A

Summary of Ph.D Thesis Entitled

PHYTOCHEMICAL INVESTIGATION AND ASSESSMENT OF BIOACTIVITY OF SOME INDIGENOUS DRUGS

Submitted by

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Use of indigenous drugs from plant origin forms a major part of complementary and alternative medicine (CAM) or traditional medicine (TM). TM is attracting more and more attention within the context of health care provision and health sector reform. Many factors are contributing to widespread use of TM. TM is a comprehensive term used to refer to TM systems like traditional Chinese medicine, Indian Ayurveda, Arabic Unani medicines and various forms of indigenous medicine. TM therapies involve use of herbs, animal parts and minerals. It also includes acupuncture, manual therapies and spiritual medicines. Ayurveda remains one of the most ancient and yet living traditions practiced widely in India, Sri Lanka and other countries and has a sound philosophical and experimental basis. According to Ayurveda every individual is different from another and hence should be considered as different entity. Ayurveda generally categorizes individuals in one of the three broad classes of Prakriti based on certain physical, physiological and psychological characters. The concept of constitutional uniqueness of human individuals leading to prescription of suitable drugs and specific diet is a remarkable feature of Ayurveda. There has been an upsurge in the clinical use of indigenous drugs. Side effects and expenses associated with allopathic drugs have provoked the need for research in herbal drugs. Natural products of plant and animal origin offer a vast resource of newer medicinal agents with potential in clinical use. In recent times, focus on plant research has been identified all over the world and large amount of evidences has been collected to show immense potential if medicinal plants used in various traditional systems.

Some plants are believed to promote positive health and maintain resistance against infection by establishing equilibrium of the body. The immune system helps an individual toward establishing an infection free state. The multidimentional response would require proper control through immunomodulation. The immune system can be stimulated or suppressed by various chemical, physical and biological agents. These include steroids, cytotoxic agents and various natural products of microbial, plant or animal origin. The concept of adaptogens relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that theses nonspecific effects give protection against different pathogens including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy.

The suppression of the immune system associated with tuberculosis, cancer, surgery or HIV infection is characterized by the reduction in number and phagocytic function of neutrophils and macrophages as well as reduction in the intracellular bactericidal capacity of these cells. This profound suppression of the individual elements of the system allows opportunistic pathogens to overwhelm the host so that secondary infection becomes most common cause of mortality in such individuals. An attempt to overcome this problem has been made by introducing the concept of prohost therapy. This approach aims at administering drugs to bolster immune defense against infection. Several natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulating agents. The major limitations are increased risk of infections and generalized effects throughout the immune system.

Against this background, the potential of Ayurvedic therapy appears to be encouraging. Ayurveda emphasizes the promotion of health with a concept of strengthening host defense against diseases. The concept of rejuvenation through Ayurveda has been used successfully in the treatment of immunocompromised conditions like AIDS, cancer and hepatic diseases.

Several drugs are mentioned in Ayurveda which possess rejuvenative properties. They are termed as Rasayana drugs. Apart from these drugs, there are several drugs which are not Rasayana drugs but mentioned in the ancient literature as rejuvenators and used in the Ayurvedic formulations along with Rasayana drugs. The present study aims to investigate such plants which are not included in Rasayana but are used in the Ayurvedic formulations along with Rasayana drugs. *P. integerrima* and *H. spicatum* attracted the attention as they are used as tonic and incorporated in several formulations like Chyavaprash along with *A. racemosus*, *W. somnifera*, *T. cordifolia* etc. Present work was carried out to evaluate the phytochemical nature of the compounds and assess the biological activity.

The studies are performed in following manner:

1. Selection of plants and literature survey.
2. Phytochemical investigation by qualitative chemical tests, TLC and HPTLC fingerprint to confirm the nature of the compounds.
3. Preparation of the Aqueous and Methanol extract of both the plants
4. Acute toxicity studied of the extract and fractions of both the plants.
5. Assessment of bioactivity of Aqueous and Methanol extract of selected plants.

6. Fractionation of bioactive extracts using different solvents and assessment of bioactivity of the fractions.
7. Isolation and characterization of compounds isolated from *P. integerrima* and *H. spicatum.*
8. In vitro antioxidant and hepatoprotective activity of fractions and isolated compounds.

The profile consisting of different parameters was prepared so as to provide means of correct identification of plant materials. The parameters include preliminary screening, studies on morphological, microscopical, physicochemical constants etc. The preliminary phytochemical studies showed presence of polar content like phenolics and flavonoids in *P. integerrima* where as *H. spicatum* showed presence of non polar components. Chemical tests showed presence of non polar components were found to be terpenoids. As *P. integerrima* showed presence of phenolics and flavonoids, the extracts and fractions were subjected to determination of total phenolic and flavonoid content.

Both the plants were identified by morphological features and were compared with the official text. Dried leaf galls of *Pistacia integerrima* were hollow, horn like, thin walled, cylindrical and tapering at both ends. The galls were grayish brown internally and reddish brown externally. Size was 25-30cm or more. Each gall contained numerous dead insects. Odor was terebinthine and taste strongly astringent and slightly bitter. Dried rhizomes of *Hedychium spicatum* possess strong aromatic odor and camphoraceous taste. They were available in form of slices 0.5 inch or less in diameter and up to 0.25inch in thickness. The rhizomes were white and starchy within covered by rough reddish brown bark with rootlets attached. The results of morphological determination pose unacceptability, because the plant materials are either available in mutilated form or sold without definite structure of the organ and hence microscopic evaluation remains indispensable and cost effective tool of the conventional analytical pharmacognosy for the identification of the medicinal plant drugs. The important microscopic features of the selected plant drugs have therefore been documented in the present study.

T.S. of *P. integerrima* showed presence of collapsed epidermis on both the sides. Epidermal cells were thin walled and tangentially elongated. Ground tissues were thin walled oval / circular. Outer two layers were tangentially elongated while between the vascular bundles radial & elongated. Outer few layers and some cells of ground tissue were filled with yellowish brown content. Vascular bundles were scattered throughout the

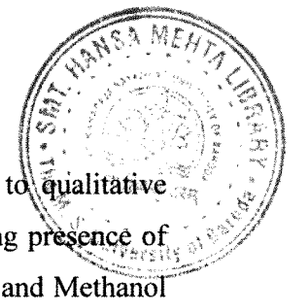
ground tissue in two rows, consisting of phloem accompanied by a large tannin sac in each vascular bundle.

T.S. of *H. spicatum* showed outermost thick layer of suberized dark brown cells of outer cork consisting of 10-15 or more layers of irregular parenchymatous cells. Inner cork consists of few layered brown, rectangular, radially arranged cells followed by wide zone of cortex which was 30-40 cells thick. Some cortical cells were filled with flattened and oval oblong starch grains. Numerous oleoresin cells were found in this region which has suberized walls. They showed presence of yellow green oil. A thin endodermal layer was present beneath the cortex. The central cylinder was distinguished by presence of peripheral plexus of irregular congested vascular bundles with poorly developed mechanical tissues. Vascular bundles were scattered irregularly throughout the ground tissue. Vascular bundles were closed and collateral possessing group of two or more xylem elements. Ground tissue was composed of parenchymatous cells with abundant starch and oil.

Powder microscopy of *P. integerrima* galls showed presence of lignified vascular bundles. Large fibers with tapering end (width 2-4 micron and length 15-20 microns) were observed. The powder showed presence of parenchymatous cells and large tannin sacs. Powder microscopy of *H .spicatum* showed presence of cork cells (width 15-20 microns and length 30-35 microns) and parenchymatous cells. In the ground tissue oleoresin cells were observed. Large numbers of starch grains (10-15micron) were observed when the powder was treated with dilute iodine solution.

Determination of physical constants indicated higher ash value (5.03%) in *H spicatum* than *P. integerrima* (3.33%). The water soluble extractive value in case of *P. integerrima* was (4.52%) and that of *H .spicatum* was (5.16%). Alcohol soluble extractive was also higher in *P. integerrima* (5.62%) where as in case of *H .spicatum* (2.26%). Volatile oil was separated by hydro distillation method and was 0.56% and 0.61% in *P. integerrima* and *H .spicatum* respectively. Both the plant drugs were subjected to evaluation of microbial content. *E. coli* content of *P. integerrima* was $4x10^3$ and that of *H .spicatum* was $7x10^3$. Foaming index was found to be less than 100, as saponins were absent in both the plant drugs. Therefore, plant extract did not show haemolysis. Theses parameters helped in establishing standards of the selected plant drugs.

Preliminary phytochemical screening showed presence of phenolics, flavonoids, carbohydrates and volatile oil in *P. integerrima* and terpenoids, volatile oil and carbohydrates in *H. spicatum*. The successive extracts were prepared with different



solvents with increasing polarity for both the plants and were subjected to qualitative chemical tests. These extracts were further subjected to TLC for confirming presence of different phytoconstituents. Based on the phytoprofiles the whole Aqueous and Methanol extract of *P. integerrima* and *H .spicatum* were subjected to fractionation. Aqueous extract was further fractionated using n Butanol, Ethyl acetate and Methanol and Methanol extract was fractionated using Chloroform, Ethyl acetate, Acetone and residual Methanol extract. Whole Methanol extract of *H .spicatum* was fractionated using Chloroform, Ethyl acetate and residual Methanol. All the fractions were subjected to TLC.

During recent past, HPTLC finger print profile has emerged out as a powerful tool. The inclusion of the profile in modern pharmaceutical herbal monographs has become a standard practice for authentication of herbal drugs especially when the active principals are not known.

All successive extracts and fractions were subjected to HPTLC using three different solvent systems of varying polarity. A complete TLC finger print profile of the resolved compounds comprising of the typical spectra, R_f value, UV absorption maxima and the percentage proportion of the individual components in the extracts are recorded and documented.

Biological screening of medicinal plants is of vital importance, not only to provide scientific basis for their continued usage but also to validate their traditional utilization. Additionally these studies aid to correlate the activity with the components in the plants. Thus biological screening along with chemical profiling provides additional means of standardization of a plant drug. The extracts and fractions were subjected to acute toxicity studies as per OECD guidelines No 423.

The studies were aimed to screen different extracts of *P. integerrima* and *H .spicatum* and their fractions for their adaptogenic activity and thereby to justify their use in the traditional systems of medicine. Whole Aqueous and Methanol extract of both *P. integerrima* and *H .spicatum* were subjected to biological screening. All the extracts except Aqueous extract of *H .spicatum* were found to possess significant activity so these extracts were considered for further fractionation. Aqueous extract of *P. integerrima* was fractionated into n- Butanol, Ethyl acetate and Methanol fractions where as Methanol extract was fractionated using Chloroform, Ethyl acetate, Acetone and remaining Methanol. Chloroform fraction of Methanol extract was not found to be effective. Methanol extract of *H .spicatum* was fractionated into Chloroform, Ethyl acetate and

remaining Methanol extract. All the extract showed significant activity as adaptogenic agent.

The data obtained revealed that extracts of both the selected plants and their fractions exhibit adaptogenic activity by various mechanisms. In case of *E.coli* induced abdominal sepsis the extracts were immunoprophylactic against abdominal sepsis. It was found that there was increase in the WBC and % neutrophils. Mortality was another parameter which was evaluated. In the control group mortality was 100% which was reduced to 33% in the treatment group.

The plants were found to offer protection in cyclophosphamide induced myelosuppression. In the cyclophosphamide treated group there was significant decrease in the WBC count where as the extracts were found to offer protection against myelosuppression. Also there was no alteration in RBC, HB, HCT and MCV values indicating the extracts can be used for long term treatment.

Phagocytosis provides the first line defense to the host against infectious microorganisms. Primary target of most of the adaptogens is believed to be macrophages which play a major role by engulfing pathogens or foreign substances and initiating innate immune response. Phagocytosis by macrophages is important against the smaller parasites. *P. integerrima and H. spicatum* showed significantly high phagocytic index which indicates marked increase in the rate of carbon clearance.

Adaptogenic activity was also evaluated by anoxia stress tolerance model and forced swim model. In the anoxic stress tolerance model, the time for first convulsion in mice was noted. It was observed that after the end of second week the time required for first convulsion to occur was prolonged. In case of forced swim model, the time required till exhaustion was noted. The procedure was repeated for seven days. On 7^{th} day the rats were sacrificed and were subjected to various determinations like biochemical parameters, organ weight and blood count. There was alteration in the biochemical parameters like glucose, triglyceride, BUN and cholesterol. But no change was observed in organ weight.

The studies on the chemical constituents of *P. integerrima* revealed the presence of rich phenolic and flavonoid content. *H .spicatum* showed presence of high terpenoid content. Therefore, flavonoid rich Ethyl acetate fraction of Methanol extract of *P. integerrima* and Methanol extract of *H. spicatum* were subjected to column chromatography and further separated by preparative TLC. Ethyl acetate fraction of Methanol extract of *P. integerrima* yielded three compounds. All the compounds were

identified based on the physical properties. The results were compared with that reported earlier for the authentic samples. All the compounds were subjected to HPLC studies for determination of % purity. PI 1 was found to be 83.6% pure. PI 2, PI 3 and HS were found to have 96-97% purity. From the spectral studies and elemental analysis, nature of the compounds was confirmed. PI 1 was found to be Quercetin, PI 2 was Leuteolin and PI 3 was identified as Gallic acid. A furanoditerpene was identified from Methanol extract of *H. spicatum*.

Preliminary investigations and qualitative evaluation of extracts and fractions of *P. integerrima* revealed presence of phenolics and flavonoids. Therefore, these extracts and fractions were subjected to antioxidant activity by DPPH scavenging assay, hydrogen peroxide assay, reducing power assay and hydroxyl radical scavenging activity. The isolated compounds from Ethyl acetate fraction of *P. integerrima* were of phenolic and flavonoid nature therefore the isolated compounds were also subjected to antioxidant activity.

Both the plants are reported to be used in liver disorders in the ancient literature. To confirm the claims endowed upon the plant drugs, the flavonoid and phenolic fraction from *P. integerrima* was subjected to in vitro screening of hepatoprotective studies against paracetamol induced hepatotoxicity in isolated rat hepatocytes. The diperpene fraction from *H .spicatum* was also subjected to in vitro screening of hepatoprotective studies against paracetamol induced hepatotoxicity in isolated rat hepatocytes. After confirming that the fractions showed significant activity, the isolated compounds were subjected to in vitro hepatoprotective studies. The evaluation was made on the basis of cell viability, SGOT, SGPT and total proteins. Activity of all the fractions and the isolated compounds was comparable to that of silymarin. The activity of flavonoid fraction and phenolic fraction of *P. integerrima* may be due to antioxidant mechanism. In case of *H. spicatum*, furanoditerpene may be responsible for the activity.

Therefore, it can be concluded that the studies carried out on the selected plants can serve as valuable tool and provide suitable standards for the identification of plant materials. Phytochemical screening and the TLC profile of detected phytoconstituents may also serve as reference standards. Studies on the biological activity provide a scientific basis to justify their usage in the traditional system of medicine.

List of Publications:

1. Evaluation of Aqueous and Methanol extracts of *Pistacia integerrima* galls as potent immunomodulator. Joshi Uttara and Mishra S. H. Pharmacognosy magazine. 2008. 4(14): 126-131
2. Preliminary evaluation of immunomodulatory and antistress activity of methanol extract of *Hedychium spicatum*. Pharmacologyonline. 2009. 1(1): 1057-1071.
3. In vitro hepatoprotective activity of isolated diterpene from *Hedychium spicatum*. (Communicated)

List of Presentations:

1. Oral Presentation: Evaluation of immunomodulatory activity of Aqueous and Methanol extracts of *Pistacia integerrima*. Joshi Uttara and Mishra S. H. GUJCOAST, March 2007.
2. Poster presentation: Evaluation of immunomodulatoty activity of Aqueous and Methanol extract of *Hedychium spicatum*. Joshi Uttara and Mishra S. H. 60th IPC, Delhi, December 2008.
3. Oral Presentation: Evaluation of adaptogenic activity of Aqueous and Methanol extract of *Hedychium spicatum*. Joshi Uttara and Mishra S. H. GUJCOAST, January 2009.