CHAPTER 5: MATERIALS AND METHOD

5.1 Materials

5.1.1 Collection and authentication of plant material

The fresh leaves of *Wrightia tinctoria* were collected from VInYY garden, Nachalloor, Karur District and the same were authenticated by Prof. P. Jayaraman of Plant Anatomy Research Centre (PARC), Chennai, and a voucher specimen was deposited at PARC.

5.1.2 Instruments

- Photographs of different magnifications were taken with Nikon labphot-2 microscopic unit.
- Water associates HPLC assembly was used using RP-18 reverse phase column as the stationary phase and PDA as detector.

5.1.3 Chemicals

All reagents and solvents used in this project work are of AR Grade obtained from M/s S.D.fine chemicals, Mumbai, India.

5.1.4 Test pathogenic microorganisms

For antimicrobial activity 5 gram positive, 5 gram negative, 3 fungal and 4 dermatophytic strains were used.


The pathogenic microorganisms were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacterial strains were grown and maintained on Muller-Hinton Agar medium (HIMEDIA), while fungi and dermatophytes were maintained on Sabouraud Dextrose Agar medium (HIMEDIA).

### 5.1.5 Animals

For primary skin irritancy test New Zealand White rabbits of either sex, between 2.0–2.5 kg were selected. For wound healing activity guinea pig weighing between 700 to 1200 g were selected. The animals were acclimatized to standard laboratory conditions (temperature 25± 2°C) and provided with food and drinking water ad libitum. The animal care and experimental
protocols were in accordance with Institutional Animal Ethical Committee of IIMT College of Medical Science whose animal house is approved by CPCSEA (1297/ac/09/CPCSEA, dated 27/10/09).

### 5.1.6 Clinical trial

The clinical study was conducted at Fathima Health Care Centre, Trichy, with INSTITUTIONAL REVIEW BOARD and duly constituted Ethics Committee by the ClinRx Laboratories, India, as per good clinical Practice (GCP) guideline.

### 5.2 Methods

#### 5.2.1 Standardization of the plant material

5.2.1.1 Pharmacognostical standardization of *Wrightia tinctoria* leaves

5.2.1.1.1 Determination of foreign matter (WHO, 1998)

*Wrightia tinctoria* leaves of 250 g were weighed and spread in a thin layer. The foreign matter was sorted into groups by visual inspection and by using a magnifying lens 6 X. Dust is regarded as mineral admixture. The content of each group (foreign plants/ foreign animals/ foreign mineral) was calculated in grams per 100 g of air-dried sample.

5.2.1.1.2 Macroscopical evaluation (Esau K, 1964)

Macroscopical evaluation refers to evaluation of drugs by colour, odour, taste, shape and special features like touch and textures. It is a technique of
qualitative evaluation based on the study of morphological and sensory profiles of the leaf.

a. Size

A graduated ruler in millimeters was used for the measurement of the length, width and thickness of the leaf.

b. Colour

The untreated *Wrightia tinctoria* leaf was examined under diffuse daylight. An artificial light source with wavelengths similar to those of daylight was also used. The colour of the leaf was compared with that of a reference sample.

c. Surface characteristics, texture and fracture characteristics

The untreated *Wrightia tinctoria* leaf was examined by using a magnifying lens 6X. The characteristics of a cut surface were observed by wetting with water. The leaf was felt by touch, bent and ruptured to obtain information regarding brittleness and the appearance of the fracture plane (whether it is fibrous, smooth, rough, granular, etc.).

5.2.1.3 Microscopical evaluation (Kokate *et al.*, 1995)

a. Fixing procedure

The *Wrightia tinctoria* leaves were cut and removed from the plant and fixed in FAA (formalin – 5mL + acetic acid – 5mL +70% ethyl alcohol-90mL). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary-
butyl alcohol (TBA) as per the schedule (Sass JE 1940) Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

b. GROUSectioning procedure

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 μm. Dewaxing of the sections was done by adopting customary procedure (Johansen DA.1940). The sections were stained with Toluidine blue as per the method published by (O Brien TP et al.,1964). Since Toluidine blue is a polychromatic stain, the staining results were remarkably good and cytochemical reactions were also obtained. The dye imparted pink colour to the cellulose walls, blue colour to the lignified cells, dark green colour to suberin, violet colour to the mucilage and blue colour to the protein bodies. Wherever necessary, sections were stained with Safranin and Fast-green and Iodine solution (for starch).

For studying the stomatal morphology, venation pattern and of trichomes distribution clearing of the leaf with 5% sodium hydroxide and epidermal peeling after partial maceration in Jeffrey’s maceration fluid (Sass JE 1940) was done. The peelings were mounted in glycerine. Powdered materials of different parts were cleared with NaOH and mounted in glycerin medium
after staining. Different cell component were studied and their dimensions were measured.

a. Procedure for Photomicrographs

Photomicrographs of different magnifications were taken with Nikon Labphot 2 Microscopic Unit. For normal observations bright field was used. For the study of crystals, starch grains, and lignified cells polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale bars. Descriptive terms of the anatomical features used were as given in the standard Plant Anatomy books. (Esau K 1964)

5.2.1.1.4 Histochemical colour reactions (Wallis TE, 1984).

The different histochemical colour reactions were performed on the leaf transverse sections to differentiate the different cell compositions and identification.

5.2.1.2 Physicochemical standardization - Proximate analysis (WHO, 1998)

5.2.1.2.1 Determination of ash values

a. Total ash

Two g of the ground air-dried leaf powder was accurately weighed in a previously ignited and tarred silica crucible. The material was spread in an
even layer and ignited by gradually increasing the heat to 500-600° C in a muffle furnace until the ash is white, indicating the absence of carbon. The crucible was cooled in desiccator and weighed. The procedure was repeated to get the constant weight. The percentage of total ash was calculated with reference to the air dried leaf powder.

a. Acid-insoluble ash

To the crucible containing the total ash, 25 mL of hydrochloric acid TS (70 g/l) was added. The crucible was covered with a watch-glass and boiled gently for 5 min. The watch-glass was rinsed with 5 mL of hot water and the washing was added to the crucible. The content of the crucible was filtered using ash less filter paper. The insoluble matter on an ash less filter paper was washed with hot water until the filtrate was neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, heated to dryness on a hot-plate and ignited to constant weight. The residue was allowed to cool in a suitable desiccator for 30 min and then weighed without delay. The procedure was repeated to get the constant weight. The content of acid-insoluble ash was calculated in mg per g of air-dried leaf powder.
b. Sulphated ash

Two g of the ground air-dried *Wrightia tinctoria* leaf was accurately weighed, in a previously ignited and tarred silica crucible. The material was moistened with 1 mL of H$_2$SO$_4$ and ignited gently and again moistened with H$_2$SO$_4$ and re-ignited by gradually increasing the heat to 800 ± 25° C until all black particles had disappeared. The crucible was cooled in desiccator and weighed. The procedure was repeated to get the constant weight. The percentage of sulphated ash was calculated with reference to the air dried leaf powder and recorded.

c. Water soluble ash

The ash obtained from total ash was boiled with 25 mL of distilled water for 5 min. The insoluble matter was collected in an ashless filterpaper and washed with hot water. The insoluble ash was transferred into silica crucible and ignited for 15 min at temperature of 450 ± 25° C and weighed. The procedure was repeated to get content weight. The weight of the insoluble matter was subtracted from the weight of the total ash taken. The difference in the weight represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air dried leaf powder and recorded.
5.2.1.2.2 Determination of calcium, potassium, sodium, phosphorous & iron

a. Determination of calcium, potassium and sodium content  (WOLF, 1982)

5 g of the powder was ashed in a furnace at 500 – 550° C. The ash was further heated with 25 mL of dilute HCl for 5 min. The solution was filtered through ashless filter paper; the container and filter paper were washed several times with distilled water. The acid filtrate and washings were made up to 100 mL in volumetric flask. Different concentrations of standards for sodium (Na\(^+\)), calcium (Ca\(^{2+}\)) and potassium (K\(^+\)) were prepared. The standard curves of these ions were drawn using a flame photometer (Systronics, India) at wave length of 590 nm for sodium, 622 nm for calcium and 770 nm for potassium. The samples were run for the determination of Na\(^+\), Ca\(^{2+}\) and K\(^+\) and the values of the ions in the unknown samples were worked out using the appropriate standard curves.

b. Determination of phosphorous content (AOAC, 1990)

Phosphorus content was determined by employing the method reported by Vanado Molybdate and read on Systronics colourimeter (AOAC 1990). An acidified solution of ammonium molybdate containing ascorbic acid and antimony is added to digested leaf powder. The phosphate in the sample reacts with the acidified ammonium molybdate to form an ammonium
molydiphosphate complex. A blue coloured solution is generated from the reduction of the ammonium molydiphosphate complex by ascorbic acid. The intensity of the blue colour is proportional to the amount of molybdophosphorus present. Antimony potassium tartrate accelerates the colour development and stabilizes the colour for several h. The amount of light absorbed by the solution at 660 nm is measured with a colourimeter. The instrument reading may be read as percent P in the sample. Phosphorus in plant material may commonly range from 0.03 % to 1.50 %. Following a 1:10,000 dilution, the blue coloured complex which is formed using this method obeys Beer's law up to a P concentration of 1.5 mg L⁻¹. To calibrate the colourimeter for routine analysis, the working solution is used as the blank and the developed 0.80 mg P L⁻¹ standard to establish the slope of the line. To check for linearity, the developed 0.20, 0.40, and 0.60 mg P L⁻¹ standards are read.

c. Determination of iron content (Vogel IGA, 1961)

The powder of 5 g was ashed in a furnace at 500 – 550° C. The ash was further heated with 25 mL of dilute HCl for 5 min. The solution was filtered through ashless filter paper; the container and filter paper were washed several times with distilled water. The acid filtrate and washings were made up to 100 mL in volumetric flask. 50 mL of the solution was kept in a
100 mL separating funnel, 10 mL of a 1 percent oxine solution in chloroform was added and shaken for 1 minute. The chloroform layer was separated and subjected for spectrophotometric estimation at 470 nm, using the solvent as a blank. The extraction is repeated with a further 10 mL of 1 percent oxine solution in chloroform and the absorbance is measured to confirm that all the iron was extracted.

5.2.1.2.3 Determination of nitrogen content (Vogel, 1989)

The leaf sample of 0.5 g was taken in a Kjeldahl tube and 2.5 g of potassium sulfate, 0.25 g copper sulfate and finally sulfuric acid were added. The tubes were heated in Kjeldahl instrument (Kjeldatherm, Gerhardt, Germany) for 1-2 h at 350° C till transparent solution. After cooling, the digested tubes were transferred to the evaporation system (Vapdest 30, Gerhardt). The system was programmed to add 30 % sodium hydroxide solution to the digested solution for 10 seconds, reaction time 2 min and steam evaporation for 3 min. The ammonia evaporated was transferred to a conical flask containing 25 mL boric acid and few drops of methyl red indicator (0.1%) was added. The ammonium borate was titrated against 0.25 M hydrochloric acid to the end point (from yellow to red colour)

\[
\%N = \frac{(T - B) \times N \times 1.401}{g \text{ sample}} \\
\text{where: } T = \text{mL of sample titrated} \\
B = \text{mL of blank titrated} \\
N = \text{acid normality}
\]
5.2.1.2.4 Determination of extractable matter (Kokate et al., 1995)

a. Determination of water soluble extractive

Powdered leaves of 5 g were added to 50 mL of water at 80° C in a stoppered flask. It was shaken well and allowed to stand for 10 min. It was cooled to 15° C and 2 g of Kieselguhr was added and filtered. 5 mL of the filtrate was transferred to a tarred evaporating basin. The solvent was evaporated on a water bath for 30 min and then dried in a steam oven. Water soluble extractive was calculated with reference to the air-dried powdered leaf.

b. Determination of alcohol soluble extractive

The air-dried coarsely powdered leaves of 5 g were macerated with 100 mL of alcohol of the specified strength in a closed flask for 24 h, shaken frequently for 6 h and allowed to stand for 18 h. It was filtered rapidly taking precautions against loss of alcohol and 25 mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105° C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air-dried powered leaf.

c. Determination of chloroform soluble extractive

The air-dried coarsely powdered leaves of 5 g were macerated with 100 mL of chloroform in a closed flask for 24 h, shaken frequently for 6 h and
allowed to stand for 18 h. It was filtered rapidly taking precautions against loss of chloroform and 25 mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105° C and weighed. The percentage of chloroform soluble extractive was calculated with reference to the air-dried powered leaf.

5.2.1.2.5 Determination of fluorescence characteristics (Kokate et al., 1995)

Fluorescence characteristics of the leaf powder as such and after treating them with chemical reagents were observed in day light as well as under UV radiation.

5.2.2 Standardization of the Wrightia tinctoria leaf extract

5.2.2.1 Collection and extraction with coconut oil

Leaves of Wrightia tinctoria were collected and cleaned. The cleaned leaves were minced to small pieces. The minced leaves were brought into contact with coconut oil in the ratio 1:5 and left for a period of 5 days in sunlight with shade. The completion of extraction was indicated through the colour of the oil turning to purplish blue. The oil was filtered and the filtrate was used for formulation.

5.2.2.2 Standardization of Wrightia tinctoria extract

5.2.2.2.1 Organoleptic evaluation (Kokate et al., 1995)

Organoleptic evaluation was done by observing the colour, odor and touch.
5.2.2.2 Preliminary physiochemical screening (Lab manual 2 DGHS, 2005)

The preliminary phytochemical screening was done through evaluation of parameters namely,

a. Volatile impurities (Kokate et al., 1995)

The extract was placed in the steam distillation flask, a few pieces of porous porcelain were added and the condenser was joined to the apparatus. Water was introduced by tube until it reaches a level. Stopper was removed and the appropriate volume of xylene R was introduced, using a graduated pipette and placing its tip at the bottom of tube. Stopper was replaced and the liquid in the flask was heated until it begins to boil and the distillation rate was adjusted to 2–3 mL per min. The tap was opened and the distillation was continued. The heating was stopped after 30 min; the heater was turned off, after 10 min the volume of solvent (xylene) collected in the graduated tube was recorded. The volatile matter content in mL per 100 g of extract was calculated.

b. Insoluble impurities (Lecrenier MC et al., 2012)

The filter to be used was dried in an oven and cooled to room temperature in desiccator. The extract, coconut oil and filter were weighed. The samples were dissolved in chloroform and then vacuum filtered. The flask and the
filter were rinsed with chloroform. The filter was vacuum dried and the filter with impurities was weighed.

\[
\text{Insoluble impurities (\%) = } \frac{\text{Gain in mass of filter}}{\text{Aliquot mass}} \times 100
\]

c. Relative density

It is the ratio of the density (mass of a unit volume) of a substance to the density of a given reference material when weighed in air at 25° C.

Method

The dry pycnometer was thoroughly cleaned and calibrated by filling it with recently boiled and cooled water at 25° C and the contents were weighed. The temperature of the extract to be examined was adjusted to 25° C and filled in the pycnometer. The temperature of the filled pycnometer was adjusted to 25° C and any excess of the extract was removed and weighed. The tare weight of the pycnometer was subtracted from the filled weight of the pycnometer.

d. Refractive index

The refractive index (\(\eta\)) of a substance with reference to air is the ratio of the sine of the angle of incidence to the sine of the angle of refraction of a beam of light passing from air into the substance.
Method

The refractive index varies with the wavelength of the light used in its measurement. Unless otherwise prescribed, the refractive index is measured at 25°C (±0.5) with reference to the wavelength of the D line of sodium (λ 589.3 nm). The temperature should be carefully adjusted and maintained since the refractive index varies significantly with temperature. The Abbe’s refractometer is convenient for most measurements of refractive index but other refractometer of equal or greater accuracy may be used. The cleanliness of the instrument should be checked frequently by determining the refractive index of distilled water, which at 25°C is 1.3325. The extract was placed on the sample plane and its refractive index was measured.

e. Acid value

The acid value is the number of mg of potassium hydroxide required to neutralize the free acids in 1 g of the substance

Method:

Accurately about 10 g of the extract was weighed into a 250 mL flask and 50 mL of a mixture of equal volumes of alcohol and solvent ether was added, which has been neutralized after the addition of 1 mL of solution of phenolphthalein. It was titrated with 0.1 N potassium hydroxide by shaking constantly until a pink colour which persists for fifteen seconds was
obtained. The number of mL required was noted. The acid value was calculated from the following formula:

\[ \text{Acid value} = \frac{56.1VN}{W} \]

where \( V \) = Volume in ml of standard potassium hydroxide
def \( N \) = Normality of the potassium hydroxide solution; and

\( W \) = Weight in g of the sample

f. Iodine number

The Iodine value of a substance is the weight of iodine absorbed by 100 part by weight of the substance.

Iodine Monochloride Method

The extract was accurately weighed and placed, in dry iodine flask, 10 mL of carbon tetrachloride was added and dissolved. 20 mL of iodine monochloride solution was added. The stopper was inserted, previously moistened with solution of potassium iodine and allowed to stand in a dark place at a temperature of about 170 ° C for thirty min. 15 mL of solution of potassium iodide and 100 mL water was added; it was shaken, and titrated with 0.1 N sodium thiosulphate, using solution of starch as indicator. The number of mL required was noted (a). At the same time the operation was carried out in exactly the same manner, but without the substance being
tested, and the number of mL of 0.1 N sodium thiosulphate required (b) was noted.

The iodine value was calculated from the formula:

\[ \text{Iodine value} = \frac{(b-a) \times 0.01269 \times 100}{W} \]

where \( W \) is the weight in g of the substance taken.

The approximate weight, in g, of the substance to be taken may be calculated by dividing 20 by the highest expected iodine value. If more than half the available halogen is absorbed, the test must be repeated, a smaller quantity of the substance being used. Iodine Monochloride Solution: The solution may be prepared by the following method: (1) 13 g of iodine was dissolved in a mixture of 300 mL of carbon tetrachloride and 700 mL of glacial acetic acid. To 20 mL of this solution, 15 mL of solution of potassium iodide and 100 mL of water were added. The solution was titrated with 0.1 N sodium thiosulphate.

\( g \). Saponification value

It is the number of mg of potassium hydroxide required to neutralize the fatty acids, resulting from the complete hydrolysis of 1 g of the oil or fat.

Method

Potassium hydroxide of 40 g was dissolved in 20 mL water, and sufficient alcohol was added to make 1,000 mL. It was allowed to stand overnight, and the clear liquor was poured off. Accurately 2 g of the extract was weighed in
a tared 250 mL flask, 25 mL of the alcoholic solution of potassium hydroxide was added, a reflux condenser was attached and boiled on a water-bath for one hour, frequently rotating the contents of the flask and then cooled. 1 mL of solution of phenolphthalein was added and the excess of alkali was titrated with 0.5 N hydrochloric acid. The number of mL required was noted (a). The experiment was repeated with the same quantities of the same reagents in the manner omitting the sample. The number of mL required was noted (b). The saponification value was calculated from the following formula

\[
\text{Saponification value (mg/g)} = (b-a) \times 0.02805 \times 1000 / W
\]

\(W\) is the weight in g of the sample taken

h. Unsaponifiable matter

The term “unsaponifiable matter” is applied to the substances non-volatile at 100-105 °C obtained by extraction with an organic solvent from the substance to be examined after it has been saponified. The result is calculated as percent mL/mL.

Method

The extract was introduced in the prescribed quantity to be examined (mg) into a 250 mL flask fitted with a reflux condenser. 50 mL of 2 M alcoholic potassiumhydroxide R was added and heated on a water-bath for 1 h,
swirling frequently. It was cooled to a temperature below 25 ° C and the contents of the flask transferred to a separating funnel with the aid of 100 mL of water. The liquid was shaken carefully with 3 quantities, each of 100 mL, of peroxide-free ether R. The ether layers were combined in another separating funnel containing 40 mL of water R. It was shaken gently for a few min, allowed to separate and the aqueous phase was rejected. The ether phase was washed with 2 quantities, each of 40 mL, of water R then washed successively with 40 mL of a 30 g/L solution of potassium hydroxide R and 40 mL of water R; this procedure was repeated 3 times. The ether phase was washed several times, each with 40 mL of water R, until the aqueous phase is no longer alkaline to phenolphthalein. The ether phase was transferred to a tared flask, washing the separating funnel with peroxide-free ether R. The ether was distilled off with suitable precautions and 6 mL of acetone R was added to the residue. Carefully the solvent was removed in a current of air. It was dried to constant mass at 100-105 ° C and was allowed to cool in a desiccator and weighed (g). The residue was dissolved in 20 mL of alcohol R, previously neutralised to phenolphthalein solution R and titrate with 0.1 M ethanolic sodium hydroxide. If the volume of 0.1 M ethanolic sodium hydroxide used is greater than 0.2 mL, the separation of the layers has been incomplete; the
residue weighed cannot be considered as “unsaponifiable matter”.

\[
\text{unsaponifiable matter} = \frac{100a \times \text{percent}}{m}
\]

i. Loss on drying at 110 °C

The extract accurately weighed was placed, in a previously dried and tared flat weighing bottle. The sample was dried in an oven at 110 °C; It was dried until two consecutive weighings do not differ by more than 5 mg, the loss of weight in mg per g of air-dried material was calculated.

j. Rancidity - Kreis Test

The test depends upon the formation of a red colour when oxidized fat is treated with concentrated hydrochloric acid and a solution of phloroglucinol in ether. The compound in rancid fats responsible for the colour reaction is epihydrin aldehyde. All oxidized fats respond to the Kreis test and the intensity of the colour produced is roughly proportional to the degree of oxidative rancidity.

Method:

The *Wrightia tinctoria* extract was mixed with 1 mL of conc. hydrochloric acid in a test tube. 1 mL of a 1 per cent solution of phloroglucinol was added in diethyl ether and mixed thoroughly with the fat-acid mixture. A pink colour formation indicates that the fat is slightly oxidized while a red colour indicates that the fat is definitely oxidized.
k. Test for presence of cotton seed oil

The extract was taken in a test tube and equal volume of the sulphur solution was added. It was mixed thoroughly by shaking and gently heated on a water bath for few min with occasional shaking until the carbon disulphide has boiled off and the sample stops foaming. The tube was placed in an oil bath maintained at 110 -115 ° C for 2.5 h. A red colour at the end of this period indicates the presence of cottonseed oil. (I.S.I. Handbook of Food Analysis, 1984)

l. Test for presence of sesame oil - Baudouin test

The extract was taken in a 25 mL measuring cylinder provided with a glass stopper, and 5 mL of conc. hydrochloric acid and 0.4 mL of furfural solution was added. The glass stopper was inserted and shaken vigorously for two min. The mixture was left to stand and allowed to separate. A red colour development in the lower acid layer indicates the presence of sesame oil. It is confirmed by adding 5 mL of water and shaking again. If the colour in acid layer persists, sesame oil is present; if the colour disappears it is absent. (As furfural gives violet tint with HCl, it is necessary to use the dilute solution specified. (I.S.I. Handbook of Food Analysis 1984)
5.2.2.2.3 Qualitative phytochemical analysis (Kokate, 1994 & Kokate et al., 1995).

It was carried out by conducting chemical testing for the presence of aquimins, alkaloids, anthroquins, cardiac glycosides, coumarins, flavonoids, leucoanthocyanins, phlobatannins, simple phenolics, steroids, saponins, tannins and terpenoids. The different qualitative chemical tests were performed for establishing profile of given extract for its chemical composition.

a. Detection of Acubins / Iridoids (monoterpene test) Trim –Hill test

The extract was treated with 5 mL of 1% aqueous HCl for 3-6 h. 0.1 mL was decanted into another test tube containing Trim Hill reagent (10 mL of acetic acid, 1mL of 0.2 % copper sulphate, and 0.5 mL concentrated hydrochloric acid) and it was heated over a flame, production of blue colour indicated the presence of iridoids (Trim AR and Hill R, 1952)

b. Detection of alkaloids

Solvent free extract, 50 mg was stirred with few mL of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows.

(i) Dragendorff’s test

To about 2 mL of filtrate, 1 mL of Dragendorff’s reagent was added. A prominent yellow precipitate indicated the test as positive.
Dragendorff’s reagent

Bismuth carbonate (5.2 g) and sodium iodide (4 g) were boiled for a few min with 50 mL glacial acetic acid. After 12 h, the precipitated sodium acetate crystals were filtered off using a sintered glass funnel. Clear, reddish brown filtrate, 40 mL was mixed with 160 mL ethyl acetate and 1 mL water and stored in amber – coloured bottle. From Stock solution, 910 mL was mixed with 20 mL of acetic acid and made up to 100 mL with water.

(ii) Mayer’s test

To a few mL of filtrate, a drop or two of Mayer’s reagent were added by the side of the test tube. A white or creamy precipitate indicated the test as positive.

Mayer’s reagent

Mercuric chloride (1.352 g) was dissolved in 60 mL of water and potassium iodide (5.0 g) was dissolved in 10 mL of water. The two solutions were mixed.

(iii) Wagner’s reagent

To a few mL of filtrate, few drops of Wagner’s reagent were added by the side of the test tube. A reddish – brown precipitate confirmed the test as positive.
Wagner’s reagent

Iodine (1.27 g) and potassium iodide (2 g) was dissolved in 5 mL of water and made up to 100 mL with distilled water.

c. Detection of anthraquinone

The extract was adjusted to pH 3 with 1N HCl, and then the solution was washed by diethyl ether. The (upper layer) ether solution was separated from the aqueous one. Ether solution was extracted with 5% sodium bicarbonate solution in separating funnel. The alkaline aqueous solution was separated, and then acidified, shaked with ether in separating funnel and the two layers were separated. If ether layer gives a rose pink colour with 10% ammonia the carboxylic-free anthraquinones are present.

d. Detection of cardiac glycoside (Keller-Killani test)

Five mL of extract was treated with 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 mL of concentrated sulphuric acid. A brown ring of interface indicated the deoxysugar characteristic of cardenolides. A violet ring could appear below the brown ring, while in the acetic acid layer, a greenish ring formed just gradually throughout the thin layer.
e. Detection of coumarins

In a test tube one g of extract was placed and covered with filter paper moistened with dil. NaOH, then heated on water bath for a few min. The filter paper was examined under UV light, yellow fluorescence is indicative for the presence of coumarins.

f. Detection of flavonoids

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

g. Detection of leucoanthocyanins

5 mL of extract added to 5 mL of isoamyl alcohol. Upper layer appears red in colour indicates for the presence of leucoanthocyanins.

h. Detection of phlobatannin

When extract was boiled with 1% aqueous hydrochloric acid, deposition of a red precipitate was to be taken as evidence for the presence of phlobatannins.

i. Detection of simple phenolics (Ferric chloride test)

The extract (50 mg) was dissolved in 5 mL of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.
j. Detection of steroids

Two mL of acetic anhydride was added to 0.5 ethanolic extract of each sample with 2 mL H₂SO₄. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

k. Detection of saponins

The extract (50 mg) was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 15 min. A two cm layer of foam indicated the presence of saponins.

l. Detection of tannins

(a) One drop of ferric chloride was added to 2 mL of the extract, the appearance of bluish or greenish black colouration indicates the presence of pyrogallol or catechol tannins, respectively.

(b) Five mL of the extract were mixed with 2 mL vanillin hydrochloric acid solution. If a precipitate was formed; this indicates the presence of gallic acid.

m. Detection of true tannins (Goldbeater’s skin test):

Goldbeater’s skin is a membrane produced from the intestine of Ox. It behaves just like untanned animal hide. A piece of goldbeaters skin previously soaked in 2% hydrochloric acid and washed with distilled water is placed in a solution of tannin for 5 min. It is then washed with distilled
water and transferred to 1 % ferrous sulphate solution. A change of the
colour of the goldbeater’s skin to brown or black indicates the presence of
tannin. Hydrolysable and condensed tannins both give the positive
goldbeater’s test while pseudotannins show very little colour or negative
test.

n. Detection of pseudo tannins (Murexide test)
Extract when heated with HCl & potassium chlorate gives a residue which
on exposure to ammonia vapours gives purple colour.

o. Detection of terpenoid
The extract was dissolved in 10 mL chloroform, filtered and the filtrate was
divided into two equal portions for proceeding the following tests

(a) Libermann-Burchard test: To the first portion of chloroform filtrate 1 mL
of acetic acid anhydride was added, followed by 2 mL of sulphuric acid
down the wall of the test tube. The appearance of a reddish violet colour
at the junction of the two layers and a bluish green colour in the acetic
acid layer indicates the presence of unsaturated sterols and
or/triterpenes.

(b) Salkowski’s test: To the second portion of chloroform filtrate an equal
volume of sulphuric acid was added. The appearance of a red colour
indicated the presence of unsaturated sterol and /or triterpenes.
5.2.3 In-vitro evaluation of anti microbial (bacterial and fungal) properties of plant extract

5.2.3.1 Extraction and fractionation of plant material

(Subramanian SS and Nagrajan S, 1969)

The leaves were collected and washed in running tap water to remove adhering dust materials. The cleaned leaves were shade-dried and finely powdered extracted with ethyl using blender for extraction. The dried and powdered leaves (100g) were soxhlet extracted (Subramanian SS and Nagrajan S, 1969) in 80 per cent methanol (500 mL) for 24 h on a water bath. The extract was concentrated and divided into 2 portions. One portion was re-extracted with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) in succession. The petroleum ether extract was rejected as being rich in fatty substance. The ethyl ether fraction was analyzed for free flavonoids while the ethyl acetate fraction was hydrolyzed to cleave glycosides by refluxing with 7% H₂SO₄ for 2 h. The resulting mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate extract thus obtained was neutralized with 5% NaOH. The other portion of methanolic extract, ethyl ether fraction (free flavonoids) and ethyl acetate fraction (bound flavonoids) were dried in vacuo and weighed.
a. The free and bound flavonoids were re-suspended in their respective solvents to get 10 mg/mL and were used for testing antimicrobial activity against skin pathogens.

b. The methanolic extract (1mg/mL) was used for the determination of total phenolic and total flavonoid content. The methanolic extract, free and bound flavonoids were re-suspended in their respective solvents to get a concentration of 1 mg/mL (100 μg/0.1mL) and were used for testing antioxidant activity.

5.2.3.2 Antimicrobial activity

5.2.3.2.1 Testing for antimicrobial activity (Andrews JM, 2001)

Disc diffusion method (Andrews JM, 2001) was performed for screening anti microbial activity. Sterile Muller-Hinton agar (for bacterial strains) and Sabouraud dextrose agar (for fungal and dermatophytes) base plates were seeded with respective inoculum (inoculum size 1×10^8 CFU/mL for bacteria and 1×10^7 cell/mL for fungi and dermatophytes.) Sterile filter paper discs of Whatmann no.1 (6mm in diameter) were impregnated with 100 μl of each of the extract of concentration (10 mg/mL) to give a final concentration of 1 mg/disc. Discs were left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination.
Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in five replicate along with standard antibiotics (positive control) streptomycin (1 mg/disc) for bacteria and terbinafine (1 mg/disc) for fungi and dermatophytes. The petri plates containing the paper discs (6 mm) dipped in ethyl ether, ethyl acetate and 80 percent methanol and water were run parallel to study the impact of the solvent itself (without plant components) on growth of the test organisms. The plates were kept at 4º C for 45 min for diffusion of extract, thereafter were incubated at 37 ± 2º C for bacteria (18-24 h) and 27 ± 2ºC for fungi (48-72 h). Growth was monitored for 24, 48 and 72 h, depending on the period of incubation time required for the visible growth. The growth in treated samples was compared with their respective control plates. Inhibition zones formed around the discs were measured with transparent ruler (in millimeters). The average of inhibition zones was recorded and compared with the standard reference antibiotics. Activity index for each extract was calculated by the standard formula as below:

\[
\text{Activity index (AI)} = \frac{\text{Inhibition Zone of the sample}}{\text{Inhibition Zone of the standard}}
\]
5.2.3.2.2 Minimum inhibitory concentration (MIC) value

(Basri DF and Fan SH, 2005)

It was determined following Broth micro dilution method (Basri DF and Fan SH, 2005). Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make 10 mg/mL final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter 100µl inoculums (for bacteria 1×10^8 CFU/ mL and 1×10^7 cell/mL for fungi) were added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 37 ± 2° C for 24 h for bacteria and 27 ± 2° C for 48 h for fungi. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4° C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bactericidal / fungicidal concentration (MBC / MFC) was determined by sub culturing 50 µl from each well showing no apparent growth. Least concentration of extract showing no visible growth on sub culturing was taken as MBC/MFC.
5.2.4 In-vitro evaluation of antioxidant properties of plant extract

5.2.4.1 Total phenolic content (Singleton VL and Rossi JA, 1965)

Total soluble phenolics in the extract were determined with Folin-Ciocalteu reagent using gallic acid (10-250 μg) as a standard phenolic compound. (Singleton VL and Rossi JA, 1965) 1.0 mL of extract solution containing 1.0 mg extract was diluted with 46 mL of distilled water in a volumetric flask. 1.0 mL of Folin-Ciocalteu reagent was added and the content of the flask mixed thoroughly. 3 min later 3.0 mL of 20% sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of the blue colour that developed was read at 760 nm using double beam spectrophotometer (UV1240, Shimadzu, Japan). The concentration of total phenols was expressed as gallic acid equivalents in mg/g of dry extract.

5.2.4.2 Total flavonoid content (Chang C, 2002)

Aluminum chloride colourimetric method was used for determination of flavonoids. (Chang C, 2002). To the 10 mL volumetric flask 2 mL of water and 1 mL of plant extract (1 mg/mL) were added. After 5 min 3 mL of 5 % sodium nitrite and 0.3 mL of 10 % aluminum chloride were added. After 6 min, 2 mL of 1 M sodium hydroxide was added and the volume made up to 10 mL with water. Absorbance was measured at 510 nm. The total flavonoids were
calculated from calibration curve of quercetin (10-250 μg) plotted by using the same procedure and was expressed as quercetin equivalents in milligrams per gram of dry extract.

5.2.4.3 Total antioxidant activity (Prieto et al., 1999)

Total antioxidant activity of the methanolic extract and its free and bound flavonoid fraction was evaluated by calculation through reduction of phosphomolybdenum according to the method of Prieto et al., 1999. An aliquot of 0.1 mL of sample (100 μg) solution was combined with 1mL of standard reagent (0.6 M Sulfuric acid, 28mM sodium molybdate and 4mM ammonium molybdate). The tubes containing the reaction mixture were capped and incubated in a boiling water bath at 95° C for 90 min. 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. Absorbance of all the samples was measured at 695 nm. The antioxidant activity were calculated from the calibration curve of ascorbic acid (10-100 μg) plotted by using the same procedure and was expressed as ascorbic acid equivalents in milligrams per gram of dry extract.
5.2.5 Statistical analysis

Three replicates of each sample were conducted and the values are reported as mean ± standard deviation.

5.3 Formulation development

The ointment was prepared using 70% of 5% *Wrightia tinctoria* oil extract. Preformulation studies were conducted and suitable ointment base consisting of 15% bees wax, 10% hard paraffin wax and 5% soft paraffin wax was selected. Butylated hydroxyl toluene was used as preservative. The ointment was prepared by melt pour and mixing technique.

5.4 Standardisation of *Wrightia tinctoria* oil extract present in the optimised formulation through modern analytical technique

(Muruganandam AV *et al.*, 2000)

HPLC method (Muruganandam AV *et al.*, 2000) was used for estimation of ointment formulation containing *Wrightia tinctoria* extract in it. This was done using Water associates HPLC assembly. The stationary column used was RP-18 reverse phase column, with Methanol-water (80:20) as mobile phase and PDA as detector. It was scanned at 300 nm with a flow rate of 1mL/min. Calibration curve was made using indirubin, AR sample. The active constituent was extracted from the formulated ointment using methanol. The methanolic
extract is injected in HPLC for estimation. Controls were run using methanolic extract of ointment base and coconut oil as control samples.

5.5 **In-vitro and in-vivo evaluation of herbal formulation for safety**

5.5.1 **In-vitro evaluation of herbal formulation for safety**

The formulated ointment was subjected for the evaluation of following parameters

5.5.1.1 Test by physical appearance

The physical appearance of the ointment including its colour and smell was noted.

5.5.1.2 Consistency

The consistency of the ointment as thick, thin, flowing, pourable was tested by squeezing the ointment from the packed ointment tube on a glass tile.

5.5.1.3 Spreadability (Mutimer MN et al., 1956)

It was determined by using a spreadability apparatus (Peppas NA and Sahlin JJ, 1989). After applying weight, time (sec) required to separate the slides was noted

\[ S = \frac{M \times L}{T} \]

Where, \( S = \text{Spreadability} \), \( M = \text{Weight tide to upper slide} \), \( L = \text{Length of glass slide} \) and \( T = \text{Time taken to separate the slide completely from each other} \).
5.5.1.4 Feel on application

The ointment was applied on the skin to check the feeling as cooling effect, tingling feel, heat generation, smoothening, etc.

5.5.1.5 Extrudability (Liberman HA et al., 1989)

A closed collapsible tube containing formulation was pressed firmly at the crimped end. When the cap was removed, formulation extruded until the pressure dissipated. Weight in grams required to extrude a 0.5 cm ribbon of the formulation in 10 seconds was determined. The average extrusion pressure in grams was reported.

5.5.1.6 Stability studies

Stability studies were performed for 6 months. Samples (packed in glass vials) were prepared in triplicates and were kept at two stability testing conditions, viz. 5°C ± 3°C serving as control and 40°C ± 2°C / 75% RH ± 5% RH (Stability chamber, Remi-2K) serving as test condition as per ICH Guidelines. Stability of samples was evaluated for physical appearance, consistency, spreadability, and feel on application, extrudability at each sampling point (0 and 6 months).

5.5.2 In-vivo evaluation of herbal formulation for safety

5.5.2.1 Primary skin irritancy test (Marzulli FN and Maibach HI, 1997)

Primary skin irritancy test, to evaluate the dermal safety of the Wrightia tinctoria ointment was conducted on rabbit model. Six healthy (N=6), previously unused
New Zealand White rabbits of either sex (2.0–2.5 kg) from Experimental Animal Centre of, IIMT Meerut, India was chosen for this study. 50 g of the *Wrightia tinctoria* ointment as applied for 24 h on 2 controlled (1 × 1 inch square) treatment sites (one site intact, and the other site abraded) on each animal. The test sites were evaluated with the standard Draize Scoring Criteria at T= 24 h and T =72 h after end of treatment.

5.5.3 In-vivo evaluation of herbal formulation for wound healing

Wound healing activity:

The protocol of the study was approved by the local animal ethical committee of IIMT College of Medical Sciences. The guinea pigs were kept in standard conditions in the animal house and were used after an acclimatization period of 7 days to get elaborated to the environment. They were provided with food and water ad libitum.
Animal model for wound healing activity:

5.5.3.1 Excision model (Esimone CO et al., 2000)

For the excision study (Esimone CO et al., 2000), 3 groups of 6 animals each were anaesthetized with diethyl ether and the hairs on the skin of the back, shaved with sterilized razor blades. A circle of diameter 2 cm was marked on each of the two sides of the skin. Circular incisions were then made on the marked areas of the skin surface and the skin carefully dissected out and the wound was left undressed to open environment. The area was measured immediately by tracing out the wound area using a transparent tracing paper and the squares counted. One group was treated with the *Wrightia tinctoria* ointment; the second group was treated with allopathy control (0.2% w/w nitrofurazone ointment); and the third group received ointment base (blank control). The test sample was applied once daily and the treatment site was assessed for wound healing on T1, T4, T7, T10, T14, T16 and T19, after surgery on intermittently basis for 19 days. Falling of scar leaving no raw wound behind was taken as an end point of complete epithelization and the days required for this was taken as period of epithelization. This model was used to monitor wound contraction and wound closure time. Wound contraction was calculated as percent contraction on wound area and was monitored planimetrically by tracing the wound margin on graph paper at pre mentioned duration.
5.5.3.2 Incision model (Krishnaveni B et al., 2000)

For the incision study (Krishnaveni B et al., 2000) 3 groups of 6 animals each were used and two paravertebral long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the guinea pig. Full aseptic measures were not taken and no local or systemic antimicrobials were used throughout the experiment. No ligature was used for stitching. After the incision was made with 5.0 cm cut the parted skin was kept together and stitched with black silk at 1 cm intervals using surgical threads (No.000) and a curved needle (No.11) for stitching after complete haemostasis, by means of interrupted sutures of 1 cm apart. The continuous threads on both wound edges were tightened for good closure of the wound. The wound was left undressed.

One group was treated with the *Wrightia tinctoria* ointment; the second group was treated with allopathy control (0.2% w/w nitrofurazone ointment); and the third group received ointment base. The test sample was applied once daily sutures were removed on 8th post wounding day and tensile strength was determined on 10th post wounding day according to the method of Lee. (Lee KH, 1969)
Tensile strength, the force required to open a healing skin wound, was used to measure healing. The instrument for this measurement is called tensiometer. It consisted of a 6x12 inc board with one post of 4 inch long fixed on each side of the longer ends. The board was placed at the end of a table. A pulley with bearing was mounted on the top of one of the posts. An alligator clamp with 1 cm width, was tied on the tip of the post without pulley by a piece of fishing line (20-lb test monofilament) so that the clamp could react at the middle of the board. Another alligator clamp was tied on a piece of fishing line with a 1-L polyethylene bottle tied on the other end. Before testing, the animal was anesthetized with ether in an open mask. The sutures of the wound were cut out with a pair of scissors; the animal was then placed on a stack of paper towels on the middle of the board. The amount of the towels could be adjusted so that the wound was on the same level of the tips of the posts. The clamps were then carefully clamped on the skin of the opposite sides of the wound at a distance of 0.5 cm away from the wound. The longer piece of fishing line was placed on the pulley and the position of the board as adjusted so that the polyethylene bottle was freely hanging in the air. Water was removed at constant rate by siphon from a large reservoir (20-L bottle) until the wound began to open up. The amount of water in the polyethylene bottle was weighed and considered as the tensile strength of the wound.
5.5.4 Statistical analysis

Three replicates of each sample were conducted and the values are reported as mean ± standard deviation.

5.6 Clinical evaluation of safety and efficacy of herbal formulation for psoriasis with positive control

The clinical study was conducted at Fathima Health Care Centre, Trichy, with INSTITUTIONAL REVIEW BOARD and duly constituted Ethics Committee by the ClinRx Laboratories, India, as per good clinical Practice (GCP) guidelines and study protocol CP-CX-BBHC-02-01.

Ethics

Institutional review board

The study protocol CP-CX-BBHC-02-01 along with the Informed consent Form (ICF), Case Report Forms (CRF), Principal Investigator’s curriculum vitae (C.V.), and relevant safety study reports / literature were submitted for review and approval to a duly constituted Ethics Committee (EC) before initiation of the study. Details on the EC membership list and their approval for the conduct of the study as per protocol CP-CX-BBHC-02-01 is provided in Appendix. The study was conducted as per Good Clinical Practice (GCP) guidelines for the ethical conduct of clinical studies. The subjects were
provided with 24-hour access to a physician in case any adverse event (AE) occurred during the course of the study.

**Ethical conduct of the study**

The study was conducted in accordance with the ethical principles included in the “Ethical Principles for Medical Research Involving Human Subjects” adopted by the 18th World Medical Association (WMA) General Assembly, Helsinki, Finland, June 1964; and amended by the 29th WMA General Assembly, Tokyo, Japan, October 1975; the 35th WMA General Assembly, Venice, Italy, October 1983; and the 41st WMA General Assembly, Hong Kong, September 4 1989. The study was also conducted according to good Clinical Practice as outlined in the ICH guidelines (1996), EN 540 and in accordance with local and national guidelines.

**Subject information and consent**

Each subject was required to sign a voluntary ICF prior to participation in the study. The ICF was approved by the Ethics Committee. The informed consent process was to fully apprise the subjects of the risks and benefits to them and to society for participating in the study. If subjects understood and agreed to participate in the study, they would sign the ICF. ICF was prepared both in English and the local language(s) applicable. If subjects had questions about
their rights, they were instructed to contact a member of the IRB. A sample ICF is provided in Appendix.

**Investigators and study administrative structure**

After approval from the Ethics Committee, the study was conducted at Fathima Health Care Centre, Trichy. The Principal Investigator was Dr. Rita, M.D, received approval and then enrolled subjects in this study.

**Description of drug and related safety information**

The *Wrightia tinctoria* ointment and one allopathy control formulation was used in the study. Description of the formulations used in the study is:

- **Herbal formulation:** *Wrightia tinctoria* extract in ointment form for topical application in 20gm tubes.

- **Allopathy:** 1% Dithranol ointment for topical application.

The active ingredients used in the herbal formulation are safe to use on humans and are cited in the traditional ayurvedic literature and have been in clinical use for many centuries.
5.6.1 Investigational plan

5.6.1.1 Overall study design and plan

It was a uni-centered two arm Clinical Trial with Positive Control, 20 subjects completed the 8 week study (10 with *Wrightia tinctoria* ointment and 10 with allopathic control). The study treatment (*Wrightia tinctoria* ointment or allopathy control) was randomly assigned to each subject with psoriasis vulgaris (confirmed by histopathology) on a first come first serve basis as per a randomization schedule generated using standard statistical procedure. There were no adverse events reported in the conduct of the study.

5.6.1.2 Selection of study population

5.6.1.2.1 Recruitment and subject confidentiality

All patients were recruited by word of mouth advertisement and satisfied the screening criteria.

5.6.1.2.2 Inclusion criteria

All the subjects are known psoriasis belonging to both sexes who are:

1. Capable of understanding and signing an informed consent
2. Between the ages of 18 and 65 years
3. Medically stable
4. Subjects with *Psoriasis vulgaris* without any systemic complication with 1-10 lesions in the glabrous skin.

5. If female, attained menopause, undergone puerperal sterilization / hysterectomy

**5.6.1.2.3 Exclusion criteria**

Persons with the following conditions or characteristics will be excluded from the study:

1. Flexural psoriasis, pustular psoriasis, arthritic psoriasis and with any systemic involvement.

2. Nursing mothers

3. Diabetes

4. VDRL positive

5. History of high blood pressure

6. History of cardio-vascular disease (MI in the past year)

7. History of fainting, arrhythmia or irregular pulse

8. Current or previous history of neurological disorders or psychosis

9. History of cerebrovascular accident, transient ischemic attacks or seizure disorder.

10. Known or suspected HIV-positive subject or subject with advanced diseases such as AIDS, hepatitis C, hepatitis B or tuberculosis.
11. Drug substance or alcohol abuse.
12. Use of alcohol or drugs within 48 h prior to and during study participation
13. Any psychological condition that will influence the conduct of the study or interpretation of results
14. Malnutrition (BMI must be over 19)
15. Smoking within 48 h prior to and during study participation
16. Use of another investigational medication / treatment in the past 30 days.

Any other condition in the opinion of the Principal Investigator would place the subject at risk or influence the conduct of the study or interpretation of results.

5.6.1.2.4. Removal of subjects from therapy or assessment

5.6.1.2.4.1. Criteria to withdraw subject from study
1. Any indication of allergic reaction to treatment.
2. Patients developing severe symptoms that were uncontrolled with the herbal drug.
3. Withdrawal of consent.
4. Administrative reasons, such as patient non-compliance or major protocol violation (e.g. Pregnancy, alcohol consumption during study)
5. Any condition, which the Principal Investigator feels that might put the subjects at undo risk. If such a situation should occur, the Principal
Investigator will clearly specify on the CRF why the subject was withdrawn from the study.

5.6.1.2.4.2. Criteria to stop a specific treatment regimen or the entire study

1. Clinically significant severe symptoms or reactions. (Note: if all AE’s occurring with specific treatment group, only that specific treatment group may be stopped).

2. Any condition, which the Principal Investigator feels, might put the subjects at undo risk. If such a situation should occur, the Principal Investigator will clearly specify why the treatment was stopped.

3. Any serious study related adverse event. (Entire study should be stopped)

5.6.1.3 Investigational product specifications

Details of the specification of the herbal formulation and the allopathic control formulation are presented below:

Herbal formulation: *Wrightia tinctoria* extract in ointment form for topical application in 20gm tubes.

Allopathic control: 1% Dithranol ointment for topical application.

5.6.1.4 Treatments

5.6.1.4.1 Treatments administered

Subjects visited the clinic for a minimum period of 7 times (7 treatments). At each visit vital signs, photographs and clinical examination results were
recorded. In addition, at the beginning (T0w) and end of treatment (T8w) Haemogram analysis, Liver Function Testing (LFT) and Renal Function Testing (RFT) were done to document the safety profile of the treatments administered. Skin biopsies were done at the beginning and end for histopathological evaluations.

5.6.1.4.2 Randomization

Randomization schedule were made available prior to start of the study and were prepared using standard statistical techniques. Each screened patient on treatment initiation day is given sequentially a subject number.

There are two arms in the study:

- group 1: Herbal ointment formulation-1 once daily
- group 2: Dithranol ointment once daily

5.6.1.4.3 Blinding

Due to the limitation on the availability of the drugs used in study in different forms viz, ointment tube form the study was only single blinded.
5.6.1.5 Screening, safety, and efficacy measurements

5.6.1.5.1 Screening measurements

Subject screening for enrollment into the study included:

1. Presentation of study information to subject and obtaining voluntary informed consent.
2. Recording demographic information
3. History and physical examination
4. Vital signs
5. Temperature was measured with a digital thermometer.
6. Weight was recorded in Kg
7. Height was recorded in cms
8. Radial pulse was observed for 60 seconds.
9. Respirations are counted for one minute. Respirations must be between 16-24 breaths/minute in order for the subject to participate in study.
10. Blood pressure was taken with sphygmomanometer using standard procedures.
11. Inclusion / exclusion criteria assessed.
5.6.1.5.2 Safety assessment

Vital signs

Vital sign measurements include systolic and diastolic BP measurements, pulse rate and respiratory rate. Systolic and diastolic BP measurements were done using manual mercury sphygmomanometer in the right upper limb in the sitting posture. Pulse rate was measured in the radial artery by palpating the artery with the middle, index and ring finger. Respiratory rate was measured by watching the expansion of abdomen with each respiration and counting them for one minute.

Haemogram measurement

It includes measurement of Total count of White blood cells (TC), Differential white blood cells count as Polymorphonuclear neutrophil (DC-P), Lymphocytes (DC-L), Eosinophils (DC-E) and Haemoglobin (Hb). TC, DC-P, DC-E, and DC-L were measured using Neubauer Counting Chamber. HB was measured using RA 50 Biochemical analyzer.

LFT measurement

Liver Function Test (LFT) includes measurement of Serum Glutamic Oxalo acetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT) and Serum Bilirubin using RA 50 Biochemical analyzer.
RFT measurement

Renal function testing (RFT) includes measurement of serum urea and serum creatinine using RA 50 Biochemical analyzer.

5.6.1.5.3 Efficacy measurements

The efficacy of the formulation was evaluated by Clinical Examination (Erythema, Scaling and New Lesions) and Histopathology analysis of skin biopsy at treatment site and analyzing the data as a function of time for the different groups of treatment. Clinical Examination was documented during every visit. Skin biopsies were taken for Histopathology analysis at the beginning and end of the treatment.

Clinical examination

Scaling was measured during each visit and scored by trained investigator and measured as No (0), Mild (1), Moderate (2), or Severe (3). Erythema was measured during each visit by trained investigator and measured as No (0), Very Slight (1), Well Defined (2), Moderate (3), or Severe (4). Presences of new lesions were documented during each visit.

Histopathology

It was done by an expert pathologist and the Stratum Granulosum parameter was measured and the results were scored 3 to 1(representing granular layer
condition). The Parakeratosis parameter was measured and the results were scored 3 to 1 (representing nuclei status in cells of stratum corneum) The Munro’s Microabscess parameter was measured and the results were scored 3 to 1 (representing organization status of abscesses within the epidermis) The Acanthosis parameter was measured and the results were scored 3 to 1 (representing status of acanthosis).

5.6.1.6 Statistical methods used in data analysis

Statistical Analysis was carried out using regular one-way ANOVA, Tukey’s test was used to do pairwise comparison evaluation where applicable.

5.6.1.7 Data analysis

5.6.1.7.1 Analysis of safety data

Data for vital signs (systolic and diastolic Blood Pressure, Pulse rate and respiratory rate), Haemogram measurements (TC, DC-P, DC-L, DC-E, and ESR), LFT measurements (SGOT, SGPT, and Serum Bilirubin) and RFT (Blood urea and Serum Creatinine) measurements were analyzed with the statistical methods described in Section 5.6.1.6 for safety assessment of Herbal Drug formulation as compared to the Allopathic control.
5.6.1.7.2 Analysis of efficacy data

Data for clinical examination including scaling, erythema, histopathology and new lesions measurements were analyzed with the statistical methods described in Section 5.6.1.6 for efficacy assessment of herbal drug formulation as compared to the allopathic control. Histopathology measurements include parakeratosis, stratum granulosum, Munro’s Microabscess and acanthosis.

5.6.2 Study subjects

5.6.2.1 Disposition of subjects

25 subjects were screened, 20 subjects were enrolled and completed the study.

5.6.2.2 Protocol violations

No major protocol violations influencing the safety or outcome of the results occurred during this study.

5.7 Investigation on indirubin as a ligand by molecular docking studies on biochemical pathogenesis of psoriasis

Psoriasis is a chronic proliferative immune mediated inflammatory skin disease with a population prevalence of 2–3%. Many bioactive substances have appeared to be related to psoriasis. Based on the current literature,
indirubin is described as a CDK/GSK-3 inhibitor. However, its affinity for the proteins mentioned in this study has not been studied in detail. Hence an attempt is made to study the various biochemical pathogenesis of psoriasis and the probable mechanism of action of indirubin present in *Wrightia tinctoria* ointment in the treatment of psoriasis by molecular docking studies.

5.7.1 The biochemical markers of psoriasis

The various biochemical markers of psoriasis namely, dihydrofolate reductase (DHFR), interleukin 17 A, interleukin 17 F, interleukin 22, tyrosine kinase, epidermal growth factor, interleukin 23, interleukin 6, interleukin 2, interleukin 8, phosphorylase kinase, keratinocyte growth factor and aryl hydrocarbon receptor (Duvic *et al.*, 1998; Schwartz *et al.*, 1992; Kristian Reich & Ulrich Mrowietz, 2007) were retrieved from the immuno biochemical pathogenesis of psoriasis.

5.7.2 Molecular docking study

Glide, a docking programme present in Maestro 9.3, a module of Schrodinger was used for the docking study. It involved protein preparation, ligand preparation, active site prediction, receptor grid generation, and running the glide docking. The structures of indirubin and known inhibitors of various proteins were obtained from PubChem database. All the 3D crystal structural
information about the target biochemical markers were obtained from the Protein Data Bank, PDB. The efficiency of indirubin present in *Wrightia tinctoria* as a ligand was studied using molecular docking to investigate its role in the treatment of psoriasis. Results were analysed with glide docking which yielded XP score of indirubin with various proteins revealing high affinity in decreasing order.