A
Summary of Ph. D Thesis Entitled

Development of Methods for the Assessment of Quality and Efficacy of some Traditional Adaptogenic Medicaments

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Summary

Adaptogens constitutes a class of metabolic regulators (of natural origin) are a new class of therapy having the unique ability to switch from stimulating to sedating effects. They are essential to assist the body by acting as stress protectors, which harmonize all body systems, normalize homeostasis, optimize metabolism, revitalize the exhausted organ systems and reduce the fatigue.

Adaptogens are now slowly but surely becoming more widely accepted. If we truly believe stress to be a main contributing factor to many illness, as well as the aging process, and if we truly want to be holistic in our approach by treating causative factors, then adaptogens should be considered as an important aspect of promoting well being. Adaptogens will have major impact on the health care only when the effective herbal formulation are developed that can safely and efficiently deliver to the patient over current synthetic treatment.

WHO in a number of resolutions emphasized the need to ensure the quality of herbs and herbal formulations by using modern techniques and also has drafted the guidelines for the assessment of herbal medicines. These guidelines define basic criteria for the evaluation of quality, safety and efficacy of herbal medicines to assist national regulatory authorities, scientific organizations and manufacturers. Therefore phytochemical standardization using sophisticated analytical techniques along with assessment of it's activity is necessary to ensure quality and efficacy of herbal drugs and this can be done at two levels.

- Standardization by chemical/biomarker compound analysis and standardization by fingerprint techniques/chemo profiling.
- Standardization by assessment of the anticipated activity.

Summary

Therefore, the present studies were conceived to explore roots of two medicinal plants *Mucuna Pruriens* and *Nymphoides macrospermum* which appear in Indian traditional medicine for their potential effects on neuro-endocrine system in order to establish the scientific data pertaining to assessment of adaptogenic activity.

Tagara, a drug mentioned in the Ayurvedic classics, forms an important ingredient of several preparations used in the treatment of diseases, such as anemia, jaundice, bleeding, hemorrhoids, tuberculosis, mental disorders, epilepsy, fever, cough, asthma, brain tonic and general tonic. The botanical source of Tagara is roots of *Valeriana jatamansi* Jones, and *V. hardwickii* Wall. belonging to family Valerianaceae. Both these species occur only in the Himalayas. Tagara is the substitute for *Valeriana wallichi DC.* The literature search revealed that in the state of Karnataka in India, and in other centers of south India, roots of another plant known as Granthika Tagara (Kannada) is used as substitute for Tagara in the markets whereas these belong to the roots of *Nymphoides macrospermum* family Menyanthaceae and no scientific information is available on phytochemical and biological aspects of this plant. However, it forms a substitution for Valerian in southern parts of the country and sometimes replaced by the vendors.

The studies performed are described in four chapters consisting of

- Introduction to Neuro-endocrine system, Stress Physiology its complications and Herbal remedies, selection of the plants and their profile, and the research envisaged.
- Development of the standardization parameters for the selected plants.
- Evaluation of the selected plants for the biological activity.
- Identification and quantification of markers from the bioactive extracts using HPTLC.

Summary

A profile consisting of distinguishing parameters for both the plant materials were developed to provide means of correct identification of individual plant material. The parameters include studies on morphological, microscopical, physico-chemical constants, preliminary phytochemical screening, inorganic elements, including the trace elements and heavy metals, microbial content etc.

Roots of *Nymphoides macrospermum* was procured from commercial vendor in the local market of Udupi .(Karnataka state).and authenticated from Botanical Survey of India, Southern Circle, Coimbatore.

Some important morphological features are documented.

T S of *Nymphoides macrospermum* roots was circular in outline showing outermost single layer Epiblema (Piliferous layer) composed of parenchyma cells with few unicellular long hairs having a very broad base, a distinct inner and outer cortex of parenchymatous cells some filled with starch grains. A single layer of Endodermis containing barrel shaped parenchyma cells few filled with larger starch grains and Stele showing a single layer of thin walled parenchymatous Pericycle, xylem and phloem units arranged alternately on different radii and large and thin walled parenchymatous Pith (Medulla).

Powder microscopy of *Nymphoides macrospermum* roots revealed the presence of sclereids, oil glands, root hairs, starch grains, wood elements and cortical parenchyma.

Determination of physico-chemical constants indicated a higher ash value (5.3%w/w) and alcohol and water-soluble extractive value (9.3 and 14% w/w respectively) The roots of *Nymphoides macrospermum* were tested for the presence of heavy metals and other essential inorganic elements. These were found devoid of cadmiuum and copper. Manganese, potassium and zinc content were

found to be 0.736, 0.706 and 0.255mg/g respectively, with high content of sodium (1.513mg/g) and Iron (4.384mg/g). Traces of lead (0.024 ppm), Mercury (0.0466 ppm) and Arsenic (1.579 ppm) were also found which are within the limits of WHO specifications for heavy metal content.

Preliminary phytochemical studies of *Nymphoides macrospermum* revealed the presence of terpenoids and phenolics as chief constituents. Pet ether, Chlorofom and Aqueous extracts were found to contain higher percentage of extractives (3.23, 1.21 and 14.63 % w/w respectively) than Ethyl acetate and Methanol extracts (0.72 and 0.93 % extractives respectively). Based on the results of preliminary phytochemical screening, the phytoconstituents of *Nymphoides macrospermum* roots can be extracted using methanol as solvent. Therefore, total methanol extract was prepared and screened for adaptogenic activity.

Biological screening of medicinal plants is of vital importance, not only to provide a scientific basis for their continued usage but also to validate their traditional utilization. Additionally these studies aid to correlate the activity with some components of the plant. Thus, biological screening along with chemo profiling provides additional means of standardization of the plant drug.

The stressor is the event that induces changes in the organism (the classical stress response). The stressor could be biological (infection), physical (immobilization, restraint, and extreme temperatures), chemicals (drugs, ethanol) or psychological (grief, conflict).on exposure to stressor, organism reacts vastly differently in terms of the neuro-endocrine responses.

The immune system also plays an important role in biological adaptation, contributing to the maintenance of homeostasis and to establishment of body integrity. Hence, the experimental work related to adaptogenic effect should

not only explore the anti-stress effect, but should also account in the improvement of defense mechanism of the host.

The studies were aimed to screen methanol extracts of roots of *Nymphoides macrospermum* for anti-stress and immunomodulatory activities using different models. Immunomodulatory activity was proved with Antibody titre, DTH response, Phagocytic function, E coli induced abdominal sepsis, and Cyclophosphamide induced myelosuppression models in mice and Anti-stress activity by swimming endurance test.

Methanol extract of the roots of *Nymphoides macrospermum* exhibited stimulatory effects on humoral immunity (HA titre), cellular immunity (DTH response) and phagocytosis (Carbon clearance and E coli induced abdominal sepsis), and also protection against Cyclophosphamide induced myelosuppression. It also reversed the stress induced elevations in the levels of Glucose, Cholesterol, SGPT, SGOT, Urea Nitrogen and reduction in Triglycerides. Thus, roots of *Nymphoides macrospermum* exhibited adaptogenic activity by altering various biochemical parameters during stress, and by stimulating the cellular defence mechanism.

Thus, the results of anti-stress and immunomodulatory activities of total methanol extract revealed the roots of *Nymphoides macrospermum* holds promise as adaptogens. Therefore, the successive extracts were investigated for the same bioactivity to identify the bioactive constituents.

In toxicity studies the successive extracts of roots of *Nymphoides macrospermum* did not show any toxicity or mortality when Dichloromethane (NMRDCME), Ethyl acetate (NMREAE) and Methanol extracts (NMRME) administered

orally up to maximum dose of 550 and 2000mg/kg respectively. Hence Dichloromethane extract at 25, 50 and 100mg/kg, Ethyl acetate and Methanol at 50, 100 and 200mg/kg dose levels respectively were screened for their adaptogenic activity using Phagocytic function, DTH response, Antibody titre and E coli induced abdominal sepsis as models.

Biological studies of successive extracts revealed that only the Dichloromethane extract of *Nymphoides macrospermum* exhibited a statistically significant increase in DTH response, HA titre, Phagocytic index and protection against Cyclophosphamide induced myelosuppression in immunomodulatory activity and also reversed swim stress induced elevations in the levels of Glucose, Cholesterol, SGPT, SGOT, Urea Nitrogen and reduction in Triglycerides while the other extracts failed to produce any such response.

This indicates that the stimulating effects on humoral immunity by NMRDCME may be due to enhanced responsiveness of macrophages and T and B lymphocytes involved in the antibody synthesis. Increase in DTH response may be due to stimulatory effect on lymphocytes and necessary cell types required for the expression reaction. Increase in carbon clearance index and protection against E coli induced peritonitis may be due to enhancement of phagocytic function of mononuclear macrophage and non-specific immunity and protection against the myelosuppressive effects of cytotoxic drug, Cyclophosphamide may be by bone marrow stimulation activity.

Swim stress is one of the most severe stressor; it involves both psychological and physical stress, which leads to stimulation of Hypothalamo-pituitary adrenal axis, triggering the release of corticotrophin releasing factor from hypothalamus with subsequent ACTH secretion. This causes adrenal glands to

secrete glucocorticoids elevating the levels of blood sugar, cholesterol, GOT, GPT, Triglycerides etc. The anti-stress activity of NMRDCME may be due to prevention of HPAA activation and can thus preventing stress-induced elevation in biochemical markers.

During the 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 principles are not known. The characteristic HPTLC finger print profiles of the important chemical constituents of the roots of *Nymphoides macrospermum* were generated using four different solvent systems of varying polarity.

HPTLC fingerprinting profile of DCM extract of *Nymphoides macrospermum*, was generated in solvent systems of different polarities in order to ascertain the total number of chemical moieties. Comparative TLC studies were performed with the DCM extract of *Nymphoides macrospermum* roots and *Valeriana wallichi* roots which revealed the presence of very closely related constituents confirming the presence of Iridoids, further from GC-MS analysis of DCM extracts of *Valeriana wallichi* and *Nymphoides macrospermum* it was observed that the gas chromatogram of DCM extract of *Nymphoides macrospermum* was different from that of the DCM extract of *Valeriana wallichi* but the mass fragmentation pattern of the constituents in both the extracts was similar to some extent suggesting the presence of a same parent moiety (iridoid) cyclopenta-(c)-pyran ring with some similar functional groups.

Summary

The phytochemical studies showed presence of terpenoidal moieties in the DCM extract of the roots and while performing co-TLC studies with some of similar compounds available the Rf of one of the components corresponded with that of Betulinic acid thus revealed its presence. Therefore, simple, sensitive, specific and reproducible HPTLC method was developed for the quantification of betulinic acid in *Nymphoides macrospermum* roots as marker constituents to ensure identity and quality of *Nymphoides macrospermum.*

DCM extract of roots of *Nymphoides macrospermum* was subjected to column chromatography over silica gel and eluted with Hexane: Ethyl acetate, three compounds NMC_1, NMC_2 and NMC_3 were isolated. NMC_1 on IR, ^{H}NMR, CHN and Mass spectral analysis was characterized to be **17-isobutyl Gonane (17-Isobutyl-hexadecahydro-cyclopenta(a)phenanthrene).**
NMC3 was identified as **Betulinic acid**. Mass fragmentation studies of NMC2 suggests it may be Valerenolic acid like compound.

In *Valeriana wallichi* DC. terpenoids and their esters named valepotriates (notably valtrate and dihydrovaltrate); their decomposition products, the baldrinals and various components of the essential oil, in particular, the valerenic acid derivatives, are considered the most important compounds responsible for the biological activities of *Valeriana wallichi* DC. Iridoids (Triterpenoid esters) are a class of secondary metabolites found in many medicinal plants. Iridoids are known to exhibit a wide range of bioactivities including cardiovascular, antihepatotoxic, cholorectic, hypoglycemic, anti-inflammatory, antispasmodic, antiviral, anti-tumor and immunomodulator and purgative activities. It is known that CNS effects of Valerian is mediated through modulation of $GABA_A$ receptor functions. Similarly, *Nymphoides*

macrospermum has also been proved to possess anticonvulsant and sedative property probably with the same mechanism.

Adaptogens modulate inflammatory cytokines: They down-regulate the tumor promoter IL-6 and TNF-α, up regulate IL-2, 10 & 12, and α and β-interferons. Stress and inflammation increase IL-6 production. Many adaptogens Ginsenosides, Withanolides etc, have demonstrated an ability to reduce IL-6 and/or TNF-α.

Nuclear factor-kappa B plays a central role in regulation of many immune, inflammatory and carcinogenic responses. The NF-kB transcription factor family represents an important group of regulators of a broad range of genes involved in cellular responses to inflammation and stress signals. Adaptogenic agents are believed to suppress the transformative, hyperproliferative and inflammatory processes that initiate carcinogenesis. Natural products interfering with NF-kB activation process are most notably curcumin, green tea, 6-gingerol (ginger) resveratrol, Ursolic acid, Lupeol esters etc Valerinic acids (Valerenal, Acetoxy valerenic acid, valerenolic acid etc) are reported to inhibit the activation of NF-kB and its regulated gene expression induced by carcinogens. Betulinic acid a pentacyclic triterpene is reported to induce apoptosis in neuroblastomas and glioblastomas through the mitochondrial activation pathway. Betulinic acid was also found to be active against HIV, and inflammation. Molecular basis for the ability of Betulinic acid to mediate apoptosis, suppress inflammation, and modulate the immune response is by inhibitng of activation of NF-kB and NF-kB-regulated gene expression induced by carcinogens and inflammatory stimuli.

Summary

The results of the above studies suggests that *Nymphoides macrospermum* roots holds promise as adaptogen and the activity may be endowed upon the presence of betulinic acid and other terpenoids .

Mucuna pruriens Linn., is another popular and important medicinal plants of India commonly called as common Cowitch, Velvet bean and Cowhage it belongs to family Leguminoseae. All parts of *Mucuna pruriens* possess valuable medicinal properties. *Mucuna* finds traditional use in number of diseases. Root is bitter, thermogenic, emolient, stimulant, purgative, aphrodisiac, diuretic, emmenagogue, anthelmintic, febrifuge and tonic. The Ayurvedic usage of roots still extends for constipation, nephropathy, strangury, dysmenorrhoea, amenorrhoea, elephantiasis, dropsy, neuropathy, ulcers, fever and delirium.

Roots of *Mucuna pruriens* were collected from the outfield of Vadodara city, Gujarat, and were authenticated from Botanical Survey of India, Southern Circle, Coimbatore.

A profile consisting of distinguishing parameters was developed to provide means of correct identification of the plant material.

Some important morphological and microscopic features are documented

Microscopic studies of the roots of *Mucuna pruriens* showed the presence of Periderm clearly differentiating into a narrow cork with tangentially elongated cells, narrow secondary cortex of thin-walled, parenchymatous cells, few containing dark brownish contents. Phloem-Secondary forming bulk in the form of long, radial strips, Xylem -Secondary very wide composed of usual elements, vessels large as well as small, surrounded by xylem parenchyma and fibres, 1-2 layers of Medullary rays extending from xylem up to phloem and a distinct cambium.

Summary

Powder microscopy of roots of *Mucuna pruriens* showed the presence of vascular elements, cork cells and sclereids.

Determination of physico-chemical constants indicated Alcohol and water-soluble extractives were found to be 4.1 and 1.28% w/w respectively. Ash content represents the mineral component of a plant material, a higher level of ash value 4.0 %w/w was observed in the roots indicating higher inorganic/mineral content like that of seeds.

Determination of inorganic elements, including the trace elements and heavy metals has gained outstanding importance in the life sciences. Studies on the mineral composition of the roots of *Mucuna pruriens* revealed that it contains potassium (0.931 mg/g), zinc (0.139mg/g), sodium (3.46 mg/g), Iron (0.919mg/g) and devoid of manganese, copper and cadmium. Seeds are reported to contain higher levels of potassium (806-2790 mg/100g), sodium (31-104mg/100g), zinc (1-15mg/100g), copper (0.33-4.34mg/100g), manganese (0.56-9.26 mg/100g) and iron (1.3-15mg/100g). The roots were found to contain traces of lead (0.02 ppm), Mercury (0.0287 ppm) and Arsenic (0.603 ppm) which are within the limits of WHO specifications for heavy metal content.

Preliminary phytochemical studies of *Mucuna* roots revealed the presence of phytosterols, phenolic compounds, amino acids and amines as chief constituents. The percentage yield of Pet ether, Methanol and Aqueous extracts were found to be higher (2.64, 3.18 and 1.22 % w/w respectively) than Chloroform and Ethyl acetate extractives (0.17 and 0.20 % w/w). Therefore, total methanol extract was prepared and screened for bioactivity.

The methanol extract of roots of *Mucuna pruriens* was screened for anti-stress and immunomodulatory activities using different models. Immunomodulatory

activity was proved with Antibody titre, DTH response, Phagocytic function, E coli induced abdominal sepsis, and Cyclophosphamide induced myelosuppression models in mice and Anti-stress activity by swimming endurance test.

The data obtained reveal that oral administration of methanol extract of the roots of *Mucuna pruriens* exhibited stimulatory effects on humoral immunity (HA titre), cellular immunity (DTH response) and phagocytosis (Carbon clearance and E coli induced abdominal sepsis), and also protection against Cyclophosphamide induced myelosuppression.

Methanol extract of roots of *Mucuna pruriens* also reversed the stress induced elevations in the levels of Glucose, Cholesterol, SGPT, SGOT, Urea Nitrogen and reduction in Triglycerides..

Based on the results of adaptogenic activity of total methanol extract the successive petroleum ether, ethyl acetate, methanol and aqueous extracts of roots of *Mucuna pruriens* were investigated for the same bioactivity to identify the bioactive constituents.

Pet ether extract at 25, 50 and 100mg/kg, Ethyl acetate, Methanol and Aqueous extracts at 50, 100 and 200mg/kg dose levels respectively were screened for the same activity.

Pet ether, and Methanol aqueous extracts of roots of *Mucuna pruriens* exhibited a statistically significant increase in DTH response, HA titre, Phagocytic index and protection against Cyclophosphamide induced myelosuppression and also

reversed swim stress induced elevations in the levels of Glucose, Cholesterol, SGPT, SGOT, Urea Nitrogen and reduction in Triglycerides while ethyl acetate extract failed to produce any such response. Pet ether extract of roots of *Mucuna pruriens* showed higher content of sterols while the Methanol and aqueous extracts of revealed the presence of phenolics, amino acids etc.

A comparative HPTLC fingerprinting profile of Pet ether, Methanol and aqueous extracts of *Mucuna pruriens* roots and seeds were generated in solvent systems of different polarities in order to ascertain the total number of chemical moieties.

L- dopa and β-sitosterol are the chemical markers reported in the seeds of *Mucuna pruriens*, co-TLC studies of roots of *Mucuna pruriens* (with β-sitosterol and L-dopa) revealed the presence of β-sitosterol and L-dopa. Therefore, simple, sensitive, specific and reproducible HPTLC methods are developed for the quantification of β-sitosterol and L-dopa in *Mucuna pruriens* roots as marker constituents to ensure identity and quality of *Mucuna pruriens*.

L dopa content of the roots by this proposed method was found to be 0.171 %.and the β-sitosterol content of the roots was found to be 0.076 %.

Mucuna pruriens is reported to contain many diverse phytochemicals like alkaloids, L-dopa, alkylamines, phenolics, saponins, proteins, minerals, fatty acids, sterols , serotonin, carbohydrates, amino acids etc.

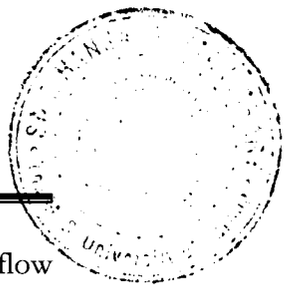
Seeds are reported to contain phytosterols like Beta-sitosterol, campesterol and stigmasterol etc. Pet ether extract of roots of *Mucuna pruriens* showed higher

content of sterols while the methanol and aqueous extracts revealed the presence of phenolics, amino acids etc.

Phytosterols are synthesized from triterpenes and are ubiquitous among angiosperm species. The role of phytosterols in adaptogenic activity has not been emphasized in the phytotherapy literature, their importance to nutrition is well recognized. Beneficial effects of phytosterols to both normal and compromised immune systems is been established.

It is been suggested that beta-sitosterol can enhance secretion of IL-2 and gamma interferon helping to promote natural killer cells, and prime TH1 helper cells to steer the focus away from the TH2 helper cells. It has been reported that beta-sitosterol has anticancer, antiulcer, antidiabetic, antiinflammatory antipyretic anthelmintic, antipyretic and analgesic properties.

In strenuous conditions, the physical performance of the organism is dependent on the availability of appropriate macro- and micronutrients required in excess on account of their increased utilization during stressful situations Amino acids like L-arginine and Glutamine supplementation enhances adrenocortical hormone, luteinizing hormone and follicle-stimulating hormone response to corticotropinreleasing hormone. Under certain metabolic, developmental or pathophysiological conditions, some of the non-essential amino acids become essential and are known as 'conditionally essential'. Arginine and glutamine are known to be conditionally essential amino acids. L-Arginine plays important roles in the urea cycle, protein synthesis, as a precursor of polyamines and creatine, and as a substrate for synthesis of nitric oxide (NO). NO was shown to be an endothelial-derived relaxation factor, a



vasodilator, which acted as a modulator of vascular tone to regulate blood flow and blood pressure. It is interesting that herbs with adaptogenic activity, e.g. *Panax ginseng*, have been shown to contain large amounts of arginine

Mucuna pruriens is reported to have L-dopa as a major constituent in the seeds as well as roots, L-DOPA is a levorotatory isomer of dihydroxyphenylalanine, a natural amino acid, is the immediate precursor of the neurotransmitter dopamine. Dopamine modulates the immune responses by influencing the cytokine network. Dopamine is an important endogenous catecholamine which exerts widespread effects both in neuronal (as a neurotransmitter) and non-neuronal tissues (as an autocrine or paracrine agent). Within the central nervous system, dopamine binds to specific membrane receptors presented by neurons and it plays the key role in the control of locomotion, learning, working memory, cognition, and emotion. Dopamine also regulates motor control, sex drive, immune function, growth hormone levels, Somatropin release, and motivational behavior.

Therefore, the adaptogenic activity of roots of *Mucuna pruriens* may be endowed upon the presence of β-sitosterol and other phytosterols, L-dopa and other amino acids.

Thus from the present studies it can be concluded that

Mucuna pruriens and *Nymphoides macrospermum* certainly possess adaptogenic activity as evidenced by stimulatory effects on humoral immunity (HA titre), cellular immunity (DTH response) and phagocytosis (Carbon clearance and E coli induced abdominal sepsis), and also provides protection against Cyclophosphamide induced myelosuppression and by prevention of HPAA activation and can thus preventing stress-induced elevation in biochemical markers in anti-stress activity. Thus, both the plants exhibit adaptogenic activity not only by altering various biochemical markers during stress, but also by stimulating the immune system.

Thus, it can be concluded that the pharmacognostical studies carried out on both the plants, can serve as valuable tool and provide suitable standards for the identification of these plant materials. Phytochemical screening and the TLC profile of detected phytoconstituents may also serve as reference standards, quantitative HPTLC assay methods developed for betulinic acid in *Nymphoides macrospermum*, beta sitosterol and L-dopa in *Mucuna pruriens* can serve for the routine quality control of commercial samples and studies on biological activity helps in justifying the traditional claims endodowed upon these drugs in scientifically accepted manner.