CHAPTER 7: SUMMARY, CONCLUSION AND RECOMMENDATIONS

A research project entitled “Development and evaluation of herbal formulation for dermatological use” was chosen with the objective of developing a formulation using *Wrightia tinctoria* (Roxb.) R.Br. for treatment of psoriasis. It included in-vitro, in-vivo and clinical evaluation followed by molecular docking study.

Psoriasis is a chronic inflammatory disease of the skin characterized by epidermal hyperplasia, dermal angiogenesis, and inflammatory cell infiltrates. *Wrightia tinctoria* (Roxb.) R.Br. is a small deciduous tree of the family Apocynaceae distributed in Central India, Burma and Timor. This plant is extensively used in the Indian system of medicine and has reported anti-psoriatic, anti-nociceptive, wound healing, immunomodulatory, and anti-inflammatory activity.

The present thesis entitled “Development and evaluation of herbal formulation for dermatological use” is organised into seven chapters. It deals with the relevant introduction in chapter 1, followed by objective and rationale in chapter 2. All the literature was complied in chapter 3 up to the latest findings related to psoriasis, *Wrightia tinctoria*, indirubin, extraction, pharmacological screening, clinical trial and molecular docking. The chapter 4 briefs the plan of work involved. Materials and Methods used in the thesis is explained in
chapter 5. Chapter 6 elaborates on the research findings and interpretation of result obtained. Chapter 7 provides a precise summary of the research findings followed by conclusion and recommendations for further work.

*Wrightia tinctoria* leaves with traditional use for the treatment of psoriasis was studied through reverse pharmacology to document a systematic clinical investigation as per GCP guidelines for effective and safe use in psoriatic patients. An investigation for pharmacognostical standardization of *Wrightia tinctoria* leaves as per WHO guidelines, preparation of extract for ointment development and analytical profiling of the ointment were made. Pharmacological investigation and molecular docking study were undertaken to proposed mechanism of action.

The *Wrightia tinctoria* leaves were collected from VInYY garden, Nachallur, Karur district, identified by Prof. P. Jayaraman of Plant Anatomy Research Centre (PARC), Chennai and a voucher specimen was deposited at PARC. In pharmacognostical standardisation of *Wrightia tinctoria* leaves macroscopical characteristics such as size, colour, surface, texture and fracture characteristics were studied. The leaves were simple, opposite, obovate-oblong, acuminate, glabrous with main nerves 6-12 pairs. Microscopical studies were performed on the transverse section of leaf. It revealed thin outline, thick semicircular abaxial midrib with less prominent adaxial hump, thin and distinct with small radially oblong thick
walled cells of epidermis, circular, thin walled, compact parenchymatous cells of ground cells and U-shaped bicollateral vascular strand in the vascular bundle.

The measurement of leaf elements revealed a maximum - minimum count for epidermal cell count as 68-130, stomatal count as 12-24, stomatal index as 18-20, vein islet number as 19-32, vein termination number as 12-17 and palisade ratio as 6-9 respectively.

It was followed by histochemical colour reactions on the leaves of *Wrightia tinctoria* and the reactions gave positive colour development for the application of aniline sulphate with sulphuric acid, weak iodine solution, sudan III/IV, Draggondroff reagent, potassium dichromate reagent, vanillin hydrochloride reagent, ferric chloride reagent, silver nitrate and hydrogen peroxide reagent. The degree of intensity of colour at the specific histological zone was observed.

Physicochemical standardization was performed by determining the chemical parameters namely ash values, elemental analysis and extractive values for the powdered leaves of *Wrightia tinctoria*.

The total ash value was 16.5 %, acid insoluble ash was 2.3 %, sulphated ash was 6.2% and water soluble ash was 3.8%. The calcium content was 0.42%, potassium content was 1.32%, sodium content was 0.52 %, phosphorous was 0.03%, Iron content was 0.03% and nitrogen content was 1.88%. The extractive
values in water was 18.48 %, alcohol was 14.62 % and chloroform was 8.24%. The fluorescence characteristics of powdered leaves of *Wrightia tinctoria* were tested by the addition of various solvents and observed under white light and UV light. The colours obtained respectively were, colourless, pale green for hexane, pale brown, green for benzene, brown, dark green for chloroform, pale yellow, green for alcohol, yellow, green for acetone and pale brown, pale green for water in white light and UV.

Extraction of *Wrightia tinctoria* leaf was made by soaking the minced leaves in coconut oil and shade drying in sunlight. The extracted oil was then standardized through organoleptic evaluation, which revealed the colour as pinkish red to deep pink colour, odour as pleasant smell of coconut oil and touch as oily. Preliminary phytochemical screening of the extracted oil was done and found to contain volatile impurities as 0.09 %, relative density as 0.932, refractive index as 1.462, acid value 18.27, iodine number as 8.22, saponification value as 274.26, unsaponifiable matter as 0.32% w/w, volatile matter as 3.82%, loss on drying as 4.92 % and passes the test for rancidity, cotton seed oil and seesame oil. The qualitative chemical analysis was carried out by conducting chemical tests which gave positive result for the presence of alkaloids using Dragendorff’s, Mayers’s and Wagner’s reagent, cardiac glycosides using Keller Killani test, flavanoids using ammonium hydroxide
test, leuco anthocyanins using isoamyl alcohol test, phlobatannins using hydrochloric acid test, simple phenolics using ferric chloride test, steroids using acetic anhydride – sulphuric acid reagent, saponins using foaming test, tannins using ferric chloride test and terpenoids using Libermann – Burchard test and Salkowiski’s test and negative result for acubins/iridoids using Trim Hill test, anthroquinins using ammonia reagent and coumarins using fluorescence test.

The antimicrobial screening was performed on methonolic extracts of *Wrightia tinctoria* leaf powder which was further subjected to successive fractional extraction using petroleum ether, ethyl ether (free flavonoids) and ethyl acetate (bound flavonoids). The free and bound flavonoid fractions were tested against 10 bacterial, 3 fungal and 4 dermatophytic strains using disc diffusion and broth micro dilution method. The free flavonoidal extract showed maximum activity against *Bacillus subtilis* (gram positive bacteria), *Proteus vulgaris* (gram negative bacteria), *Candida albicans* (fungus) and *Trichophyton rubrum* (dermatophyte). The bound flavonoidal extract showed maximum activity against *Staphylococcus aureus* (gram positive bacteria), *Escherichia coli* (gram negative bacteria), *Candida albicans* (fungus) and *Epidermophyton floccosum* (dermatophytes) among the various tested organisms. The bound flavonoidal extract (activity index 0.58) had strong activity against dermatophytes.
Broth micro dilution method was used to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC).

The range of MIC and MBC / MFC of the extracts was recorded as 0.31-2.5 mg/mL. In the present investigation for free flavonoids, MIC value was 0.312 mg/mL, and MBC value was 0.625 mg/mL for all the gram positive bacterial strains and *Proteus vulgaris* in gram negative strain, proving the free flavonoidal extract to be bacteriostatic in nature.

For the bound flavonoidal extract the lowest MIC value of 0.312 mg/mL was observed for all the gram positive strains tested except *Bacillus cereus* and the MBC value for all the strains were higher than MIC values indicating bacteriostatic effect of the extract. For gram negative organisms the lowest MIC value of 0.312 mg/mL was observed for *Proteus vulgaris* and *Pseudomonas aeruginosa* and on comparison of MIC with MBC values, it indicated the bound flavonoidal extract to be bacteriostatic except against *Salmonella paratyphi* for which it showed bactericidal effect. The least MIC of bound flavonoids extract against fungal strains was 0.312 mg/mL for *Candida albicans* and for all fungal strains it exhibited fungi static effect.

The data suggest that the extracts expressed bacteriostatic, fungistatic and fungicidal activity against specific organisms indicating their broad spectrum of
action. Thus, this investigation documents valid evidence that flavonoids which are poly hydroxy phenols have effective antimicrobial activity against a wide range of microorganisms.

In-vitro antioxidant properties of the methanolic extract of leaves of *Wrightia tinctoria* was determined by estimating the total phenolic, total falvonoid content and total antioxidant activity. Total phenolic content of $30.17 \pm 2.36$ mg/g (gallic acid equivalents /g of dry extract) and total flavonoid content of $21.33 \pm 0.94$ mg/g (quercetin equivalents /g dry extract) were obtained when estimated through Folin Ciocalteu reagent method and aluminum chloride colourimetric method respectively. The total antioxidant activity determined by phosphomolybdenum reduction method was found to be maximum for free flavonoid fraction of the methanolic extract as $86.56 \pm 2.20$ mg/g (ascorbic acid equivalents /g of dry extract) when compared to the bound flavonoid fraction of the methanolic extract as $43.88 \pm 2.56$ mg/g (ascorbic acid equivalents /g of dry extract).

The *Wrightia tinctoria* ointment was prepared using 70% *Wrightia tinctoria* leaf extract in coconut oil. Preformulation studies were conducted and suitable ointment base consisting of 15% bees wax, 10% hard paraffin wax and 5% soft paraffin wax. Butylated hydroxyl toluene was used as preservative. The ointment was prepared by melt pour and mixing technique.
HPLC method using Water Associates HPLC assembly, stationary column as RP-18 reverse phase column, with methanol-water (80:20) as mobile phase, PDA as detector and scanned at 300 nm with a flow rate of 1mL/min was utilised for estimation of indirubin in ointment formulation containing *Wrightia tinctoria* extract in it. The ointment contained 0.14 % w/w of indirubin.

For the in-vitro evaluation, the formulated ointment was subjected to test by physical appearance which was pinkish red with pleasant smell of coconut oil, thick in consistency and spreadability was smooth and easily spreadable. The feel on application was with mild soothing effect and extrudability was freely extrudable.

The stability studies as per ICH guidelines was conducted for an observation period of 6 months at 5° C ± 3° C and at 40° C ± 2° C/75% RH ± 5% RH and the changes in the physical appearance, consistency, spreadability, feel on application and extrudability as parameters were observed at the initial and at the end of 6 months.. The changes were in appearance with colour towards brown and less smell of coconut oil, consistency was slightly thin, spreadability was smooth and easily spreadable, the feel on application was with mild soothing effect and extrudability was freely extrudable.

For in-vivo evaluation, primary skin irritancy test to evaluate the dermal safety of the *Wrightia tinctoria* ointment was conducted on rabbit model. Six healthy
previously unused New Zealand white rabbits from experimental animal centre of, IIMT. Meerut, India was chosen for this study. The *Wrightia tinctoria* ointment was applied for 24 h on 2 controlled treatment sites on each animal. The test sites were evaluated with the standard Draize scoring criteria at time 24 h and 72 h after end of treatment. The primary irritation index was determined to be zero suggesting “no irritation” potential for the drug formulation tested.

Preclinical evaluation of *Wrightia tinctoria* ointment for efficacy of wound healing was done on guinea pig model with incision and excision wounds. For the excision study 3 groups of 6 guinea pigs each were excised with 2 cm wound and treated with the *Wrightia tinctoria* ointment; allopathy control as nitrofurzone 0.2% w/w ointment; and ointment base as blank control, respectively. The test sample was applied once daily and assessed for wound healing on specific days as T1, T4, T7, T10, T14, T16 and T19, after surgery on intermittent basis for 19 days. Wound healing was observed for all the 3 groups of animals with the wound healing rates for allopathy control greater than *Wrightia tinctoria* ointment which was greater than ointment base.

For the incision study 3 groups of 6 guinea pigs each were incised with 5.0 cm cut and then sutured after complete haemostasis by means of interrupted sutures of 1 cm apart. One group was treated with the *Wrightia tinctoria*
ointment; the second group was treated with nitrofurozone 0.2% w/w ointment as allopathy control and the third group received ointment base as blank control. The test sample was applied once daily, sutures were removed on 8th post wounding day and tensile strength, which is the force required to open a healing skin wound was determined on 10th post wounding day according to the method of Lee. Wound healing was observed with all the 3 groups with the wound healing rates for allopathy control greater than Wrightia tinctoria ointment which was greater than ointment base.

A randomized controlled clinical study was conducted for the evaluation of clinical safety and efficacy of Wrightia tinctoria ointment as group 1 and with dithranol ointment which is positive control as group 2. The improvement in the clinical condition of psoriasis patients was observed. Twenty subjects completed the 8 week study where random assignment of 10 each were with Wrightia tinctoria ointment and allopathic control respectively on first come first serve basis. There were no adverse events reported in the conduct of the study.

Safety of the Wrightia tinctoria ointment was assessed by monitoring vital signs, haemogram measurements, liver function measurements and renal function test measurements. The vital sign measurements namely, BP-systolic, BP-diastolic, pulse rate and respiratory rate during every visit and haemogram including total count of white blood cells, differential white blood cells count
as polymorphonuclear neutrophil, lymphocytes, eosinophils and haemoglobin were measured. The liver function test includes measurement of serum glutamic oxalo acetic transaminase, serum glutamine pyruvic transaminase and serum bilirubin. The renal function tests includes measurement of serum creatinine and serum urea. The above tests were conducted and estimated using biochemical analyser at the beginning and end of the study. The findings of all the above physiological parameters were observed to be within the prescribed normal limits (p>0.05) for the *Wrightia tinctoria* ointment and the allopathy control.

Efficacy of the *Wrightia tinctoria* ointment was assessed by clinical examination using scoring scale for erythema, scaling and occurrence of new lesions during every clinical visit. In addition skin biopsy was taken at the treatment site at the beginning and end of treatment for histopathology analysis. Scaling scores were observed to decrease from 2.0 to 0.8 for group 1 and 1.90 to 0.10 for group 2. The erythema scores were observed to reduce from 2.50 to 1.50 for group 1 and from 2.0 to 0.4 for group 2. The scaling and erythema were observed to be decreased with time (p < 0.05) for all groups and no treatment effects (p=0.07) were observed. No statistically significant occurrence of new lesions was observed with *Wrightia tinctoria* ointment and allopathic control (P> 0.05).
Histopathology of the skin biopsies was done for parakeratosis which represents the nucleated cells of the stratum corneum. The other parameters as stratum granulosum representing the granular layer condition, Munro’s microabscess representing the organisation status of abscesses within the epidermis and acanthosis which represents the status of acanthosis were also studied. For parakeratosis the score was decreased from 3 to 1.5 for group 1 (p=0.001) and decreased from 2.8 to 1.6 for group 2 (p=0.003). For stratum granulosum the score was decreased from 2 to 1 for group 1 (p=0.008) and decreased from 1.6 to 1.2 for group 2 (p=0.309). For the case of Munro’s microabscess the score was decreased from 2.42 to 1.2 for group 1 (p=0.005), and decreased from 2.6 to 1.3 for group 2 (p=0.002). For the case of acanthosis the score was decreased from 2.8 to 2.00 for group 1 (p=0.011) and was decreased from 2.90 to 2.10 for group 2 (p=0.011). Statistically significant positive response was observed with time for both treatment groups. However, there were no statistically significant treatment (group) effects. But for stratum granulosum, statistically significant (p=0.008) positive response with time was observed with the Wrightia tinctoria ointment and no time effect (p=0.309) was observed with allopathy formulation.

In molecular docking study, based on current literature, the possible role of aryl hydro carbon receptor (AHR), dihydrofolate reductase (DHFR), interleukin 17a,
interleukin 17 f, interleukin 22, tyrosine kinase, epidermal growth factor, interleukin 23, interleukin 6, interleukin 2, interleukin 8, phosphorylase kinase, keratinocyte growth factor in psoriasis were studied.

The efficiency of indirubin present in Wrightia *tinctoria* as a ligand by molecular docking studies was then carried out using Glide, a docking programme present in Maestro 9.3, a module of Schrodinger. By the steps of protein preparation, ligand preparation, active site prediction, receptor grid generation and running the glide docking the docking study was carried out. Results were analysed with glide docking which yielded XP score of indirubin with various proteins revealing high affinity in decreasing order. Using this and the available literature an attempt to establish the probable mechanism of action of *Wrightia tinctoria* leaf oil extract in ointment for treating psoriasis was made.

The highest affinity of indirubin was observed with Aryl Hydrocarbon Receptor (-7.509 K cal/mol) followed by DHFR (-4.772 K cal/mol) and Interleukin 17A (-4.503 K cal/mol). When the glide score of Indirubin was compared with that of known inhibitors for these targets it was observed that indirubin exhibits a better binding affinity to Aryl Hydrocarbon Receptor and Interleukin 17A when compared with the known inhibitor bilirubin at the protein TYR A : 135 and biotin at the protein GLN A : 94 respectively, through
their pyrole nucleus. The known inhibitor methotrexate for DHFR showed higher affinity for it than indirubin. So indirubin binding with DHFR was neglected. This suggests that the activity of Indirubin most probably could be through binding with aryl hydro carbon receptor and interleukin 17A.

The immuno biochemical literature indicate that Interleukins (IL-2, IL-6, IL-8, IL-17A, IL-17F, IL-22, IL-23) play a vital role in stimulating the immune system, immunological cell differentiation and inflammation. AHR activation can trigger immunosuppressive responses through the generation of regulatory cells. Indirubin is an AHR agonist. Through its AHR signalling affinity it is able to compete with variable proteins involved in immunological disturbance. This is clearly visible through the varied research conducted in literature cited and its correlation with our XP glide score between indirubin and AHR.

The IL-17 A establishes a self-sustaining inflammatory feedback loop by activating core inflammatory elements namely Th 17, IL- 23, keratinocytes and proinflammatory factors that establish a self-reinforcing cycle. . The indirubin has a greater affinity for IL-17 A which is clearly visible through the varied research conducted in literature cited and its correlation with our XP glide score between indirubin and IL-17A. Thus Indirubin could be proposed to
have its antipsoriatic activity through the AHR signalling pathway and self-sustaining and self reinforcing inflammatory feedback loop of IL-17 A.

Hence in the present study we have concluded the following:

Through the research methodology and findings of work the primary objectives of this thesis namely, Standardization of the *Wrightia tinctoria* leaf, Standardization of the *Wrightia tinctoria* oil extract, In-vitro evaluation of anti microbial (bacterial and fungal) properties of *Wrightia tinctoria* methonolic against skin pathogens and antioxidant properties were achieved. Following them development of ointment formulation for dermatological use with leaf extract of *Wrightia tinctoria*, preclinical evaluation of *Wrightia tinctoria* ointment for safety and wound healing were done. Finally, evaluation for clinical safety and efficacy of *Wrightia tinctoria* ointment for treatment of psoriasis with positive control and molecular docking study were performed. Thus a *Wrightia tinctoria* leaf extract based ointment formulation for the treatment of psoriasis has been successfully formulated and standardised as follows: As per guidelines of WHO for pharmacognostical standardisation, as per OIE guidelines for antimicrobial screening, GMP guidelines for ointment formulation, NACLAR guidelines for animal experimentation, ICH guidelines for stability studies and GCP guidelines for clinical study.
For future research the following recommendations are suggested:

1. An elaborate clinical study by involving patients of different geographical origin and varied psoriasis type and severity may be carried out.

2. A large scale manufacturing of the developed formulation may be studied and standardization of controlling parameters could be attempted.

3. Elaborate study on large scale cultivation methods could be made.