Annual Report

Minor Research Project

From

22.02.2012 to 22.02.2014

Standardisation of *Putranjiva roxburghii* Wall. and *Dioscorea bulbiferra* Linn. and evaluation of its immunomodulatory activity.

Principal Investigator

Madhavi Badole
Assistant Professor
Dept. of Chemistry
Ramnarain Ruia College,
Matunga, Mumbai.

General profile of plant

Putranjiva roxburghii Wall. is a moderate sized evergreen tree. The height is upto 12m with a pendent branches and dark grey bark having horizontal lenticels. Leaves are simple, alternate, dark green, shiny, elliptic-oblong. *Putranjiva roxburghii* Wall. is found wild or cultivated in almost all parts of India, widely grown in Thailand, Nepal, Bangladesh, Indochina, Myanmar and Srilanka.

Uses and pharmacology of plant

Leaves of *Putranjiva roxburghii* Wall. are bitter, astringent, refrigerant and procreant. The leaves are useful in the treatment of catarrh, skin disease, fever and sterility. The leaves are given in decoction for cold and fever, and are also used in rheumatism. The leaves of *Putranjiva roxburghii* Wall. posses analgesic, antipyretic and anti-inflammatory activity. *Putranjiva roxburghii* Wall. is one of the constituents of "Y-Spur" an ayurvedic formulation, prepared by Vilco Laboratories, which is found to be very effective in male infertility. Seeds are sweet, acrid, procreant, refrigent, conceptive, ophthalmic, laxative, anti-inflammatory and diuretic. It is useful in vatta and pita, syphilis, cold, fever, burning sensation, hyperdipsia, opthalmopathy, constipation, inflammations strangury, azospermia, sterility, swelling, rheumatism, and habitual abortion.

Authentication

A herbarium of *Putranjiva roxburghii* Wall. was prepared and authenticated from botanical Survey of India, Pune.

Dioscorea bulbifera Linn. is common throughout India, ascending upto 1800 m. It is found mainly in Himalayas, Chota Nagpur, Bihar, Orissa, Konkan and found wild on W.coast.

Dioscorea bulbifera Linn. is a perennial, slender, bulb bearing twinner with a tuberous root. Leaves are broadly ovate, cordate, alternate and simple. It propagates by seeds, bulbils and tubers.

Uses and pharmacology of plant

The bulbils of *Dioscorea bulbifera* Linn. are anorexiant, Diuretic, hunger suppressant. It is anthelmintic, amphrodiasic, diuretic, antiseptic, it is also used in the treatment of thyroid and cancer, bitter tubers are used in treatment of leprosy and tumers. It has diuretic and anti-

inflammatory activity. It is used for sore throat and struma. Bulbs are used to treat piles, dysentery, syphilis, ulcer.

A herbarium of *Dioscorea bulbifera* Linn. was prepared and authenticated from Balasaheb Sawant Kokan Vidyapeeth, Dapoli.

Need for selection of Putranjiva *roxburghii* Wall and *Dioscorea bulbifera* Linn.

Ayurvedic system of medicine describes a concept called “rasayana plants”, which are used in delaying the ageing process, improvement of mental health and removal of diseased condition. It is found that these rasayana plants have immunomodulatory activity.

Ayurvedic concept of preventive health is attributed to immunostimulant activity of rasayanas. Immuonosupressors are widely used in transplantation surgery. As these plants modulate the immune response through stimulation or suppression, they are widely used in treatment of diseases like cancer or in grafting.

An immunomodulator is a substance that helps to regulate the immune system and maintain a disease free state. In many diseases, the immune system is impaired and these drugs modulate the immune response, by either its stimulation or suppression.

It has been found that the plants described in Ayurveda as “rasayana” plants have immunomodulatory activity.

Immunomodulation can thus be used as an alternative to conventional therapy for variety of diseased conditions like Acquired Immuno Deficiency Syndrome (AIDS), when host defense mechanism has to be activated in under the conditions of impaired immuno response when selective immuno suppression has to be induced in disease like auto immune disorder and organ transplantation. Due to importance of immunomodulators in treatment of diseases like Acquired Immuno Deficiency Syndrome (AIDS), Cancer etc. A rasayana plant which may be a potential immunomodulator was selected for the present research work.

Putranjiva roxburghii Wall. and *Dioscorea bulbifera* Linn. is described in Charak Samhita as a “rasayana” plant and is thus expected to show immunomodulatory activity.

Method in brief

HPTLC method for simultaneous determination of β-amyrin and stigmasterol

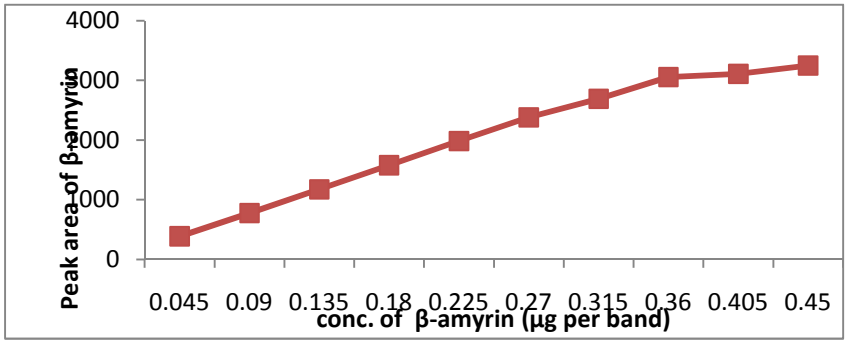
HPTLC method for simultaneous determination of β-amyrin and stigmasterol in *Putranjiva roxburghii* wall. has been developed and validated. The analytes were separated on silica gel $60F_{254}$ HPTLC plates with n - hexane: chloroform: methanol (3: 6.5:0.5 v/v/v) as mobile phase after chamber saturation for 10 min. The development distance was 80 mm. The derivatization was done by using anisaldehyde – sulphuric acid reagent. Detection and quantification were performed by densitometry, with a tungsten lamp, at 580 nm. The response of β-amyrin and stigmasterol was linear in the concentration range 0.045 to 0.360 µg per band and 0.041 to 0.328 µg per band respectively. The validated method was used for quantitative analysis of β-amyrin and Stigmasterol in *Putranjiva roxburghii* wall. and can be used for routine quality-control analysis of leaf powder of *Putranjiva roxburghii* wall.

Optimised chromatographic conditions used for quantification of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Parameters	Description
Stationary phase	Aluminium backed HPTLC Silica gel 60 F_{254} (Cat No. 1.0.5554) (E. Merck, Germany), 200µm thickness.
Mobile phase	n-hexane: chloroform: methanol (3: 6.5:0.5 *v/v/v*).
Sample applicator	CAMAG Automatic TLC Sampler 4(ATS4), equipped with 25µL –Hamilton syringe.
Speed of application	Methanol-150nL/second.
Band length	8 mm.
Distance from edges of plate(X-position)	15 mm.
Distance from bottom of plate(Y-position)	8 mm.
Development chamber	CAMAG glass twin trough chamber (20 x 10 cm and 10 x 10 cm).
Chamber saturation	10 minutes with filter paper.
Development distance	60 mm from lower edge of plate.
Densitometer Scanner	CAMAG TLC Scanner 3 with win CATS software, version 1.4.4.
Derivatizing reagent	Anisaldehyde sulphuric acid.
Wavelength of detection(λ)	580 nm.

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Graph of Linear dynamic range of β-amyrin

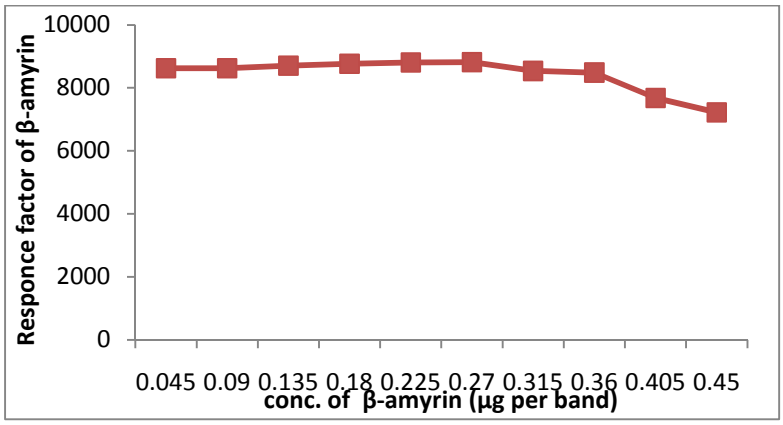


The graph shows that the response of β-amyrin is linear in the concentration range of 0.045µg per band to 0.360 µg per band.

A graph of response factor of β-amyrin (Y-axis) against the corresponding concentration of β-amyrin (X-axis) was plotted and is shown in Figure.

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

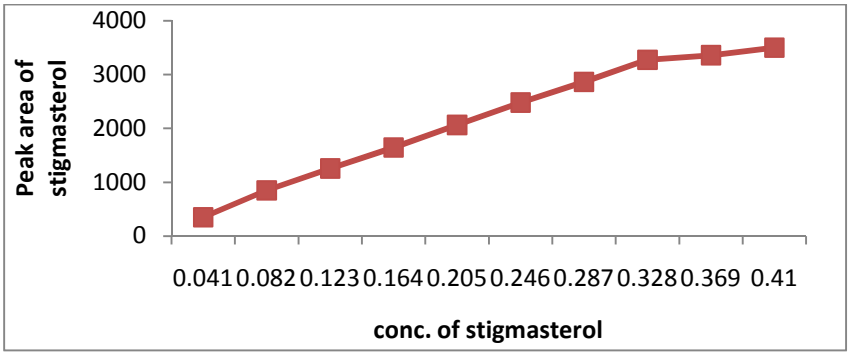
Graph of response factor of β-amyrin against corresponding applied concentration of β-amyrin



From the above graph, it is observed that the response factor of β-amyrin was found to be constant in the concentration range of 0.045µg per band to 0.360 µg per band.

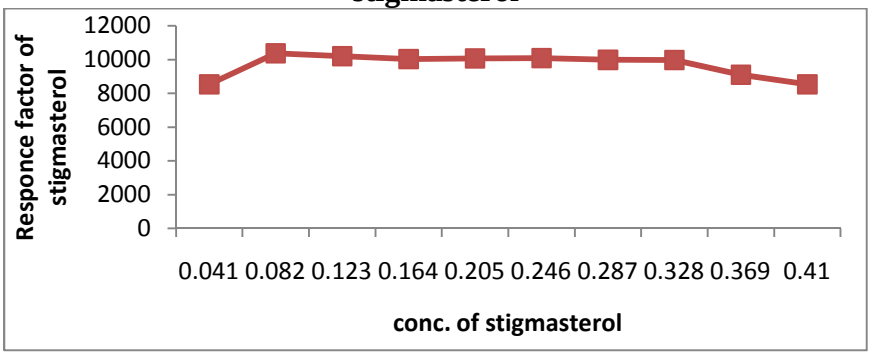
High performance thin layer chromatographic determination of β-amyrin from leaf powder of *Putranjiva roxburghii* Wall.

Graph of Linear dynamic range of stigmasterol



High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Graph of response factor of stigmasterol against corresponding applied concentration of stigmasterol



From the above graph, it is observed that the response factor of stigmasterol was found to be constant in the concentration range of 0.082µg per band to 0.328 µg per band.

Liner Working Range for β-amyrin and stigmasterol

Working standard solutions of β-amyrin and stigmasterol in the concentration range of 0.045 to 0.360 µg per band and 0.082 to 0.328 µg per band respectively were applied, in triplicate, to three different plates and developed and scanned using the optimized conditions described above. The densitograms were then acquired and the peak areas were recorded for each concentration of β-amyrin and stigmasterol.

The values of mean peak areas, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) for each of its applied concentration were calculated.

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Results of Linear working range of β-amyrin

Obs. No.	Concentration of β-amyrin (µg per band)	Peak areas of β-amyrin			Mean	S.D.	%R.S.D.
		I	II	III			
1	0.045	380	385	390	385.0	5.00	1.298
2	0.09	780	790	795	788.3	7.63	0.968
3	0.135	1148	1152	1160	1153.3	5.77	0.500
4	0.180	1540	1550	1555	1548.3	7.63	0.493
5	0.225	1960	1975	1980	1971.6	10.40	0.527
6	0.270	2373	2380	2377	2376.6	2.88	0.121
7	0.315	2685	2690	2682	2685.6	4.04	0.150
8	0.360	3040	3045	3050	3045.0	5.00	0.160

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Results of Linear working range of stigmasterol

Obs. No.	Concentration of stigmasterol (µg per band)	Peak areas of stigmasterol			Mean	S.D.	%R.S.D.
		I	II	III			
1	0.041	415	425	410	416.6	7.637	1.833
2	0.082	825	835	815	825.0	10.00	1.212
3	0.123	1225	1235	1228	1229.3	5.131	0.417
4	0.164	1615	1627	1632	1624.6	8.736	0.537
5	0.205	2035	2042	2048	2041.6	6.506	0.318
6	0.246	2450	2455	2445	2450.0	5.000	0.204
7	0.287	2840	2856	2835	2843.6	10.969	0.385
8	0.328	3245	3252	3258	3251.6	6.506	0.200

Regression analysis

The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (peak area of β-amyrin and stigmasterol) and independent variable (concentration of β-amyrin and stigmasterol).The regression equation is:

$y = mx + c$

where,
y = Mean peak area of β-amyrin and stigmasterol.
m = slope of the regression line.
x= Concentration of β-amyrin and stigmasterol (µg per band)
c = Intercept on y – axis.
The values of correlation co-efficient, intercept and slope were determined from the graph of mean peak area of β-amyrin and stigmasterol (Y–axis) against corresponding applied concentration of β-amyrin and stigmasterol (X–axis).

The regression equation for β-amyrin was found to be,
$y = 8518.5 x + 19.25$
This indicates that 99.94% (correlation coefficient (r) × 100) of the variation in the response is explained by the variation in concentration of β-amyrin. The results of the regression analysis are given in Table.

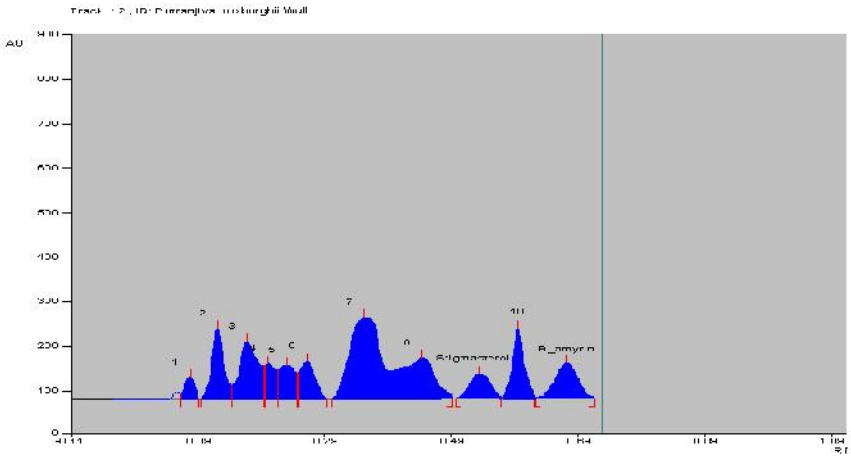
The regression equation for stigmasterol was found to be,
$y = 9864.9 x + 11.25$
This indicates that 99.99% (correlation coefficient (r) × 100) of the variation in the response is explained by the variation in concentration of stigmasterol.

Limit of detection (LOD) and limit of Quantification (LOQ)

The values of LOD and LOQ were determined at a signal to noise ratio of 3:1 and 10:1 respectively. The values of **Limit of detection** (LOD) and **Limit of Quantification** (LOQ) obtained for β-amyrin were 0.045 and 0.09 µg per band and stigmasterol were 0.041 and 0.082 µg per band respectively

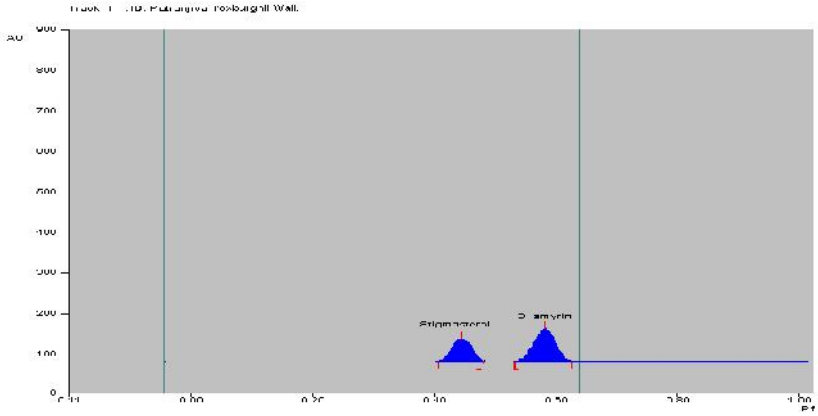
High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

A typical HPTLC chromatogram of methanol extract of leaf powder of *Putranjiva roxburghii* Wall. at λ = 580 nm



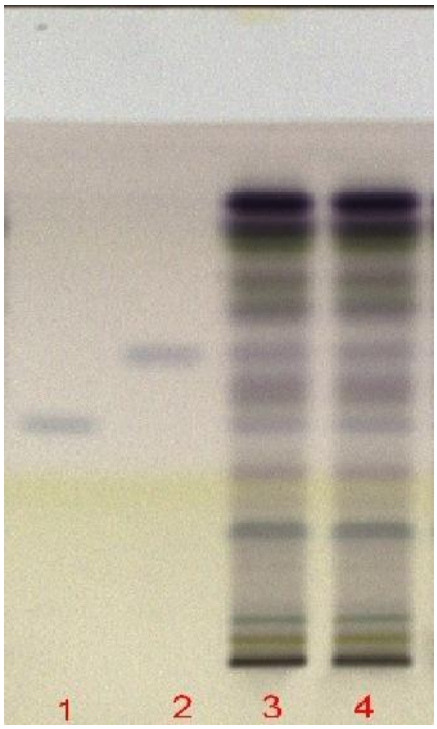
High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

A typical HPTLC chromatogram of reference standard β-amyrin and stigmasterol. at λ = 580 nm



High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

TLC plate showing separation of standard β-amyrin and stigmasterol and β-amyrin and stigmasterol present in methanol extract of leaf powder of *Putranjiva roxburghii* Wall.



Photograph of developed TLC plate showing chromatography of stigmasterol (1), β-amyrin (2) and methanol extract of leaf powder of *Putranjiva roxburghii* Wall. (3, 4)

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Results of assay experiment β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Obs no	Mass of plant powder (mg)	β-amyrin peak area	Amount of β-amyrin found	Stigmasterol Peak area (mg)	Amount of stigmasterol found (mg)
1	1007	1245	0.072	1345	0.068
2	1005	1268	0.073	1325	0.067
3	1004	1264	0.073	1348	0.068
4	1007	1260	0.072	1350	0.068
5	1005	1258	0.072	1342	0.067
6	1009	1263	0.073	1355	0.068
7	1006	1268	0.073	1335	0.067
Mean	1006.1	1260.8	0.0623	1342.8	0.0675
S.D.	1.676	7.925	0.0004	10.090	0.0005
% R.S.D.	0.166	0.628	0.721	0.751	0.791

The amount of β-amyrin and stigmasterol present in each sample solution and the values of standard deviation (S.D.), percent relative standard deviation (% R.S.D.) were calculated. The results of assay experiment of β-amyrin and stigmasterol present in leaf powder solutions are given in Table.

Calculations of assay of average content of β-amyrin and stigmasterol in 1000.0 mg of leaf powder of *Putranjiva roxburghii* Wall.

The linear regression analysis data used for the quantitation of β-amyrin and stigmasterol in leaf powder of *Putranjiva roxburghii* Wall. is as follows:

$y = mx + c$

Where, y = mean peak area of β-amyrin and stigmasterol

m = slope of the regression line.

c= Intercept on Y-axis.

x = concentration of β-amyrin and stigmasterol (µg per band).

From the sample solution, mean peak area of β-amyrin in leaf powder of *Putranjiva roxburghii* Wall. , y = 1260.8

From the sample solution, mean peak area of stigmasterol in leaf powder of *Putranjiva roxburghii* Wall. , y = 1342.8

Mean weight of the sample = 1006.1 mg

From the plot of concentration of β-amyrin (X-axis) against peak area of β-amyrin (Y-axis), the linear regression equation obtained for β-amyrin was,

$$y = 8518.5x + 19.25$$

From the plot of concentration of stigmasterol (X-axis) against peak area of stigmasterol (Y-axis), the linear regression equation obtained for stigmasterol was,

$$y = 9864.9x + 11.25$$

Where,

	β-amyrin	stigmasterol
Slope(m)	8518.5	9864.9
Intercept(c)	19.25	11.25
Correlation coefficient(r)	0.9994	0.9999

Unknown concentration of β-amyrin in 10 µl sample solution

$$= (y - c) / m$$

$$= (1260.8 - 19.25) / 8518.5$$

$$= 0.145 \mu g.$$

Since, 10 µl of sample on plate contains 0.145 µg of β-amyrin, 5000 µl contains 72.5µg of β-amyrin.

Therefore, amount of β-amyrin in 1006.1 mg of leaf powder of *Putranjiva roxburghii* Wall. = 0.072mg/g.

Unknown concentration of stigmasterol in 10 µl sample solution

$$= (y - c) / m$$

$$= (1342.8 - 11.25) / 9864.9$$

$$= 0.133 \mu g.$$

Since, 10 µl of sample on plate contains 0.133 µg of stigmasterol, 5000 µl contains 66.5µg of stigmasterol.

Therefore, amount of β-amyrin in 1006.1 mg of leaf powder of *Putranjiva roxburghii* Wall. = 0.066 mg/g

Recovery Experiment

Accuracy of the experiment was determined by recovery experiment at three different levels, using standard addition method. The recovery experiment was carried out to determine if there is any interference of other constituents present in methanol extract of leaf powder of *Putranjiva roxburghii* Wall. with respect to the separation, detection and quantification of β-amyrin and stigmasterol.

To the accurately weighed about 1000mg each of leaf powder of *Putranjiva roxburghii* Wall., known amounts of β-amyrin and stigmasterol were added in solution form, at three different

levels. Each sample solution was then analysed by the developed HPTLC method under optimised chromatographic conditions. The recovery experiment for leaf powder of *Putranjiva roxburghii* Wall. was carried out in seven replicates at every level. The amounts of β-amyrin and stigmasterol recovered from leaf powder solution for each level were determined. From the amounts of β-amyrin and stigmasterol obtained, values of percent recovery were determined for leaf powder of *Putranjiva roxburghii* Wall

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* wall.
Results of recovery experiment for β-amyrin after addition of standard β-amyrin to leaf powder of *Putranjiva roxburghii* Wall.

Level	Wt. of sample*	Wt. of standard added (mg)	Amount of β-amyrin found(mg)								S. D.	%R. S.D.
			1	2	3	4	5	6	7	Mean** (mg)		
0	1005	0	0.073	0.072	0.071	0.073	0.073	0.071	0.072	0.072	0.00089	1.24
1	1007	0.073	0.140	0.135	0.138	0.142	0.137	0.137	0.141	0.138	0.0025071	1.80
2	1004	0.091	0.160	0.162	0.159	0.163	0.158	0.166	0.161	0.161	0.0026904	1.66
3	1003	0.109	0.180	0.175	0.178	0.179	0.180	0.172	0.175	0.177	0.0030551	1.72

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* wall.
Results of recovery experiment for stigmasterol after addition of standard stigmasterol to leaf powder of *Putranjiva roxburghii* Wall.

Level	Wt. of sample*	Wt. of standard added (mg)	Amount of stigmasterol found(mg)								S. D.	%R.S.D.
			1	2	3	4	5	6	7	Mean** (mg)		
0	1005	0	0.068	0.067	0.066	0.068	0.065	0.071	0.069	0.067	0.00089	1.24
1	1007	0.067	0.126	0.125	0.124	0.123	0.124	0.137	0.126	0.122	0.0025071	1.80
2	1004	0.083	0.142	0.140	0.141	0.143	0.140	0.166	0.138	0.142	0.0026904	1.66
3	1003	0.100	0.155	0.150	0.153	0.151	0.148	0.172	0.146	0.1145	0.0030551	1.72

* Sample: Leaf powder of *Putranjiva roxburghii* wall.

** Mean amount of β-amyrin and stigmasterol found (mg)

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.
Results of recovery experiment for β-amyrin after addition of standard β-amyrin to leaf powder of *Putranjiva roxburghii* Wall.

Level	**X**	**Y**	**X^2**	**XY**
0	7 x 0	7 x 0.073	7 $x(0)^2$	7 x 0 x 0.073
1	7x 0.073	7 x 0.143	$7x(0.073)^2$	7 x 0.073 x 0.143
2	7 x0.091	7 x 0.162	$7x(0.091)^2$	7 x 0.091 x 0.162
3	7 x 0.109	7 x 0.180	$7x(0.109)^2$	7 x 0.109 x0.180
∑X	**1.9159**	**3.906**	**0.1794**	**0.314464**
$(\sum X)^2$	**3.6706**			

No. of observations = 28.

Therefore, Percent recovery = $\frac{(28 \times 0.3144) - (1.9159 \times 3.906)}{28 \times (0.1795) - (3.671)}$

= 97.59 %

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Results of recovery experiment for stigmasterol after addition of standard stigmasterol to leaf powder of *Putranjiva roxburghii* Wall.

Level	**X**	**Y**	**X^2**	**XY**
0	7 x 0	7 x 0.951	7 $x(0)^2$	7 x 0 x 0.951
1	7x 0.47	7 x 1.414	$7x(0.47)^2$	7 x 0.47 x 1.414
2	7 x 0.71	7 x 1.641	$7x(0.71)^2$	7 x 0.71 x 1.641
3	7 x 0.95	7 x 1.896	$7x(0.95)^2$	7 x 0.95 x 1.896
∑X	**14.91**	**41.314**	**11.3925**	**25.4161**
$(\sum X)^2$	**222.308**			

No. of observations = 28.

Therefore, Percent recovery = $\frac{(28 \times 0.2639) - (1.75 \times 3.591)}{28 \times (0.1496) - (3.0625)}$

= 98.0%

Results:

High performance thin layer chromatographic determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

Results of validation parameters for β-amyrin and stigmasterol

Parameters	**β-amyrin**	**stigmasterol**
Linear working range(µg per band)	0.045-0.360	0.082 - 0.328
Correlation coefficient(r)	0.9994	0.9998
Limit of Detection(LOD)(µg per band)	0.045	0.041
Limit of Quantification(LOQ)(µg per band)	0.09	0.082
Instrument precision (% R.S.D, n=10)	0.574	0.496
Repeatability (% R.S.D, n =6) Leaf powder of *Putranjiva roxburghii* wall.	0.81	0.80
Intermediate precision (% R.S.D, n= 18) Leaf powder of *Putranjiva roxburghii* Wall.	0.693	0.734
Stability of standard solution	Stable for minimum 24 hours	Stable for minimum 24 hours
System suitability		
R_F (% R.S.D. , n =6)	0.818	0.918
Peak area (% R.S.D., n=6)	0.2999	0.465
Assay (mg/g) Leaf powder of *Putranjiva roxburghii* Wall.		
Percent recovery (%) Leaf powder of *Putranjiva roxburghii* Wall.	0.072 97.59	0.066 98.0

Discussion

In the present research work, a HPTLC method has been developed for the quantitative determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall. To achieve quantitative extraction of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall., the influence of extracting solvents like methanol, chloroform and diethyl ether was determined. The maximum percent extractive value for leaf powder of *Putranjiva roxburghii* Wall. was obtained using methanol. Therefore, methanol was selected as the solvent for the quantitative determination of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall. Further, parameters like volume of solvent and time needed for extraction of β-

amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall. was optimized, using methanol as the extracting solvent. It was found that 10.0 cm^3 of methanol and extraction time of 15 minutes was sufficient for complete extraction of β-amyrin and stigmasterol from leaf powder of *Putranjiva roxburghii* Wall.

In the reported method C_{18} RP HPTLC plate has been used whereas in the present research work normal mode of separation has been used for quantitation of β-amyrin and stigmasterol.

An overlay of UV spectra of standard β-amyrin and stigmasterol with β-amyrin and stigmasterol present in leaf powder of *Putranjiva roxburghii* Wall. shows that the peak of β-amyrin and stigmasterol is not masked by the peak of any other component present in the sample. The peak area of β-amyrin and stigmasterol in leaf powder solution increased after addition of standard β-amyrin and stigmasterol standard to the leaf powder solution without interference from other peaks. The percent recovery values for leaf powder solutions obtained were high indicating good accuracy of the method.

Conclusion

An HPTLC method developed for the quantitation of β-amyrin and stigmasterol from the leaf powder of *Putranjiva roxburghii* Wall. is simple, precise and accurate and can be used for routine quality control analysis of leaf powder of *Putranjiva roxburghii* Wall.

High Performance Liquid Chromatographic determination of diosgenin from bulbils powder of *Dioscorea bulbifera* Linn.

Method in brief

A simple, rapid and precise reverse phase high performance liquid chromatographic method has been developed for the determination of diosgenin from methanolic extract of dried bulbils powder of *Dioscorea bulbifera* Linn. Chromatographic analysis was carried out on Zorbax C_{18} column (150mm x 4.6mm, 5µm), with a mobile phase of mixture of methanol and water, in the volume ratio of 95:5, at a flow rate of 1.0 cm^3/min. quantitation was performed using a UV-visible detector at 210 nm.

The proposed HPLC method was validated and applied for the quantitative determination of diosgenin from *Dioscorea bulbifera* Linn.

High performance Liquid Chrmatographic determination of diosgenin.
Optimized Chromatographic conditions

PARAMETERS	CHROMATOGRAPHIC CONDITONS
Pump	Jasco PU-980 pump
Stationary phase	Zorbax C_{18}, (150 mm x 4.6 mm, 5µm)
Mobile phase	Methanol : Water (95:5) v/v
Flow rate	1.0 cm^3/min
Detector	Jasco UV-970 UV/V is detector
Wavelength	210 nm
Recorder	Borwin chromatography software 1.21

Method Validation:

Linear dynamic range of diosgenin:

This experiment was carried out to demonstrate the range over which the response of the detector is linear with respect to concentration of diosgenin.

Aliquots of (10.0 µL, 20.0 µL, 30.0 µL, 50.0 µL, 100.0 µL, 200.0 µL, 400.0 µL, 600.0 µL, 800.0 µL, 1000.0 µL and 1200.0 µL) were drawn from diosgenin standard solution of concentration (100.0 µg/cm^3) and transferred to separate 10.0 cm^3 volumetric standard flask. The volume of each standard flask was adjusted to 10.0 cm^3 with the mobile phase used, to obtain standard solutions of diosgenin with concentrations of 0.10 µg/cm^3, 0.20 µg/cm^3, 0.30 µg/cm^3, 0.50 µg/cm^3, 1.00 µg/cm^3, 2.00 µg/cm^3, 4.00 µg/cm^3, 6.00 µg/cm^3, 8.00 µg/cm^3, 10.00 µg/cm^3, and 12.00 µg/cm^3 respectively.

Twenty microlitres of each of the standard solutions of diosgenin in the concentration range of 0. 10 µg/cm^3 to 14.00 µg/cm^3 were injected into the chromatographic system under the optimized chromatographic conditions. The chromatograms were recorded and the peak areas of diosgenin for each injected concentration of diosgenin, were noted. The response factors were calculated for each concentration of diosgenin by dividing each peak area by concentration of diosgenin at that level. The values of peak areas and response factors of diosgenin for each injected concentration are tabulated in Table.

High Performance Liquid Chromatographic determination of diosgenin.

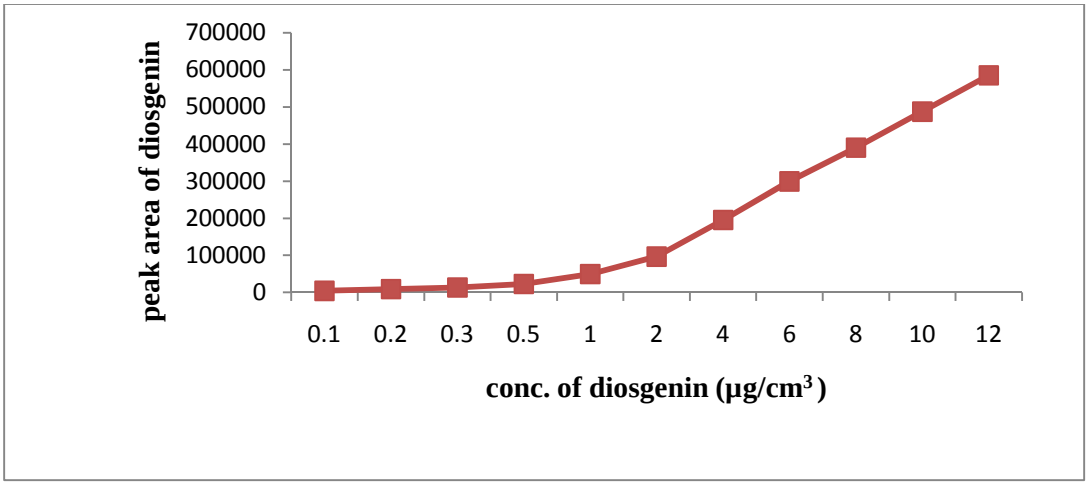
Results of the linear Dynamic Range of diosgenin

Obs. No	Concentration of diosgenin (µg/cm^3)	Peak area of diosgenin	Response factor
1	0.1	4250	42500
2	0.2	8721	43605
3	0.3	12952	43173.3
4	0.5	22480	44960
5	1	49560	49560
6	2	96520	48260
7	4	195078	48769.5
8	6	299405	49900.8
9	8	390580	48822.5
10	10	487521	48752.1
11	12	585450	48787.5

A graph of peak area values of diosgenin (Y-axis), against the corresponding concentrations of diosgenin (X-axis), was plotted and is shown in Figure.

High Performance Liquid Chromatographic determination of diosgenin

Linear Dynamic Range of diosgenin

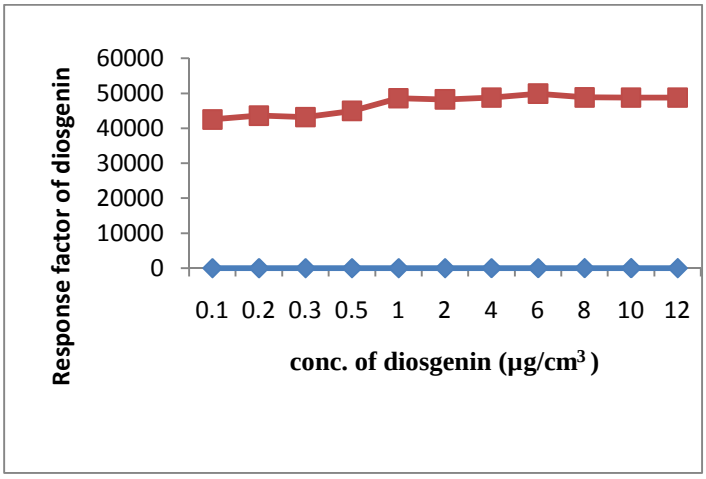


The graph shows that the response of diosgenin is linear in the concentration range of 1.00 µg/cm^3 to 12.00 µg/cm^3.

A graph of response factor of diosgenin (Y-axis), against corresponding concentration of diosgenin (X-axis), was plotted as shown in Figure.

High Performance Liquid Chromatographic determination of diosgenin

Graph of response factor for diosgenin against the corresponding concentration of diosgenin



From the above graph, it is observed that the response factor was found to be constant in the concentration range of 1.0 µg/cm^3 to 12.00 µg/cm^3 of diosgenin.

Linear Working Range of diosgenin :

The concentration range of diosgenin solution selected for linearity was 1.00 µg/cm^3 to 12.00 µg/cm^3 respectively.

Into a series of 10.0 cm^3 standard volumetric flask, aliquots of (100.0 µL, 200.0 µL, 400.0 µL, 600.0 µL, 800.0 µL, 1000.0 µL and 1200 µL) were drawn from diosgenin stock solution of

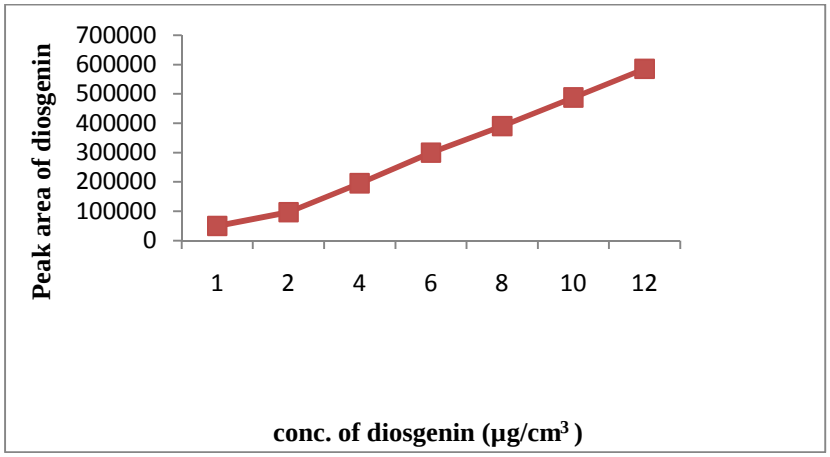
concentration (100.0 µg/cm^3) and the contents of each flask were diluted up to the mark with the mobile phase used, to obtain a concentration range of 1.00 µg/cm^3 to 12.00 µg/cm^3 respectively. Twenty microlitres, of each of these solutions, were injected into the chromatographic system under the optimized chromatographic conditions, in triplicate. The chromatograms were recorded and the peak areas of diosgenin were noted for each concentration of the working standard solutions of diosgenin, applied in triplicate. The values of mean peak areas, standard deviation and the percent relative standard deviation of a diosgenin for each injected concentration were calculated. The results are tabulated in Table.

High Performance Liquid Chromatographic determination of diosgenin
Results of the Linear Working Range of diosgenin

Obs. No	**Concentration of diosgenin (µg/cm^3)**	**Peak area of diosgenin**			**Mean peak area**	**S.D**	**%R.S.D**
1	1.0	49850	49652	49923	49808.3	140.2	0.28
2	2.0	96786	96574	96445	96601.6	172.1	0.17
3	4.0	195120	194832	196252	195401.3	750.6	0.38
4	6.0	299457	299206	299560	299407.6	182.0	0.06
5	8.0	390596	390289	390496	390460.3	156.5	0.04
6	10.0	487557	487239	487953	487583.0	357.7	0.07
7	12.0	585473	585460	585840	585591.0	215.7	0.03

A graph of mean peak area values of diosgenin (Y-axis) against the corresponding concentration of diosgenin (X-axis) was plotted, which showed a linear response in concentration range of 1.00 µg/cm^3 to 12.00 µg/cm^3.

High Performance Liquid Chromatographic determination of diosgenin
Calibration curve of standard diosgenin



From the above graph, it is observed that a linear response exists in the concentration range of 1.00 µg/cm^3 to 12.00 µg/cm^3 of diosgenin. The results of the linearity experiment were subjected to regression analysis.

Regression analysis:

The regression analysis was carried out to determine the relationship between the mean peak area of diosgenin and concentration of diosgenin (µg/cm^3). The calibration curve was represented by the regression equation y= mx + c.

Where, y= mean peak area of diosgenin

M = slope of the regression line

X = concentration of diosgenin (µg/cm^3)

C = intercept on the y-axis

The values of correlation coefficient, intercept and slope were determined from the graph of mean peak area (y-axis), against applied concentration of diosgenin (x-axis)

The regression equation for diosgenin was found to be :

Y = 48760.6 x + 1163.9

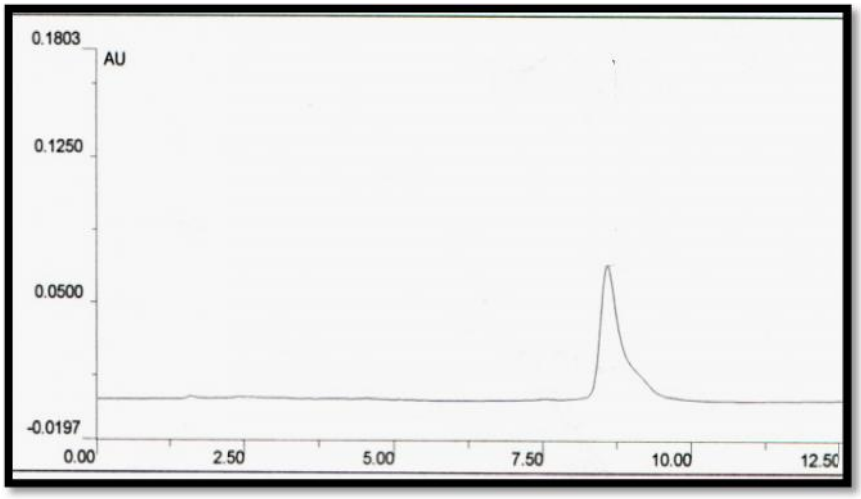
This indicates that 99.99 % (correlation coefficient r x 100) of the variation in the response is explained by the variation in concentration of diosgenin. The results of the regression analysis are given in Table.

High Performance Liquid Chromatographic determination of diosgenin
Regression analysis data of diosgenin

Slope (m)	48760.6
Intercept (c)	1163.9
Correlation coefficient (r)	0.9999

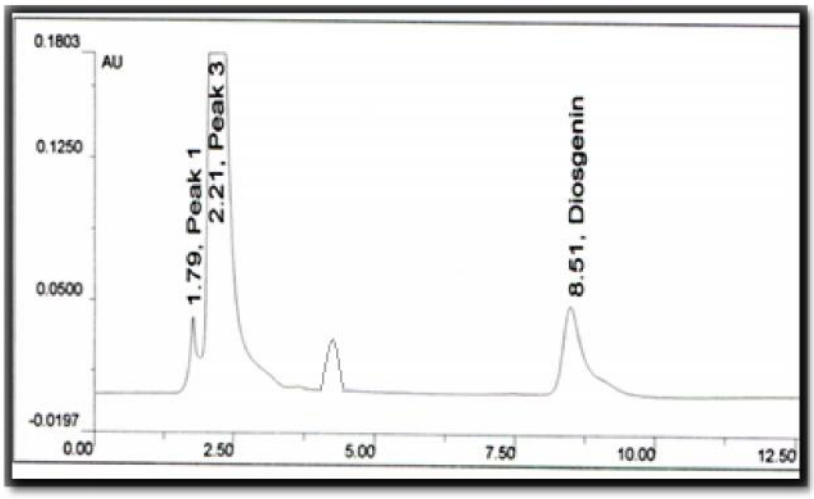
High Performance Liquid Chromatographic determination of diosgenin

A typical HPLC chromatogram of standard diosgenin.



High Performance Liquid Chromatographic determination of diosgenin.

A typical chromatogram of methanolic extract of dried bulbils powder of *Dioscorea bulbifera* Linn.



Limit of Detection (LOD) & Limit of Quantitation (LOQ) for diosgenin:

The limit of detection (LOD) can be defined as the peak, whose signal to noise ratio is atleast 3:1. The limit of detection for diosgenin was 0.01µg/cm^3. The limit of quantitation (LOQ) can be defined as the peak whose signal to noise ratio is at least 10:1. The limit of quantitaion for diosgenin was 0.30 µg/cm^3.

Application of the proposed method for the determination of Diosgenin from *Dioscorea bulbifera* Linn.

Sample preparation:

About100.0 mg of bulbils powder was accurately weighed and transferred to a stoppered test tube and 10.0 cm of methanol was then added to it. It was then sonicated for 20 min. The extract was then filtered through whatman filter paper no 41 and the filtrate were further used as a sample solution for the assay experiment.

Assay procedure

The quantitation of diosgenin was done using above validated HPLC method. The optimized chromatographic conditions were set on the HPLC system and the system was monitored to

attain a stable base line. Twenty microlitres of solution was injected into the chromatographic system under the optimized chromatographic conditions.

The identity of peak of diosgenin in the sample solution was confirmed by comparing the chromatogram of the sample with that of the diosgenin standard solution having retention time as 8.51minutes. Amount of diosgenin present in the sample solution was determined from the calibration curve by using the peak area of diosgenin in the sample solution.

To ascertain the repeatability of the method, the assay experiment was repeated seven times. The values of amount of diosgenin present in *Dioscorea bulbifera* Linn., standard deviation and the percent relative standard deviation were calcultated. The results of assay are given in Table.

High performance Liquid Chromatographic determination of diosgenin
Results of assay Experiment

Obs.No.	**weight of bulbils powder**	**Peak area of diosgenin**	**Amount of diosgenin present mg/g**
1	100.5	303789	0.620
2	100.8	304198	0.621
3	100.3	303952	0.620
4	100.7	304315	0.621
5	100.6	304489	0.622
6	100.5	304096	0.621
7	100.4	303948	0.620
Mean	**100.5**	**304112.4**	**0.621**
S,D	**0.171**	**240.76**	**0.004**
%RSD	**0.170**	**0.079**	**0.079**

Calculation of assay of mean content of diosgenin in 100.5mg dried bulbils powder of *Dioscorea bulbiferra* Linn. :

According to the calibration curve, $y = mx + c$

Where, y = peak area of Diosgenin.

M = slope of the regression line.

X = concentration of diosgenin in µg/cm^3.

C = Intercept on Y-axis.

For the sample solution, mean peak area of diosgenin, y = 304112.4

Average weight of the sample = 100.5mg

From results of regression analysis of calibration data

M = 48760.6, c = 1163.9
Unknown concentration of diosgrnin in 1.0 cm^3 of sample solution

= (y-c) / m

= (304112.4 - 1163.9) / 48760.6
Unknown concentration of diosgenin in 10.0 $cm^3$of sample solution =

62.0 µg or 0.0620 mg

Concentration of diosgenin in 100.5 mg of bulbils powder on *Dioscorea bulbifera* Linn. = 0.062mg

Concentration of diosgenin in 1.0 g of bulbils powder of *Dioscorea bulbifera* Linn. = 0.62mg

Recovery :

The recovery experiment was carried out by standard addition method. A fixed amount of sample was weighed three times. Three different levels of standards were added to each weighed amount of sample which were 100%, 125% and 150% of the standard in the plant powder. Each set of analysis was repeated to check if there is any interference of other constituents present in *Dioscorea bulbifera* Linn. by HPLC under optimized chromatographic conditions. The value of percentage recovery was calculated.

High performance Liquid Chromatographic determination of diosgenin
Results of recovery experiment.

Level	Wt. of sample (mg)*	Wt. of std. added (mg)	Amount of diosgenin found (mg)							Mean** (mg)	S.D	% R.S.D
			1	2	3	4	5	6	7			
0	100.5	0	0.062	0.061	0.063	0.062	0.063	0.061	0.060	0.061	0.001	1.802
1	100.7	0.06	0.120	0.121	0.122	0.119	0.117	0.118	0.124	0.120	0.002	2.006
2	100.4	0.08	0.142	0.140	0.138	0.141	0.137	0.139	0.138	0.139	0.002	1.291
3	100.6	0.09	0.152	0.151	0.149	0.148	0.150	0.147	0.149	0.149	0.001	1.149

* Sample: Dried bulbils of *Dioscorea bulbiferra* Linn.
**Mean amount of Diosgenin found.

High Performance Liquid Chromatographic determination of diosgenin
Results of Recovery Experiment

Level	X	Y	X^2	XY
0	7x 0	7x 0.061	7 x $(0.00)^2$	7 x 0x 0.061
1	7x 0.06	7x 0.120	7 x $(0.06)^2$	7 x 0.06 x 0.120
2	7x 0.08	7x 0.139	7 x $(0.08)^2$	7 x 0.08 x 0.139
3	7x 0.09	7x 0.149	7 x $(0.09)^2$	7 x 0.09 x 0.149
Σ	**1.61**	**3.283**	**0.126**	**0.222**

Number of observations = 28

Therefore,

$$\% \text{ Recovery} = \frac{(28 \times 0.222) - (1.61 \times 3.283)}{(28 \times 0.126) - (2.592)} \times 100$$

$$= 97.69$$

Results:

High Performance Liquid Chromatographic determination of diosgenin

Results of validation parameters for diosgenin.

Parameters	**Diosgenin**
Linear working range (µg/cm^3)	**1µg/cm^3 to 12.00 µg/cm3**
Correlation coefficient(r)	**0.9999**
Limit of Detection(LOD)(µg/cm3)	**0.01 µg/cm^3**
Limit of Quantification (LOQ) (µg/cm3)	**0.30 µg/cm^3**
Instrument precision (% R.S.D, n=10)	**0.086**
Repeatability (% R.S.D, n =6)	**0.067**
Bulbils powder of *Dioscorea bulbifera* Linn.	
Intermediate precision (% R.S.D, n= 18)	**0.077**
Bulbils powder of *Dioscorea bulbifera* Linn.	
Stability of standard solution	**Stable for minimum 48 hours**
System suitability	
Retention time of diosgenin	
(% R.S.D. , n =6)	**0.369**
Peak area (% R.S.D., n=6)	**0.195**
Assay (mg/g)	
bulbils powder of *Dioscorea bulbifera* Linn.	**0.62**
Percent recovery (%)	**97.69**

Discussion

The mobile phase used in the present research work for quantitaion of diosgenin from methanolic, dried bulbils powder extract of *Dioscorea bulbiferra* Linn. is methanol and water in the volume ratio of 95:5 (v/v) which is relatively simpler as compared to the mobile phase used in the reported methods.

The retention time for diosgenin was found to be 8.54 minutes which is relatively less than the retention time reported in the literature (18.06 min., 11.08 min., and 15.05 min. respectively.).

The method used in the present research work was also found to be sensitive to measure the concentration as low as 0.01 $\mu g/cm^3$, whereas in the reported method [1-3], the detection limit was 0.037 $\mu g/cm^3$, 0.04 $\mu g/cm^3$ and 10.0 $\mu g/cm^3$ respectively.

The column used in the present research work, comprised of octadecyl bonded to silica phase. Due to the length of the column, (250.0 mm) and small particle size of silica (5.0 µm), a good resolution of diosgenin from different components of bulbils powder of *Dioscorea bulbiferra* Linn. was obtained.

Hence the HPLC method used in the present research work was found to be simpler, sensitive and accurate than other reported methods.

Conclusion

An HPLC method developed for the quantitation of diosgenin from the bulbils powder of *Dioscorea bulbifera* Linn. is simple, precise and accurate and can be used for routine quality control analysis of bulbils powder of *Dioscorea bulbiferra* Linn.

Study of Immunomodulatory activity of *Putranjiva roxburghii* Wall.Using cyclophosphamide induced immune suppressed mice

Study Protocol

Study title	Immunomodulatory effect of *Putranjiva roxburghii* Wall. on Cyclophosphamide Induced Immune Suppressed Swiss Albino Mice
Testing facility	Animal Testing Unit (CPCSEA/315) Ramnarain Ruia College, Matunga, Mumbai- 400 019
Drugs tested in the study	Leaf powder of *Putranjiva roxburghii* Wall. Stem powder of *Tinospora cordifolia* Miers. Lithium carbonate powder
Storage container for Drugs	Polycarbonate Container
Storage conditions of Drugs	Room Temperature (30 ± 2^{o} C)

Test system	
Species	Swiss Albino mice
Sex	Male
Animal Source	Haffkine Bio- Pharma. Corpn. Ltd., Mumbai
Number of Groups	Four
Number of animals	Six per group
Age of animals at start of study	3- 4 weeks
Body weight at start of study	20- 23 g
Identification of animals	By cage tag and marking on the inner surface of the ear
Acclimatization period	One week in the experimental room

Administration of leaf powder of *Putranjiva roxburghii* Wall. and powder of *Tinospora cordifolia* Miers. and powder of Lithium carbonate	
Route of administration of drug	Intragastric by gavage using a metal cannula, (No. 16) attached to a graduated syringe
Justification for route of drug administration	Oral route is the intended therapeutic route of test material administration in humans
Concentration of drug dose	100 mg /kg bodyweight /animal /day
Vehicle for administration of drug	Distilled water
Volume of drug administration	1 cm^3 per animal
Duration of administration of drug	15 days

Cyclophosphamide administration	
Route of administration of Cyclophosphamide	Subcutaneous by using sterile latex free BD 1 cm^3 syringe with 26G needle
Justification for route of Subcutaneous administration	Subcutaneous route is the recommended route of administration
Dose administered	200 mg /kg bodyweight /animal
Vehicle for administration	Sterile Normal Saline
Volume of administration	0.5 cm^3 per animal
Time for administration	16^{th} day i.e., after 15 days of drug treatment

Blood sample withdrawals	1. On 1st day of study before drug administration
	2. On 16th day before cyclophosphamide administration
	3. On 3rd day after cyclophosphamide administration
	4. On 7th day after cyclophosphamide administration

Animal Husbandry	
Environmental conditions	The animal room was maintained at a temperature of 28- 30° C and kept at relative humidity of 65- 70 %.
	The rate of air exchange was continuous using exhausts.
	The lighting was controlled by a timer to give a cycle of 12 hours continuous light and 12 hours continuous darkness.
Accommodation	Animals were housed in groups of six in solid floor polypropylene cages with rice husk bedding and facilities for food and water.
Food	Rat feed pellets supplied by Amrut Animal Feed Laboratory, Chakan Oil Mills Ltd., Maharashtra
Water	Potable water was provided *ad libitum* in glass bottles with stainless steel sipper caps.
Parameters used for evaluation of Immunomodulatory effect	Total leukocyte count and neutrophil counts

Approval for animal study

The study was approved by the Institutional Animal Ethics Review Committee, Ramnarain Ruia College, Matunga, Mumbai- 400 019. Experimental animals were handled according to the University and Legalization, regulated by the Committee for the Purpose of Control and

Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Animal grouping for the present animal study

The animals were randomly divided into five groups of six male mice each.

Group (I): Control Group (1.0 cm^3 distilled water administered during the entire study)

Group (II): Lithium carbonate 100 mg/kg + Cyclophosphamide 200 mg/kg administered subcutaneously after first fifteen days of treatment with lithium carbonate.

Group (III): *Tinospora cordifolia* Miers. 100 mg/kg, total aqueous extract of stem powder + Cyclophosphamide 200 mg/kg administered subcutaneously after first fifteen days treatment with stem powder of *Tinospora cordifolia* Miers.

Group (IV): *Putranjiva roxburghii* Wall. 100 mg/kg, total aqueous extract of leaf powder + Cyclophosphamide 200 mg/kg administered subcutaneously after first fifteen days treatment with leaf powder of *Putranjiva roxburghii* Wall.

Drug treatment administered to various groups

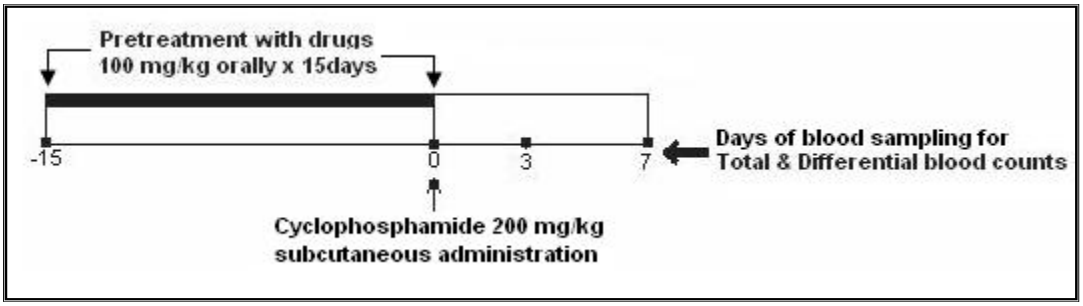
Group **n = 6/ group**	**Drug Treatment**	**Concentration of Dose** **and Duration**
I (Control Group)	Distilled water	1.0 cm^3 of distilled water/animal, for 15 days
II **(Positive Control)**	Lithium Carbonate	100 mg/ kg bodyweight/ animal, in 1.0 cm^3 of distilled water, for 15 days
III **(Positive Control)**	Stem powder of ***Tinospora cordifolia* Miers.**	
IV	Leaf powder of *Putranjiva roxburghii* Wall.	

n- Number of animals

Note- Drug treatment was given prior to single dose of cyclophosphamide administration (200 mg/kg bodyweight) subcutaneously.

Dosage regimen

Schedule for the present study



Observations made during the study

Clinical signs	Animals were observed daily for signs of toxicity throughout the study period
Body weights	Individual animal body weights were recorded daily throughout the study period
Food and water intake	Animals were supplied with known amounts of food, water and their daily food and water intake were recorded
Mortality	Mortality rate of animals (if any) was recorded. Animals that died (if any) during the course of the experiment were subjected to autopsy
Cage side observations	Daily cage side observations for condition of fur skin, subcutaneous swelling, abdominal distention, eye dullness/ pupil diameter, ptosis (drooping of eyelids), colour and consistency of faeces, wetness or soiling of the perineum, condition of teeth, breathing abnormalities and gait were recorded throughout the study period

Results

The results of blood counts viz. total leukocyte counts and absolute neutrophil counts for six animals belonging to respective groups are tabulated in Table. The total leukocyte counts and the absolute neutrophil counts in the drug treated group were compared with the values of the control group.

Results of leukocyte counts and absolute neutrophil counts for mice

belonging to Group I (Control Group)

Days for blood counts	-15th day Counts (cells/mm^3)		0 day Counts (cells/mm^3)		3rd day Counts (cells/mm^3)		7th day Counts (cells/mm^3)	
Animal no.	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil
1	5200	2496	5500	2970	3200	1344	19800	12276
2	6500	3315	6200	2852	2900	1160	12900	7611
3	5900	3127	5800	3306	3300	1518	10100	7676
4	4950	3317	6500	3900	3100	1612	19900	11343
5	5200	3120	6020	3371	2700	1242	18200	9464
6	7050	3243	6300	2646	2600	1014	12500	7875
Mean	5800.0	3102.9	6053.3	3174.2	2966.7	1310.3	15566.7	9573.5
S.D.	837.26	309.66	361.48	448.88	280.48	223.70	4243.43	2027.07

The animals belonging to Control Group (Group I) administered with distilled water for first fifteen days showed similar blood counts on both days of blood count measurements, -15th Day and on 0 Day prior to cyclophosphamide administration. However the values of blood counts post cyclophosphamide administration on 3rd Day dropped to almost half the initial values due to immunosuppressive effects of cyclophosphamide. Thereafter a rebound increase occurred in blood counts on Day 7.

Results of leukocyte counts and absolute neutrophil counts for mice **belonging to Group II (Treated with Lithium carbonate)**

Days for blood counts	-15th day Counts (cells/mm3)		0 day Counts (cells/mm3)		3rd day Counts (cells/mm3)		7th day Counts (cells/mm3)	
Animal no.	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil
1	7900	3318	9500	4750	6300	2709	14800	8436
2	6300	3024	10200	5202	7900	3634	18900	11151
3	5500	2585	8700	3654	6500	2600	15000	9600
4	5700	3420	9100	4368	6100	2806	16400	10004
5	7000	3710	9800	5586	5900	2773	12050	8194
6	6500	2795	8600	3698	5700	2850	13500	8505
Mean	6483.3	3166.0	9316.7	4518.6	6400.0	2901.3	15108.3	9367.2
S.D.	881.85	418.44	630.61	787.39	787.40	372.18	2370.32	1149.98

Results of leukocyte counts and absolute neutrophil counts for mice **belonging to Group III (Treated with *Tinospora cordifolia* Miers.)**

Days for blood counts	-15th day Counts (cells/mm3)		0 day Counts (cells/mm3)		3rd day Counts (cells/mm3)		7th day Counts (cells/mm3)	
Animal no.	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil
1	4600	2392	9700	5238	6200	2976	13300	8246
2	6500	3120	9200	5888	5900	3186	16200	10692
3	7100	2911	8400	4200	6400	3328	15000	8700
4	6700	3015	9300	5394	7700	4928	17400	11832
5	5500	2640	8420	4294	7500	4200	18500	11100
6	6400	3328	10200	6426	7200	3600	16000	9120
Mean	6133.3	2923.6	9203.3	5215.2	6816.7	3681.0	16066.7	9934.6
S.D.	917.97	337.81	708.28	874.80	746.77	734.12	1817.32	1453.96

Results of leukocyte counts and absolute neutrophil counts for mice

belonging to Group IV (Treated with *Putranjiva roxburghii* Wall.)

Days for blood counts	-15th day Counts (cells/mm3)		0 day Counts (cells/mm3)		3rd day Counts (cells/mm3)		7th day Counts (cells/mm3)	
Animal no.	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil	Leukocyte	Absolute Neutrophil
1	4500	1665	10100	5564	5124	2306	18500	9620
2	5300	2067	9400	4982	5831	2332	17100	9405
3	6500	2665	10500	5670	6124	2633	14800	8880
4	7200	2880	10200	5490	5675	2384	16600	10790
5	4600	2024	9600	6726	5894	2240	15500	9920
6	4500	1890	10300	5940	5546	2163	16200	9720
Mean	5433.3	2198.5	10016.7	5728.6	5699.0	2342.9	16450.0	9722.5
S.D.	1158.73	471.08	426.22	580.40	343.71	161.56	1291.12	632.40

The animals of Group II to IV were treated with the respective drugs at concentration 100 mg/kg bodyweight, lithium carbonate powder, stem powder of *Tinospora cordifolia* Miers., leaf powder of *Putranjiva roxburghii* Wall., for fifteen days prior to the administration of cyclophosphamide. After fifteen days of drug treatment to the animals belonging to Group II to Group IV, on "Day 0" it was observed that there was a rise in the total leukocyte count and the absolute neutrophil count, as compared to the blood counts measured on -15th Day. Thus all four drug treated groups showed a significant leukocytosis (increase in total leukocyte count) with predominant neutrophilia (increase in absolute neutrophil count). The drug treated mice, belonging to Group II to Group IV thus showed an increased number of cell counts revealing the immune stimulated condition.

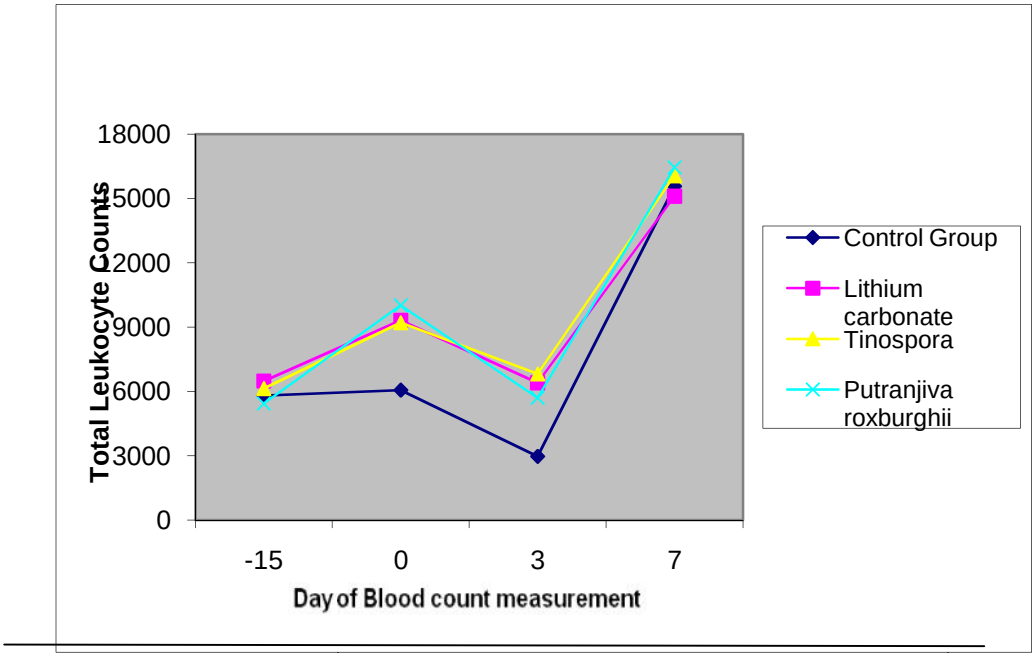
The administration of single subcutaneous dose of cyclophosphamide, at concentration of 200 mg/kg bodyweight of animals to the drug treated groups produced a fall in total

leukocyte count and absolute neutrophil count on "3rd Day" of blood count measurement as shown in Figure.

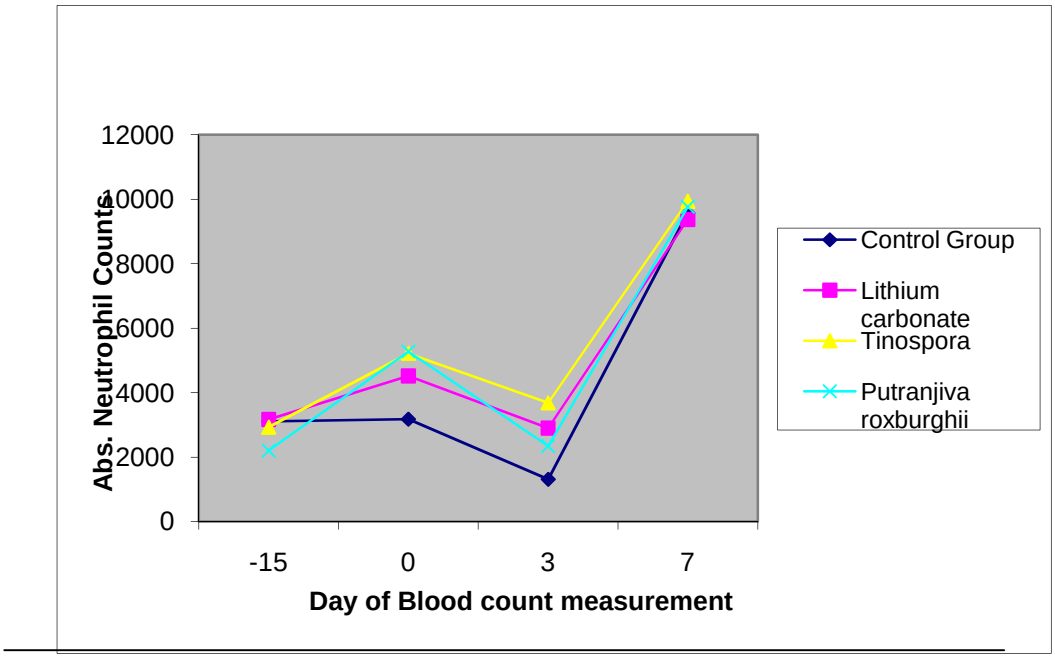
However, it was observed that the total leukocyte counts and the absolute neutrophil counts of the drug treated animals belonging to Group II to Group IV were not as low as that of Control animals (Group I). This effect occurred due to the counteractivity of the respective drugs administered for the first fifteen days to mice belonging to Group II to Group IV, against the immunosuppressive action of cyclophosphamide.

Thereafter an increase in the total leukocyte count and absolute neutrophil count was observed on Day 7, in all the animals of drug treated groups, similar to that of the control group. This increase in blood counts is attributed to the irreversible toxic effects produced by cyclophosphamide.

Mean graph of Total leukocyte counts against the day of blood count measurement



Mean graph of Absolute neutrophil counts against the day of blood count measurement



Discussion

The result of the present study demonstrates that plant under investigation, *Putranjiva roxburghii* Wall. protected the mice from leucopenic/ neutropenic effects of Cyclophosphamide administered subcutaneously. Also protection afforded by *Putranjiva roxburghii* Wall. was comparable to two known immunomodulators, lithium carbonate and traditional medicinal plant *Tinospora cordifolia* Miers.

The present study uses aqueous extracts of leaf powders of *Putranjiva roxburghii* Wall. to evaluate the immunomodulatory activity against the toxic effects of subcutaneously administered Cyclophosphamide.

During the entire study period there was no mortality in any of the groups, during the drug treatment or following the subcutaneous injection of cyclophosphamide. Also there were no

signs of toxicity in any of the experimental animals, during the first 15 days of drug treatment ensuring a high degree of safety of the drugs used in the study at a dose of 100 mg/kg.

The findings of the present study thus encourages and demands the future research using aqueous extracts of leaf powder of *Putranjiva roxburghii* Wall. on different experimental models for exploring its potential benefits.

Conclusion

The leaf powder of *Putranjiva roxburghii* Wall. produced leucocytosis with neutrophilia on pretreatment. Also when compared to control group the total aqueous extract of leaf powder of *Putranjiva roxburghii* Wall. prevented, leucopenia and neutropenia produced by cyclophosphamide in mice. Therefore on the basis of results of the present study it is concluded, that the leaf powder of *Putranjiva roxburghii* Wall. have potent immunostimulanting effect comparable to known immunostimulants, lithium carbonate and *Tinospora cordifolia* Miers. The leaf powders of *Putranjiva roxburghii* Wall. can thus be recommended for use in certain herbal formulations with immune enhancing activity.

References

1. Dobriyal R.M. and Narayana D.B. Ayurvedic herbal raw material, The Eastern Pharmacist, Delhi, India, 1998.
2. Ibanez E, Kubatova A, Senorans F.J., Cavero S., Reglero G., Hawthorne S.B., *Journal of Agricultural Food Chemistry*, **51**, 375-382, 2003.
3. Bartram T. Encyclopedia of Herbal Medicine, Robinson, Grace: Dorset, 1995.
4. Swartz M. E., Krull I. S., Analytical Method Development and Validation, Dekker Inc., Marcel, 1997.
5. Snyder L. R., Kirkland J. J., Glajch J. L., Practical HPLC Method Development, 2nd Ed., John Wiley and Sons, New York, 1997.
6. Skoog D.A,Holler F.J., Crouch S.R., Principles of Instrument Analysis, Thomson Brooks/Cole 6th Ed. 196-197, 2007.
7. J. Goldstein, D. Newbury, D. Joy, C. Lyman, P. Echlin, E. Lifshin, L. Sawyer, and J. Michael, Scanning Electron Microscopy and X-ray Microanalysis, 3rd Ed. Plenum Publishers, New York, 2002.
8. Dahanukar SA, Thatte UM, Rege NN. Immunostimulants in Ayurveda medicine. Immunomodulatory Agents from plants., Birkhauser Verlag Basel, Switzerland, 1999.