CHAPTER – 2

Materials & Methods

(Nymphoides macrospermum)

Present section deals with detailed description of methods employed for carrying out different studies on the roots of *Nymphoides macrospermum* categorized into following headings.

Pharmacognostical parameters

- Macroscopical studies
- Microscopical studies
- Determination of physicochemical constants

Determination of inorganic elements including the heavy metals

Determination of Microbial content

Phytochemical parameters

- Preliminary phytochemical studies
- HPTLC fingerprint profile of the successive extracts of the selected plant materials
- Determination of Phenolic content.
- Estimation of suitable marker content in the extract.
- Isolation and characterization of the compounds from bioactive extract/fraction.
- Development of HPTLC method for quality control of the selected plant materials using a suitable marker.

Biological parameters

- Toxicity studies of the extracts of the selected plant materials
- Evaluation of the extracts for anti-stress and immunomodulatory activitites using suitable models .

2.1 Pharmacognostical parameters

2.1.1 Collection and identification of plant material

Roots of *Nymphoides macrospermum* was procured from commercial vendor in the local markets of Udupi (Karnataka), and authenticated from Botanical Survey of India, Southern Circle, Coimbatore. Voucher specimens(NM/05-06/04/KM) have been deposited in the Pharmacy Department of The M.S University of Baroda, Vadodara, India.

2.1.2 Preparation of powdered material

The roots of the selected plants were first properly cleaned and then dried under shade. The shade dried plant materials were then subjected to size reduction using a mechanical pulverizer to a coarse powder, which was used in further studies.

2.1.3 Macroscopical studies

The roots of *Nymphoides macrospermum* were examined macroscopically using reported methods (Wallis, 1985)

2.1.4 Microscopical studies

Transverse sections of roots were taken using microtome, stained with different stains and examined under microscope. Permanent slides were prepared as per methods described by Johansen (1940) and the photomicrographs of the sections were taken at different magnifications, depending upon the anatomical details to be brought out, using Olympus BX 40 microscope attached with Olympus DP12 digital camera.

Lignified elements: For observing the lignified tissues the sections were treated with a mixture of Phloroglucinol –Concentrated HCl (1:1) on a slide, drain off the reagent and were mounted in glycerin-water mixture, lignified elements take pink or red color (Lala,1981).

Starch: the presence of starch was examined by treating the sections of the plant materials (or powder) with N/50 iodine solution. Starch grains stain blue color (Lala 1981).

Microscopy of powdered roots: Powdered roots was subjected to preliminary tests as described below

Table.2.1 Preliminary tests for powder anaysis.

Preliminary Tests
Odour
Color
Taste
Touch
Test with water/ Aqueous extract
Test for Tannins Extract powder with water , treat filtrate with $FeCl_3$
Test for Mucilage Mount powder on a slide with 1-2 drops of water
Test with H_2SO_4 Treated powder with few drops of H_2SO_4
Test with caustic alkali Treated powder with 5% alkali
Test for oils Pressed a little quantity of powder between two filter papers.

Powdered roots pretreated, with suitable clearing reagents, were placed in glycerin-water mixture on a slide under microscope and observed for presence of diagnostic characters, which were recorded using Olympus BX 40 microscope attached with Olympus DP12 digital camera.

2.1.5 Proximate analysis

Roots of *Nymphoides macrospermum* were subjected to determination of physico-chemical constants using reported methods (Indian Pharmacopoeia, 1996: WHO, 1998)

Following determinations were made

1. Loss on drying: An excess of water in medicinal plant material will encourage microbial growth and deterioration following hydrolysis. Limits for water content should therefore be set for every given plant material. Loss on drying was determined as mentioned below

Accurately weighed (5g) of plant material was placed in a weighing bottle which was previously dried and tarred. The samples were dried at 100-105^{0}C until two consecutive weighing do not differ by more than 5 mg. The loss in weight was calculated with reference to the air-dried plant material.

2. Total Ash:

Controlled incineration of plant drugs results in an ash residue. It usually represents the mixture of inorganic salts and silica naturally occurring in the plan drugs and adhering to it, but it may also include inorganic matter added for the purpose of adulteration.

For determining the total ash about 2g of the powdered drug was weighed accurately in a weighed silica crucible and spread as a fine layer at the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450 ^{0}C until free from carbon then cooled and weighed. The procedure was repeated till a constant weight was obtained. The percentage of the total ash was calculated with reference to the air-dried drug.

3. Acid insoluble ash:

The ash obtained above (total ash) was boiled with 25 ml of hydrochloric acid for 5 minutes. The insoluble ash was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a weighed silica crucible, ignited, cooled and weighed. The procedure was repeated to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried plant material.

4. Water soluble ash: The total ash (obtained as above) was boiled for 5 min with 25 ml of hot water. The insoluble matter was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a weighed silica crucible and ignited at a temperature not exceeding 450 ^{0}C. The procedure was repeated till a constant weight of the insoluble matter was deducted from the weight of the total ash. The difference in weight was considered as water soluble ash. The percentage of water soluble ash was calculated with reference to air dried drug.

5. Alcohol soluble extractives: Extraction of plant (drug) material with solvent yields a solution of different components and the composition of this solution will depend upon the constituents present in the drug and the solvent used.

Method: Powdered plant material (5 g) was macerated with 100ml of methanol (90% v/v) in a closed flask for 24 hrs. The contents of the flask were shaken for first six hours and then set aside for 18 hrs. The contents were filtered and 25 ml f the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 ^{0}C and weighed. The percentage of methanol soluble extractive was calculated with reference to the air dried plant material.

6. Water soluble extractives:

Method: powdered plant material (5g) was macerated with 100 ml of chloroform water in a closed flask for 24 hrs. Contents of the flask were shaken for first six hours and then set aside for 18 hrs. The contents were filtered, and 25 ml of the filtrate was evaporated to dryness in a weighed flat bottom shallow dish, finally dried at 105 ^{0} C and weighed. Percentage of water soluble extractive was calculated with reference to the air dried plant material.

2.1.6 Estimation of in-organic elements including the heavy metals

Elemental content of roots of *Nymphoides macrospermum* was estimated on atomic absorption spectrophotometer at Vaibhav Analytical Laboratory, Ahmedabad.

Instrumentation

The atomic absorption spectrophotometer (AAS) (SYSTRONIC 128), coupled with hydride generator and hollow cathode lamps for different elements including heavy metals was used.

2.1.7 Determination of Microbial content

Roots of *Nymphoides macrospermum* were subjected to determination of Microbial content (Total viable count, Total bacterial and fungal count, E coli, Salmonella spp, S aureus and P aeruginosa) using reported methods (WHO, 1998)

2.2 Preliminary phytochemical studies

2.2.1 Preparation of extracts

Petroleum ether (60-80^0) extract

The coarsely powdered roots of *Nymphoides macrospermum* (100g) were extracted with Petroleum ether (60-80^0) by hot extraction process (Soxhlet) for 4 hrs. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Chloroform extract

The marc left after petroleum ether extraction was dried in air and extracted with chloroform by hot extraction process (Soxhlet) for 4 hrs. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Ethyl acetate extract

The marc left after Chloroform extraction was dried in air and extracted with Ethyl acetate by hot extraction process (Soxhlet) for 4 hrs. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Methanol extract

The marc left after Ethyl acetate extraction was dried in air and extracted with Methanol by hot extraction process (Soxhlet) for 4 hrs. After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Aqueous extract

The marc left after methanol extraction was dried and extracted with chloroform water by maceration process. After completion of extraction, the solvent was removed by evaporation and the residue was concentrated *in vacuo.* Color, consistency and percentage yield of the extracts were noted. The extracts were preserved under vacuum for further phytochemical studies.

2.2.2 Preliminary phytochemical analysis of successive extracts

Following qualitative chemical tests were carried out on vacuum dried successive extracts of the roots of *Nymphoides macrospermum* to identify the presence of various chemical constituents. (Kokate 2005)

Tests for alkaloids:
Mayer's reagent
Dragendorff's reagent
Wagner's reagent
Hager's reagent

Test for steroids/terpenoids
Liebermann Burchard test
Salkowaski test

Test for Glycosides (Anthraquinones)
Borntrager Test
Modified Borntrager Test

Test for tannins and phenolic compounds
Dilute ferric chloride solution
Lead acetate solution
Test with gelatin solution.

Test for flavonoids
Shinoda test (Mg/HCl test- Magnesium turnings test)

Test for Carbohydrates
Fehling's Test
Benedict's test

Test for proteins and amino acids
Biuret test
Ninhydrin test

Test for Saponins
Foam Test

Test for Gums and Mucilage
Precipitation with alcohol
Molisch's test

TLC profile of the extracts obtained by successive solvent extraction.

All the successive extracts of selected plant materials were subjected to TLC studies using various solvent systems to determine presence of various phytoconstituents. The R_f values of observed compounds were noted for all the extracts.

Preliminary phytochemical screening showed the presence of terpenoids/ steroids and phenolic compounds all of which can be extracted using methanol. Therefore, total methanol extract containing the above constituents was prepared and screened for bioactivity.

2.2.3 Preparation of Total Methanolic extract

Coarsely powdered roots of *Nymphoides macrospermum* (500g) was extracted with Methanol by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo*.

2.3 Evaluation for Adaptogenic Activity

2.3.1 Acute toxicity studies

Toxicity study was carried out on total Methanolic extract as per OECD guidelines in female albino mice. Animals were dosed with single oral dose of 2000 mg/kg body weight and observed for mortality. Mice were observed for any reactions like tremors, convulsions, salivation, and diarrhoea. 1/10th of the highest tolerable dose was used as a safe dose for further in vivo studies.

- **Evaluation for Anti-stress activity**

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.

- ❑ Forced swimming stress induced changes in rats.
    1. Changes in the weight of different organs.
    2. Biochemical Estimations

- **Evaluation for Immunomodulatory activity**

The immune system 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

Immunomodulatory activity using following models and parameters.

- Cyclophosphamide induced myelosuppression assay.
- Carbon clearance assay in mice.
- Delayed type hypersensitivity response in rats.
- Haemagglutination antibody titre value.
- E coli induced abdominal sepsis

Animals

Swiss albino mice of either sex, weighing 25-30 g, and Albino Rats, Wistar strain weighing 180-200g housed in standard conditions of temperature, humidity and light were used. They were fed with standard rodent diet and water ad libitum.

Chemicals

Carbon ink suspension Pelican AG, Germany, ink was diluted eight times with saline and used for carbon clearance test in a dose of 10μl/gm body weight of mice. Cyclophosphamide Injection(Endoxan 200mg vial of Zydus Biogen, Cadila healthcare LTD). Dextrose, EDTA, Sodium chloride, Potassium dihydrogen orthophosphate, Disodium hydrogen orthophosphate 96 well micro-titre plates, Micropipette, Vernier calipers, Neubaurs chamber, Microscope , Cylindrical vessel (50 x 40 cm) etc

Bacterial suspension

E coli (Hospital strain, 1x 10^8 cells) are sub cultured overnight in a shaker water bath at 50 rpm. The culture was centrifuged (200 x 9) for 15 mins and the cell pellet was washed 3 times with phosphate buffered saline. The cell count was adjusted turbidimetrically and by haemocytometer. The viability of the culture was confirmed by serial dilution and surface spreading techniques.

Preparation of 20 v/v SRBC suspension

The blood was collected from a healthy sheep from the Baroda municipal slaughterhouse, India, in a mixture of 0.49% EDTA and 0.9% of sodium chloride solution. It was preserved at a temperature of 2–8 °C. On the day of immunization, the blood sample was centrifuged at 5000 rpm for 10 min and then washed three times, to remove plasma, with 0.9% sodium chloride solution. The SRBC (20% v/v) suspension was then prepared in 0.9% sodium chloride solution.

Collect sheep blood in 0.49% EDTA in saline (Equal Volume)

↓

Centrifuge blood to get thick pellet of SRBC

↓

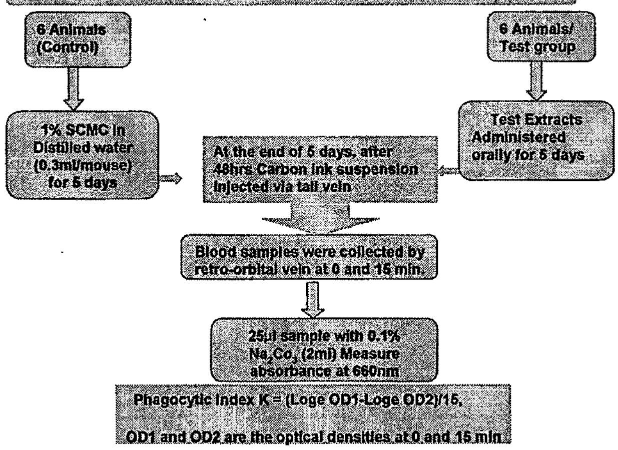
Wash the pellet continuously with saline till u get clear supernatant.

↓

Prepare 20% SRBC (2ml SRBC in 10ml of saline)

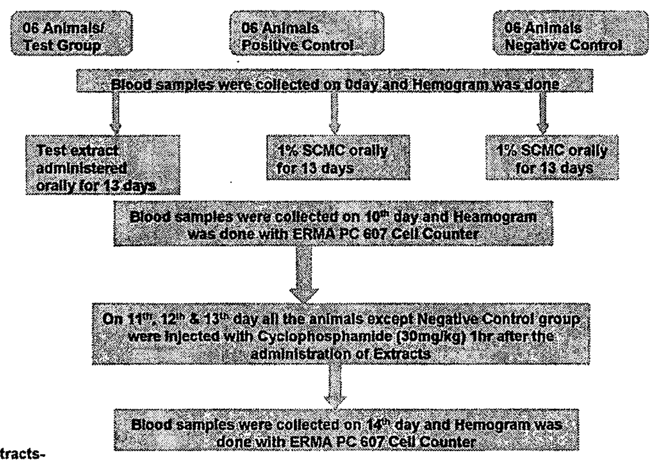
2.3.2 Evaluation for Immunomodulatory activity

Fig. 2.1 Carbon Clearance Test (Gonda et al)



Test Extracts-
NMR (Total methanol extract of roots of *N. macrospermum*) 50,100 & 200mg/kg,
NMDCME (Dichloromethane extract of roots of *N. macrospermum*) 25,50 & 100 mg/kg,
NMEAE (Ethyl acetate extract of roots of *N. macrospermum*) 50,100 & 200mg/kg,
NMRME (successive methanol extract of roots of *N. macrospermum*) 50,100 & 200mg/kg body weight

Fig. 2.2 Cyclophosphamide induced Myelosuppression Assay (Manjrekar et al., 2000)



Test Extracts-
NMR(Total methanol extract of roots of *Nymphoides macrospermum*) 50,100 & 200mg/kg,
NMDCME (Dichloromethane extract of *Nymphoides macrospermum*) 25,50 & 100 mg/kg, body weight

Fig. 2.3 Humoral antibody titre (Puri et al., 1994)

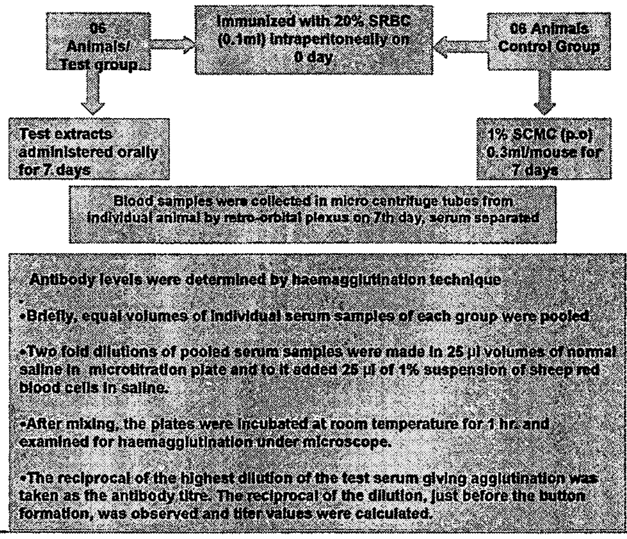
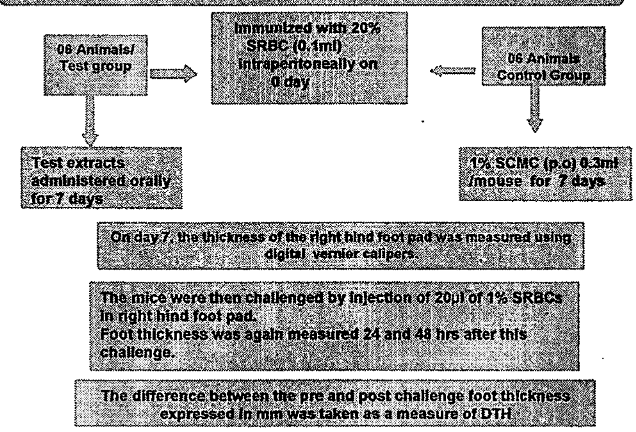


Fig. 2.4. Delayed Hypersensitivity (Puri et al., 1994)



Test Extracts-

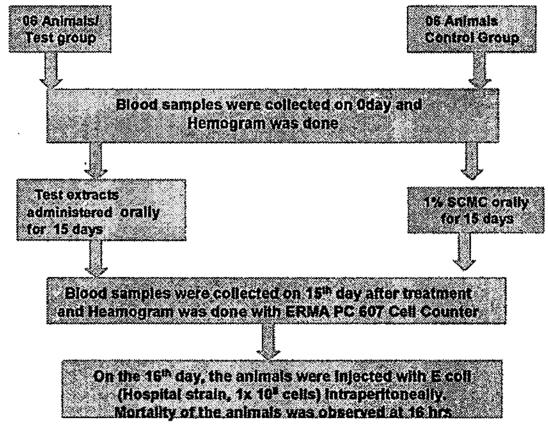
NMR (Total methanol extract of roots of ***N. macrospermum*****) 50,100 & 200mg/kg,**

NMDCME (Dichloromethane extract of roots of ***N. macrospermum*****) 25,50 & 100 mg/kg,**

NMEAE (Ethyl acetate extract of roots of ***N. macrospermum*****) 50,100 & 200mg/kg,**

NMRME(Successive methanol extract of roots of *N. macrospermum*) 50,100 & 200mg/kg body weight

Fig.2.5 E Coli induced abdominal sepsis (Mungantiwar et.al, 1997)

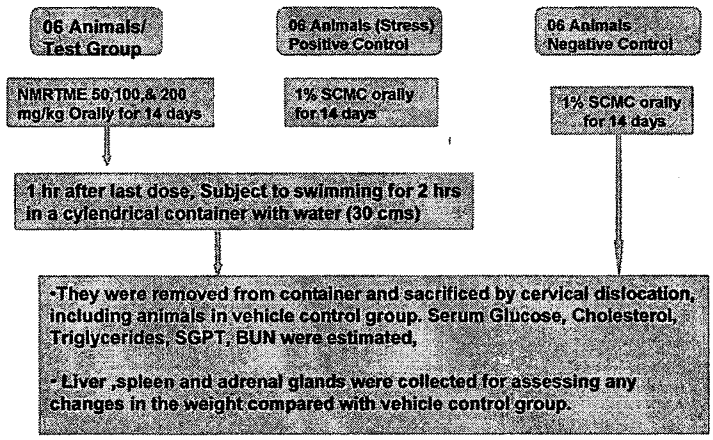


Test Extracts-

NMRTME(Total methanol extract of roots of *N. macrospermum*) 50,100 & 200mg/kg b w.t
NMDCME(Dichloromethane extract of roots of *N. macrospermum*) 25,50 & 100 mg/kg b. w.t
NMEAE (Ethyl acetate extract of roots of *N. macrospermum*) 50,100 & 200mg/kg b. w.
NMRME(Successive methanol extract of roots of *N. macrospermum*) 50,100 & 200mg/kg b. w.t

2.3.3 Evaluation for Anti-stress activity

Fig.2.6 Swimming endurance test
(Bhatwadekar et.al., 1999)



Test Extracts-
NMR (Total methanol extract of roots of *N. macrospermum*) 50,100 & 200mg/kg,
NMDCME (Dichloromethane extract of roots of *N. macrospermum*) 25,50 & 100 mg/kg,

2.3.4 Biochemical Estimations

SGOT (Serum glutamate oxaloacetate transaminase), SGPT (Serum glutamate transaminase), Blood Glucose, Blood urea Nitrogen, total cholesterol and serum triglycerides were determined to assess the effect of swimming endurance stress.

2.3.4a SGPT (Serum Glutamate Pyruvate Transaminase or Alanine transaminase ALT)

2,4-DNPH method of Reitman & Frankel. Autospan Reagent kit (SPAN DIAGNOSTICS)

Principle

GPT (ALT) catalyses the following reaction

α- Ketoglutarate + L-Alanine $\longleftrightarrow$ L-glutamate +Pyruvate

Pyruvate so formed is coupled with 2,4 DinitroPhenyl hydrazine to give the corresponding Hydrazone which gives Brown color in alkaline medium and this can be measured colorimetrically.

Pipette into Tube marked	Test (T)
Reagent 1(Buffered Aspartate)	0.25ml
Incubated at 37^{0}C for 5min	
Serum	0.05ml
Mixed well and incubated at 37^{0}C for 1hr	
Reagent 2(DNPH reagent)	0.25ml
Mixed and allowed to stand for 20min at root temp.	
Solution 1(NaoH)	2.5ml

Similarly prepared standard plot with given standards

Mixed well and allowed to stand for 20min and read the O.D against Purified water on a colorimeter at 505nm. From the standard plot, determined the concentration of the sample.

2.3.4b GOT (Aspartate Transaminase AST)

2,4-DNPH method of Reitman & Frankel. Autospan Reagent kit (SPAN DIAGNOSTICS)

Principle

GOT catalyses the following reaction

α- Ketoglutarate + L-Aspartate $\longleftrightarrow$ L-glutamate +Oxaloacetate

Oxaloacetate so formed is coupled with 2,4 DinitroPhenyl hydrazine to give the corresponding Hydrazone which gives Brown color in alkaline medium and this can be measured colorimetrically.

Pipette into Tube marked	Test (T)
Reagent 1(Buffered Aspartate)	0.25ml
Incubate at 37^{0}C for 5min	
Serum	0.05ml
Mixed well and incubated at 37^{0}C for 1hr	
Reagent 2(DNPH reagent)	0.25ml
Mixed well and allowed to stand for 20min at root temp.	
Solution 1(NaoH)	2.5ml

Similarly prepare standard plot with given standards

Mixed well and allowed to stand for 20min and read the O.D against Purified water on a colorimeter at 505nm. From the standard plot determined the concentration of the sample.

2.3.4c HDL-CHOLESTEROL

(PTA-METHOD) Reagent kit (Reckon Diagnostics) for quantitative estimation of high density lipoprotein (HDL) cholesterol in serum.

Principle

High density lipoproteins (HDL) are separated from other lipoprotein fractions by treating serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipated. Cholesterol content of which is estimated by enzymatic method.

Serum + PTA reagent $\longrightarrow$ Supernatant(HDL) + Ppt (other fractions)

Procedure

Separation of HDL-fraction.

PIPETTE INTO TEST TUBES TEST	
Sample (ml)	0.2
3-HDL cholesterol (ml) (Precipitating reagent)	0.2

Mixed well and centrifuged at 3500-4000rpm for 10min. Separated the clear supernatant immediately to determine cholesterol content.

Reaction parameters	
Type of Reaction	End point
Wavelength	505nm
Flow cell temperature	37^0C
Sample volume	0.05ml
Reagent volume	1ml
Incubation time	10min at 37^0C
Light path	1.0 cm
Zero setting	Reagent

Pipette into Test Tubes	BLANK	STD	TEST
Cholesterol Reagent(ml)	1.0	1.0	1.0
HDL standard (ml)	-	0.1	-
Supernatant (ml)	-	-	0.1
Mixed well and incubated at 37^0C for10min			
Distilled Water (ml)	2.0	2.0	2.0

Mixed well and read absorbance of test and standard against reagent blank at 505nm.

Results

$$\text{Serum HDL-Cholesterol} = \frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times \text{Conc. of Std} \times \text{Dilution factor}$$

$$= \frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 50 \times 2$$

2.3.4d CHOLESTEROL LIQUID

Liquid Reagent for quantitative estimation of Cholesterol in serum or plasma ENZOPAK Reagent kit (Reckon Diagnostics)

Principle

The cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidized by cholesterol oxidase (CO) to cholesten 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compounds in the presence of peroxidase to yield a colored complex which is read at 505nm.

$$\text{Cholesterol esters } \xrightarrow{CE} \text{ Cholesterol + Fatty acids}$$

$$\text{Cholesterol } \xrightarrow{CO} \text{ Cholesterol-4-en-3-One} + H_2O_2$$

$$2\,H_2O_2 + 4AAP \longrightarrow \text{Quinoneimine dye} + 4H_20$$

Phenolic compound

The intensity of the color produced is directly proportional to the concentration of total cholesterol in the sample.

Reaction parameters	
Type of Reaction	End point
Wavelength	505nm
Flow cell temperature	37^0C
Sample volume	0.01ml
Reagent volume	1ml
Incubation time	10min at 37^0C
Standard concentration	200mg/dl
Light path	1cm
Zero setting	Reagent

Pipette into Test Tubes	BLANK	STD	TEST
Cholesterol Reagent(ml)	1.0	1.0	1.0
Standard (ml)	-	0.1	-
Sample (ml)	-	-	0.1

Mixed well and read absorbance of test and standard against reagent blank at 505nm.

Results:

Cholesterol concentration (mg/dl)= $\frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 200$

2.3.4e TRIGLYCERIDES

GPO method ENZOPAK Reagent kit (Reckon Diagnostics) for quantitative estimation of triglycerides in serum .

Principle

Lipase hydrolyses triglycerides sequentially to Di and monoglycerides and finally to glycerol. Glycerol Kinase (GK) using ATP as PO4 source converts glycerol liberated to Glycerol-3-Phosphate(G-3-Phosphate), G-3 phosphate Oxidase (GPO) oxides G-3-Phosphate formed to Dihydroxy acetone phosphate and hydrogen peroxide is formed. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidase 4-aminoantipyrine and DHBS (3,5 Dichloro-2-hydroxy benzene sulphate) to a red colored complex. The absorbance of the colored complex is measured at 520nm which is proportional to triglyceride concentration.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GP}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{Glycerol-3-Phosp.} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone Phosphate} + H_2O_2$$

$$2\,H_2O_2 + 4\text{AAP} + \text{DHBS} \longrightarrow \text{Quinoneimine dye} + 4H_20$$

Reaction parameters	
Type of Reaction	End point
Wavelength	520nm
Flow cell temperature	37^0C
Sample volume	0.01ml
Reagent volume	1ml
Incubation time	5min at 37^0C
Standard concentration	200mg/dl
Light path	1cm
Zero setting	Reagent

Pipette into Test Tubes	BLANK	STD	TEST
Working Reagent(ml)	1.0	1.0	1.0
Standard (ml)	-	0.1	-
Sample (ml)	-	-	0.1
Mix well and incubate at 37^0C for10min			
Distilled Water (ml)	1.5	1.5	1.5

Mixed well and read absorbance of test and standard against reagent blank at 520nm.

Results: $\text{Triglycerides (mg/dl)} = \dfrac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 200$

2.3.4f GLUCOSE

GOD-POD METHOD Autospan Reagent kit (SPAN DIAGNOSTICS) for quantitative estimation of Glucose .

Principle:

Glucose oxidase (GOD) oxides glucose to gluconic acid and Hydrogen peroxide, in presence of enzyme Peroxidase, released Hydrogen peroxide is coupled with Phenol and 4-aminoantipyrine (4-AAP) to form colored quinoneimine dye. Absorbance of colored dye is measured at 505 nm and is directly proportional to the concentration of glucose in the sample.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O$$

$$H_2O_2 + \text{Phenol} + \text{4-AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + H_2O$$

Reaction parameters	
Type of Reaction	End point
Wavelength	505nm
Flow cell temperature	37^0C
Sample volume	0.01ml
Reagent volume	1ml
Incubation time	10 min at 37^0C
Standard concentration	100mg/dl
Light path	1cm
Zero setting	Reagent

Procedure

Pipette into Test Tubes	BLANK	STD	TEST
Working Reagent(ml)	1.5	1.5	1.5
Standard (ml)	-	0.01	-
Sample (ml)	-	-	0.01
Mix well and incubate at 37^0C for10min			
Distilled Water (ml)	1.5	1.5	1.5

Mixed well and read absorbance of test and standard against reagent blank at 505nm.

Results: Glucose (mg/dl) = $\frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 100$

2.3.4g SERUM UREA NITROGEN

DAM (Diacety monoxime) method Autospan Reagent kit (SPAN DIAGNOSTICS)

Principle

Urea reacts with hot acidic Diacetylmonoxime in presence of Thiosemicarbazide and produces a rose purple colored complex which is measured colorimetrically.

Pipette into Test Tubes	BLANK	STD	TEST
Solution I (ml)	2.5	2.5	2.5
Standard (ml)	-	0.01	-
Sample (ml)	-	-	0.01
Mix well			
DAM(ml)	0.25	0.25	0.25

Mixed well and kept the tubes in boiling water exactly for 10min. Cool ed immediately under running water for 5min, mixed by inversion and color intensity was measured against blank.

Result

$$\text{Serum urea nitrogen (mg/100ml)} = \frac{\text{Abs. of Test}}{\text{Abs. of Std}} \times 30$$

Statistical analysis

To determine statistically significant differences among the treated groups, data were analyzed by using One-way analysis of variance ANOVA.

2.4 Evaluation of selected successive extracts of roots of *Nymphoides macrospermum*

Preliminary phytochemical investigation of roots of *Nymphoides macrospermum* revealed the presence of a fairly high content of Terpenoids in Pet ether and Chloroform extract and higher phenolic content in Ethyl acetate and Methanol extract, so the roots were successively extracted with Dichloromethane, Ethyl acetate , Methanol and lastly with water for further chemical and biological studies.

Dichloromethane extract

The coarsely powdered shade dried plant material (500g) was extracted exhaustively with DCM by hot extraction process (Soxhlet), After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Ethyl acetate extract

The marc left after DCM extraction was dried in air and extracted exhaustively with Ethyl acetate by hot extraction process (Soxhlet) , After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Methanol extract

The marc left after Ethyl acetate extraction was dried in air and extracted exhaustively with Methanol by hot extraction process (Soxhlet). After completion of extraction, the solvent was removed by distillation and concentrated *in vacuo.*

Aqueous extract

The marc left after methanol extraction was dried and extracted with chloroform water by maceration process. After completion of extraction, the solvent was removed by evaporation and the residue was concentrated *in vacuo.*

2.5 Acute toxicity studies

Toxicity study was carried out on all the successive extracts of both the selected plant materials as per OECD guidelines in female albino mice. Animals were dosed with single oral dose of 2000 mg/kg body weight and observed for mortality. Mice were observed for any reactions like tremors, convulsions, salivation, and diarrhoea. 1/10th of the highest tolerable dose was used as a safe dose for further in vivo studies.

2.6 Evaluation for Adaptogenic Activity

After determination of the safe dose of each successive extract of the selected plant material, the extracts were screened for Adaptogenic activity as per the method described in section 2.3.2 and 2.3.3.

2.7. Development of comparative HPTLC fingerprint profile of the extracts of roots of *Nymphoides macrospermum* and *Valeriana wallichi.*

Preliminary phytochemical screening showed the presence of terpenoids, phytosterols and phenolic compounds all of which can be extracted using methanol. Therefore total methanol extract containing the above constituents and the successive extracts were used for fingerprint studies. A comparative fingerprinting study was also performed with extracts of roots of Valerian. Compounds of varying polarity in the extracts were separated using various solvent systems on TLC. The HPTLC fingerprint profile comprising of typical spectra, Rf values, UV λ_{Max} and relative percentage of the separated compounds were then recorded.

Table.2.2 Solvent systems used for recording the HPTLC finger print profiles of extracts of roots of *Nymphoides macrospermum*

	Solvent system
1	Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03)
2	Hexane:Ethyl acetate: Acetic acid (7:3:0.3)
3	Ethyl acetate: Formic acid:Acetic acid:Water (8:1:0.4:1)
4	Toluene: Formic acid:Ethyl formate (5:1:4)
5	Hexane:Ethyl acetate (65:35)

Solvent systems 1 & 2 were used to resolve the non polar compounds and the separated compounds (steroids & terpenoids) were detected by derivatization with anisaldehyde sulphuric acid whereas solvent systems 3 & 4 were used to resolve medium polar and polar compounds in the extract on a HPTLC plate. The characteristic finger print profiles for various chemical constituents in each extract under UV light of different wavelengths (UV-254, UV-366) and after derivatization with suitable reagents were recorded on a CAMAG-HPTLC system

Sample solutions

A stock solution (10 mg/ml) of each extract was prepared in respective solvent and was used for further investigation.

Chromatographic conditions

A Camag HPTLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20x10cm), Camag scanner 3 and integrated winCATS 4 software was used for the analysis. HPTLC was performed on a pre-coated TLC plates silica gel 60F $_{254}$ (20cm x 20cm). Samples were applied to the plate as 8mm wide bands with an automatic TLC sampler (Linomat V) under a flow of N_2 gas, 10mm from the bottom and 10 mm from the side and the space between two spots was 15 mm of the plate. The linear ascending development was carried out in a CAMAG twin trough chamber (20cm x 10 cm) at room temperature (25^0 ± 2 ^{0} C and 40% relative humidity). The length of the chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried in current air with the help of an hair dryer and then scanned under UV light at 254 nm and 366nm using a slit width of 6 x 0.45mm and data resolution 100µm/step and scanning speed 20mm/s with a computerized CAMAG TLC scanner-3 integrated with winCATS 4TLC scanner 3 (CAMAG). The plates were derivatized, wherever necessary, with suitable reagents (Stahl, 1969: Wagner, 1996) to detect the presence of specific chemical constituents and the characteristic peaks of the detected compounds were recorded at 540 nm. The photographs were taken with the help of Reprostar 3 (CAMAG) digital camera.