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Original Research Article

Velvet bean roots stimulates humoral and cell mediated immunity and offers protection against Cyclophosphamide induced myelosuppression.

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Abstract

The objective of the study was to investigate the immunomodulatory activity of roots of *Mucuna pruriens* on cellular and humoral immunity. Extraction and phytochemical screening of the roots were performed using standard methods, HPTLC fingerprinting profiles of the methanol extract were developed on CAMAG system, to resolve components, Quantification of β-Sitosterol was done by validated HPTLC method. Immunomodulatory activity of the extract was assessed by Cyclophosphamide-induced myelosuppression assay, Macrophage phagocytosis by Carbon clearance method, Humoral antibody response and Delayed type hypersensitivity reaction using SRBC as an antigen. Oral administration of methanolic extract of Mucuna roots at 100, 200 and 400 mg/kg in mice dose dependently potentiated the delayed-type hypersensitivity reaction induced by sheep red blood cells. It significantly enhanced the circulating antibody titre in response to SRBC and macrophage phagocytosis. Cyclophosphamide induced myelosuppression was counteracted in a dose dependent manner with increase in the levels of WBC compared to cyclophosphamide group. The results of the present study suggests that the developed HPTLC fingerprint profiles of the major constituents in methanolic extract along with their Rf values would serve as a reference standard, methods developed for the quantification of L-dopa and β-Sitosterol can be used to ensure the identity and quality of the plant and the results of the biological studies indicates that the roots of *Mucuna pruriens* influenced both humoral and cell mediated immunity and offered protection against immunosuppression induced by cytotoxic agent Cyclophosphamide holds promise as an immunomodulator.

Keywords: *Mucuna pruriens*, Myelosuppression, Immunomodulatory, Velvet bean, cell mediated immunity

Introduction

Natural products, including plants, animals and minerals have been the basis of treatment of human diseases. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists. However, the basis of its development remains rooted in traditional medicine and therapies [1]. Modulation of immune response by using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations. Immunomodulation could provide an alternative to conventional chemotherapy under the conditions of impaired immune responsiveness. Medicinal plants are a rich source of substances that are claimed to induce paraimmunity, the non specific immunomodulation of granulocytes, macrophages, natural killer cells and complement functions [2].

Mucuna pruriens Linn, (Leguminoseae) one of the popular and important medicinal plants of India is a constituent of many indigenous drug formulations. The plant is commonly called as Cowitch, Velvet bean and Cowhage and it is indigenous to tropical countries. All parts of *Mucuna pruriens* possess valuable medicinal properties [3]. *Mucuna pruriens* finds traditional use in number of diseases. Seeds have a long history of use in Indian Ayurvedic medicine, where it is used for worms, dysentery, diarrhea, snakebite, sexual debility, cough, tuberculosis, impotence, rheumatic disorders, muscular pain, gonorrhea, sterility, gout, delirium, dysmenorrhea, diabetes, and cancer [4,5]. The plant is reported to have L-Dopa as a major constituent mainly in seeds [6]. *Mucuna pruriens* contains many diverse phytochemicals like L-DOPA, Amino acids, 1-methyl-3-carboxy-6, 7-dihydroxy-1,2,3,4

tetrahydroisoquinolone,5-hydroxy tryptamine, 5-methoxy-n,n-dimethyltryptamine-noxide, 5-oxyindole-3-alkylamine, 6-methoxyharman, arachidic acid, behenic acid, β -carboline, β -sitosterol, bufotenine, choline, cystine, leucine, linoleic acid, myristic acid, n,ndimethyltryptamine, n,n-dimethyltryptamine-n-oxide, nicotine, oleic acid, palmitic acid, palmitoleic acid, phenylalanine, proline, protein, saponins, serine, stearic acid, threonine, tryptamine, tyrodine, valine and vernolic acid Lecithin. Alkaloidal constituents viz., mucunadine, mucunine, prurienidine, prurienine. Four 1,2,3,4 tetra hydroisoquinoline alkaloids from the seed [7, 8]. The seeds of *Mucuna pruriens* have been reported to possess anti-parkinson, androgenic, antidiabetic, hypotensive & hypo-cholesterolaemic, antivenin, myotoxic, cytotoxic and anticoagulant, analgesic, antipyretic, anti-inflammatory [9] anti-tumor and antioxidant activities [10, 11] Anticancer activity of Mucuna seeds on hepatoma cells [12]. Seeds of *Mucuna pruriens* have been investigated exhaustively on phytochemical, pharmacological and clinical aspects. But according to the available literature, all parts of *Mucuna pruriens* possess valuable medicinal properties. Root is bitter, thermogenic, emolient, stimulant, purgative, aphrodisiac, diuretic, emmenagogue, anthelmintic, febrifuge and tonic. The Ayurvedic usage of roots still extends for constipation, nephropathy, strangury, dysmenorrhoea, amenorrhoea, elephantiasis, dropsy, neuropathy, ulcers, fever and general debility [4 & 5].

Very little or no information regarding the scientific studies on roots of *Mucuna pruriens* is available.

The present studies were aimed at exploring the influence of roots of *Mucuna pruriens* on humoral and cell mediated immune responses and phagocytic functions of the cells of reticulo-endothelial system.

Materials & Methods

Plant material

The roots of *Mucuna pruriens*, were collected from the outfield of Vadodara city, Gujarat, India and were authenticated from Botanical Survey of India, Southern Circle, Coimbatore. A voucher specimen (No.Pharmacy/MP/05-06/05/KM) has been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

Reagents and chemicals

L-dopa was procured from M/s Hi media, β-Sitosterol from M/s Acros Organics (New Jersey USA), HPTLC plates silica gel 60F254 (20cm x 20cm) from E Merck (Darmstadt. Germany), other solvents and chemicals used were of analytical grade. Carbon ink suspension: Pelican AG, Germany, ink was diluted eight times with saline and used for carbon clearance test in a dose of 10µl/gm body weight of mice. Cyclophosphamide (Endoxan, Cadila Healthcare Ltd, Goa) was used as a standard immunosuppressant.

Preparation of 20 v/v SRBC suspension

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

Animal strain used

Swiss albino mice of either sex, weighing 17–25 g housed in standard conditions of temperature, humidity and light were used. They were fed with standard rodent diet and water ad libitium.

Preparation of Extract

The root powder was exhaustively extracted using methanol (1000ml) in a soxhlet extractor. Concentration under reduced pressure and low temperature afforded 3.7 % (w/ w) of methanolic extract (MPR) which was suspended in 1% Sodium Carboxy Methylcellulose and used for experimental studies.

Phytochemical analysis of Methanol extract of roots of *Mucuna pruriens* (MPR)

The extract was subjected to chemical tests to detect the presence of various phytoconstituents[13] . For HPTLC fingerprinting of the extract; three solvent systems of different polarity were developed to resolve polar, medium polar and non polar components on a precoated silica gel TLC plate (Merck). Solvent system 1 (Toluene: Ethyl acetate: Methanol: Water (10:5:2.5:1) was used to resolve the non polar compounds and solvent system 2 (Toluene: Formic acid: Ethyl formate (5:1:4) was used to resolve medium polar compounds. The characteristic finger print profile for various components in the extract under UV light (UV-254, UV-366) and after derivatization with suitable reagents was recorded on a CAMAG-HPTLC system. In brief suitably diluted stock solution was spotted on precoated silica gel G60 F254 TLC plates with the help of CAMAG Linomat V applicator and the plates were developed in solvent systems. The developed plates were scanned using TLC Scanner 3 (CAMAG). The non polar components (steroids and terpenoids) in the extract were resolved and the plate was derivatized using Anisaldehyde Sulphuric acid reagent characteristic peaks of the detected compounds were recorded at

540 nm. Similarly the polar components (Phenolic compounds) in the extract were separated using another solvent system and the plate was derivatized using Ferric Chloride reagent characteristic peaks of the detected compounds were recorded at 540 nm.
Seeds of *Mucuna pruriens* is reported to contain L-dopa, etc therefore, co-TLC studies were performed with L dopa, β-Sitosterol and other available markers in our laboratory, identical spot as that of standard L dopa and β-Sitosterol were observed, further confirmation was done by RF comparison, multi wavelength scanning, and spectral overlay.

Quantification of L-dopa in Methanol extract of roots of *Mucuna pruriens* (MPR)

L dopa content of the extract was determined by CAMAG HPTLC system.

Preparation of standard and test solution:

A stock solution of MPR (1mg/ml) and L-dopa (100µg/ml) were prepared separately by dissolving 10 mg of MPR and 1mg of L-dopa in methanol and the volume was made up 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 N2 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(250 ±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 1100 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 MPR 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 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 L-dopa in MPR

10 µL of MPR 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.

Quantification of β-Sitosterol in Methanol extract of roots of *Mucuna pruriens* (MPR)

β-Sitosterol content of MPR was determined by CAMAG HPTLC system [14]. In brief, 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 The linear ascending development was carried out in a CAMAG twin trough chamber (20cm x 10cm) 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 and sulphuric acid followed by heating at 110^0 C for 3 min. 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 for preparing six point calibration graph of peak area vs. concentration. Quantification of β-Sitosterol in MPR was performed by external standard method, using pure β-Sitosterol as standard.

Evaluation of Immunomodulatory activity of Methanol extract of roots of *Mucuna pruriens* (MPR)

Acute toxicity studies

Acute toxicity studies were performed with Swiss albino mice as per OECD guidelines.

Cyclophosphamide-induced myelosuppression assay

Animals were divided into 5 groups of 6 animals each. Animals in the treated group were administered MPR (100, 200 and 400 mg/kg, p.o.) in 1.0 % Sodium Carboxy Methyl cellulose daily for 13 days. Positive control and negative control group animals received 1.0% SCMC in water (0.3 ml/mouse, orally, daily for 13 days). On days 11, 12 and 13, all the animals except in the negative control group were given cyclophosphamide (30mg/kg, i.p.) 1 h after the administration of extract. Blood samples were collected on day 1 and then on days 10 and 14, Total white blood cell (WBC) was determined using Erma PC-607 cell counter (Erma Inc, Japan).[15]

Macrophage phagocytosis by Carbon clearance Method

Mice were divided into five groups, containing 6 animals in each group.

Group I (control) was given 1.0% SCMC in water (0.3 ml/mouse) for 5 days. Group II was treated MPR (100,200 & 400 mg/kg) orally for 5 days. At the end of five days, after 48 h, mice were injected via the tail vein with carbon ink suspension (10 µl/gm body wt.) (Pelican AG, Germany).

Blood samples were drawn) from the retro-orbital vein at 0 and 15 min, 25-µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and its absorbance was measured at 660 nm.

The phagocytic index K was calculated using the following equation:

$$K = (\text{Log e OD1} - \text{Log e OD2})/15,$$

where OD1 and OD2 are the optical densities at 0 and 15 min, respectively. Results are expressed as the arithmetic mean ± S.E.M. of five mice.(16)

Humoral antibody response and Delayed type hypersensitivity reaction using SRBC as an antigen.

Method described by Mahesh et al. was adopted. Animals were divided into 4 groups of six animals each. Animals in treatment groups were given MPR (100, 200 & 400 mg/kg p.o.) in 1.0 % Sodium Carboxy Methyl Cellulose daily for 7 days. The animals were immunized by injecting 0.1 ml of 20% of fresh sheep red blood cells suspension intraperitonially on 0 day. Blood samples were collected in micro centrifuge tubes from individual animal by retro-orbital plexus on 7th day. Blood samples were centrifuged to obtain serum. Antibody levels were determined by Haemagglutination technique.

Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 µl volumes of normal saline in microtitration plate and to it added 25 µl of 1% suspension of sheep red blood cells in saline. After mixing, the plates were incubated at room temperature for 1 hr. and examined for haemagglutination under microscope.

The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

On day 7, the thickness of the right hind foot pad was measured using digital vernier calipers. The mice were then challenged by injection of 20µl of 1% SRBCs in right hind foot pad. The contra lateral paw received equal volume of saline. Foot thickness was again measured 24 and 48 hrs after this challenge. The difference between the pre and post challenge foot thickness express in mm was taken as a measure of Delayed type hypersensitivity DTH reaction.

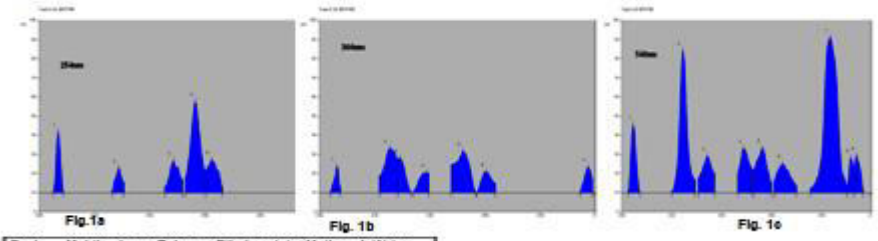
Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was carried out employing ANOVA.

Results and Discusssion

Soxhlet extraction of roots of *Mucuna pruriens* afforded methanol extract (3.7 % w/w).The preliminary phytochemical analysis and HPTLC finger printing revealed the presence of steroids and terpenoids, phenolics, amino acids and L dopa and absence of alkaloids, saponins gums and mucilage etc.

HPTLC fingerprinting of Methanol extract of roots of *Mucuna pruriens* for non-polar constituents.



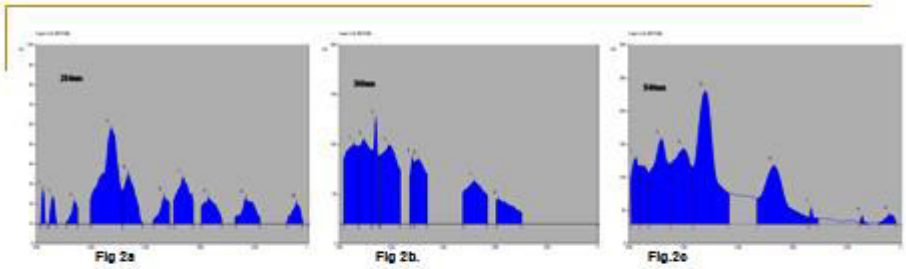
Peak	Mobile phase: Toluene :Ethyl acetate: Methanol: Water (10:5:2.5:1)					
	254nm		366nm		540nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.07	12.98	0.06	5.64	0.05	6.19
2	0.29	8.06	0.26	25.71	0.25	19.37
3	0.49	15.77	0.29	12.10	0.34	6.71
4	0.57	45.53	0.39	8.92	0.49	7.85
5	0.63	17.66	0.52	28.57	0.57	8.77
6			0.61	10.63	0.65	6.93
7			0.98	8.44	0.84	37.63
8					0.92	2.89
9					0.94	3.66

Fig. 1a TLC chromatogram after densitometric scan under UV-254nm;
Fig. 1b. TLC chromatogram after densitometric scan under UV-366nm;
Fig. 1c TLC chromatogram after densitometric scan at 540 nm.

Fig.1 TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM

Figure. 1 HPTLC fingerprint profile of MPR in Solvent system 1 (Toluene: EA: Methanol :Water (10:5:2.5:1).
Fig. 1a TLC chromatogram after densitometric scan under UV-254nm;
Fig. 1b. TLC chromatogram after densitometric scan under UV-366nm;
Fig. 1c TLC chromatogram after densitometric scan at 540 nm.

HPTLC fingerprinting of Methanol extract of roots of *Mucuna pruriens* for polar constituents.



Peak	Mobile phase: Toluene: Formic acid: Ethyl formate (5:1:4)					
	254nm		366nm		366nm	
	Max R_f	Relative %	Max R_f	Relative %	Max R_f	Relative %
1	0.03	1.86	0.06	17.50	0.02	6.32
2	0.06	2.44	0.09	15.21	0.05	6.56
3	0.14	2.98	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. 2a TLC chromatogram after densitometric scan under UV-254nm;
Fig. 2b. TLC chromatogram after densitometric scan under UV-366nm;
Fig. 2c TLC chromatogram after densitometric scan at 540 nm.

Fig.2 TLC CHROMATOGRAM OF METHANOL EXTRACT OF ROOTS OF *Mucuna pruriens* AFTER DENSITOMETRIC SCAN AT 254,366 AND 540NM

Figure. 2 HPTLC fingerprint profile of MPR in Solvent system 2 (Toluene: Formic acid: Ethyl formate (5:1:4).
Fig. 2a TLC chromatogram after densitometric scan under UV-254nm;
Fig. 2b. TLC chromatogram after densitometric scan under UV-366nm;
Fig. 2c TLC chromatogram after densitometric scan at 540 nm.

Quantification of L-dopa

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. MPR 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 3 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 1a.

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 1b)

β-Sitosterol content of MPR method was found to be 0.076 %.

Table 1. Method validation parameters for quantification of L dopa using proposed HPTLC Densitometric method.

Table 1.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

Table 1b: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

Table 1c. 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	

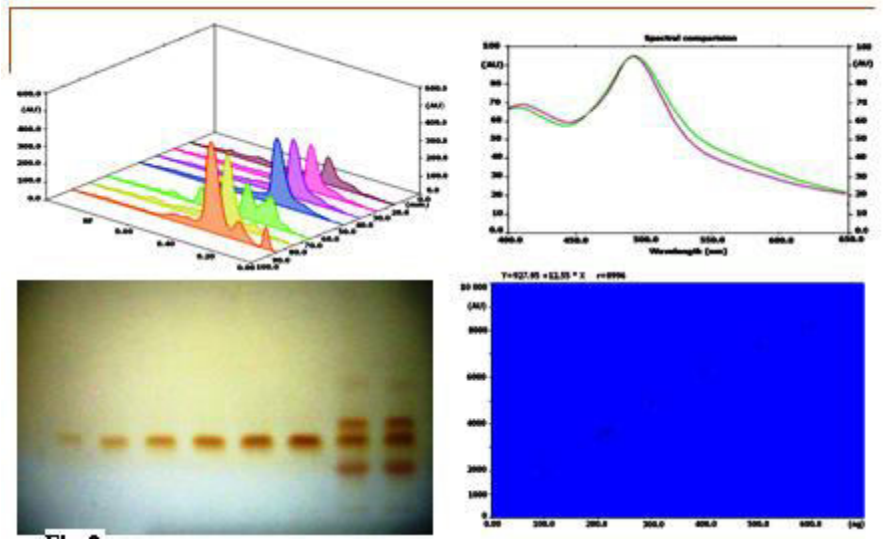


Figure 3a & b. UV visible spectrum of standard L-dopa and L-dopa in sample. 3D Overlay Chromatogram of Standard and sample track.

Quantification of β-Sitosterol in Methanol extract of roots of *Mucuna pruriens* (MPR)

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 (MPR) 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 1c)

Quantification of β-Sitosterol in Methanol extract of roots of *Mucuna pruriens* (MPR)

β-Sitosterol content of MPR method was found to be 0.076 %.

Evaluation of Immunomodulatory activity of Methanol extract of roots of *Mucuna pruriens* (MPR)

Acute toxicity studies

From acute toxicity studies MPR was found safe up to of 2000mg/kg dose level therefore MPR was screened for immune function activity at 100, 200 and 400mg/kg dose levels.

Cyclophosphamide-induced myelosuppression assay

In Cyclophosphamide induced myelosuppression assay, mice when pretreatment with MPR 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/mm3) prior Cyclophosphamide administration and further treatment with extract till 14th day followed by administration of Cyclophosphamide on 11th, 12th and 13th 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/mm3) respectively.

Table 2. Effect of Methanol extract of roots of *Mucuna pruriens* on Cyclophosphamide induced myelosuppressoion

Treatment	TOTAL LEUCOCYTE COUNT(mm^3)		
	Day 1	Day 10	Day 14
Negative control	7.66±1.03	8.16±1.03	8.25±1.015
Positive control (CPM)	8.46±1.058	8.53±1.052	1.05±0.133
MPR 100	7.93±0.889	11.88±1.62*^{a}	3.68±0.212*^{b}
MPR 200	8.05±1.094	13.85±1.41*^{a}	6.366±0.761***^{b}
MPR 400	7.35±0.658	12.46±1.52*^{a}	4.01±0.336*^{b}

Values are Mean± SEM, n= 6 mice per group, *p <0.05, ***p< 0.001. 'a' vs Negative control; 'b' vs Positive control.

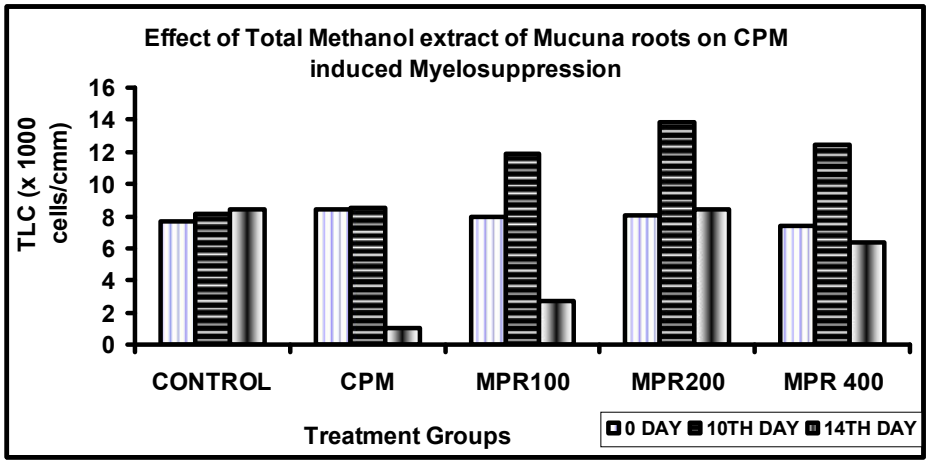


Figure 4: Effect of Total methanol extract of Mucuna roots CPM induced Myelosuppression

Humoral antibody response and Delayed type hypersensitivity reaction

Humoral response to SRBCs was checked by haemagglutination antibody (HA) titre. The HA titre value in control group was found to be 80 ±16, administration of MPR at 100, 200 and 400 mg/kg produced statistically significant an increase in Humoral antibody titre as evident by haemagglutination at that dilution. A dose dependant increase was established only at 100 & 200mg/kg dose levels (277.33± 51.37, 426.66 ± 53.97). (Table 3)

DTH response was assessed by measuring food pad thickness using digital vernier calipers. DTH response of Normal control group was 0.545±0.033 while that of MPR 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. (Table 3)

Table 3: 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*	362.66±69.45**	0.903±0.041**

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

Macrophage phagocytosis by Carbon Clearance Method

The phagocytic activity is generally measured by the rate of removal of carbon particles in the blood stream. MPR 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). (Table 3)

The results of immune function activity 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 also protection against Cyclophosphamide induced myelosuppression.

Discussion

Extraction of roots afforded reddish brown colored methanol extract (3.7 % w/w). The preliminary phytochemical analysis and HPTLC finger printng profiles to resolve polar, medium polar and non polar components revealed the presence of steroids and terpenoids, phenolics, amino acids and L-dopa this would serve as a reference standard. β-Sitosterol and L-dopa content of the extract was found to be 0.076 % and 0.171 % respectively. HPTLC methods developed for Quantification of L-dopa and β-Sitosterol are simple, precise, specific, sensitive and accurate and can be employed to ensure the identity and quality of the plant.

Immumomodulatory activity of methanol extract of roots of *Mucuna pruriens* was explored by evaluating its effect on Cyclophosphamide induced myelosuppression, Phagocytic function in mice, antibody titre and DTH response.

Cyclophosphamide is a potent immunosuppressive agent, capable of inhibiting immune response. Bone marrow is the organ most affected during any immunosuppressant 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 [18]. Administration of MPR increased the total WBC count which was lowered by Cyclophosphamide. Thus it can be established from the study that MPR possess the ability to counteract the myelosuppressive effects of cytotoxic drug, Cyclophosphamide by stimulating the bone marrow activity.

Phagocytosis and killing of invading microorganisms by macrophages constitute body's primary line of defense against infections [19]. Macrophages 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 [20]. Increased phagocytic activity is indicative of increased functioning of reticulo-endothelial system. MPR was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance compared to control group.

Foot pad thickness test or DTH response was measured to assess the cell-mediated immune response. 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 [21]. Increase in DTH response as evidenced by increased paw thickness in mice revealed stimulatory effect of MPR on T cells and accessory cell types required for the expression of reaction.

Haemagglutination antibody titre was determined to establish the humoral response. The antibody production to T dependent antigen SRBC requires the co-operation of T and B lymphocytes and macrophages which interacts with SRBC and proliferates into antibody secreting plasma cells. MPR was found effective in increasing HA titre which indicates the enhanced responsiveness

of macrophages and T and B lymphocytes involved in the antibody production [22].

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.[23]. β-sitosterol has an amazing array of scientifically acknowledged benefits for key areas of health in immune dysfunctions, inflammatory disorders and rheumatoid arthritis benign prostatic hypertrophy,[24], colon cancer [25], breast cancer [26].

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 [27]. 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. Extremely little doses of β-sitosterol have been reported to elevate the proliferative activity of T-lymphocytes, increasing the activity of NK-cells [28].

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 [29].

Thus, the developed HPTLC fingerprint profiles of the major constituents in MPR along with their Rf values would serve as a reference standard, methods developed for the quantification of L-dopa and β-Sitosterol can be used to ensure the identity and quality of the plant and the results of the biological studies indicates that the roots of *Mucuna pruriens* holds promise as an immunomodulator and the activity may be endowed upon the presence of L dopa, β-Sitosterol and other constituents and provides a scope for further detailed investigation on the fraction/individual constituents.

Conclusion

Roots of *Mucuna pruriens* influenced both humoral and cell mediated immunity and offered protection against immunosuppression induced by cytotoxic agent Cyclophosphamide holds promise as an immunomodulator.

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Authors Contribution

Krishna Murthy is the research fellow who performed the experiments and wrote the manuscript Dr.S.H Mishra is the research supervisor who gave his advices and inputs by editing the manuscript.

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