CHAPTER - 5

Results & Discussion

(Mucuna pruriens)

The systematic studies were undertaken on roots of *Mucuna pruriens* 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**
- **Identification and quantification of constituents as bioactive marker.**

5.1 Pharmacognostic Evaluation

Macroscopic studies

Shape:	Angular
Size:	Long, 7mm or more in thickness, hard.
Color:	Dark Brown to Black
Branching:	Lateral
Fracture:	Fibrous
Odour:	Characteristic.
Taste:	Characteristic.

Microscopic studies

The results of microscopic studies are as under
T.S of the root presents a circular outline. Following are the tissues seen from the periphery to the centre.

Periderm

Roots shows a narrow cork consisting of 4 or 5 rows of tangentially elongated cells, secondary cortex narrow consisting of 2 to 5 rows of thin-walled, parenchymatous cells, a few containing dark brownish contents.

Phloem

Secondary phloem wide, forming bulk of the in the form of long, radial strips that are conical due to the medullary rays funneling out in the phloem region.

Phloem fibers are arranged in groups or occasionally single. Phloem rays uni to biseriate, cambium distinct 1 or 2 layered.

Xylem

Secondary xylem very wide composed of usual elements, vessels large as well as small, surrounded by xylem parenchyma and fibres.

Medullary rays

Medullary rays 1-2 layers extending from xylem up to phloem. Widening of the ray cells into the cortical region towards cortical region the medullary ray cells become radially elongated in cortex.

Powder study of the roots of *Mucuna pruriens*

Preliminary Tests	Observation	Inference
Odour	characteristic	
Color	Grey	
Taste	Characteristic	
Touch	Smooth	
Test with water/ Aqueous extract	No Frothing	May be due to absence of saponins
Test for Tannins	Brown ppt	due to presence of Tannins
Test for Mucilage	Non-mucilaginous	Absence of Mucilage
Test with H_2SO_4	Brown coloration	
Test with caustic alkali	---	
Test for oils	Oily stain,	Presence of oils

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

Vascular elements:

Large number of vessel elements in entire or fragmented form showing types of thickening like helical, simple, pitted, bordered etc are found.

Tracheids:

Fragments of tracheids with linear pits are found commonly in the powder.

Parenchyma:

Cortical parenchyma consisting of thin walled polyhedral cells containing starch are seen.

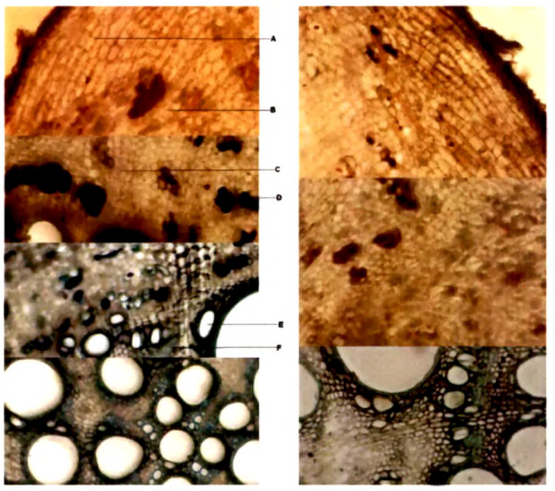


Fig.5.01 T.S of the roots of *Mucuna pruriens* (X10)
A-Cork; SL-Sclereids; B-Cortex; C-Medullary rays; D-Tannin containing cells
E & F-Vascular elements.

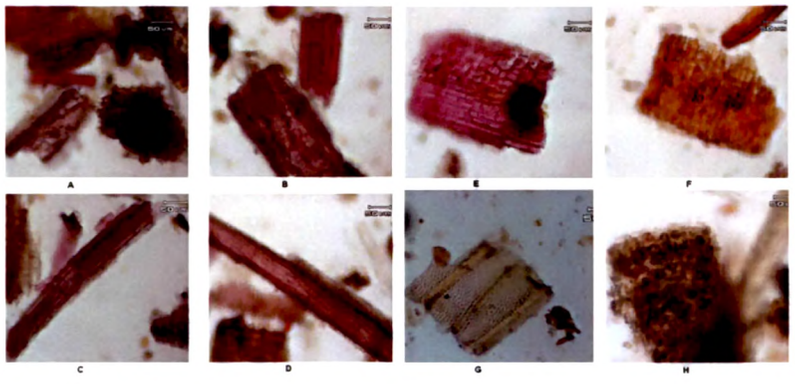


Fig. 5.02. Diagnostic features for the powder microscopy of the roots of *Mucuna pruriens* roots (X40). A-G-Vascular elements; F- Cork cells.

Table 5.2 Physico-chemical constants, Content of Heavy metals and other inorganic trace elements including Microbial content of roots of *Mucuna pruriens*

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	3.2	Sodium (mg/g)	3.46	Total Viable count	9560
Loss on drying	7.0	Potassium (mg/g)	0.931	Total Bacterial Count	5460
Total Ash	4.0	Zinc (mg/g)	0.139	Total Fungal count	4100
Acid insoluble ash	2.38	Manganese (mg/g)	Nil	E-coli	Absent
Water soluble ash	1.5	Copper (mg/g)	Nil	Salmonella spp	Absent
Alcohol soluble extractive	4.1	Cadmium (ppm)	Nil	S. aureus	Absent
Water soluble extractive	1.28	Iron (mg/g)	0.919	P. aeruginosa	Absent
		Lead (ppm)	0.02		
		Mercury (ppm)	0.0287		
		Arsenic (ppm)	0.6037		

The plant material under study viz., roots of *Mucuna pruriens* was subjected to proximate analysis as described in section 2.1. Proximate analysis helps to set up certain standards for dried drugs in order to judge their quality. Alcohol and water-soluble extractives were found to be 4.1 and 1.28% w/w respectively. Ash content represents the mineral component of a plant material, a higher level of ash content (3.3-5.5%) is reported in seeds of *Mucuna pruriens* (Vadivel et.al.,) similarly a higher ash value of 4.0 %w/w was observed in the roots indicating higher inorganic/mineral content like that of seeds. Results summarized in Table 5.2.

Studies on the mineral composition of the roots of *Mucuna pruriens* revealed that it contains potassium (0.931 mg/g), zinc (0.139mg/g), sodium (3.46 mg/g), Iron (0.919mg/g) and devoid of manganese, copper and cadmium. Seeds are reported to contain higher levels of potassium (806-2790 mg/100g), sodium (31-104mg/100g), zinc (1-15mg/100g), copper (0.33-4.34mg/100g), manganese (0.56-9.26 mg/100g) and iron (1.3-15mg/100g). The roots were found to contain traces of lead (0.02 ppm), Mercury(0.0287 ppm) and Arsenic (0.603 ppm) which are within the limits of WHO specifications for heavy metal content. Results summarized in Table 5.2.

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

5.2. Preliminary phytochemical analysis

The roots of *Mucuna pruriens* were subjected to successive solvent extraction. Percentage yield, color and consistency of the selected successive extracts are recorded in Table 5.3. Higher extractive values were obtained with methanol, pet. ether and aqueous extracts.

Successive Solvent Extraction of *Mucuna pruriens* roots

Table 5.3 Physical properties of successive Extracts

Sl.No	Extract	% yield	Color & Consistency
1	Pet.Ether	2.64	Straw yellow Semi solid
2	Chloroform	0.17	Reddish brown semisolid
3	Ethyl acetate	0.20	Reddish brown semisolid
4	Methanol	3.18	Reddish brown-semisolid
5	Aqueous	1.22	Black-solid

Table 5.4 Phytoconstituents present

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

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

Preliminary phytochemical screening revealed the presence of phytosterols terpenoids and phenolic compounds, amino acids as constituents in the roots of *Mucuna pruriens* (Results are summarized in Table 5.4). Seeds are reported to

contain alkaloids like Prurinine, Prurienidine, Mucunine, Mucunadine etc while no alkaloids were found in the roots, fatty acids like saturated (stearic and palmitic acid), unsaturated (oleic and linoleic acid) are reported in seeds, the pet ether and chloroform extracts of roots confirmed presence of fats/fatty acids, a phytosterol (β-sitosterol) is reported from seeds and the pet ether and chloroform extract of roots showed postitive tests for steroids, Therefore, total methanol extract of roots of *Mucuna pruriens* was prepared and screened for bioactivity.

Acute toxicity studies

Toxicity study was carried out on total Methanolic extract 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 to be the safe dose the extract was screened for adaptogenic activity at three different dose levels 100, 200 and 400mg/kg body weight.

5.3 Evaluation for Adaptogenic Activity

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.

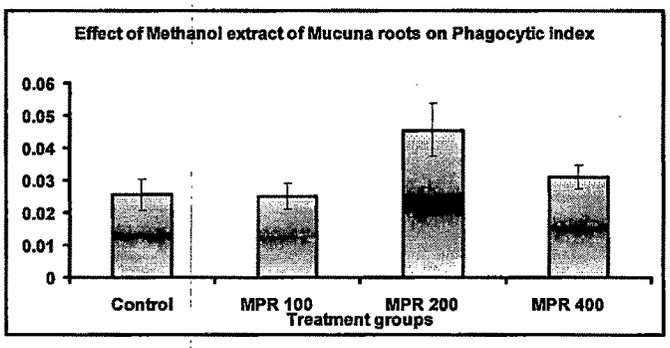
Immunomodulatory activity was proved with Antibody titre, DTH response, Phagocytic function, E coli induced abdominal sepsis, and Cyclophosphamide induced myelosuppression models in mice and Anti-stress activity by swimming endurance test.

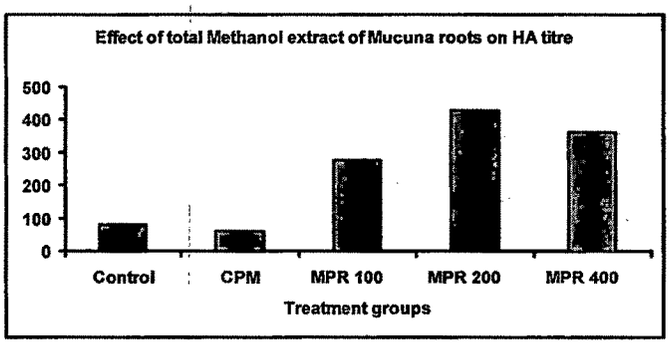
- Evaluation of Immunomodulatory activity of total methanol extract of roots of *Mucuna pruriens*

Table 5.5 : Effect of Methanol extract of roots of *Mucuna pruriens* and 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
MPR 100	0.025±0.0039^{ns}	277.33±51.37*	0.798±0.03ns
MPR 200	0.0455 ± 0.0083***	426.66±53.97***	1.81±0.069***
MPR 400	0.031 ± 0.0037^{ns}	362.66±69.45**	0.903±0.041**

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





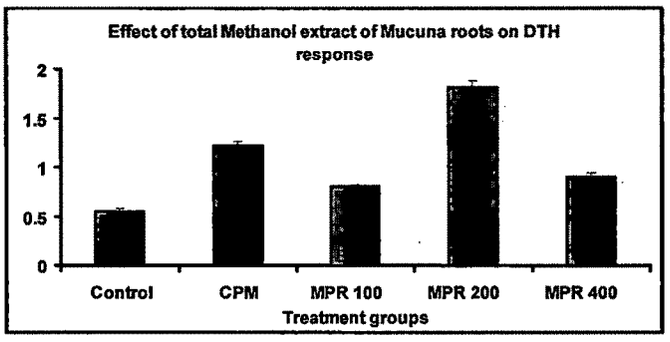
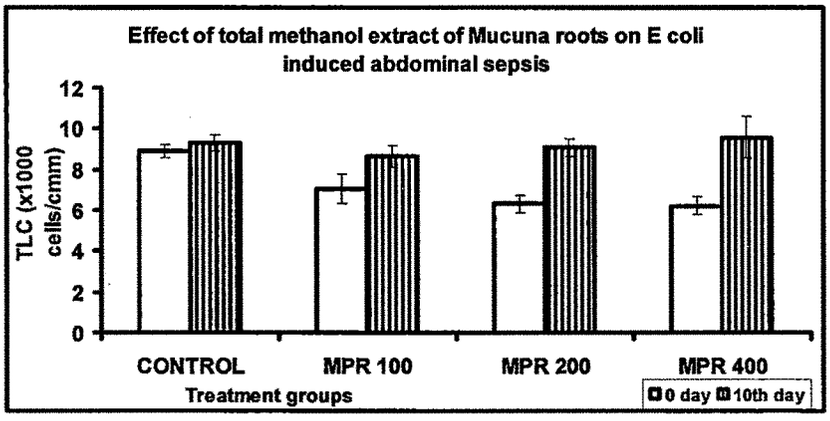


Table 5.6 : Effect of Methanol extract of roots of *Mucuna pruriens* on Leucocyte count and % Mortality in E coli induced abdominal sepsis

GROUPS	TOTAL LEUCOCYTE COUNT (Thousands/cmm) Mean ±SEM		% MORTALITY
	0 DAY	15th DAY	
CONTROL	8.91±0.336	9.28±0.39	100
MPR 100	7.06±0.74	8.61±0.53^{ns}	50
MPR 200	6.3±0.44	9.08±0.44*	37.5
MPR 400	6.23±0.41	9.58±1.03**	25

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 roots of *Mucuna pruriens* was 0.798±0.03, 1.81±0.069 and 0.903±0.041 at 100, 200 and 400mg/kg dose respectively. A statistically significant response ($P<0.001$) was obtained at 200mg/kg dose level. Increase in DTH response as evidenced by increased paw thickness in mice revealed stimulatory effect of roots of *Mucuna pruriens* on T cells and accessory cell types required for the expression of reaction.

Methanol extract of roots of *Mucuna pruriens* at 100 200 and 400 mg/kg showed HA titre of 277.33±51.37, 426.66±53.97 ($P<0.001$) and 362.66±69.45 ($P<0.01$) respectively was found effective in increasing HA titre value indicating the enhanced responsiveness of macrophages and T and B lymphocytes involved in the antibody production. (Benacerraf et al.,1978)

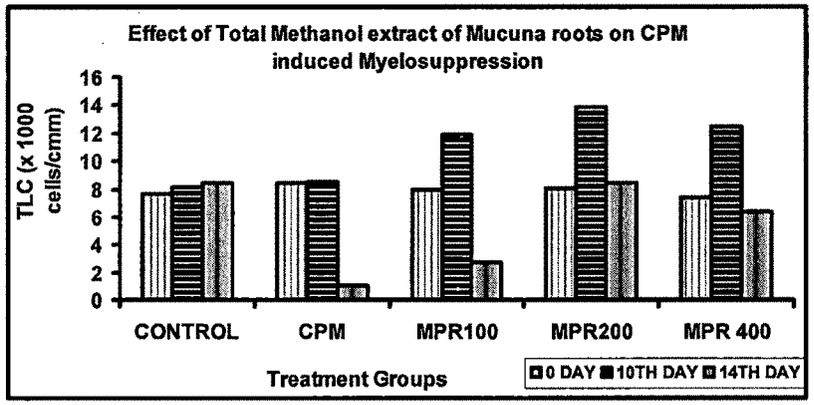
Total methanol extract of roots of *Mucuna pruriens* was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance 0.0455 ± 0.0083 at 200mg/kg dose level compared to control group (0.0256±0.0048).

In E coli induced abdominal sepsis model (Infectious /Biological stress), oral administration of methanol extract of roots of *Mucuna pruriens* in mice for ten days at 50, 100 and 200mg/kg dose levels produced a significant increase in total leucocyte count and decreased mortality rate, at 400mg/kg dose level mortality rate was reduced to 25 % at compared to control group. Thus the findings of this study further substantiates the results of phagocytic activity.

Table 5.7 Effect of Methanol extract of roots of *Mucuna pruriens* 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±1.058	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
MPR100	7.93±0.889	11.88±1.62 **	2.71±0.596 ns	8.568±0.49	9.358±0.28	7.63±0.34	15.1±0.816	16.7±0.584	12.61±0.72
MPR200	8.05±1.094	13.85±1.41 ***	8.38±3.459 ***	8.591±0.49	9.788±0.25 ns	7.15±0.3 ns	14.91±0.80	17±0.507 **	12.2±0.456
MPR 400	7.35±0.658	12.466±1.5 ***	6.366±0.761 ***	9.02±0.484	9.725±0.36	7.74±0.64 ns	15.91±1.02	17.233±0.5* *	12.91±1.19

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



In Cyclophosphamide induced myelosuppression assay, mice when pretreatment with methanol extract of roots of *Mucuna pruriens* at 100, 200 and 400 mg/kg for 10 days increased the total WBC count from 7.93±0.889 to 11.88±1.62 at 100mg/kg, 8.05±1.094 to 13.85±1.41 at 200mg/kg and 7.35±0.658 to 12.466±1.5 (thousand cells/mm^3) prior Cyclophosphamide administration and further treatment with extract till 14^{th} day followed by administration of Cyclophosphamide on 11^{th}, 12^{th} and 13^{th} days, the extract at 100mg/kg did not show any statistically significant change in TLC count 2.71±0.596 whereas at 200 and 400mg/kg dose levels the TLC values were almost restored to normal 8.38±0.459 and 6.366±0.761 (thousand cells/mm^3) respectively.

The results of immunomodulatory screening of methanol extract reveals that roots of *Mucuna pruriens* exhibits stimulatory effects on humoral immunity (HA titre), cellular immunity (DTH response) and phagocytosis (Carbon clearance and E coli induced abdominal sepsis), and also protection against Cyclophosphamide induced myelosuppression.

- **Evaluation of Anti-stress activity of total methanol extract of roots of *Mucuna pruriens***

Table: 5.8 Effect of Methanol extract of roots of *Mucuna pruriens* 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
MPRTME 100	0.193±0.019*	6.53±0.12 ns	0.613±0.18*
MPRTME 200	0.163±0.016***	5.96±0.07***	0.556±0.17***
MPRTME 400	0.166±0.013***	6.07±0.14**	0.553±0.2***

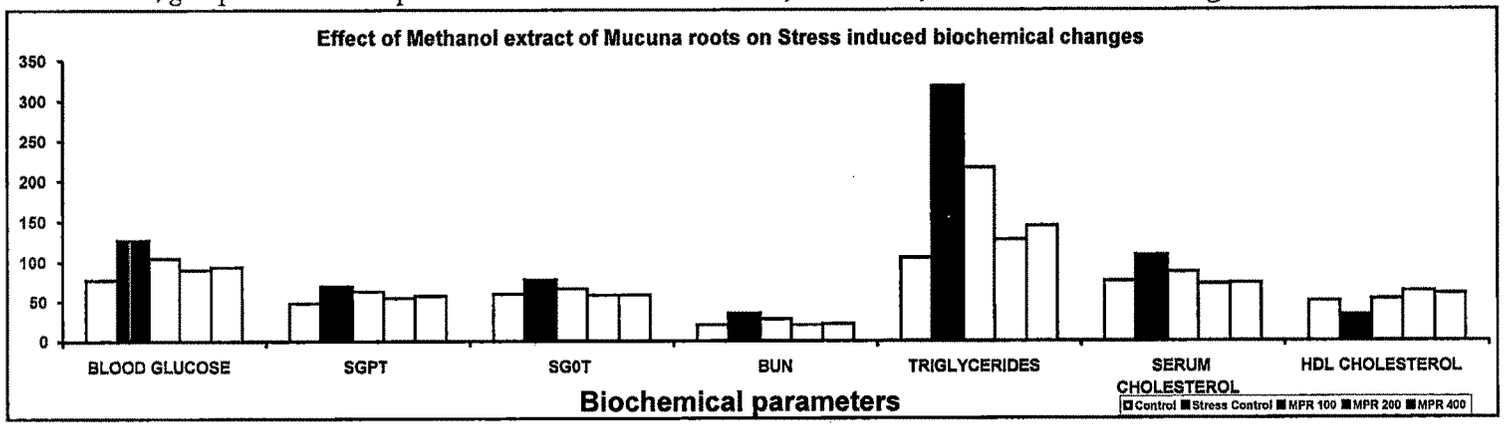
n=6 rats /group Values are expressed as Mean ± SEM

*** P< 0.05, ** P<0.01 , ***P<0.001 Ns= non significant**

Table: 5.9 Effect of Methanol extract of roots of *Mucuna pruriens* on Stress induced changes in biochemical markers.

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	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
MPR 100	103.98±2.58	62.4±0.58 ns	65.47±0.897 ns	26.85±0.647 *	216.13±1.99*	85.83±0.991 *	52.76±0.544**
MPR 200	89.53±1.77 ***	54.72±0.66 ***	57.59±0.605 ***	20.97±1.537 ***	126.88±6.39 ***	71.73±0.581 ***	62.96±0.508***
MPR 400	93.45±2.95 ***	56.92±1.82 **	57.39±1.69 ***	21.5±0.526 ***	144.27±2.43 ***	72.54±0.859 ***	59.54±0.781**

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 compared to normal control group. 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 compared to stress control group. 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.

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

At first, the successive petroleum ether, ethyl acetate, methanol and aqueous extracts were subjected for toxicity studies (Results are as summarized in the Table 5.10)

Table 5.10
Toxicity studies of successive extracts of roots of *Mucuna pruriens*

Extract	Dose level mg/kg body weight			
	2000mg/kg	550mg/kg	175mg/kg	55mg/kg
MPRPE	Died after 5 days	Non-toxic	-------	-------
MPREAE	Non-toxic	-------	-------	-------
MPRME	Non-toxic	-------	-------	-----
MPRWE	Non-toxic	------		

MPRPE = Pet. Ether extract of roots of *Mucuna pruriens*.
MPREAE=Ethyl acetate extract of roots of *Mucuna pruriens*
MPRME= Methanol extract of roots of *Mucuna pruriens*
MPRWE= Aqueous extract of roots of *Mucuna pruriens*

Pet ether, methanol and aqueous extracts when administered orally (as per OECD guidelines) at a maximum dose of 550 mg and 2000 mg/kg body weights respectively did not show any toxicity or mortality hence at three different dose levels Pet ether extract at 25, 50 and 100mg/kg , Ethyl acetate, Methanol and Aqueous extracts at 50, 100 and 200mg/kg dose levels respectively were screened with the above mentioned models.

- **Evaluation of Immunomodulatory activity of successive extracts of roots of *Mucuna pruriens***

Table 5.11: Effect of successive solvent extracts of roots of *Mucuna pruriens* on Phagocytic index, HA titre and DTH response

GROUP	PHAGOCYTIC INDEX (Mean ±SEM)	HA TITRE Mean ±SEM	DTH RESPONSE Mean ±SEM
Control	0.0288±0.0027	138.66±25.68	0.665±0.022
CPM		80±16	1.2±0.059
MPRPE 25	0.0413±0.0034 **	405.33±69.45 **	1.661±0.061 ***
MPRPE 50	0.0516±0.0016 ***	512±114.59 ***	1.735±0.038 ***
MPRPE 100	0.0491±0.0025 ***	469.33±42.66 ***	1.753±0.0844 ***
MPREAE 50	0.0243±0.0019 ns	64±14.31 ns	1.241±0.053 ns
MPREAE 100	0.0336±0.0014 ns	133.33±29.11 ns	1.105±0.066 ns
MPREAE 200	0.033±0.002 ns	138.66±25.68 ns	1.051±0.09 ns
MPRME 50	0.0383±0.002*	144±38.53 ns	1.535±0.0325 **
MPRME100	0.043±0.002 **	277.33±51.37 *	1.635±0.0182**
MPRME 200	0.041±0.002 **	234.66±61.08 *	1.573±0.04 **
MPRWE 100	0.0393±0.002 *	160±32 *	1.596±0.028 **
MPRWE 200	0.0385±0.003 *	181.33±34.72 *	1.351±0.096 *

Table 5.12 : Effect of successive solvent extracts of Mucuna roots 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
MPRPE 25	6.65±0.23	9.21±0.29**	16.6
MPRPE 50	6.4±0.2	10.45±0.24***	0
MPRPE 100	6.85±0.24	10.65±0.52 ***	0
MPREAE 50	6.16±0.32	6.41±0.29 ns	100
MPREAE 100	6.63±0.29	7±0.3 ns	100
MPREAE 200	7.21±0.44	7.51±0.61 ns	100
MPRME 50	7.6±0.46	9.1±0.56 *	33
MPRME100	7.98±0.47	11.39±0.68***	33
MPRME 200	6.68±0.28	8.93±0.57 *	16.6
MPRWE 100	7.21±0.36	9.4±0.26**	50
MPRWE 200	7.48±0.37	10.18±0.21***	50

n=6 mice /group 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 *Mucuna pruriens* on Phagocytic activity**

Phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic micro organisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophills and tissue macrophages. Once the particulate matter isingested into phagosomes, the phagosomes fuse with lysosomes and the ingested matter is then digested. (Tiwari e al 2004) 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). The phagocytic indices of MPRPE was assessed at 25, 50 and 100mg/kg b.w., MPREAE and MPRME at 100, 200 and 400 mg/kg dose and MPRAE at 100 and 200 mg/kg dose levels. The phagocytic index of control group was 0.0288±0.0027 whereas MPRPE at all the three dose levels showed a highly significant phagocytic activity 0.0413±0.0034, 0.0516±0.0016 and 0.0491±0.0025 ($P<0.001$). Administration of MPREAE at 50, 100 and 200 mg/kg dose levels did not show any phagocytic activity. The phagocytic response of MPRME ($P<0.01$) and MPRAE at 100 and 200mg dose levels were also found to be less significant ($P< 0.05$). Increased phagocytic activity is indicative of increased functioning of reticulo-endothelial system.
(Results are summarized in Table 5.11)

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

- **Effect of successive extracts of roots of *Mucuna pruriens* on HA titre and DTH response.**

The humoral antibody titre value in control was found to be 138.66±25.68. Administration of MPRPE produced increase in humoral antibody titre as evident by haemagglutination at that dilution. The HA titre value obtained at 25, 50 and 100 mg/kg dose were 405.33±69.45, 512±114.59 and 469.33±42.66 respectively. Similarly DTH response was found to be 1.661±0.061, 1.735±0.038 and 1.735±0.038 a dose dependant increase in response was observed. Statistically significant increase was observed at all three dose levels ($P<0.001$). In case of MPREAE at all three dose levels showed a non-significant HA titre value and DTH response. MPRME at 100 and 200 mg/kg dose levels showed HA titre value of 277.33±51.37 & 234.66±61.08 respectively but at 50mg/kg dose it produced non-significant response but DTH response at all the three dose levels were found significant ($P<0.01$). In case of MPRAE at 100 and 200mg/kg dose levels produced HA titre value of 160±32 and 181.33±34.72 respectively which was less significant compared to MPRME and MPRPE.

(Results are summarized in Table 5.11)

- **Effect of successive extracts of roots of *Mucuna pruriens* on E coli induced abdominal sepsis.**

The protective effect against E coli induced abdominal sepsis is assessed by the total leukocyte count and mortality rate. In case of control group the total leukocyte count on 0 and 15th days was found to be 7.56±0.33 and 7.86±0.37($x10^3$ cells/mm^3) and 100% mortality was observed after E coli administration, whereas administration of MPRPE at 25, 50 and 100mg/kg dose levels produced a highly significant increase in TLC from 6.65±0.23 to 9.21±0.29, 6.4±0.2 to 10.45±0.24 and 6.85±0.24 to 10.65±0.52 ($x10^3$ cells/mm^3) respectively with 0 % mortality at 50 and 100mg/kg dose levels. MPREAE at all the three dose levels did not affect the TLC count hence 100 % mortality was observed with all the dose levels. MPRME and MPRAE also produced a highly significant increase in TLC at 100 and 200mg/kg dose with 33 and 50% mortality. (Results are summarized in Table 5.12)

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

- **Effect of successive extracts of roots of *Mucuna pruriens* on Cyclophosphamide induced Myelosuppression assay.**

In Cyclophosphamide induced myelosuppression assay, mice when pretreatment with MPRPE at 25, 50 and 100mg/kg, MPRME at 50,100 & 200 mg/kg and MPRWE at 100& 200mg/kg for 10 days increased the total WBC count from 7.25±0.81 to 10.4±0.42 at 25mg/kg, 7.57±0.52 to 11.51±0.63 at 50mg/kg and 7.1±0.29 to 11.37±0.38 (thousand cells/mm^3) prior Cyclophosphamide administration and further treatment with extract till 14^{th} day followed by administration of Cyclophosphamide on 11^{th}, 12^{th} and 13^{th} days, the extract at 50 & 100mg/kg showed statistically significant change in TLC count 6.61±0.93 and 6.02±0.47 ($P<0.001$) TLC values were close to normal 7.57±0.52 and 7.1±0.29 (thousand cells/mm^3) respectively compared to CPM control. In case of RBC and Hb count MPRPE at all the three doses did not make much difference. But in case of MPRME and MPRWE both the extracts at all dose levels produced an increase in TLC on pretreatment upto 10 days but on co-administration of Cyclophosphamide though at all dose levels it offered protection against CPM induced myelosuppression only MPRME at 200mg/kg dose level produced significant increase in TLC count and did not make much difference in Hb and RBC counts. (Results as summarized in Table 5.13)

Table 5.13 Effect of successive extract of roots of *Mucuna pruriens* on on Leucocyte, RBC and Hb count in Cyclophosphamide induced Immunosuppression

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

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	7.52±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
MPRPE 25	7.25±0.81	10.4±0.42	5.2±0.87*	7.58±0.49	9.23±0.82	9.01±0.65	14.5±0.56	14.7±0.92	13.3±0.61
MPRPE 50	7.57±0.52	11.51±0.63	6.61±0.93 ***	8.21±0.91	10.67±0.62	10.43±0.41	14.2±0.47	15.3±0.59	15.2±0.43
MPRPE 100	7.1±0.29	11.37±0.38	6.02±0.47 ***	8.13±0.73	10.17±0.65	9.94±0.37	12.5±0.31	13.2±0.43	13.15±0.33
MPRME 50	7.33±0.59	9.89±0.81	4.91±0.41*	8.4±0.14	9.51±0.66	8.66±0.31	13.4±0.47	13.7±0.59	12.9±0.42
MPRME100	7.82±0.54	10.52±0.64	5.31±0.52*	7.8±0.54	8.9±0.71	8.4±063	14.1±0.28	15.68±0.49	14.93±0.82
MPRME 200	7.17±0.37	10.97±0.52	6.1±0.33***	7.93±0.71	10.02±0.37	9.74±0.77	14.7±0.59	15.92±0.38	14.71±0.19
MPRWE100	8.11±0.81	11.06±0.73	4.36±0.61 *	8.32±0.55	9.1±0.73	8.9±0.47	13.8±0.82	14.1±0.52	13.6±0.31
MPRWE200	6.90±0.46	9.86±0.65	4.19±0.86*	9.83±0.44	10.31±0.43	10.1±0.61	14.5±0.37	14.7±0.49	13.2±0.73

- **Evaluation of Anti-stress activity of successive extracts of roots of *Mucuna pruriens***

Since MPREAE failed to exhibit Immunomodulatory response only the other extracts MPRPE, MPRME and MPRAE were subjected to anti-stress activity

Table 5.14 : Effect of successive extracts of roots of *Mucuna pruriens* 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
MPRPE 25	0.177±0.008**	6.338±0.152**	0.63±0.012*
MPRPE 50	0.152±0.004***	5.968±0.07***	0.53±0.01 ***
MPRPE 100	0.18±0.004**	6.208±0.02**	0.551±0.014 ***
MPRME 50	0.204±0.002 *	6.698±0.071 *	0.645±0.009*
MPRME100	0.171±0.007**	6.383±0.079 *	0.646±0.01 *
MPRME 200	0.17±0.006**	6.733±0.103 ns	0.688±0.008 ns
MPRWE 100	0.86±0.002***	6.556±0.081*	0.701±0.009 ns
MPRWE 200	0.191±0.007*	6.818±0.125 ns	0.681±0.009 ns

n=6 rats /group 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 *Mucuna pruriens* on Stress induced changes in organ weight**

Swim stress produced a highly significant increase in the weight of Adrenals, liver and spleen (0.257±0.009, 7.938±0.211 & 0.8±0.01 respectively) compared to vehicle control group. Significant reductions in the weight of these organs were observed in groups pretreated with MPRPE at 25, 50 and 100mg/kg dose levels. The results were highly significant at 50 and 100mg/kg dose levels. MPRME at 200and MPRAE at 100mg/kg dose levels produced significant reduction in weight of adrenal glands. (Results are summarized in Table 5.14)

Table 5.15: Effect of successive extract of roots of *Mucuna pruriens* 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	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
MPRPE 25	94.28±1.76 *	55.72±0.89 **	64.64±0.4 *	27.52±0.483 *	140.6±1.88***	74.16±0.808**	58.54±0.74***
MPRPE 50	86.19±1.82 ***	49.73±1.26 ***	57.76±0.8 ***	21.3±0.985 ***	129.38±6.7 ***	66.36±0.899***	64.46±0.77***
MPRPE 100	86.98±0. 783 ***	47.5±0.434 ***	57.73±1.58 ***	22.72±0.607 ***	111.38±3.3***	66.72±1.31***	54.93±0.688***
MPRME 50	82.73±1.45 ***	61.94±0.63 **	70.2±0.452 ns	26.55±0.584 *	209.8±1.41 **	92.03±2*	45.27±0.951*
MPRME100	79.01±0.899 ***	63.55±0.71 *	65.24±1.04 *	23.47±1.29 **	188.81±2.5**	94.71±1.57*	45.39±1.22*
MPRME200	81.76±1.22 ***	50.88±0.52 ***	62.37±0.511 ***	21.47±1.56 ***	186.15±4.2***	87.67±1.45**	44.01±0.516*
MPRWE100	92.7±0.676*	60.71±0.74 **	70..92±0.543 *	28.02±0.395 *	285.56±5.3ns	93.9±1.55*	51.21±0.785*
MPRWE200	102.82±2.95*	66.28±0.68 ns	66.9±1.477 ns	29.35±0.396 ns	264.18±3.0*	90.18±2*	42.79±1.087*

n=6 rats /group Values are expressed as Mean ± SEM * P< 0.05, ** P<0.01 , ***P<0.001 Ns= non significant. All treated groups are compared with stress control

Swim stress produced significant increase in the level of the biochemical markers compared to normal group. Pretreatment with MPRPE at all three dose levels showed reduction in the level of these markers. But statistically significant reduction was observed only at 50 and 100 mg.kg In MPRME treated groups the activity was significant only at 200mg/kg. but in case of MPRWE , though there was reduction in these biochemical levels but the activity was not so significant like that of MPRPE and MPRME treated groups. (Results summarized in Table 5.15)

Pet ether, and Methanol extracts of roots of *Mucuna pruriens* exhibited a statistically significant increase in DTH response, HA titre, Phagocytic index and protection against Cyclophosphamide induced myelosuppression and also reversed swim stress induced elevations in the levels of Glucose, Cholesterol, SGPT, SGOT, Urea Nitrogen and reduction in Triglycerides while ethyl acetate extract failed to produce any such response. Evaluation of successive extracts of roots of *Mucuna pruriens* suggests the presence of bioactive constituents in Pet ether and Methanol extracts. Qualitative chemical analysis of Pet ether extract of roots of *Mucuna pruriens* showed higher content of sterols while the Methanol and aqueous extracts of revealed the presence of phenolics, amino acids etc.

The adaptogenic activity of roots of *Mucuna pruriens* may be endowed upon the higher content of sterols, phenolics, amino acids etc.

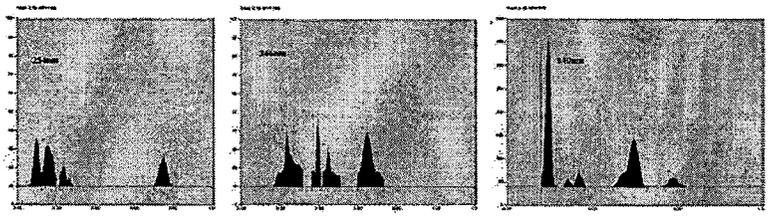
5.4 Development of comparative HPTLC fingerprint profile of the extracts of roots and seeds of *Mucuna pruriens*

Preliminary phytochemical screening showed the presence of terpenoids, phytosterols and phenolic compounds and amino acids. 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 seeds of *Mucuna pruriens*. Compounds of varying polarity in the extracts were separated using various solvent systems on TLC. The HPTLC fingerprint profile comprising of typical spectra, Rf values, UV λ_{Max} and relative percentage of the separated compounds were then recorded.

Table 5.16.

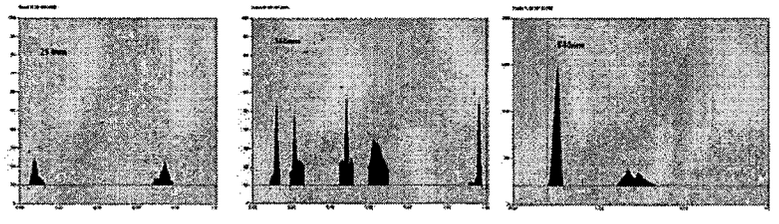
Solvent systems used for recording the HPTLC finger print profiles of methanol extract of roots of *Mucuna pruriens*	
	Solvent system
1	**Toluene: Chloroform: Ethyl acetate: Acetic acid (10:2:1:0.03) v/v**
2	**Toluene :Ethyl acetate: Methanol: Water (10:5:2.5:1)**
3	**Ethyl acetate: Formic acid:Acetic acid:Water (8:1:0.4:1)**
4	**Toluene: Formic acid:Ethyl formate (5:1:4)**
5	**n-Butanol:Acetic acid:Water (4:1:1)**

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 amino acids and amines, which were detected by derivatization with Ninhydrin.



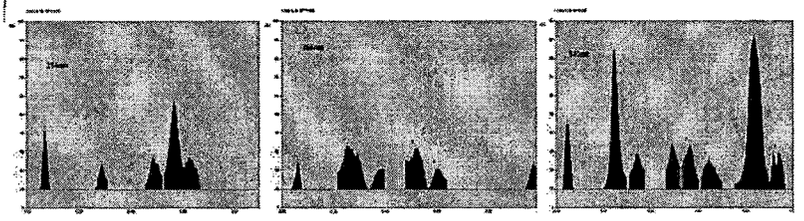
Peak	Mobile phase: Toluene:Chloroform:Ethyl acetate: Acetic acid (10:2:1:0.03 v/v)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.10	25.73	0.24	32.38	0.01	52.22
2	0.16	37.09	0.40	13.56	0.11	2.13
3	0.24	12.67	0.45	16.54	0.17	4.77
4	0.70	24.51	0.65	37.51	0.49	35.92
5	-	-	-	-	0.73	4.95

Fig. 5.03a TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



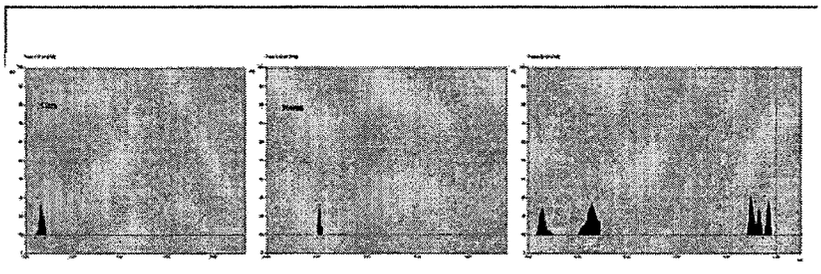
Peak	Mobile phase: Toluene:Chloroform:Ethyl acetate: Acetic acid (10:2:1:0.03 v/v)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.09	44.97	0.13	13.56	0.01	72.22
2	0.75	55.03	0.22	19.96	0.42	15.58
3	--	--	0.49	23.77	0.48	12.20
4	--	--	0.63	30.85	---	----
5	-		-	-		

Fig. 5.03b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF SEEDS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



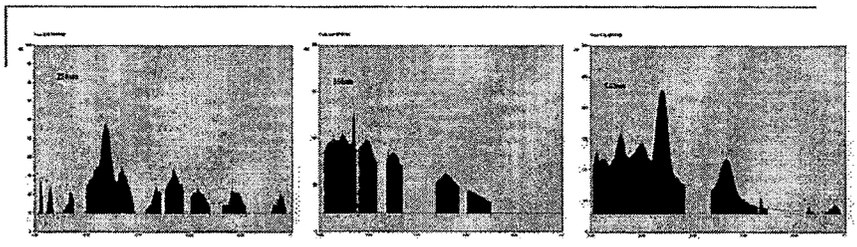
Peak	Mobile phase: Toluene :Ethyl acetate: Methanol: Water (10:5:2.5:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.07	12.98	0.06	5.54	0.05	6.19
2	0.29	8.06	0.26	25.71	0.25	19.37
3	0.49	15.77	0.29	12.10	0.34	6.71
4	0.57	45.53	0.39	8.92	0.49	7.85
5	0.63	17.66	0.52	28.57	0.57	8.77
6			0.61	10.63	0.65	6.93
7			0.98	8.44	0.84	37.53
8					0.92	2.89
9					0.94	3.66

Fig.5.04a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



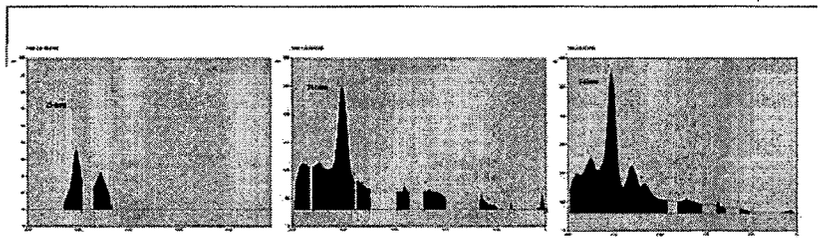
Peak	Mobile phase: Toluene :Ethyl acetate: Methanol: Water (10:5:2.5:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.06	100.0	0.21	100.00	0.06	18.53
2					0.26	36.33
3					0.90	19.45
4					0.93	11.27
5					0.97	14.41

Fig. 5.04b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF SEEDS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



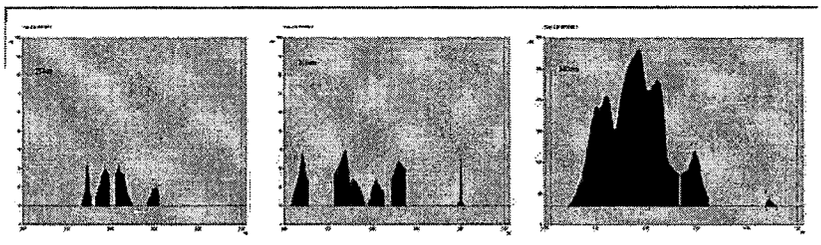
Peak	Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.03	1.86	0.06	17.60	0.02	6.32
2	0.06	2.44	0.09	15.21	0.05	6.56
3	0.14	2.99	0.14	9.67	0.12	17.00
4	0.28	38.44	0.19	21.15	0.20	18.53
5	0.34	13.24	0.28	4.17	0.28	31.73
6	0.47	5.69	0.30	11.70	0.53	17.52
7	0.54	14.75	0.52	13.54	0.67	0.94
8	0.63	8.27	0.62	7.06	0.86	0.28
9	0.77	8.17			0.96	1.11
10	0.96	4.17				

Fig. 5.05a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



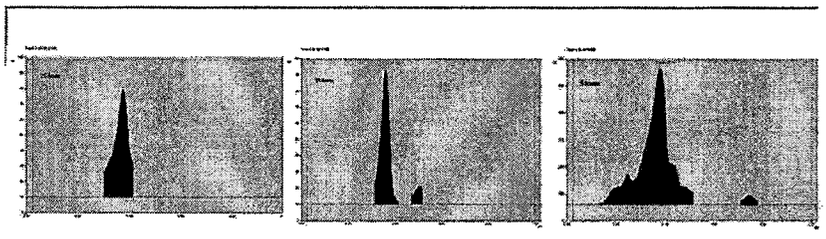
Peak	Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.19	56.44	0.04	14.27	0.04	9.07
2	0.29	43.56	0.11	16.17	0.10	17.31
3			0.20	42.03	0.19	39.16
4			0.26	8.33	0.28	14.04
5			0.44	5.75	0.34	11.11
6			0.54	9.27	0.52	6.27
7			0.74	2.99	0.65	1.59
8			0.86	0.33	0.76	0.82
9			0.98	0.85	0.97	0.64

Fig.5.05b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF SEEDS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



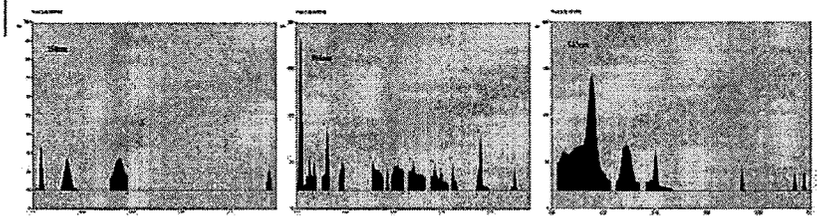
Peak	Mobile phase: n Butanol:Acetic acid: Water (4:1:1)					
	254nm		366nm		540nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.30	16.77	0.08	21.63	0.21	13.80
2	0.38	33.50	0.28	29.01	0.25	13.51
3	0.43	35.53	0.31	11.43	0.38	40.35
4	0.57	14.20	0.42	11.74	0.45	22.76
5			0.52	23.11	0.60	9.17
6			0.81	3.09	0.89	0.40

Fig. 5.08a. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



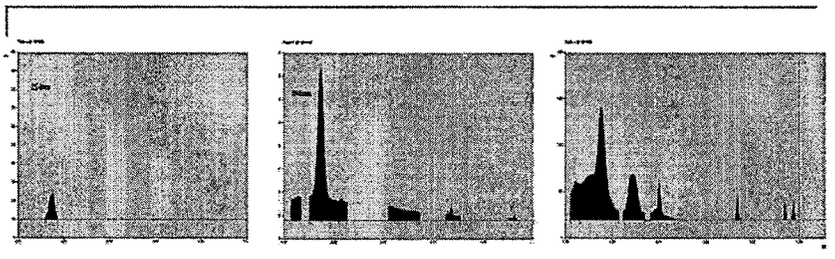
Peak	Mobile phase: n Butanol:Acetic acid: Water (4:1:1)					
	254nm		366nm		540nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.38	100.00	0.36	89.65	0.25	13.84
2			0.61	10.35	0.38	83.24
3					0.74	2.92

Fig. 5.08b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF SEEDS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



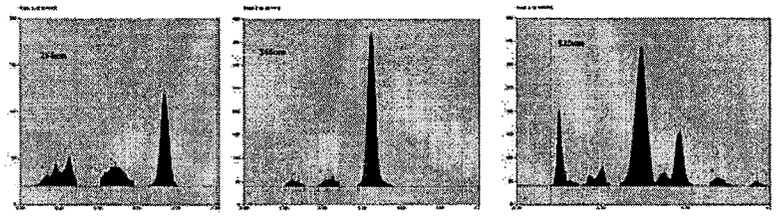
Peak	Mobile phase: Ethyl acetate: Formic acid: Acetic acid: Water (8:1:0.4:1)					
	254nm		366nm		540nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.03	15.72	0.02	15.92	0.09	11.53
2	0.14	27.77	0.05	6.41	0.16	59.96
3	0.35	47.00	0.07	3.14	0.29	17.91
4	0.96	9.51	0.13	8.69	0.40	7.09
5			0.19	4.74	0.74	1.41
6			0.32	10.41	0.94	1.01
7			0.39	1.91	0.98	0.99
8			0.42	12.14		
9			0.48	12.79		
10			0.57	5.61		
11			0.60	4.57		
12			0.66	3.37		
13			0.76	9.15		
14			0.90	2.26		

Fig.5.06. TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



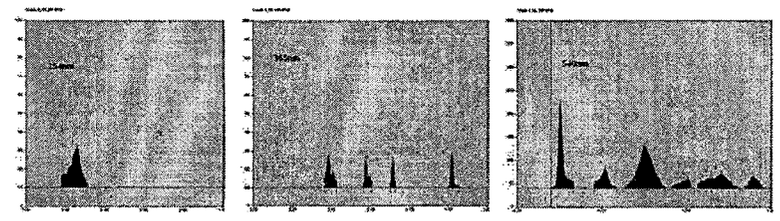
Peak	Mobile phase: Ethyl acetate: Formic acid: Acetic acid: Water (8:1:0.4:1)					
	254nm		366nm		366nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.15	100.0	0.05	11.47	0.05	14.61
2			0.14	85.27	0.16	54.81
3			0.74	2.26	0.30	21.11
4			0.98	1.00	0.40	9.47

Fig. 5.06b. TLC CHROMATOGRAM OF METHANOL EXTRACT OF SEEDS OF Mucuna pruriens AFTER DENSITOMETRIC SCAN AT 254 AND 366 NM



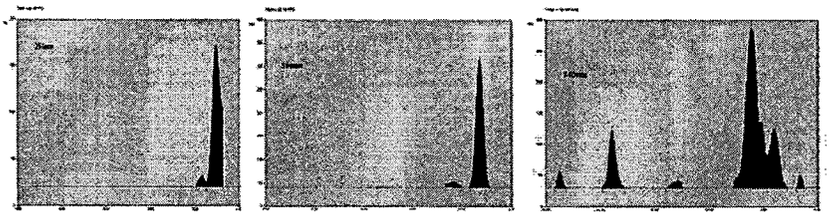
Peak	Mobile phase: Toluene:Chloroform:Ethyl acetate: Acetic acid (10:2:1:0.03 v/v)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.14	6.91	0.25	3.30	0.07	13.20
2	0.19	9.05	0.28	1.97	0.19	1.31
3	0.26	15.05	0.42	2.85	0.26	2.27
4	0.49	23.63	0.48	5.08	0.49	4.60
5	0.74	46.32	0.65	86.79	0.63	54.69
6					0.72	4.02
7					0.94	15.44

Fig. 5.09a. TLC CHROMATOGRAM OF PET ETHER EXTRACT OF ROOTS of *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



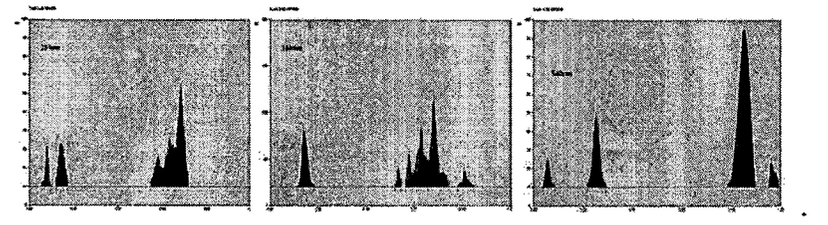
Peak	Mobile phase: Toluene:Chloroform:Ethyl acetate: Acetic acid (10:2:1:0.03 v/v)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.26	100.0	0.39	41.71	0.01	23.48
2	---		0.58	23.79	0.27	10.33
3			0.71	15.32	0.51	37.38
4			0.92	19.18	0.76	5.26
5			-	-	0.90	5.75
					0.95	11.67

Fig.5.09b. TLC CHROMATOGRAM OF PET ETHER EXTRACT OF SEEDS of *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



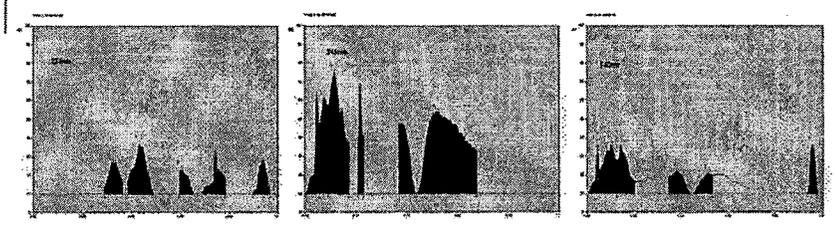
Peak	Mobile phase: Toluene :Ethyl acetate: Methanol: Water (10:5:2.5:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.83	5.48	0.77	5.72	0.05	2.46
2	0.90	94.52	0.88	94.28	0.24	12.51
3					0.49	1.99
4					0.76	63.75
5					0.84	17.61
6					0.94	1.67

Fig. 5.10a TLC CHROMATOGRAM OF PET ETHER EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



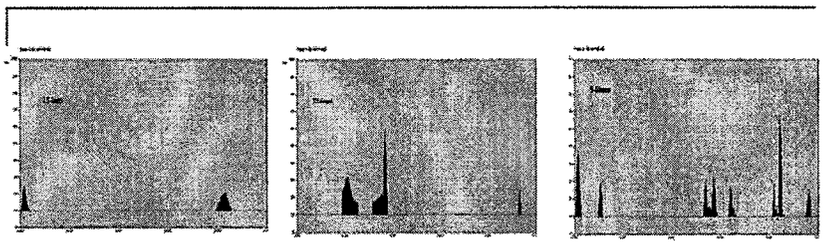
Peak	Mobile phase: Toluene :Ethyl acetate: Methanol: Water (10:5:2.5:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.08	7.87	0.14	18.62	0.06	4.77
2	0.14	17.57	0.54	3.88	0.25	18.98
3	0.58	12.73	0.58	7.97	0.85	71.26
4	0.63	23.97	0.63	27.35	0.96	4.98
5	0.68	37.85	0.68	31.86		
6			0.72	6.09		
7			0.81	5.22		

Fig. 5.10b TLC CHROMATOGRAM OF PET ETHER EXTRACT OF SEEDS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



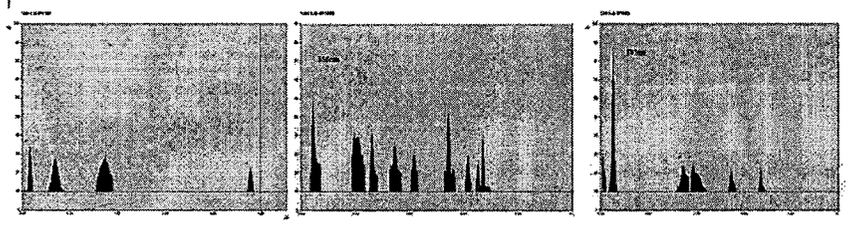
Peak	Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4)					
	254nm		366nm		540nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.32	20.09	0.05	5.91	0.04	8.98
2	0.44	35.31	0.08	10.07	0.10	28.52
3	0.61	11.45	0.12	23.75	0.14	24.62
4	0.75	18.40	0.22	5.69	0.38	14.79
5	0.94	14.76	0.38	9.91	0.52	11.13
6			0.52	44.68	0.96	11.96

Fig.5.11a TLC CHROMATOGRAM OF ETHYL ACETATE EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



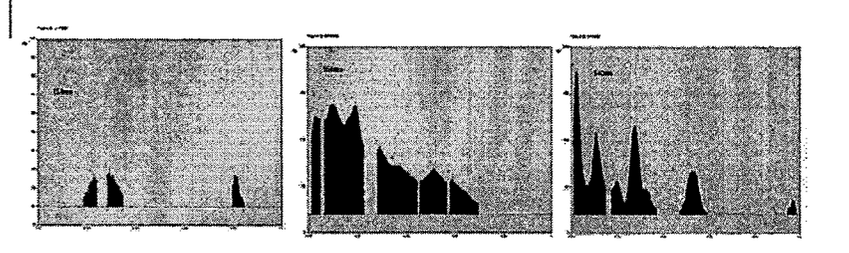
Peak	Mobile phase: Ethyl acetate: Formic acid: Acetic acid: Water (8:1:0.4:1)					
	254nm		366nm		540nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.02	33.85	0.21	48.04	0.02	21.98
2	0.83	66.45	0.37	45.75	0.11	10.39
3			0.93	6.21	0.54	11.78
4					0.58	11.43
5					0.64	8.33
6					0.82	7.88
7					0.85	20.03
8					0.96	8.18

Fig. 5.11b. TLC CHROMATOGRAM OF ETHYL ACETATE EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



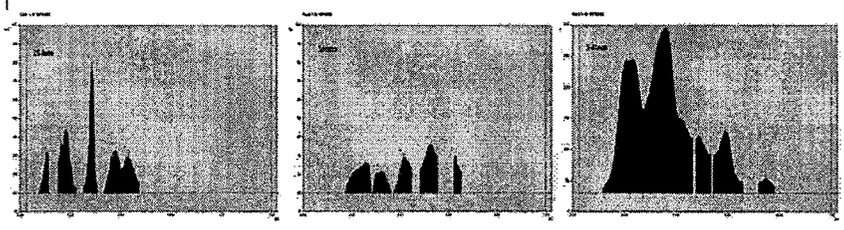
Peak	Mobile phase: Ethyl acetate: Formic acid: Acetic acid: Water (8:1:0.4:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.03	15.53	0.04	17.80	0.01	8.66
2	0.14	36.15	0.20	23.55	0.05	36.04
3	0.34	48.32	0.26	9.51	0.34	18.37
4			0.35	13.49	0.39	21.23
5			0.42	7.31	0.55	7.42
6			0.55	13.87	0.67	6.27
7			0.62	5.41		
8			0.65	2.96		
9			0.67	6.10		

Fig. 5.12a. TLC CHROMATOGRAM OF SUCCESSIVE METHANOL EXTRACT OF ROOTS OF Mucuna pruriens AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



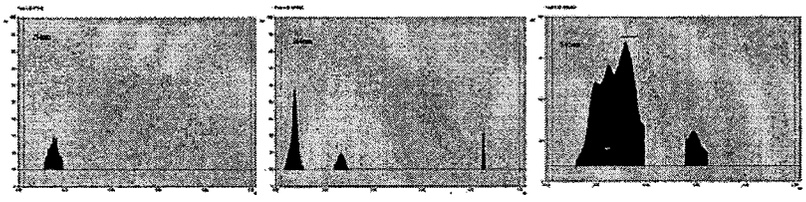
Peak	Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.23	32.47	0.03	9.78	0.02	26.51
2	0.30	42.34	0.10	23.04	0.11	21.41
3	0.81	25.19	0.19	22.65	0.20	8.21
4			0.30	11.11	0.28	23.10
5			0.36	12.71	0.32	4.77
6			0.52	12.80	0.53	14.17
7			0.59	7.91	0.97	1.84

Fig. 5.07a. TLC CHROMATOGRAM OF SUCCESSIVE METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



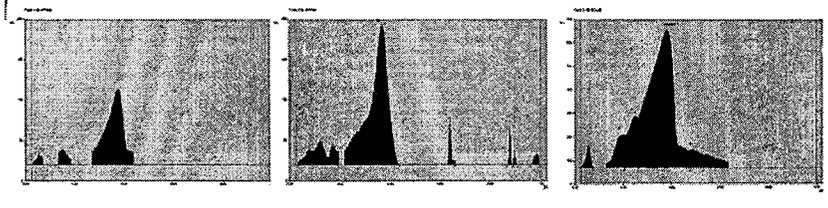
Peak	Mobile phase: n Butanol:Acetic acid: Water (4:1:1)					
	254nm		366nm		540nm	
	Max R_F	Relative %	Max R_F	Relative %	Max R_F	Relative %
1	0.11	9.03	0.26	25.72	0.21	17.25
2	0.18	26.70	0.31	4.96	0.23	14.66
3	0.28	26.63	0.32	8.71	0.35	49.45
4	0.38	18.64	0.42	19.27	0.49	6.71
5	0.43	18.89	0.53	31.43	0.59	10.25
6			0.63	9.91	0.75	1.69

5.13a. TLC CHROMATOGRAM OF SUCCESSIVE METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



Peak	Mobile phase: n Butanol:Acetic acid: Water (4:1:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.12	100.0	0.08	74.69	0.20	19.23
2			0.28	17.56	0.25	23.44
3			0.86	7.75	0.32	47.28
4					0.59	10.05

Fig. 5.14a. TLC CHROMATOGRAM OF AQUEOUS EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM



Peak	Mobile phase: n Butanol:Acetic acid: Water (4:1:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.06	3.93	0.09	4.22	0.06	2.16
2	0.15	8.35	0.12	6.59	0.20	6.43
3	0.38	87.71	0.17	4.40	0.25	11.12
			0.37	79.16	0.38	71.74
			0.64	2.27	0.49	4.20
			0.88	1.51	0.54	2.54
			0.90	0.45	0.59	1.82
			0.99	1.41		

Fig. 5.14b. TLC CHROMATOGRAM OF AQUEOUS EXTRACT OF SEEDS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM

Comparative HPTLC fingerprinting studies of extracts of roots and seeds were performed.

The TLC chromatogram of methanol extract of roots (in solvent system 1) at 254nm and 366nm showed the presence of 4 peaks each while at 540nm 5 peaks were observed. In case of methanol extract of seeds at 254nm 2peaks were observed, at 366nm 4peaks and at 540nm only 3 peaks were found. Peak for the compound with Rf 0.01 at 540nm and peak with Rf 0.65 at 366nm were found to be common in both chromatogram. (See Fig. 5.03 a & b)

The TLC chromatogram of methanol extract of roots (in solvent system 2) at 254nm revealed presence of 4 components, 7 peaks at 366nm and 9 peaks at 540nm, where as the chromatogram of seeds extract only one peak eachwas found at 254nm and 366nm and 5 peaks at 540nm. In both the chromatograms 3 peaks with Rf 0.06, 0.25 and 0.92 were found common. (see Fig.5.04 a & b)

Thus, the comparative HPTLC fingerprinting studies of total methanol extracts of roots and seeds for non-polar constituents was found to be quite similar.

The TLC chromatogram of methanol extract of roots (in solvent system 3) at 254nm revealed presence of 4 components, 14 peaks at 366nm and 7 peaks at 540nm, where as the chromatogram of seeds extract single peak was found at 254nm, 4 peaks each at 366nm and 540nm respectively. In both the chromatograms 3 peaks for the compounds with Rf 0.05, 0.16, 0.29 and 0.40 at 540nm, peak with Rf 0.05 and 0.13 at 366nm and peak for the compound with Rf 0.14 at 254nm were found common. (Fig.5.05 a & b)

The TLC chromatogram of methanol extract of roots (in solvent system 4) at 254nm revealed presence of 10 components, 8 peaks at 366nm and 9 peaks at 540nm, where as the chromatogram of seeds extract 2 peaks were found at 254nm, 9peaks at 366nm and 9 peaks at 540nm. In both the chromatograms 5 peaks for the compounds with Rf 0.05, 0.20, 0.28, 0.53 and 0.96 at 540nm, peak with Rf 0.2 at 366nm and peak for the compound with Rf0.28 were found common. (Fig. 5.07 a & b)

Similarly the TLC chromatogram of methanol extract of roots (in solvent system 5) at 254nm revealed presence of 4 components, 6 peaks at 366nm and 6 peaks at 540nm, where as the chromatogram of seeds extract single peak was found at 254nm, 2peaks at 366nm and 3 peaks at 540nm. In both the chromatograms 2 peaks for the compounds with Rf 0.25 & 0.38 at 540nm, peak with Rf 0.52 at 366nm and peak for the compound with Rf 0.38 were found common. (Fig. 5.08 a &b)

The TLC chromatogram of Ethyl acetate extract of roots of *Mucuna pruriens* (in solvent system 4) at 254nm revealed the presence of 5peaks, at 366nm showed presence of 6 peaks and at 540nm showed the presence of 6 peaks.3 peaks with Rf 0.05, 0.53 and 0.96 were found common when compared with the chromatogram of total methanol extract of roots. Similarly the TLC chromatogram of ethyl acetate extract (in solvent system 3) at 254nm showed 2 peaks and at 366nm 3 peaks and at 540nm 8 peaks were. (See fig.5.11a & b)

TLC chromatogram of pet ether extract of roots of *Mucuna pruriens* (MPRPE) (in mobile phase 1) at 254nm showed presence of 5 peaks for compounds with Rf 0.14, 0.19, 0.26, 0.49 and 0.74 respectively. At 366nm showed 5 peaks for compounds with Rf 0.25, 0.28, 0.42, 0.46 and 0.65. At 540nm after derivatization 7 peaks with Rf 0.07, 0.19, 0.26, 0.49, 0.63, 0.72 and 0.94 were found. The chromatogram of Pet ether extract of seeds of *Mucuna pruriens* at 245nm revealed the presence of single peak with Rf 0.26, at 366nm 4 peaks with Rf 0.39, 0.58, 0.71 and 0.92 were found. While at 540 nm it showed the presence of 6 peaks for compounds with Rf 0.01, 0.26, 0.51, 0.76, 0.90 and 0.94. Two peaks with Rf 0.26 and 0.94 were found common in both the chromatograms. (See fig. 5.09 a & b)

TLC chromatogram of pet ether extract of roots of *Mucuna pruriens* (MPRPE) (in mobile phase 2) at 254nm showed presence of 2 peaks for compounds with Rf 0.83 and 0.90 respectively similarly at 366nm also it showed 2 peaks for compounds with Rf 0.77 and 0.88. At 540nm after derivatization, 5 peaks with Rf 0.05, 0.24, 0.49, 0.76, 0.84 and 0.94 were found. The chromatogram of Pet ether extract of seeds of *Mucuna pruriens* at 245nm revealed the presence of 5 peaks with Rf 0.08, 0.14, 0.58, 0.63 and 0.68, at 366nm 7 peaks with Rf 0.14, 0.54, 0.58, 0.63, 0.68, 0.72 and 0.81 were found. While at 540 nm it showed the presence of only peaks for compounds with Rf 0.06, 0.25, 0.84 and 0.96. Three peaks with Rf 0.06, 0.25 and 0.84 were found common in both the chromatograms. (See fig. 5.10 a & b)

HPTLC comparative fingerprinting studies of Pet ether extract of roots and seeds of *Mucuna pruriens* suggests the presence of some similar constituents in both the extracts.

The chromatogram of successive methanol extract of roots (in solvent system 4) at 254nm revealed the presence of 3 peaks, 7 peaks each at 366 and 540 nm. Similarly the chromatogram (in solvent system 5) revealed the presence of 5 peaks at 254nm, 6 peaks each at 366 and 540 nm. (See fig. 5.07a, 5.12a, 5.12b)

The chromatogram of aqueous extract of roots in (solvent system 5) showed presence of single peak at 254nm, 3 peaks at 366nm and 4 peaks at 540nm but in case of seed 3 peaks were found at 254nm, 8 at 366nm and 7 peaks at 540nm. Peaks for the compounds with Rf 0.20, 0.25 and 0.59 were found in common. (See fig.5.14 a & b)

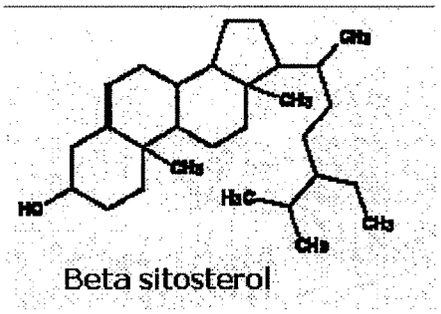
Thus, the HPTLC fingerprint profiles of the important chemical constituents in the bioactive extracts of the roots *Mucuna pruriens* have been established. A complete HPTLC finger print profile of the resolved compounds comprising of the typical spectra, Rf value, and the percentage proportion of the 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.

L-dopa and β-Sitosterol are the main constituents reported from seeds of *Mucuna pruriens* The phytochemical studies showed presence of steroidal moieties in the Pet ether extract and methanol and aqueous extracts revealed the presence of phenolics, amino acids etc. While performing co-TLC studies with some of similar compounds available the Rf of the components corresponded with that of sitosterol and L-dopa thus revealed their presence. Therefore, simple, sensitive, specific and reproducible HPTLC methods were developed for the quantification of β-Sitosterol and L-dopa in *Mucuna pruriens* roots as marker constituents to ensure identity and quality of *Mucuna pruriens.*

5.5 Determination of content of marker in methanol extract of roots of *Mucuna pruriens*

β-Sitosterol is one of the most prevalent vegetable-derived phytosterols, which is found in numerous plants including rice, wheat, corn, nut, peanut etc. It is structurally related to cholesterol.(Karl H. Pegel, 1997) β-sitosterol has an amazing array of scientifically acknowledged benefits for key areas of health in immune dysfunctions, inflammatory disorders and rheumatoid arthritis (Bouic et al., 1996), hypercholesterolemia (Law MR. 2000), breast cancer (Awad A, 2000), colon cancer (Awad et al, 2000) and benign prostatic hypertrophy. (Awad, AB et al.2001)

Previously β-sitosterol has been quantified by liquid chromatography and tandem mass spectrometry using atmospheric pressure photoionization (APPI-LC-MS/MS), (Jan Lembcke et al., 2005) while there are other reports using high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD), (Nair, 2006) online liquid chromatography- gas chromatography (LC-GC) (Willibald Kamm et al., 2002) and Gas chromatography.(Wendy R. Sorenson et al., 2006)



Hence considering its wide therapeutic applications, alternative quantification techniques 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 β-sitosterol in *Mucuna pruriens* roots.

Quantification of β-sitosterol from *Mucuna* roots

Reagents and chemicals

Pure β-sitosterol was obtained from M/s Acros Organics(New Jersey USA), 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 5 g of the coarse powder of *Mucuna pruriens* roots and extracted separately with Methanol (4x 50mL) 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 β-sitosterol (100μg mL^{-1}) was prepared by dissolving 1 mg of accurately weighed sitosterol 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 Toluene: Chloroform: Methanol (4:4:1 *v/v*) for 20 min at room temperature(25^0 ±2 ^{0} C and 40% relative humidity) . The length of the chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried in current air with the help of an hair dryer .The post chromatographic derivatization was carried out in anisaldehyde - 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 527 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 β-sitosterol in extract of *Mucuna* root was performed by external standard method, using pure β-sitosterol as standard.

Calibration curve for β-Sitosterol

Stock solution of β-sitosterol (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.

Quantification of β-Sitosterol in Test sample

5μL of sample solution was applied in triplicate on a TLC plate, developed and scanned as above. Peak areas were recorded and the amount of β-Sitosterol was calculated using the calibration plot.

Specificity

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

Method validation

The method was validated for precision, accuracy and repeatability.(ICH guideline) Instrumental precision was checked by repeated scanning of the same spot 200 & 600ng three times and was expressed as coefficient of variance (%RSD). Method precision was studied by analyzing the standards 200 & 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 (136.8, 152 and 167.2 μg mL^{-1}), % recovery was calculated.

Table 5.17. Method validation parameters for quantification of β-sitosterol using proposed HPTLC Densitometric method.

a) Lineartity regression Data

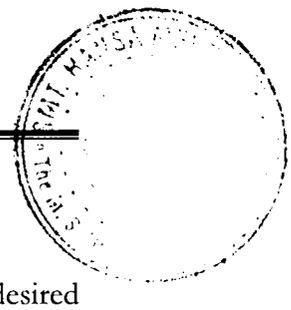
Sl No.	Parameter	Results
1	R_F	0.55
2	Dynamic range (ng spot^{-1})	100-600
3	Equation	Y=59.708+9.816x
4	Slope	9.816
5	Intercept	59.708
6	Limit of Detection	5.55ng
7	Limit of Quantification	18.5 ng
8	Linearity (Correlation coefficient)	0.9998
9	Specificity	Specific

b) Precision studies data

Concentration (ng spot^{-1})	Instrumental Precision (% RSD)	Method Precision (% RSD)	
		Intra day	Inter day
200	0.46	0.78	0.69
600	0.56	0.55	0.60

c) Recovery studies of β-sitosterol

Sl No.	Amount of β-sitosterol in the sample(µg)	Amount of β-sitosterol added (µg)	Amount of β-sitosterol found (µg)	Recovery (%)
1	76	60.8	140.4	102.63
2	76	76.0	150.6	99.07
3	76	91.2	168.0	100.47



HPTLC Separation optimization

Different compositions of the mobile phase were tested and the desired resolution of β-Sitosterol with symmetrical and reproducible peaks was achieved by using mobile phase of Toluene: Chloroform: Methanol (4:4:1 *v/v*) with 20 min of chamber saturation with the mobile phase and 10 min of development. A peak corresponding to β-sitosterol was seen at R_f 0.55. The methanolic extract of the roots of Mucuna roots when subjected to HPTLC as per the methodology described above, showed the presence of β-sitosterol peaks. A comparison of the spectral characteristics of the peaks for standard β-sitosterol and that of the sample (fig 1) revealed the identity of β-sitosterol 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 β-sitosterol 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 β-sitosterol 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 β-sitosterol is $Y=59.708+9.816x$ and the correlation coefficient of calibration plot was 0.9998 indicating good linearity.

Results of regression analysis on calibration curve and detection limits are presented in table 5.17a.

Precision studies

Instrumental precision was checked by repeated scanning of the same spots (200 & 600ng spot $^{-1}$) of standard β-sitosterol three times and the RSD values were 0.46 and 0.56 for 200 and 600 ng spot $^{-1}$ respectively. To determine the precision of the developed assay method 200 and 600ng spot $^{-1}$ of β-sitosterol standard was analyzed three times within the same day to determine the intra-day variability. The RSD values were 0.78 and 0.55 for 200 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 0.69 and 0.60 respectively. (Table 5.17 b)

Sample analysis and recovery studies

The developed HPTLC method was subsequently applied for the analysis of β-Sitosterol in methanolic extract of *Mucuna pruriens* rooots the β-sitosterol content of the roots by this proposed method was found to be 0.076 %.

For the examination of recovery rates, 80, 100 and 120 % of pure β-sitosterol were added in pre-analyzed sample and quantitative analysis was performed. The recoveries were between 99.07-102.63 %. (Table 5.17 c).

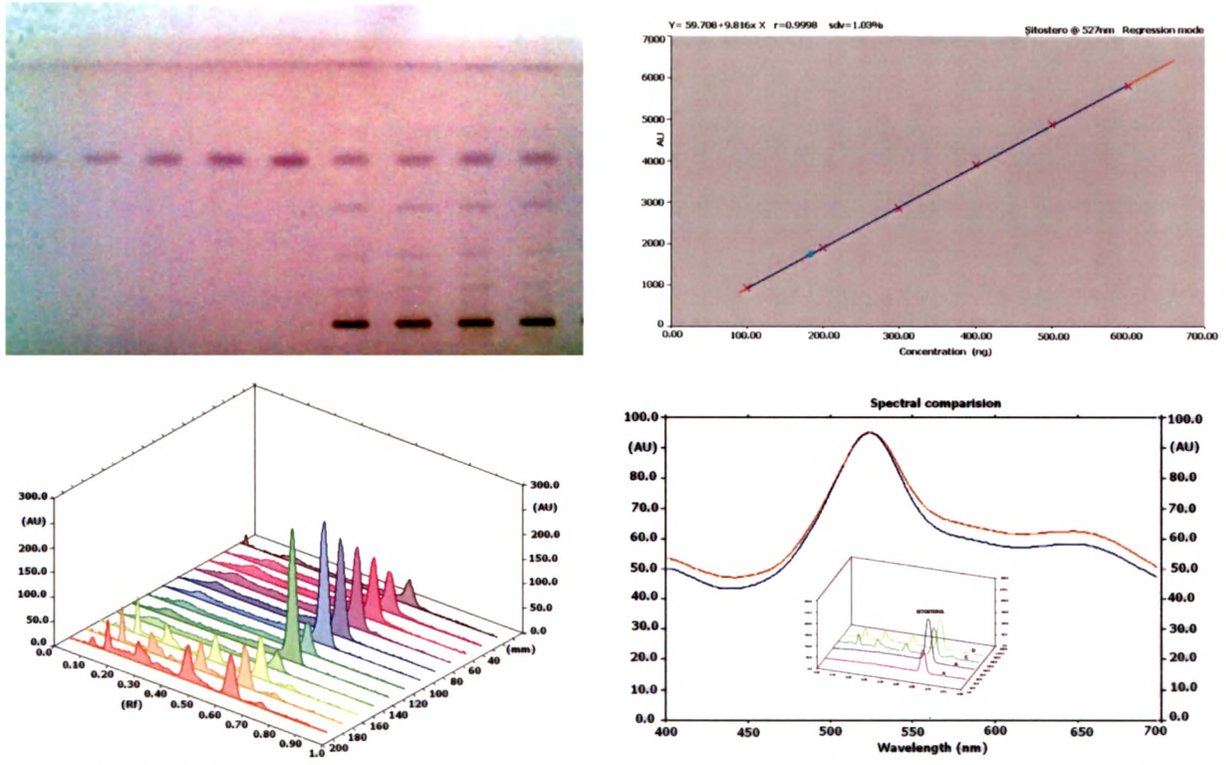


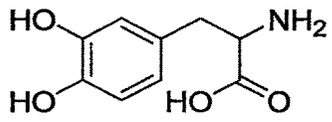
Fig.5.15. UV visible spectrum of standard β-sitosterol and β-sitosterol in sample. 3D Overlay Chromatogram of Standard and sample track.

Quantification of L-Dopa from roots of *Mucuna pruriens*

L-DOPA, the levorotatory isomer of dihydroxyphenylalanine, a natural amino acid, is the inmediate precursor of the neurotransmitter dopamine. The actions of L-DOPA are mainly those of dopamine. Unlike dopamine, L-DOPA can readily enter the Central Nervous System and is used in the treatment of conditions, such as Parkinson's disease, which are associated with depletion of dopamine in the brain. L-DOPA is considered by many clinicians the drug of choice in the management of idiophatic parkinsonian syndrome. L-DOPA is rapidly decarboxylated in the human body, so that very little unchanged drug is available to cross the blood-brain barrier for central conversion into dopamine.(Peter J. Houghtona et al 2005) Consequently L-DOPA is usually given together with a peripheral dopa-decarboxylase inhibitor such as carbidopa or benserazide to increase the proportion of L-DOPA that can enter the brain and to reduce its adverse effects.

Scientific literature reports several methods for the determination of L-DOPA in biological fluids and in pharmaceutical preparations, such as spectrophotometry, mostly in pharmaceuticals , (Karimi et al 2006, Saxer et al., 2004, Tolokán et al., 1997, Sagar KA et al., 2000, Blandini et al., 1997, Wang et al., 2006) high performance liquid chromatography (HPLC), mostly in biological fluids, electrophoresis (Zhang et al., 2001) and voltammetry (Maia, MS et al 2005) . Nevertheless, HPLC and other mentioned techniques has often suffered from diverse disadvantages with regard to cost or selectivity, with complex sample preparation procedures, and long analysis time.

High performance thin-layer chromatography (HPTLC) is a technique carried out within a short period, requires few mobile phase and allows for the analysis of a large number of samples simultaneously. The ability of HPTLC to analyse many samples in parallel has the advantage over techniques because separation of ten or twenty samples takes the same time as the separation of one sample. Amount of the order of nanograms (UV detection) and smaller than picograms (fluorescence detection) can be detected.



3,4-Dihydroxyphenyl alanine

Reagents and chemicals

Pure L-dopa was obtained from M/s Hi media, other solvents and chemicals were of analytical grade and HPTLC plates silica gel $60F_{254}$ (20cm x 20cm) were purchased from E Merck.

Sample preparation:

Accurately weighed 2.5 g of the coarse powder of *Mucuna* 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 L-dopa (100μg mL^{-1}) was prepared by dissolving 1 mg of accurately weighed L-dopa in a mixture of water & methanol (7:3 v/v) and making up the volume of the solution to 10 mL with the same solvent mixture.

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 Butanone: DCM: Isopropanol: Acetic acid:Water (6:4:4:4:3.6) for 20 min at room temperature($25^0 \pm 2\ ^0$ C and 40% relative humidity) . The length of the chromatogram run was 6 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 Ninhydrin followed by heating at 110 ^{0} C for 2 min. Quantitative evaluation of the plate was performed in absorption-reflection mode at 492 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 L-dopa in extract of *Mucuna* root was performed by external standard method, using pure L-dopa as standard.

Calibration curve for L-dopa

Stock solution of L-dopa (100μg mL^{-1}) was prepared in a solvent mixture of water & methanol (7:3 v/v) 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 L-dopa was 925.95+12.55X and co-relation coefficient (r) was 0.995.

Quantification of L-dopa 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 L-dopa was calculated using the calibration plot.

Specificity

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

Method validation

The method was validated for precision, accuracy (10) 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 % recovery and average % recovery was calculated.

Table 5.18 Method validation parameters for quantification of L dopa using proposed HPTLC Densitometric method.

a) Lineartity regression Data

Sl No.	Parameter	Results
1	R_F	0.32
2	Dynamic range (ng spot^{-1})	100-600
3	Equation	Y=927.95+12.55x
4	Slope	12.55
5	Intercept	927.95
6	Limit of Detection	1.312ng
7	Limit of Quantification	4.37 ng
8	Linearity (Correlation coefficient)	0.995
9	Specificity	Specific

b) Precision studies data

Concentration (ng spot^{-1})	Instrumental Precision (% RSD)	Method Precision (% RSD)	
		Intra day	Inter day
200	0.057	0.76	0.75
600	0.044	0.22	0.21

c) Recovery studies of L dopa

Sl No.	Amount of L dopa present in the sample(µg)	Amount of L dopa added (µg)	Amount of L dopa found (µg)	Recovery (%)
1	95.0	76.0	168.5	
2	95.0	95.0	186.9	98.36-99
3	95.0	114.0	206.9	

HPTLC Separation optimization

Different compositions of the mobile phase were tested and the desired resolution of L-dopa with symmetrical and reproducible peaks was achieved by using mobile phase of Butanone: DCM: Isopropanol: Acetic acid: Water (6:4:4:4:3.6 v/v) with 20 min of chamber saturation with the mobile phase and 18 min of development. A peak corresponding to L-dopa was seen at R_F 0.32. The methanolic extract of the roots of Mucuna when subjected to HPTLC as per the methodology described above, showed the presence of L-dopa peaks. A comparison of the spectral characteristics of the peaks for standard L-dopa and that of the sample revealed the identity of L-dopa 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 L-dopa 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 L-dopa 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 L-dopa is Y=925.95+12.55x and the correlation coefficient of calibration plot was 0.995 indicating good linearity.

Results of regression analysis on calibration curve and detection limits are presented in table 5.18 a.

Precision studies

Instrumental precision was checked by repeated scanning of the same spots (200 & 600ng spot $^{-1}$) of standard L-dopa five times and the RS.D values were 0.057 and 0.044 for 200 and 600 ng spot $^{-1}$ respectively. To determine the precision of the developed assay method 200 and 600ng spot $^{-1}$ of L-dopa standard was analyzed five times within the same day to determine the intra-day variability. The RSD values were 0.76 and 0.22 for 200 and 600 ng spot $^{-1}$ respectively. Similarly the inter-day precision was tested on the same concentration levels on two days and the R.S.D values were 0.75 and 0.21 respectively. (Table 5.18b)

Sample analysis and recovery studies

This developed HPTLC method was subsequently applied for the analysis of L-dopa in methanolic extract of *Mucuna pruriens* roots (MPRME) and the free L dopa content of the roots by this proposed method was found to be 0.171 %.

For the examination of recovery rates, 80, 100 and 120 % of pure L dopa were added in pre analyzed sample and quantitative analysis was performed. The recoveries were between 98.36-99 %. (Table 5.18 c)

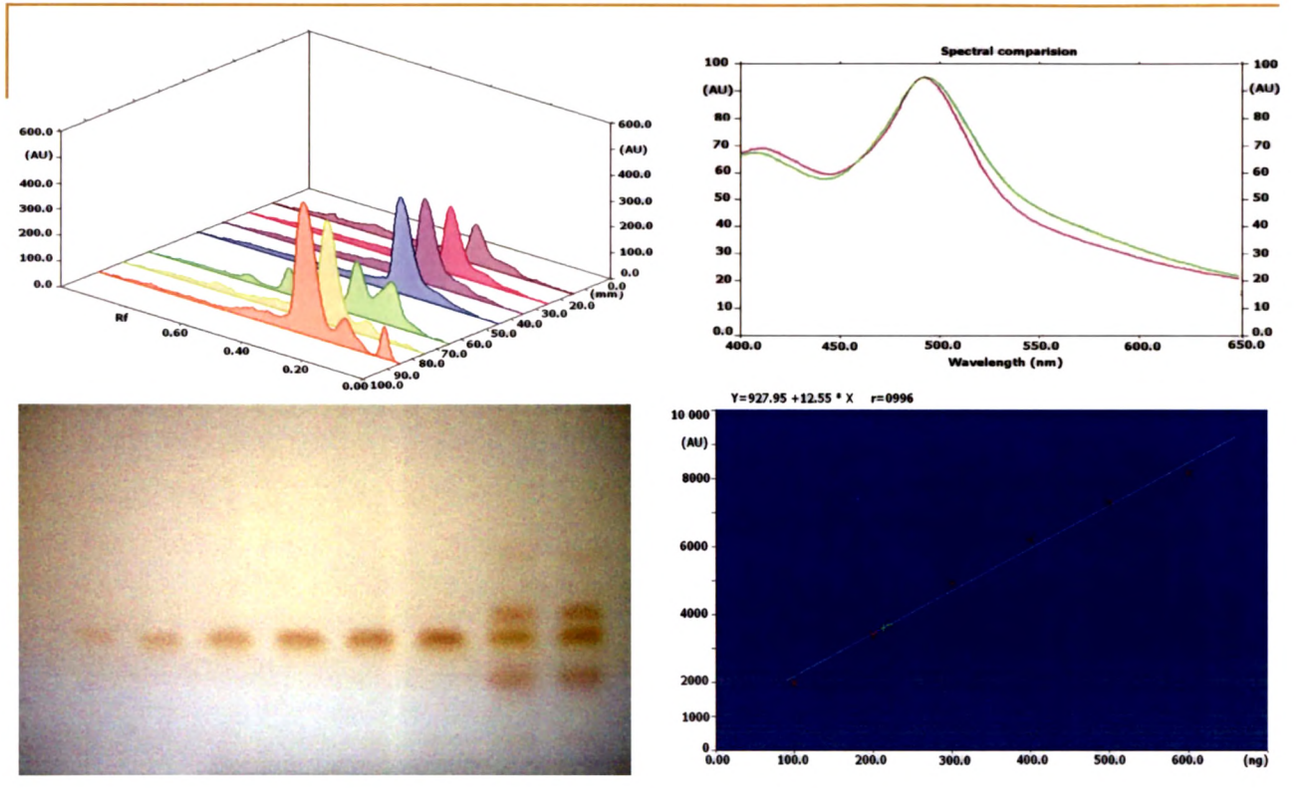


Fig.5.16. UV visible spectrum of standard L-dopa and L-dopa in sample. 3D Overlay Chromatogram of Standard and sample track.

Mucuna pruriens is reported to contain many diverse phytochemicals like alkaloids, L-dopa, alkylamines, phenolics, saponins, proteins, minerals, fatty acids, sterols , serotonin, carbohydrates, fiber, etc. Vadivel et al have reported amino acids like Alanine, Arginine, Aspartic-acid, Cystine, , Glutamic-acid, Glycine, Histidine, L-DOPA, Leucine, isoleucine, Lysine, Methionine, Proline, Phenyalanine Threonine, Tryptamine, Tyrosine, Valine. Several *in vivo* studies have been reported the blood-sugar-lowering effect of Velvet bean(Grover JK et al., 2001). Pant ML et al., (1968) have reported hypo-cholesterolaemic activity of seeds, Iauk et al., (1993) have reported analgesic, antipyretic, and anti-inflammatory effects of alcoholic extracts of the leaves and fruits of *Mucuna pruriens.* Anti-tumor activity of methanolic extract of seeds on tumor growth and host survival time in Ehrlich ascitic carcinoma bearing mice is reported by Y Rajeswar et al., The methanol extract of *Mucuna pruriens* seeds has proved to possess a strong antioxidant activity by inhibiting DPPH & hydroxyl radicals, Nitric oxide and Superoxide scavenging and reducing power activities when compared with Curcumin, Quercetin, alpha –Tocopherol and L-Ascorbic acid. (Yerra Rajeshwar 2005).

Seeds are reported to contain phytosterols like Beta-sitosterol, campesterol and stigmasterol etc. Pet ether extract of roots of *Mucuna pruriens* showed higher content of sterols while the methanol and aqueous extracts revealed the presence of phenolics, amino acids etc.

Phytosterols are synthesized from triterpenes and are ubiquitous among angiosperm species. The role of phytosterols in adaptogenic activity has not been emphasized in the phytotherapy literature, their importance to nutrition is well recognized. Beneficial effects of phytosterols to both normal and compromised immune systems is been established. (Bouic, 2002).

Phytosterols often occur free or as glycosides or esters with fatty acids. (Chappell, 2002). Sterols are always present in plants as a mixture. Beta-sitosterol, along with campesterol and stigmasterol, are the most common sterols found in plants (Lindsey et al., 2003). They serve primarily as structural components of cell and organelle membranes, regulating the fluidity and permeability of these membranes. Dietary plant sterols are extremely bioactive in humans. They are well known for their ability to inhibit absorption of cholesterol and lowering of serum cholesterol by two main processes, preferential uptake in the gut for plant sterols versus cholesterol, and improving elimination of cholesterol. It has been reported that beta-sitosterol has anticancer, antiulcer, antidiabetic, antiinflammatory antipyretic anthelmintic, antipyretic and analgesic. Properties. (Gupta et al., 1996; Bouic, 2002).

It has been suggested that beta-sitosterol can enhance secretion of IL-2 and gamma interferon helping to promote natural killer cells, and prime TH1 helper cells to steer the focus away from the TH2 helper cells (Bouic, 2001). The lipophilicity of Beta-sitosterol may be a potent inhibitor of cholesterol synthesis and has been shown to cause apoptosis in cancer cells. That is, beta-sitosterol seems to decrease the total cholesterol content in cancer cells and this causes the cell to stop dividing and die.

Phytosterol compounds found in plants like *Astragalus membranaceus* (Fabaceae), *Bryonia alba* (Cucurbitaceae), *Codonopsis pilosula* (Campanulaceae), *Eleutherococcus senticosus* (Araliaceae), *Lepidium meyenii* (Brassicaceae), *Ocimum sanctum* (Lamiaceae) and *Rhodiola rosea* (Crassulaceae). are thought to play an important role in their adaptogenic properties. (Pannosian 2003)

Mucuna pruriens is reported to have L-dopa as a major constituent in the seeds as well as roots, L-DOPA is a levorotatory isomer of dihydroxyphenylalanine, a natural amino acid, is the immediate precursor of the neurotransmitter dopamine. Dopamine modulates the immune responses by influencing the cytokine network. Dopamine is an important endogenous catecholamine which exerts widespread effects both in neuronal (as a neurotransmitter) and non-neuronal tissues (as an autocrine or paracrine agent). Within the central nervous system, dopamine binds to specific membrane receptors presented by neurons and it plays the key role in the control of locomotion, learning, working memory, cognition, and emotion. Dopamine also regulates motor control, sex drive, immune function, growth hormone levels, Somatropin release, and motivational behavior.(Grietje ch beck 2004)

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

Panax ginseng, have been shown to contain large amounts of arginine (Vanita Gupta., 2004) Both these amino acids are also been reported in *Mucuna pruriens*

The roots of *Mucuna pruriens* certainly possess adaptogenic activity as evidenced by stimulatory effects on humoral immunity (HA titre), cellular immunity (DTH response) and phagocytosis (Carbon clearance and E coli induced abdominal sepsis), and also provides protection against Cyclophosphamide induced myelosuppression and by prevention of HPAA activation and can thus preventing stress-induced elevation in biochemical markers in anti-stress activity. Thus, adaptogenic activity is not only by altering various biochemical markers during stress, but also by stimulating the immune system. The activity may be endowed upon the presence of β-sitosterol and other phytosterols, L-dopa and other amino acids..