:-

Coarsely powdered drug (10 gm) was weighed accurately and macerated with 100 ml of water in a closed flask for 24 hours. It was shaken frequently during the first 6 hours and allowed to stand. After 18 hours it was filtered rapidly. Then 25 ml of the filtrate was evaporated to dryness in a tarred flat-bottomed shallow dish, dried at 105°C and weighed. The percentage of water-soluble extractive value was calculated with reference to the air-dried drug.

4.2.2 Ash Values[4]:-

Ash values are helpful in determining the quality and purity of crude drug, especially in the powdered form. It usually represents the inorganic salts naturally occurring in the drug and adhering to it. Hence, an ash determination furnishes a basis for judging the identity and cleanliness of a drug. Procedure given in Indian Pharmacopoeia was used to determine the different ash values such as total ash, acid insoluble ash, and water soluble ash etc.

Determination of total ash value:-

Exact 3 grams of air dried powdered drug was taken in a tared silica crucible. It was incinerated by gradually increasing the temperature to make it dull red hot until free from carbon. The crucible was cooled and weighed, repeated for constant value. Then the percentage of total ash was calculated with reference to the air-dried drug.

Determination of acid insoluble ash value:-

The ash obtained as directed under total ash value was boiled with 25 ml of 2N HCl for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water, ignited and weighed, then the percentage of acid insoluble ash was calculated with reference to the air dried drug.

Determination of water soluble ash value:-

The total ash obtained was boiled with 25 ml of water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and

ignited for 15 minutes at a temperature above 250°C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water-soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug. All the ash values were calculated and recorded.

4.3 PREPARATION OF EXTRACT[6]:

A number of factors were taken into consideration in choosing solvents that were to be used for successive extraction. The choice of solvent was also decided by considering what was planned with the extract. The effect of solvent on subsequent bioassay was an important factor.

The extraction process by using *n*-hexane is important in the isolation process, since non polar compounds can be extracted rapidly in this process. Therefore, successive extraction was used with *n*-hexane as starting solvent, followed by chloroform and ethanol. The polarity of solvents was gradually increased and ranged from non polar solvent (*n*-hexane) to polar solvent (ethanol). This was to ensure that wide polarity range of compounds could be extracted in the process.

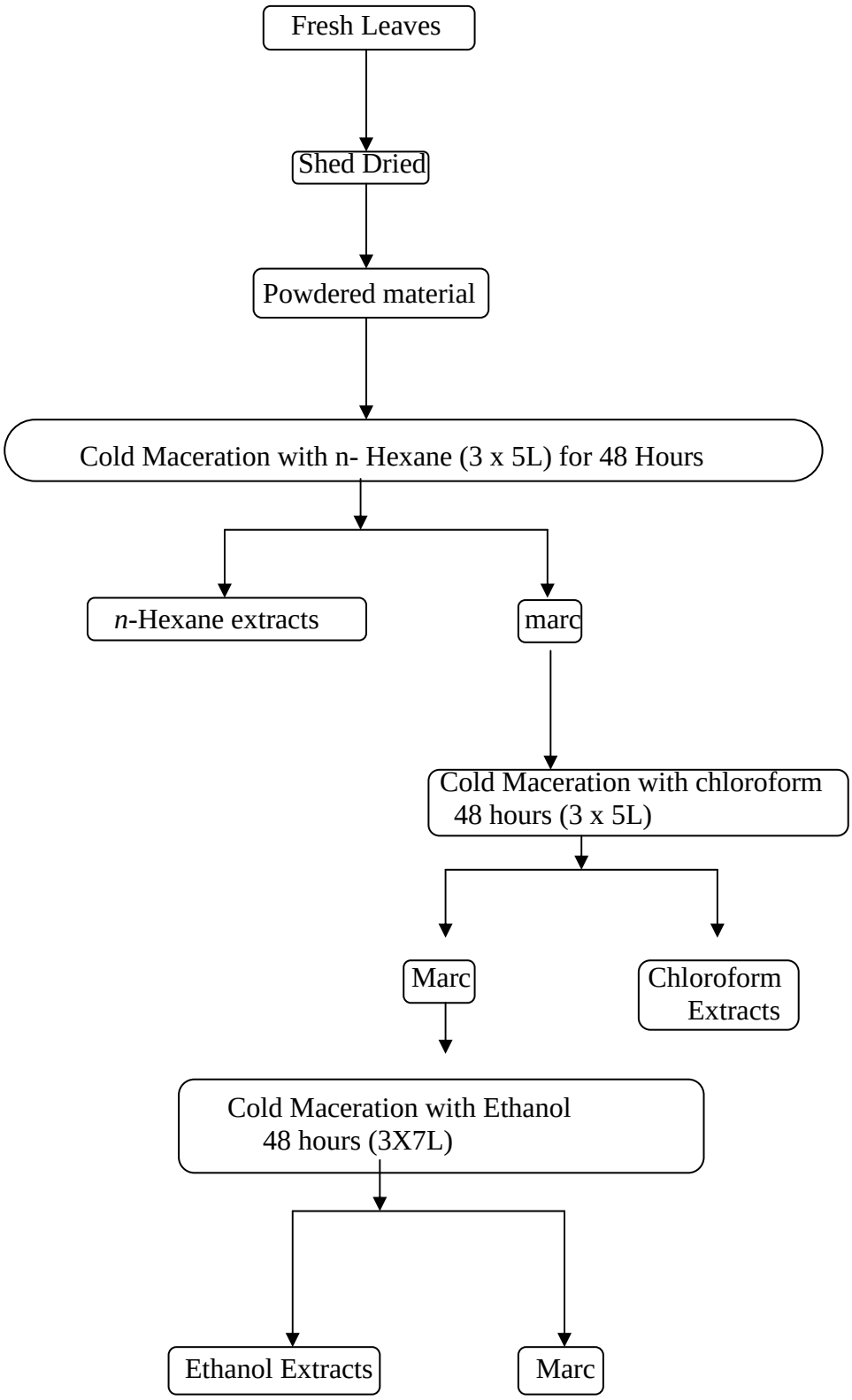
The leaves were carefully examined and old, insect damaged, fungus-infested leaves and twigs were removed. Healthy leaves were spread out and air dried at room temperature until they broke easily by hand. Leaf material was ground using mechanical grinder and the powder was sieved through sieve No.14 (Mesh size-1410 µ) and stored in air tight containers. The sieved powder was used for extraction and evaluation purpose.

The powdered leaves (3 kg) of *Corchorus fascicularis* Lam. were extracted with *n*- Hexane with three times each by cold maceration at room temperature, in cold maceration whole powdered plant(leaves)-drug is kept in contact with the solvent in a stoppered container for a 48 hour with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermo labile drugs[7]. For extraction, the powder was taken in conical flask and macerated 48 hours at room temperature with all material defatted completely[8]. The extracts thus obtained were

concentrated by distilling off the solvent under reduced pressure by using Rota Evaporator (Buchi, Germany). The defatted marc thus obtained was air dried successively extracted with chloroform and ethanol i.e. three times each. These extracts were concentrated separately under reduced pressure[9]. The progress of the extraction was evaluated by applying spot of extract on thin layer chromatography plate.

The thin layer chromatography was performed using silica gel plates and the plates were visualized in iodine chamber. Completion of extraction was confirmed by non appearance of spot on TLC plate by visualizing in iodine chamber.

Flow chart of extraction methodology of leaves of *Corchorus fascicularis* Lam.



4.4 IDENTIFICATION OF PHYTOCONSTITUENTS BY CHEMICAL TESTS (PHYTOCHEMICAL SCREENING):

The individual extracts were subjected to qualitative chemical investigations for the identification of the phytoconstituents such as sterols, flavonoids, triterpenes, alkaloids, glycosides, tannins, proteins, carbohydrates. The Preliminary Phytochemical tests were performed for each extract[10-19].

Following tests were performed and the results of phytochemical tests are tabulated in Table-3 (Page No.-74).

4.5 PROXIMATE CHEMICAL ANALYSIS: -

4.5.1 Test for carbohydrates: -

Molish's test (General test): -

To 2-3 ml aqueous extract, few drops of α-naphthol solution in alcohol was added and concentrated H_2SO_4 was added from sides of the test tube which formed violet ring at the junction of two liquids.

A. For reducing sugars: -

a) Fehling's test:

1ml Fehling's A and 1ml Fehling's B Solution was mixed and boiled for one minute. To this equal volume of test solution was added. On heating in boiling water bath for 5-10 min., initially it formed a yellow and then brick red precipitate.

b) Benedict's test:

Equal volume of Benedict's reagent and test solution were mixed in test tube and heated on boiling water bath for 5 min. Solution appears green, yellow or red color depending on the amount of reducing sugar present in test solution.

B. For Monosaccharide: -

a) Barfold's test:

Equal volume of Barfold's reagent and test solution were added and heated on boiling water bath and cooled. Red precipitate was observed indicating the presence of monosaccharide.

C. For Hexose sugars: -

Cobalt- chloride test:

3ml of test solution was mixed with 2 ml cobalt chloride, boiled and cooled. On addition of few drops of NaOH solution, it formed greenish blue (glucose), purple (fructose) or upper layer greenish blue and lower layer purplish (mixture of glucose and fructose).

D. Test for Non-reducing sugars:-

a) Test solution did not give response to Fehling's and Benedict's test.

b) Tannic acid test for starch: 20 % tannic acid was added to test solution and precipitate was observed if positive.

4.5.2 Test for proteins:-

a) Biuret test (general test):

To 3 ml test solution, 4 % NaOH and few drops of 1 % $CaSO_4$ solution was added and observed for violet or pink color.

b) Million's test (for proteins):

3 ml of test solution was mixed with 5 ml Million's reagent to obtain white precipitate. The precipitate was further warmed which turned to brick red or precipitate was dissolved giving red color.

c) Xanthoprotein test: (for protein containing tyrosine or tryptophan):

3 ml of test solution was mixed with 1 ml conc. H_2SO_4, which was observed for the presence of white precipitate. After boiling precipitate turns yellow. Addition of NH_4OH precipitate turns orange.

d) Precipitation test:

The test solution gave white colloidal precipitate with following reagents:

i. Absolute alcohol.
ii. 5% $HgCl_2$ solution.
iii. 5% $CuSO_4$ solution.
iv. 5% lead acetate.
v. 5% ammonium sulphate.

4.5.3 Test for steroids:-

a) Salkowski test:

2 ml chloroform and 2 ml conc. H_2SO_4 was added to 2 ml of extract. After well shaking, the chloroform layer appeared red and acid layer showed greenish yellow fluorescence. This indicates presence of steroids.

b) Liebermann- Burchard reaction:

The 2 ml extract was mixed with chloroform and 1-2 ml acetic anhydride and 2 drop of conc. H_2SO_4 was added from the side of test tube which formed first red, then blue and finally green color. This indicates presence of steroids.

c) Liebermann's reaction:

The mixture of 3 ml extract with 3 ml of acetic anhydride was heated and cooled, and then few drops of conc. H_2SO_4 were added and observed for blue color. This indicates presence of steroids.

4.5.4 Test for amino acids:-

a) Ninhydrin test (general test):

To 3 ml test solution 3 drops of 5 % Ninhydrin solution was added and heated in boiling water bath for 10 min. It was then observed for purple or bluish color.

b) Test for Tyrosine:

3 ml test solution was heated with 3 drops of Million's reagent. This solution was observed for dark red color.

4.5.5 Test for glycosides:-

A. For cardiac glycosides:-

a) Balijet's test:

A test solution observed for yellow to orange color with sodium picrate.

b) Legal's test (for cardenoloids):

To aqueous or alcoholic test solution, 1 ml Pyridine and 1 ml sodium nitroprusside were added and then observed for pink to red color.

c) Test for deoxysugars (Kellar- killiani test) :

To 2 ml extract, glacial acetic acid, one drop of 5 % $FeCl_3$ and conc. H_2SO_4 was added and observed for reddish brown color at the junction of the two liquid and upper layers appears as bluish green.

B. For Anthraquinone glycoside:-

a) Bontrages's test:

Powder drug was boiled with 5 ml of 10% sulfuric acid for two minutes. It was filtered while hot. After cooling the filtrate, it was shaken gently with equal volume of benzene. Separated benzene layer was treated with half of its volume of solution ammonia (10%).It was allowed to separate. Ammoniacal layer acquired pink or red color due to the presence of anthraquinones.

b) Modified Bontrager's test: -

C-Glycosides of anthraquinones require more drastic conditions for hydrolysis. Hydrolysis of drug was carried out with 5 ml of dilute HCl and 5 ml of 5 % solution of ferric chloride. Remaining procedure was carried out as described for Bontrager's test.

C. For Saponin glycosides:

a) Foam test:

The drug extract or dry powder was shaken vigorously with water. It was then observed for persistent foam.

4.5.6 Test for Flavonoids:

a) Shinoda test:

To an extract, 5 ml 95% ethanol, few drops concentrated HCl and 0.5 g magnesium turnings were added. Pink color was observed. This indicates that presence of flavonoids.

b) Lead acetate test:

To small quality of residue, lead acetate solution was added and observed for yellow colored precipitate. This indicates that presence of flavonoids.

c) Ferric chloride test:

To a test solution, few drops of ferric chloride solution were added. Then it was observed for intense green coloration. This indicates that presence of flavonoids.

4.5.7 Test for Alkaloids:-

a) Dragendroff's test:

To 2-3 ml filtrate, few drops Dragendroff's reagent was added. Then it was observed for the formation of orange brown precipitate.

b) Mayer's test:

2-3 ml of filtrate was mixed with few drops Mayer's reagent then it was observed for the formation of precipitate.

c) Wagner's Reagent:

2-3 ml filtrate was treated with Wagner's reagent then it was observed for the formation of reddish brown precipitate.

4.5.8 Test for Tannins and phenolic compounds:-

To 2-3 ml test solution, few drops of following reagents were added and the changes in color were observed:

a) 5 % $FeCl_3$ solution: - deep blue- black color.
b) Lead acetate solution: - white precipitate.
c) Bromine water: - discoloration of bromine water.
d) Acetic acid solution: - red color solution.
e) Potassium dichromate: - red precipitate.
f) Dilute $HNO_{3:}$- reddish to yellow color.

4.5.9 Test for carotenoids:-

Carr-Price test:

The solution of antimony trichloride in chloroform added to extract, it did not show blue coloration which indicated absence of carotenes.

4.5.3 Test for Triterpenoids:-

a) Salkowski test:

2 ml chloroform and 2 ml concentrated H_2SO_4 was added to 2 ml of extract. After well shaking, the chloroform layer appeared red and acid layer showed yellow fluorescence.

b) Liebermann- Burchard reaction:

The 2 ml extract was mixed with chloroform and 1-2 ml acetic anhydride and 2 drop of conc. H_2SO_4 was added from the side of test tube which formed deep red, color.

c) Liebermann's reaction:

The mixture of 3 ml extract with 3 ml of acetic anhydride was heated and cooled, and then few drops of concentrated H_2SO_4 were added and observed for red color.

4.6 SEPARATION OF UNSAPONIFIABLE MATTER FROM *n*-HEXANE EXTRACT:

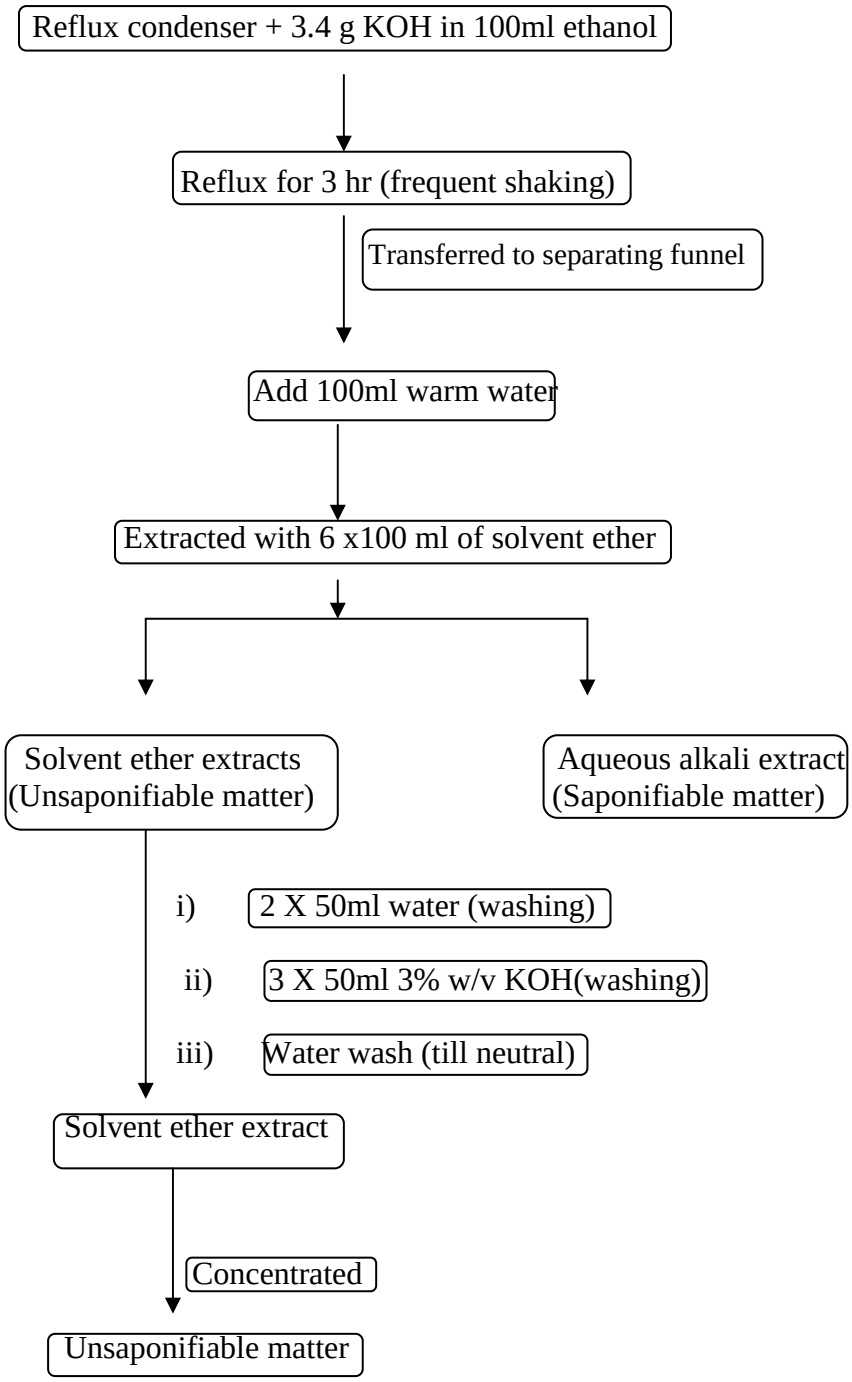
n- Hexane extract contains waxy fatty acids and other phytocomponents along with sterol, triterpenes. These compounds are unsaponifiable and it can be fractionated from waxy saponifiable matter as by using following procedure. Saponification of *n*- Hexane extract was performed as per IP 1996 and separated into two parts[20].

Saponification:

The *n*-Hexane extract (10 g) of *Corchorus fascicularis* Lam. was transferred into 250 mL round bottom flask fitted with reflux condenser. A solution of 3.4g of KOH in 120 ml of ethanol (95%) was added to the extract and refluxed for one and half-hour with frequent shaking. The content of the flask was transferred to separating funnel with the aid of 100 ml of hot water, while the liquid was still warm; it was shaken carefully with 3 quantities each of 100 ml of peroxide free ether (solvent ether). All the three ether extracts were combined and transferred to second separating funnel. It was swirled with 40 ml of water and allowed to separate. Aqueous part was rejected. Again ether extract was washed with two quantities each of 40ml of 3% w/v solution of KOH and each washing of KOH was followed with 40ml of water wash. Finally 50% ether layer was washed with successive quantities each of 40ml of water until aqueous layer was not alkaline to phenolphthalein indicator. Solvent ether layer was concentrated by distillation under reduced pressure, dried and weighed the residue. The yield of unsaponifiable matter of *Corchorus fascicularis* Lam. was 7.3 g.

Separation of saponifiable and unsaponifiable matter from n- Hexane extracts

n- Hexane extracts (10 g)


Reflux condenser + 3.4 g KOH in 100ml ethanol
Reflux for 3 hr (frequent shaking)
Transferred to separating funnel
Add 100ml warm water
Extracted with 6 x100 ml of solvent ether
Solvent ether extracts (Unsaponifiable matter)
Aqueous alkali extract (Saponifiable matter)
i) 2 X 50ml water (washing)
ii) 3 X 50ml 3% w/v KOH(washing)
iii) Water wash (till neutral)
Solvent ether extract
Concentrated
Unsaponifiable matter

4.7 QUALITATIVE ANALYSIS OF EXTRACT PERFORMED BY CHEMICAL TEST WAS FURTHER SUPPORTED BY TLC STUDIES:

TLC is a widely used separation technique in plant chemistry research. It can be used in a search for the optimum extraction solvents for identification of known and unknown compounds and at least equally important for selection of biologically active compounds. TLC also plays key role in preparative isolation of compounds, purification of crude extracts and control of separation efficiency of the different chromatographic techniques and systems. TLC has many advantages in plant chemistry research and development; these include single use of stationary phase, wide optimization possibilities with the chromatographic systems, special development modes and detection methods, storage function of chromatographic plates, low cost in routine analysis and availability of purification and isolation procedures.

TLC separations can also be used to select column chromatography conditions. TLC conditions that give a useful R_f i.e. compounds separate from the majority of other components without staying at the origin or with the solvent front and we can approximately transfer to column chromatography. Identification of target compound on TLC plate can be carried out by comparison with standard, by an overlay assay carried out on top of developed plate in the case of an unknown biologically active component, or by scraping off, extracting, and assaying portions of adsorbent. Unlike column chromatography, TLC is non equilibrium technique, which means that the conditions of mobile and stationary phases are not constant throughout plate but show a discrepancy during the run and according to the position on the plate.

The extracts were subjected to thin layer chromatographic studies using reported methods to confirm presence of various phytoconstituents[13, 14, 15].

Preparation of TLC plates:-

Slurry of silica gel G was made with the help of mortar and pastel using

suitable solvent (alcohol or chloroform). The slurry was then poured and spread on to the plates. These plates were left for some time open to air and then placed in an oven to activate at 105^0C for one hour. This is time consuming process and results obtained are not reproducible. Therefore now a day's TLC profile of extracts are developed on readymade coating plates of Silica gel G of E-Merck, aluminum backed thin layer chromatography plates. Layers containing an indicator that fluoresces when irradiated with 254 or 366 nm ultraviolet light are designated as ''F'' or ''UV'' layers. These layers are used to facilitate detection of compounds that absorb at these wavelengths and give dark zones on a bright background (fluorescence quenching). After performing the separation with the optimum layer, mobile phase, and development technique combination, the zones must be detected. If the zones are not naturally colored or fluorescent, or do not absorb 254 nm UV light so they can be viewed as fluorescence-quenched zones on special F-plates containing a fluorescent indicator, a detection reagent must be applied by spraying or dipping, usually followed by heating. This derivatization is mainly used in the post chromatographic mode for localization of the separated component zones on the layer. Very often universal reagents are used, such as iodine vapors or sulfuric acid. These reagents can locate almost all of the existing organic compound classes. Selective reagents can be used as derivatizing reagents for individual or group identification of the analytes[13].

In each case very tiny drop was chromatographed with the help of capillary tube. Development of chromatogram was done in closed tanks in which the atmosphere had been saturated with solvent vapor by wetting a filter paper lining. Samples were applied rapidly and developed without delay to minimize the possibility of oxidation or photo-oxidation of constituents. The separated components were visualized under visible and ultraviolet light (short UV-254 nm and long UV360 nm). The TLC plates were subsequently sprayed with different spraying reagent according to the class of the compound. The extracts were subjected to thin layer chromatographic studies using reported methods to confirm presence of various phytoconstituents[14, 15].

Thin layer chromatographic study of extracts of leaves of *Corchorus fascicularis* Lam.:-

TLC profile of extracts were developed on readymade coating plates of Silica gel G of E-Merck as well as some times the plates are prepared on glass plates using silica gel G slurry in suitable solvent by pouring method.

4.7.1 TLC pattern for unsaponifiable matter of n-hexane extract of leaves of *Corchorus fascicularis* Lam. -

Preliminary phytochemical analysis (Chemical tests) showed triterpenoids test positive for n-Hexane extract. Hence, the TLC patterns of n- Hexane extract, for triterpenoids was studied.

Stationary phase: Silica gel G 254 TLC Plates (Merck).

Mobile phase: Different solvent systems such as Benzene: Ethyl acetate (9:1), Benzene: chloroform (9.5:0.5), n-Hexane: ethyl acetate has been tried for optimization of better resolution.

Better resolution was observed in n-Hexane: Ethyl acetate (7:3)

Spraying reagent: Anisaldehyde (0.5ml) + Glacial Acetic acid (10ml) + Methanol (85ml) + Conc. Sulphuric Acid (5ml) followed by heating at 100 ^{0}C for 7 minutes

4.7.2 TLC pattern for saponifiable matter of n-hexane extract of leaves of *Corchorus fascicularis* Lam. –

TLC profile of saponifiable matter was developed for the presence of fatty acids using several solvent systems like Toluene:ethyl acetate (9.0:1.0), Hexane:diethyl ether (9.5:0.5), Hexane: diethyl ether (8.5:1.5). Spots on TLC plates were visualized with the help of spraying reagent bromothymol blue as well as with iodine chamber.

4.7.3 TLC pattern for chloroform extract of leaves of *Corchorus fascicularis* Lam. -

Preliminary phytochemical analysis (Chemical tests) showed steroids test positive for chloroform extract. Hence, the TLC pattern of chloroform extract for steroids was studied.

Stationary phase: Silica gel G 254 TLC Plates (Merck).

Mobile phase: Different solvent systems such as Petroleum ether: Ethyl acetate (9.0:1.0), Benzene: chloroform (9.5:0.5), Chloroform: Ethyl acetate in different ratio has been tried for optimization of better resolution.

Better resolution was observe in Chloroform: Ethyl acetate (5:5)

Spraying reagent: 70% Ethanolic Sulphuric acid, followed by heating at 50^0C for 10 minutes

4.7.4 TLC pattern for ethanol extract of leaves of *Corchorus fascicularis* Lam. -

Preliminary phytochemical analysis (Chemical tests) showed flavonoids test positive for ethanol extract. Hence, the TLC pattern of ethanol extract was studied.

Stationary phase: Silica gel G 254 for TLC Plates (Merck).

Mobile phase**:** Different solvent systems such as Chloroform: Ethyl acetate (9.2:0.8), Petroleum ether: Chloroform (9.5:0.5), Toluene: Ethyl acetate and Methanol in different ratio have been tried for optimization of better resolution.

Better resolution was observed in Chloroform: Methanol: Ethyl acetate (7:2:1)

Spraying reagent**:** 70 % Ethanolic Sulphuric acid, followed by heating at 50^0C for 10 minutes

4.8SEPARATION AND ISOLATION OF PHYTOCONSTITUENTS BY COLUMN CHROMATOGRAPHY, PREPARATIVE THIN LAYER CHROMATOGRAPHY:

Introduction:

Plant extracts are usually a complex mixture containing many different constituents. The separation and determination of the active components in medicinal plant extracts provide solution in studying their pharmacological, toxicological and other biological mechanisms. The presence of other plant constituents like proteins, fats, and carbohydrates (sugar) in a crude extract make the isolation and measurement of active constituents extremely difficult.

Different chromatographic techniques such as thin layer chromatography, preparative thin layer chromatography, column chromatography, high performance liquid chromatography, gas chromatography and flash chromatography are frequently used for the analysis of plant medicines, but TLC is an important method which is considered reproducible and accurate.

4.8.1 Column Chromatography[21,22]:

Column chromatography in chemistry is a method used to purify individual chemical compounds from mixtures of compounds. It is often used for preparative applications on scales from micrograms up to kilograms.

The classical preparative chromatography column is a glass tube with a diameter from 5 to 50 mm and a height of 50 cm to 1 m with a tap at the bottom. Slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. Care must be taken to avoid air bubbles. A solution of the organic material is pipetted on top of the stationary phase. This layer is usually topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the velocity of newly added eluent. Eluent is slowly passed

through the column to advance the organic material. Often a spherical eluent reservoir or an eluent-filled and stoppered, separating funnel is put on top of the column.

The individual components are retained by the stationary phase differently and separated from each other while they are running at different speeds through the column with the eluent. At the end of the column they elute one at a time. During the entire chromatography process the eluent is collected in a series of fractions. The composition of the eluent flow can be monitored and each fraction is analyzed for dissolved compounds, e.g. by analytical chromatography, UV absorption, or fluorescence. Colored compounds (or fluorescent compounds with the aid of an UV lamp) can be seen through the glass wall as moving bands.

Column chromatography is one of the most frequently used techniques in the isolation of natural plant constituents. In principal, plant constituents are distributed between the solid phase (example- silica gel or sephadex) and the mobile phase, which comprises an eluting solvent. In silica gel separation of compounds in an extract is based on number of factors including the polarity of compounds, hence compounds eluted from column with solvent system of differing polarity. Silica gel constitutes polar ends which interact strongly with polar compounds and they are eluted later from the column. In sephadex gel filtration the separation of constituents in an extract depends on the size of the molecules. Constituents with the small molecular weight interact strongly with the matrix of the gel and tend to move slowly through the gel and they are eluted later while the large molecular weight constituents are eluted early because they move fast through the column.

The successive isolation of bioactive compounds from indigenous medicinal plants will validate indigenous knowledge adding value to the plants and support plant conservation and knowledge preservation. It may also contribute to research and development in the production of new pharmaceutical drugs for the treatment of various diseases.

The separation of constituents from extract was done by column chromatography:

The clean and dried column was used for separation of different chemical constituents present in extracts. The column was cleaned and washed with acetone and dried. The silica gel for column chromatography (Mesh size 60-120) was activated at 110 ^{0}C. The column was fixed to stand; about 3/4th height of column was packed by preparing slurry of silica gel with n- Hexane. The sample was prepared by vigorously mixing the extract with silica gel and solvent, the solvent was then evaporated to form a free flowing material with the help of rotary evaporator. This sample was charged in the column. The column was allowed to stand for 1 hour for absorption of material after that the sample was eluted and fractions were collected. The samples eluted in the form of fractions were collected. Fractions with similar separation pattern on TLC plates were mixed and studied further. Fractions showing mixtures of two or more compounds were purified further. However, fractions showing inseparable mixture of several compounds were rejected. The fraction showing single spot were mixed and considered as single compound.

Separation of different chemical constituent of unsaponifiable matter, chloroform extract and ethanol extract were carried out by column chromatography [13]. Most of the fractions obtained from column chromatography most of them were mixtures of 2-3 components. This required further fractionation. This can be done by preparative TLC [14] or by re-column method [15, 16]. We have selected preparative TLC method for isolation of pure compound from fractions because it is less expensive, time consuming; gives more pure compounds used for spectral analysis.

Column chromatography of unsaponifiable Matter obtained from n-Hexane Extract of leaves of *Corchorus fascicularis* Lam:

Stationary phase : Silica gel for Column Chromatography 60-120#

Mobile phase : n-hexane: ethyl acetate (7: 3)

Fractions collected : 25 ml

No. of fractions collected : 130

Column chromatography of chloroform extract of leaves of *Corchorus fascicularis* Lam:

Stationary phase : Silica gel for Column Chromatography 60-120#

Mobile phase : chloroform: ethyl acetate (1:1)

Fractions collected : 30 ml

No. of fractions collected : 180

Column chromatography of methanol extract of leaves of *Corchorus fascicularis* Lam:

Stationary phase : Silica gel for Column Chromatography 60-120#

Mobile phase : methanol: chloroform: ethyl acetate (7:2:1)

Fractions collected : 27 ml

No. of fractions collected : 160

4.8.2 Preparative TLC:

The fractions showing single spots were mixed and considered as single compound but after mixing of these fractions and when TLC was taken it showed certain impurity. It may be due to other compound. Therefore, for isolation of pure compound it is necessary to perform preparative thin layer chromatography. Further purification was carried out by preparative thin layer chromatography.

Preparative TLC is one of the simplest and cheapest methods available for the isolation of compound or compounds from the mixture. The plates used in this method were 0.5-1.0 mm thick which allowed a greater amount of sample to be loaded on the plate. 20X20 cm or 5X20 cm glass plates were coated with the thick layer of silica gel or any other adsorbent material. The plates were then activated at 110 ^{0}C. Readymade coating plates of silica gel G of Merck, aluminum backed thin layer chromatography plates can be used.

Sample Application In preparative Layer Chromatography[23]:

Sample application for preparative separations in planar chromatography usually requires spotting larger volumes of the sample solution on the plate. Its solution is usually deposited on almost the whole width of the chromatographic plate in the shape of band, streak or rectangle. Adsorbent layers used for preparative separations are thicker than analytical separations. These procedures of sample application can be performed manually using capillary or micro syringe. Then the sample solution is spotted side by side on the start line of the chromatographic plate. This mode is tedious and needs lot of manual operations. Shape of starting band is often not appropriate leading to lower resolution of the zones on final chromatogram. More experience is necessary when sample application is performed by moving tip of pipette or syringe needle over a start line without touching the layer surface.

The sample containing single compound or mixture of two compounds was applied in the form of thin band on the plate. The plate was then developed in respective solvent system. A non destructive detection method was used in detecting separated compounds. The most common method of visualizing developed chromatogram was UV light, to detect all quenching compounds. However there are large numbers naturally occurring compounds that do not fluorence or quench, so other detection methods were used. A simple method for non water soluble compounds is to spray the layer with a fine mist of water so that it was wet enough to become transparent. Non water soluble compounds appeared as dark areas with transmitted light and as lighter areas when the plate is viewed in reflected light. Chromatograms were also sprayed with vanillin sulphuric acid to view vanillin active compounds. The different bands separated on the plate were scratched and recovered with methanol. Purity of dried sample was checked by TLC method.

PTLC of Unsaponifiable matter of *n*-Hexane extract:

The preparative thin layer chromatography of unsaponifiable matter was developed in the same solvent system which was applied in column chromatography; the bands were identified, scraped and extracted with chloroform. Two pure

compounds were isolated from unsaponifiable matter of n-hexane extract of leaves of *Corchorus fascicularis* L. they are designated as **HEC-1** and **HEC-2.**

PTLC of Chloroform extract:

The chloroform extract was separated by PTLC using same solvent system which was applied for column chromatography. In preparative thin layer chromatography of this extract, the bands were identified, scraped and extracted with chloroform. Two solid pure compounds were isolated from chloroform extract of leaves of *Corchorus fascicularis* L. they are designated **CEC-1** and **CEC-2**.

PTLC of Ethanol extract:

The ethanol extract was separated by PTLC using same solvent system which was applied for column chromatography. In preparative thin layer chromatography of this extract, the bands were identified, scraped and extracted with chloroform. Two solid pure compounds were isolated from ethanol extract of leaves of *Corchorus fascicularis* L. they are designated **EEC-1** and **EEC-2.**

4.9 CHARACTERIZATION OF PHYTOCONSTITUENTS USING SPECTROSCOPIC TECHNIQUES:

All isolated compounds from different extracts of leaves of *Corchorus fascicularis* L. has been characterized and their structures were identified by using physical methods, chemical methods, elemental analysis and spectroscopic techniques like UV, IR, ^{1}H-NMR, ^{13}C-NMR, LC-Mass.

Physical and chemical methods include determination of description, solubility, melting point, boiling point and R_f value [24].

STRUCTURE ELUCIDATION AND CHARACTERIZATION OF ISOLATED COMPOUNDS:

Introduction:

The plant kingdom has an estimated 200,000 primary and secondary metabolites with various sets of atomic arrangements. This property allows for wide

variations in the chemical properties such as molecular weight, solubility and physical properties like volatility. The analysis of drug metabolites and natural products requires the utilization of rapid assays and techniques to screen a maximum number of active components in small quantities of biological mixtures within shortest possible time. Lack of rapid assays, techniques and inefficient methods of identification of unknown components result gives valuable information on potential new drug neglected or inaccurately interpreted. The identification of compounds involves a combination of different techniques including ultraviolet (UV), infrared (IR), nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR), mass spectroscopy (MS) and Liquid Chromatography –mass spectroscopy (LC-MS).

4.9.1 Spectroscopic Characterization of compounds isolated from unsaponifiable matter of n-Hexane extract, Chloroform extract and Ethanol extract.

Different spectroscopic methods were used to elucidate the structure of isolated compounds. The compounds isolated from unsaponifuable matter of *n*-hexane extract, chloroform extract and methanol extract were analyzed at Wochardt Research Centre, Aurangabad.

In spectroscopic techniques, the ultraviolet spectrum was recorded on 1601 UV-Visible spectrophotometer (Shimadzu), The infrared spectrum was recorded on FT-IR Spectrum one (Perkin Elmer, USA), ^{1}H-NMR spectra were recorded on a Varian-400 MH_Z NMR spectrometer; Mercury Plus (Switzerland), ^{13}C-NMR spectra were recorded on a Varian-400 MH_Z NMR spectrometer; Mercury Plus (Switzerland) at Wockhardt R & D Ltd, Aurangabad, India. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded using $CDC1_3$ and DMSO as solvent with Tetramethyl silane (TMS) as an internal standard. Mass spectrum was recorded at high resolution on a mass spectrometer (Perkin Elmer Autosystem XL with Turbomass) at Research & Development centre; the data are given in m/z values. Elemental analysis was recorded on Elementar instrument model Vario Micro Cube using oxygen and helium as combustion and carrier gases respectively at a temperature of 1150°C at Wockhardt Research and Development Centre, Aurangabad, India.

4.10 BIOLOGICAL SCREENING OF LEAVES EXTRACTS OF *Corchorus fascicularis* Lam.

4.10.1 ANTI-OXIDANT ACTIVITY OF LEAVES EXTRACTS OF *Corchorus fascicularis* Lam:

Plant foods are well known sources of vitamins, such as vitamin C and folic acid, carotenoids, fiber and these are naturally free saturated fat and cholesterol. In addition, these foods contain significant amounts of polyphenols, a group of phytochemicals recognized as the most abundant antioxidants in our diet.

Antioxidants components are micro constituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation of oxidizing chain reactions and are also involved in scavenging free radicals[25].

Natural antioxidants, particularly found in fruits and vegetables have been of increasing interest to both consumers and scientists such as epidemiologists, food scientists, chemists and plant scientists because epidemiological studies have indicated that frequent consumption of natural antioxidants are associated with a lower risk of cardiovascular diseases and different types of cancers. The available data also supports to the protective role for fruits and vegetables in protection against pancreas, bladder and breast cancer[26, 27, 28].

Now a day the use of synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effects. This concern has resulted in an increased interest in the investigation of the effectiveness of naturally occurring compounds with antioxidant properties. Thus, the natural antioxidants present in foods and other biological materials have attracted considerable interest because of their presumed safety and potential nutritional and therapeutic effects[29].

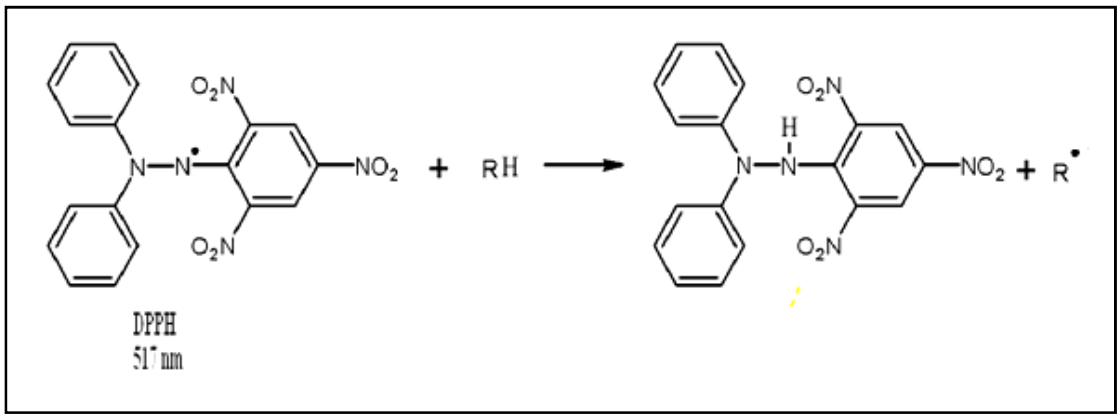
Oxidative stress induced Reactive oxygen species (ROS) and free radicals are believed to be major cause of physiological disorders like Alzheimer's, Parkinson's, arthritis, atherosclerosis, coronary heart diseases, emphysema, gastric ulcer, diabetes mellitus, cirrhosis, aging and cancer[30, 31, 32].

ROS are highly reactive molecules which include free radicals such as superoxide ions (O_2^-), hydroxyl radicals (OH^-), singlet molecular oxygen radicals and hydrogen peroxide (H_2O_2). Superoxide anion radical is one of the strongest reactive oxygen species among free radicals that are generated first after oxygen is taken into living cells[33]. Therefore, in recent years, significant attention has been paid to explore the potential of antioxidant property of plant extracts of plant origin, which may be used for human consumption. Crude extracts from plant materials rich in phenolic compounds are increasingly of interest in the food industry, because they can retard oxidative degradation of lipids and thereby improve the quality and nutritive value of food. Some studies have shown that the increased dietary intake of natural antioxidants such as flavonoids and other phenolic compounds present in most plants, may act as potent candidates in preventing diseases related to oxidative stress[34].

4.10.1a Determination of DPPH Radical Scavenging Activity[35, 36]:

Principle:

A simple method that has been developed to determine the antioxidant activity of foods utilizes the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH) radical. The structure of DPPH and its reduction by an antioxidant is shown in following mechanism,



The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptive of DPPH radical at 517 nm reduces as the odd electron of DPPH radical

undergoes pairing with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is absorbance produced at 517 nm has been used as a measure of radical scavenging activity.

Materials and methods:

Plant material:

The green leaves of *Corchorus fascicularis* Lam. Were collected from the stony Hilly area of village Tande, of Shirpur Tahasil in Dhule District of Maharashtra, India in July 2010. Dr. L. K. Kshirsagar, Taxonomist, Department of Botany, S. S. V. P. S. College, Dhule; Maharashtra; authenticated the plant material.

Preparation of Extract:

The green leaves of *Corchorus fascicularis* Lam. were collected from the stony Hilly area of village Tande, of Shirpur Tahasil in Dhule District of Maharashtra, India and were dried under shade for 7 - 10 days, after that dried of leaves were pulverized. The powdered leaves were extracted by using *n*-hexane, chloroform and ethanol. The powdered leaves (1 kg) of *Corchorus fascicularis* Lam. were extracted with n-hexane with three times each by cold maceration at room temperature. For extraction, the powder was taken in conical flask and macerated for 48 hours at room temperature till all the material defatted completely. The extracts thus obtained were concentrated by distilling off the solvent under reduced pressure by using Rota Evaporator. The defatted marc thus obtained was successively extracted with chloroform (3 x 1L) and ethanol (3 x 1L) i.e. three times each. These extracts were concentrated separately under reduced pressure.

Chemicals

DPPH- (1, 1- dipheny l-2- pieryl-hydrazyl) (Sigma chemicals Ltd), Ascorbic Acid (S. D. Fine Chemicals)

Reagents:

DPPH Solution (100 mM):

DPPH solution was prepared by dissolving 33 mg of DPPH in 100 ml of methanol. From this stock solution, 10 ml was taken and diluted to 100 ml using methanol to obtain 100 mM DPPH solution and kept in amber colored bottle to protect from sunlight.

Ascorbic Acid Standard Solution:

100 µg /ml stock solution was prepared by dissolving 10 mg of ascorbic acid in 100 ml of distilled water, from this 10, 20, 40, 60, 80, 100 µg /ml of ascorbic acid solution was prepared.

Preparation of test solutions:

A stock solution of concentration 1 mg/ml was prepared by adding 10 mg of n- Hexane, Chloroform, Ethanol extracts in 10 ml methanol and solutions of various concentrations of different extracts such as 50, 100, 200, 400, 800, 1000 µg /ml were prepared.

Procedure:

100 µl of various concentrations (50-1000 µg /ml) of different extracts and 100 µl solution of DPPH (100 mM in methanol) was incubated at 37 ^{0}C for 30 minutes and change in absorbance of reaction mixture was read at 517 nm using micro titer plate ELIZA reader (BIOTEK power wave XS, Model-96 well micro plate). An equal amount of methanol and DPPH was served as control. The experiment was performed in triplicate and percentage radical scavenging activity was calculated by formula given below:

$$\% \text{ of inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

The IC_{50} value was calculated for each sample and standards and results were expressed as is the concentration of the sample required to scavenge 50% of DPPH free radicals.

4.10.1b Determination of Total antioxidant Capacity[37]:

Principle:

Phosphomolybdenum assay used to determine the total antioxidant capacity is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate Mo (V) complex at acid $P^H = 9$

Chemicals:

Ascorbic Acid (SD fine chemicals Ltd., Mumbai), Ammonium Molybdate, Sodium Phosphate, Sulphuric Acid (Loba Chem Ltd., Mumbai)

Reagents:

The reagent was prepared by mixing 28 mM sodium Phosphate (0.042 gm in 100 ml water), 4 mM Ammonium molybdate (3.72 gm in 100 ml water) and 0.6 M Sulphuric Acid (5.88 ml in 100 ml water).

Procedure:

The total antioxidant capacity of the extracts was estimated using the phosphomolybdenum reduction assay according to Saleh and Hameed and Preito et al. was used to determine the total antioxidant capacity of the plant extract. The taste tubes containing 0.1 ml (100 µg) of sample solution combined with 1 ml of reagent (0.6 m sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95 ^{0}C for 90 minutes. After mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm using against blank in Genesys-5-UV spectrophotometer (Milton Roy, New York). A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The antioxidant capacity of the extracts was expressed as the ascorbic acid equivalent (AAE)

4.10.2 ANTI-INFLAMMATORYACTIVITY OF LEAVES EXTRACTS OF *Corchorus fascicularis* Lam:

Introduction:

Inflammation is the body's reaction to invasion by an infectious agent, antigen challenge or even just physical, chemical or traumatic damage inflammation is a complex stereotypical reaction of the body expressing the response to damage of its cell and vascularized tissue. The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and immune response. Inflammation is the first response of the immune system to infection.

The five basic symptoms of inflammation- redness, swelling, heat and pain function have been known since the ancient Greek and Roman era. These signs are due to extravasations of plasma and infiltration of leukocytes into the site of inflammation. Early investigation considered inflammation a primary host defense system. From this point of view inflammation is the key reaction of innate immune response but in fact, inflammation is more than this, since it can lead to death.

Materials and Methods:

Standard drugs/ chemicals solutions:

Diclofenac sodium (10 mg/kg) (Lupin), used for the purpose of comparison, were prepared by dissolving them in saline: CMC. Carrageenan (λ4) (C3889-5G), Histamine (H-7375), were obtained from Sigma Aldrich, USA.

Pharmacological studies:

Animals:

Wistar rats (150-200 g; 8-11 weeks old) and Swiss albino mice (25-30 g; 7-10 weeks old) were obtained from R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur and Government Veternary College, Mahu, MP (India). The animals were housed in Animal house of R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, India in polycarbonate cages, in a room maintained

under controlled room temperature 22 ± 2^0C, relative humidity 60 -70% and provided with food and water *ad libitum*. All the experimental procedures and protocols used in the study were reviewed by the Institutional Animal Ethics Committee (Register Number: RCPIPER/IAEC/2010-11/13) and the care of laboratory animals were taken as per the guidance of CPCSEA, Ministry of Forests and Environment, Government of India. The animals were deprived of food for 24 h before experimentation but allowed free access to water throughout. All studies were carried out by using six animals in one group for anti- inflammatory activity.

4.10.2.1 Acute toxicity test:

Acute toxicity tests were performed according to OECD-2006 guideline no. 425. Animals were weighed and marked; a single high dose of n-hexane, chloroform and ethanol as recommended by the OECD guidelines was administered to the first animal. After single administration, animals were observed for the sign of toxicity up to 24 h. If mortality was observed, one step lesser dose of previous administration was given to the next animals. If the animal survived, the same dose was given to the next five animals. Further all the animals were observed for the presence of signs of toxicity and mortality for 14 days. The body weights of the animals were also recorded. Additional observations like changes in skin, eyes and mucous membranes, and also respiratory circulatory, autonomic and central nervous system and behavior pattern. Attention was also given to observe precipitation of tremors and convulsions. The LD50 of the test drug was calculated using a computer assisted statistical programme-AOT425statPgm[38].

4.10.2.2 Carrageenan-induced paw edema:

Wistar Rats of either sex (150-200 g) were divided into eight groups containing six animals in each. The rats were fasted for 12 h prior to induction of edema. Rats were deprived of water only during the experiment to ensure uniform hydration and minimize variability in edematous response. Inflammation of hind paw was induced by injecting 0.1 ml of 1% w/v carrageenan in normal saline into the subplantar region of right hind paw[39]. The negative control group received Saline: CMC (0.5%) solution[40] and the positive control group received Diclofenac sodium

(10 mg/kg) p.o.[41]. Three groups orally received petroleum ether extract at doses 100, 200 and 400 mg/kg, respectively. The remaining three groups orally received methanol extract of both the plants at doses 100, 200 and 400 mg/kg, respectively. All the drug treatments were given 1 hr before the carrageenan injection; edema was expressed as the increase in paw volume due to carrageenan injection. The paw volume was measured with a digital plethysmometer (Ugo Basile, 7140) before and 1, 2, 3, 4, 5 and 6 h after carrageenan injection[42, 43]. The extracts and the reference drugs were dissolved in 0.5% carboxy methyl cellulose solution just before use.

Percentage rise in paw volume was calculated by using following formula[44]

% Rise = Vt – Vc / Vc x 100

Where, Vt = Paw volume post carrageenan/histamine injection t

Vc = Paw volume before carrageenan/histamine injection o

Statistical analysis

The statistical analysis of all the results was carried out using one-way ANOVA followed by Dunnet's multiple comparisons using graph pad prism and the level of significance was determined in comparison with the control group.

4.10.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING:

4.10.3.1 Introduction:

Antibiotics are one of the most important weapons in fighting No. of bacterial infections. However, from few decades these health benefits are under hazard. Many antibiotics became less effective against certain illnesses not only because many of them produce toxic reactions hence, in many developing countries traditional medicine is in the primary health care system[45, 46] due to their less toxicity and higher affectivity.

Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. The most common bacteria causing illness are *S. aureus*, *E. coli* and others[47, 48]. Natural products of higher plants may give new source of antimicrobial agents with novel mechanisms of action[49, 50, 51].

4.10.3.2 Principle:

The principles of determining the affectivity of a noxious agent to a bacterium were well enumerated by Rideal ,Walker and others at the turn of the century, the discovery of antibiotics made these tests(or their modification) too cumbersome for the large numbers of tests necessary to be put up as a routine..The Oxford Group used these methods initially to assay the antibiotic contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface.

4.10.3.3 Antimicrobial activity of Leaves extracts of *Corchorus fascicularis* Lam.:

Disc Diffusion Method[52-54]:-

Reagents for the Disc Diffusion Test:

1. Mueller-Hinton Agar Medium

Of the many media available, Müeller-Hinton agar is considered to be the best for routine susceptibility testing of non fastidious bacteria for the following reasons:

* It shows acceptable batch-to-batch reproducibility for susceptibility testing.
* It is low in sulphonamide, trimethoprim, and tetracycline inhibitors.
* It gives satisfactory growth of most non fastidious pathogens.
* A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Method:

In *vitro* antibacterial and antifungal activity were examined for ethanol, n-Hexane, chloroform and water extracts. Antibacterial and antifungal activities of these extracts against two pathogenic bacteria and one pathogenic fungi were investigated by the Agar Disc Diffusion method. All the extracts were screened for their antibacterial and antifungal activities against the *S.aureus*, *E. coli* and fungi strain *C. albicans.*

The dilutions of *C. fascicularis* L. extracts and standard drugs were prepared in double distilled water using nutrient agar tubes. Muller Hinton sterile agar plates were seeded with bacterial strains (1×10^8 bacteria/ ml and 1×10^7 fungi/ ml) and allowed to stay at 37 ^{0}C for 3 hrs. Control experiments were carried out under similar condition by using Chloroamphenicol for antibacterial activity and Nyastatin for antifungal activity as standard drugs. All the plates were incubated at 37°C for 18 to 24 hrs for bacteria and at 28°C for 48 to 96 hrs for fungi.

The zones of growth inhibition around the disks were measured after 18 to 24 hrs of incubation at 37°C for bacteria and 48 to 96 h for fungi at 28°C, respectively. The sensitivity of the microorganism species to the plant extracts was determined by measuring the sizes of inhibitory zones (including the diameter of disc) on the agar surface around the discs.

4.10.3.4 Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentrations (MBC) and Minimum fungicidal concentration (MFC) of isolated Compounds[52]:

Broth Dilution Method or Tube Dilution Method:

The Broth Dilution method or tube dilution method is a simple procedure for testing a small number of isolated compounds. It has added advantage is that the same tubes can be taken for MBC tests.

Material:

Sterile graduated pipettes of 10ml, 5ml, 2ml and 1ml; Sterile capped 7.5 x 1.3 cm tubes / small screw-capped bottles, Pasteur pipettes, overnight broth culture of test and control organisms (same as for disc diffusion tests), required antibiotic in powder form (either from the manufacturer or standard laboratory accompanied by a statement of its activity in mg/unit or per ml. Clinical preparations are not used for reference technique.) required solvent for the antibiotic, sterile Distilled Water - 500ml and suitable nutrient broth medium.

Method:

Broth micro-dilution method or tube dilution method was followed for determination of Minimum inhibitory concentration (MIC) values for each isolated compounds showing antimicrobial activity against tested microorganisms. To measure the MIC values, various concentrations of the stock 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.13, 0.06µg/ml were assayed against the tested microorganisms. Isolated compounds from extracts were re-suspended in DMSO (which has no activity against tested microorganisms) to make 10 mg/ml final concentration and then two fold serially diluted; 5 ml of each isolated compound was added to test tubes containing 5 ml of sterile DSNB media (for bacteria) and SDA (for fungi). The tubes were then inoculated with standard size of microbial suspension (for bacteria 1×10^8 CFU/ml and 1×10^7 cell/ml for fungi) and the tubes were incubated at 37°C for 24 h for bacteria and 28°C for 48 h for fungi in an incubator and observed for change in turbidity after 24 h compared with the growth and in controls. A tube

containing nutrient broth and inoculums but no isolated compound was taken as control. The least isolated compound concentration which inhibited the growth of the tested microorganisms was taken as MIC. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Each isolated compound was assayed in triplicate and each time two sets of tubes were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the test tubes. The MIC values were taken as the lowest concentration of the each isolated compounds in the test tubes that showed no turbidity after incubation. The turbidity of the test tube was interpreted as visible growth of microorganisms.

Reading of results:

MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculums itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition. Standard strain of known MIC value run with the test is used as the control to check the reagents and conditions.

The growth in the tube was observed visually for turbidity and inhibition was determined by the absence of growth. MIC was determined by the lowest concentration of sample that prevented the development of turbidity. The procedure was performed for three bacterial species for six isolated compounds. The procedure was repeated to confirm the MIC.

Determination of Minimum bactericidal concentration (MBC):

Method:

Dilutions and inoculations were prepared in the same as for the determination of MIC values. The control tube which were containing no antibiotic is immediately sub cultured (Before incubation) by spreading a loopful uniformly over a quarter of the plate (disc) on a medium suitable for the growth of the test organism and incubated at 37^{o}C overnight in incubator. The tubes were also incubated overnight at

37^{o}C. These include tube-containing compound without inoculum and the tube containing the growth medium and inoculum.

The MBC of compounds were determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 h. The highest dilution that yielded refuse single bacterial colony was taken as the Minimum bactericidal Concentration (MBC). MBC was calculated for some of the pure isolated compounds showed high antimicrobial activity against highly sensitive organisms.

Reading of results:

These subcultures showed,

- Similar number of colonies indicated bacteriostatics.
- A reduced number of bacterial colonies indicated a partial bactericidal activity
- No growth was observed, if the whole inoculums have been killed.
- The highest dilutions showed minimum 99% inhibition was taken as MBC.

Determination of Minimum fungicidal concentration (MFC):

Method:

Dilutions and inoculations were prepared in the same as for the determination of MIC values. The control tube which were containing no antibiotic is immediately sub cultured (Before incubation) by spreading a loopful uniformly over a quarter of the plate (disc) on a medium suitable for the growth of the test organism and incubated at 28^{o}C overnight in incubator. The tubes were also incubated overnight at 28^{o}C. These include tube-containing compound without inoculum and the tube containing the growth medium and inoculum.

The MFC of compounds were determined by sub culturing the test dilution on MGYP and then further incubated for 24 h. The highest dilution that yielded refuse single fungal colony was taken as the Minimum fungicidal Concentration (MFC). MFC was calculated for some of the pure isolated compounds showed high antimicrobial activity against highly sensitive organisms.

Reading of results:

These subcultures showed,

- A reduced number of bacterial colonies indicated a partial fungicidal activity.
- No growth was observed, if the whole inoculums have been killed.
- The highest dilutions showed minimum 99% inhibition was taken as MFC.

4.11 REFERENCES

1. Khandelwal, K.R. Practical Pharmacognosy Techniques and Experiments.19th ed. Pune, Nirali Prakashan. 2008, 157-159.
2. WHO. Quality Control Methods, for Medicinal Plant Material, Geneva. 1998, 28-33.
3. Ewans, W.C. Treas and Evans Pharmacognocy.15th ed. New-York, Saunders. 2004, 98-99.
4. Government of India Ministry of Health and Family Welfare. Indian Pharmacopeia, Vol-II. New Delhi, Controller of Publication. 1996, A-52-A-54.
5. Wallis, T.E. Practical Pharmacognocy. J and Churchill Ltd. London 1953, 132-133.
6. Harborne, J.B. Phytochemical Methods- A Guide to Modern Techniques of Plant Analysis. 3rd edition London, Chapman and Hall. 1996, 4-7.
7. Prashant, Tiwari; Bimlesh, Kumar; Mandeep, Kaur; Gurpreet, Kaur; Harleen, Kaur. Internationale Pharmaceutica Sciencia. 2011, vol.1, No. 1, 98-106.
8. Handa, S. S.; Suman, Preet, Singh Khanuja; Gennaro, Longo; Dev, Dutt, Rakesh. Extraction Technologies for Medicinal and Aromatic Plants. International Centre for Science and High Technology, Trieste. 2008, 68-73.
9. Suffness M., Douros J. Cancer Chemother Pharmacology. 1978, 1: 91–100.
10. Pulok K. Mukerjee, "Quality Control of Herbal Drugs". 2002, 540- 42, 580.
11. Harborne, J. B. Phytochemical Methods: A guide to modern techniques of plant analysis, 3rd edition, Springer, London. 1998, 129-138.
12. Anonymous. The wealth of India: Raw Materials. Publication and Information Directorate, CSIR, New Delhi, India. 1976.
13. Stahl, E. (1969). Thin Layer Chromatography: A Laboratory handbook, 2nd edition, Springer Ltd. 1969.
14. Gupta, R. N., Zewig G., Sherma J. Handbook of Chromatography, Vol. I, CRC Press, Florida. 1981, 12-20.
15. Vogel, A.I. A text book of practical organic chemistry including Quantitative organic analysis, 6th edition, Longman group Ltd. 2006, 454-455.

16. Adamonics, J. A. Chromatographic Analysis of Pharmaceuticals, Marcel Dekker, New York. 1997, 57-72.
17. Still, W. C.; Kahn, M.; Mitra, A. Journal of Organic Chemistry. 1978, 43: 2923-2925.
18. Scott, R.P.W. Liquid Chromatography Column Theory, John Willey and Sons, New York. 1992, 1- 38.
19. Peach, K.; Tracey, M.V. Modern methods of plant analysis, Narosa Publishing house, New Delhi, India. 1979, 471,627,658.
20. Government of India Ministry of Health and Family Welfare. Indian Pharmacopeia, Vol-II. New Delhi: Controller of Publication; 1996. Page-A-52.
21. Laboratory Handbook of Chromatographic Methods. Van Nostrand Reinhold Company London. Chief Editor–O. Mikes (Zechoslovak Academy of Sciences, Prague.) Page No. 157 – 168, 189 – 228, For PTLC Page No. 239 - 244.
22. http://en.wikipedia.org/wiki/Column_chromatography
23. Monika,W. H.; Teresa, J.; Kowalska, S.; Chromatographic Science Series. Thin Layer Chromatography in Phytochemistry. CRS Press, Taylor and Francis group. Page number part No.1-166.
24. Wagner, H. Plant Drug Analysis: A Thin Layer Chromatography, 2nd edition, Springer –Verlag Berlin, New York. 1996, 212.
25. Othman, A.; Ismail A.; Ghani, N. A.; Adenan, I. Food Chemistry. 2007, 100, 1523-1530.
26. Thaipong, K.; Boonprakob, U.; Zevallos, L.C.; Brayne, D. H. Asian Journal of Tropical Medicine and Public Health, 2005, 36(4), 254-257.
27. Kaur, C.; Kapoor, H.C. International Journal of Food Science and Technology. 2001, 36,703-725.
28. Blomhoff, R. Current Opinion in Lipodology. 2005, 16, 17-54.
29. Kaur, C.; Kapoor, H. C. Antioxidant activity and total phenolic content of some Asian vegetables, 2002, 37, 153-161.
30. Singh, L.; Kaur, N.; Kumar, P. Biochemistry Cell Archiology. 2009, 9, 135-144.
31. Saumya, S. M.; Mahaboob, B.P. International Journal of Pharmcy and Pharmaceutical Sciences. 2011, 3(1), 165-169.

32. Pracheta; Sharma, V.; Paliwal, R.; Sharma, S. International Journal PharmTech Research. 2011, 3 (1), 124-132.
33. Jun, L.; Wang, C.; Wang, Z.; Zhang, C.; Lu, S.; Liu, J. Food Chemistry. 2011, 126,261–269.
34. Mohammad, A., M.; Koji, Y.; Toshiki, M.; Yumi, N.; Katsumi, S.; Hiroaki, S.; Yoshifumi, T. International Journal of Biological Sciences. 2007, 3, 349-355.
35. Ebrahimzadeh, M. A.; Nabavi, S. M.; Nabavi, S. F.; Bahramian, F.; Bekhradnia, A. R. Pakistan Journal of Pharmacological Sciences. 2010, 23, 29-34.
36. Kalaskar, M. G.; Surana, S. J. Journal of Natural Medicines. 2011, 65,633-640.
37. Saleh, E. S.; Hameed, A. Food Chemistry. 2009, 114, 1271-1277.
38. OECD. Guidelines for Testing of Chemicals, Revised draft Guidelines 425: Acute oral toxicity-up and down method, revised document. 2006.
39. Winter, C.A.; Risley, E. A.; Nuss, W.G. Proceeding of the Society for Experimental Biology and Medicines. 1962, 11: 544-547.
40. Moraes, B.M.; Amaral, C.D.; Morimoto, M.S.S.; Vieira, C.L.G.; Perazzo, F.F.; Carvalho, J.C.T. Inflammopharmacology. 2007, 15(4): 175-178.
41. Saneja, A.; Kaushik, D.; Khokra, S. L.; Kaushik, P.; Sharma, C.; Aneja, K.R. Journal of Natural Product. 2009, 2:49-54.
42. Amresha, G.; Reddy, G.D.; Rao, C.H.V.; Singh, P.N. Journal of Ethnopharmacology. 2007, 110: 526–531.
43. Gupta, M.; Mazumder, U.K.; Kumar, S.R.; Gomathi, U.; Rajeshwar, Y.; Kakoti, B.B.; Tamil, S.V. Journal of Ethnopharmacology. 2005, 98: 267–273.
44. Patil, C.R.; Gadekar, A.R.; Patel, P.N.; Rabhade, A.; Surana, S. J.; Gaushal, M. H. Homeopathy. 2009, 98 (2): 88-91.
45. Fransworth, N.R. Journal of Ethnopharmacology. 1993, 38,145-152.
46. Houghton, P. J. Journal of Altern and Complement Medicine. 1995, 1,131-143.
47. Van, T. T. H.; Moutafis, G.; Tran, L.T.; Coloe, P.J. Journal of Veternary Medicinal Science. 2007, 70, 873-879.
48. Gerner-Smidt, P.; Whichard, J.M. Foodborne diseases trends and report: Foodborne Pathogens Diseases. 2008, 5: 551-554.
49. Runyoro, D.; Matee, M.; Olipa, N.; Joseph, C.; Mbwambo, H. BMC Complement Alternative Medicines. 2006, 6 (11).

50. Shahidi, B. H. Asian Journal of Plant Sciences. 2004, 3, 82-86.
51. Rajput, A.P.; Rajput, T.A. International Journal of PharmTech Research. 2011, 3: 2195-2198.
52. National Committee for Clinical Laboratory Standards. *Performance Standards for antimicrobial susceptibility testing*. 8th Informational Supplement. M100 S12. National Committee for Clinical Laboratory Standards, 2003. Villanova, PA, USA.
53. Ira, R. Bacteriology, Standard Operative procedure manual for microbiology laboratories, National Institute of Biologicals. 1995, 73-97.
54. John, D.T.; James, H.J. Antimicrobial Susceptibility testing: General Considerations. Manual of Clinical Microbiology 7th edition, Murray P.R, Baron E.J, Pfaller M.A, Tenover F.C, Yolken R, American Society for Microbiology, Washington DC, 1999, P. 1469-1473.