# CHAPTER - 4

## Results & Discussion

## (Nymphoides macrospermum)

The systematic studies were undertaken on roots of *Nymphoides macrospermum* in order to evolve parameters for standardization and to assess their adaptogenic activity.

The results of different studies are compiled and discussed under following headings.

- **Pharmacognostic Evaluation**
- **Preliminary Phytochemical Evaluation**
- **Biological evaluation**
- **Separation and identification of bioactive marker constituents.**

## 4.1. Pharmacognostic Evaluation

The traditional healers, use *Nymphoides macrospermum* roots in the treatment of various ailments. Some important morphological features are documented for this plant material.

### 4.1.1 Macroscopic studies:

Two types of roots which are clustered and run vertically downwards from a horizontal rhizome are available as

1) Spongy, long roots;
2) Stout, short roots with pointed ends and brick red fur.

| | |
|---|---|
| *Shape:* | Angular |
| *Size:* | Long, 5cm or more |
| *Color:* | Brick red to brown |
| *Branching:* | Lateral |
| *Odour:* | Characteristic |
| *Taste:* | Characteristic |

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. Sympatric existence of some species of *Nymphoides* may be responsible for the inadvertent mixing of other species during collection. Spongy, long and stout, short roots (pointed ends and brick red fur) running vertically downwards from the horizontal rhizome can serve as dominant morphological character of *Nymphoides*, but it also closely resembles *Nymphoides indica* and makes it difficult in identification.

### 4.1.2. Microscopic studies

Microscopic evaluation of medicinal herbs is an indispensable and cost effective tool in the conventional analytical pharmacognosy for the identification of medicinal herbs (Wallis, 1965). Use of microscopic characteristics has been the mainstay of classical pharmacognosy and remains as one of the essential component of the modern monograph. In this regard, the important microscopic features of the roots have been documented.

T S of root presents a circular outline. The following are the important tissues seen from the periphery to the centre.

**Epiblema (Piliferous layer or Rhizodermis)**

It is the outermost single layered; protective covering made up of compactly arranged thin walled parenchyma cells, some of which are developed into unicellular long hairs having a very broad base.

**Cortex**

It is below the epiblema, cortical cells are parenchymatous, arranged in radial series/concentric form. Cortex is distinguished into an outer and inner region.

**Outer cortex**

It comprises of 2 layers made up of patches of sclereids separated by a layer of parenchyma. The sclereids distinctly show presence of pits.

**Inner cortex**

It is made up of large polygonal to rounded parenchyma filled with large sized starch grains. The starch grains are accumulated centrally or towards the base of the cells.

**Endodermis**

Innermost layer of cortex, made up of single layered barrel shaped parenchyma cells. Cortical cells close to endodermis filled with larger starch grains.

**Stele**

It is the central cylinder shows the following regions

**Pericycle.**

It is a single layer of thin walled parenchyma cells enclosing vascular tissues

**Vascular elements**

It consists of xylem and phloem units alternately on different radii. Polyarch (in groups of 10) and exarch and protoxylem face towards periphery

**Pith (Medulla)**

It is the centre of the stele, large and made up of thin walled parenchyma.

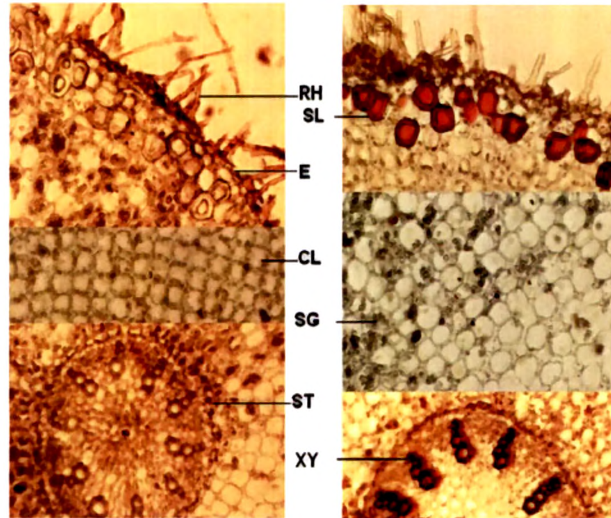



Fig.4.01. T.S of the roots of *Nymphoides macrospermum* (X10)
RH-Root hairs; SL-Sclereids; E-Epiblema; CL-Cortical Layer; SG-starch grains
ST-Stele region; XY-Xylem

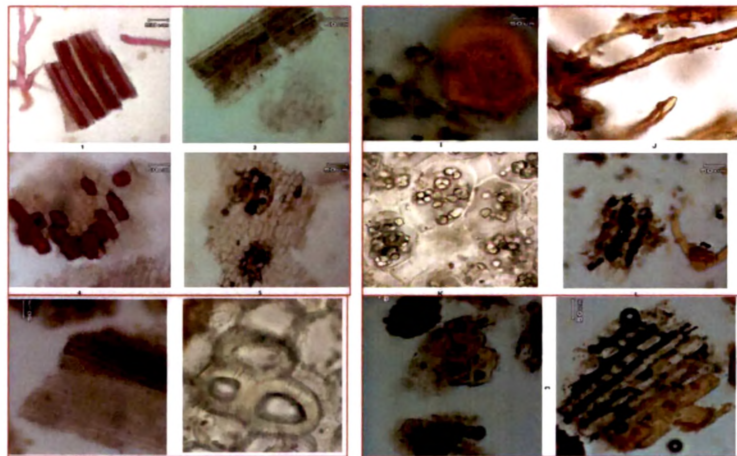


Fig.4.2. Diagnostic features for the powder microscopy of the roots of *Nymphoides macrospermum* (X40).1& 2-Wood elements; 3,4-Sclereids; 5,6-Parenchyma. I-Oil gland; J-Unicellular long root hair; K,L- Cortical cells containing compound starch grains.

When the same organ of closely allied taxa forms the source for traditional medicine and substituted with similar organ of other plant, microscopy alone may be inadequate for the purpose of an unequivocal diagnosis. In such case, powder characters are of great value for identifying the powdered crude drugs.

**Table 4.1 Powder study of the roots of *Nymphoides macrospermum***

| Preliminary Tests | Observation | Inference |
|---|---|---|
| Odour | Faintly malodorous | |
| Color | Brownish | |
| Taste | Characteristic | |
| Touch | Smooth | |
| Test with water/ Aqueous extract | Frothing | May be due to saponins |
| Test for Tannins | Brown ppt | due to presence of Tannins |
| Test for Mucilage | Non-mucilaginous | Absence of Mucilage |
| Test with $H_2SO_4$ | Reddish-brown color | |
| Test with caustic alkali | Brown color | |
| Test for oils | Faint oily stain, disappears on heating | Presence of volatile oils |

The various diagnostic characters of the root powder are depicted in figure.

Sclereids
From the outer cortex are oval to sub rectangular, thickened walls having distinct pits.

Oil glands
Fragments of parenchyma containing entire or a portion of oil glands.

Root hair
Unicellular long hairs having a very broad base.

Starch grains
Compound starch grains within parenchyma cells which are large and round.

Wood elements
Xylem vessels and fibers inter-lock with each other to form a spindle shaped structure.

Parenchyma
Cortical parenchyma consisting of thin walled polyhedral cells with intercellular space.

*Nymphoides macrospermum* roots revealed presence of some dominating diagnostic features like sclereids, oil glands, root hairs, starch grains, wood elements and cortical parenchyma. In addition, microscopy does not reveal much about the deterioration during storage. The regulations on herbal medicines include TLC as powerful tool for true identification of the plant material in terms of its chemical constituents (WHO1998).

Table 4.2 Physico-chemical constants, Content of Heavy metals and other inorganic trace elements including Microbial content of roots of *Nymphoides macrospermum*

| Physico chemical constants | | Content of heavy metals and other inorganic elements | | Microbial Content | |
|---|---|---|---|---|---|
| Parameter | Percentage (w/w) | Metals/Elements | Content | Parameter | Content (cfu/g) |
| Foreign organic matter | 4.1 | Sodium (mg/g) | 1.513 | Total Viable count | 8540 |
| Loss on drying | 5.0 | Potassium (mg/g) | 0.736 | Total Bacterial Count | 4240 |
| Total Ash | 5.3 | Zinc (mg/g) | 0.255 | Total Fungal count | 4300 |
| Acid insoluble ash | 3.06 | Manganese (mg/g) | 0.706 | E-coli | Absent |
| Water soluble ash | 2.2 | Copper (mg/g) | Nil | Salmonella spp | Absent |
| Alcohol soluble extractive | 9.30 | Cadmium (ppm) | Nil | S. aureus | Absent |
| Water soluble extractive | 14.0 | Iron (mg/g) | 4.384 | P. aeruginosa | Absent |
| | | Lead (ppm) | 0.024 | | |
| | | Mercury (ppm) | 0.0466 | | |
| | | Arsenic (ppm) | 1.5792 | | |

### 4.1.3. Proximate analysis

Proximate analysis was carried out as per reported methods. Proximate analysis helps to set up certain standards for dried drugs in order to avoid batch-to-batch variation and to judge their quality. A higher ash value (5.3%w/w) was observed indicating higher inorganic content. Alcohol and water-soluble extractives were also found to be higher (9.3 and 14% w/w respectively).

Determination of inorganic elements, including the trace elements and heavy metals has gained outstanding importance in the life sciences. Elements present even at minimal concentrations in biological and environmental matrices, in fact, can exert fundamental influence on vital functions in the human body proportionally to their amounts.

All the living organisms require inorganic elements for their growth and survival. Medicinal plants contain considerable amounts of mineral constituents, in particular, the presence of essential elements (Mg, Mn, Zn and many others) is a prerequisite for correct growth and development of plants. Inorganic elements in plants also plays role in the accumulation of secondary metabolites such as alkaloids, glycosides, terpenoids, phenolic compounds etc (Andrijany, 1998; Lokova et al.,1998) as they are responsible for the activity of a number of enzymatic systems, which in turn regulate the metabolic pathways leading to the synthesis of these compounds.

The reports on the potential deleterious effects of some Ayurvedic medicines, (Saper et al., 2004) due to presence of unacceptable levels of metals and metalloids such as Lead, Arsenic, Mercury etc, and their possible chronic toxicological effects, has caused much concern not only among the herbal

practitioners, but also among large population who still depend on the medicinal plants for their healthcare needs. Thus it has become necessary that

all the medicinal plant materials should be ensured for their content of heavy metals and other inorganic elements. The plant material under study viz., 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.

The pharmacognositcal studies carried out on these roots therefore serve as valuable tool and provide suitable standards for the identification of the plant materials.

## 4.2. Preliminary phytochemical analysis of roots of *Nymphoides macrospermum*

The roots of *Nymphoides macrospermum* were subjected to successive solvent extraction. Percentage yield, color and consistency of the selected successive extracts are recorded in Table 4.3. Results indicate maximum extractive values attained with Water, petroleum ether and chloroform, whereas with Ethyl acetate and methanol these extractive values were found to be very less.

**Successive Solvent Extraction of *Nymphoides macrospermum roots***

**Table 4.3 Physical properties of successive Extracts**

| Sl. No | Extract | Yield in % | Color & Consistency |
|---|---|---|---|
| 1 | PET.ETHER | 3.23 | Reddish brown semisolid |
| 2 | CHLOROFORM | 1.21 | Greenish black semisolid |
| 3 | ETHYL ACETATE | 0.725 | Reddish brown semisolid |
| 4 | METHANOL | 0.93 | Yellowish brown-semi solid |
| 5 | AQUEOUS | 14.63 | Off white-solid |

**Table 4.4 Phytoprofile of roots of *Nymphoides macrospermum***

| Class of Constituents | Successive extracts | | | | |
|---|---|---|---|---|---|
| | P | C | E | M | W |
| Alkaloids | - | - | - | - | - |
| Carbohydrates | - | - | - | + | + |
| Oils/Fats | + | + | - | - | - |
| Flavonoids | - | - | - | - | - |
| Phenolics | - | - | + | + | + |
| Proteins & Amino acids | - | - | - | + | + |
| Steroids/Terpenoids | + | + | - | - | - |
| Saponins | - | - | - | - | - |
| Tannins | - | - | - | + | + |
| Gums & Mucilage | - | - | - | - | - |

**P-Pet ether extract, C-Chloroform extract, E- Ethyl acetate extract, M-Methanol extract & W-Aqueous extract.**

The extracts obtained in successive extraction process were subjected to various qualitative chemical tests to determine the presence of various phytoconstituents. Pet. ether and Chloroform extracts showed presence of steroids/terpenoids and fixed oils/fats, Ethyl acetate extract showed the presence of phenolics whereas methanol and water extracts revealed the presence of tannins, proteins & amino acids and carbohydrates. Alkaloids, gums & mucilage and saponins were not found. Results are summarized in Table 4.4

Based on the results of preliminary phytochemical screening total methanol extract (NMR) containing the above constituents was prepared and screened for bioactivity.

## 4.3 Evaluation for Adaptogenic Activity

Stress is a non-specific response of body known to alter the physiological homeostasis of the organism resulting in various endocrinal and visceral responses. 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 antistress effect, but also account in the improvement of defense mechanism of the host.

Experimental evidence suggests that, chronic repeated stress is known to cause a wide range of physiological, psychological and neuro-endocrinal changes (Mc. Even, 2000). Research has confirmed that adaptogens have various effects on organisms. Adaptogens are substances that normalize the body functions, strengthen systems and functions compromised by stress and have a protective

effect against wide variety of environmental and emotional stress. They seem to increase overall resistance of the body.

Brekhman and Dardymov have explained that if the original concept of adaptogenic function is kept in mind, an adaptogen has to be screened not only for its anti-stress activity but also for immuno competance activities.

In the present study, the investigations for anti-stress and immunomodulatory activities were done using different models. The effect of adaptogen was studied on physical stress, stress induced biochemical changes and a few models to prove immunomodulatory ability of the selected plants.

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.

### 4.3.1. Acute toxicity studies

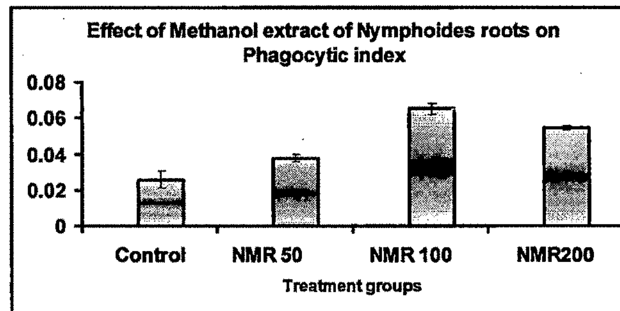
Toxicity study was carried out on total methanolic extract of *Nymphoides macrospermum* (NMR) as per OECD guidelines in female albino mice. Animals when administered with single oral dose of 2000 mg/kg body weight did not show any toxicity or mortality. Hence considering this the safe dose, the extract was screened for adaptogenic activity at three different dose levels 100, 200 and 400mg/kg body weight.

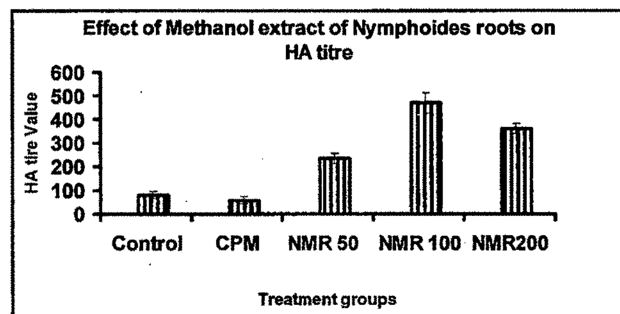
## 4.3.2. Evaluation of Immunomodulatory activity of total methanol extract of roots of *Nymphoides macrospermum*

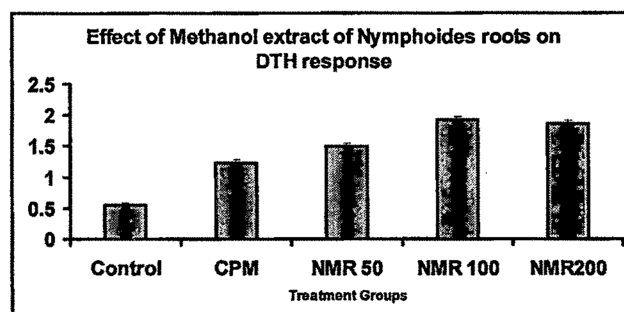
**Table 4.5 Effect of methanol extract of *Nymphoides* macrospermum roots on Phagocytic index, HA titre and DTH response**

| GROUP | PHAGOCYTIC INDEX Mean ±SEM | HA TITRE Mean ±SEM | DTH RESPONSE Mean ±SEM |
|---|---|---|---|
| Control | 0.0256±0.0048 | 80±16 | 0.545±0.033 |
| CPM | | 58.66±15.27 | 1.22±0.047 |
| NMR 50 | 0.0375 ±0.002* | 234.66±21.33$^{ns}$ | 1.49±0.0415** |
| NMR 100 | 0.0651 ± 0.003*** | 469.33±42.66*** | 1.92±0.0495*** |
| NMR200 | 0.0546± 0.001 *** | 362.66±21.66** | 1.85±0.054*** |

n=6 mice /group Values are expressed as Mean ± SEM
P< 0.05, ** P<0.01, ***P<0.001 Ns= non significant






DTH response of Normal control group was 0.545±0.033 while that of methanol extract of *Nymphoides macrospermum* roots was 1.49±0.0415, 1.92±0.0495 and 1.85±0.054 at 50, 100 and 200mg/kg dose respectively. A statistically significant response ($P<0.001$) was obtained at 100 and 200 mg/kg dose levels. Increase in DTH response as evidenced by increased paw thickness in mice revealed stimulatory effect of *Nymphoides macrospermum* roots on T cells and accessory cell types required for the expression of reaction. (Results in Table 4.5)

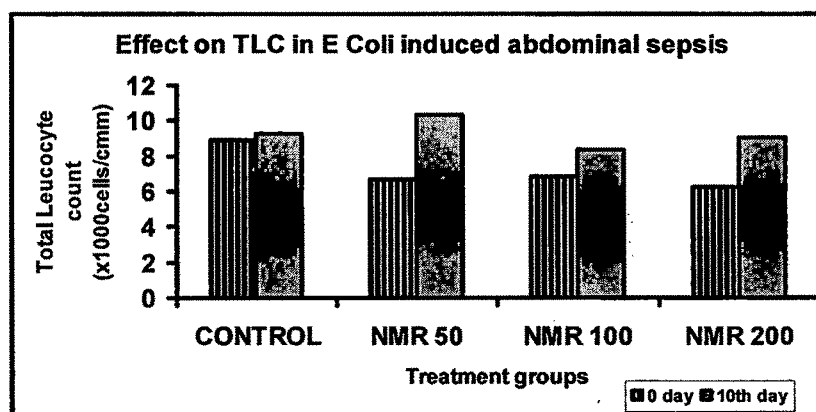
Methanol extract of *Nymhoides macrospermum* roots at 50, 100 and 200 mg/kg showed HA titre of 234.66±21.33(ns), 469.33±42.66 ($P<0.001$) and 362.66±21.66 ($P<0.01$) respectively was found effective in increasing HA titre indicating the enhanced responsiveness of macrophages and T and B lymphocytes involved in the antibody production. (Benacerraf et al.,1978)

Total methanol extract of *Nymphoides macrospermum* roots was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance 0.37±0.002, 0.65± 0.003 and 0.54± 0.001 at 50, 100 and 200mg/kg dose levels compared to control group (0.0256±0.0048). (Results in Table 4.5)

**Table 4.6**
**Effect of Methanol extract of *Nymphoides macrospermum* roots on Leucocyte count and % Mortality in E coli induced abdominal sepsis**

| GROUPS | TOTAL LEUCOCYTE COUNT (Thousands/cmm) Mean ±SEM | | % MORTALITY |
|---|---|---|---|
| | 0 DAY | $15^{th}$ DAY | |
| CONTROL | 8.91±0.336 | 9.28±0.39 | 100 |
| NMR 50 | 6.71±0.32 | 10.34±0.31*** | 25 |
| NMR 100 | 6.8±0.32 | $8.39±0.45^{ns}$ | 37.5 |
| NMR 200 | 6.21±0.33 | 9.05±0.34* | 37.5 |

Values are expressed as Mean ± SEM
* P< 0.05, ** P<0.01, ***P<0.001 ns= non significant

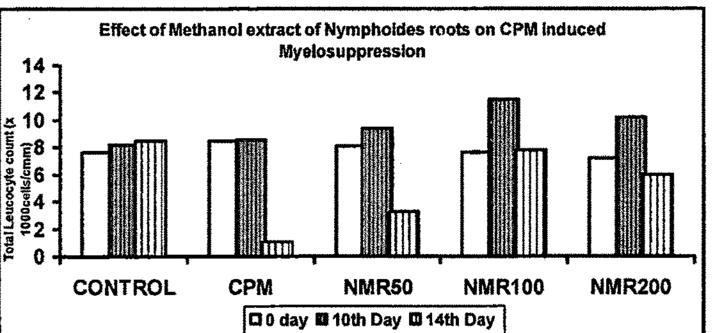



Stimulation of macrophages as evidenced by increase in phagocytic index in carbon clearance test is further substantiated by the results of the studies of *Nymphoides macrospermum* roots on E coli induced abdominal sepsis (Infectious /Biological stress) where oral administration of extract for ten days at 50, 100 and 200mg/kg dose levels produced a significant increase in total leucocyte count and mortality rate was reduced to 25 % at 50mg/kg dose level compared to control group. (Results are shown in Table 4.6)

Table 4.7 Effect of Methanol extract of roots of *Nymphoides macrospermum* on Leucocyte, RBC and Hb count in Cyclophosphamide induced Immunosuppression.

| GROUP | TOTAL LEUCOCYTE COUNT $10^3$ cells/mm$^3$ Mean ±SEM | | | RBC COUNT $10^6$ cells/mm$^3$ Mean ±SEM | | | HAEMOGLOBIN (g%) Mean ±SEM | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 DAY | 10th DAY | 14th DAY | 0 DAY | 10th DAY | 14th DAY | 0 DAY | 10th DAY | 14th DAY |
| CONTROL | 7.66±1.03 | 8.166±1.03 | 8.466±1.05 | 8.5±0.174 | 8.738±0.17 | 9.62±0.519 | 14.7±0.468 | 15.2±0.474 | 17.2±1.221 |
| CPM | 8.466±1.05 | 8.533±1.05 | 1.05±0.133 | 9.583±0.49 | 9.835±0.48 | 7.1±0.297 | 16.8±0.909 | 17.266±0.9 | 11.8±0.57 |
| NMR50 | 8.1±0.28 | 9.41±0.35 | 3.25±0.307 | 6.6±0.26 | 8.18±0.33 | 8.57±0.38 | 12.1±0.34 | 14.38±0.53 | 12.23±0.72 |
| NMR100 | 7.65±0.46 | 11.5±0.73 | 7.8±0.51*** | 8.44±0.19 | 10.67±0.25 | 10.21±0.25 | 10.66±0.66 | 14.06±0.65 | 13.33±0.65 |
| NMR200 | 7.21±0.44 | 10.25±0.64 | 5.97±0.36** | 8.24±0.23 | 9.58±0.43 | 9.1±0.421 | 9.56±0.36 | 12.61±0.7 | 11.66±0.65 |

n=6 mice /group Values are expressed as Mean ± SEM , * $P< 0.05$, ** $P<0.01$ , ***$P<0.001$ ns= non significant



The Total Leucocyte Count of methanol extract of *Nymphoides macrospermum* treated groups at 50, 100 and 200mg/kg dose levels were increased from 8.1±0.28 to 9.41±0.35 on pretreatment for 10 days and 3.25±0.307 on 14 th day at 50mg/kg and 7.65±0.46 to 11.5±0.73 and 7.8±0.51 at 100mg/kg and 7.21±0.44 to 10.25±0.64 and 5.97±0.36 at 200mg/kg dose levels on 0, $10^{th}$ and $14^{th}$ days respectively. No changes were obsereved with respect to RBC and Hemoglobin values. Thus, it can be established from the study that *Nymphoides macrospermum* roots possess the ability to counteract the myelosuppressive effects of cytotoxic drug, Cyclophosphamide by stimulating the bone marrow activity.(Results summarized in Table 4.7)

### 4.3.3 Evaluation of Anti-stress activity of total methanol extract of roots of *Nymphoides macrospermum*

Table 4.8
Effect of methanol extract roots of *Nymphoides macrospermum* on Stress induced changes in organ weight.

| Treatment group | Changes in organ weight(g) | | |
|---|---|---|---|
| | Adrenal gland | Liver | Spleen |
| | Mean ±SEM | Mean ±SEM | Mean ±SEM |
| **Vehicle Control** | **0.155 ± 0.02** | **5.35±0.11** | **0.496±0.17** |
| **Stress Control** | **0.252±0.018** | **7.6±0.2** | **0.768±0.2** |
| **NMRTME 50** | **0.174±0.036 ***** | **6.28±0.07**** | **0.63±0.011** |
| **NMRTME 100** | **0.18±0.078***** | **6.22±0.09**** | **0.616±0.016**** |
| **NMRTME 200** | **0.174±0.059***** | **6.28±0.01**** | **0.63±0.0163** |

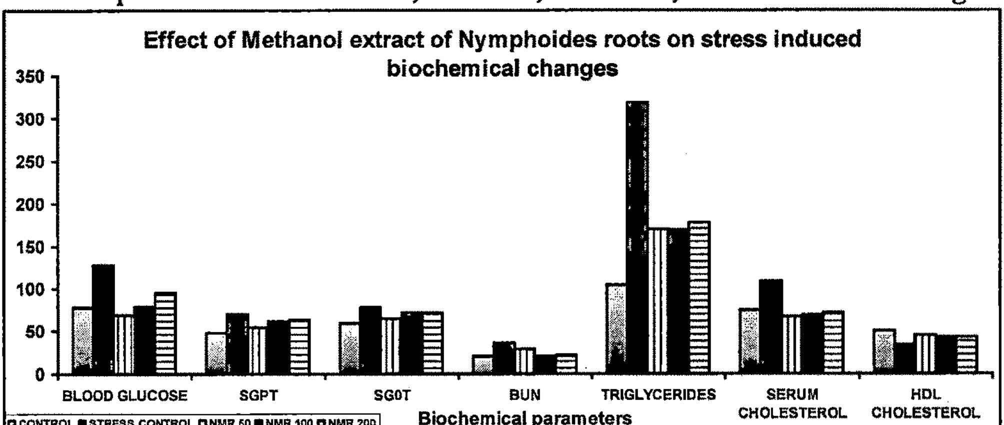
**n=6 rats /group Values are expressed as Mean ± SEM , * P< 0.05, ** P<0.01 , ***P<0.001 ns= non significant**

Table: 4.9

Effect of Methanol extract of roots of *Nymphoides macrospermum* on Stress induced changes in biochemical parameters.

| GROUPS | BLOOD GLUCOSE (mg/dl) Mean ±SEM | SGPT IU/ml Mean ±SEM | SGOT IU/ml Mean ±SEM | UREA NITROGEN (mg/dl) Mean ±SEM | TRI GLYCERIDE (mg/dl) Mean ±SEM | SERUM CHOLESTROL (mg/dl) Mean ±SEM | HDL CHOLESTEROL (mg/dl) Mean ±SEM |
|---|---|---|---|---|---|---|---|
| CONTROL | 77.17±1.75 | 48.07±1.43 | 58.82±1.134 | 20.08±0.545 | 104.15±3.27 | 74.59±0.648 | 50.21±0.626 |
| STRESS CONTROL | 127.36±6.47 | 69.02±1.2 | 77.15±1.973 | 35.12±0.501 | 318.73±10.45 | 107.92±1.589 | 33.28±0.519 |
| NMR 50 | 67.92±2.54*** | 54.38±1.44** | 63.6±0.79** | 28.68±0.525* | 170.25±4.18*** | 65.88±1.167*** | 45.27±0.951** |
| NMR 100 | 78.67±4.29*** | 60.63±0.73** | 71.18±0.9 | 20.32±0.699*** | 169.18±4.553*** | 68.19±0.875** | 42.51±0.596** |
| NMR 200 | 94.9±3.05* | 62.97±1.22 | 71.18±1.26 | 21.55±0.689*** | 177.97±3.94*** | 71.17±0.709** | 42.89±0.74** |

n=6 rats /group Values are expressed as Mean ± SEM , * $P< 0.05$, ** $P<0.01$ , ***$P<0.001$ ns= non significant



An increase in weigh of liver, spleen and adrenal glands was observed in the Stress control group while a significant reduction in the weights of these organs was observed in the groups treated with methanol extract at 50, 100 and 200mg/kg dose levels. An increase in Blood glucose, SGPT, SGOT, Blood Urea Nitrogen, Triglycerides and serum cholesterol levels were found in stress control group while normal levels of these biochemical parameters were observed in extract treated groups.

The results of the biological studies of methanol extract of roots of *Nymphoides macrospermum* indicates preliminarily that *Nymphoides macrospermum* roots hold promise as adaptogen and provides a scope for further detailed investigation on the fraction/individual constituents.
Considering the results of studies of methanol extract of roots of *Nymphoides macrospermum* the successive extracts were investigated for the same bioactivity.

### 3.3.4. Acute toxicity studies

The successive extracts were subjected for toxicity studies following OECD guidelines. (Results are summarized in the Table 4.10 )

**Table 4.10**
**Effect of successive extracts of roots of *Nymphoides macrospermum* on mice under toxicity studies**

| Extract | Dose level mg/kg body weight | | | |
|---|---|---|---|---|
| | 2000mg/kg | 550mg/kg | 175mg/kg | 55mg/kg |
| NMDCME | Died after 3 days | Non-toxic | ------- | ------- |
| NMEAE | Non-toxic | -------- | ------- | ------- |
| NMME | Non-toxic | ------- | ------- | ----- |
| NMWE | Died within 12hrs | Died within 24 hrs | Died after 3 days | ------ |

**NMDCME= Dichloromethane extract of roots of *Nymphoides macrospermum.***
**NMEAE= Ethyl acetate extract of *Nymphoides macrospermum***
**NMME= Methanol extract of *Nymphoides macrospermum***
**NMWE= Aqueous extract of *Nymphoides macrospermum.***

Studies on the successive extracts of roots of *Nymphoides macrospermum* did not show any toxicity or mortality when Dichloromethane, Ethyl acetate and Methanol extracts 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. The results of these studies are recorded in Table 4.11 and 4.12

## 4.3.5. Evaluation of Immunomodulatory activity of successive extract of roots of *Nymphoides macrospermum*

Table 4.11

**Effect of successive solvent extracts of roots of *Nymphoides macrospermum* on Phagocytic index, HA titre and DTH response**

| GROUP | PHAGOCYTIC INDEX Mean ±SEM | HA TITRE Mean ±SEM | DTH RESPONSE Mean ±SEM |
|---|---|---|---|
| Control | 0.027±0.0019 | 138.66±25.68 | 0.665±0.022 |
| CPM | | 64±14.31 | 1.2±0.059 |
| NMRDCME 25 | 0.06±0.0019 | 384±57.24 | 1.8±0.035 |
| NMRDCME 50 | 0.067±0.0022 | 597.33±85.33 | 1.97±0.035 |
| NMRDCME 100 | 0.064±0.002 | 469.33±42.66 | 1.8±0.066 |
| NMREAE 50 | 0.022±0.0019 | 138.66±25.69 | 0.948±0.042 |
| NMREAE 100 | 0.029±0.019 | 192±28.62 | 1.141±0.042 |
| NMREAE 200 | 0.03±0.013 | 192±28.62 | 1.211±0.069 |
| NMRME 50 | 0.0193±0.0008 | 96±14.31 | 1.001±0.064 |
| NMRME100 | 0.0263±0.002 | 80±16 | 0.776±0.028 |
| NMRME 200 | 0.025±0.0025 | 80±16 | 0.79±0.034 |

**Table 4.12: Effect of successive extracts of roots of *Nymphoides macrospermum* on Leucocyte count and % Mortality in E coli induced abdominal sepsis**

| GROUPS | TOTAL LEUCOCYTE COUNT (Thousands/cmm) Mean ±SEM | | % MORTALITY |
|---|---|---|---|
| | 0 DAY | 15th DAY | |
| CONTROL | 7.56±0.33 | 7.86±0.37 | 100 |
| NMRDCME 25 | 7.85±0.21 | 12.11±0.26 | 33 |
| NMRDCME 50 | 7.83±0.28 | 12.53±0.67 | 33 |
| NMRDCME 100 | 7.8±0.36 | 12.46±0.32 | 33 |
| NMREAE 50 | 7.8±0.3 | 7.78±0.32 | 100 |
| NMREAE 100 | 8.05±0.13 | 7.96±0.13 | 100 |
| NMREAE 200 | 7.93±0.32 | 7.76±0.32 | 100 |
| NMRME 50 | 6.18±0.45 | 6.58±0.44 | 100 |
| NMRME100 | 6.55±0.27 | 6.96±0.27 | 100 |
| NMRME 200 | 6.15±0.46 | 6.21±0.64 | 100 |

Values are expressed as Mean ± SEM
* P< 0.05, ** P<0.01 , ***P<0.001 ns= non significant

- **Effect of successive extracts of roots of *Nymphoides macrospermum* on HA titre and DTH response.**

DTH is antigen specific and causes erythema and induction at the site of injection in immunized animals. The histology of DTH can be different for different species, but the general characteristics are like influx of immune cells at the site of injection, macrophages and basophills in mice and induction becomes apparent within 24-72 hrs (Poulter, et al., 1982). T Cells are required to initiate the reaction. DTH is a part of the process of graft rejection, tumor immunity and most important, immunity to many intracellular infectious microorganism. DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity. (Kuby et al., 1997).

Footpad thickness test or DTH response was measured to assess the cell-mediated immune response. The DTH response was not significant in the groups treated with NMREAE and NMRME at all the different dose levels, while in case of NMDCME treated groups at 25, 50 and 100 mg/kg dose levels, it was highly significant being maximum at 50mg/kg 1.97±0.035 ($P<0.001$ ) dose when compared with control group.

Thus, increase in DTH response by NMRDCME may be due to stimulatory effect on lymphocytes and necessary cell types required for the expression reaction.

Haemagglutination antibody titre was determined to establish the humoral response. The antibody production to T dependant antigen SRBC requires the co-operation of T and B-lymphocytes and macrophages, which interacts with SRBC and proliferates into antibody secreting plasma cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross linking to form clusters that are readily ingested by phagocytic cells. (Benacerraf, 1978). To evaluate the effects of selected extracts on humoral response their influence was tested on sheep erythrocyte-specific haemagglutination titre in mice.

The HA titre value of control group was 138.66±25.68 while that of Cyclophosphamide treated control group was 64±14.31. An increase in HA titre was observed in the groups treated with NMDCME. Increase in HA titre was highly significant (***P<0.001) at 50 and 100mg/kg dose levels (597.33±85.33 and 469.33±42.66 respectively) was observed, when compared to control. Oral administration of NMREAE and NMRME at all the different dose levels did not produce much difference in the HA titre values compared with control group.

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.

- **Effect of successive extracts of roots of *Nymphoides macrospermum* on Phagocytic activity**

Phagocytosis and killing of invading microorganisms by macrophages constitute body's primary line of defense against infections Macrophages (Van Furt R., 1982) are an integral part of the immune system, acting as phagocytic, microbicidal and tumoricidal effector cells. Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies leading to a more rapid clearance of foreign particulate matter from the blood. (Kende M.,1982) Increased phagocytic activity is indicative of increased functioning of reticuloendothelial system.

The results of Phagocytic activity was much similar to that of DTH response and HA titre. Phagocytic index of Control group was found to be 0.027±0.0019, The groups treated with NMREAE and NMRME proved to be poorly effective by producing a similar phagocytic index like that of control group while the groups treated with NMRDCME at all the three different dose levels produced a statistically significant response.

Thus, increase in carbon clearance index by NMRDCME may be due to the enhancement of phagocytic function of mononuclear macrophage and non-specific immunity.

- **Effect of successive extracts of roots of *Nymphoides macrospermum* on E coli induced abdominal sepsis.**

Stimulation of macrophages as evidenced by increase in phagocytic index in carbon clearance test is further substantiated by the results of the studies of *Nymphoides macrospermum* roots on E coli induced abdominal sepsis (Infectious /Biological stress) . Activated macrophages secrete a number of cytokines such as IL-1 etc which in turn stimulates other immunocytes like neutrophils this gives the ability to counter the infectious stress. Thus the protection offered by the NMRDCME against E coli peritonitis could be attributed to secretion of Cytokines from activated macrophages.

Pretreatment with NMRDCME at 25, 50 and 100 mg/kg dose levels for 10 days produced a significant increase in leukocyte count at all the three dose levels with reduced mortality rate when compared to control. Administration of NMREAE and NMRME extracts did not affect the Leukocyte count and the mortality rate was maximum similar to that of control group.

Thus, protection against E coli induced peritonitis by NMRDCME may be due to the enhancement of phagocytic function of mononuclear macrophage and non-specific immunity.

The results of above studies revealed potential of DCM extract of *Nymphoides macrospermum* roots, therefore ,this was taken up for further studies.

- **Effect of NMRDCME on Cyclophosphamide induced myelosuppression**

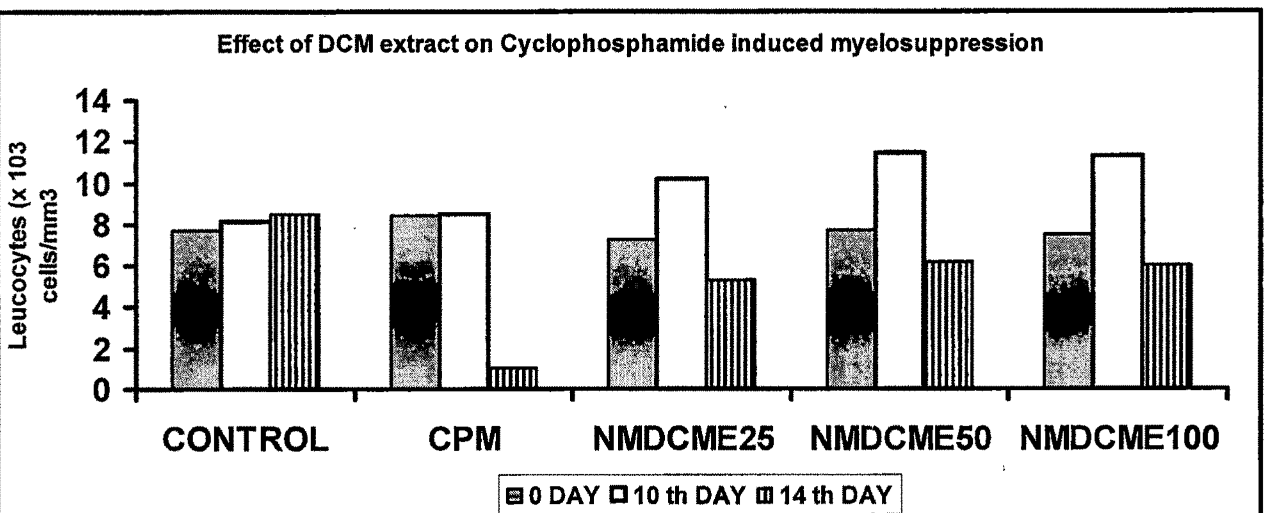
Cyclophosphamide is a potent immunosuppressive agent, capable of inhibiting immune response. Bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in leucopenia or decrease in leukocyte count. (Agarwal et al., 1999). In case of cyclophosphamide induced myelosuppression, administration of NMRDCME was found to increase the total WBC count which was lowered by cyclophosphamide, a cytotoxic drug.

Thus, it can be established from the study that *Nymphoides macrospermum* roots possess the ability to counteract the myelosuppressive effects of cytotoxic drug, Cyclophosphamide by stimulating the bone marrow activity.

Table 4.13 Effect of successive extracts of roots of *Nymphoides macrospermum* on Leucocyte, RBC and Hb count in Cyclophosphamide induced Immunosuppression.

| GROUP | TOTAL LEUCOCYTE COUNT $10^3$ cells/$mm^3$ Mean ±SEM | | | RBC COUNT $10^6$ cells/$mm^3$ Mean ±SEM | | | HAEMOGLOBIN (g%) Mean ±SEM | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 DAY | $10^{th}$ DAY | $14^{th}$ DAY | 0 DAY | $10^{th}$ DAY | $14^{th}$ DAY | 0 DAY | $10^{th}$ DAY | $14^{th}$ DAY |
| CONTROL | 7.52±0.93 | 8.23±0.73 | 8.18±0.63 | 8.26±0.74 | 8.38±0.26 | 8.22±0.18 | 14.2±0.51 | 14.4±0.54 | 14.7±0.46 |
| CPM | 8.18±0.60 | 8.47±0.57 | 1.18±0.33 | 9.35±0.49 | 9.63±0.41 | 7.4±0.26 | 15.2±0.70 | 15.26±0.25 | 10.21±0.45 |
| NMDCME25 | 7.29±0.597 | 10.18±0.86 | 5.28±0.31* | 7.75±0.54 | 10.03±0.31 | 9.12±0.54 | 15.2±0.54 | 16.8±0.84 | 14.9±0.71 |
| NMDCME50 | 7.73±0.486 | 11,43±0.72 | 6.19±0.45 *** | 7.03±0.28 | 9.68±0.29 | 8.89±0.53 | 14.8±0.52 | 16.2±0.75 | 15.1±0.56 |
| NMDCME100 | 7.51±0.772 | 11.29±0.69 | 6.02±0.4 2*** | 6.94±0.26 | 8.95±0.45 | 8.03±0.61 | 15.6±0.61 | 17.0±0.83 | 15±0.49 |

n=6 mice /group Values are expressed as Mean ± SEM , * P< 0.05, ** P<0.01 , ***P<0.001 ns= non significant



## 4.3.6. Evaluation of Anti-stress activity of successive extract of roots of *Nymphoides macrospermum*

Table 4.14 : Effect of Successive extracts of roots of *Nymphoides macrospermum* on Stress induced changes in organ weight

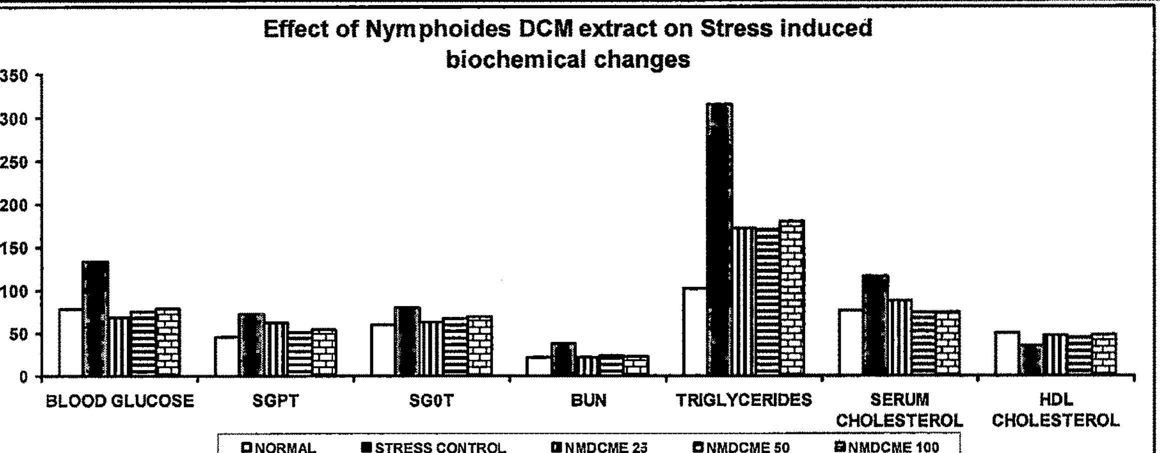
| Treatment group | Changes in organ weight | | |
|---|---|---|---|
| | Adrenal gland | Liver | Spleen |
| | Mean ±SEM | Mean ±SEM | Mean ±SEM |
| Vehicle Control | 0.158±0.006 | 5.216±0.084 | 0.505±0.0164 |
| Stress Control | 0.257±0.009 | 7.938±0.211 | 0.8±0.01 |
| NMRDCME 25 | 0.171±0.003*** | 6.47±0.076 | 0.643±0.011* |
| NMRDCME 50 | 0.183±0.003** | 5.993±0.067*** | 0.553±0.012*** |
| NMRDCME 100 | 0.197±0.003** | 6.03±0.06* | 0.628±0.015* |

n=6 rats /group Values are expressed as Mean ± SEM ,
* $P< 0.05$, ** $P<0.01$ , ***$P<0.001$ ns= non significant

Table 4.15 Effect of NMRDCME on Stress induced changes in biochemical parameters

n=6 rats /group Values are expressed as Mean ± SEM , * $P < 0.05$, ** $P < 0.01$ , *** $P < 0.001$ ns= non significant

| GROUPS | BLOOD GLUCOSE (mg/dl) Mean ±SEM | SGPT IU/ml Mean ±SEM | SGOT IU/ml Mean ±SEM | UREA NITROGEN (mg/dl) Mean ±SEM | TRI GLYCERIDES (mg/dl) Mean ±SEM | SERUM CHOLESTROL (mg/dl) Mean ±SEM | HDL CHOLESTEROL (mg/dl) Mean ±SEM |
|---|---|---|---|---|---|---|---|
| CONTROL | 78.2±1.42 | 46.23±1.83 | 59.82±0.793 | 21.08±0.504 | 102.48±1.968 | 76.09±0.7 | 50.21±0.626 |
| STRESS CONTROL | 134.03±3.31 | 72.43±1.3 | 79.15±1.25 | 37.35±0.658 | 315.4±7.23 | 116.09±1.63 | 34.28±0.32 |
| NMDCME 25 | 68.59±2.08*** | 61.75±0.87* | 62.76±0.686*** | 21.15±0.366*** | 171.29±4.4*** | 87.67±0.645* | 47.27±0.749** |
| NMDCME50 | 75.34±3.98*** | 51.08±1.59*** | 67.18±0.605** | 23±0.472** | 169.98±3.1*** | 74.17±1.529*** | 45.01±0.789** |
| NMDCME 100 | 78.67±3.41** | 54.57±1.07*** | 69.68±0.902** | 22.83±0.724*** | 179.47±4.9*** | 74.54±1.118*** | 47.72±0.913** |



- **Effect of NMRDCME on Stress induced changes in biochemical parameters and organ weights.**

Swim stress is one of the most severe stressor, since it involves both psychological and physical stress.

In the present study swim stress resulted in significant increase in the weights of liver, spleen and adrenal glands. The increase in adrenals weight could be attributed to stress induced adrenomedullary response. Adrenalin stimulates $\beta_2$- receptors on the pituitary gland causing greater release of ACTH. ACTH stimulates adrenal medulla as well as cortex. Swim stress being physical as well as psychological causes greater release of adrenalin leading to increase in weight of adrenal glands. Increase in Liver weight may be due to increased m-RNA levels in liver cells following swim stress, since the protein required for repair of wear and tear is great during swim stress and metabolic changes are more, therefore liver showed increase in weight following swim stress. This is in accordance with the results reported by Sardesai et al (1993) and Bhattacharya et al., (2000).
An increase in weigh of liver, spleen and adrenal glands was observed in the Stress control group while a significant reduction in the weights of these organs were observed in the groups treated with NMRDCME at 25, 50 and 100mg/kg dose levels. A highly significant response was observed at 50mg/kg dose level.

It is known that stress leads to stimulation of Hypothalamo-pituitary adrenal axis, which will trigger the release of corticotrophin releasing factor from hypothalamus with subsequent ACTH secretion. This causes adrenal glands to secrete glucocorticoids. The blood glucose level is strongly influenced by

glucocorticoids. They cause increase in blood glucose by mobilizing glycogen from liver and muscles due to enhancement of metabolic process. This glucose is available as rapid source of energy for physical work and metabolic requirements, which is high during swim stress (Sethi et al., 2003). The adaptogens prevent HPA activation and can thus prevent stress-induced elevation of blood glucose.

Similarly the data of the present study indicates that swim stress induced an increase in SGOT, SGPT, (probably due to alterations in the membrane permeability which might occur in the cells during stress) BUN, TRIGLYCERIDES, CHOLESTEROL levels in stress control group. The mechanisms by which stress raises serum cholesterol is likely to be related to the enhanced activity of hypothallamo-hypophyseal axis resulting in increased liberation of catecholamines and corticosteroids. This could lead to increase in blood cholesterol level since epinephrine is known to mobilize lipids from adipose tissue. (Bijlani et al 1985) similarly effect of stress on triglycerides has been shown to be variable. The stress associated with motor racing and public speaking results in acute elevation of triglycerides. Since a close relationship has been established between catecholamines and triglycerides it could be suggested that the change in the level of serum triglycerides is possibly mediated via adrenal medullary secretions and through activation of sympathetic nervous system. (Carlson et.,al 1968). where as these biochemical parameters were almost normalized like that of the values of Normal control group in NMRDCME treated groups at different dose levels. (as summarized in Table)

Thus, oral administration of DCM extract of *Nymphoides macrospermum* roots, exhibited stimulatory effects on humoral immunity (HA titre), cellular immunity (DTH response) and phagocytosis (Carbon clearance and E coli induced abdominal sepsis), protection against Cyclophosphamide induced myelosuppressionm and also reversed the stress induced elevations in the levels of Glucose, Cholesterol, SGPT, SGOT, Urea Nitrogen and reduction in Triglycerides. Qualitative chemical analysis revealed presence of fairly high content of terpenoids in NMRDCME.

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

In the recent years, HPTLC has emerged as a powerful tool for the establishment of TLC fingerprint profile (IHP, 2002: Pandey et al., 2006). The parameters to establish a complete TLC fingerprint profiles include , distinctive pattern of chromatogram, migration distances of the compounds separated (Rf), bands as observed with naked eye, as examined under UV (254 & 366), the UV absorption spectra of the resolved compounds, densitometric/flourimetric measurements of the resolved compounds for the calculation of their relative percentage and finally response to several reagents during derivatization.

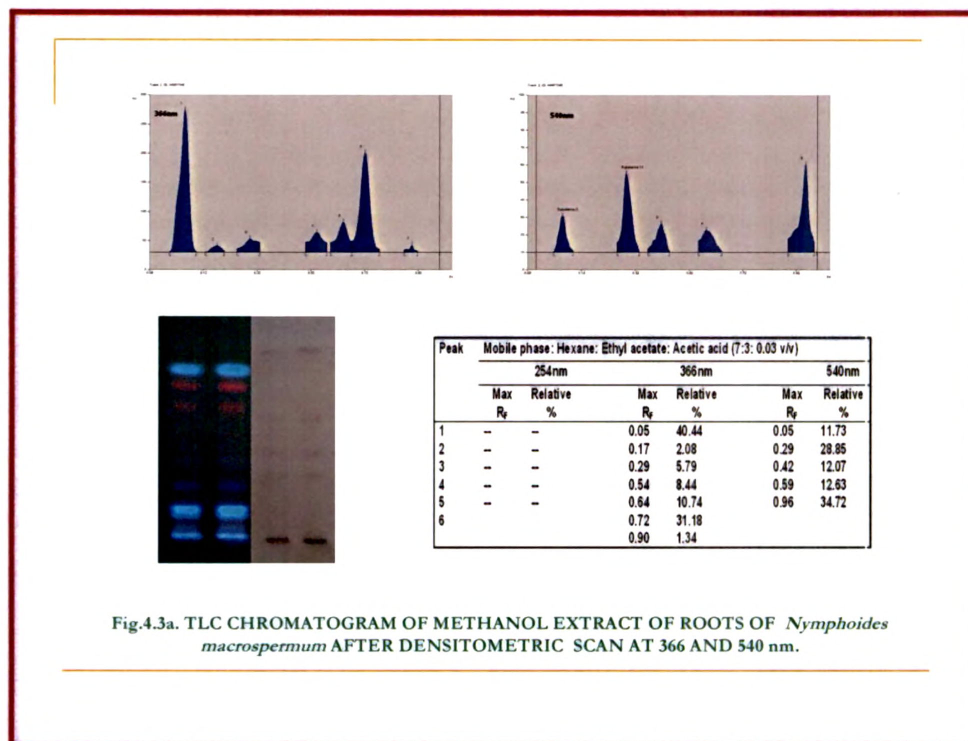
The main advantage of the fingerprint techniques is that the herbal drugs can be authenticated especially when the active principles are not known or when chemical markers are not available for the analysis. In the absence of known chemical markers the distinctive TLC fingerprint profile would form a characteristic identity for the drug which can be used to ascertain the quality of the herbal drug. Moreover, in those cases where chemical markers/biomarkers are known and method of analysis established for those compounds, it is still advisable and essential to develop fingerprint profiles to further characterize the herbal drug, since it is believed and in certain cases established that many compounds other than the marker compounds present in the herb have a role in the final therapeutic activity of the drug (Bruneton, 1995; Pugh and Sambo, 1988).

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, and relative percentage of the separated compounds were then recorded.

**Table 4.16**
**Solvent systems used for recording the HPTLC finger print profiles of methanol extract of roots of *Nymphoides macrospermum***

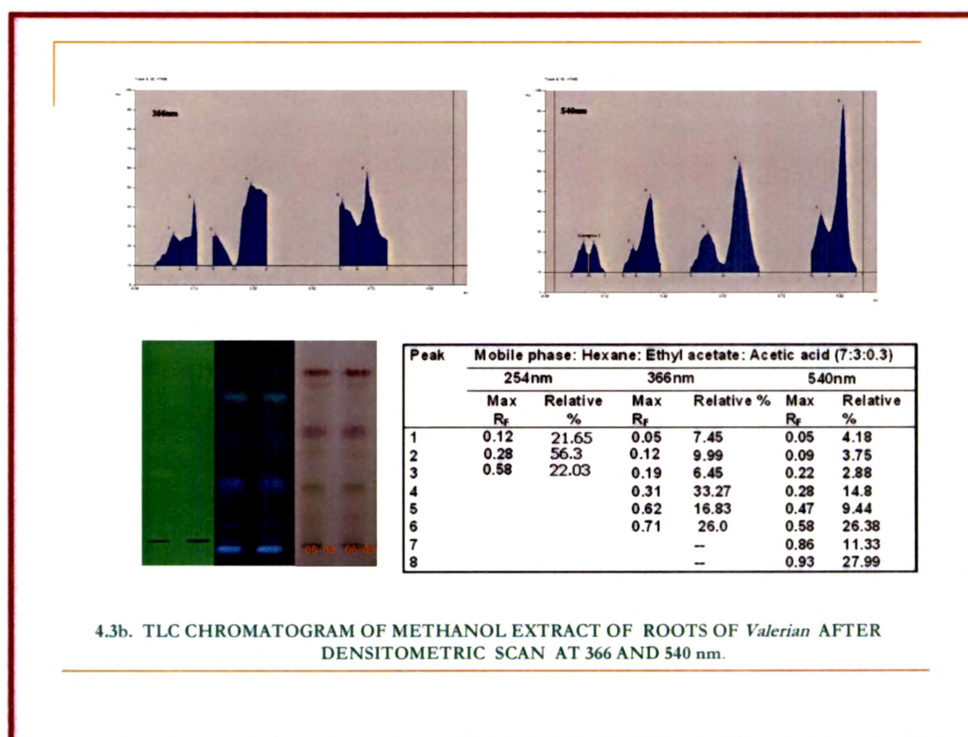
| | Solvent system |
|---|---|
| 1 | Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03) v/v |
| 2 | Hexane:Ethyl acetate: Acetic acid (7:3:0.3) |
| 3 | Ethyl acetate: Formic acid:Acetic acid:Water (8:1:0.4:1) |
| 4 | Toluene: Formic acid:Ethyl formate (5:1:4) |
| 5 | Hexane:Ethyl acetate (65:35)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

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, solvent system 5 was used to resolve iridoids, which were identified with HCl-Acetic acid reagent.



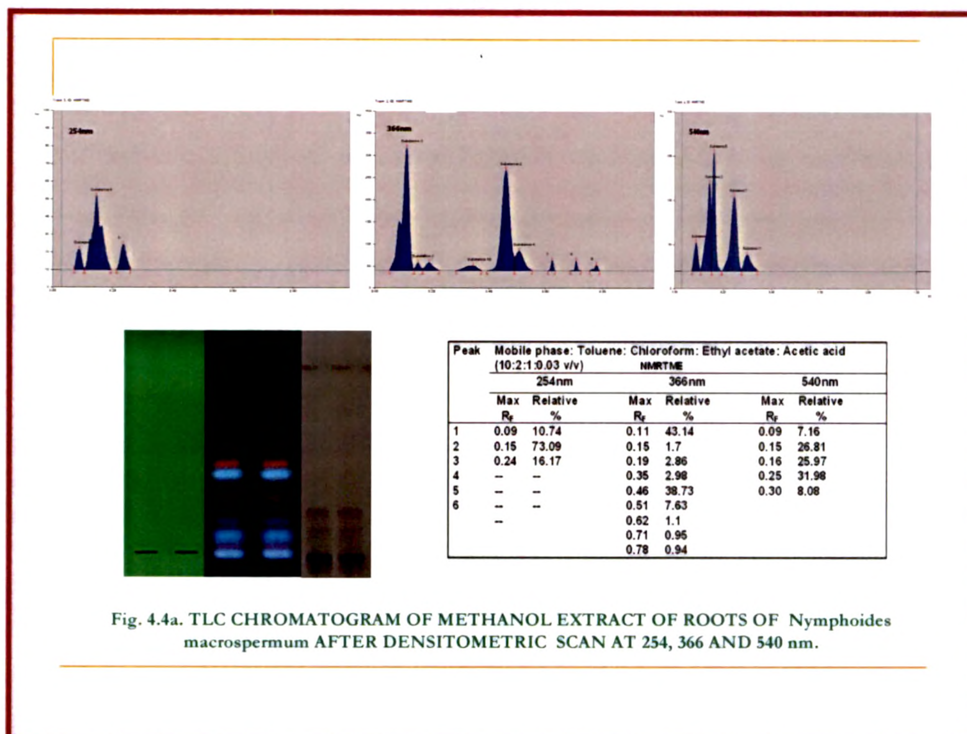

| Peak | Mobile phase: Hexane: Ethyl acetate: Acetic acid (7:3: 0.03 v/v) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_f$ | Relative % | Max $R_f$ | Relative % | Max $R_f$ | Relative % |
| 1 | -- | -- | 0.05 | 40.44 | 0.05 | 11.73 |
| 2 | -- | -- | 0.17 | 2.08 | 0.29 | 28.85 |
| 3 | -- | -- | 0.29 | 5.79 | 0.42 | 12.07 |
| 4 | -- | -- | 0.54 | 8.44 | 0.59 | 12.63 |
| 5 | -- | -- | 0.64 | 10.74 | 0.96 | 34.72 |
| 6 | | | 0.72 | 31.18 | | |
| | | | 0.90 | 1.34 | | |

Fig.4.3a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Nymphoides macrospermum* AFTER DENSITOMETRIC SCAN AT 366 AND 540 nm.



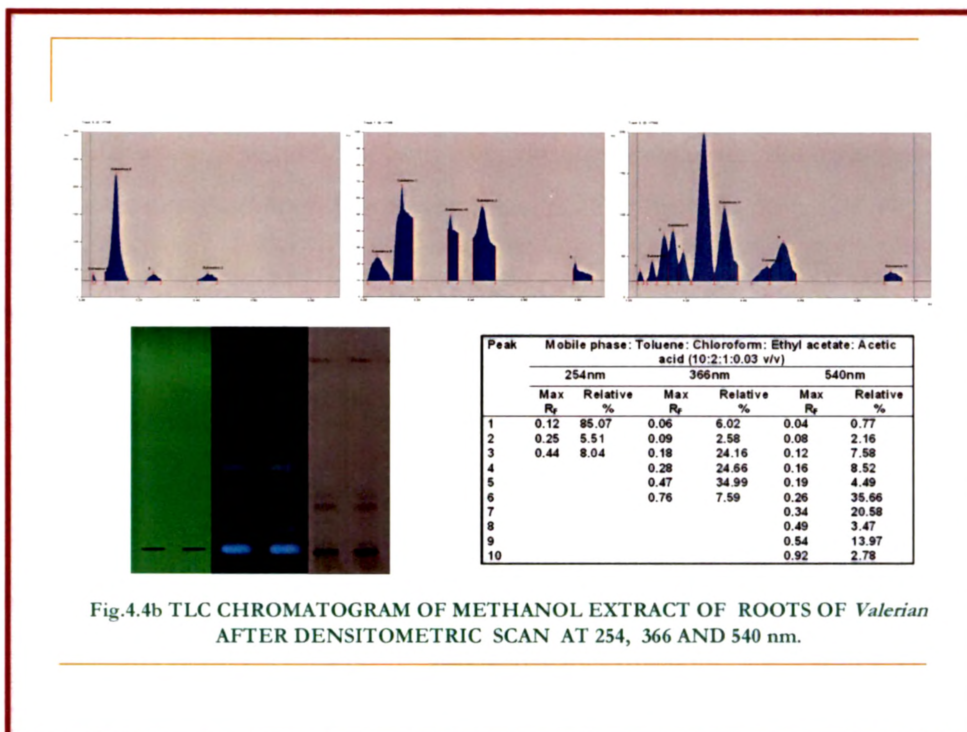

| Peak | Mobile phase: Hexane: Ethyl acetate: Acetic acid (7:3:0.3) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.12 | 21.65 | 0.05 | 7.45 | 0.05 | 4.18 |
| 2 | 0.28 | 56.3 | 0.12 | 9.99 | 0.09 | 3.75 |
| 3 | 0.58 | 22.03 | 0.19 | 6.45 | 0.22 | 2.88 |
| 4 | | | 0.31 | 33.27 | 0.28 | 14.8 |
| 5 | | | 0.62 | 16.83 | 0.47 | 9.44 |
| 6 | | | 0.71 | 26.0 | 0.58 | 26.38 |
| 7 | | | | -- | 0.86 | 11.33 |
| 8 | | | | -- | 0.93 | 27.99 |

4.3b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 366 AND 540 nm.




| Peak | Mobile phase: Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03 v/v) NMRTME | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.09 | 10.74 | 0.11 | 43.14 | 0.09 | 7.16 |
| 2 | 0.15 | 73.09 | 0.15 | 1.7 | 0.15 | 26.81 |
| 3 | 0.24 | 16.17 | 0.19 | 2.86 | 0.16 | 25.97 |
| 4 | -- | -- | 0.35 | 2.98 | 0.25 | 31.98 |
| 5 | -- | -- | 0.46 | 38.73 | 0.30 | 8.08 |
| 6 | -- | -- | 0.51 | 7.63 | | |
| | -- | | 0.62 | 1.1 | | |
| | | | 0.71 | 0.95 | | |
| | | | 0.78 | 0.94 | | |

Fig. 4.4a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF Nymphoides macrospermum AFTER DENSITOMETRIC SCAN AT 254, 366 AND 540 nm.



| Peak | Mobile phase: Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03 v/v) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.12 | 85.07 | 0.06 | 6.02 | 0.04 | 0.77 |
| 2 | 0.25 | 5.51 | 0.09 | 2.58 | 0.08 | 2.16 |
| 3 | 0.44 | 8.04 | 0.18 | 24.16 | 0.12 | 7.58 |
| 4 | | | 0.28 | 24.66 | 0.16 | 8.52 |
| 5 | | | 0.47 | 34.99 | 0.19 | 4.49 |
| 6 | | | 0.76 | 7.59 | 0.26 | 35.66 |
| 7 | | | | | 0.34 | 20.58 |
| 8 | | | | | 0.49 | 3.47 |
| 9 | | | | | 0.54 | 13.97 |
| 10 | | | | | 0.92 | 2.78 |

Fig.4.4b TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 254, 366 AND 540 nm.

TLC comparative study of methanol extracts of *Nymphoides macrospermum* and Valerian were performed by developing the plate with (Solvent system 1) Hexane :Ethyl acetate : Acetic acid (7:3:0.03 v/v) and derivatizing with AS and scanned densitometrically at 254, 366 and 540nm. In case of *Nymphoides* scanning at 366nm showed 7 spots with Rf 0.05, 0.17, 0.29,0.54, 0.64, 0.72 and 0.90 and scanning at 540nm showed only 5 spots with Rf 0.05,0.29, 0.42, 0.59 and 0.96 while in case of Valerian at 366nm the chromatogram showed 6 spots with Rf 0.05,0.12,0.19,0.31,0.62 and 0.71 and at 540nm 8 spots were observed with Rf 0.05, 0.09.0.22,0.28,0.47,0.58,0.86 and 0.93.

Chromatogram of both the extracts when scanned at 366nm showed some peaks with very close Rf values 0.05, 0.29, 0.64 and 0.72 and similarly at 540 nm scan peaks with Rf 0.05, 0.29 and 0.58 Chromatograms of both the extracts when screened at 254nm did not exhibit any peaks, but some peaks with identical Rf were observed in both the extracts indicating the presence of similar class of constituents in both the extracts. (See fig. 4.3 a & b)

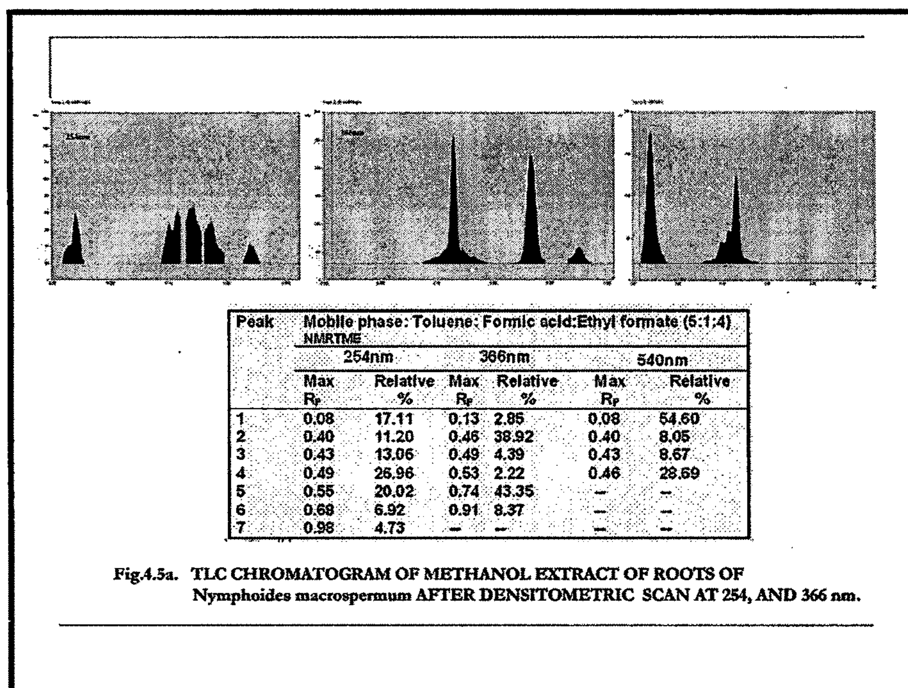
In HPTLC fingerprinting studies of methanol extracts of roots of *Nymphoides macrospermum* and *Valerian wallichi* with solvent system 2 (Toluene:Chloroform:Ethyl acetate: Acetic acid 10:2:1:0.03 v/v) at 254nm scan showed 3 spots with Rf 0.09, 0.15 and 0.24, at 366nm scan 9 spots were observed with Rf 0.11, 0.15, 0.19, 0.35, 0.46, 0.51, 0.62, 0.71 and 0.78 respectively and at 540nm scan showed only 5 spots with Rf 0.09, 0.15, 0.16, 0.25 and 0.30. while in case of Valerian at 254nm it showed 3 spots with Rf 0.12, 0.25 and 0.44 and scanning at 366nm 6 spots were observed with Rf 0.06,0.09, 0.18, 0.28, 0.47 and 0.76 and at 540nm scan it showed 10 spots with Rf 0.04, 0.08, 0.12, 0.16,0.19,0.26, 0.34, 0.49, 0.54 and 0.92.

Peaks with almost same Rf 0.24 (254nm scan), 0.19 & 0.46 (366nm scan) and 0.09, 0.16 & 0.25 (540nm scan) were observed in both the extracts. (See fig. 4.4 a & b)

Similarly the TLC chromatogram of methanol extract of *Nymphoides macrospermum* and *Valeriana wallichi* with solvent system 3 a t 254nm revealed the presence of 3peaks, at 366nm showed the presence of 9 peaks and at 540nm it showed the presence of 7 peaks, whereas in case of Valerian at 254nm 3 peaks were found, at 366nm and 540nm revealed the presence of 7 and 5 peaks. Peaks for the compounds with Rf 0.1, 0.12, 0.24 and 0.44 were found common in both the extracts.

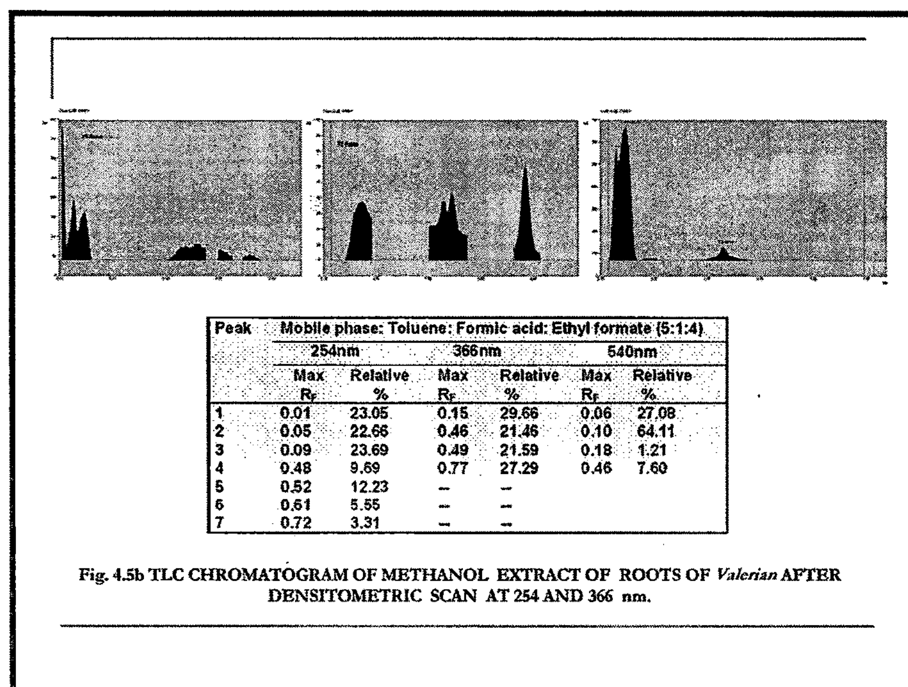
In HPTLC fingerprinting studies of methanol extracts of roots of *Nymphoides macrospermum* and *Valerian wallichi* with solvent system 4 (Toluene: Formic acid: Ethyl formate 5:1:4 v/v) at 254nm scan showed 7 spots, at 366nm showed the presence of 6 spots and at 540 it showed the presence of only 4 spots. Whereas in case of Valerian at 254nm 7 peaks were found , at 366nm and 540 nm revealed the presence of 4peaks. In both the extracts peaks for the compounds with Rf 0.46 and 0.49 were found common.( See fig. 4.5a & b).
Results of the comparative HPTLC fingerprinting studies indicates the presence of some similar class of constituents both the extracts.

Similarly, HPTLC fingerprint profile for the successive Dichloromethane, ethyl acetate extract and successive methanol extracts of roots of *Nymphoides macrospermum* were generated. (See fig. 4.10 a & b and 4.11 a & b).



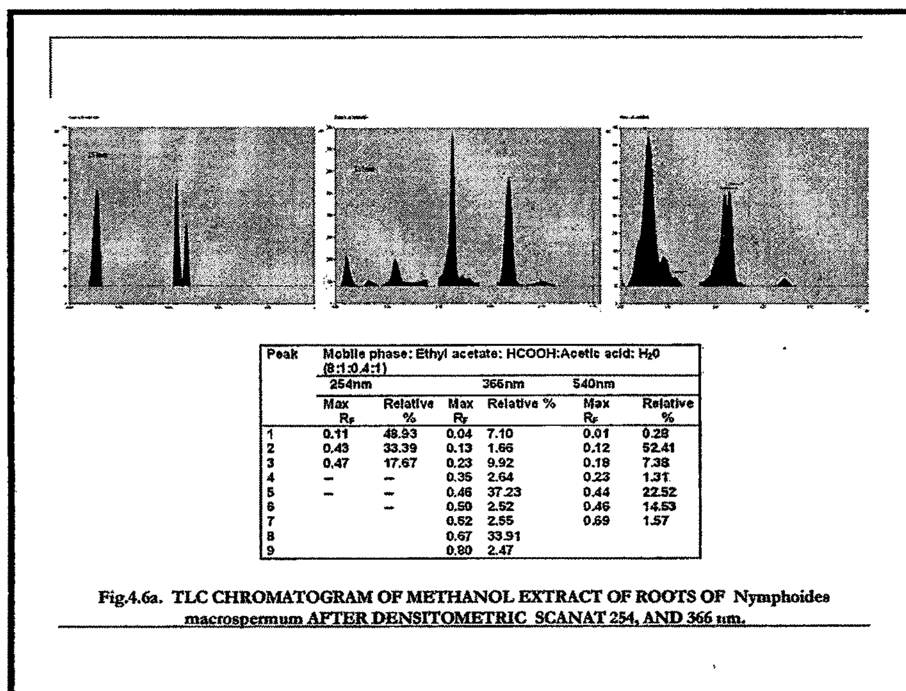
| Peak | Mobile phase: Toluene: Formic acid:Ethyl formate (5:1:4) NMRTME | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.08 | 17.11 | 0.13 | 2.85 | 0.08 | 54.60 |
| 2 | 0.40 | 11.20 | 0.46 | 38.92 | 0.40 | 8.05 |
| 3 | 0.43 | 13.06 | 0.49 | 4.39 | 0.43 | 8.67 |
| 4 | 0.49 | 26.96 | 0.53 | 2.22 | 0.46 | 28.69 |
| 5 | 0.55 | 20.02 | 0.74 | 43.35 | – | – |
| 6 | 0.68 | 6.92 | 0.91 | 8.37 | – | – |
| 7 | 0.98 | 4.73 | – | – | – | – |

**Fig.4.5a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF Nymphoides macrospermum AFTER DENSITOMETRIC SCAN AT 254, AND 366 nm.**



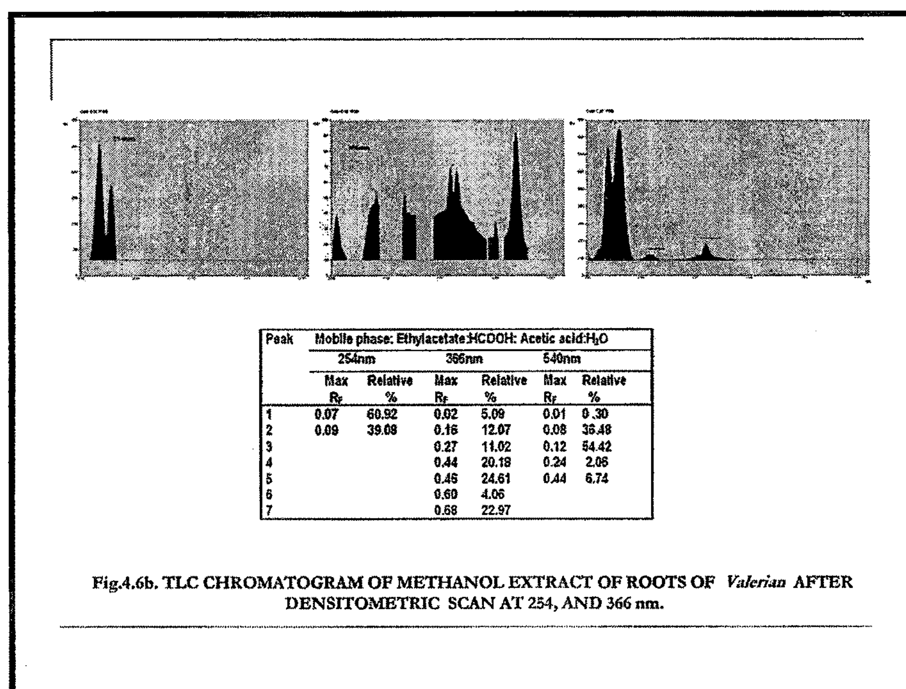
| Peak | Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.01 | 23.05 | 0.15 | 29.66 | 0.06 | 27.08 |
| 2 | 0.05 | 22.66 | 0.46 | 21.46 | 0.10 | 64.11 |
| 3 | 0.09 | 23.69 | 0.49 | 21.59 | 0.18 | 1.21 |
| 4 | 0.48 | 9.69 | 0.77 | 27.29 | 0.46 | 7.60 |
| 5 | 0.52 | 12.23 | – | – | | |
| 6 | 0.61 | 5.55 | – | – | | |
| 7 | 0.72 | 3.31 | – | – | | |

**Fig. 4.5b TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 254 AND 366 nm.**



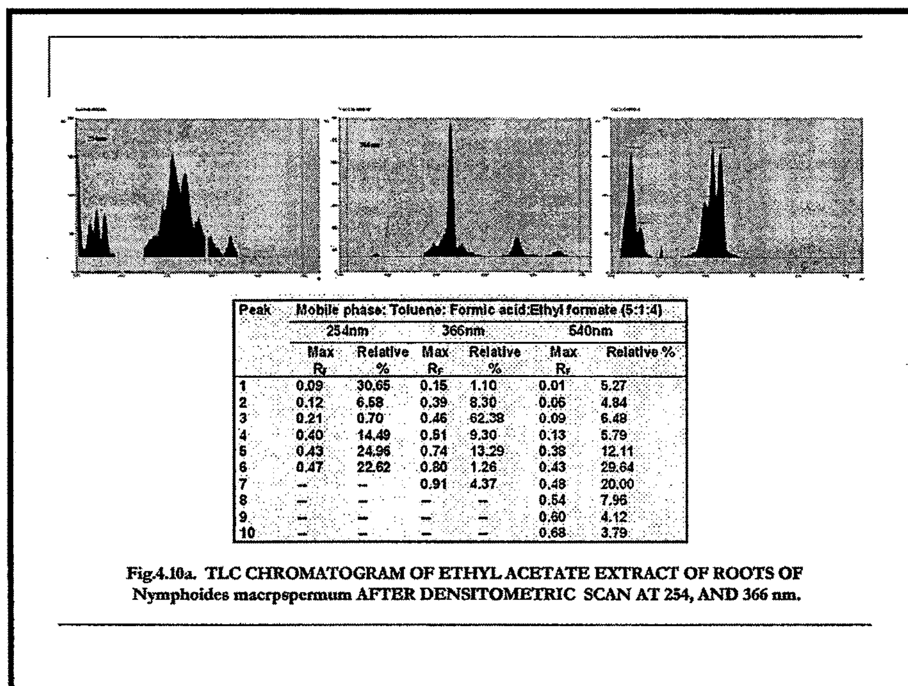
| Peak | Mobile phase: Ethyl acetate: HCOOH:Acetic acid: $H_2O$ (8:1:0.4:1) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.11 | 48.93 | 0.04 | 7.10 | 0.01 | 0.28 |
| 2 | 0.43 | 33.39 | 0.13 | 1.66 | 0.12 | 52.41 |
| 3 | 0.47 | 17.67 | 0.23 | 9.92 | 0.18 | 7.38 |
| 4 | – | – | 0.35 | 2.64 | 0.23 | 1.31 |
| 5 | – | – | 0.46 | 37.23 | 0.44 | 22.52 |
| 6 | | – | 0.50 | 2.52 | 0.46 | 14.53 |
| 7 | | | 0.62 | 2.55 | 0.69 | 1.57 |
| 8 | | | 0.67 | 33.91 | | |
| 9 | | | 0.80 | 2.47 | | |

**Fig.4.6a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF Nymphoides macrospermum AFTER DENSITOMETRIC SCANAT 254, AND 366 nm.**



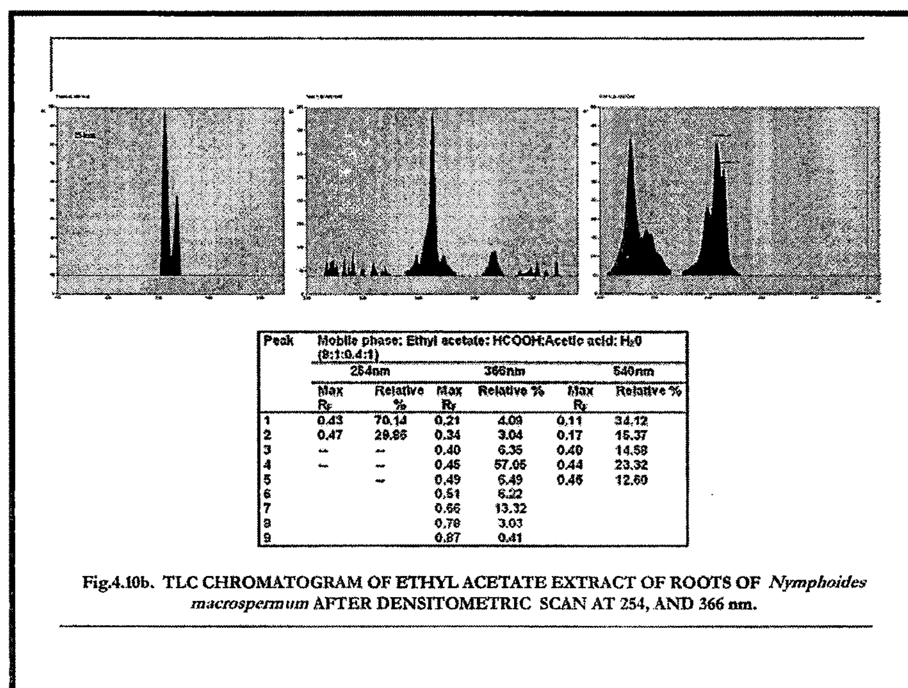
| Peak | Mobile phase: Ethylacetate:HCOOH: Acetic acid:$H_2O$ | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.07 | 60.92 | 0.02 | 5.09 | 0.01 | 0.30 |
| 2 | 0.09 | 39.08 | 0.16 | 12.07 | 0.08 | 36.48 |
| 3 | | | 0.27 | 11.02 | 0.12 | 54.42 |
| 4 | | | 0.44 | 20.18 | 0.24 | 2.06 |
| 5 | | | 0.46 | 24.61 | 0.44 | 6.74 |
| 6 | | | 0.60 | 4.06 | | |
| 7 | | | 0.68 | 22.97 | | |

**Fig.4.6b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 254, AND 366 nm.**



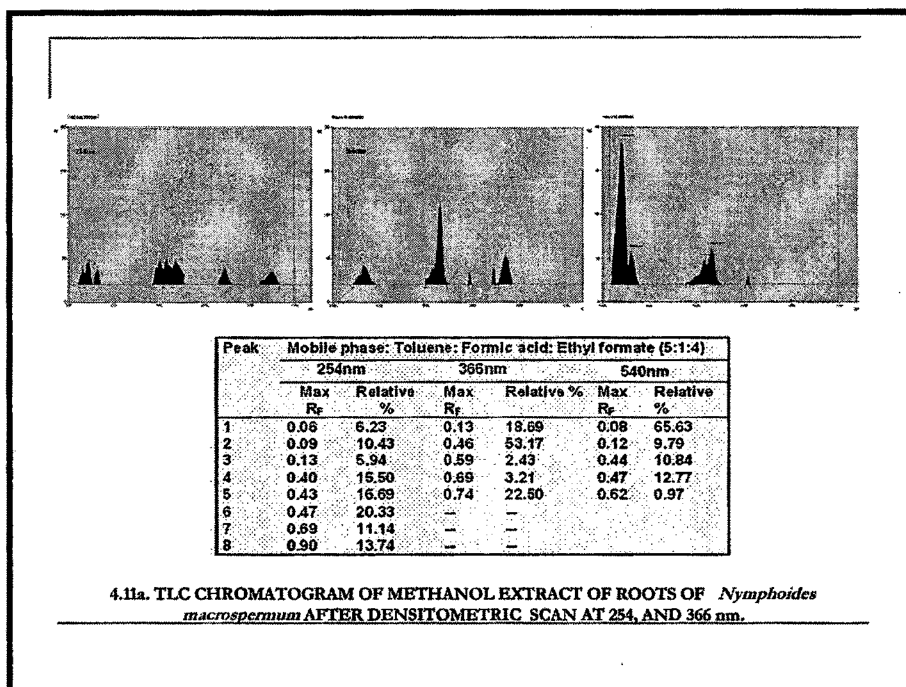
| Peak | Mobile phase: Toluene: Formic acid:Ethyl formate (5:1:4) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_f$ | Relative % | Max $R_f$ | Relative % | Max $R_f$ | Relative % |
| 1 | 0.09 | 30.65 | 0.15 | 1.10 | 0.01 | 5.27 |
| 2 | 0.12 | 6.58 | 0.39 | 8.30 | 0.06 | 4.84 |
| 3 | 0.21 | 0.70 | 0.46 | 62.38 | 0.09 | 6.48 |
| 4 | 0.40 | 14.49 | 0.51 | 9.30 | 0.13 | 5.79 |
| 5 | 0.43 | 24.96 | 0.74 | 13.29 | 0.38 | 12.11 |
| 6 | 0.47 | 22.62 | 0.80 | 1.26 | 0.43 | 29.64 |
| 7 | – | – | 0.91 | 4.37 | 0.48 | 20.00 |
| 8 | – | – | – | – | 0.54 | 7.96 |
| 9 | – | – | – | – | 0.60 | 4.12 |
| 10 | – | – | – | – | 0.68 | 3.79 |

**Fig.4.10a. TLC CHROMATOGRAM OF ETHYL ACETATE EXTRACT OF ROOTS OF Nymphoides macrpspermum AFTER DENSITOMETRIC SCAN AT 254, AND 366 nm.**



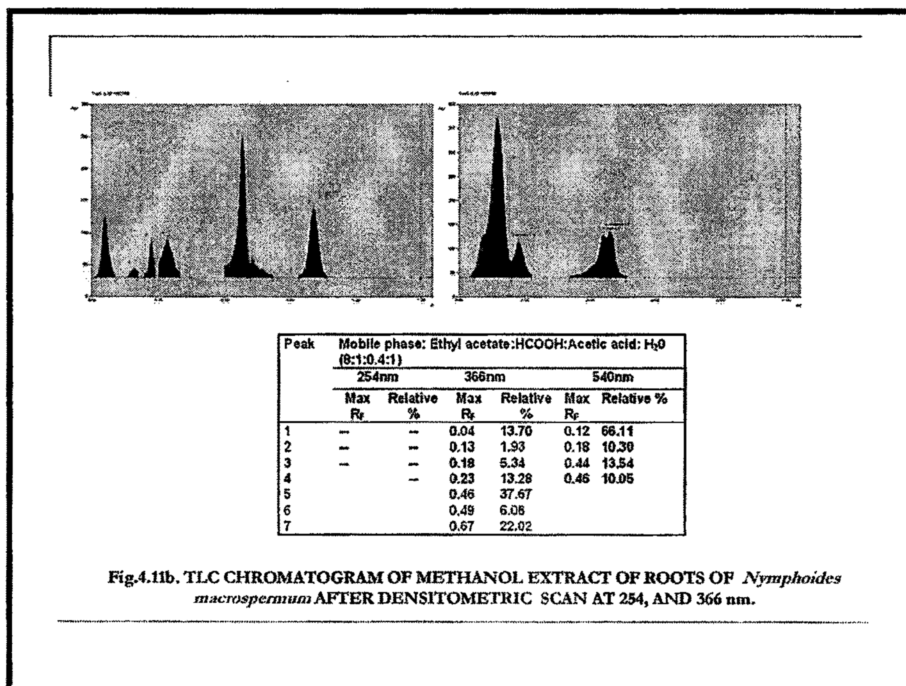
| Peak | Mobile phase: Ethyl acetate: HCOOH:Acetic acid: $H_2O$ (8:1:0.4:1) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_f$ | Relative % | Max $R_f$ | Relative % | Max $R_f$ | Relative % |
| 1 | 0.43 | 70.14 | 0.21 | 4.09 | 0.11 | 34.12 |
| 2 | 0.47 | 29.86 | 0.34 | 3.04 | 0.17 | 15.37 |
| 3 | – | – | 0.40 | 6.35 | 0.40 | 14.58 |
| 4 | – | – | 0.45 | 57.05 | 0.44 | 23.32 |
| 5 | | – | 0.49 | 6.49 | 0.46 | 12.60 |
| 6 | | | 0.51 | 6.22 | | |
| 7 | | | 0.66 | 13.32 | | |
| 8 | | | 0.78 | 3.03 | | |
| 9 | | | 0.87 | 0.41 | | |

**Fig.4.10b. TLC CHROMATOGRAM OF ETHYL ACETATE EXTRACT OF ROOTS OF *Nymphoides macrospermum* AFTER DENSITOMETRIC SCAN AT 254, AND 366 nm.**



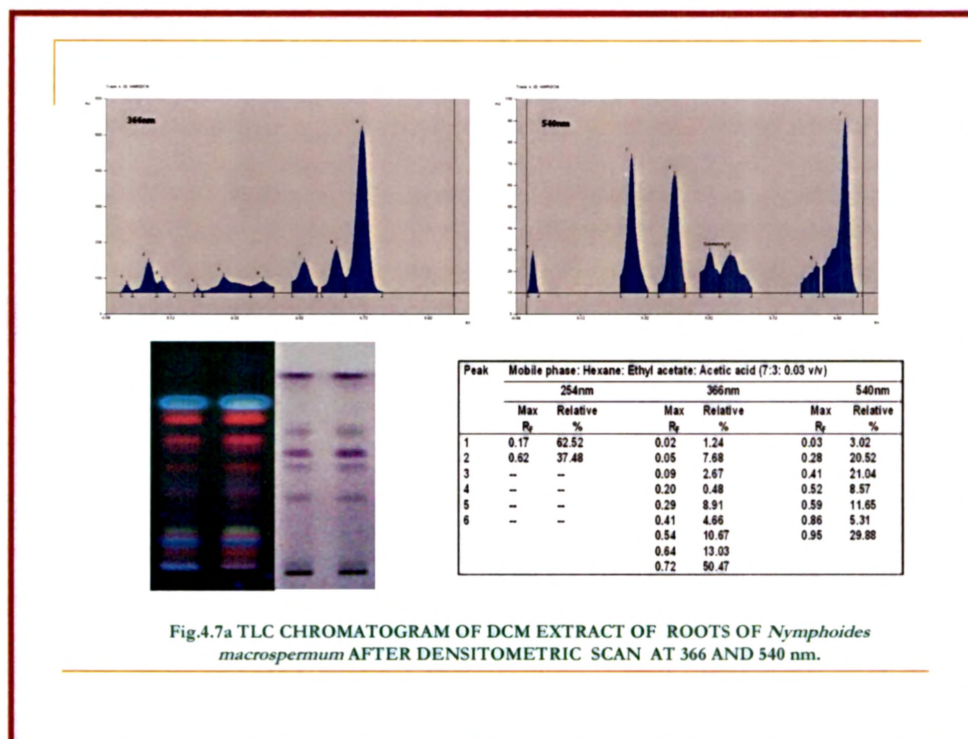
| Peak | Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.06 | 6.23 | 0.13 | 18.69 | 0.08 | 65.63 |
| 2 | 0.09 | 10.43 | 0.46 | 53.17 | 0.12 | 9.79 |
| 3 | 0.13 | 5.94 | 0.59 | 2.43 | 0.44 | 10.84 |
| 4 | 0.40 | 15.50 | 0.69 | 3.21 | 0.47 | 12.77 |
| 5 | 0.43 | 16.69 | 0.74 | 22.50 | 0.62 | 0.97 |
| 6 | 0.47 | 20.33 | – | – | | |
| 7 | 0.69 | 11.14 | – | – | | |
| 8 | 0.90 | 13.74 | – | – | | |

4.11a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Nymphoides macrospermum* AFTER DENSITOMETRIC SCAN AT 254, AND 366 nm.



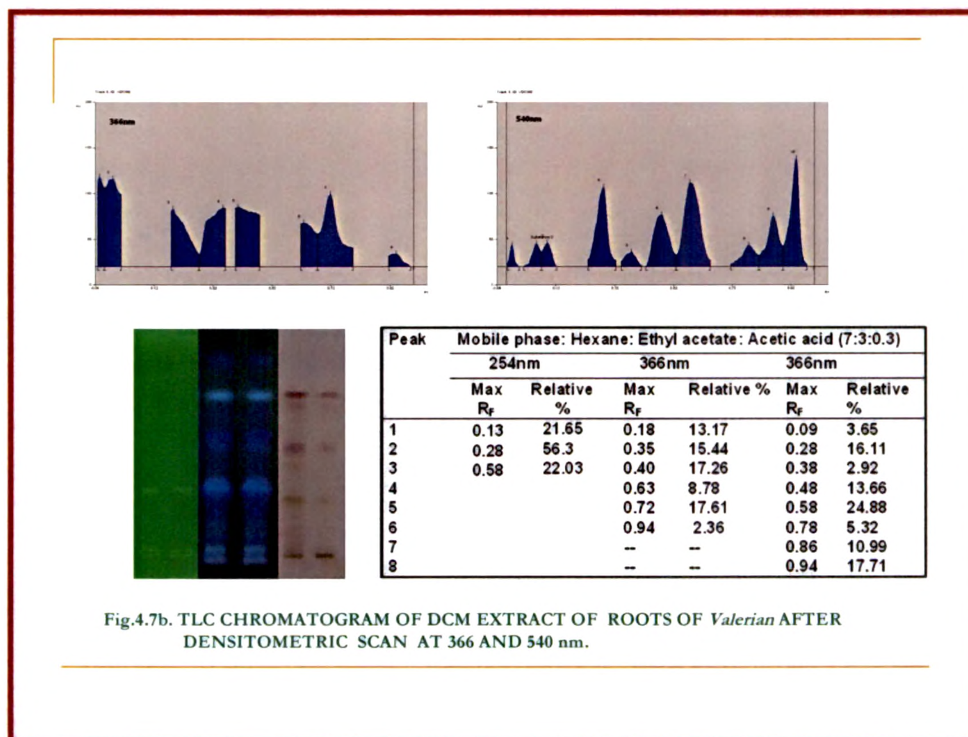
| Peak | Mobile phase: Ethyl acetate:HCOOH:Acetic acid: $H_2O$ (8:1:0.4:1) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | – | – | 0.04 | 13.70 | 0.12 | 66.11 |
| 2 | – | – | 0.13 | 1.93 | 0.18 | 10.30 |
| 3 | – | – | 0.18 | 5.34 | 0.44 | 13.54 |
| 4 | | – | 0.23 | 13.28 | 0.46 | 10.05 |
| 5 | | | 0.46 | 37.67 | | |
| 6 | | | 0.49 | 6.06 | | |
| 7 | | | 0.67 | 22.02 | | |

Fig.4.11b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Nymphoides macrospermum* AFTER DENSITOMETRIC SCAN AT 254, AND 366 nm.



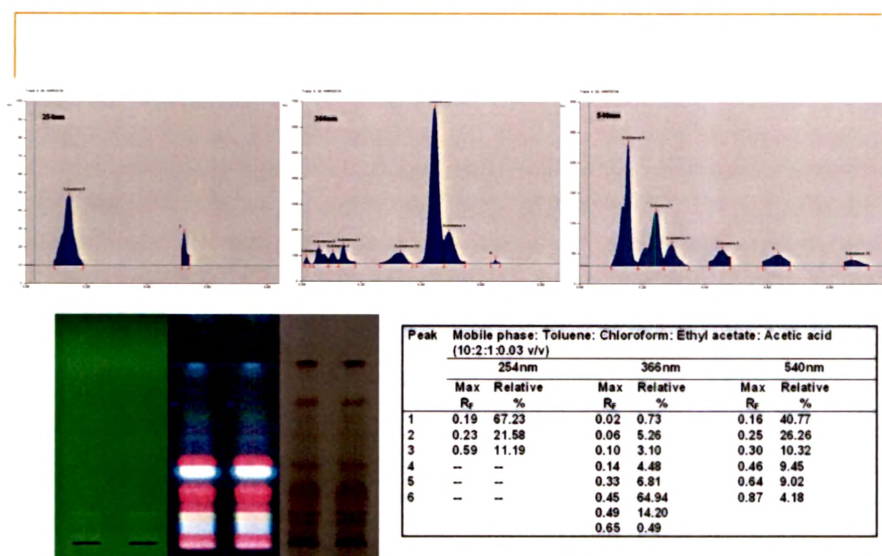

| Peak | Mobile phase: Hexane: Ethyl acetate: Acetic acid (7:3: 0.03 v/v) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.17 | 62.52 | 0.02 | 1.24 | 0.03 | 3.02 |
| 2 | 0.62 | 37.48 | 0.05 | 7.68 | 0.28 | 20.52 |
| 3 | -- | -- | 0.09 | 2.67 | 0.41 | 21.04 |
| 4 | -- | -- | 0.20 | 0.48 | 0.52 | 8.57 |
| 5 | -- | -- | 0.29 | 8.91 | 0.59 | 11.65 |
| 6 | -- | -- | 0.41 | 4.66 | 0.86 | 5.31 |
| | | | 0.54 | 10.67 | 0.95 | 29.88 |
| | | | 0.64 | 13.03 | | |
| | | | 0.72 | 50.47 | | |

Fig.4.7a TLC CHROMATOGRAM OF DCM EXTRACT OF ROOTS OF *Nymphoides macrospermum* AFTER DENSITOMETRIC SCAN AT 366 AND 540 nm.



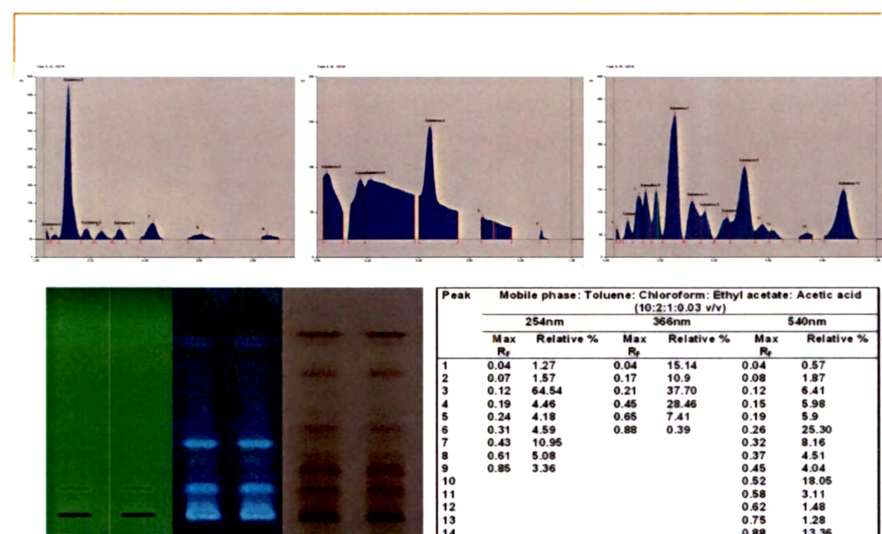

| Peak | Mobile phase: Hexane: Ethyl acetate: Acetic acid (7:3:0.3) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 366nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.13 | 21.65 | 0.18 | 13.17 | 0.09 | 3.65 |
| 2 | 0.28 | 56.3 | 0.35 | 15.44 | 0.28 | 16.11 |
| 3 | 0.58 | 22.03 | 0.40 | 17.26 | 0.38 | 2.92 |
| 4 | | | 0.63 | 8.78 | 0.48 | 13.66 |
| 5 | | | 0.72 | 17.61 | 0.58 | 24.88 |
| 6 | | | 0.94 | 2.36 | 0.78 | 5.32 |
| 7 | | | -- | -- | 0.86 | 10.99 |
| 8 | | | -- | -- | 0.94 | 17.71 |

Fig.4.7b. TLC CHROMATOGRAM OF DCM EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 366 AND 540 nm.



| Peak | Mobile phase: Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03 v/v) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_f$ | Relative % | Max $R_f$ | Relative % | Max $R_f$ | Relative % |
| 1 | 0.19 | 67.23 | 0.02 | 0.73 | 0.16 | 40.77 |
| 2 | 0.23 | 21.58 | 0.06 | 5.26 | 0.25 | 26.26 |
| 3 | 0.59 | 11.19 | 0.10 | 3.10 | 0.30 | 10.32 |
| 4 | -- | -- | 0.14 | 4.48 | 0.46 | 9.45 |
| 5 | -- | -- | 0.33 | 6.81 | 0.64 | 9.02 |
| 6 | -- | -- | 0.45 | 64.94 | 0.87 | 4.18 |
| | | | 0.49 | 14.20 | | |
| | | | 0.65 | 0.49 | | |

Fig.4.8a TLC CHROMATOGRAM OF DCM EXTRACT OF ROOTS OF Nymphoides macrospermum AFTER DENSITOMETRIC SCAN AT 254, 366 AND 540 nm.



| Peak | Mobile phase: Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03 v/v) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 540nm | |
| | Max $R_f$ | Relative % | Max $R_f$ | Relative % | Max $R_f$ | Relative % |
| 1 | 0.04 | 1.27 | 0.04 | 15.14 | 0.04 | 0.57 |
| 2 | 0.07 | 1.57 | 0.17 | 10.9 | 0.08 | 1.87 |
| 3 | 0.12 | 64.54 | 0.21 | 37.70 | 0.12 | 6.41 |
| 4 | 0.19 | 4.46 | 0.45 | 28.46 | 0.15 | 5.98 |
| 5 | 0.24 | 4.18 | 0.65 | 7.41 | 0.19 | 5.9 |
| 6 | 0.31 | 4.59 | 0.88 | 0.39 | 0.26 | 25.30 |
| 7 | 0.43 | 10.95 | | | 0.32 | 8.16 |
| 8 | 0.61 | 5.08 | | | 0.37 | 4.51 |
| 9 | 0.85 | 3.36 | | | 0.45 | 4.04 |
| 10 | | | | | 0.52 | 18.05 |
| 11 | | | | | 0.58 | 3.11 |
| 12 | | | | | 0.62 | 1.48 |
| 13 | | | | | 0.75 | 1.28 |
| 14 | | | | | 0.88 | 13.36 |

Fig.4.8b. TLC CHROMATOGRAM OF DCM EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 254, 366 AND 540 nm.

TLC comparative study of DCM extracts of *Nymphoides macrospermum* and Valerian were performed with (Solvent system 2) Hexane :Ethyl acetate : Acetic acid (7:3:0.03 v/v) chromatogram in case of *Nymphoides* at 254nm showed presence of 2 peaks, at 366nm showed 9 peaks with and at 540nm showed the presence of 7 peaks. In case of Valerian no peaks were detected at 254nm, at 366nm 6 peaks were found and at 540nm 8 peaks were found. In both the extracts peaks for the compounds with Rf 0.28, 0.41, 0.59, 0.72, 0.86 and 0.94 were found common. (See fig. 4.7 a & b)

Similarly DCM extracts of *Nymphoides macrospermum* and Valerian when resolved with (Solvent system 1 ) Toluene: Chloroform: Ethyl acetate : Acetic acid (10:2:1:0.03 v/v) chromatogram in case of *Nymphoides* at 254nm showed presence of 3 peaks, at 366nm showed 8 peaks with and at 540nm showed the presence of 8 peaks. In case of Valerian 9 peaks were detected at 254nm, at 366 nm 6 peaks were found and at 540nm 14 peaks were found. In both the extracts peaks for the compounds with Rf 0.16, 0.25, 0.46, 0.64, and 0.87 were found common. (See fig. 4.8 a & b)

The chromatograms of DCM extracts of both the drugs were very much similar suggesting the presence of very closely related constituents in both the extracts.

## 4.5. Identification of Valepotriates from roots of *Nymphoides macrospermum*

Valerian contains over 150 chemical constituents; many are physiologically active. There is substantial variation in the chemical constituents in plants from different sources, growing conditions, processing methods and storage conditions. Although the sedative effect of the roots is known for centuries, the exact chemical compounds responsible are yet to be identified. Valerian's effects on the central nervous system is generally attributed to the presence of valepotriates, and their breakdown products (baldrinals), valerenic acid, valerenal and valeranone, and also other constituents in the essential oil. Valepotriates: These compounds are chemically unstable iridoid triesters in which the various hydroxyl groups are esterified with acetic, isovaleric, hydroxyisovaleric, and a-methylvaleric acids. The valepotriates are divided into the monoene valepotriate didrovaltrate and several diene valepotriates (valtrate, isovaltrate, homovaltrate, and acevaltrate). Valepotriates are triesters of a terpenoid, trihydric alcohol. This alcohol has the structure of an iridoid cyclopenta-(c)-pyran with an attached epoxide ring. A complete HPTLC finger print profile of the resolved compounds comprising of the typical spectra, Rf value, UV absorption maxima and the percentage proportion of the individual components in the extract are recorded and documented. The reported data could be of great value as a reference standard for evaluation of this plant material.

A comparative HPTLC fingerprinting of the Dichloromethane extracts of roots of *Nymphoides macrospermum* and Valerian for Valepotriates was performed as per the method described in American Herbal Pharmacopoeia

**Chromatographic conditions:**

**Stationary Phase:** HPTLC plates 10x10 cms Silica gel with florescent indicator.

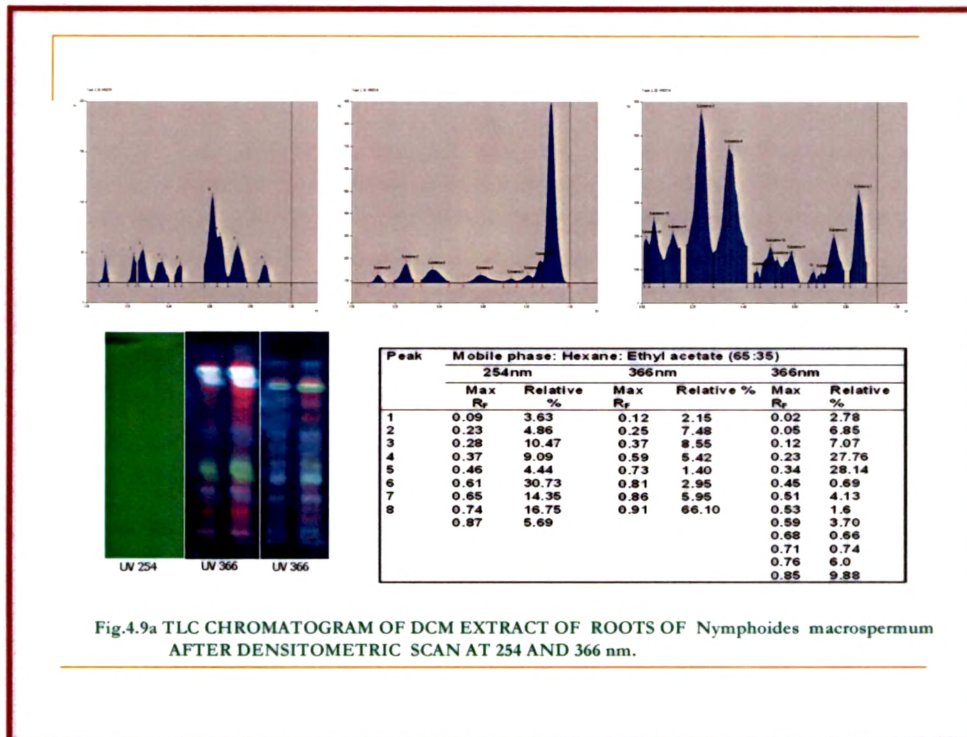
**Mobile Phase:** Hexane: Ethyl acetate: Acetic acid (6.5:3.5:0.05)

**Sample application:** 10 ul of DCM extract of Nymphoides and Valerian are applied each as 8mm bands

**Detection:**
1. UV- 254nm
2. UV-366nm
3. Spray the plate with HCl- Acetic acid reagent, dry in stream of cold air, heat at 110 $^{0}$ C for 5 min. and scan at UV-366nm

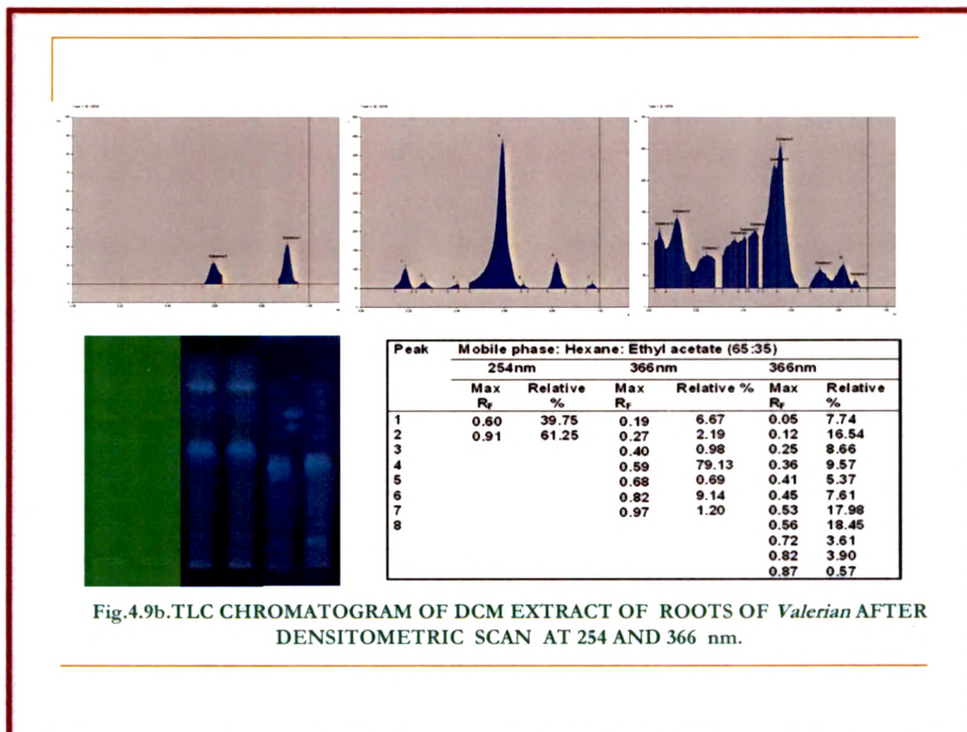
TLC chromatogram of DCM extract of *Nymphoides macrospermum* roots at 254nm showed presence of 9 peaks for compounds with Rf 0.09, 0.23, 0.28, 0.37, 0.46, 0.61, 0.65, 0.74 and 0.87 and at 366nm after derivatization produced 13 peaks with Rf 0.02, 0.05, 0.12, 0.23, 0.34, 0.45, 0.51, 0.53, 0.59, 0.68, 0.71, 0.76 and 0.85 respectively. The chromatogram of Valerian at 254nm revealed the presence of 7 peaks with Rf 0.19, 0.27, 0.40, 0.59, 0.68, 0.82 and 0.87 while at 366nm 11 peaks with Rf 0.05, 0.12, 0.25, 0.36, 0.41, 0.45, 0.53, 0.56, 0.72, 0.82 and 0.87.A common peak at Rf 0.45 ( blue fluorescence) corresponds to Valerenic acid revealed, its presence in *Nymphoides macrospermum* also.

Thus, the HPTLC fingerprint profiles of the important chemical constituents in the bioactive extracts of the roots of *Nymphoides macrospermum* are recorded and documented. The reported data could be of great value as a reference standard for evaluation of this plant material.




| Peak | Mobile phase: Hexane: Ethyl acetate (65:35) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 366nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.09 | 3.63 | 0.12 | 2.15 | 0.02 | 2.78 |
| 2 | 0.23 | 4.86 | 0.25 | 7.48 | 0.05 | 6.85 |
| 3 | 0.28 | 10.47 | 0.37 | 8.55 | 0.12 | 7.07 |
| 4 | 0.37 | 9.09 | 0.59 | 5.42 | 0.23 | 27.76 |
| 5 | 0.46 | 4.44 | 0.73 | 1.40 | 0.34 | 28.14 |
| 6 | 0.61 | 30.73 | 0.81 | 2.95 | 0.45 | 0.69 |
| 7 | 0.65 | 14.35 | 0.86 | 5.95 | 0.51 | 4.13 |
| 8 | 0.74 | 16.75 | 0.91 | 66.10 | 0.53 | 1.6 |
| | 0.87 | 5.69 | | | 0.59 | 3.70 |
| | | | | | 0.68 | 0.66 |
| | | | | | 0.71 | 0.74 |
| | | | | | 0.76 | 6.0 |
| | | | | | 0.85 | 9.88 |

Fig.4.9a TLC CHROMATOGRAM OF DCM EXTRACT OF ROOTS OF Nymphoides macrospermum AFTER DENSITOMETRIC SCAN AT 254 AND 366 nm.

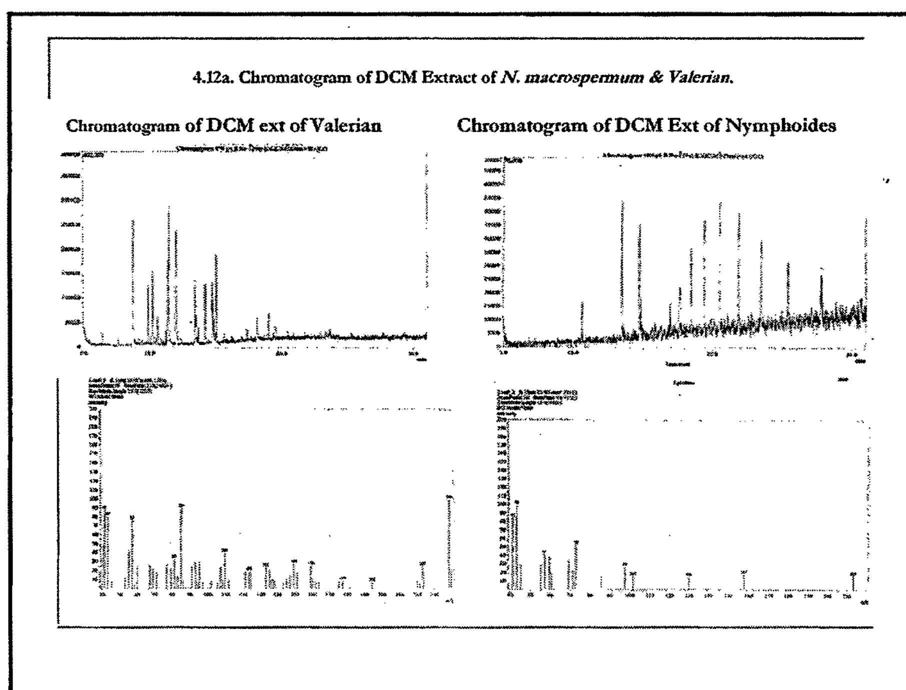


| Peak | Mobile phase: Hexane: Ethyl acetate (65:35) | | | | | |
|---|---|---|---|---|---|---|
| | 254nm | | 366nm | | 366nm | |
| | Max $R_F$ | Relative % | Max $R_F$ | Relative % | Max $R_F$ | Relative % |
| 1 | 0.60 | 39.75 | 0.19 | 6.67 | 0.05 | 7.74 |
| 2 | 0.91 | 61.25 | 0.27 | 2.19 | 0.12 | 16.54 |
| 3 | | | 0.40 | 0.98 | 0.25 | 8.66 |
| 4 | | | 0.59 | 79.13 | 0.36 | 9.57 |
| 5 | | | 0.68 | 0.69 | 0.41 | 5.37 |
| 6 | | | 0.82 | 9.14 | 0.45 | 7.61 |
| 7 | | | 0.97 | 1.20 | 0.53 | 17.98 |
| 8 | | | | | 0.56 | 18.45 |
| | | | | | 0.72 | 3.61 |
| | | | | | 0.82 | 3.90 |
| | | | | | 0.87 | 0.57 |

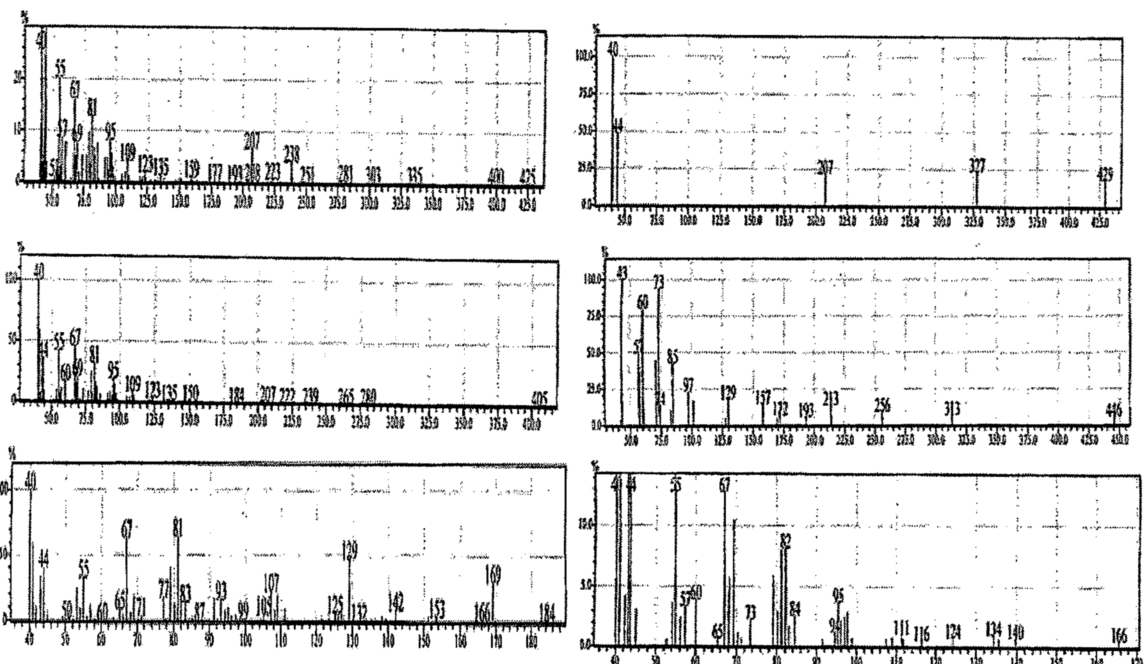
Fig.4.9b.TLC CHROMATOGRAM OF DCM EXTRACT OF ROOTS OF *Valerian* AFTER DENSITOMETRIC SCAN AT 254 AND 366 nm.

Further the DCM extracts of roots of Nymphoides macrospermum and Valeriana wallichi were subjected to GC-MS analysis. GC-MS analysis was obtained from a Hewlett- Packard 6890D5973 system operating on EI mode, equipped with a capillary column HP-5 MS 30 m \0.25 mm; film thickness: 0.25 μm; temperature program: 60 $^0$C (5 min) to 280 $^0$ C at a rate of 3$^0$ C/min; inj. temp. 200$^0$C.

The chromatogram of DCM extract of *Nymphoides macrospermum* was different from that of the DCM extract of *Valerian* 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. (See fig. 4.12 a,b & c)




**4.12a. Chromatogram of DCM Extract of *N. macrospermum* & *Valerian*.**



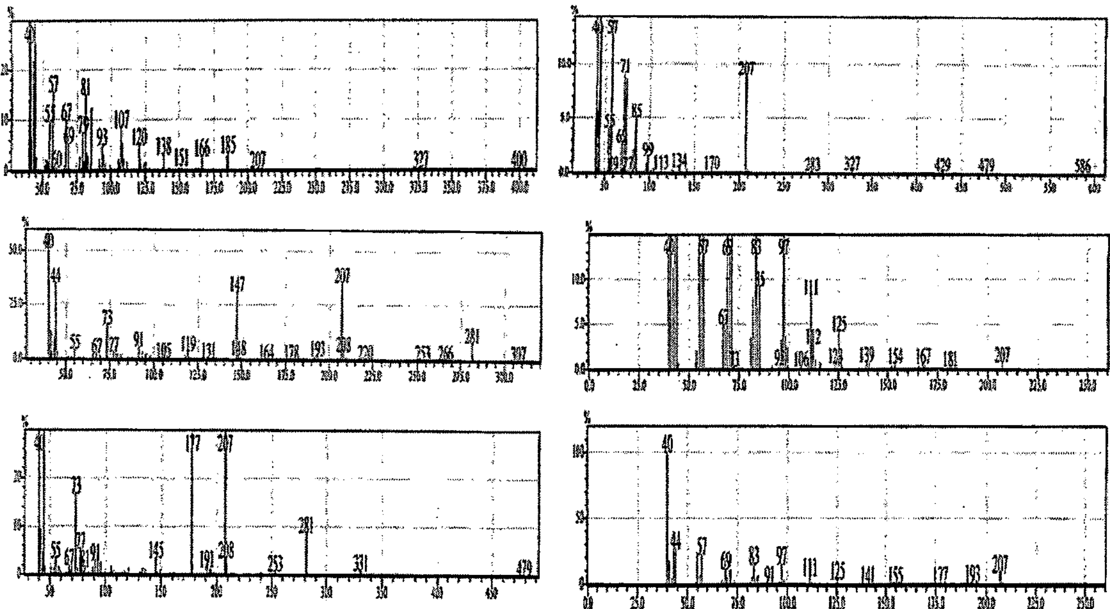
4.12b. Chromatogram of DCM Extract of *N. macrospermum* & *Valerian.*

Chromatogram of DCM ext of Valerian

Chromatogram of DCM Ext of Nymphoides

4.12c. Chromatogram of DCM Extract of *N. macrospermum* & *Valerian*.

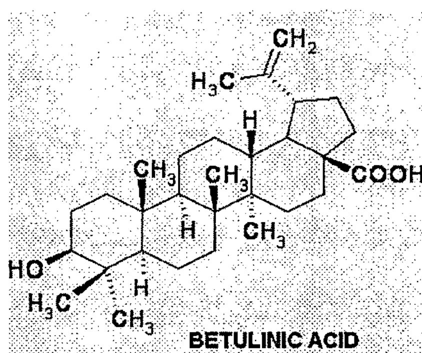
Chromatogram of DCM ext of Valerian | Chromatogram of DCM Ext of Nymphoides



## 4.6. Determination of content of marker in methanol extract of roots of *Nymphoides macrospermum*

Marker compounds along with chromatographic profiles may be used to standardize the herbal raw materials. The clinical efficacy and pharmacological effects of a plant material will depend strongly on the amounts of biologically active ingredients present, and these must be accurately measured if a plant material is to be chemically standardized. Thus for the evaluation of identity and determination of quality of a medicinal plant material a validated analytical method of analysis for the active ingredients has to be developed. 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.

**Betulinic acid** (3β-hydroxy-lup-20(29)-en-28-oic acid) a naturally occurring triterpene was originally extracted from the bark of an African tree, *Ziziphus mauritiana* (Chowdhury AR et al., 2002), later from other plants like *Trifillum peltatum, Ancistocladuis heyeneaus, Diospyros leucomelas, Tetracera boliviana, Sizigium formosanum,* (Zuco et al., 2002) *Chaenomeles sinensis* (Gao et al., 2003). Betulinic acid and its derivatives have been discovered as a new class of compounds as potential anti-cancer and anti-HIV agents.(Soler F et al., 1996, Schmidt et al., 1997) Previously, betulinic acid has been determined quantitatively in white birch bark by HPLC (Guoling Zhao et al.,2007) while there is another report of an HPTLC method for its estimation in an ayurvedic preparation using Chloroform: Methanol: Formic acid (98:2:2) solvent system.( Uppuluri V et al., 2004)




Hence considering wide therapeutic applications of betulinic acid and as one of the marker constituent to ensure identity and quality of this plant a simple, sensitive, specific and reproducible HPTLC method was developed for the quantification of betulinic acid in *Nymphoides macrospermum* roots.

## Quantification of Betulinic acid from *Nymphoides* roots

### Reagents and chemicals

Pure Betulinic acid was obtained from M/s Sigma-Aldrich Chemicals, (Steinheim, Germany) other solvents and chemicals were of analytical grade and HPTLC plates silica gel $60F_{254}$ (20cm x 20cm) were purchased from E Merck (Darmstadt. Germany).

### Preparation of crude extract:

Accurately weighed 2.5 g of the coarse powder of *Nymphoides macrospermum* roots and extracted separately with Methanol (4x 25mL) under reflux (30 min each time) on a water bath. The combined extracts were filtered and concentrated, and transferred to 25 mL volumetric flask and the volume was made up with methanol.

**Preparation of Standard solution**

A stock solution of betulinic acid (100μg $mL^{-1}$) was prepared by dissolving 1 mg of accurately weighed betulinic acid in methanol and making up the volume of the solution to 10 mL with methanol.

**Chromatography**

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 and standards were applied on 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) which was presaturated with 20 mL mobile phase Hexane: Ethyl acetate: Acetic acid (7:3:0.03 *v/v*) for 30 min at room temperature($25^0 \pm 2\ ^0$ 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 .The post chromatographic derivatization was carried out in anisaldehyde and sulphuric acid followed by heating at 110 $^0$ C for 3 min (Wagner). Quantitative evaluation of the plate was performed in absorption-reflection mode at 540 nm, 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 4 software. Quantification of betulinic acid in extract of *Nymphoides* root was performed by external standard method, using pure betulinic acid as standard.

**Calibration curve for Betulinic acid**

Stock solution of betulinic acid (100μg $mL^{-1}$) was prepared in methanol and different amounts (100-600ng $spot^{-1}$) were applied on a TLC plate, using Linomat V for preparing six point calibration graph of peak area vs. concentration. The regression equation for betulinic acid was39.366+12.165$X$ and co-relation coefficient (r) was 0.998.

**Specificity**

Specificity of the method was determined by analyzing sample of standard betulinic acid and the unknown sample. The spot for betulinic acid in sample was confirmed by comparing the $R_F$ and spectra of the spot with that of the standard. The peak purity of betulinic acid was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot.

**Quantification of Betulinic acid in Test sample**

10 μL of sample solution was applied in triplicate on a TLC plate and developed, scanned as above. Peak areas were recorded and the amount of betulinic acid was calculated using the calibration plot.

**Method validation**

The method was validated for precision, accuracy (ICH guidelines) and repeatability. Instrumental precision was checked by repeated scanning of the same spot 100 & 600ng five times and was expressed as coefficient of variance (%RSD). Method precision was studied by analyzing the standards 100 & 600 ng per spot under the same analytical procedure and lab conditions on the same day and on the different days (inter day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of preanalyzed sample with standard at three levels (24.4, 30.6 and 36.7 μg $mL^{-1}$), % recovery and average % recovery was calculated.

**HPTLC Separation optimization**

Different compositions of the mobile phase were tested and the desired resolution of betulinic acid with symmetrical and reproducible peaks was achieved by using mobile phase of Hexane: Ethyl acetate: Acetic acid (7:3:0.03 *v/v*) with 30 min of chamber saturation with the mobile phase and 18 min of development. A peak corresponding to betulinic acid was seen at $R_F$ 0.60. The methanolic extract of the roots of *Nymphoides macrospermum* when subjected to HPTLC as per the methodology described above, showed the presence of betulinic acid peaks. A comparison of the spectral characteristics of the peaks for standard betulinic acid and that of the sample revealed the identity of betulinic acid present in the sample. It can be seen from fig 2 that good separation can be achieved by the conditions described above. Peak purity test of betulinic acid was done by comparing its UV-visible spectra in standard and sample track.

**System suitability test**

Linearity and detection limit

Linearity was checked by applying standard solutions of betulinic acid at six different concentration levels. The calibration curve was drawn in the concentration range of 100-600 ng $spot^{-1}$. The equation for calibration curve of betulinic acid is $Y=39.366+12.165x$ and the correlation coefficient of calibration plot was 0.998 indicating good linearity.

Results of regression analysis on calibration curve and detection limits are presented in Table 4.17.

**Table 4.17** Method validation parameters for quantification of Betulinic acid using proposed HPTLC Densitometric method.

### a) Lineartity regression Data

| Sl No. | Parameter | Results |
|---|---|---|
| 1 | $R_F$ | 0.60 |
| 2 | Dynamic range (ng spot$^{-1}$) | 100-600 |
| 3 | Equation | Y=39.366+12.165x |
| 4 | Slope | 12.165 |
| 5 | Intercept | 39.366 |
| 6 | Limit of Detection | 15.24ng |
| 7 | Limit of Quantification | 50.82 ng |
| 8 | Linearity (Correlation coefficient) | 0.998 |
| 9 | Specificity | Specific |

### b) Precision studies data

| Concentration (ng spot$^{-1}$) | Instrumental Precision (% RSD) | Method Precision (% RSD) | |
|---|---|---|---|
| | | Intra day | Inter day |
| 100 | 1.89 | 1.59 | 2.0 |
| 600 | 1.38 | 1.32 | 0.86 |

### c) Recovery studies of Betulinic acid

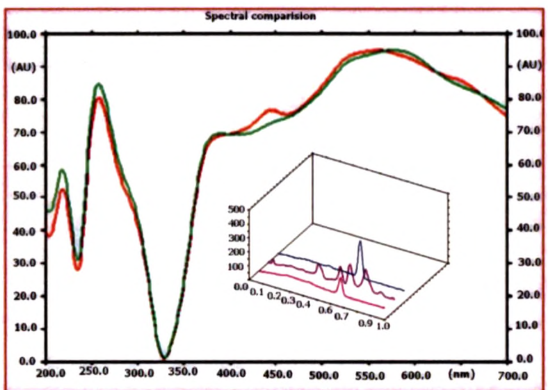
| Sl No. | Amount of Betulinic acid present in the sample(μg) | Amount of Betulinic acid added (μg) | Amount of Betulinic acid found (μg) | Recovery (%) |
|---|---|---|---|---|
| 1 | 30.6 | 24.4 | 57.9 | 98.99-105 |
| 2 | 30.6 | 30.6 | 60.9 | |
| 3 | 30.6 | 36.7 | 66.6 | |

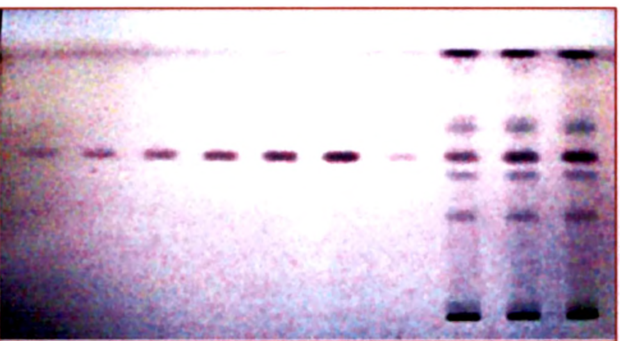
**Precision studies**

Instrumental precision was checked by repeated scanning of the same spots (100 & 600ng spot $^{-1}$) of standard betulinic acid five times and the RSD values were 1.89 and 1.38 for 100 and 600 ng spot $^{-1}$ respectively. To determine the precision of the developed assay method 100 and 600ng spot $^{-1}$ of betulinic acid standard was analyzed five times within the same day to determine the intra-day variability. The RSD values were 1.59 and 1.32 for 100 and 600 ng spot $^{-1}$ respectively. Similarly, the inter-day precision was tested on the same concentration levels on two days and the RSD values were 2.0 and 0.86 respectively. (Table 4.17)

**Sample analysis and recovery studies**

This developed HPTLC method was subsequently applied for the analysis of betulinic acid in methanolic extract of *Nymphoides macrospermum* and the free betulinic acid content of the roots by this proposed method was found to be 0.030 %. The recovery rates, 80, 100 and 120 % of pure betulinic acid were added in pre-analyzed sample and quantitative analysis was performed. The recoveries were between 98.99-105 %.



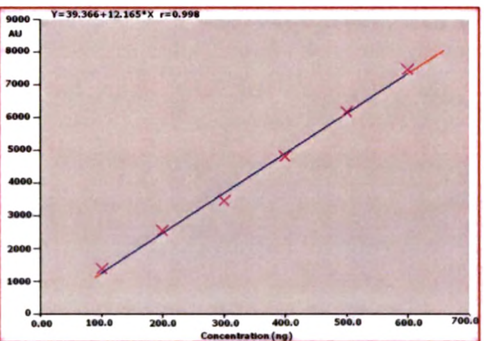



Fig.4.13 UV-Visible spectrum of standard betulinic acid and betulinic acid in sample and 3D overlaid chromatogram of standard tracks and sample track.

## 4.7. Isolation and Characterization of compounds from the fractions having bioactivity

DCM extract (5g) of roots of *Nymphoides* was subjected to column chromatography over silica gel and eluted with Hexane: Ethyl acetate (7:3). Around 130 fractions of 50ml each were collected. TLC of Fractions 6-8 when observed at 366nm exhibited blue fluorescence afforded a yellow amorphous solid on evaporation of solvent. Similarly fractions 75-90 when observed under UV 366nm showed an intense yellowish-green fluorescence, while upon TLC all the fractions were found to contain 4 bands while the one with intense fluorescence was found to be rich in these fractions. While the fractions in between were found to contain black-green sticky mass.

TLC of Fractions 119-127 showed an intense pink band on treatment with anisaldehyde-Sulphuric acid reagent hence all these fractions were pooled up and upon concentration a white amorphous substance was obtained.

The yellow amorphous substance obtained from fractions 6-8 was eluted with hexane: ethyl acetate 7:3 in a silica packed column, fractions of 25ml each were collected and analyzed. Fractions 1 & 2 when observed under UV 366 showed blue fluorescence while on concentration yielded a white amorphous solid which on TLC was found impure. Further it was again subjected to purification in a column packed with silica and eluted with Pet ether (60-80) and Hexane (9:1) again the 1$^{st}$ and 2$^{nd}$ fractions were found to exhibit blue fluorescence. This was further purified by preparative TLC on silica using Pet ether as mobile phase. The silica coating of the solvent front was scraped and the compound was recovered washing with Pet ether. This upon TLC gave a single spot confirming its purity. 125mg of this white amorphous compound was obtained from 5g of the extract and it was named NMC1.

The fractions 75-90 containing yellowish green fluorescent compound was separated by preparative TLC on silica coated plates using Hexane: Ethyl acetate (7:3) as mobile phase. Fluorescent area of the silica layer was scrapped and the fluorescent compound was recovered with the same solvent and was found to be single confirming its purity by TLC was named NMC2

Since the yield of NMC2 was very less it was subjected to GC-MS analysis for characterization.

The white amorphous substance obtained from fractions 119-127 was named NMC3.

**Characterization of NMC1**

NMC1 was subjected to IR, CHN, NMR and Mass spectral analysis for characterization.The compound,with molecular composition: C- 87% and H- 13%, mp 65-$67^0$C,showed IR (KBr): 2956.39, 2918.40 and 2848.96 (alkyl C-H stretching), 1464.02 (C-H bending), 1024 (in plane C-H bending),$^1$H NMR ($CDCl_3$) $\delta$:0.777, 0.810, 0.839, 0.939, 1.086, 1.184, 1.285, 1.504 has probable proton assignment as follows:

**Fig.4.14. IR Spectra of NMC1**

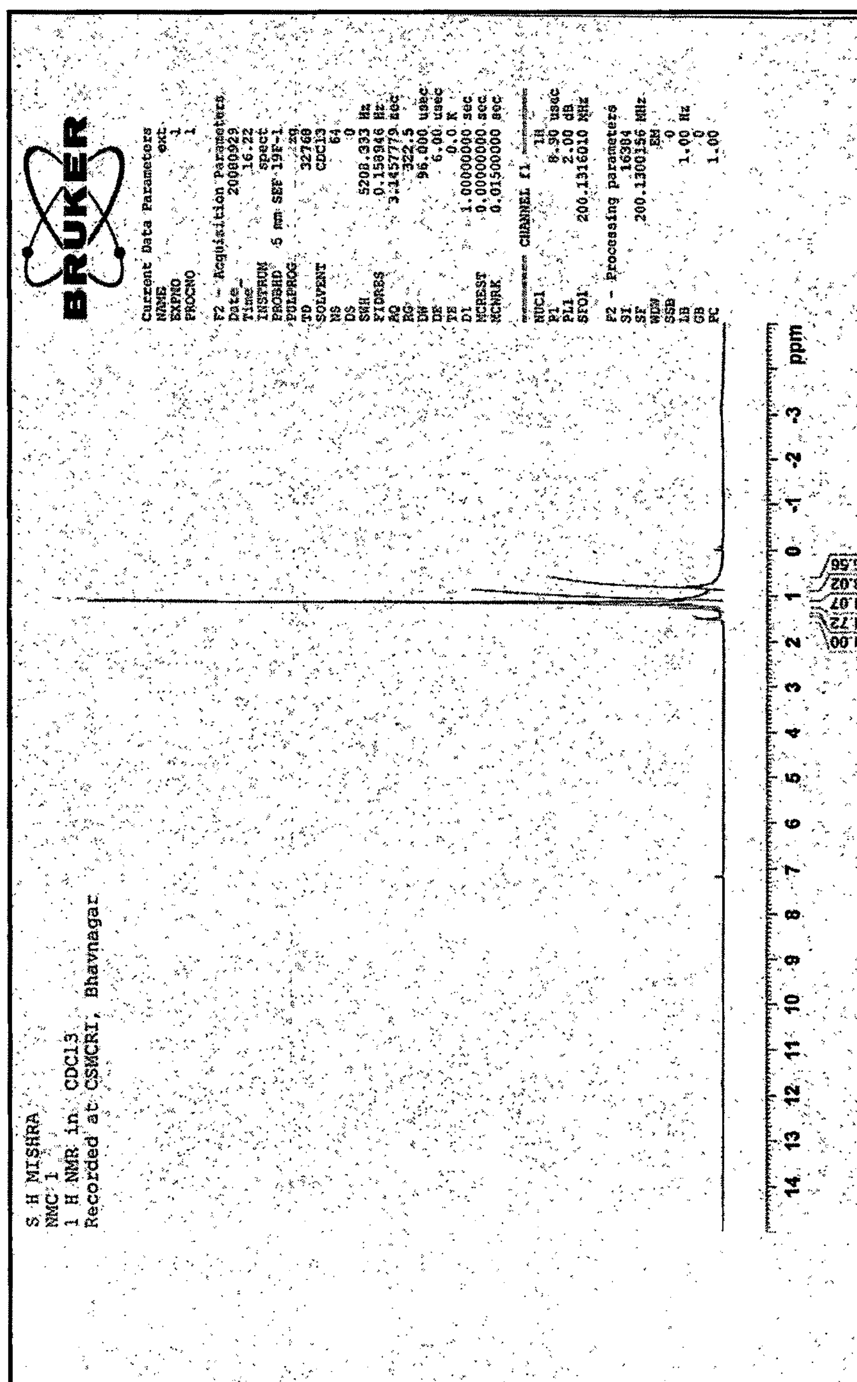


Fig. 4.15a. 1H NMR spectra of NMC1

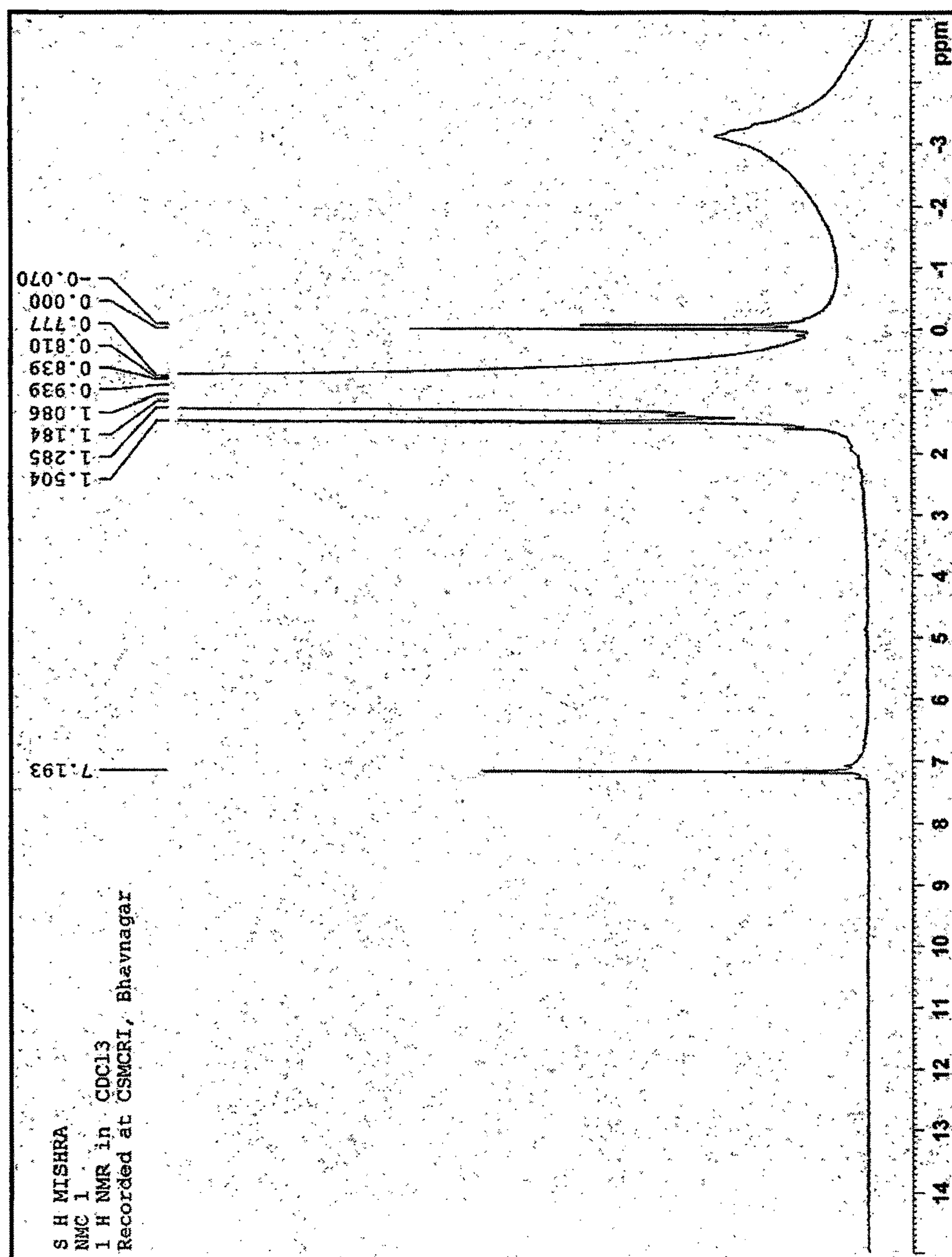



**Fig. 4.15b. $^1$H NMR spectra of NMC1**

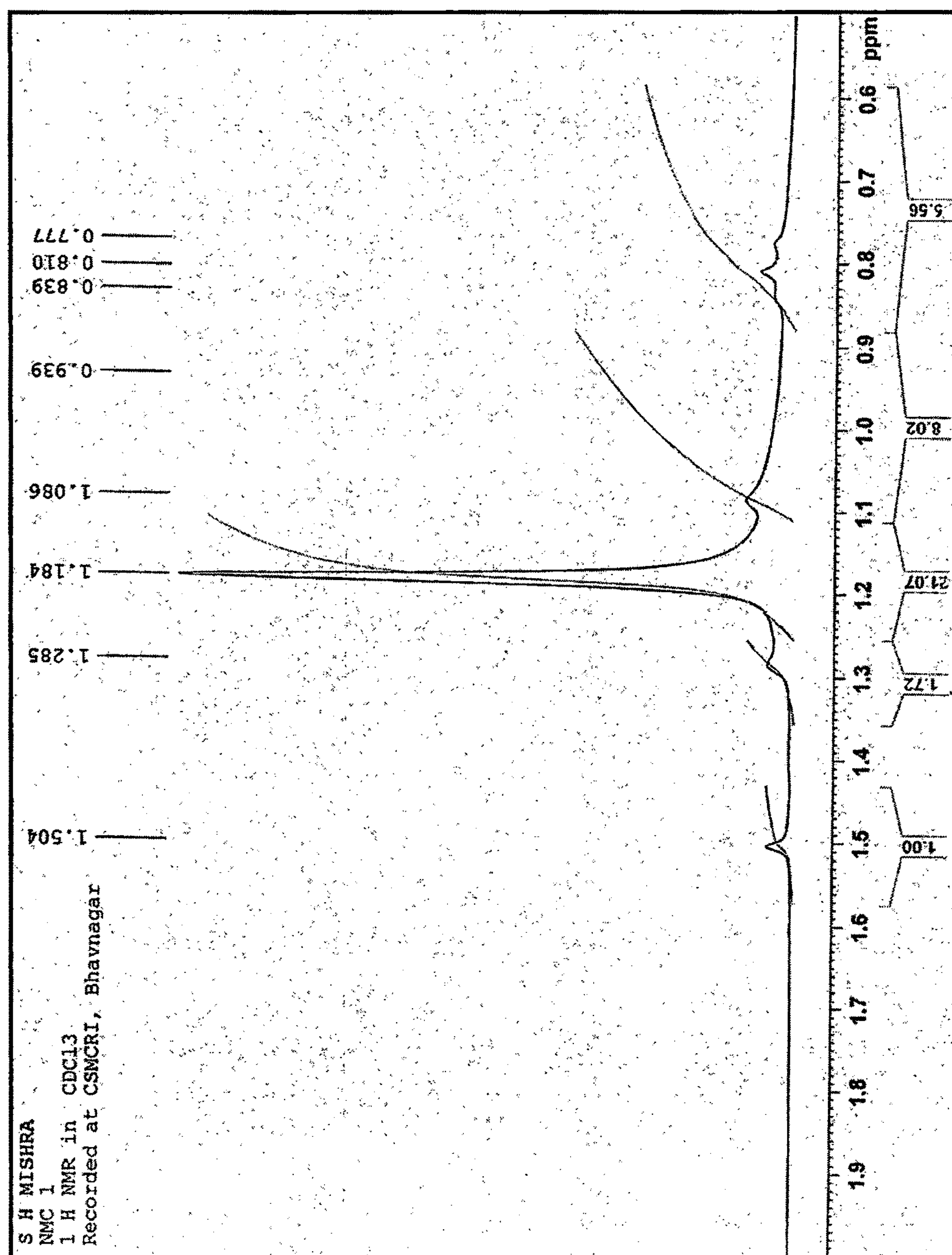
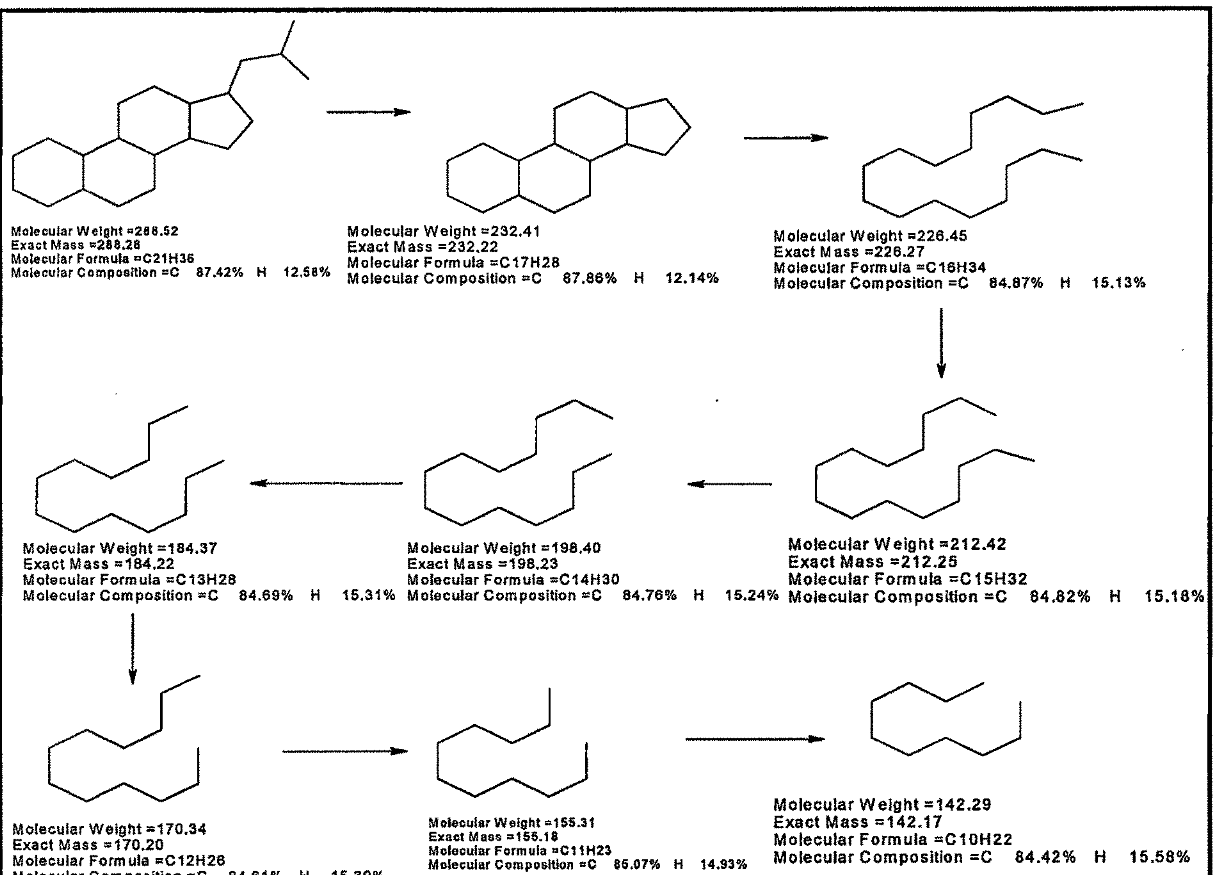
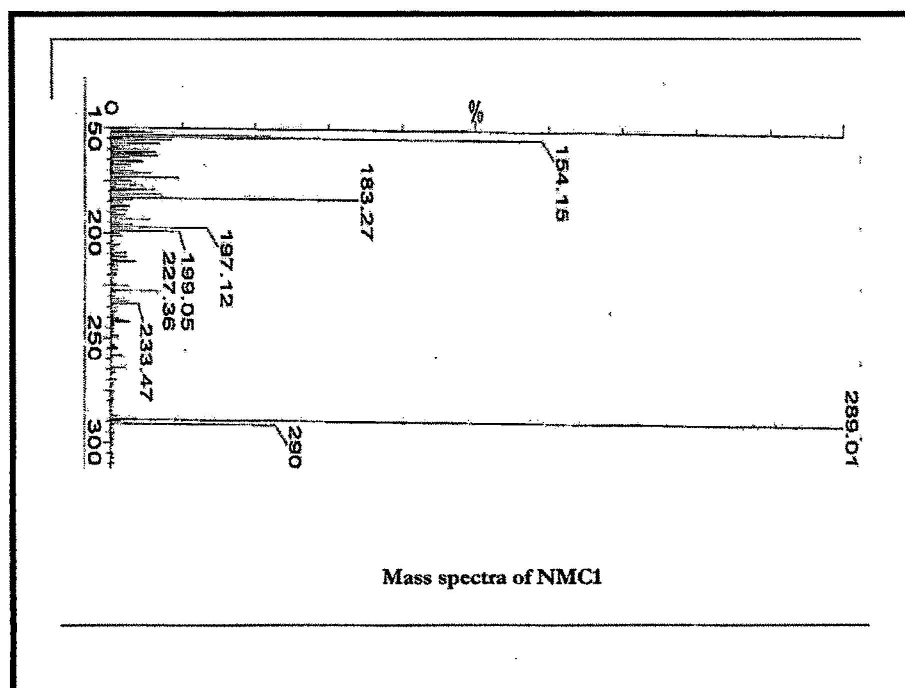



**Fig. 4.15c. $^{1}$H NMR spectra of NMC1**

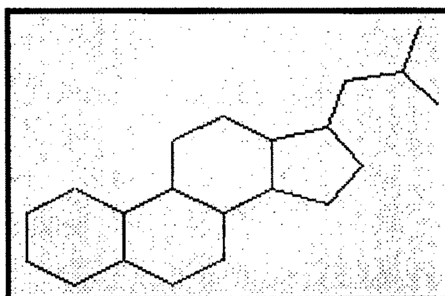
Fig. 4.16a. Proposed Mass fragmentation pattern of NMC1





**Mass spectra of NMC1**

Thus from the above IR, $^{1}H$ NMR and Mass fragmentation pattern analysis the structure of compound NMC1 may be

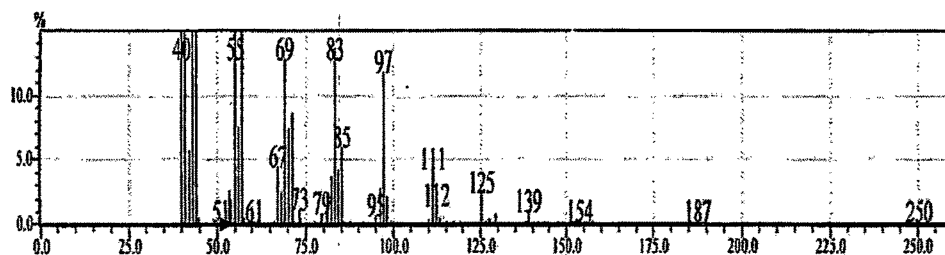


**17-Isobutyl -hexadecahydro-cyclopenta(a)phenanthrene**

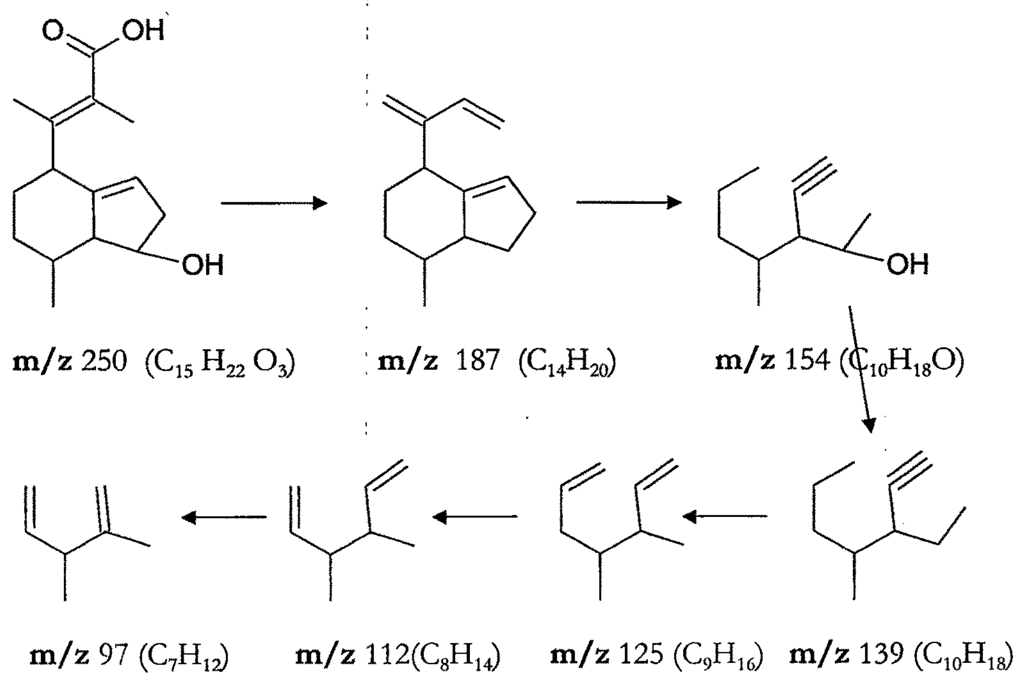
**17-ISOBUTYL GONANE**

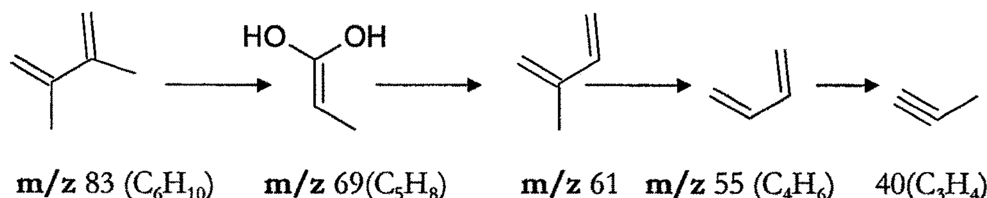
## Characterization of NMC2

NMC2 was subjected to GC-MS analysis on Hewlett- Packard 6890D5973 system operating on EI mode, equipped with a capillary column HP-5 MS 30 m \0.25 mm; film thickness: 0.25 μm; temperature program: 60 $^0$C (5 min) to 280 $^0$ C at a rate of 3$^0$ C/min; inj. temp. 200$^0$C.




Mass fragmentation pattern of NMC2 was compared with the fragmentation of Valerenolic acid($C_{15}H_{22}O_3$).






**m/z** 83 ($C_6H_{10}$) **m/z** 69($C_5H_8$) **m/z** 61 **m/z** 55 ($C_4H_6$) 40($C_3H_4$)

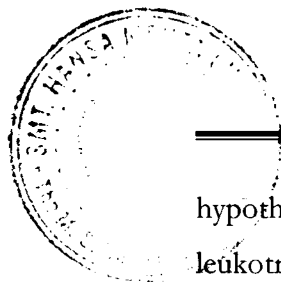
The fragmentation pattern of NMC2 was found to be very much similar to that of Valerenolic acid Hence the structure of NMC2 may be Valerenolic acid like Compound .

### Characterization of NMC3

NMC3 was obtained as a white amorphous substance.It was subjected to co-TLC studies with betulinic acid using Hexane: Ethyl acetate :Acetic acid (7:3:0.03) % v/v as mobile phase and derivatized with Anisaldehyde-$H_2SO_4$ reagent, NMC3 showed an identical spot like that of betulinic acid (reference) confirming NMC3 to be Betulinic acid.

Gonane is the precursor for the biosynthesis of Gonane derivatives like Sitoindoside (*Withania somnifera*), Ginsenosides (Panax ginseng) etc Similarly the isolated Isobutyl Gonane may also be such precursor in biosynthesis of Gonane derivatives.

Most plants reported as adaptogens are also reported to have estrogenic, tonic, antioxidant, nootropic and aphrodisiac properties. These properties can be linked, however, to the neuroendocrine systems involved in the response to stress. Natural products having adaptogenic properties fall into three diverse classes of compounds ubiquitous to the Plant Kingdom; triterpenes, phenylpropanes, and oxylipins (Hydroxylated fatty acids) These compounds are similar in structure to endogenous mammalian stress mediators of the





hypothalamus-pituitary adrenal (HPA) axis: catecholamines, corticosteroids, leukotrienes and lipoxines and they may function by a sparing action or by competitive binding. (Panossian, 2003).

Isoprenoid compounds (also called terpenes) are made from isopentenyl pyrophosphate (IPP) or isoprene units. The more than 23,000 isoprenoid compounds identified so far in nature have many essential biological functions in prokaryotes and eukaryotes. In mammals and humans being isoprene units are used to make steroidal hormones and bile acids. Many isoprenoids have significant biological functions in nature. The classes of terpene compounds found in plants are monoterpenes, diterpenes, sesquiterpenes, triterpenes, tetraterpenes, and polyterpenes. Triterpenes belong to a very large group of compounds arranged in a four or five ring configuration of 30 carbons with several oxygens attached. Triterpene compounds most often found in plants belong to the ursane/oleanane class (Oleolanic acid, ursolic acid, Amyrin). Most triterpenoid compounds in plants believed to be responsible for adaptogenic activity are found as saponin glycosides. Ginsenosides*(Panax ginseng,)* Elutherosides *(Elutherococcus senticosus,)* Withanolides *(Withania somnifera),* Asiaticosides *(Centella asiatica),* Bacoposide *(Bacopa monera), Asparagus racemosus,* Tinosporoside *(Tinospora cordifolia), Ocimum sanctum.* (Robyn Clein, 2006)

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. (Lamberto Tomassini et al.,) It is known that CNS effects of Valerian is mediated through modulation of GABA $_A$ receptor functions.(Chun-su Yan 2002) Similarly *Nymphoides macrospermum* has also been proved to possess anticonvulsant and sedative property probably with the same mechanism.(Anita et al.,)

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. (Paul Bremner., 2002) 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 inhibiting of activation of NF-kB and NF-kB-regulated gene expression induced by carcinogens and inflammatory stimuli.

The results of the above studies suggests that *Nymphoides macrospermum* roots holds promise as adaptogen by altering various biochemical markers during stress, and also by stimulating the cellular defence mechanism and the activity may be endowed upon the presence of betulinic acid and other terpenoids .