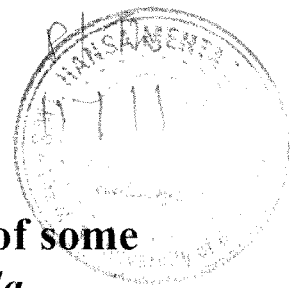


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A summary of Ph.D thesis entitled  
**Development of Methods for Standardization of some  
Adaptogenic *Rasayana* Drugs of *Ayurveda***

A THESIS SUBMITTED TO  
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### CERTIFICATE

This is to certify that the thesis entitled, “**Development of Methods for Standardization of some Adaptogenic Rasayana Drugs of Ayurveda**”, submitted by Mr. Manan A Raval for the degree of **Doctor of Philosophy in Pharmacy** to The M.S. University of Baroda, Vadodara has been carried out under my supervision and guidance. The matter presented in this thesis has not been submitted for the award of any other degree or other academic qualification anywhere.

Guide

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## Summary

Although, plants are used frequently in many traditional systems throughout the globe, their acceptability in modern medicine is remarkably low, largely due to lack of standardization parameters. Since the markers are yet to be identified and established for majority of plant drugs used in Herbal/*Ayurvedic* products pose a great difficulty in getting them accepted in modern therapeutics. It also becomes difficult to standardize them due to lack of information pertaining to Active Pharmaceutical Ingredient.

The texts of *Ayurveda* contain a full section on *Rasayana* drugs, recommended for enhancement of body's resistance being one of the main therapeutic strategies. The description of ancient hypothesis on *Rasayana* therapy matches with currently accepted mechanisms of Adaptogenic drugs of modern medicine.

*Ayurveda* consists mainly plants as drugs, although, many of these plants still lack standards for identification of correct sources and generate controversy. Often, the plant material provided of a particular botanical source does not possess claimed therapeutic activity or sometimes plant supplied has no resemblance whatsoever with the actual one. This type of controversy generally is encountered in case of extensively used plants.

The studies were, therefore, aimed to evolve certain parameters for the quality assessment of the selected herbal drugs mentioned in *Ayurveda* as *Rasayana* and generally utilized to improve the resistance of the body under the stress conditions. One of these drugs *Leptadenia reticulata*, termed as *Jivanti* in *Ayurveda*, is extensively exploited as lactogenic in dairy cattle feed, so much so that it has reached to the level of extinction while other plants, *Pentatropis microphylla* and *Dregea volubilis*, are used as substitutes for *L.reticulata* commercially in the name of *Jivanti*.

These are obtained from the different genus of the family Asclepiadaceae, therefore, form the controversial sources for *Jivanti*. The experiments were designed to perform the comparative phytochemical and biological studies of these controversial plants and evolve the differentiation parameters for the selected plants.

*Leptadenia reticulata* Wight & Arn. (*Asclepiadaceae*) is termed as *Jivanti* in central and western part of India. Tribes utilize this plant as stimulant, tonic and in treatment of diseases related to reproductive system. Traditionally the plant is reputed as lactogenic agent and studies have showed an enhanced development of mammary glands in lactating rats. Clinical studies showed lactogenic properties of *L.reticulata* and one of its herbal formulations. Stigmasterol, tocopherol, triterpene alcohol, pregnane glycosides, flavonoidal aglycones and flavonoidal glycosides were isolated from the aerial parts of *L.reticulata*.

*Dregea volubilis* (*Asclepiadaceae*) is also described as *Jivanti* in *Ayurveda* sometimes supplied as substitute for *L. reticulata*. The plant is described as tonic, coolent, aphrodisiac and as a cure for burning sensation. The seeds of *D.volubilis* possess ester glycosides with sterol genins, while stems, leaves and bark showed presence of taraxerol and taraxerol benzoate, two steroidal compounds, kaempferol along with other unknown phytoconstituents are also reported with pregnane aglycones. Different extracts of flowers of *D.volubilis* showed presence of volubiloside A, B, C and new polyhydroxy pregnane glycosides dregelol, volubilogenone and volubilol along with pregnane derivatives- drevogenin, isodrevogenin and 17- alpha-marsdenin. The juice of the leaves was found effective in treatment of experimentally induced diabetes in rats. The plant showed antifibrotic potential due to presence of pregnane and steroidal derivatives. The aqueous extract of the leaves were found to be hepatotoxic in rats.

Another plant of *Pentatropis microphylla* (*Asclepidaceae*) is known as *Kakanasa* in Ayurveda and is also used as substitute of *Jivanti* in the treatment of diseases of reproductive system. Traditionally aqueous extract of roots is used in treatment of gonorrhea. Different extracts of the aerial parts of the plant showed presence of octacosenol, alpha-amyrin, friedelin, beta-sitosterol, salicylic acid and some unidentified triterpenoids.

The whole plants of *Leptadenia reticulata* Wight and Arn., *Dregea volubilis* Benth. Ex Hook. and *Pentatropis microphylla* Wight. and Arn. were collected from near by tribal areas and were compared with the authentic samples preserved in the Botany Department, The M.S. University of Baroda, Vadodara. The voucher specimens were preserved in Herbal Drugs Technology Laboratory, Pharmacy Department, The M. S. University of Baroda, Vadodara bearing specimen no. HDT/MR/20041801 for *L. reticulata*, HDT/MR/20042503 for *D. volubilis* and HDT/MR/20041101 for *P. microphylla*. The collected plant materials were first dried in sun and then under the shade. The aerial parts were then powdered using pulverizer and used for further studies.

The literature review indicated the plants were subjected to preliminary phytochemical and microscopic studies individually. As the plants form the controversial sources of a traditionally used plant drug, the comparative studies were performed which were thought to be helpful in differentiation of such controversial plants.

All the selected plants, belong to the same family, the microscopic studies of transverse section of different parts of the plants did not reveal much information of differentiation. The microscopic evaluation of powdered aerial parts revealed that *P. microphylla* is devoid of trichomes, while *D. volubilis* and *L. reticulata* consisted of

multicellular, unicellate covering trichomes. The powdered aerial part of *L.reticulata* consisted of collapsed cell covering trichomes which were absent in *D.volubilis*. This is the main microscopic feature useful to differentiate *L.reticulata* from other two plants.

Preliminary comparative Phytochemical studies revealed absence of the alkaloids and volatile oil in all the plants while the flavonoids, triterpenoids, steroids, amino acids, sugars and saponins were found present. The confirmation of phytoconstituents detected in qualitative analysis after postchromatographic derivatization using HPTLC technique was undertaken. The reported chromatograms confirmed the nature and content of different phytoconstituents in all the three plant drugs. The extracts of the plants, when subjected to Correlative –TLC (Co-TLC), revealed the presence of some of the compounds, especially of steroidal/triterpenoidal nature on the similar  $R_f$  value. The UV spectra of these compounds was recorded using TLC scanner, showed similarity among the nature of phytoconstituents of both the extracts of all the three plants. Relative amount of these common compound was also determined in Pet. ether and chloroform extract of all the three plants.

The results of HPTLC studies indicated Ethyl acetate fraction of the methanolic extract revealed the presence of flavonoidal aglycones only, while Ethyl acetate fraction of aqueous extract showed the presence of triterpenoidal/steroidal and also flavonoidal aglycones. These studies indicated that triterpenoidal/steroidal and flavonoidal glycosides are present in all the selected plants.

Detailed analysis of the chromatograms indicated that there are more four compounds present in the Pet. ether extract of *L.reticulata* and *P.microphylla* on the similar  $R_f$  value. The area of three common peaks of Pet.ether extract of *L.reticulata* was higher than those of *P.microphylla* indicating the higher content in former drug while in

*D.volubilis* they were not present. Similarly, in chloroform extract also, the compounds present on the similar  $R_f$  value were in higher amount in case of *L.reticulata* than other two plant extracts.

The results justify that *P.microphylla* and *D.volubilis* can serve as substitutes of *L.reticulata* as far as the chemical constituents of steroidal and triterpenoidal in nature are concerned.

However, interestingly, there have been no similarities observed in the components of methanolic extract of all three different plant extracts indicating that the flavonoids of each plant are different in nature. This may be quite uncommon that the plants of same family do not show similarity in constituents of common nature. These extracts were subjected to quantification of separated components, based on the area proportion of the peaks. The results; however did not show any similarities in the content of flavonoidal compounds.

*L.reticulata* is recommended in different diseases and many times is substituted by *P.microphylla* or by *D.volubilis* in practice. Although, the identification standards for *L.reticulata* are available, such systematic standardization parameters are yet to be developed for other two plants used as substitutes. The experiments were, therefore, planned to evolve parameters for establishing the standard value that could be used for comparing these three plant drugs.

The air dried, powdered plant materials were subjected to the determination of various physico chemical constants as per the methods described in WHO guidelines. The results are summarized in comparative manner which provide few of the differentiation and standardization parameters for the selected drugs.

As the metal ions generally act as catalyst or sometimes directly initiate degradation cycle of the phytoconstituents, it is of vital importance to develop the qualitative and quantitative profiles of the inorganic content present in plant materials.

The results indicated maximum amount of the calcium and sodium is present in *P.microphylla*. The results also suggested the absence of unusually high accumulation of the inorganic content in the plants studied. The plant of *P.microphylla* generally found near the sea coast. It is, therefore, not surprising to find comparatively higher amount of sodium and potassium in it. Calcium was present in all the plants in the amount that could not be estimated quantitatively. *L.reticulata* and *P.microphylla* are rich in the magnesium content while *P.microphylla* consists of the maximum amount of zinc. Manganese was estimated in trace amounts in all three plants. Results indicated absence of heavy metals in the plants analyzed.

Direct methanolic extract of the different parts of all the three selected plants was subjected to generate HPTLC fingerprint profile represented as chromatograms. The developed chromatograms differentiated the aerial parts and underground parts of the selected plants from each other. There were certain peaks found considered to be characteristic peaks, as far as the comparative identification is concerned, in the plants studied.

In order to proceed for chemical standardization, some important compounds are identified and selected as markers. These marker compounds should have a consistent presence although, in a variable content in the extracts. These extracts are processed on the basis of constant value of these markers to become standardized extract. Identification of marker, therefore, is an important task, generally achieved by fingerprinting technique and correlative TLC in case of compounds of known chemical nature.



- - Since the presence of salicylic acid was reported in *P.microphylla*, which is used traditionally in the treatment of cuts and burns topically and is also a substitute of *L.reticulata* which is also utilized similarly, having *in vitro* antimicrobial, anti-inflammatory used in inflammation also. There have been no reports of presence of any active compounds for this activity in *L.reticulata*. During detection of active compound presence of **Salicylic acid** in the methanolic extract of *L.reticulata* was **first time noted**, confirmed by Co-TLC studies. However Salicylic acid was not detected in *D.volubilis* extract. Salicylic acid was, therefore, used as biomarker, for assessment of quality of the plant material.

Presence of Stigmasterol was already reported in *L.reticulata*, being responsible for its lactogenic potential. The methanolic extract of aerial parts of *L.reticulata*, *P.microphylla* and *D.volubilis* being the substitutes of *L.reticulata*, were subjected to detection of stigmasterol using Co-TLC. The overlapping spectra of standard and the spectra of the corresponding sample track were recorded, confirmed the presence of Stigmasterol in these plants. All the three plants were, thus, found containing stigmasterol as one of the constituents.

Stigmasterol isolated from the ether fraction of Pet ether extract of the plant *L.retiulata* showed lactogenic potential in rats suggested the potential of Stigmasterol as lactogenic agent in such plants. The compound like Stigmasterol, widely distributed in higher plants, may not be accepted as marker as such, but when the special consideration is taken to the lactogenic potential of the plant extracts, it can be selected as one of the chemical markers which represent the chemical integrity of particular group of compounds responsible for biological potential of the plants.

There are no reports regarding a method on HPTLC which could detect both these compounds in a complex matrix using single mobile phase and TLC run. The studies

were planned to develop a simple, accurate and precise analytical method to estimate both the markers from the selected plants using HPTLC for routine analysis.

The maximum amount of salicylic acid was present in aerial parts of *P.microphylla* while the maximum amount of stigmasterol was present in aerial parts of *L.reticulata*. Since, *L.reticulata* is also used traditionally, as a remedy for diseases related to vision, detection of some compounds responsible for this activity was, therefore, undertaken to estimate  $\beta$  Carotene being a precursor for retinol, which may serve as main agent responsible for the activity. The acetone extract of *L.reticulata* along with other two plants, *P.microphylla* and *D.volubilis* were subjected to estimate the content of  $\beta$  carotene using Co-TLC. The studies confirmed the presence of  $\beta$  Carotene in acetone extract of aerial parts of all the three plants. **The presence of  $\beta$  Carotene is reported for the first time in the aerial parts of *L.reticulata*, *D.volubilis*, *P.mcirophylla*.**

$\beta$  Carotene was selected as one of the biomarkers and an analytical method was developed for content determination in the plant extracts. The results indicated aerial parts of *L.reticulata* possesses the highest amount of  $\beta$  Carotene among the selected plants.

The magnitude of information generated regarding herbal drugs increases day by day and at times it becomes rather impossible to retrieve any data, new Software, therefore, was developed as a Microsoft Visual Basic Application having Windows XP interface to compile the data suggesting the probable interface and designing the report pattern as per administrator need. Market samples of *L.reticulata* were subjected to evaluation and data generated were compared with stored data for authentic *L.reticulata* using software.

Complementary and alternative medicines containing radical scavengers exhibit efficient anti oxidant activity owing to their phenolic and flavonoids content. The result of the preliminary phytochemical analysis showed the presence of flavonoids and other phenolic constituents in the methanolic extract. The flavonoids were determined by adopting two complimentary methods. Methanolic and aqueous extract of aerial parts and stem were subjected to these estimations.

The results indicated that the amount of total flavonoidal and phenolics content was maximum in the methanolic extracts of *L.reticulata* among the extract assessed of selected plants.

Since, methanolic extract of *L.reticulata* is available commercially to be incorporated in some topical herbal formulations; stability studies were performed to ascertain the chemical integrity of the phytoconstituents present during long time storage of this extract. Methanolic extract of the selected plants were subjected to the accelerated stability studies as per the regulatory guide lines. Morphological evaluation and estimation of total phenolics and flavonoidal content were selected as evaluation parameters. The results of the experiments showed the trend of decrease in the content of total phenolic and total flavonoids present with time.

Five Different samples under the name of *Jivanti* were purchased from local market. The powdered aerial parts, stem of *L.reiculata*, aerial parts of *D.volubilis* and aerial parts *P.microphylla* were used as positive control and the samples were subjected to the process of standardization using the determination of certain standardization parameters and the results were, then, compared with that of the standard. The results thus revealed that *D.volubilis* and *P.microphylla* both are sold with the name of *L.reticulata* being *Jivanti* in the practice and the standardization parameters developed, are capable of differentiating the plants from each other.

An attempts to standardize marketed and laboratory formulations, was made on the basis of parameters mentioned in official *Ayurveda* books. In addition, peroxide value, total retinol content and moisture content of ghee were also determined. The peroxide value and Acid value of marketed formulations was high compared to that of the formulation prepared in laboratory. The content of Vitamin A estimated from the products was comparatively lesser than that of the lab formulation. Saponification value and Iodine value of market formulations remains almost same as that of the lab formulation. Moisture content of lab formulation was lesser than that of the market formulations. The market samples were also assessed to detect the presence of adulteration in the *ghee*. Since acid value and peroxide value was high even in case of fresh product prepared in laboratory, it appears that some of the ingredients, added may be contribute for this increase. The values in case of marketed and fresh product were almost were close, indicating no degradation during storage conditions.

During preparation, the effect of heat on the chemical integrity of the product was determined on the basis of the values of few of the selected chemical parameters. The results indicated that Acid value and Peroxide value were high when the temperature and time of the heating was enhanced, alongwith reduction in content of total vitamin A. Heating at 115<sup>0</sup> C for 15 minutes, however, be considered optimum with least amount of degradation of the product.

The presence of flavonoidal and steroidal moieties was confirmed in the methanolic extract of all the selected plants respectively. These phytoconstituents are believed to be therapeutically active; however, these two extracts were selected for further evaluation of the plants for adaptogenic properties.

Acute toxicity study of selective extracts indicated that, these were well tolerated up to 1000 mg/kg body wt of the animals.

The Rejuvenation therapy is known as *Rasayan chikitsa* in *Ayurveda*. Traditionally, *Rasayana* drugs are used against a plethora of seemingly diverse disorders with no pathophysiological connections according to modern medicine. The presence of flavonoidal and compounds are correlated with the antioxidant potential of the plants. There are several flavonoidal compounds isolated from the different extract of *L.reticulata* and *D.volubilis*. Phytochemical studies revealed the presence of flavonoidal and phenolics component in methanolic and aqueous extracts of all three selected plant drugs.

These plants are also used as stimulant and tonic in traditional medicine, the methanolic and aqueous extracts, of selected plants, were screened for antioxidant potential by adopting reported methodologies *in vitro*. The results confirmed the ability of extracts of the selected plant drugs, in scavenging the generated radicals.

The literature already reported the presence of flavonoids in *L.reticulata* and in *D.volubilis*, while there was detected for the first time in methanolic extract of *P.microphylla*. There are many reports appeared in literature regarding the hepatoprotective potential of flavonoidal type of compounds and these are believed to act due to their radical scavenging activity. The methanolic extracts of all the three selected drugs were, therefore, subjected for evaluation of hepatoprotective activity using reported model in rats.

Paracetamol, being hepato-toxin, acting by generation of free radicals; was used to induce the hepatic toxicity in assessment of preliminary hepatoprotective potential of the plant extract, with a view that, if the results show positive trends then other agents could be tried. The assessment of activities was done by measuring the elevated levels of various biochemical markers as indicators of the liver function.

The results indicated test extracts did not possess significant activity at lower dose level, hence, the studies were repeated using moderately higher dose level, but the activity did not improve.

The extracts were also subjected to determine their hepatoprotective activity *in vitro*, with a view to eliminate a possibility that some biologically active phytoconstituents were destroyed or could not permeate due to complex structure in the *in vivo* studies. The experiments were, therefore, performed to observe effects of the three plant drug test samples, on isolated rat hepatocytes culture *in vitro*.

The results suggested sharp decrease in viability of hepatocytes after treatment with paracetamol. The test extracts however did not show significant improvement in the viability of the hepatocytes.

Although the selected plants are not described as hepatoprotective, but used as general tonic, these were subjected to ascertain the probable role of the plants as liver tonic. The plants, however, did not provide the reasons of usage as hepatoprotective against chemical induced liver toxicity; these may have indirect effect as adaptogenic agents.

The entire plant of *L.reticulata* (*Asclepidaceae*) is extensively used as lactogen traditionally in veterinary practice and has almost reached to a level of extinction due to excessive consumption. The plants of *Dregea volubilis* and *Pentatropis microphylla* (*Asclepidaceae*) are now used as its substitute and sometimes replace the original drug as lactogen. The lactogenic potential of these drugs was studied in female rats using, pup weight, weight of mother, parenchyma percentage, secretary rating, estimation of total protein content, glycogen content of mammary glands tissues and serum prolactin as assessment parameters.

The increase in formation of milk requires more amount of normal sugar and proteins, therefore proportionate increase in reserves of tissue glycogen and proteins are

necessary. The arithmetic mean amount of glycogen and total protein content in mammary tissues of treatment groups were found higher than those of the control groups, showing higher metabolic state of the tissues analyzed.

The intensity of observed alterations in histology slides of the mammary glands, expressed in the form of secretory ratings in case of all the treated groups the secretory ratings of the tissues increased significantly in a dose dependant manner, when compared to the respective control groups. The parenchyma percentage is calculated as the ratio of area of parenchyma cell to the ground tissues in mammary gland stated in percentage. In present studies there has also been an increase observed in all groups.

The increment in body weight of pups and decrease in mothers' weight were observed and reported in percentage. The decreasing trend towards loss in mother rats' body weights leads towards a conclusion that the effect may be secondary to lactogenesis in mothers.

The results indicated *P.microphylla* is better substitute of *L.reticulata* when compared to *D.volubilis*. The studies also justify the selection of Stigmasterol as marker for lactogenic potential of the plants and plant extracts.

The serum level of prolactin in female rat was estimated on 3<sup>rd</sup>, 8<sup>th</sup> and 13<sup>th</sup> day of the pup birth. The experiments were performed using the principle of bioluminescence in Immuno assay using the kit meant for estimation of prolactin in human. The experiments were performed using the facilities available at commercial pathology laboratory by simulating the determination of prolactin in human serum. The results obtained were, however, not convincing and not statistically significant may be due to nonspecificity of the antibody used, to the rat prolactin.

The permeation studies in Franz diffusion cell and using rat intestine as membrane were performed to detect the compounds which permeate through the membrane which may be responsible for the lactogenic action.

The results of the permeation studies revealed that the triterpenoids/steroids from Pet ether extract and flavonoids from methanolic extract were able to permeate through the membrane. The compounds could pass *in vitro* through the membrane confirming the lactogenic potential.

The comparative lactogenic potential of laboratory formulations prepared using modern method as well as those prepared by traditional method gave interesting results. The Pet. ether extract of *L.reticulata*, when formulated as o/w emulsion, administered to animals, showed increased activity, than that of the traditional formulation.

The enhancement of the activity as shown by Pet. ether extract in lab formulation and presence of Stigmasterol in considerable amount in this, clearly indicate the efficacy of formulation and justifies its traditional methodology of preparation.

The plants selected are termed as *Rasayana* in *Ayurveda*, which are correlated with adaptogens of modern therapy. In order to access adaptogenic potential of the selected plants, a well accepted model of swim endurance test was used. The methanolic extracts of *L.reticulata*, *P.microphylla* and *D.volubilis* were used in stress induced rats. The stress condition was induced by enforcing the animals to swim and the changes in various biochemical parameters under the stressed conditions were measured.

In general, the level of Serum GPT was increased while the level of other selected markers like, serum glucose, serum cholesterol, serum triglycerides and serum blood urea nitrogen was appeared to be decreased during the stress condition, observed in



control group. The animals treated with methanolic extracts of these drugs showed marked changes in these parameters, thus, protecting the animals in the stress conditions. The methanolic extract showed this effect in dose dependant manner.

It is well accepted fact that, *Rasayana* drugs act through diversified mechanisms, as some of these are radical scavengers, hepatoprotective agents and offering improvement of positive metabolic drives.

All the drugs presently studied, prevented the animals from stress generated during swim endurance test due to physical strain. These drugs also improved the post partum lactation in the rats, thereby, exhibited the capacity of the drugs to improve the positive metabolic drive

The animal activity, thus, indicated all the three selected drugs described as *Rasayana* in *Ayurveda* are adaptogenic and they act by improving the metabolic drives and offer the protection to the living system against the physical strains.

The studies undertaken could be consolidated to provide convincing facts on the usage of all the three selected plant drugs, described as *Rasayana* in *Ayurveda*, effective as adaptogenic agents.