Results and Discussion

Chapter III- Results and Discussion

The section describes the results of the experiments performed. The results are summarized in the tabulated form with necessary statistical analysis. The graphs of the results are also provided whenever the results are compared to each other. The results are followed by the discussion.

3.1 Pharmacognostic Studies:

3.1.1Morphological and Microscopic Evaluation of Plants:

Different parts of *L.reticulata*, *D.volubilis* and *P.microphylla* were subjected to morphological examinations. The histology of different parts was examined and the observations were recorded. The recorded observations were compared to that of the published results and found comparable. The results, thus, confirmed the identity of the collected plants.

3.1.2 Comparative Microscopic Studies:

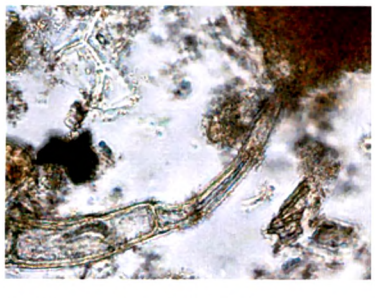
As *P.microphylla* and *D.volubilis* are used as substitutes of *L.reticulata,* the comparative microscopic studies of the different parts of the plants were performed and results are summarized as in Table 3.1

The microscopic examination of aerial parts of the powdered plant materials was performed to detect and establish various identifying microscopic characteristics which will be helpful in differentiation of substitutes from *L.reticulata*, supplied in the form of dried powder. The photomicrograph of the identifying features of the plant materials are shown in Fig 3.1-3.3.

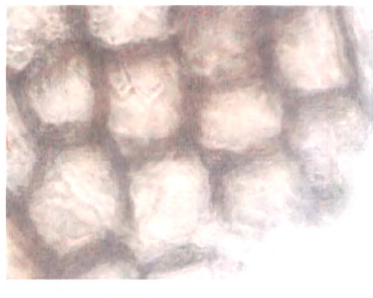
The powdered aerial parts of the plant *P.microphylla* are devoid of trichomes while the aerial parts of *D.volubilis* and *L.reticulata* consisted of multicellular, uniceriate covering trichomes. The powdered aerial part of *L.reticulata* consisted of collapsed cell covering trichomes which were absent in *D.volubilis.* Anomocytic types of

stomatas are present in the samples of aerial parts of *L.reticulata D.volubilis* and *P.microphylla*. The cork cells of *P.microphylla* were hexagonal in shape and striated (Fig.3.3) while that of *L.reticulata* were pentagonal to hexagonal in shape and having thick lignified cell wall (Fig 3.1). Powdered aerial parts of *L.reticulata* consisted of abundant prismatic crystals of calcium oxalate. Stone cells are present as scattered mass, where one or two cells are joined to each other in *L.reticulata* while in *D.volubilis* stone cells are present in the form of groups where the cells are attached to each other and form a raw. *D.volubilis* and *L.reticulata* possess pitted xylem vessels while spiral vessels were seen along with the pitted ones in *P.microphylla.*

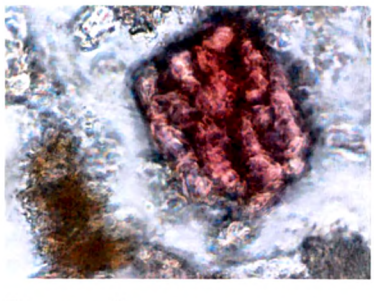
Fig.3. 1 Photomicrograph of microscopic studies of aerial parts of *L.reticulata:*



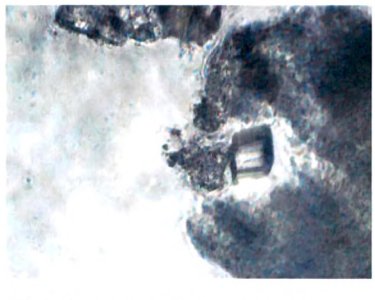
Collapsed cell Trichome



Cork cells



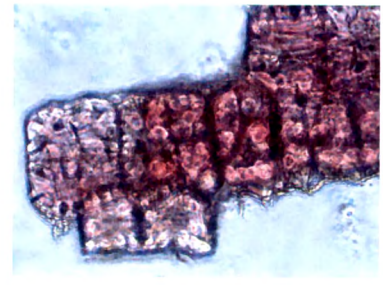
Stone cell



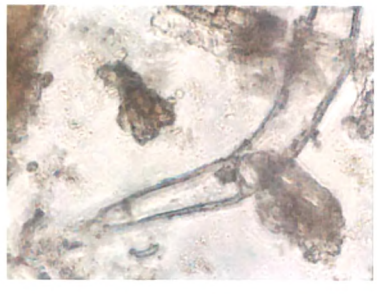
Calcium oxalate crystal

Fig.3.2 Photomicrograph of microscopic studies of aerial parts *D.volubilis:*

Xylem Fiber

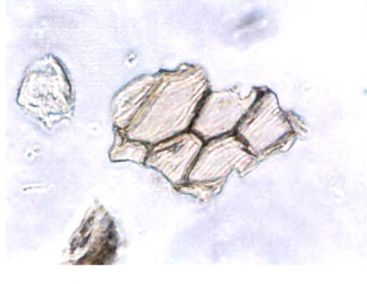


Stone cells

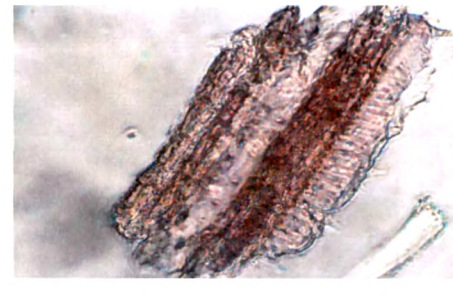


Multicellular Trichome

Fig 3.3 Photomicrograph of microscopic studies of aerial parts of *P.microphylla:*



Cork cells



Vascular tissues and xylem vessel

Table: 3.1Summary of comparative microscopic studies:

Sr. No	Description of the Microscopic structure	Aerial parts of *L.reticulata*	Aerial parts of *D.volubilis*	Aerial parts of *P.microphylla*
1	Stomatal index	6.72	5.81	7.95
2	Palisade Ratio	4.59	5.29	3.59
3	Trichomes	Collapsed cell multicellular trichomes are present	Multicellular covering trichomes are present	Trichomes are absent
4	Cork cells	Rectangular or Polyhedral in shape, Thick lignified cell wall	Same as *L.reticulata*	Cork cells are polyhedral in shape with striations seen in section view
5	Stone cells	Abundant in powder Present in pairs (2475 X 20 μ in size)	Abundant in powder, cells are attached to each other in a long raw. (1025 X 17 μ in size)	Few stone cells are seen

3.2 Phytochemical studies

3.2.1 Preliminary Phytochemical screening of the plant extracts:

Powdered samples of aerial parts of *L. reticulata*, *D.volubilis* and *P.microphylla* were subjected to successive solvent extraction. The consistency, color and percentage extractive values so obtained are tabulated in Table.3.2.

The studies revealed that the maximum extractive value was of methanolic extract of all the extracts among all the extracts. The extracts were then subjected to preliminary chemical tests to detect the presence of various phytoconstituents. Preliminary comparative Phytochemical studies revealed absence of the alkaloids and volatile oil in the plants selected while the flavonoids, triterpenoids, steroids, amino acids, sugars and saponins were found present. The results of the studies performed are tabulated as in Table 3.3

Phytochemical evaluation of the plant extracts may provide the information regarding various types of the phytoconstituents present. Presence or absence of particular type of phytoconstituents in the plants of the interests may be helpful, partly, in development of analytical profile and in differentiation of such controversial plants. The plants studied could not be differentiated from the each other on the basis of such preliminary phytochemical studies.

Table 3.2: Extractive values of successive solvent extraction of aerial parts of *L. reticulata*, *D.volubilis* and *P.microphylla*:

Serial number	Aerial Parts of *L.reticulata*			Aerial parts of *D.volubilis*		Aerial Parts of *P.microphylla*	
	Solvent used for getting extract	Colour and state of extract	Average Extractive value (% w/w on dried wt basis of powdered material)	Colour and state of extract	Average Extractive value (% w/w on dried wt basis of powdered material)	Colour and state of extract	Average Extractive value (% w/w on dried wt basis of powdered material)
1	Petroleum ether	Dark green, and sticky	2.1	Dark green and dry solid	3.12	Dark Green and semi solid, sticky	2.81
2	Benzene	Dark green, sticky	0.98	Dark green and sticky	0.91	Greenish semisolid , sticky	1.15
3	Chloroform	Dark Green and sticky	2.3	Dark Green and sticky	1.68	Dark Green semisolid non sticky	0.83
4	Ethyl acetate	Dark Brown semisolid	6.3	Dark Brown semisolid	3.69	Dark Green , semi solid, sticky	1.79
5	Methanol	Dark Green semisolid	7.2	Dark Green semisolid	8.52	Dark Green , Granular and dry	13.71
6	water	Brown, dry solid	3.2	Yellowish green and dry solid	4.82	Reddish Brown , semi solid , sticky	7.44

Table 3.3: Comparative phytoprofile of the extract in successive solvent extractions:

Serial Number	Class of compound	Aerial Parts of *L.reticulata*						Aerial parts of *D.volubilis*						Aerial Parts of *P.microphylla*					
Extract	---------->	P	B	C	EA	M	W	P	B	C	EA	M	W	P	B	C	EA	M	W
1	Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Carbohydrates and Sugars	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+
3	Phytosterol and Terpenoids	+	+	+	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-
4	Cardiac Glycosides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Amino Acids	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+
6	Saponins	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+
7	Fixed oil	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
8	Flavonoids	-	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	+	+
9	Volatile oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	Phenolics	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+

Key:
P: Petroleum ether, M: Methanol, B : Benzene, W: Water, C: Chloroform, EA : Ethyl Acetate

(+): Presence of specific type of Compounds , (-) Absence of specific type of

3.2.2 Development of Comparative Phytochemical profiles of the plant extracts using HPTLC:

The successive extracts were further subjected to confirmation of phytoconstituents detected in qualitative analysis after postchromatographic derivatization using HPTLC technique.

The reported chromatograms confirmed the nature and content of different phytoconstituents in all the three plant drugs. The extracts of the plants, when subjected to Correlative –TLC (Co-TLC), revealed the presence of some of the compounds, especially of steroidal/triterpenoidal nature on the similar R_f value as shown in Fig 3.4-3.9. The presence of phytosterol, saponins, flavonoids, phenolics, sugar and amino acids in different extracts were also detected and confirmed.

In order to reveal the presence of the phytosterols as detected in Pet. ether and Chloroform extracts qualitatively, the extracts were subjected to HPTLC studies. Both the extracts were first taken up for saponification and the separated unsaponifiable fraction showed the presence of phytosterols. Many other compounds of similar R_f values were also detected. The UV spectra of these compounds showed similarity among the nature of phytoconstituents of both the extracts of all the three plants, as mentioned in Table 3.5 and in 3.6. The extracts of all the three plants showed the presence of one compound at position number 6 in Pet. ether extract and another at position number 1 in Chloroform extract being common while there were considerable similarity of common compounds observed in the Pet.ether extract of *L.reticulata* and *P.microphylla* than that of *D.volubilis*. The content of such compounds; it was higher in *L.reticulata* extract than that of the extract of other two plants.

Ethyl acetate fraction of the methanolic extract revealed the presence of flavonoidal aglycones only, while Ethyl acetate fraction of aqueous extract showed the presence of

triterpenoidal/steroidal and also flavonoidal aglycones. These studies indicated that triterpenoidal/steroidal and flavonoidal glycosides are present in all the selected plants.

Detailed analysis of the chromatograms indicated that there are more four compounds present in the Pet. ether extract of *L.reticulata* and *P.microphylla* on the similar R_f value. The comparison of UV spectra recorded is shown in Fig. 3.5 confirms the structure similarity of these compounds. The area of three common peaks of Pet.ether extract of *L.reticulata* was higher than those of *P.microphylla* indicating the higher content in former drug while in *D.volubilis* they were not present.

The chromatograms were also run using the optimized solvent system mentioned in the Table 3.4 and then scanned at 254 and 366 nm to record UV spectra of the peaks of separated compounds. The spectra obtained, represented a separated constituents of the plant extract/fraction studied. Finally their nature was also found by post chromatographic derivatization.

Similarly, in chloroform extract also, the compounds present on the similar R_f value were in higher amount in case of *L.reticulata* than other two plant extracts. The corresponding UV spectra recorded for the purpose of comparison are shown in fig 3.7.

The methanolic extract of all three plants when partitioned in ethyl acetate, after hydrolysis showed, the presence of different compounds other than those observed in Pet.ether and Chloroform extract. The nature of the compounds revealed to be flavonoidal aglycones as shown in Fig. 3.9 and 3.10.

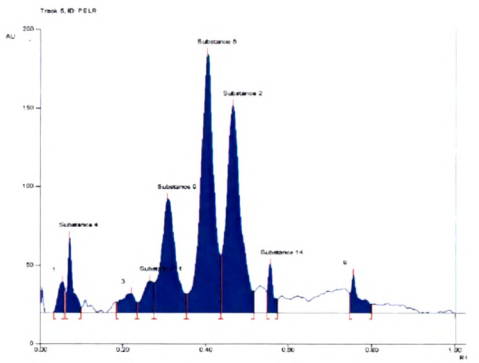
Table: 3.4 Summary of Chromatographic conditions for development of Chromatograms using HPTLC:

Sr No.	Extract	Solvent system	Type of Phytoconstituents present	Detection method
1.	Pet ether extracts	Toluene:Ethylacetate :Formic Acid (90:10:1.0)	Steroids and Triterpenoids	Fluorescent detection and then Post chromatographic derivatization using LB reagent.
2	Chloroform Extract	Toluene:Ethylacetate :Formic Acid (90:10:1.0)	Steroids and Triterpenoids	Fluorescent detection and then Post chromatographic derivatization using LB reagent.
3	Ethyl acetate extracts	Et Ac: MeOH: Water (10:1.3:0.1)	Flavonoidal aglycone	Fluorescent detection and then Postchromatographic derivatization using NP reagent
4	Methanol extract	Et Ac: MeOH: Water (10:1.3:0.1)	Flavonoidal glycosides	Fluorescent detection and then Postchromatographic derivatization using NP reagent
5	Aqueous Extract (After Hydrolysis)	Et Ac: MeOH: Water (10:1.3:0.1)	Flavonoidal glycosides	Fluorescent detection and then Postchromatographic derivatization using NP reagent
		Toluene:Ethylacetate :Formic Acid (90:10:1.0)	Steroids and Triterpenoids	Fluorescent detection and then Post chromatographic derivatization using LB reagent.

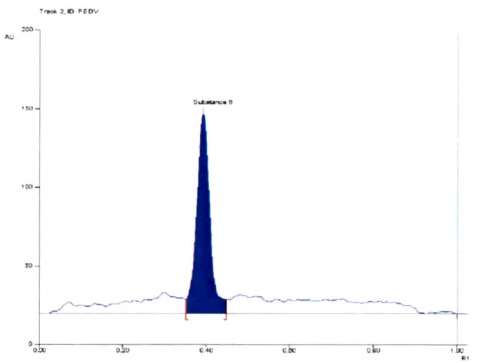
LB Reagent: Liebermann Burchard Reagent

NP Reagent: Natural Product /Poly Ethylene Glycol Reagent

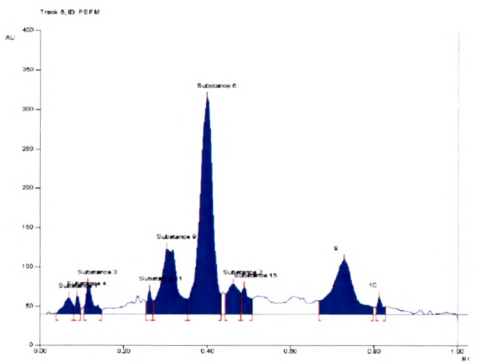
Fig.3.4 Chromatogram of triterpenoidal and steroidal compounds in Pet Ether extract of Aerial parts of the Plants:



(a)L.reticulata



(b)*D.volubilis*

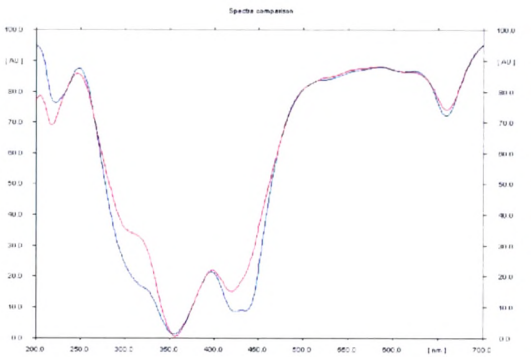


(c)*P.microphylla*

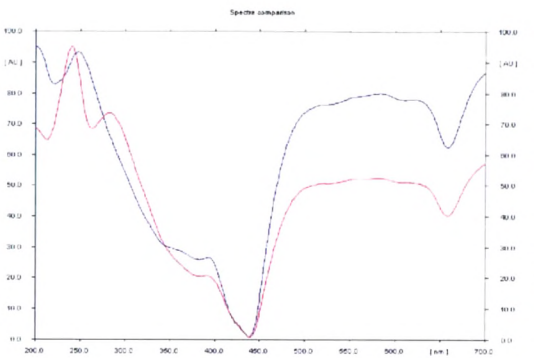
Table: 3.5 Comparative Peak Analysis of triterpenoidal and steroidal compounds in Pet Ether extract:

Sr.No	Extract	R_f	Peak Area	% Area	Substance code	**Chromatographic Conditions:**
1	PELR	0.07	413.1	4.07	Substance 4	Stationary Phase: SilicagelG 60 F254, Mobile phase:Toluene:Ethyl Acetate:Formic Acid (9:1.0:0.1,v/v/v), Post Chromatographic Derivatization: LB Reagent, Scanning Wavelenght:366 nm.
		0.23	368.0	3.62	Substance 11	
		0.28	1716.9	16.90	Substance 9	
		0.36	3575.6	34.60	Substance 6	
		0.44	3045.5	29.97	Substance 2	
2	PEDV	0.35	2733.80	100	Substance 6	
3	PEPM	0.08	155.6	1.24	Substance 4	
		0.25	226.4	1.76	Substance 11	
		0.27	2198.8	17.12	Substance 9	
		0.35	5681.4	44.246	Substance 6	
		0.44	743.3	5.79	Substance 2	

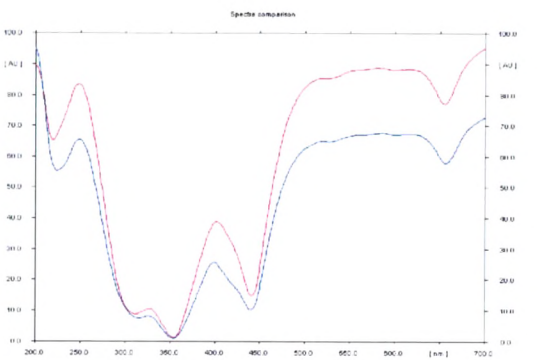
Fig 3.5 Comparative UV spectra of the peaks represents the spots on the same R_f values:



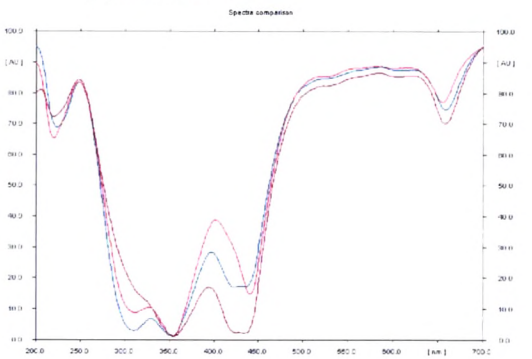
(a)Substance 4



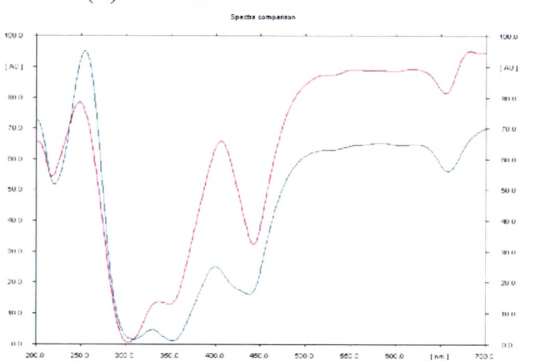
(b)Substance 11



(c)Substance 9

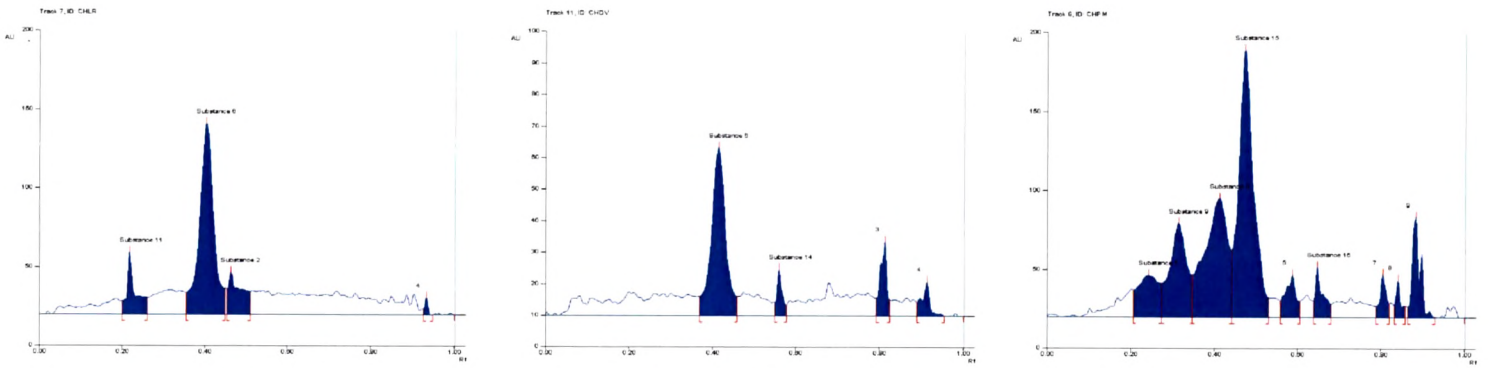


(d)Substance 6



(e)Substance 2

Fig.3.6 Chromatogram of triterpenoidal and steroidal compounds in Chloroform extract of Plants:

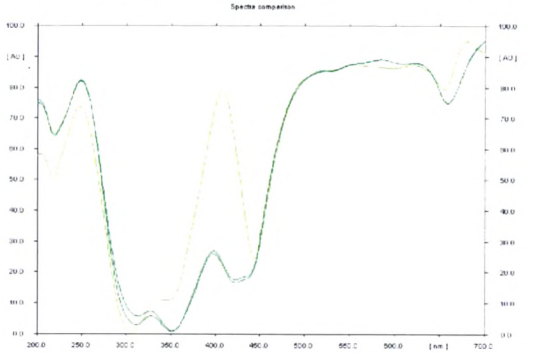


(a)L.reticulata *(b)D.volubilis* *(c)P.microphylla*

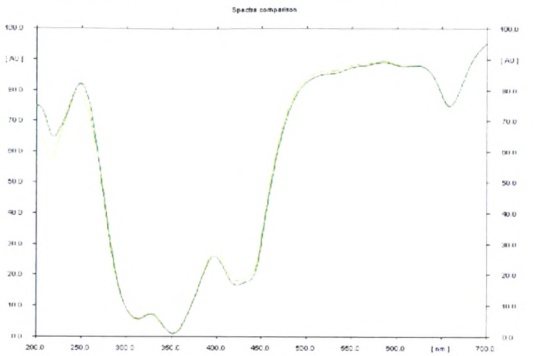
Table: 3.6 Comparative Peak Analysis of triterpenoidal and steroidal compounds in Chloroform extract:

Sr.No	Extract	R_f	Peak Area	% Area	Substance code	**Chromatographic Conditions:** Stationary Phase: SilicagelG 60 F254, Mobile phase:Toluene:Ethyl Acetate:Formic Acid (9:1.0:0.1,v/v/v), Post Chromatographic Derivatization: LB Reagent, Scanning Wavelenght:366 nm.
1	PELR	0.36	2876.8	70.31	Substance 1	
2	PEDV	0.36	1146.1	51.17	Substance 1	
		0.63	360.6	16.10	Substance 2	
3	PEPM	0.35	2842.7	24.10	Substance 1	
		0.64	413.7	3.51	Substance 2	

Fig 3.7 Comparative UV spectra of the peaks represents the spots on the same R_f values:

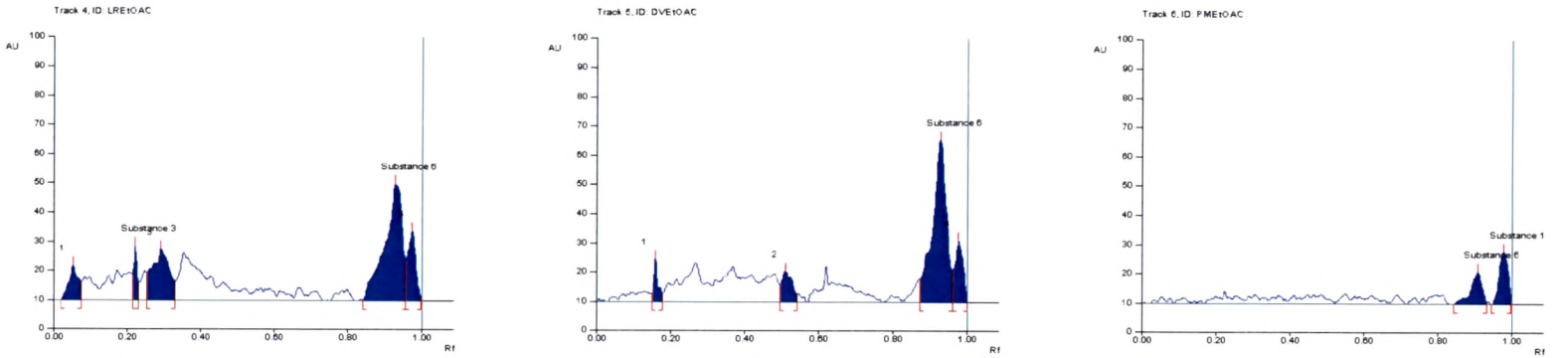


(a)Substance 1



(b)(aSubstance 2

Fig: 3.8 Chromatogram of Flavonoidal Compounds in Ethyl Acetate extract of the selected plants:

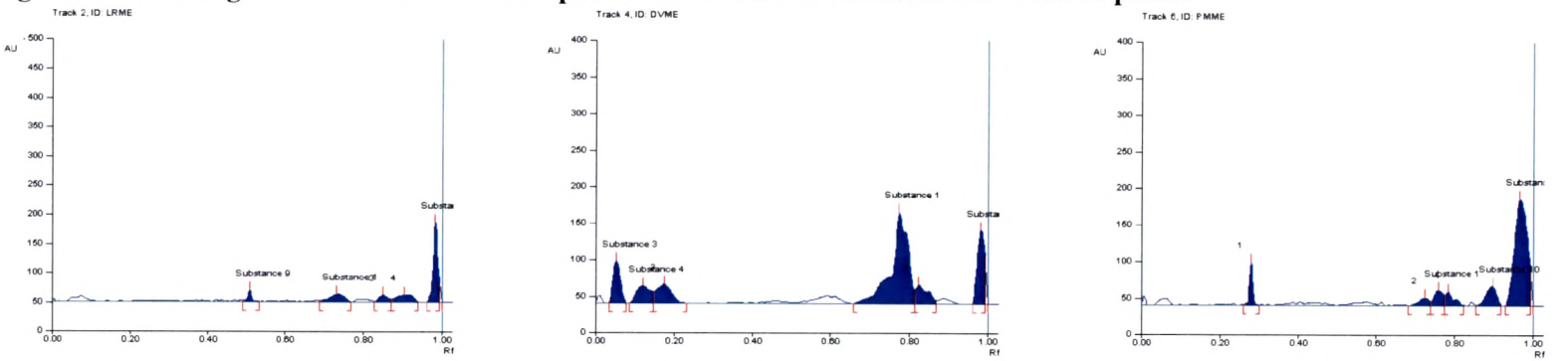


(a)L.reticulata *(b)D.volubilis* *(c)P.microphylla*

Table: 3.7 Comparative Peak Analysis of Flavonoidal compounds in ethyl acetate extract:

Sr.No	Extract	R_f	Peak Area	% Area	**Chromatographic Conditions:**
1	ETOACLR	0.21	153.5	5.50	Stationary Phase: SilicagelG 60 F254, Mobile phase:Ethyl Acetate: Methanol: Water (10:1.3:0.1,v/v/v), Post Chromatographic Derivatization: NP Reagent Scanning Wavelenght:366 nm.
		0.25	616.3	22.08	
		0.84	1408.2	50.44	
		0.96	389.3	13.95	
2	ETOACDV	0.15	128.0	5.89	
		0.50	221.4	9.85	
		0.87	1557.4	69.28	
3	ETOACPM	0.84	253.7	44.30	
		0.95	318.9	55.70	

Fig: 3.9 Chromatogram of Flavonoidal Compounds in Methanol extract of the selected plants

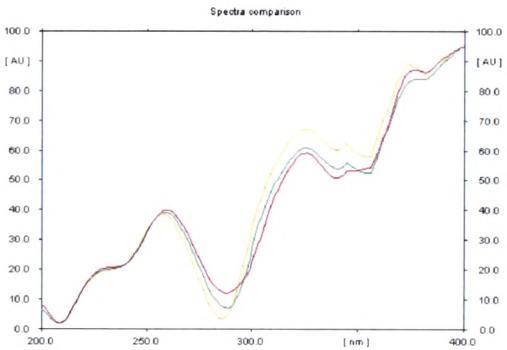


(a)L.reticulata *(b)D.volubilis* *(c)P.microphylla*

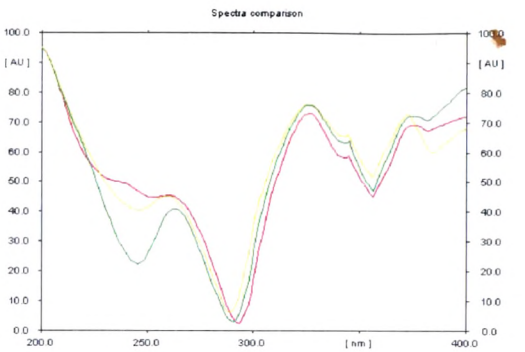
Table: 3.8 Comparative Peak Analysis of flavonoidal compounds in methanol extract

Sr.No	Extract	R_f	Peak Area	% Area		**Chromatographic Conditions:**
1	MELR	0.51	135.6	6.56		Stationary Phase: SilicagelG 60 F254, Mobile phase:Ethyl Acetate: Methanol: Water (10:1.3:0.1,v/v/v), Post Chromatographic Derivatization: NPReagent, Scanning Wavelenght:366 nm.
		0.73	336.2	16.26	Peak 1	
		0.85	183.4	8.87		
		0.91	344.9	16.68		
		0.98	1067.5	51.63	Peak 2	
2	MEDV	0.12	575.6	7.85		
		0.18	658.9	8.98		
		0.77	3602.2	44.10	Peak 1	
		0.82	557.4	7.60		
		0.98	1208.3	16.47	Peak 2	
3	MEPM	0.28	316.4	6.57		
		0.73	192.4	4.0	Peak 1	
		0.76	337.8	7.02		
		0.79	271.3	5.64		
		0.97	3210.0	66.58	Peak 2	

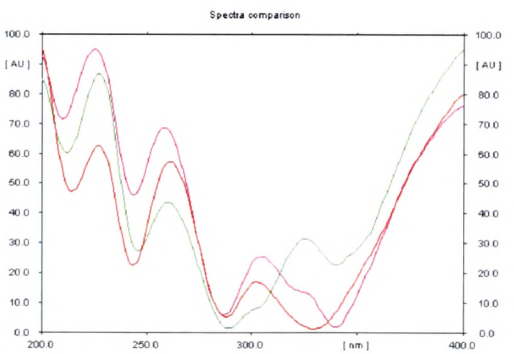
Fig: 3.10 Comparative UV spectra of the peaks represents the spots on the same R_f values:



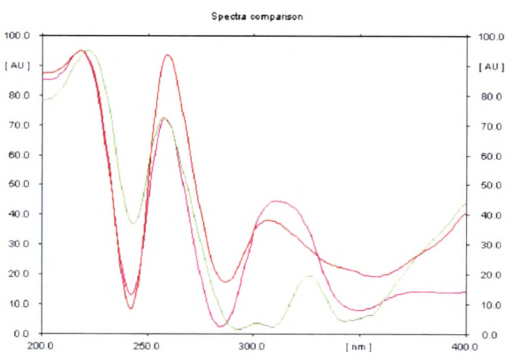
(a)Peak 1 Before Derivatization



(b) Peak 1 After Derivatization



(c)Peak 2 Before Derivatization



(d) Peak 2 After Derivatization

The compounds representing Peak 1 and Peak 2 were common in methanolic extract of all the three selected plants (Fig 3.9). The comparative UV spectra of these compounds are shown in Fig. 3.10 after and before derivatization.

The results justify that *P.microphylla* and *D.volubilis* can serve as substitutes of *L.reticulata* as far as the chemical constituents of steroidal / triterpenoidal and flavonoidal nature are concerned.

However, interestingly, there have been no similarities observed in the flavonoidal components of ethyl acetate and aqueous extracts of all three different plant extracts indicating that the flavonoids present in these extracts are different in nature. This may be quite uncommon that the plants of same family do not show similarity in constituents of common nature. These extracts were hence subjected to quantification of these components. The results, however, did not have any similarity in content also.

3.3 Development of standardization parameters for different plant drugs:

L.reticulata is recommended in different diseases and many times is substituted by *P.microphylla* or by *D.volubilis* in practice. Although, the identification standards for *L.reticulata* are available[34], such systematic standardization parameters are yet to be developed for other two plants used as substitutes. The experiments were, therefore, planned to evolve parameters for establishing the standard value that could be used for comparing these three plant drugs.

3.4.1 Determination of various physico chemical constants as per WHO guidelines:

The air dried, powdered plant materials were subjected to the determination of various physico chemical determinations as per the methods described in WHO guidelines for the purpose.

The content of moisture and foreign matter found were within the prescribed limits without bitterness and having low values of haemolytic and foaming index. The amount of heavy metals determined was within the prescribed limits. The total bacterial and fungal count was also found within the prescribed limits. The Ash value, Acid Insoluble Ash values, Water soluble extractive value were also found to be in the moderate range. Absence of unusually high values of such physical determinations confirms the absence of the impurities; especially those originate from earthy matter. Since the plant materials were collected from the wild habitats and from the place far from the site of any radioactive elements, the experiments involving the determination of pesticides and radioactive entities were skipped. Volatile oils were found absent in all three plants. The comparative account of various physico chemical parameters is given in Table 3.9.

Table: 3.9 WHO parameters for aerial parts of *L.reticulata, D.volubilis* and *P.microphylla:*

Sr No.	WHO Parameters	Aerial parts of *L.reticulata*	Aerial parts of *D.volubilis*	Aerial parts of *P.microphylla*
		Results	Results	Results
1	Moisture content	5.8% w/w	8.9%w/w	2.1 % W/W
2	Foreign matter	0.058 % w/w of dried material	0.041 % w/w of dried material	0.023 % w/w of dried material
3	Ash value(%w/w)	9.604 ±0.260	6.632 ±0.260	11.06 ± 0.723
4	Acid Insoluble Ash value (%w/w)	0.634± 0.045	0.933 ±0.03	1.712 ±0.110
5	Water Soluble Ash Value	0.07%w/w	0.04%w/w	0.05%w/w
6	Extractive Value	5.605 ± 0.543	20.109±0.942	14.64 ±0.394
7	Powder fineness	All the particles pass through a No. 710 sieve, and not more than 40% through a No. 180 sieve	All the particles pass through a No. 710 sieve, and not more than 40% through a No. 180 sieve	All the particles pass through a No. 710 sieve, and not more than 40% through a No. 180 sieve
8	Foaming index	Less than 100	Less than 100	Less then 100
9	Hemolytic activity	100mg/ml of water extract not show haemolysis	100mg/ml of water extract not show haemolysis	100mg/ml of water extract not show haemolysis
10	Heavy Metal Content	Within the prescribed limits		
11	Total bacterial count	$4.7X10^{3}$ /g	$8.1X10^{3}$ /g	$9.8X10^{3}$ /g
12	Total fungal count	$79 X 10^{2}$ /g	$46 X 10^{2}$ /g	$73 X 10^{2}$ /g

3.3.2 Quantitative Determination of various Inorganic ions in plant materials:

The estimation of metallic content is important, if the sample of the powdered plant material is processed in any of the commercial processing house. Various processes like, Drying, Pulverizing, Packaging may incorporate the inorganic impurities in the product. As the metal ions may act as catalyst or may directly initiate degradation cycle of the phytoconstituents, it is of vital importance to develop the qualitative and quantitative profiles of the inorganic content present in plant materials. The plants are collected from costal region where the soil is generally heavily loaded with various inorganic salts. These plants grow, though, in many regions but the market supply come majority from the costal region where there is alteration in inorganic content which could give a differentiation feature for standardization.

The Ash obtained from aerial parts of three selected plants was subjected to the estimation of various metal ions using Atomic Absorption Spectroscope. The results obtained are summarized as in Table 3.10

The results indicated maximum amount of the calcium and sodium is present in *P.microphylla*. The results also suggested the absence of unusually high accumulation of the inorganic content in the plants studied. The plant *P.microphylla* is a mangrove, generally found near the sea coast. It is, therefore, not surprising to find comparatively higher amount of sodium and potassium in these plants. Calcium was present in all the plants in the amount that could not be estimated quantitatively. *L.reticulata* and *P.microphylla* are rich in the magnesium content while *P.microphylla* consists of the maximum amount of zinc. Manganese was estimated in trace amounts in all three plants.

As the heavy metals are generally accumulated in plants, the estimation/detection of such metal is mandatory as per the regulatory guidelines to avoid probable toxic effects due to the usage of such metals. Results indicated absence of such heavy metals in the plants analyzed.

Table3.10: Estimation of various elemental ions from plants:

Element	Standard used	Calibration range (µg/ml)	Wavelength used (nm)	Sensitivity (µg/ml)	*L.reticulata*	*D.volubilis*	*P.microphylla*
					Amount present (Average± SD) (µg/ml)	Amount present (Average± SD) (µg/ml)	Amount present (Average± SD) (µg/ml)
Sodium	NaCl	0.4 – 1.5	589.6	0.008	0.762	0.099	1.19
Potassium	KCl	0.4 – 1.5	766.5	0.008	0.928	0.264	1.027
Magnesium	Mg metal	0.1 – 0.4	285.2	0.02	1.872	1.101	2.249
Manganese	$MnSO_4$	1.0 – 1.4	279.8	0.02	0.036	0.077	0.065
Cobalt	$CoCl_2$	2.5 – 9.0	240.7	0.05	----	----	----
Copper	Cu metal	1.0 – 5.0	324.7	0.025	----	----	----
Zinc	$ZnSO_4$	0.4 – 1.5	213.9	0.008	0.034	0.028	0.705
Lead	$Pb(NO_3)_2$	2.5 – 20.0	217	0.06	-----	-----	-----
Mercury	$HgSO_4$	73 - 290	253.7	1.6	--------	-----	------

3.3.3 HPTLC finger printing profile of Methanolic extract of selected plants:

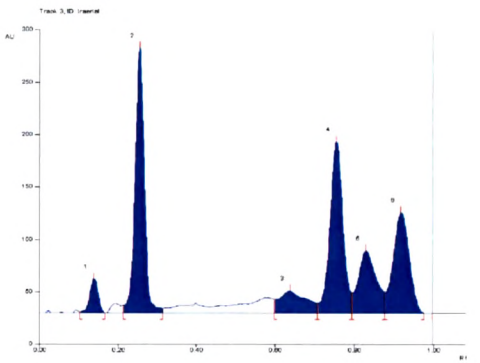
Direct methanolic extract of the different parts of all the three selected plants was subjected to generate HPTLC fingerprint profile represented as chromatograms.

Methanolic extract of root and aerial parts of *L.reticulata* when separately subjected to HPTLC (Fig. 3.11) showed presence of one compound common at R_f 0.80.The methanolic extract of root and aerial parts of *L.reticulata* also showed presence of compounds with a variation in the content as depicted by the peak areas. Majority of them are common. Hence, the complete profile could only, serve as preliminary tool for identification.

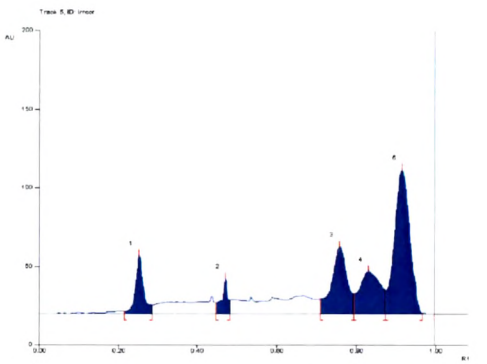
In case of methanolic extract of aerial parts and roots of *D.volubilis* and *P.microphylla* are concerned, the chromatograms again provided a profile of separated components in a fixed number that could serve as means of identification.(Fig: 3.12,3.13)

When the chromatograms were compared among each other the presence of only one compound was found in common between R_f 0.20-0.25.

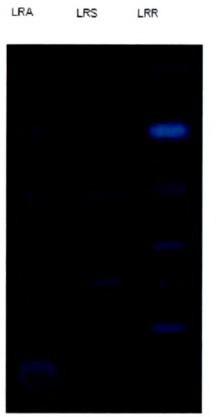
Fig. 3.11 Chromatographic Fingerprint of methanolic extract to differentiate aerial parts and root of *L.reticulata* from each other:



(a)Aerial parts of *L. reticulata*



(b) Roots of *L.reticulata*

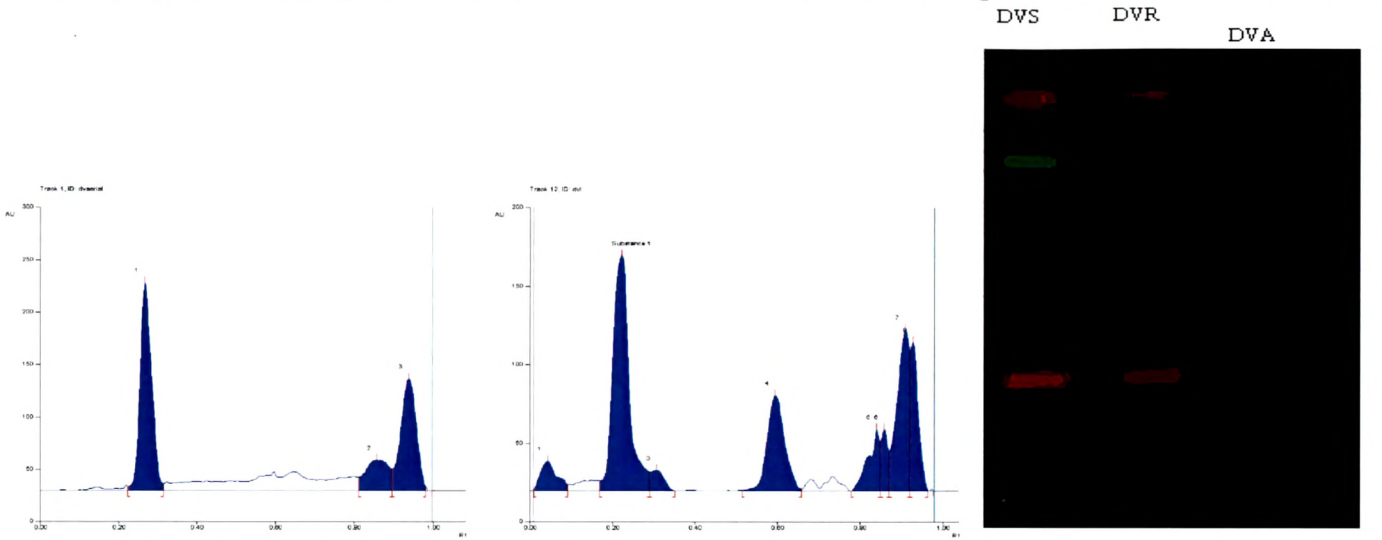


(c)Photograph of TLC plate after derivatization

Table: 3.11 Analysis of chromatogram of Aerial parts and roots of *L.reticulata:*

Sr. No	Extract	R_f Value	Peak Area	% Area	**Chromatographic Conditions:**
1	LRA	0.10	617.7	3.54	Stationary Phase: SilicagelG 60 F254
		0.21	5186.2	29.69	
		0.60	1262.1	7.23	Mobile phase:Toluene: Ethyl Acetate: Formic Acid
		0.71	4665.8	26.71	
		0.80	2359.4	13.51	(9:4:0.05, v/v)
		0.88	3375.2	19.32	
2	LRS	0.55	195.3	1.15	Scanning Wavelenght:366 nm
		0.79	16718.9	98.85	
3	LRR	0.25	665.9	9.93	Post Chromatographic Derivatization: 10% Sulphuric acid and Heating for 10 min and photograph of the plate was recorded
		0.47	294.0	4.38	
		0.76	1453.4	21.67	
		0.80	1260.7	18.80	
		0.92	3031.3	45.22	

Fig.3.12 Chromatographic Fingerprint of methanolic extract to differentiate aerial parts and roots of *D.volubilis* from each other:



(a)Aerial parts of *D.voluhilis* (b) Roots of *D.volubilis* (c)Photograph of TLC plate after Derivatization

Table: 3.12 Analysis of chromatograms of Aerial parts and roots of *D.volubilis:*

Sr. No	Extract	R_f Value	Peak Area	% Area
1	DVA	0.27	5064.9	49.59
		0.86	1521.2	14.89
		0.94	3627.2	36.52
2	DVS	0.22	4720.7	36.54
		0.59	2032.3	15.73
		0.84	857.9	6.64
		0.91	2372.9	18.37
		0.93	1461.8	11.32
3	DVR	0.22	8107.6	35.70
		0.61	4565.70	20.16
		0.81	743.0	3.28
		0.85	195.4	0.86
		0.92	3872.1	17.10

Chromatographic Conditions:

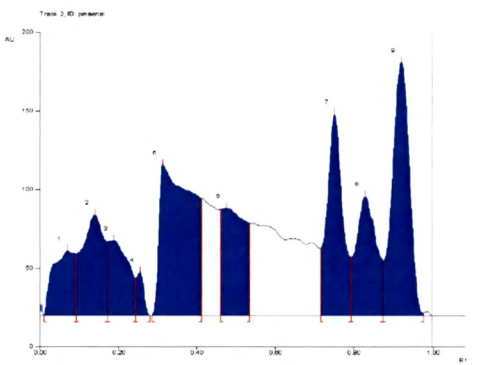
Stationary Phase: SilicagelG 60 F254

Mobile phase:Toluene: Ethyl Acetate: Formic Acid (9:4:0.05, v/v)

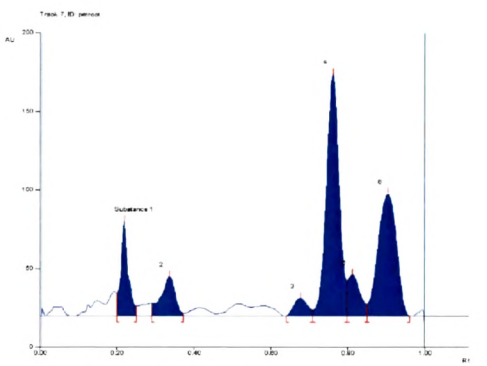
Scanning Wavelenght:366 nm

Post Chromatographic Derivatization: 10% Sulphuric acid and Heating for 10 min and photograph of the plate was recorded.

Fig.3.13 Chromatographic Fingerprint of methanolic extract to differentiate aerial parts and roots of *P.microphylla* from each other:



(a)Aerial parts of *P.microphylla*



(b)Roots of *P.microphylla*

*(c)*Photograph of TLC plate after derivatization

Table: 3.13 Analysis of chromatograms of aerial parts and roots of *P.microphylla:*

Sr. No	Extract	R_f Value	Peak Area	% Area	**Chromatographic Conditions:**
1	PMA	0.19	2141.9	6.68	Stationary Phase: SilicagelG 60 F254
		0.25	456.3	1.42	
		0.31	6889.0	21.50	Mobile phase:Toluene: Ethyl Acetate: Formic Acid (9:4:0.05, v/v)
		0.48	11.29	3617.3	
		0.75	4482.7	13.99	Scanning Wavelenght:366 nm
		0.83	3328.6	18.76	
2	PMR	0.22	988.6	9.66	Post Chromatographic Derivatization: 10% Sulphuric acid and the image of the
		0.34	782.0	7.64	
		0.68	343.8	3.36	plate was recorded
		0.76	4297.4	42.0	
		0.81	709.8	6.94	
		0.91	3109.7	30.39	

3.3.4 Development of methods for detection and estimation of some markers in selected plant materials.

The extracts from botanicals are generally found with a variable consistency so far as the chemical constituents are concerned. These extracts, therefore, may be standardized on the basis of either content of chemical compound or the bioactivity exhibited by them.

In order to proceed for chemical standardization, some important compounds are identified and selected as markers. These marker compounds should have a consistent presence although, in a variable content in the extracts. These extracts are processed on the basis of constant value of these markers to become standardized extract. Identification of marker, therefore, is an important task, generally achieved by fingerprinting technique and correlative TLC in case of compounds of known chemical nature. So far as unknown compounds are concerned, components are first detected qualitatively and subsequently separated, isolated and finally quantified after due characterization and structure elucidation. These marker compounds are helpful not only in assessing the extracts but also provide a measure of quality assurance of raw materials and finished products. The experiments were, therefore, designed to detect few of such chemicals which could be utilized as markers for standardization of the selected plant drugs.

3.3.4.1 Detection and estimation of Salicylic acid and Stigmasterol in the plant extracts using HPTLC:

Since the presence of salicylic acid was reported in *P.microphylla*, used traditionally in the treatment of inflammation, cuts and burns topically and also is a substitute of *L.reticulata* which is also utilized similarly[30], having *in vitro* antimicrobial[47] activity but did not have any reports of presence of biologically active compounds. The experiments, therefore, were planned to detect the presence of biologically active components from the plant extracts.

Presence of salicylic acid from the extract of *L.reticulata* was detected in Co-TLC studies. The UV spectra of the corresponding spots were recorded at three positions 1. Peak start 2. Peak maxima 3. Peak end for their superimposability using HPTLC.(Fig. 3.14)

The result indicates the presence of salicylic acid for the first time in the extract of the aerial parts of the plant *L.reticulata* while in case of *D.volubilis*, salicylic acid was absent.

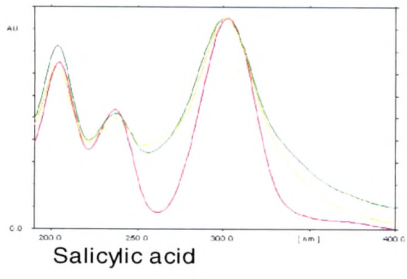
Stigmasterol was reported in *L.reticulata* being responsible for its lactogenic potential. The aerial parts of *L.reticulata, P.microphylla*, being the substitutes of and *D.volubilis* were also subjected to detection of stigmasterol using Co-TLC. The overlapping spectra of standard and the spectra of the corresponding sample track were recorded (Fig. 3.14), confirmed the presence of Stigmasterol in these plants.

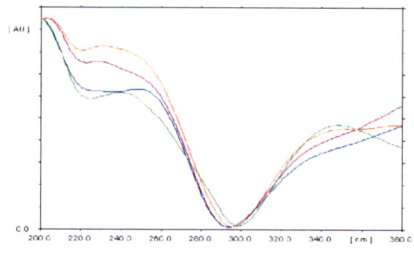
Traditionally, the selected plants are used in the treatment of cuts and burns[30]. Presence of Salicylic acid in plant extracts, therefore, confirms the traditional claim and hence Salicylic acid can be termed as biomarker helpful in the assessment of quality of the plant material employed for such purposes.

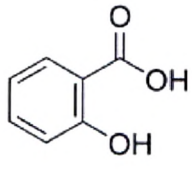
The plant *L.reticulata* is used as lactogenic in lactating mothers. Ether fraction and Stigmasterol isolated from the ether fraction of Pet ether extract of the plant *L.retciulata* showed lactogenic potential in rats suggested the potential of Stigmasterol as lactogenic agent in such plants[40]. The structure similarity of the phytosterol with steroidal hormone may be responsible for such physiological response. Stigmasterol, as present in the plants when quantified, represents the content of phytosterols which may be responsible for lactogenic potential of plant. The compound like Stigmasterol, widely distributed in higher plants, may not be accepted as marker as such, but when the special consideration is taken to the lactogenic potential of the plant extracts, it can be selected as one of the chemical markers,

which represent the chemical integrity of particular group of compounds responsible for biological potential of the plants.

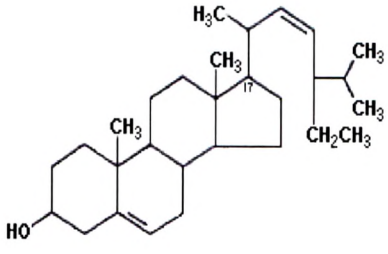
Fig 3.14 Overlapping UV spectra of salicylic acid and stigmasterol with the corresponding spots in sample track







Salicylic acid



Stigmasterol

Isolation/Detection of bioactive phytoconstituents from the medicinal plants responsible for biological actions is very important on regulatory point of view. Such compounds after proper justification may be considered as biological marker in assessment of quality of the plants and therapeutic products of the plants. The standardization of the plants is generally performed to assure the presence of biologically important component and uniformity of content of such compounds to get reproducible biological response. The estimation of markers, thus, becomes important aspect of standardization procedure. Presence of Stigmasterol and Salicylic acid was confirmed in the selected plants and their presence could

be correlated with the traditional therapeutic claims. The plants, therefore, evaluated for the content of both the marker compounds.

Fig: 3.15 Detection and Estimation of Salicylic acid:

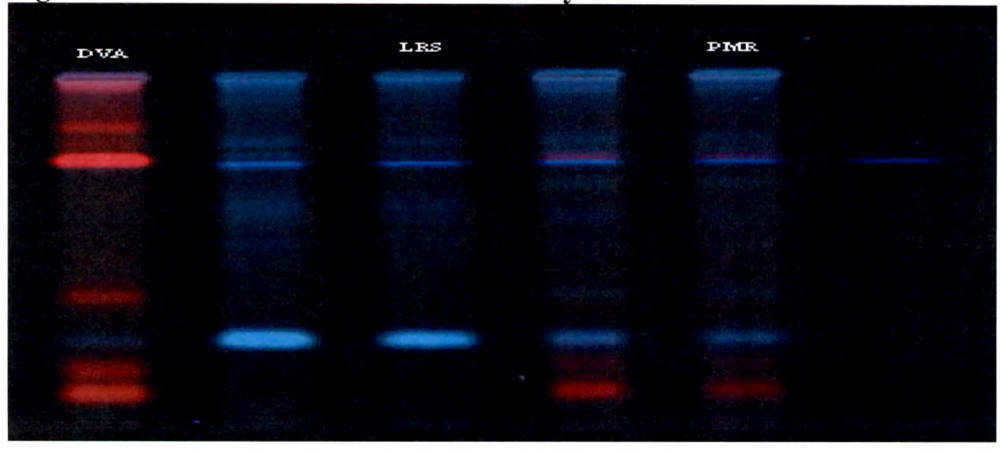


Fig:: 3.16 Detection and Estimation of Salicylic acid:

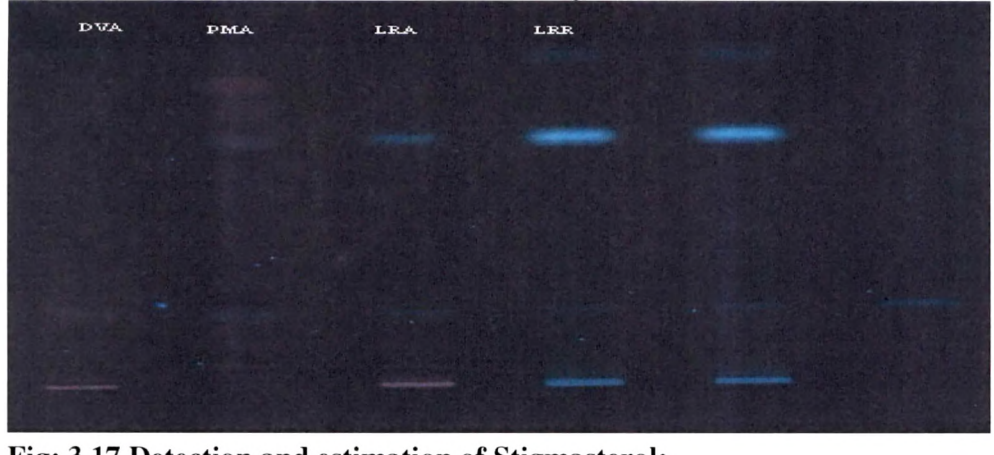
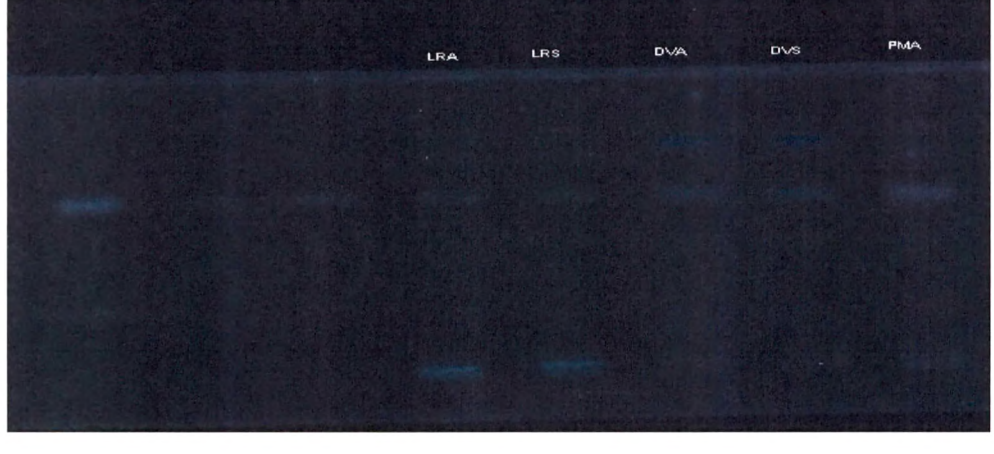


Fig: 3.17 Detection and estimation of Stigmasterol:



3.3.3.4 Estimation of Salicylic acid and Stigmasterol in the Plant extracts:

Although, various methods are available to estimate both stigmasterol and salicylic acid using HPLC and GC, these methods, pose difficulty in selection of stationary and mobile phases and sample preparation technique in case of plant extracts containing multi components and therefore are usually used for estimating a single compound. Some methods are already reported for estimating salicylic acid[109] and stigmasterol[110] in synthetic intermediates and herbal extracts respectively using HPTLC.

However there are no reports regarding a method on HPTLC which could detect both these compounds in a complex matrix using single mobile phase and TLC run. The present study was aimed towards development of a simple, accurate and precise analytical method to detect and estimate both the markers from the selected plants using HPTLC for routine analysis.

A reported solvent system, Toluene: Ethyl acetate: Formic acid (9.0:1.0:0.1, v/v/v) was adopted for detection of stigmasterol from plant sources. Preliminary studies suggested good resolution of both the markers using this solvent system but R_f value of salicylic acid was too low for quantitative analysis. The method, therefore, was modified by altering the composition. The developed system Toluene: Ethyl acetate: Formic acid (9.0:4.0: 0.1, v/v/v) required higher level of tank saturation. The method finally adopted was Toluene: Ethyl acetate: Formic acid (9.0:4.0: 0.05, v/v/v), which required comparatively moderate level of tank saturation. The optimized mobile phase could resolve both the compounds at reasonably apart from each other and the bands obtained were compact too. R_f values obtained for salicylic acid and stigmasterol were 0.25± 0.009 and 0.59 ± 0.01 respectively.

Presence of the polar component like formic acid along with non polar solvent created the problem of fronting of the spots during the development of TLC plate. The spots near to the edge of the plate were at higher R_f values than the corresponding spots towards the centre.

The problem of fronting arises due to difference in the evaporation rate of the solvents at room temperature. The part role of the vapor of solvents in separation of the component is known and such inhomogeneous composition of the components in the vapor of the tank created fronting phenomenon. It was observed that as the saturation increased R_f value of salicylic acid decreased markedly. Appropriate saturation is required to develop a system with reproducible R_f value of the spots of the interest. The experiments, thus, were performed to optimize the R_f value and chamber saturation. Another factor which might alter the reproducibility was heating time after derivatization of the plate with methanolic sulfuric acid. Steroidal moieties fluoresce after heating, when postchromatogrphically derivatized with methanolic sulfuric acid. The minimum heating period should be mentioned which is requires to complete the reaction. The plates were scanned at different time intervals ranges from 5-15 minutes at $\overline{100}^0$ C at the interval of 2 minutes. The time period after which the intensity of the spot remained constant was selected as the heating time of the plate. The product developed after the postchromatographic derivatization with sulfuric acid is not considered to be stable in presence of atmospheric oxygen and the intensity of the spot decreases with the time. The experiments were performed to establish the time period for which the spot intensity has not been decreased.

Salicylic acid can be detected at 200nm and 306 nm using Deuterium lamp. To achieve more specificity in the estimation fluorescent mode of detection was selected. Salicylic acid fluoresces at 366 nm (mercury lamp) but couldn't be detected by scanner while the K 400 filter was selected. The plate was then scanned at various scanning wavelengths ranging from 250-370nm in fluorescence mode at the interval of 10 nm. The maximum response was obtained at 300 nm; hence, this wavelength was selected for detection and estimation of salicylic acid. Stigmasterol absorbs light at 207 nm in UV region. The intensity of the light

emitted from lamp is very low at this wavelength hence the sensitivity level required could not be achieved. Postchromatographic derivatization of the plate was performed using 10 % v/v sulphuric acid. The plate was heated for 10 min at 110^0 C on heater and then scanned at 366 nm in fluorescence mode using mercury lamp.

The results of robustness studies suggested that the amount of formic acid in the proposed system is critical. Alterations in the relative proportion of formic acid affect the miscibility, sharpness of salicylic acid spot and separation of the compounds of the interest. Alteration in ethyl acetate proportion in the system did not affect the resolution much, except the alteration in R_f values. The results were not affected much by altering the other parameters except the saturation timing for the tank. As saturation time increased R_f values for salicylic acid decreased. Saturation time less than 30 min could not be able to prevent the fronting effect. Any volume less than 15 ml might develop the lesser amount of saturation in the tank within the 30 min period and hence the fronting effect was observed.

Heating time of the plate is very critical. Heating of the plates beyond 15 min resulted in complete charring of the spots. 10 min is the optimistic time period for heating. The area under the peak remains unaltered up to 20 min when plates were exposed to the room temperature. It is preferable that after cooling the plates should be scanned immediately. Salicylic acid was stable for 30 min on TLC plate at room temperature on the plate. The temperature of the area was maintained constant at 27^0 C throughout the experiments.

The standard deviation of peak areas was calculated for each parameter and %RSD was found to be less than 2.0%. Low RSD values indicated robustness of the method.

Calibration range selected for the present experiment was 10-120 ng/spot and 100-1400ng/spot respectively for stigmasterol and salicylic acid. Peak areas and concentrations were subjected to least square linear regression analysis to calculate the calibration equation

and correlation coefficients. Linear Regression coefficient was found to be 0.9927 ±0.002 and 0.9953 ± 0.002 in case of salicylic acid and stigmasterol respectively. The linearity of calibration graphs and adherence of the system to beer's law was validated by high value of correlation co-efficient and Standard Deviation (SD) for intercept value. The results are tabulated as in Table3.14.

The linearity of calibration graphs and adherence of the system to beer's law was validated by high value of correlation co-efficient and Standard Deviation (SD) for intercept value.

The proposed method when employed for estimation of the amount of both the components in extracts spiked at three different levels afforded recovery in the range of 90-110% as listed in Table 3.16

Limit of detections were found to be 0.8 ng/ spot and 6.0 ng/spot in case of stigmasterol and salicylic acid respectively. Limit of quantifications were 10 ng/spot-120 ng/spot and 100 ng/spot to 1400 ng/spot in the case of stigmasterol and salicylic acid respectively.

The results shown in Table 3.15 are the data of intra day and inter day variation of both the components at three different levels. *The* % RSD for within and day-to-day variation was found to be less than 2.0% in all cases.

Result of recovery studies and interday-intraday analysis proves the accuracy and precision respectively of the developed analytical method.

The purity of the spot was confirmed by overlaying the spectra of both the standards with the spectra of samples recorded on TLC plates in UV region with the help of scanner. (Fig 3.14)

The results confirm the presence of Salicylic acid in *L.reticulata*. Salicylic acid could not be detected from *D.volubilis* extract. All the three plant possess stigmasterol. The maximum amount of salicylic acid was present *in* aerial parts of *P.microphylla* while the maximum

amount of stigmasterol was present in aerial parts of *L.reticulata*. The comparative account of the content of salicylic acid and stigmasterol is shown in Table 3.17.

Fig.3.18 Peak of Salicylic acid and Stigmasterol in track of standard and sample:

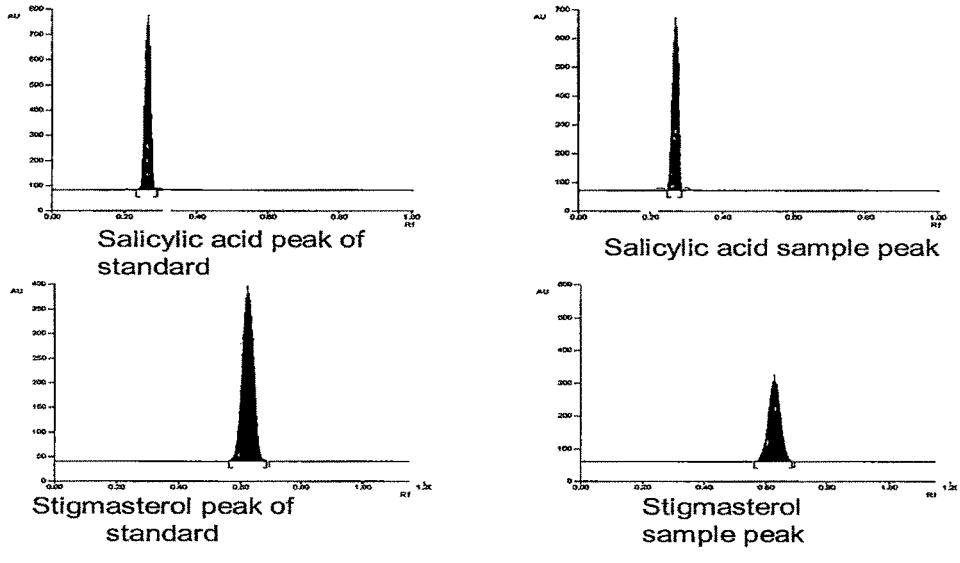


Table: 3.14 Linear Regression Data of calibration curve for salicylic acid and Stigmasterol:

	Linearity range (ng/spot)	r ± S.D.	Slope ± S.D.	Confidence limit of slope	Intercept ± S.D.	Confidence limit of intercept
Salicylic acid	100-1400	0.9950± 0.001	5.566± 0.121	± 0.096	1877.80± 10.61	± 8.489
Stigmasterol	10-120	0.9878± 0.003	43.76 ±0.923	±0.738	736.51±25.38	±20.307

n= 6, confidence limit = 0.95

Table: 3.15 Intra-day and Inter-day precision of HPTLC method:

Name of the Compound	Amount (ng/spot)	Intra-day Precision			Inter- day Precision		
		S.D.	RSD	S.E.	S.D.	RSD	S.E.
Salicylic acid	400	37.95	0.863	15.493	20.29	0.463	5.865
	800	71.16	1.129	29.050	27.48	0.436	7.932
	1000	96.03	1.281	39.204	38.17	0.509	11.018
Stigmasterol	40	47.59	2.004	19.428	52.82	2.222	15.247
	80	58.09	1.267	23.715	15.55	0.335	4.488
	100	80.47	1.489	32.85	78.48	1.446	13.163

n=6

Table 3.16: Recovery studies:

Name of the compound	Amount of standard spiked (ng)	Average of Amount Recovered(ng)	Recovery (%)	R.S.D	S.E.
Salicylic acid	0	254.68	---	---	---
	120	370.91	99.81± 1.28	1.33	0.74
	150	412.46	104.66± 1.68	1.61	0.96
	300	558.63	100.76± 0.73	0.73	0.42
Stigmasterol	0	31.09	---	---	---
	30	62.43	104.48± 4.00	3.82	2.22
	35	68.42	106.67±1.12	1.05	0.60
	45	77.70	101.98± 3.99	3.91	2.25

n= 3

Table 3.17: Estimation of Salicylic acid and Stigmasterol in Methanolic extracts of plants:

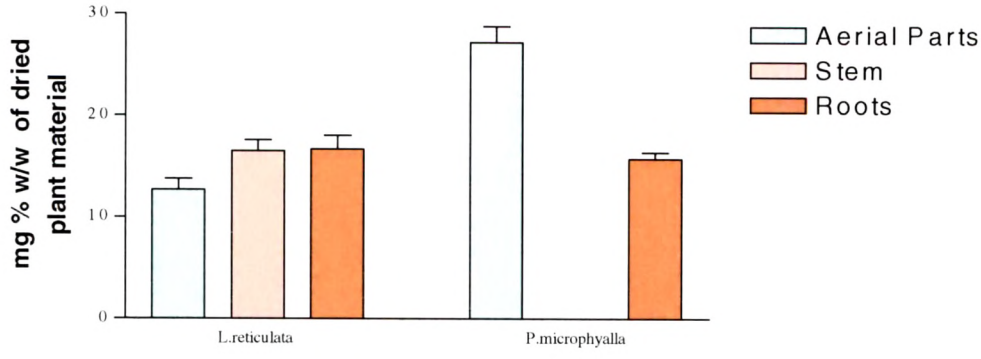
Sr. No	Name of the plant	Plant organ	Amount of Salicylic acid ±SD (% mg w/w, on basis of dried wt of the plant material)	Confidence limit	Amount of Stigmasterol ±SD (% mg w/w, on basis of dried wt of the plant material)	Confidence limit
1	*L.reticulata*	Aerial Parts	12.72± 1.04	11.54-13.90	777.312 ± 9.87	766.20-784.41
2		Stem	16.52±1.03	15.35-17.69	117.81±5.71	111.34-124.28
3		Roots	16.69± 1.33	15.18-18.19	101.00±2.10	98.63-103.37
4	*P.microphylla*	Aerial Parts	27.20±1.55	25.44-28.95	208.64±2.84	205.42-211.86
5		Roots	15.73±0.64	15.00-16.45	58.27±1.30	56.79-59.75
6	*D.volubilis*	Aerial parts	---	---	516.73±28.54	484.44-549.02
		Stem	---	---	165.63±4.32	160.74-170.52

n=3, confidence limit=0.95

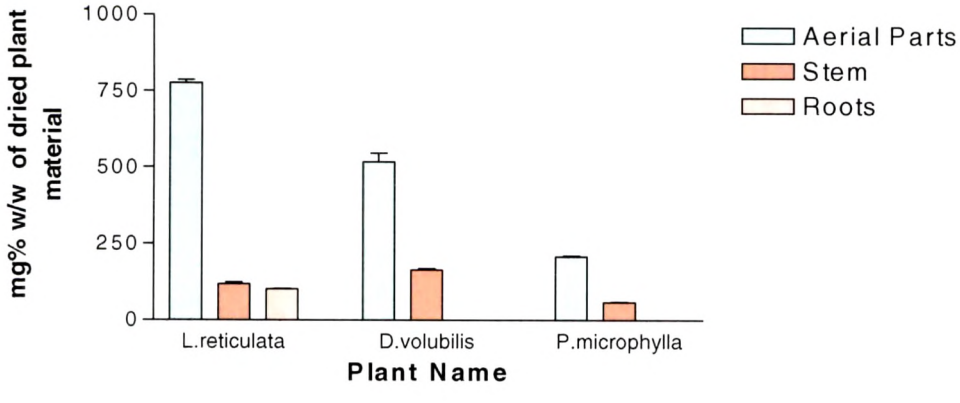
Table: 3.18 Summary of Validation of the developed analytical method:

Sr No.	Parameters		Results	
			Salicylic acid	Stigmasterol
1	Precision	Interday Precision(RSD)	0.469	1.33
		Intraday Precision (RSD)	1.09	1.58
2	Accuracy(Accuracy was ascertained by Recovery studies by spiking the samples with three different quantities of respective standard)		101.74	104.37
3	Specificity		Specific	Specific
4	Limit of Detection (ng/spot)		6.0	0.8
5	Limit of Quantification (ng/spot)		100-1400	10-120
6	Robust ness		Robust	Robust

Graph 3.1: Content of salicylic acid in different parts of the selected plants:



Graph 3.2: Content of Stigmasterol in different parts of the selected plants:



3.3.5 Estimation of β-carotene from the plant extracts using HPTLC:

As *L.reticulata* is used traditionally, as a remedy for diseases related to vision[26], detection of some compounds responsible for this activity was under taken

β carotene being a precursor for retinol, may serve as main agent responsible for the activity. The acetone extract of *L.reticulata* along with other two plants, *P.microphylla* and *D.volubilis* were also subjected to estimate the content of β carotene using CO-TLC.

All the tracks were first scanned *in situ* in range of 400-800 nm using TLC scanner and then were subjected to post chromatographic derivatization using antimony trichloride.

The comparative spectra of standard β carotene and that of spot in the tract of plant extract were recorded, which confirmed the presence of β carotene in acetone extract of aerial parts of all the three plants. (Fig. 3.20).

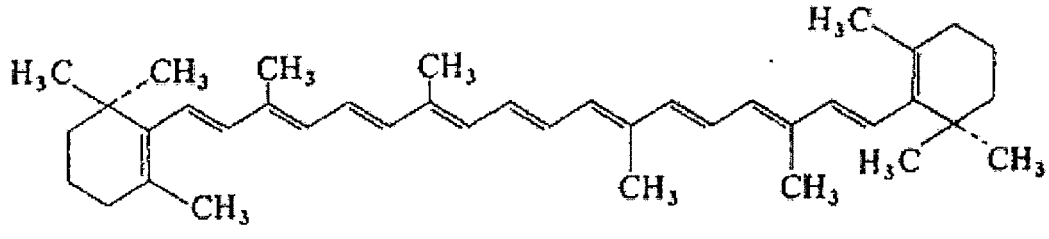


Fig. 3.18 shows overlain spectra of β carotene in standard and sample track. The spectra showed a peak at 444 nm. The peak obtained was such in the shape that practically ranging from 439 to 450 nm wavelengths can be selected as the scanning wavelength. The peak purity of β carotene was assessed by comparing the spectra at peak start, peak apex and peak end position of the spot. Good correlation was obtained between standard and sample overlain spectra of β carotene. The result indicated specificity of the method. Limit of detection and Limit of quantification determined was 100 ng and 400 ng respectively.

Calibration range selected for the present experiment was 400-1400 ng. Peak areas and concentrations were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. Linear Regression coefficient was found to be 0.9987. The linearity of calibration graphs and adherence of the system to beer's law was validated by high value of correlation co-efficient and Standard Deviation (SD) for intercept value. No significant difference was observed in the slopes of standard curves (ANOVA; $p< 0.05$) indicates repeatability of the method.

Inter day and intraday analysis data recorded showed Relative Standard Deviation less than 2 %. Statistical analysis of the data revealed no significant difference between the

corresponding datasets (ANOVA; $p< 0.05$). The results, thus, indicate the method developed is specific and precise for estimation of β-carotene from the matrix utilized. Recovery studies performed at three different levels (Table 3.21).The results are expressed as % recovery ± SD The summary of the results obtained for different validation parameter is tabulated as in Table 3.23.

The amounts of β carotene in the acetone extracts of the plant materials was estimated and reported as average of three estimations along with standard deviation in Table 3.22.*L.reticulata* possesses maximum amount of β carotene amongst the plant analyzed.

Table: 3.19 Linear Regression Data for calibration curves for β carotene:

Component name	Linearity range (ng)	r ± S.D.	Slope ± S.D.	Confidence limit of slope	Intercept ± S.D.	Confidence limit of intercept
β Carotene	400-1400	0.9952±0.052	3.539±0.0471	±0.037	-288.595±76.745	±61.411

n= 6, confidence limit = 0.95

Table: 3.20 Intra-day and Inter-day precision of HPTLC method:

Name of the Compound	Amount (ng/spot)	Inter-day Precision			Intra- day Precision		
		S.D.	RSD	S.E.	S.D.	RSD	S.E.
β Carotene	600	38.50	2.04	15.71	14.45	0.766	8.34
	1000	51.15	1.53	20.88	19.12	0.579	11.03
	1200	13.46	0.33	5.49	22.94	0.580	13.24

n=3

Table: 3.21 Recovery studies for estimation of β carotene :

Name of the compound	Amount of standard spiked (ng)	Average of Amount Recovered(ng)	Recovery (%)	R.S.D	S.E.
β Carotene	0	35.005	---	---	---
	30	69.664	97.715	4.579	2.583
	35	81.090	100.390	2.996	1.729
	45	70.116	99.095	9.393	5.686

n= 3

Table: 3.22 Estimation of β Carotene in Acetone extracts of plants:

Sr. No	Name of the plant	Plant organ	Amount of β Carotene ±SD (%w/w, on basis of dried wt of the extract)	Confidence limit
1	*L.reticulata*	Aerial Parts	0.017±0.001	±0.0017
2	*P.microphylla*	Aerial Parts	0.015±0.003	±0.0027
3	*D.volubilis*	Aerial parts	0.013±0.004	±0.0041

n=3, confidence limit=0.95

Table: 3.23 Summary of Validation studies:

Sr No.	Parameter studied		Results
1	Precision	Interday Precision(RSD)	1.30%
		Intraday Precision (RSD)	0.64%
2	Accuracy(Accuracy was ascertained by Recovery studies by spiking the samples with three different quantities of standard β carotene)		Average % recovery 99.82 ± 4.090
3	Specificity		Specific
4	Limit of Detection		90 ng/spot
5	Limit of Quantification		400-1400 ng/ spot
6	Robust ness		Robust

Fig: 3.19 Detection and Estimation of β carotene:

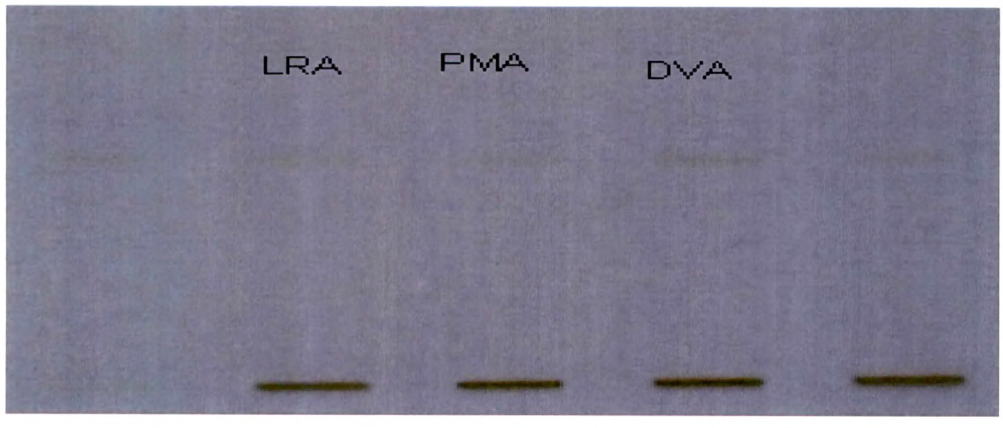
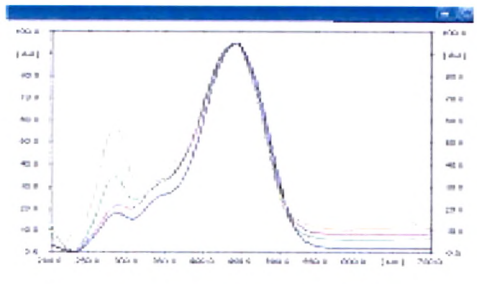
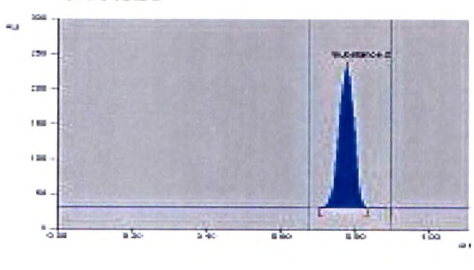


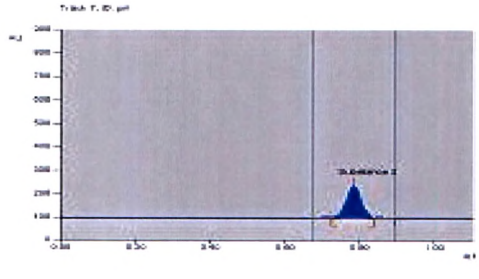
Fig: 3.20 Peak of β carotene in track of sample and standard:



Overlaying spectra of spot of Beta carotene in track of sample



Peak of β carotene in track of standard



Peak of β carotene in sample track

3.3.5 Determination of total Phenolics and Flavonoidal content of Plant extracts:

3.3.5.1 Estimation of Total Flavonoid content:

Complementary and alternative medicines containing radical scavengers exhibit efficient anti oxidant activity owing to their phenolic and flavonoids content. The result of the preliminary phytochemical analysis showed the presence of flavonoids and other phenolic constituents in

the methanolic extract. The flavonoids were determined by adopting two **complementary** methods.

Aluminum chloride forms acid stable complexes with the C-4 keto or C-3 or C-5 hydroxyl group of flavones and flavonols. The intensity of the colour of the complex formed was estimated at 415 nm using UV spectrophotometer[74].

Table 3.24: Calibration Curve for determination of Flavonoidal content by $AlCl_3$ Method:

Concentration of Quaercetin (µg/ml)	Abs.
10	0.024
20	0.065
30	0.1
40	0.133
50	0.172

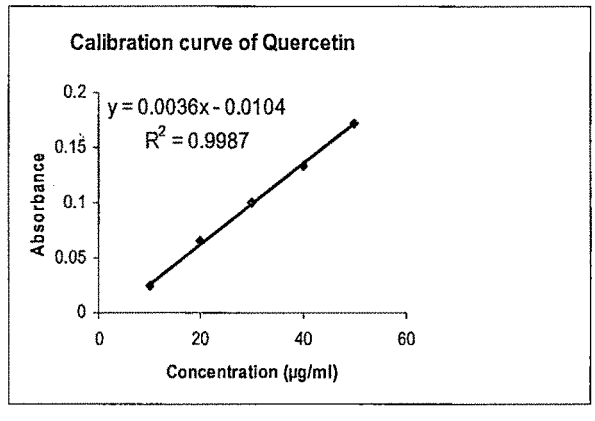
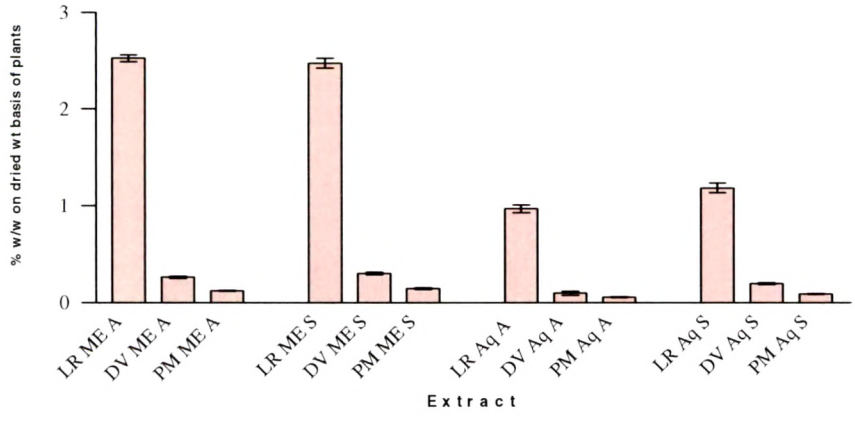


Table 3.25: Estiamtion of flavonoid content by Aluminium chloride method:

Extract	Flavonoid content (%w/w of dried wt of extract)
Methanolic extract of aerial parts of *L.reticulata*	2.525±0.058
Methanolic extract of stem of *L.reticulata*	2.475±0.089
Methanolic extract of aerial parts of *D.volubilis*	0.261±0.021
Methanolic extract of stem of *D.volubilis*	0.301±0.025
Methanolic extract of aerial parts of *P.microphylla*	0.121±0.007
Methanolic extract of stem of *P.microphylla*	0.145±0.016
Aqueous extract of aerial parts of *L.reticulata*	0.971±0.072
Aqueous extract of stem of *L.reticulata*	1.186±0.088
Aqueous extract of aerial parts of *D.volubilis*	0.098±0.037
Aqueous extract of stem of *D.volubilis*	0.197±0.015
Aqueous extract of aerial parts of *P.microphylla*	0.054±0.008
Aqueous extract of stem of *P.microphylla*	0.089±0.004

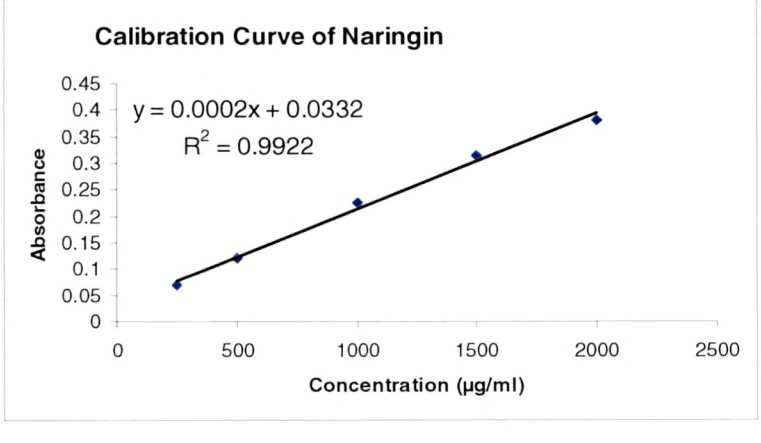
Graph3.3: Content of Flavonoids estimated using Aluminium Chloride method in Methanolic Extract of Plant:



3.3.5.2 Determination of Flavonoid content by DNPH Method:

2, 4-dinitrophenylhydrazine reacts with ketones and aldehydes to form 2,4-dinitrophenylhydrazones. It was also observed that flavones, flavonols and isoflavones with the C2-C3 double bond could not react with 2,4-dinitrophenylhydrazine, while the hydrazones of all flavanone standards, i.e. naringin, (±)- naringenin and hesperetin, showed maximum absorbance at 495 nm (data not shown). Therefore, the wavelength 495 nm was selected for all measurements in the 2, 4-dinitrophenylhydrazine reaction[74].

Table 3.26: Calibration curve of Estimation of Flavonoid contend by 4-DNPH method:



Conc.of Naringin (µg/ml)	Abs.
250	0.07
500	0.121
1000	0.226
1500	0.315
2000	0.381

Table 3.27: Estiamtion of flavonoid content by 2,4-DNPH method:

Extract	Flavonoid content (%w/w of dried wt of extract)
Methanolic extract of aerial parts of *L.reticulata*	0.916±0.02
Methanolic extract of stem of *L.reticulata*	1.483±0.26
Methanolic extract of aerial parts of *D.volubilis*	0.194±0.01
Methanolic extract of stem of *D.volubilis*	0.450±0.02
Methanolic extract of aerial parts of *P.microphylla*	0.308±0.05
Methanolic extract of stem of *P.microphylla*	0.745±0.16
Aqueous extract of aerial parts of *L.reticulata*	0.514±0.098
Aqueous extract of stem of *L.reticulata*	0.981±0.098
Aqueous extract of aerial parts of *D.volubilis*	0.078±0.003
Aqueous extract of stem of *D.volubilis*	0.139±0.12
Aqueous extract of aerial parts of *P.microphylla*	0.081±0.004
Aqueous extract of stem of *P.microphylla*	0.079±0.003

The quantitative determination of flavonoid was performed in various samples by 2,4-dinitrophenylhydrazine method was performed in addition to aluminum chloride method to avoid biases and summation of the values obtained by such methods provide the amount of the total flavonoids present as indicated in Table 3.28[74].

Graph 3.4: Content of Flavonoids estimated using 2,4-DNPH method in Methanolic Extract of Plant:

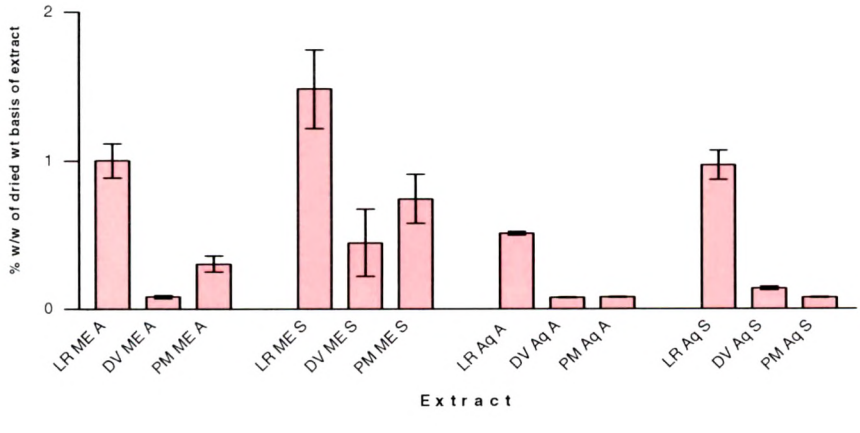


Table 3.28: Total Flavonoid Content of plant materials:

Extract	Total Flavonoid content (%w/w of dried wt of extract)
Methanolic extract of aerial parts of *L.reticulata*	3.041
Methanolic extract of stem of *L.reticulata*	3.958
Methanolic extract of aerial parts of *D.volubilis*	0.751
Methanolic extract of stem of *D.volubilis*	0.755
Methanolic extract of aerial parts of *P.microphylla*	0.429
Methanolic extract of stem of *P.microphylla*	1.195
Aqueous extract of aerial parts of *L.reticulata*	1.542
Aqueous extract of stem of *L.reticulata*	2.167
Aqueous extract of aerial parts of *D.volubilis*	0.176
Aqueous extract of stem of *D.volubilis*	0.336
Aqueous extract of aerial parts of *P.microphylla*	0.135
Aqueous extract of stem of *P.microphylla*	0.168

3.3.5.3Estimation of Total Phenolic content:

The plant contains salicylic acid and other phenolic components. The phenolics are considered to be useful in treatment of the topical infections. As the plants are traditionally recommended in treatment of cuts and burns the amount of total phenolic content in the plants were selected as one of the standardization parameter. The experiments were designed to estimate the amount of total phenolic content from the methanolic extract of the various parts of the plants.

The content of total phenolic was estimated using Folin-Ciocalteu's reagent form methanolic extract of the plants. The results suggested stem of *L.reticulata* consists of maximum amount of the phenolic components. The amount of total phenolic content estimated from different parts of the plants is reported in Table 3.30.

Table 3.29: Development of calibration curve for estimation of Total Phenolics:

Conc. Of Gallic acid µg/ml	Abs.
2	0.21
4	0.381
6	0.575
8	0.785
10	0.999

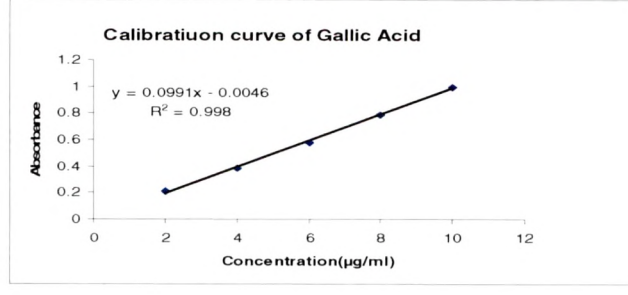


Table 3.30: Estimation of Total Phenolic Content of the plant extracts:

Extract	Total Phenolic Content (% w/w of dried wt of extract)
Methanolic extract of aerial parts of *L.reticulata*	1.013±0.026
Methanolic extract of stem of *L.reticulata*	1.168±0.084
Methanolic extract of aerial parts of *D.volubilis*	1.069±0.060
Methanolic extract of stem of *D.volubilis*	1.201±0.081
Methanolic extract of aerial parts of *P.microphylla*	0.999± 0.094
Methanolic extract of stem of *P.microphylla*	1.269±0.070
Aqueous extract of aerial parts of *L.reticulata*	0.125±0.018
Aqueous extract of stem of *L.reticulata*	0.714±0.098
Aqueous extract of aerial parts of *D.volubilis*	0.191±0.012
Aqueous extract of stem of *D.volubilis*	0.094±0.013
Aqueous extract of aerial parts of *P.microphylla*	0.078±0.011
Aqueous extract of stem of *P.microphylla*	0.143±0.012

Graph 3.5: Estimation of Total Phenolic content of Methanolic Extract of the Plants:

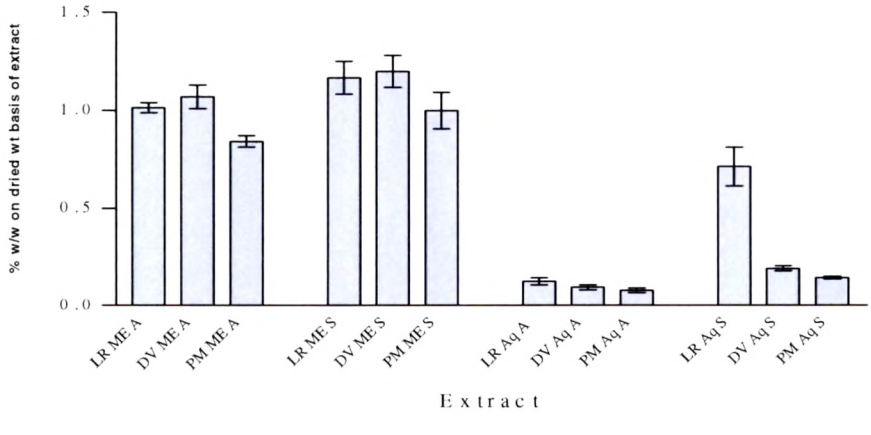


Table: 3.31 Comparative summary of physico chemical parameters evolved for aerial parts of *L.reticulata, D.volubilis* and *P.microphylla.*

Sr No.	Standardization Parameter	Aerial parts of *L.reticulata*	Aerial parts of *D.volubilis*	Aerial parts of *P.microphylla*
1	Moisture content	0.58% w/w	0.89%w/w	0.21 % W/W
2	Foreign matter	0.058 % w/w of dried material	0.041 % w/w of dried material	0.023 % w/w of dried material
3	Ash value	9.604 ±0.260%w/w	6.632 ±0.260	11.06 ± 0.723
4	Acid Insoluble Ash value	0.634± 0.045 %w/w	0.693 ±0.03	1.712 ±0.110
5	Water Soluble Ash Value	0:07%w/w	0.04%w/w	0.05%w/w
6	Extractive Value	19.805 ± 0.415	20.109±0.942	14.64 ±0.394
7	Powder fineness	All the particles pass through a No. 710 sieve, and not more than 40% through a No. 180 sieve	All the particles pass through a No. 710 sieve, and not more than 40% through a No. 180 sieve	All the particles pass through a No. 710 sieve, and not more than 40% through a No. 180 sieve
8	Foaming index	Less than 100	Less than 100	Less then 100
9	Hemolytic activity	100mg/ml of water extract not show haemolysis	100mg/ml of water extract not show haemolysis	100mg/ml of water extract not show haemolysis
10	Heavy Metal Content	Found within the prescribed limit		
11	Total bacterial count	$4.7X10^{3}$ /g	$8.1X10^{3}$ /g	$9.8X10^{3}$ /g
12	Total fungal count	$79 X 10^{2}$ /g	$46 X 10^{2}$ /g	$73 X 10^{2}$ /g
13	Total flavonoid content in methanolic extract of aerial parts of the plants	3.041	0.751	0.429
14	Total Phenolic content in methanolic extract of aerial parts of the plants	1.013±0.26%w/w	1.069±0.060%w/w	0.999±0.094%w/w
15	Content of Salicylic acid in selected plants on dried wt basis	12.72±1.04 mg% w/w	Absent	27.20±1.55 mg%w/w
16	Content of Stigmasterol in selected plants on dried wt basis	777.31±9.87 mg% w/w	516.73±28.54 mg%w/w	208.64±2.84 mg%w/w
17	Content of β carotene in acetone extract on dried wt basis	0.017±0.0017 %w/w	0.015±0.003 %w/w	0.013±0.004 %w/w

3.4 Stability studies:

Methanolic extract of the plants selected were subjected to the Accelerated stability studies as per the regulatory guide lines. Morphological evaluation and estimation of total phenolics and flavonoidal content were selected as evaluation parameters. The results of the experiments showed the trend of decrease in the content of total phenolic and total flavonoids present tabulated as in Table 3.32 indicates the time of the collection of the sample and the amount of the analyte remained.

There were no alterations observed in color and consistency of the extracts studied.

As methanolic extract of *L.reticulata* is available commercially to be incorporate in some topical herbal formulations, Stability studies were performed to ascertain the chemical integrity of the phytoconstituents present during long time storage of the extract.

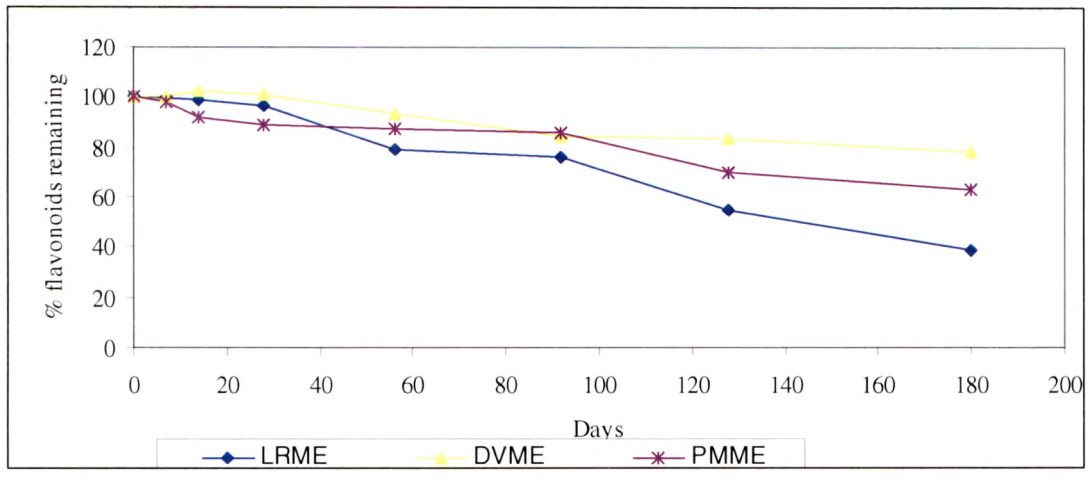
Therapeutic potential of the plant has never been assigned to the single moiety but it is the group of the substances that are responsible for therapeutic action of the plants. The estimation of a single moiety, thus, does not represent the stability of the whole extract. The extracts form a very complex matrix for analyzing a single moiety especially for development of analytical method to generate stability profile. Being related to the therapeutic action of the plants, content of total Phenolics and total flavonoids were selected as the parameters for the evaluation of the stability of the extracts. The studies indicated that the content of the phytoconstituents were affected by accelerated stability conditions.

Table 3.32: Stability studies of the Methanolic extract of Aerial parts of *L.reticulata*, *D.volubilis* and *P.microphylla:*

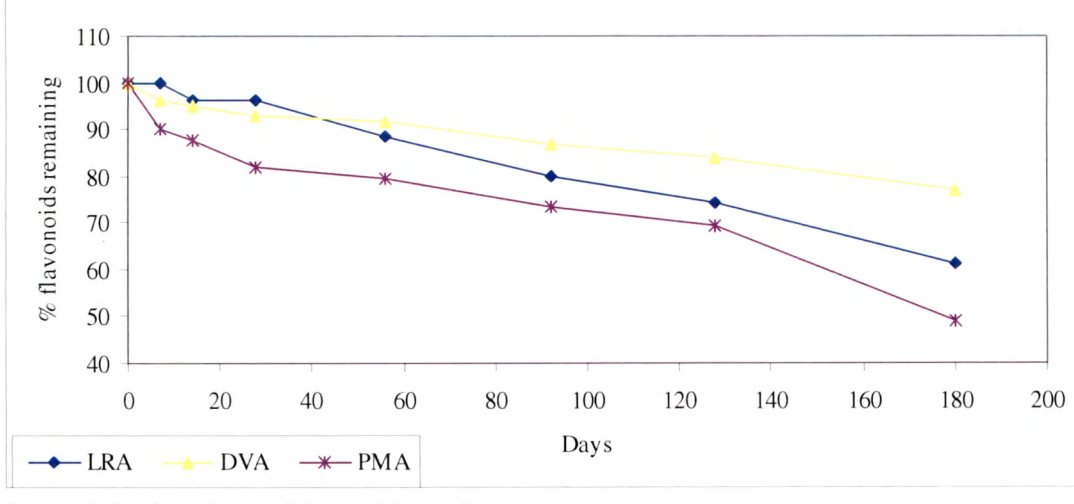
Sr. No	Day	*L.reticulata*			*D.volubilis*			*P,microphylla*		
		2,4-DNPH	Aluminium Chloride	Total Phenolic	2,4-DNPH	Aluminium Chloride	Total Phenolic	2,4-DNPH	Aluminium Chloride	Total Phenolic
1	0	0.5168±0.029	2.525±0.057	1.013±0.026	0.4862±0.024	0.2606±0.020	1.250±0.171	0.3080±0.055	0.121±0.007	0.842±0.029
2	7	0.5152±0.007	2.517±0.400	0.9755±0.042	0.4873±0.048	0.2507±0.020	1.065±0.128	0.3003±0.011	0.1088±0.005	0.8432±0.030
3	14	0.5099±0.014	2.431±0.385	1.275±0.241	0.4991±0.038	0.2478±0.002	1.021±0.227	0.2821±0.024	0.1050±0.050	0.8577±0.0037
4	28	0.4981±0.009	2.231±0.180	1.200±0.117	0.4911±0.049	0.2419±0.030	0.9768±0.025	0.2740±0.024	0.099±0.003	0.8401±0.042
5	56	0.4101±0.018	2.181±0.109	0.9877±0.099	0.4525±0.04	0.2387±0.020	0.9534±0.056	0.2701±0.007	0.096±0.002	0.8131±0.021
6	92	0.3913±0.035	2.014±0.219	0.9523±0.056	0.4113±0.063	0.2259±0.029	0.9361±0.024	0.2642±0.036	0.089±0.001	0.8010±0.010
7	128	0.2817±0.005	1.875±0.342	0.9102±0.038	0.4046±0.015	0.2190±0.008	0.8413±0.046	0.2142±0.0015	0.084±0.003	0.7013±0.052
8	180	0.2003±0.020	1.543±0.298	0.8502±0.033	0.3817±0.008	0.2010±0.010	0.7530±0.036	0.1943±0.015	0.059±0.008	0.6132±0.055

The values are represented as Mean of three estimations. The data sets are statistically different to each other. ($p < 0.05$, 95% confidence interval). The results are expressed as % w/w on dried wt basis of the plant extract.

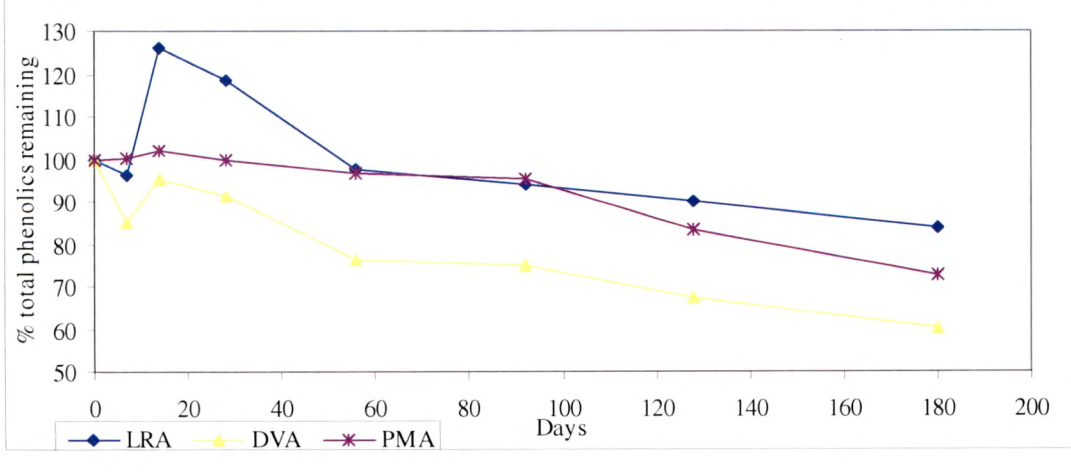
Graph 3.6: Stability of flavonoidal content estimated using Aluminium chloride Method:



Graph3.7: Stability of flavonoidal content estimated using 2,4-DNPH method:



Graph3.8: Stability of Total Phenolic content:



3.5 Evaluation of different marketed samples of *L.reticulata*:

Five Different samples under the name of *Jivanti* were purchased from local market. The powdered aerial parts, stem of *L.reiculata*, aerial parts of *D.volubilis* and aerial parts *P.microphylla* were used as positive control and then subjected to evaluation.

The results of microscopic examination of the samples were compared with the microscopic features observed from the authentic materials. The materials were subjected to detection of salicylic acid and stigmasterol and estimation of β carotene using HPLTC and determination of other physicochemical parameters (Table: 3.34).The results of these experiments were compared to the values the authentic materials mentioned in Table :3.33.

The HPTLC studies revealed the presence of stigmasterol and salicylic acid in all the samples but β carotene was not present in the market samples indicated faulty processing or preservation of materials.

The chromatographic fingerprint of the test sample were developed and then compared with that of obtained from authentic materials (Fig. 3.21(a), 3.21(b)).

The results indicated that the physicochemical and chromatographic profiles of sample number 3 and 6 were matched respectively with that of authentic materials LRA and DVA. The values of the parameters studies for sample no 7 and 8 (SMP 7 and 8) were not matched with any of the authentic materials studied, but the HPTLC fingerprints of these materials were identified as the mixture of the pattern obtained of the fingerprints of DVA + LRA and PMA+LRA respectively. Sample 4 (SMP 4) could not be identified.

The results thus revealed that *D.volubilis* and *P.microphylla* both are sold with the name of *L.reticulata* being *Jivanti* in the practice and the standardization parameters developed, are capable of differentiating the plants from each other.

Table 3.33: Physico chemical Evaluation of authentic plant material:

Sr No.	Sample Code	Ash value	Confidence interval	Acid Insoluble Ash	Confidence interval	Extractive Value	Confidence interval	Presence of stigmasterol in extract + Yes - No	Presence of salicylic acid in extract + Yes - No	Content of β carotene in samples (µg per 100 g of dried plant material) Average ±S.D.
1	LRA	9.604 ±0.260	9.440-9.768	0.634± 0.045	0.594 -0.673	5.605 ±0.543	5.129-6.082	+	+	158.36 ±0.02
2	LRS	6.829± 0.069	6.769-6.889	0.518± 0.019	0.501-0.535	4.380 ±0.038	4.3466-4.413	+	+	---
3	DVA	6.632 ±0.260	6.743-6.860	0.633 ±0.03	0.600-0.664	20.109±0.942	19.282-20.935	+	-	95.24 ±0.05
4	PMA	11.06 ± 0.723	10.06-12.06	1.712 ±0.110	1.166-1.762	14.64 ±0.394	13.84-15.44	+	+	104.25 ±0.03

n= 5, The values are represented as average ± S.D., Confidence interval is determined at 95.0%.

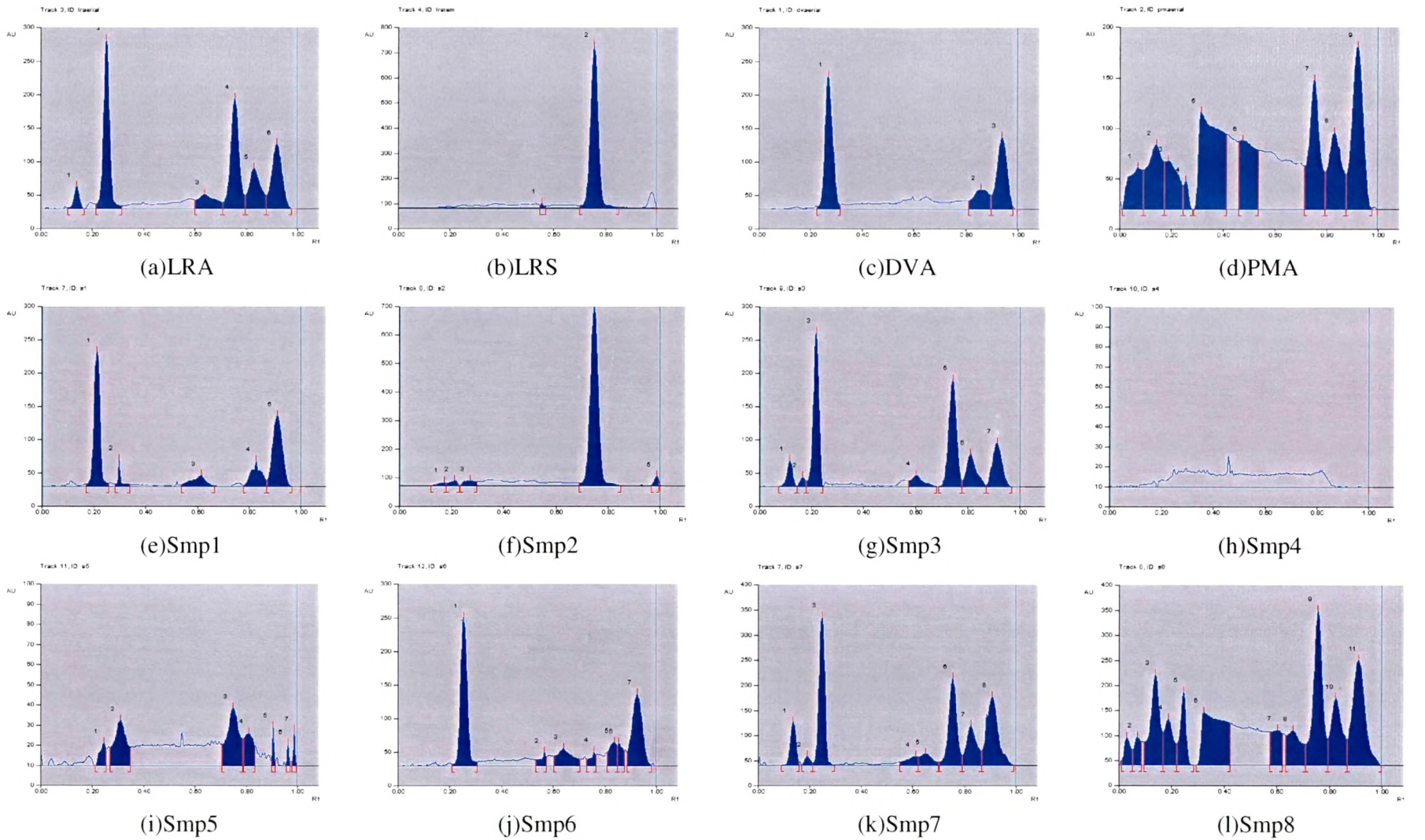
Table 3. 34: Physico chemical evaluation of Market samples and coded standards:

Sr No.	Sample Code	Ash value	Acid Insoluble Ash	Extractive Value	Presence of stigmasterol in extract + Yes - No	Presence of salicylic acid in extract + Yes - No	Content of β carotene in samples (µg per 100 g of dried plant material) Average ±S.D.)	Identification of samples
5	**SMP1**	**6.812 ±0.249**	**0.633 ±0.040**	**18.816±0.383**	+	-	**97.35±0.02**	**DVA**
6	SMP2	7.037± 0.136	1.275 ±0.437	4.530± 0.226	+	+	---	LRS
7	**SMP3**	**9.576 ±0.354**	**0.754 ±0.040**	**19.12± 0.293**	+	+	**168.59 ±0.03**	**LRA**
8	**SMP4**	**5.413± 0.399**	**0.875 ±0.057**	**11.17± 0.641**	+	-	---	**Sample 1**
9	SMP5	11.08 ±0.584	1.815 ±0.110	14.85 ±0.298	+	+	91.42 ±0.07	PMA
10	SMP6	6.666 ±0.038	0.633 ±0.040	19.13 ±0.468	+	-	----	DVA
11	**SMP7**	**7.619 ±0.227**	**0.570 ±0.121**	**20.899 ±0.397**	+	+	**----**	**DVA+LRA**
12	**SMP8**	**10.525 ±0.219**	**0.981 ±0.237**	**15.651 ±0.581**	+	+	----	**PMA+LRA**
14	SMP10	9.747±0.114	0.699 ±0.026	5.337 ±0.739	+	+	----	LRA

n= 5, The values are represented as average ± S.D.Key: LRA: *L.reticulata* aerial parts, LRS: *L.reticulata* stem, DVA : *D.volubilis* aerial parts, PMA: *P.microphylla* aerial parts, LRS :

L.reticulata stem, + indicates presence of particular compound, - indicate absence of particular compound. The bold letters indicated the market samples.

Fig: 3.21 (a) Chromatogram of positive control and market samples:

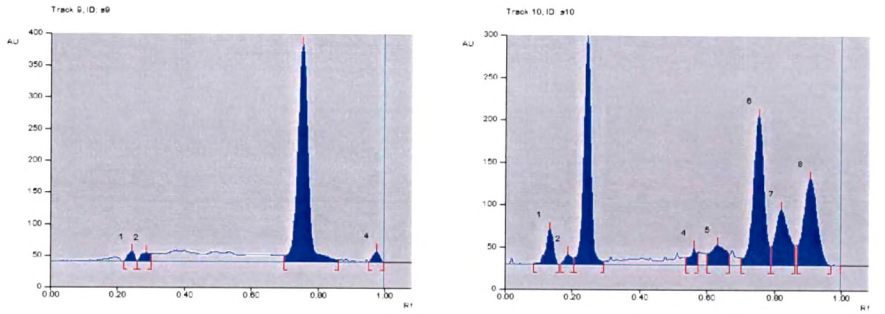


(a)LRA (b)LRS (c)DVA (d)PMA

(e)Smp1 (f)Smp2 (g)Smp3 (h)Smp4

(i)Smp5 (j)Smp6 (k)Smp7 (l)Smp8

Fig: 3.21(b) Chromatogram of positive control and market samples:



(a)Smp9 (b)Smp10

3.6 Evaluation of *Ayurvedic* formulation- *Jivantyadi Ghrita* and comparison of various market samples.

3.6.8.1 Evaluation of *Jivantyadi Ghrita*:

In order to assess the purity of the 'ghee' being animal fat, which serves in many *Ayurvedic* preparations as base, generally Acid number, Iodine number and Saponification value are determined.

The peroxide value and Acid value of marketed formulations was high compared to that of the formulation prepared in laboratory. The content of Vitamin A estimated from the products was comparatively lesser than that of the lab formulation. Saponification value and Iodine value of market formulations remains almost same as that of the lab formulation. Moisture content of lab formulation was lesser than that of the market formulations. The market samples were also assessed to detect the presence of *vanaspati ghee*. The results revealed the absence of *vanaspati ghee* in the market samples. The comparative results of the experiments performed are tabulated as in Table 3.35.

The peroxide value is the number of miliequivalents of active oxygen that expresses the amount of peroxide contained in 1000 g of the substance[78] Peroxide value indicates amount of active oxidizing oxygen present which is responsible to propagate the auto oxidation of the product which may finally act on therapeutically active components of the formulation. Thus rigorous control over peroxide value is necessary to formulate the product having the least deterioration.

The peroxide value of marketed formulations was high compared to the formulation prepared in laboratory indicating the production of active radicals. The chemical integrity of the product may be affected due to reaction of active radicals with other added ingredients and finally deteriorating the quality.

Acid value is directly an indication of the amount of free acids present in sample and an increment in acid value as compare to the blank as shown from Table I indicates the degradation of fats and liberation of free acids. The phenomenon generally appears due to ageing of the product during storage which renders liberation of free acid and thereby making the product unstable chemically. Since higher acid value obtained even in case of fresh product prepared in laboratory, it appears that some of the ingredients added may be contributing for this increase. The values in case of marketed and fresh product were almost were close, indicating proper storage conditions applied.

3.6.8.2 Assessment of effect of Heating on the formulation:

The experiments were performed to access the effect of heating during the preparation on the chemical integrity of the product by determining the values of few of the selected chemical parameters.

The formulation of medicated *ghrita* requires removal of water, which otherwise support the microbial growth during storage, involves heating of the mixture of butter oil, milk, decoction of plants and other plants added in the form of dried powder.

Heating of butter oil with milk and decoction of extract simulates the process of *Ghee* making, which may be divided in three basic phases. 1. Raising the temperature up to boiling point of water. 2. Removal of water in the form of vapor requires higher amount of heat. 3. The removal of the water bound to non fat solid. The second and especially final stage is important for quality of the product formed[111].

The experiments indicated that acid value and peroxide value were increased drastically in last phase during the preparation of *Ghrita*. The experiments, therefore, designed to observe the effect of alteration of temperature on the product during this phase by determining the level of selected chemical parameters.

The results suggested that the Acid value and Peroxide value were increased as the temperature and time of the heating was increased accompanied by reduction in total vitamin A content. The result are tabulated as in Table 3.36

The moisture content of the product should be minimized to protect the product from the microbial growth. An increase in temperature dramatically raises the rate at which fatty acids react with oxygen, promoting rancidity, and therefore raising the peroxide value. If the contents are heated at higher temperature the formulation can be prepared in lesser time but the product may undergo excessive chemical degradation which may affect the stability and therapeutic potential. There must be an optimistic temperature and time of heating selected for especially the later part of the process to produce the product with the least degradation. The results in the present investigation, suggest that heating at 115^0 C for 15 minutes in the last stage can be considered as an optimum heating temperature and time to achieve the minimum level of moisture with the least degradation of the product.

Table 3.35: Evaluation of Marketed and freshly prepared samples of *Jivantaydi Ghrit:*

Sr No.	Code No. of product	Acid value (Not more than 3)	Peroxide value	Iodine value (30-40)	Saponification value (220-232)	Total Vitamin A content(µg/100 g sample)	Moisture content (Not more than 1% w/w)
1	AN	2.223±0.095	2.670±0.187	31.823±0.792	271.351±0.873	220.100±11.404	0.880±0.157
2	BN	2.183±0.153	2.610±0.299	33.413±1.384	227.714±7.493	185.780±9.176	0.720±0.098
3	MN	1.213±0.191	2.463±0.595	39.207±0.476	219.432±0.973	222.710±37.013	0.470±0.066
4	L.F.	1.480±0.050	1.197±0.239	33.560±0.717	224.477±0.980	310.440±15.017	0.520±0.142
5	Control	0.338±0.010	0.548±0.043	33.787±1.007	225.952±1.075	311.823±20.87	0.420±0.113

Values mentioned in the brackets indicate specified limits. Results express as a mean of three estimations ±Standard Deviation (SD)

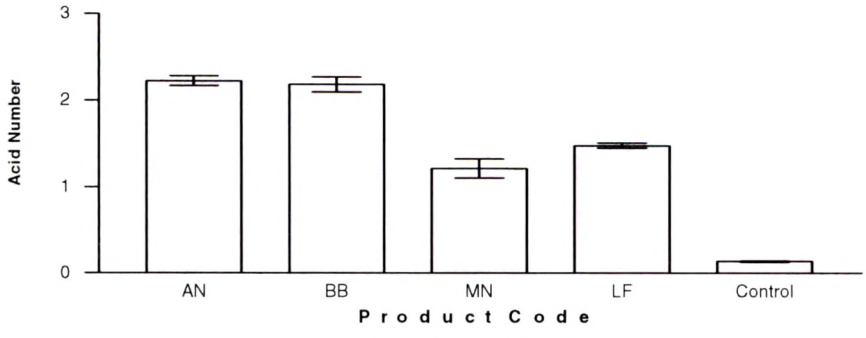
Table 3.36: Effect of Heating Time and Temperature on the selected parameters:

Temp.	Time of Heating											
	10 minutes				15 minutes				20minutes			
	AV	PV meq/kg	Total Vitamin A content(µg/100 g sample	Moisture content %w/w	AV	PV meq/kg	Total Vitamin A content(µg/100 g sample	Moisture Content %w/w	AV	PV meq/kg	Total Vitamin A content(µg/100 g sample	Moisture content %w/w
105^0 ± 2^0 C	0.773±0.086	0.801±0.064	325.721±27.461	9.913±1.409	1.538±0.353	1.007±0.306	318.458±27.461	3.189±1.291	3.277±0.940	2.116±0.468	290.110±33.575	2.170±1.370
110^0 ± 2^0 C	0.881±0.043	0.987±0.133	317.372±15.363	5.767±0.487	1.762±0.169	1.204±0.206	296.432±4.222	1.164±0.579	5.426±0.360	2.408±0.363	273.429±14.652	1.530±0.677
115^0 ± 2^0 C	0.950±0.039	0.967±0.020	305.452±6.723	1.933±0.630	1.871±0.144	1.354±0.107	278.143±9.589	0.387±0.114	6.496±0.179	2.302±0.150	257.334±27.446	0.520±0.238

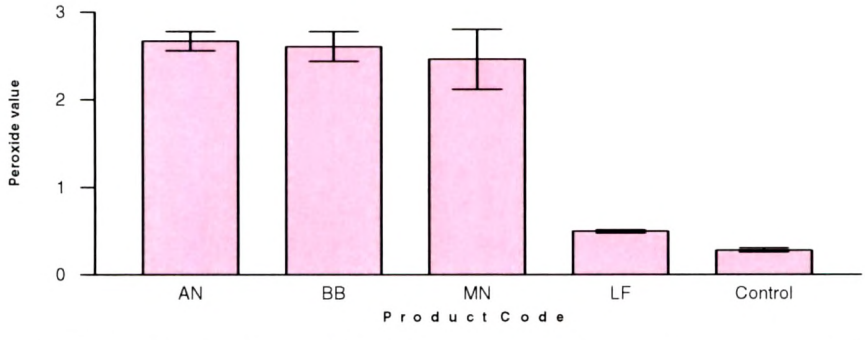
AV: Acid Number PV: Peroxide value

Results indicate the average of the determination of the three batches ± Standard Deviation (SD).

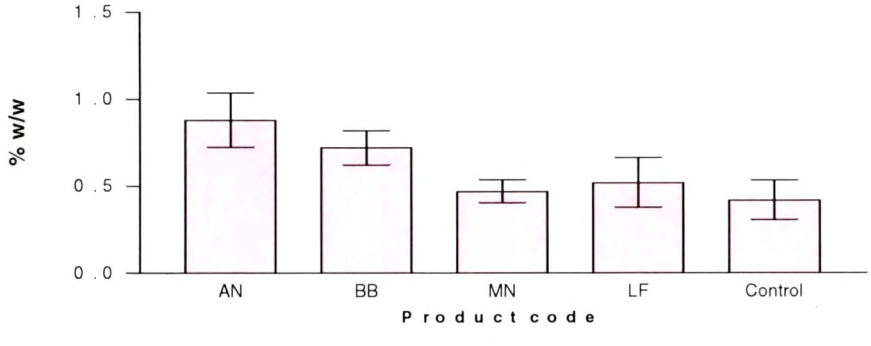
Graph: 3.9 Comparison of Acid value of market sample with Lab formulation:



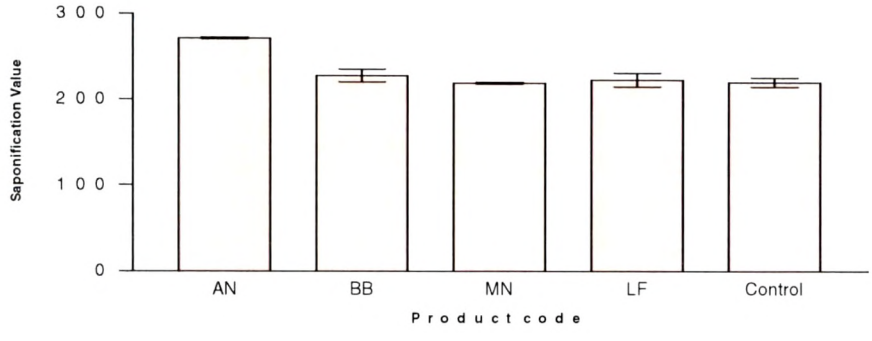
Graph: 3.10 Comparison of Peroxide value of market sample with Lab formulation:



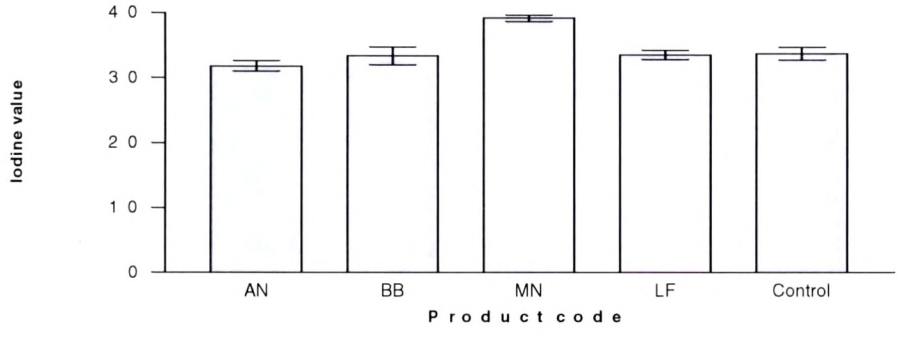
Graph:3.11 Comparison of Moisture content of market sample with Lab formulation:



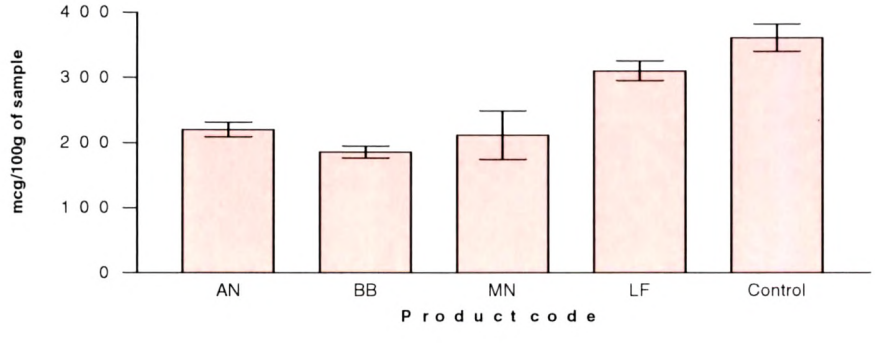
Graph: 3.12 Comparison of Saponification value of market sample with Lab formulation:



Graph: 3.13 Comparison of Iodine value of market sample with Lab formulation



Graph: 3.14 Comparison of Total Vitamin A content of market sample with Lab. formulation



3.7 Biological assessment of different extracts from the selected plants.

3.7.1 *In vitro* antioxidant activity:

The revitalization and rejuvenation is known as the *'Rasayan chikitsa'* (rejuvenation therapy) in *Ayuveda*. Traditionally, Rasayana drugs are used against a plethora of seemingly diverse disorders with no pathophysiological connections according to modern medicine[112]. Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals[113].Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism[114]. As free radicals play a major role in many chronic ailments and the plant has also been described as tonics and stimulant, an attempt has been made to investigate free radical scavenging activity of the selected plant drugs. Phytochemical studies revealed the methanolic extract of the plants possesses flavonoids and phenolic compounds.

As the plants are used as stimulant and tonic in traditional medicine, the methanolic and aqueous extract of aerial parts and stem were screened for antioxidant *potential in vitro* by adopting reported methodologies like, DPPH radical scavenging activity, NBT super radical scavenging activity, Nitrous oxide radical scavenging activity and reducing power.

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine[82]. The results indicated that, the methanolic extract of the stem of all the selected plants were more potent as compared to the methanolic extract of the aerial parts. The DPPH radical scavenging activity was, therefore, performed on TLC plates to detect the compounds responsible from the methanolic extract of the stem part of all the selected plant drugs, for the scavenging activity of the extracts.

The TLC plate derivatized with DPPH solution indicated the development of the yellow color zones, due to destruction of the formed radical. The spot was comparatively intense at R_f 0.54 in case of the *L.reticulata,* while the compound at R_f 0.73 being also efficient in destruction of the radicals in all the extracts of the selected plant drugs.

The TLC system was developed to separate flavonoids and other phenolics. The TLC plate was derivatized with Natural product reagent.

The results of DPPH radical scavenging activity indicated the potential of the selected extracts in scavenging the free radicals of all the three plant drugs. Methanolic extract of *D.volubilis* possess the comparatively low EC_{50} value of all the extracts evaluated.

Solution of Nitro Blue Tetrazolium (NBT) in phosphate buffer in the presence of riboflavin and EDTA produces free radicals on illumination fro 5 min., having blue color. If the substance of interest has radical scavenging activity, the color reduces after addition of substance to the solution of NBT in phosphate buffer along with riboflavin and EDTA and illumination for stipulated time period. The test extracts of the plants showed very high values of EC_{50} as compare to that of Ascorbic acid, selected as standard compound for the present study. The results suggested the extracts studied possess mild to moderate super oxide suppressing activity[83].

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Oxygen reacts with the excess NO to generate nitrite and peroxy nitrite anions, which act as free radicals. In the present study the nitrite produced by the incubation of solutions of sodium nitoprusside in standard phosphate buffer at 25^0 C was reduced by the extracts selected. The higher EC_{50} value of the extracts studied as compare to that of curcumin suggested moderate Nitrous oxide scavenging activity of the extracts studied[84].

The results of DPPH radical scavenging activity, NBT superoxide scavenging activity and NO radical scavenging activity are recorded in Table: 3.37-3.38.

Fe3+ – Fe2+ transformation was investigated in the presence of test extracts to estimate reducing ability of the compounds in the test extracts. The results are displayed in graphical form (3.39, 3.40). The graph shows the intensity of color produced by the standard at the selected concentrations was higher than that of the color produced by the extracts itself. The result, thus, indicated very mild reducing potential of the test extracts studied [85].

The analytical studies revealed higher amount of phenolics and flavonoids are present in stem part of the plants. The methanolic extract of the aerial part of the plants showed higher antioxidant potential as compare to that of the stem extract. The results thus indicated the probable role of phenolics and flavonoidal components in this activity.

The result of the antioxidant activity suggested mild to moderate antioxidant potential of the plant extracts studied. The methanolic extracts of the stem of the selected plants were found to be more active against DPPH radicals than the other radicals studied. The results indicated the methanolic extract of the stem was comparatively more potent than the extracts of the aerial parts in all three selected plants.

Phenolic/flavonoidal compounds act as antioxidants with mechanisms involving both free radical scavenging and metal chelation. They have ideal structural chemistry for free radical-scavenging activities, and have been shown to be more effective antioxidants *in vitro* than vitamins E and C on a molar basis[116].

The antioxidant potential is generally correlated with the content of flavonoids and phenolics. The correlation of the antioxidant potential with these analytical potential, therefore, provide a scope of comparing the analytical and biological potential of the extracts of the selected plants in the process of the standardization.

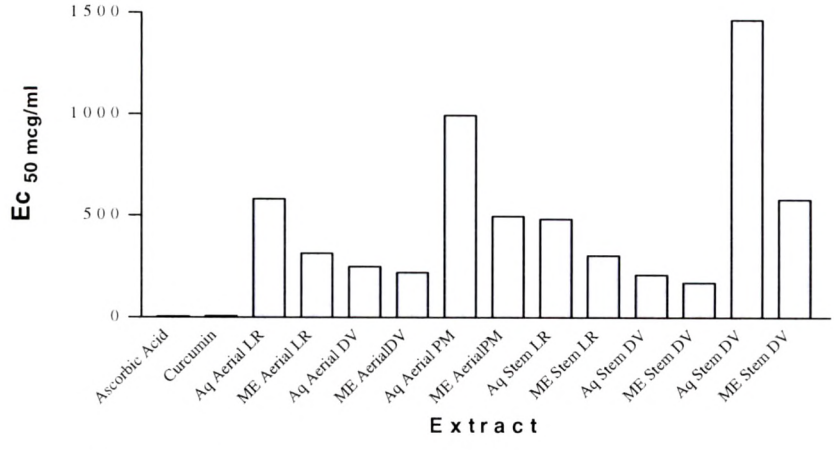
Table 3.37: *In vitro* radical scavenging activity of Methanolic and Aqueous extract of the stem of the selected plants:

Extract	DPPH radical scavenging activity	NBT superoxide scavenging activity	Nitrous Oxide scavenging activity
	EC_{50} µg/ml	EC_{50} mg/ml	EC_{50} mg/ml
Ascorbic acid	7.2	0.002	---
Curcumin	8.061		0.0087
Aqueous extract of *Leptadenia reticulata*	483.171	62.905	12.417
Methanolic extract of *Leptadenia reticulata*	323.592	0.788	8.378
Aqueous extract of *Dregea volubilis*	208.895	0.314	>100 mg
Methanolic extract of *Dregea volubilis*	170.802	0.293	0.3415
Aqueous extract of *Pentatropis microphylla*	1465.287	>100 mg	29.317
Methanolic extract of *Pentatropis microphylla*	580.644	>100mg	>100 mg

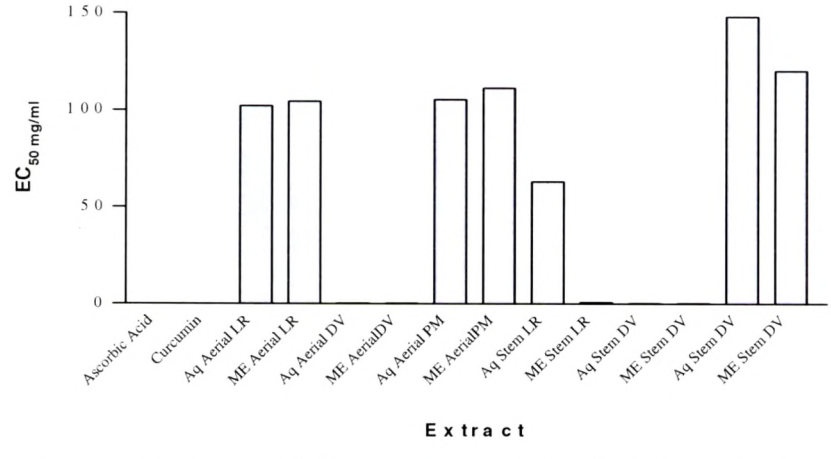
Table 3.38: *In vitro* radical scavenging activity of the Methanolic and Aqueous extract of the aerial parts of the selected plants:

Extract	DPPH radical scavenging activity	NBT superoxide scavenging activity	Nitrous Oxide scavenging activity
	EC_{50} µg/ml	EC_{50} mg/ml	EC_{50} mg/ml
Ascorbic acid	7.24	0.002	
Curcumin	8.06	----	0.0087
Aqueous extract of *Leptadenia reticulata*	581.712	>100	17.366
Methanolic extract of *Leptadenia reticulata*	314.55	>100	19.854
Aqueous extract of *Dregea volubilis*	250.87	0.475	>100
Methanolic extract of *Dregea volubilis*	221.59	0.375	59.544
Aqueous extract of *Pentatropis microphylla*	993.87	>100 mg	>100
Methanolic extract of *Pentatropis microphylla*	497.01	>100mg	68.957

Graph: 3.15 DPPH Radical Scavenging activity of methanolic and aqueous extract of aerial parts of selected plants:



Graph: 3.16 NBT Super oxide Scavenging activity of methanolic and aqueous extract of aerial parts of selected plants:



Graph: 3.17 Nitrous oxide Scavenging activity of methanolic and aqueous extract of aerial parts of selected plants:

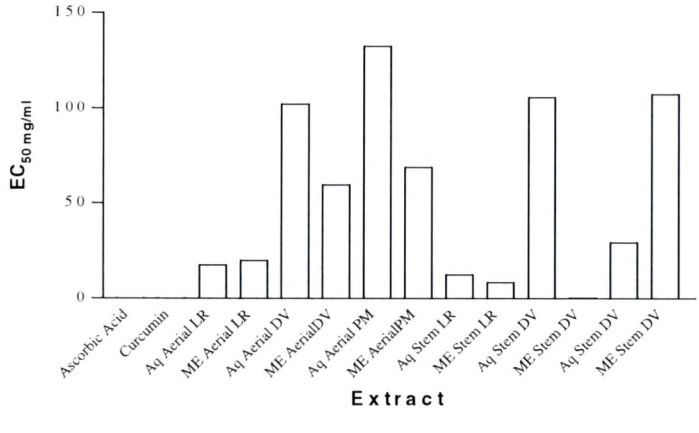


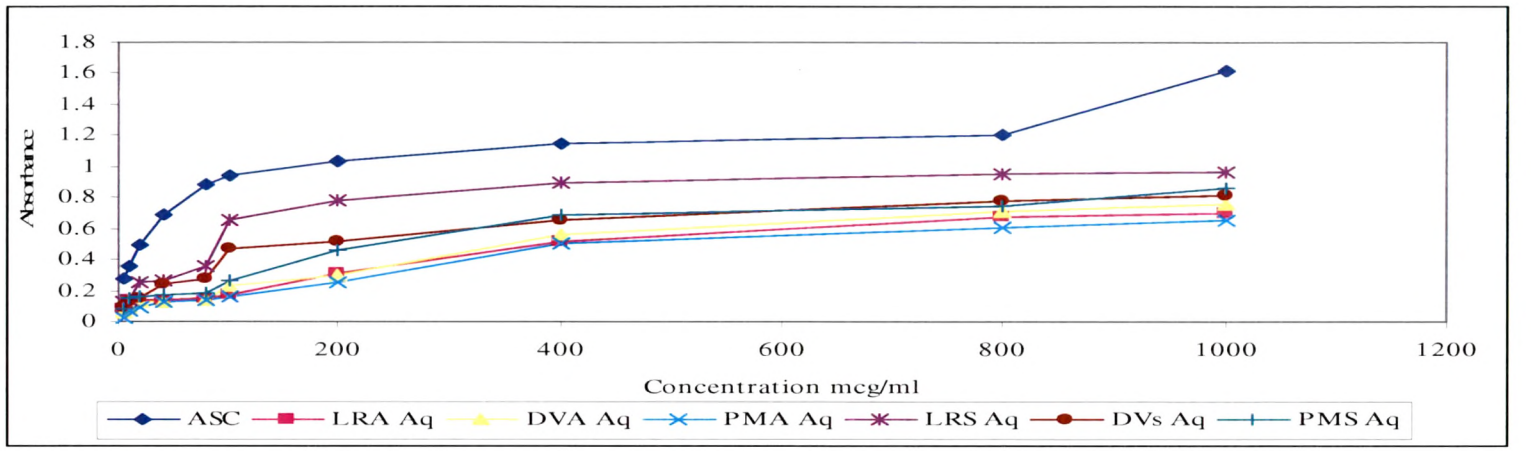
Table 3.39: Reducing Power of Aqueous extract of the selected plants:

Sr No	Concentration µg/ml	Absorbance Ascorbic acid	Absorbance of Methanolic Extract of Aerial parts of the plants			Absorbance of Methanolic Extract of stem of the plants		
			L.reticulata	*D.volubilis*	*P.microphylla*	*L.reticulata*	*D.volubilis*	*P.microphylla*
1	5	0.28	0.08	0.06	0.02	0.125	0.09	0.08
2	10	0.36	0.135	0.09	0.07	0.139	0.131	0.151
3	20	0.49	0.139	0.121	0.09	0.252	0.147	0.161
4	40	0.69	0.141	0.129	0.130	0.259	0.241	0.175
5	80	0.88	0.149	0.143	0.142	0.351	0.276	0.183
6	100	0.94	0.174	0.230	0.159	0.658	0.469	0.269
7	200	1.03	0.311	0.296	0.253	0.783	0.519	0.458
8	400	1.15	0.512	0.561	0.501	0.895	0.651	0.692
9	800	1.20	0.681	0.715	0.612	0.955	0.783	0.744
10	1000	1.62	0.695	0.755	0.650	0.962	0.811	0.861

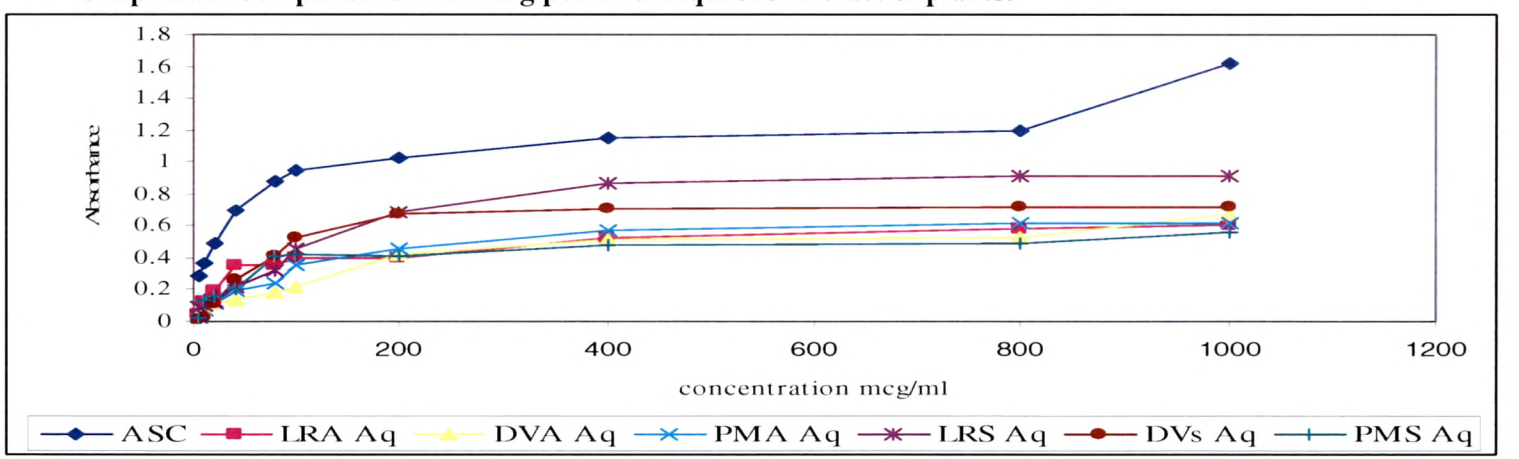
Table: 3.40 Reducing Power of Methanolic extract of the selected plants:

Sr No	Concentration µg/ml	Absorbance Ascorbic acid	Absiorbance of Methanolic Extract of Aerial parts of the plants			Absorbance of Methanolic Extract of stem of the plants		
			L.reticulata	*D.volubilis*	*P.microphylla*	*L.reticulata*	*D.volubilis*	*P.microphylla*
1	5	0.28	0.042	0.051	0.027	0.091	0.009	0.026
2	10	0.36	0.123	0.094	0.071	0.105	0.028	0.135
3	20	0.49	0.194	0.115	0.114	0.124	0.115	0.157
4	40	0.69	0.354	0.137	0.193	0.217	0.259	0.201
5	80	0.88	0.358	0.186	0.242	0.321	0.411	0.405
6	100	0.94	0.398	0.214	0.351	0.459	0.519	0.419
7	200	1.03	0.401	0.424	0.454	0.682	0.672	0.411
8	400	1.15	0.527	0.511	0.571	0.871	0.701	0.475
9	800	1.20	0.579	0.523	0.611	0.907	0.719	0.485
10	1000	1.62	0.601	0.667	0.617	0.911	0.723	0.557

Graph:3.18 Comparative Reducing power of Methanolic extract of plants:



Graph:3.19 Comparative Reducing power of Aqueous extract of plants:



3.7.2 Toxicity Studies:

Acute toxicity study was performed for selective extracts according to the acute toxic classic method (OECD, 1996) using the female albino rats. The maximum dose of the particular extract found not to be toxic are mentioned in following Table3.41

Table: 3.41 The highest tolerable level of the extracts evaluated:

Sr No	Descriptions		Dose in mg/kg body wt
	Name of the plants	Extract	
1	Aerial parts of *L.reticulata*	Pet. ether extract	1500
		Methanolic extract	1000
2	Aerial parts of *D.volubilis*	Pet. ether extract	1000
		Methanolic extract	1000
3	Aerial parts of *P.microphylla*	Pet. ether extract	3000
		Methanolic extract	1000

The toxicity studies suggested that the extract intended to be studied were toxic beyond the dose level mentioned. The dose level at which the activities performed were selected from the values obtained in Table 3.41. The screening of the extract for particular activity was started, with the dose, ten times lesser than that of the maximum safe dose as mentioned (Table 3.40). If the response was not observed the activity was performed at another dose level which is almost double of that of the dose selected initially. The physiological action of the extract was then ascertained by performing the same experiments at the dose which is again double to that of the initial dose elicited the response.

3.7.3 Assessment of Hepatoprotective activity of plant extracts (*in vivo* and *in vitro).*

3.7.3.1 *In-Vivo* Hepatoprotective activity:

The selected drugs are classified as *Rasayana* in *Ayurveda* which may exhibit their protective effect against different stress conditions. The phytochemical studies revealed the presence of flavonoids in the methanolic extracts of the all three plant drugs. Methanol extract during chemical investigations showed the presence of flavonoids and other phenolic compounds. There are many reports appeared in literature regarding the hepatoprotective potential of these compounds. These are believed to act due to their radical scavenging activity. Silymarine – a flavonoidal moiety is used as a positive control in many determinations of hepatoprotective activities. Liver is the organ which is highly sensitive to the stress conditions, especially due to the attack of chemical moieties and liver toxicity is also correlated with the depleted of antioxidants in animals The role of active radicals in induction and propagation of liver damage is well established. Different extracts of the *L.reticulata* showed presence of Luteoline, Diosmentine[37, 38], Isoquercetin, Hyperoside and Rutin[39] while Kaempferol[51] was detected in *D.volubilis*. The methanolic extracts of all the three selected drugs were, therefore, subjected for evaluation of hepatoprotective activity using reported models in rats.

Paracetamol was used as hepato-toxin, being an agent acting by generation of free radicals; to induce the hepatic toxicity in assessment of preliminary hepatoprotective potential of the plant extract with a view that if the results show positive trends then other agents could be tried. The assessment of activities was done by measuring the elevated levels of various biochemical markers as indicators of the liver function. The results of the experiments are tabulated as in Table 3.42.

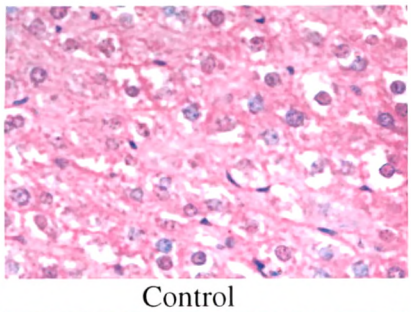
The serum level of GPT, GOT, Alkaline Phosphatase (AKP), Protein(TP) and Total Bilirubin (TB) was increased in group administered with only toxicant. The positive control has capability to inhibit such biochemical alterations. The results indicated test extracts did not possess significant activity. As, the test extract did not show statistically significant activity in lower dose level studies were performed at moderately higher level to assess any possible activity. Histopathology showed that all tissues, cell and central vein which were intact in the control animals, while incase of Paracetamol treated liver the damage to the central vein, generation of sinusoidal hemorrhage and disturbed integrity of the microstructures was observed. In case of the liver in the animals treated with Paracetamol and sylimarine as positive control partial improvement in damaged conditions was seen while in case of test group considerable protection in the damaged condition like liver hemorrhages or tissue integrity was not seen. The photomicrograph of representative slide from each group is shown in Fig. 3.22

Table 3.42: Results of Assessment of *In vivo* Hepatoprotective Activity:

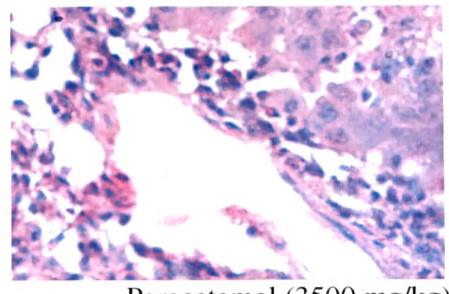
Sr No	Group	Dose (mg/kg body wt of animal)	SGOT Units/ml	SGPT Units/ml	ALKP Units/ml	TBL mg/dl	TP g/dl
1	Normal	0.2 ml 0.1% solution of Na carboxy methayl cellulose	65.433±2.457	36.591±1.404	71.074±1.464	1.185±0.116	5.416±0.038
2	Pracetamol	3500	178.113±6.113	147.0122±3.890	155.680±9.084	1.669±0.072	7.968±0.416
3	Positive control (Silymarine)	200	81.591±3.266	92.122±2.039	90.254±1.765	1.303±0.115	6.161±0.202
4	Methanolic Extract of *L.reticulata*	200	1763.950±6.136	166.946±7.322	146.138±1.765	1.683±0.101	7.078±0.339
		400	169.226±6.652	149.011±5.296	155.364±5.176	1.613±0.091	7.018±0.399
5	Methanolic Extract of *D.volubilis*	200	164.535±7.488	149.373±3.609	138.088±5.583	1.555±0.081	6.787±0.530
		400	169.756±6.425	153.235±7.234	137.425±9.362	1.685±0.077	7.466±0.959
6	Methanolic Extract of *P.microphylla*	200	161.814±6.790	156.228±8.542	151.380±11.863	1.632±0.048	8.459±0.506
		400	168.101±8.593	146.191±4.722	150.351±7.336	1.547±0.069	7.301±0.726

n=6, Values are presented as mean ± SEM. The data sets are not significantly differ to each other

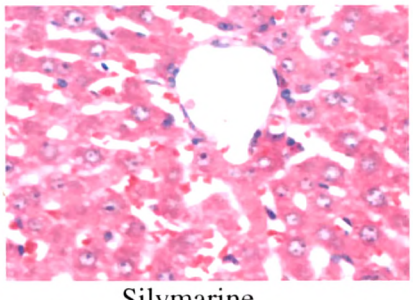
Fig: 3.22 Histological Evaluation of Hepatoprotective potential of the selected extracts:



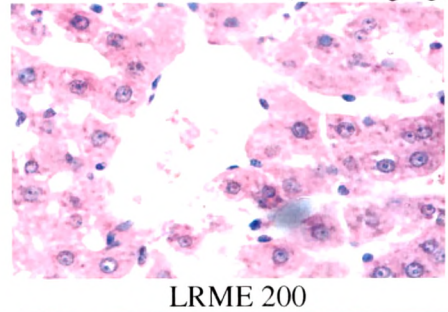
Control



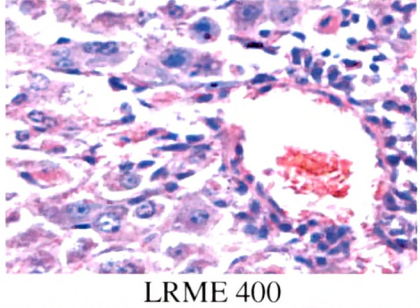
Paracetamol (3500 mg/kg)



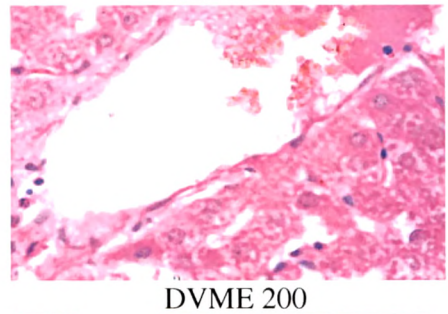
Silymarine



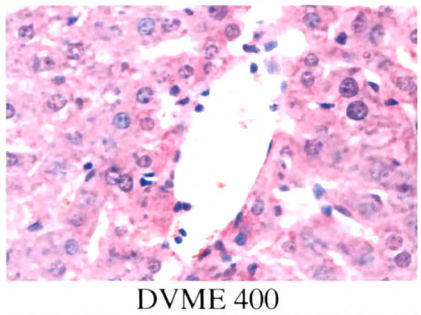
LRME 200



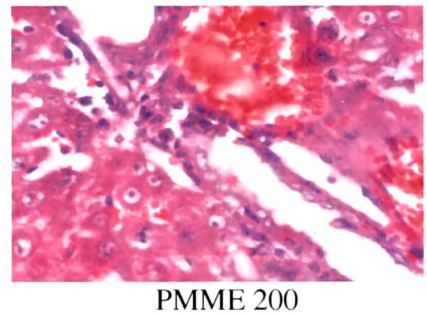
LRME 400



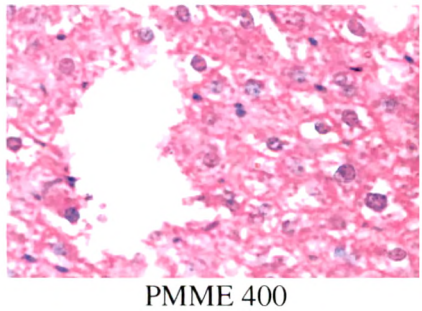
DVME 200



DVME 400



PMME 200



PMME 400

3.7.3.2 *In Vitro* Hepatoprotective Activity:

The results of *in vivo* hepatoprotective studies using paracetamol as toxicant, the methanolic extracts, however, did not show significant promising potential. These were again subjected to determine their hepatoprotective activity *in vitro* with a view to eliminate a possibility that some biologically active phytoconstituents were destroyed or could not permeate due to complex structure in the *in vivo* studies. The experiments were, therefore, performed to observe effects of the three plant drug test samples, on isolated rat hepatocytes culture *in vitro*. The isolated hepatocytes were treated with Paracetamol which induces mortality, while the level of protection afforded by the extracts, was assessed by determining the percentage viability of the hepatocytes in presence of toxicants. The results suggested sharp decrease in viability of hepatocytes after treatment with paracetamol. The test extracts however did not show significant improvement in the viability of the hepatocytes.

The results of the *in vivo* and *in vitro* experiments, however, did not provide convincing reasons of using these plants as hepatoprotective agents against chemical induced liver toxicity and may have indirect effect as adaptogenic agents.

Table 3.43 : *In vitro* Hepatoprotective activity:

Sr NO.	Description	% Viability
1	Control	83.740±3.964
2	Control + Paracetamol	15.150±6.197
3	Control + Paracetamol + Silymarine	48.359±6.385
4	Control+ Paracetamol+ Methanolic extract of *L.reticulata*	14.326±3.117
5	Control+ Paracetamol+ Hydrolyzed Methanolic extract of *L.reticulata*	14.887±2.972
6	Control+ Paracetamol + Methanolic extract of *D.volubilis*	12.997±2.535
7	Control+ Paracetamol+ Hydrolyzed Methanolic extract of *D.volubilis*	12.044±2.924
8	Control+ Paracetamol + Methanolic extract of *P.microphylla*	12.133±2.937
9	Control+ Paracetamol + Hydrolyzed Methanolic extract of *P.microphylla*	12.421±2.135

n=6, Values are presented as mean ± SEM. The data sets are not significantly differ to each other

3.8.4 Assessment of comparative adaptogenic potential shown by methanolic extracts in swim endurance test[98]:

All the three plants selected in the present study are described under the category of *Rasayana* in *Ayurveda* texts. *Rasayana* are generally, agents which protect the body from nonspecific stress conditions. In modern therapy such type of substances are called as adaptogens. In order to access adaptogenic potential of the selected plants, a well accepted model of swim endurance test was used.

The methanolic extracts of *L.reticulata*, *P.microphylla* and *D.volubilis* were used in stress induced rats. The stress was induced by enforcing the animals to swim and the changes in various biochemical parameters under the stressed conditions were measured.

Various biochemical parameters were altered during stress conditions. Hyperglycemic response was observed on early days but with increased duration of stress, such alterations could not be observed as homeostatic mechanism regulates the blood glucose level. Enhanced activity of the hypthaloamo-hypophysal axis regulates the level of serum cholesterol and it is directly linked to the level of catecholemines and corticosteroids[117]. The extracts studied minimize the alterations in the level of cholesterol, thus, it might inhibit the increment in the level of catecholamine in the animals. The altered level of triglycerides in the shock conditions depends on the type of the shock. The stress associated with motor racing or public speaking induces the triglyceride level in subjects[118] while electric shock suppressed the level of triglycerides [119]. The living system can not differentiate the stress conditions and thus such conflicting responses could be observed [120]. Since, a close relationship has been established between catecholamine and triglycerides it could be suggested that the alteration in the level of serum triglyceride is possibly mediated via adrenal medullary secretions and through activation of sympathetic nervous system. The significant rise of SGPT indicates

generation of the stress[98]and the ability of the extracts in minimizing such rise suggests ability of extracts in protecting the animals from stress conditions.

In general, the level of Serum GPT was increased while the level of other selected parameters, serum glucose, serum cholesterol, serum triglycerides and serum blood urea nitrogen was appeared to be decreased during the stress condition, observed in control group. The animals treated with methanolic extracts of these drugs showed marked changes in these parameters, thus, protecting the animals in the stress conditions. The methanolic extract showed this effect in dose dependant manner.

Table 3.44: Assessment of Comparative Potential of Methanolic Extract against Swim Endurance Test:

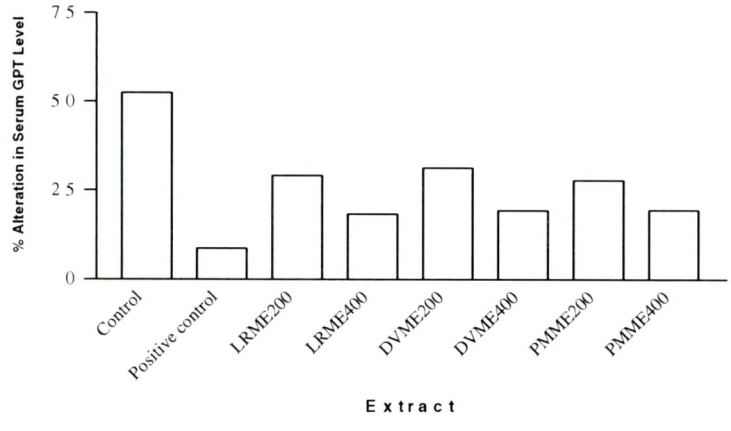
Sr No			SGPT Units/ml		Serum Glucose mg/dl		Triglyceride mg/dl		Cholesterol mg/dl		Blood Urea Nitrogen mg/ml	
Days		Dose in mg/kg body wt of animal (p.o.)	1	7	1	7	1	7	1	7	1	7
1	Blank (without stress)		46.918± 1.939		111.165± 1.823		53.169 ±3.807		36.809± 4.057		20.028 ±2.406	
2	Control	0.2 ml solution of 0.1% SCMC	56.656± 1.267##	86.414± 6.028	121.143± 4.523##	83.958± 4.016	56.425± 1.924#	34.300± 3.579*	50.105± 3.065##	19.858 ±2.689	32.163 ±1.883##	16.458 ±1.03**
3	Positive Control (*Borhaevia diffusa* aqueous extract)	200	61.235± 1.451##	66.594± 3.181**	103.120± 2.912##	93.990± 2.537**	58.960± 1.643#	48.241± 2.640*	50.056± 2.956##	38.800 ±9.307**	26.368 ±1.584##	25.161 ±2.656**
4	Methanolic extract of *L.reticulata*	200	58.647± 1.779###	75.719± 3.056**	98.086± 4.030##	91.306± 2.077**	57.208± 2.467#	36.880± 3.046*	55.073± 1.044##	37.465 ±6.926***	26.155 ±0.516##	20.025 ±2.242**
		400	60.593± 6.369###	71.701± 2.413**	101.794± 2.566##	97.014± 3.123**	55.751± 4.146#	43.175± 4.678*	53.205± 0.871##	46.163 ±2.522**	25.435 ±0.729##	21.441 ±0.729**
5	Methanolic Extract of *D.volubilis*	200	59.915± 5.962##	78.646± 2.654*	104.22± 3.938#	100.335± 1.980**	58.566± 1.484#	32.380± 2.267*	72.906± 7.789##	54.611 ±4.726***	38.630 ±2.775##	21.301 ±1.851***
		400	61.406± 4.564##	73.336± 2.346**	101.038± 5.701#	98.0383± 5.411**	53.966± 2.612#	37.753± 4.051*	67.721± 2.753###	56.240 ±6.738**	28.896 ±1.554##	19.365 ±1.513***
6	Methanolic Extract of *P.microphylla*	200	60.581± 4.803##	77.443± 2.271*	113.041± 5.770#	100.938± 5.810***	50.711± 1.468#	34.696± 3.641**	80.025± 7.565###	59.191 ±3.492***	27.131 ±0.845###	20.313 ±2.159***
		400	59.819± 4.394##	71.469± 1.779**	110.750± 4.400#	105.746± 2.582**	56.603± 2.302#	44.436± 2.521*	79.170± 5.160###	62.348 ±6.752*	27.955 ±1.067##	22.976 ±0.715***
	Two Way ANOVA	F	7.313		4.928		10.41		9.236		16.451	
		p	<0.001		<0.001		<0.0001		<0.0001		<0.001	

Values are mean ± SEM. *p<0.5, **p<0.05, ***p<0.001, #p<0.5, ##p<0.05, ###p<0.001as compared to respective controls

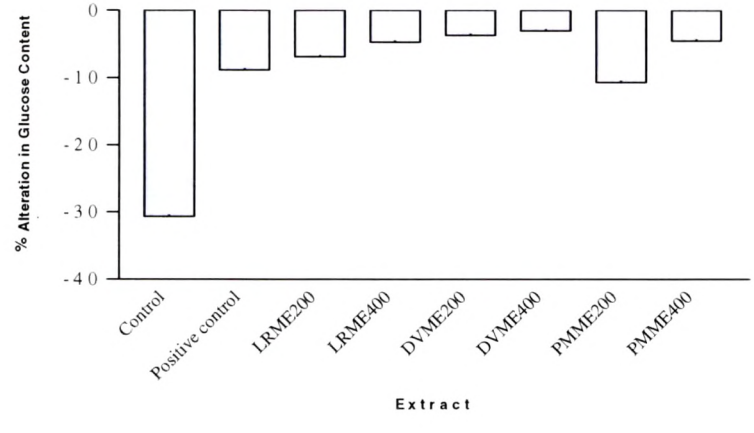
Table: 3.45 Percentage alterations in selected biochemical markers in swim endurance test:

			SGPT(%)	Serum Glucose (%)	Triglyceride (%)	Cholesterol (%)	Blood Urea Nitrogen (%)
1	Control	0.2 ml Vehicle	52.506	-30.695	-39.734	-60.367	-48.829
2	Positive Control (Borhaevia diffusa aqueous extract)	200	8.736	-8.853	-11.690	-22.486	-4.557
3	Methanolic extract of *L.reticulata*	200	29.104	-6.906	-35.538	-31.977	-23.137
		400	18.336	-4.692	-67.127	-13.235	-15.702
4	Methanolic Extract of *D.volubilis*	200	31.258	-3.713	-44.711	-16.953	-32.983
		400	19.426	-2.969	-30.042	-25.093	-44.858
5	Methanolic Extract of *P.microphylla*	200	27.830	-10.706	-31.580	-21.247	-25.129
		400	19.475	-4.518	-21.490	-26.034	-17.810

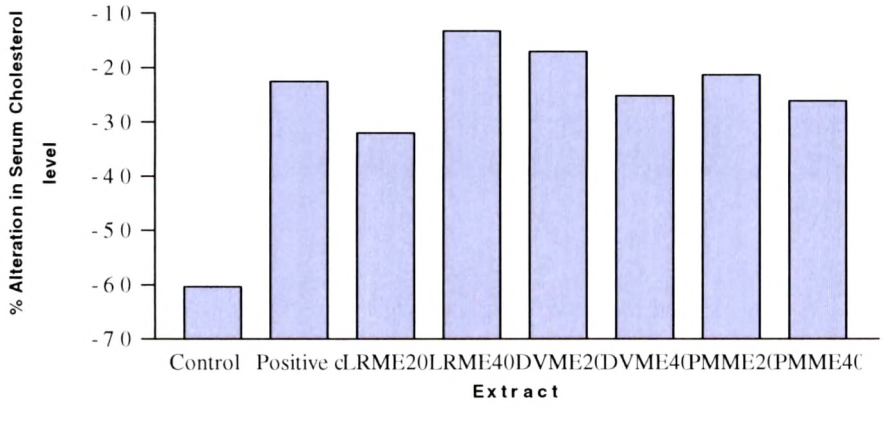
Graph: 3.20 Comparative alteration of serum GPT level in test groups:



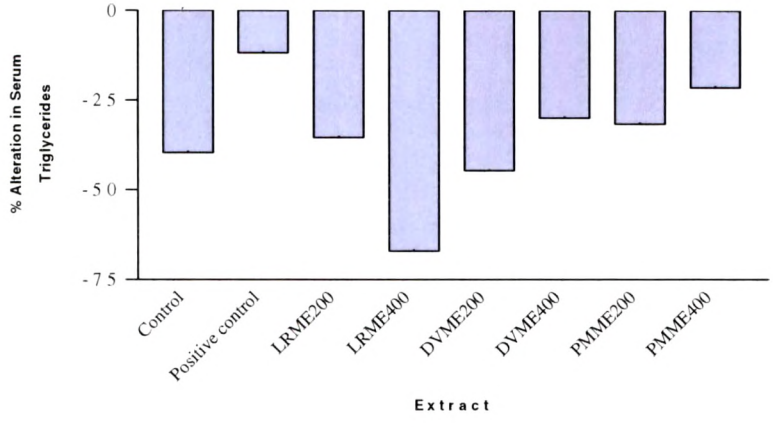
Graph: 3.21 Comparative alteration of serum Glucose level in test groups:



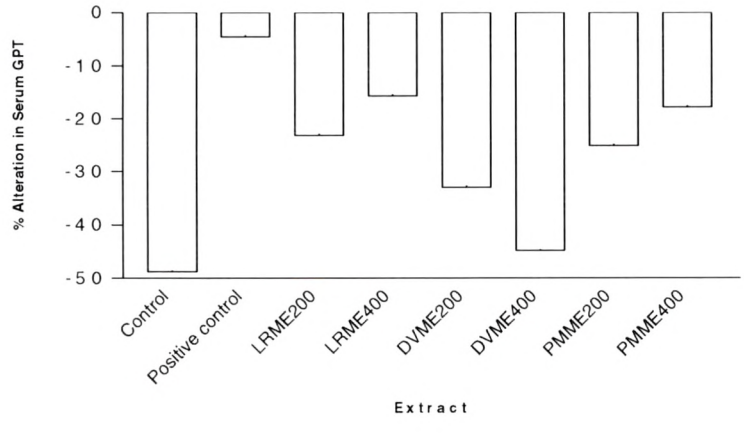
Graph: 3.22 Comparative alteration of serum Cholesterol in test groups:



Graph: 3.23 Comparative alteration of serum Triglyceride level in test groups:



Graph: 3.24 Comparative alterations in serum level of BUN in test groups:



3.7.5 Assessment of lactogenic potential of extracts of the selected plants.

3.7.5.1 Assessment of lactogenic potential of extracts of *D.volubilis* and *P.microphylla* in lactating rats and comparison with extracts of *L.reticulata*:

The entire plant of *L.reticulata (Asclepidaceae)* is extensively used as lactogen traditionally in veterinary practice and has almost reached to a level of extinction due to excessive consumption. The plants of *Dregea volubilis* and *Pentatropis microphylla (Asclepidaceae)*

are now used as its substitute and sometimes replace the original drug as traditional lactogen. The lactogenic potential of these drugs was studied in rats using ,pup weight, weight of mother, parenchyma percentage, secretary rating, estimation of total protein content, glycogen content of mammary glands tissues and prolactin level in serum as assessment parameters.

The results of animal experiments are tabulated in Table 3.46; indicate dose dependant increment in total protein content and glycogen content of the biopsiesd mammary glands in all treated groups.

The increase in formation of milk requires more amount of normal sugar and proteins, therefore proportionate increase in reserves of tissue glycogen and proteins are necessary. The arithmetic mean amount of glycogen and total protein content in mammary tissues of treatment groups were found higher than those of the control groups, showing higher metabolic state of the tissues analyzed.

The histological observations, from photomicrographs of the slides of each group shown as in Fig. 3.23 to Fig. 3.25 were recorded and quantitative determinations were made. The intensity of observed alterations is expressed in the form of secretory ratings case of all the treated groups the secretory ratings of the tissues increased significantly in a dose dependant manner, when compared to the respective control groups.

The lactation stage in mammary glands is characterized by enlargement of the alveoli and thus increment in parenchyma area with subsequent decrease in stroma is observed. The appearance of epithelial cells is cuboidal while the myoepithelial cells are flattened in shape[20].

The parenchyma percentage calculated as the ratio of area of parenchyma cell to the ground tissues in mammary gland stated in percentage. In present studies there has also been an

increase observed in percentage of the area covered by the parenchyma tissues in mammary glands.

The yield of milk in any animal is the product of the output per active secretory cell and to the total number of such cells .The parenchyma percentage represents the relative amount of the cells concerned with the lactation to the stroma[21].

At the end of the 13th day, increment in body weight of pups and decrease in mothers' weight were observed and reported in percentage. According to the principles of bio energetic, with the increase in the output of milk, the lactating mother rats lose body weights if increased energy output is not compensated by extra ration. The decreasing trend towards loss in mother rats' body weights leads towards a conclusion that the effect may be secondary to lacto genesis in mothers.

The results indicated *P.microphylla is better* substitute of *L.reticulata* when compared to *D.volubilis*. The studies also justify the selection of Stigmasterol as marker for lactogenic potential of the plants and plant extracts.

The results of the permeation studies revealed that the triterpenoids/steroids from Pet ether extract and flvaonoids from methanolic extract of the plants were able to permeate through the membrane. The compounds could pass *in vitro* through the membrane might be responsible for the physiological action of the extract studied.

The studies supports the traditional claims of *D.volubilis* and *P.microphylla* as therapeutic substitutes of *L.reticulata*, both of them can be utilized in place of *L.reticulata* as regular veterinary lactogens after through efficacy trials. Presence of stigmasterol in the extract studied was confirmed, indicates Stigmasterol and other phytosterols are the components responsible for lactogenic potential of the Pet. ether extract of the extracts studied.

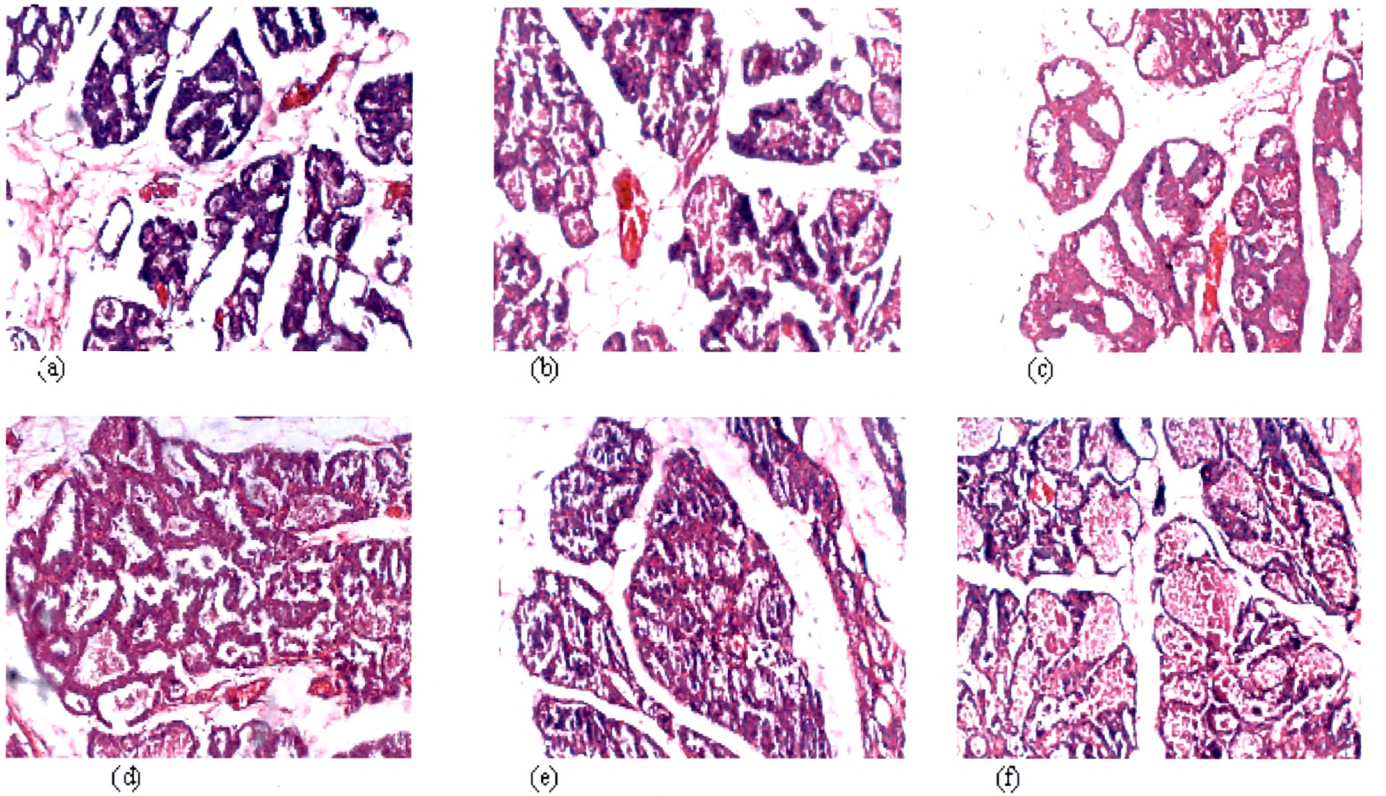
The serum level of prolactin in female rat was estimated on 3rd, 8th and 13th day of the pup birth. The experiments were performed using the principle of bioluminescence in Immuno assay using the kit meant for estimation of prolactin in human. The experiments were performed using the facilities available at commercial pathology laboratory by simulating the determination of prolactin in human serum. The results obtained were, however, not convincing and not statistically significant may be due to nonspecificity of the antibody used, to the rat prolactin.

Table: 3.46 Biochemical and Histological Studies on mammary glands of lactating rats:

	Description	Dose Mg/kg body wt of animal per day p.o using pea nut oil as vehicle	Protein content mg/100 mg of mammary glands tissue	Glycogen content mg/100 g of mammary glands tissue	% parenchyma	Secretary rating	% Wt decrease in mother's weight	% Wt increase in pups' weight
1	Control	0.2 ml pea nut oil	9.993 ±0.311	36.178 ±0.832	51.774 ±2.152	1.167 ±0.105	-3.33 ±1.46	110.71 ±7.204
2	Positive control	4.5	12.014** ±0.778	45.935** ±1.816	64.232** ±4.973	2.667** ±0.247	-2.98* ±0.43	159.77* ±4.301
3	Stigmasterol	1.0	14.267*** ±0.300	41.167** ±1.095	65.991** ±5.940	2.000** ±0.182	-1.63* ±0.22	140.47* ±31.63
		2.00	16.983*** ±1.154	53.562*** ±2.125	77.895*** ±4.161	2.750*** ±0.214	-1.31* ±1.10	178.75* ±9.77
4	*L.reticulata* Pet.ether extract	100	11.301* ±0.837	43.891** ±2.562	62.954** ±3.782	2.167** ±0.210	-3.38* ±1.11	158.47* ±32.27
		200	12.365** ±0.835	53.624** ±4.633	69.498*** ±1.670	2.250** ±0.250	-2.91* ±0.97	136.21* ±11.14
5	*D.volubilis* Pet.ether extract	100	10.986** ±0.230	49.785** ±3.644	57.836* ±2.822	2.333*** ±0.217	-1.73* ±0.90	107.65* ±5.27
		200	12.681*** ±0.729	52.901** ±3.815	63.905** ±4.407	2.667*** ±0.115	-0.79* ±0.15	100.03* ±7.53
6	*P. microphylla* Pet.ether extract	100	10.968** ±0.295	46.266** ±2.247	59.003** ±2.140	2.000** ±.223	-0.94* ±0.20	152.31* ±16.09
		200	12.158*** ±0.293	55.337*** ±2.766	59.526** ±2.321	2.250*** ±0.170	-0.45* ±0.31	154.69* ±7.67
		400	12.457** ±0.755	67.901*** ±3.093	65.984** ±3.610	2.583*** ±0.153	0.21* ±0.29	148.13* ±11.72
7	*P. microphylla* Methanolic extract	50	11.114** ±0.3015	58.684** ±3.905	71.053*** ±2.167	2.167** ±0.380	-0.69* ±0.16	175.77* ±14.12
		100	11.769** ±0.379	65.901*** ±3.778	65.639** ±2.545	2.500*** ±0.516	0.14* ±0.34	165.38* ±27.78
		150	12.701*** ±0.532	71.065*** ±1.736	75.516** ±2.635	3.250** ±0.559	0.88* ±0.25	198.73* ±9.64
8	One way ANOVA	F	7.554	12.225	4.077	2.813	3.762	2.846
		df	83	83	83	83	83	83
		p	<0.001	<0.001	<0.001	<0.01	<0.05	<0.05

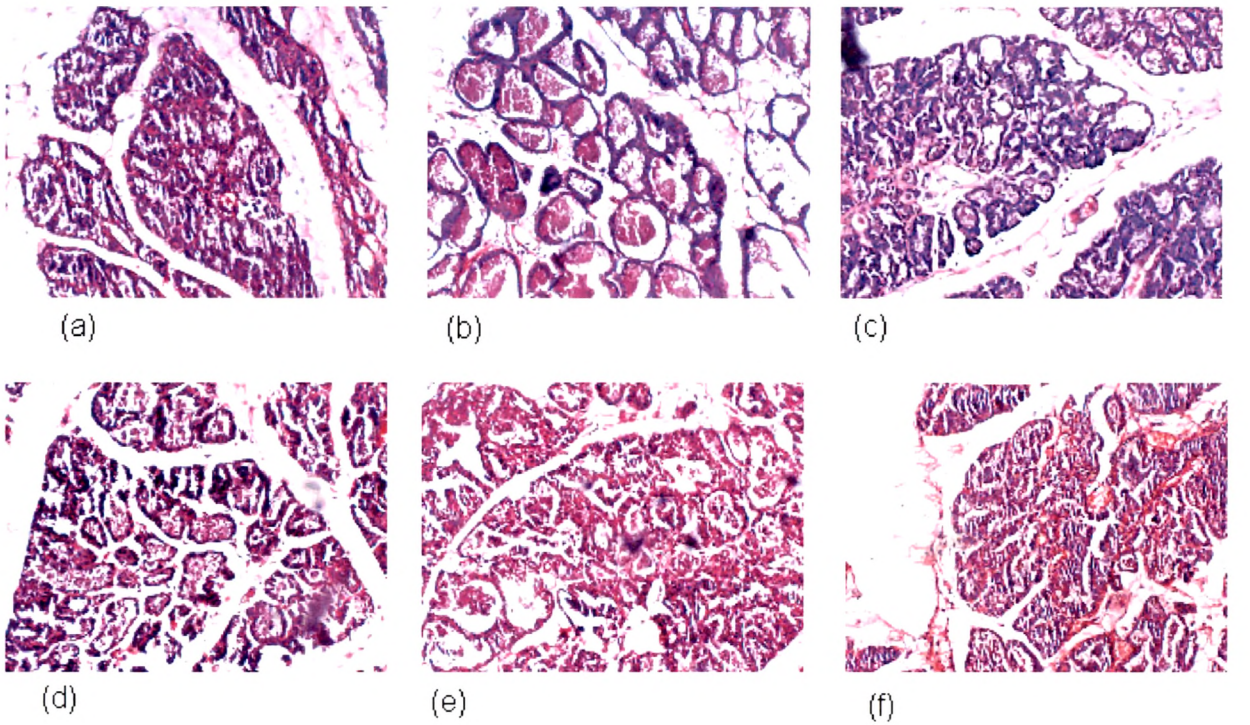
Values are mean ± SEM. *p<0.5, **p<0.05, ***p<0.01, ****p<0.001 as compared to respective controls.

Fig: 3.23 Photomicrograph of mammary glands tissues (400 X):



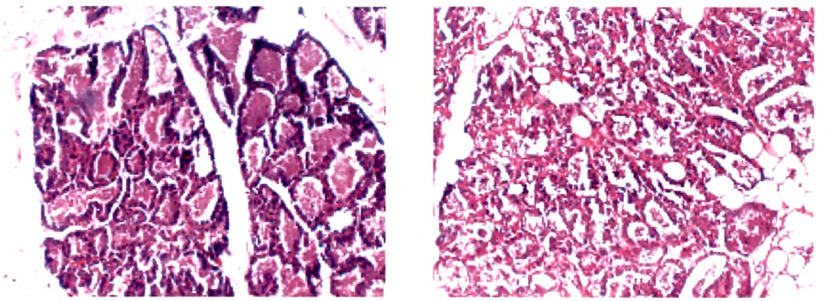
a- Blank (0.2 ml Arachis oil) , b- 4.5 mg/kg Domperidone, c-1.0 mg/kg stigmasterol, d- 2.0 mg/kg stigmasterol, e-Pet.ether extract of *L.reticulata*, 100 mg/kg , f- Pet.ether extract of *L.reticulata* 200 mg/kg

Fig:3.24 Photomicrograph of mammary glands tissues (400 X):



a- Pet.ether extract of *D.volubilis*, b- 100 mg/kg, b- Pet.ether extract of *D.volubilis*, 200 mg/kg, c- Pet.ether extract of *P.microphylla*, 100 mg/kg, d- Pet.ether extract of *P.microphylla*, e- 200 mg/kg, Pet.ether extract of *P.microphylla*, 400 mg/kg, f- Methanol extract of *P.microphylla*, 50 mg/kg

Fig:3.25 Photomicrograph of mammary glands tissues (400 X):



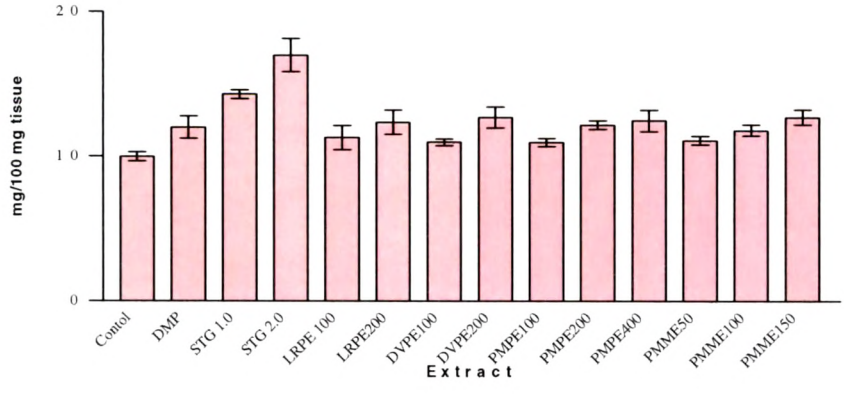
(a) (b)

a- Methanolic extract of *P.microphylla*, 100 mg/kg, b- Methanolic extract of *P.microphylla*, 150 mg/kg

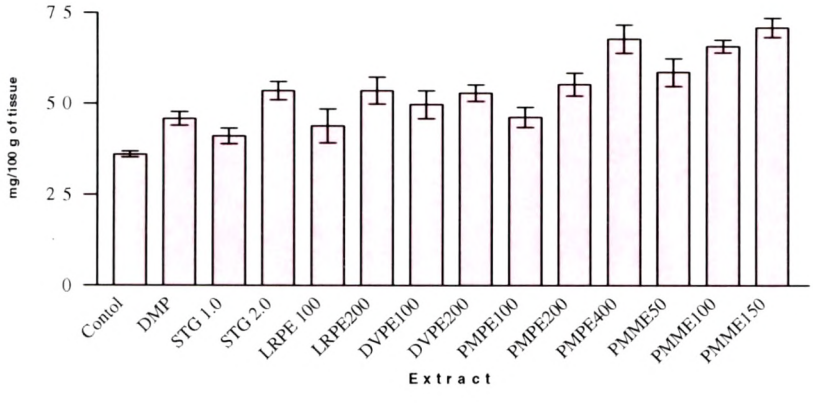
Table: 3.47 Percentage alterations in Biochemical and Histological parameters as compared to control:

Sr .No	Description	Dose Mg/kg body wt of animal per day p.o using pea nut oil as vehicle	% Increase in Protein content as compared to control	% Increase in Glycogen content as compared to control	% Increase in area covered by parenchymatous cells as compared to control	% Increase in Secretory rating as compared to control	% Wt decrease in mother's weight as compared to control	% Wt increase in pups' Weight as compared to control
1	Positive control	4.5	20.85	26.96	24.06	128.53	10.51	49.06
2	Stigmasterol	1.0	42.76	13.79	27.45	71.37	51.05	26.88
		2.00	69.94	48.05	50.455	135.64	60.66	61.45
3	*L.reticulata* Pet.ether extract	100	13.07	21.31	21.59	85.68	1.50	43.13
		200	23.72	48.22	34.23	92.80	12.61	23.03
4	*D.volubilis* Pet.ether extract	100	9.92	37.61	60.62	99.91	48.04	2.76
		200	26.89	46.22	23.43	128.53	76.27	9.64
5	*P. microphylla* Pet.ether extract	100	9.75	27.77	15.03	71.37	71.77	37.57
		200	21.66	52.95	14.97	85.68	86.48	39.72
		400	24.655	87.68	27.44	121.33	93.69	33.80
6	*P. microphylla* Methanolic extract	50	11.210	62.20	17.92	85.68	79.27	58.76
		100	17.77	82.15	27.35	114.22	95.79	49.38
		150	27.09	95.43	45.85	178.49	126.42	79.50

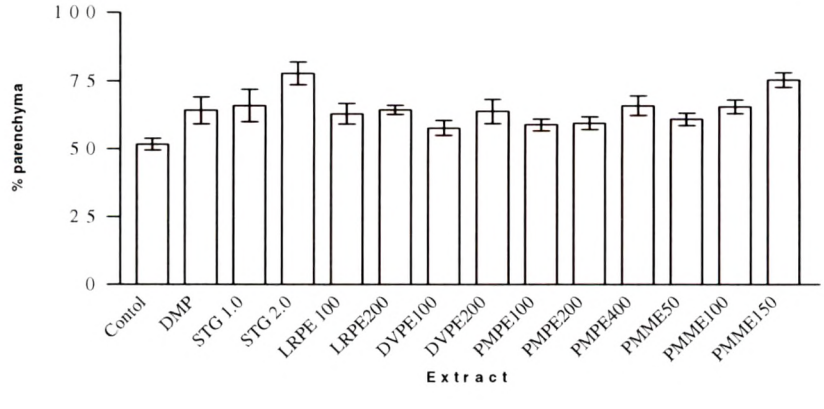
Graph: 3.25 Total glycogen content of mammary glands tissue as compared to control:



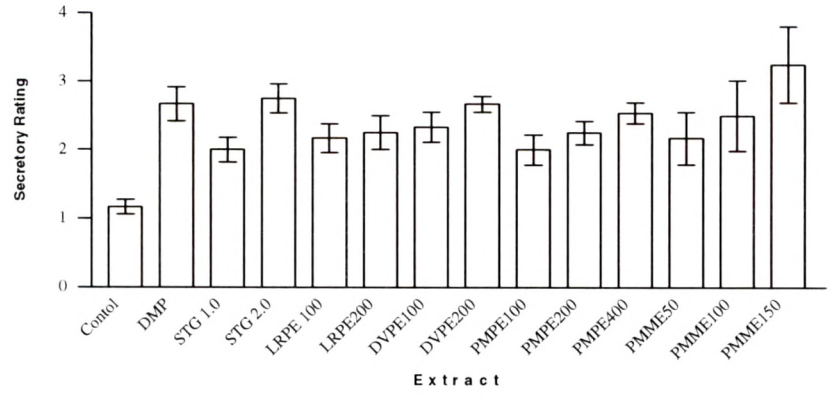
Graph: 3.26 Total glycogen content of mammary glands tissue as compared to control:



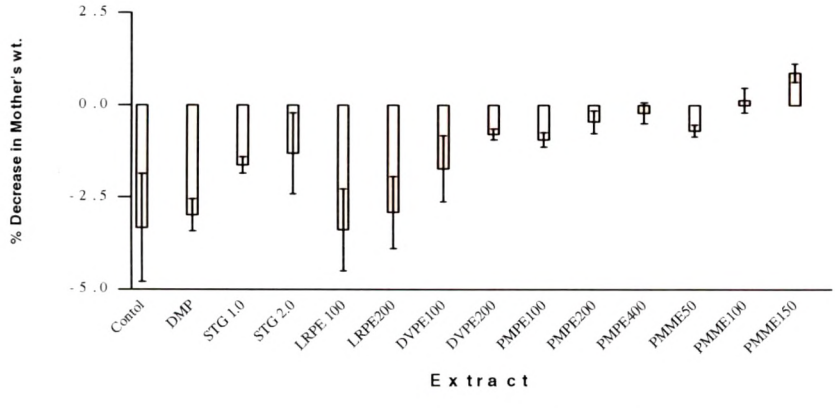
Graph: 3.27 Percentage of parenchyma present in mammary glands tissue as compared to control:



Graph: 3.28 Secretory Rating of mammary glands tissue as compared to control:



Graph: 3.29 Decrease in mother's weight from 3rd to 13th day of the pup birth:



Graph: 3.30 Increase in pup's weight from 3rd to 13th day of the birth:

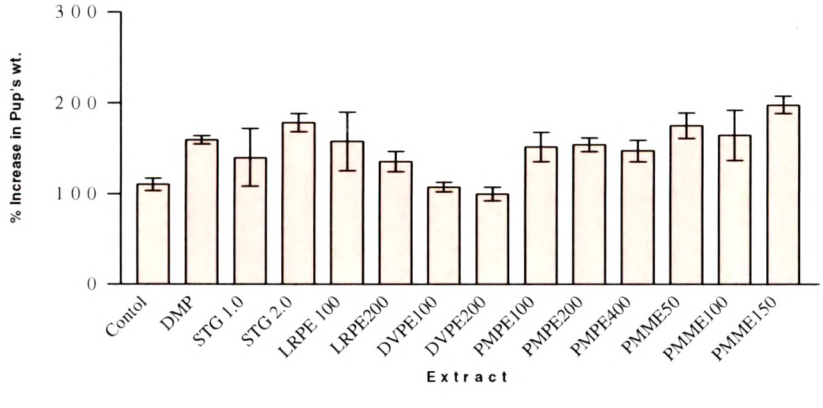
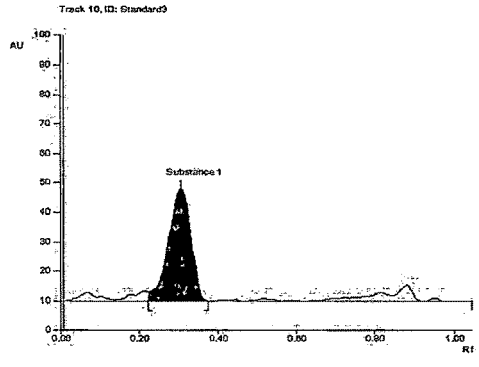
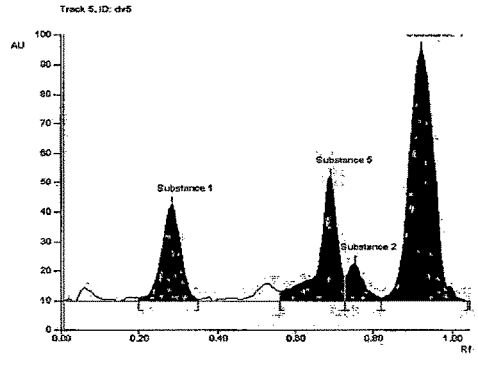


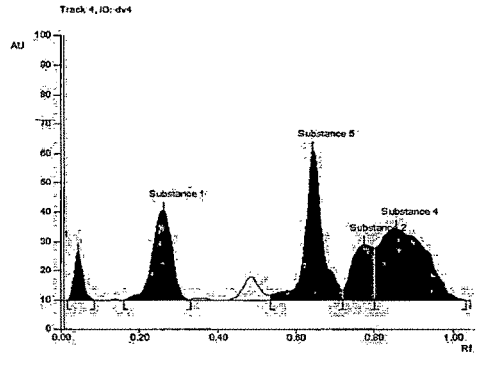
Fig. 3.26 Chromatogram of the content permeated from the extract through intestine *in vitro:*



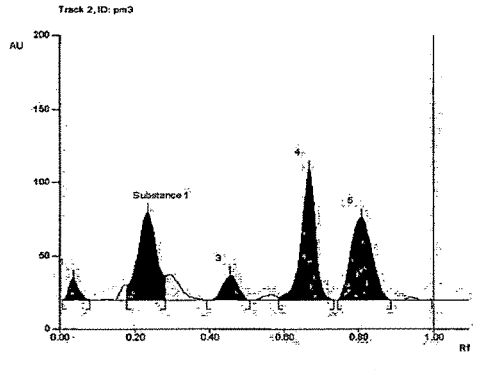
(a) Stigmsterol standard



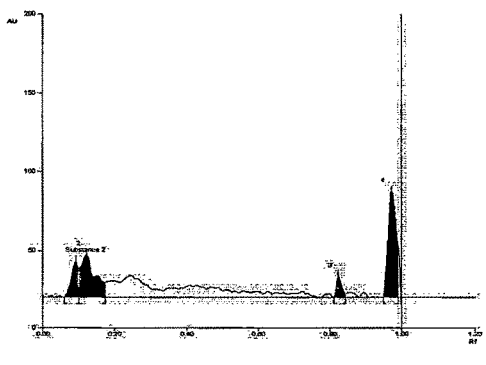
(b) *D.volubilis* Pet .ether extract



(c)*L.reticulata* Pet .ether extract



(d)*P.microphylla* Pet .ether extract



(e) *P.microphylla* Methanolic extract

3.7.5.2 Assessment of lactogenic potential of the formulations prepared from P.ether extract of *L.reticulata*:

The comparative lactogenic potential of formulations prepared by modern method as well as traditional method gave interesting results. The Pet. ether extract of *L.reticulata,* when administered to animals, the activity was found to be increased significantly, than that of the traditional formulation. The results are tabulated in Table 3.48

The traditional use of butter oil, itself is an indicator of extracting more non polar components which may be considered to be responsible activity. The enhancement of the activity as shown by Pet. ether extract in lab formulation and presence of Stigmasterol in considerable amount in this, clearly indicate the efficacy of formulation and justifies its traditional methodology of preparation.

Table3.48 Lactogenic Potential of formulations assessed:

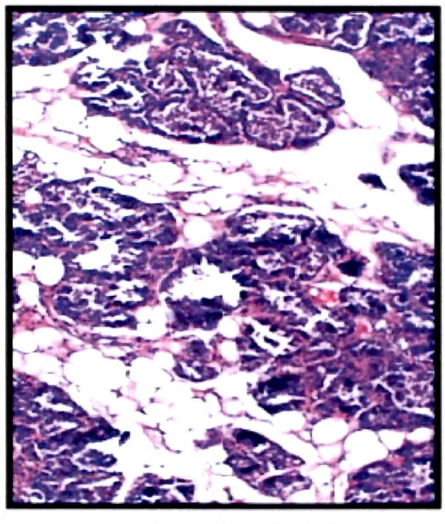
Sr .No	Description		Protein content mg/100 mg of wet mammary glands tissues	Glycogen content mg/100 g of wet mammary glands tissues	% parenchyma	Secretary rating	% increase in pups' wt.	%decrease in mother's wt
1	O/W emulsion Blank		7.040 ±0.390	36.516 ±4.027	53.403 ±1.227	1.083 ±0.1537	117.88 ±6.45	-7.63 ±1.17
2	O/W Emulsion [100 mg/kg body wt P.ether extract (p.o.) incorporated in oil phase]		10.840** ±0.498	50.010*** ±1.483	78.391*** ±3.4355	2.333** ±0.2108	197.57* ±13.72	0.62* ±1.17
3	Ghee (control)		6.566 ± 0.338	52.896 ±1.702	50.932 ±2.67	1.583 ±0.2713	113.67 ±3.46	-2.85 ±0.61
4	Traditional fromulation [100 mg/kg body wt P.ether extract (p.o.) incorporated in *ghee* by heating]		8.714*** ±0.235	58.017** ±2.511	53.403** ±1.227	2.500** ±0.1291	139.48** ±7.04	6.07** ±0.98
5	One way ANOVA	F	25.936	24.211	24.5016	11.052	5.614	0.7239
		df	3,20	3,20	3,20	3,20	3,20	3,20
		p	0.0001	0.0012	0.0001	0.002	0.0228	0.5656

Values are mean ± SEM. *p<0.5, **p<0.05, ***p<0.01, ****p<0.001 as compared to respective controls.

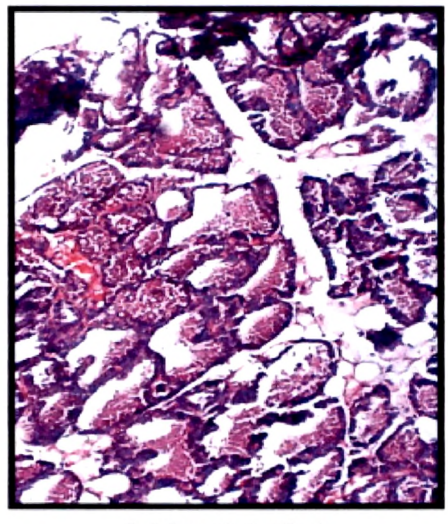
Table3.49 Comparison of Lactogenic Potential of formulations with respective controls:

Sr .No	Description	Protein content mg/100 mg of wet mammary glands tissues	Glycogen content mg/100 g of wet mammary glands tissues	% parenchyma	Secretary rating	% increase in pups' wt.	%decrease in mother's wt
2	O/W Emulsion [100 mg/kg body wt P.ether extract (p.o.) incorporated in oil phase]	53.977	36.953	47.114	115.420	67.755	11.205
4	Traditional fromulation [100 mg/kg body wt P.ether extract (p.o.) incorporated in *ghee* by heating]	32.713	9.681	4.851	57.927	22.706	312.98

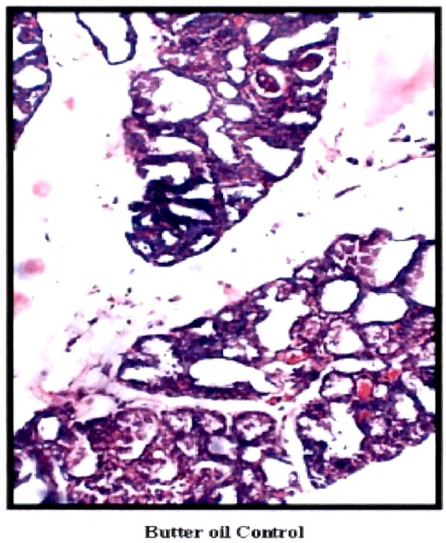
Fig:3.27 Photomicrograph of histology of mammary glands in rats after treatement with lactogenic formulation (400 X):



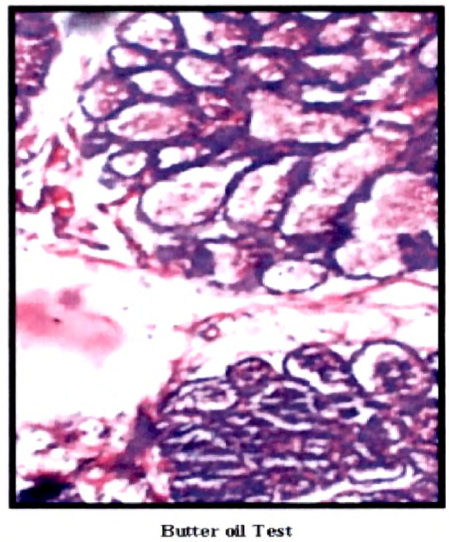
Lab Formulation Control



Lab Formulation Test



Butter oil Control



Butter oil Test

3.8 Implementation of Developed Software in assessment and retrieving of the Data related to Standardization of Plants:

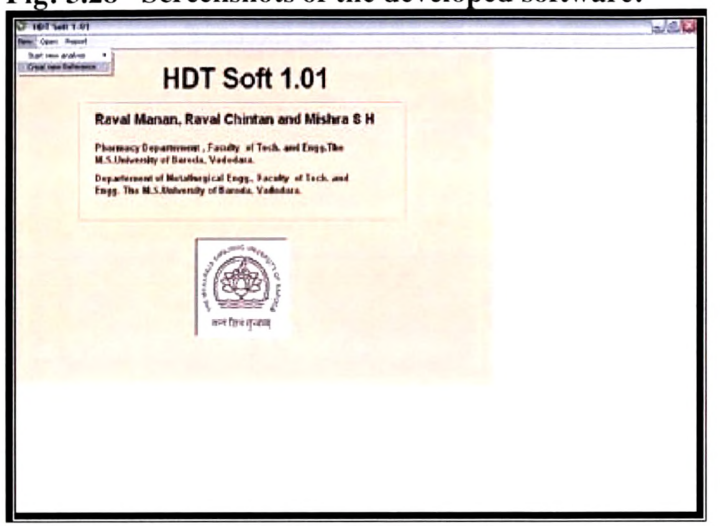
The Software was developed as Visual Basic application which is capable of generation of two types of the files:

1. Reference files created by the administrator
2. Analysis files created by the chemist.

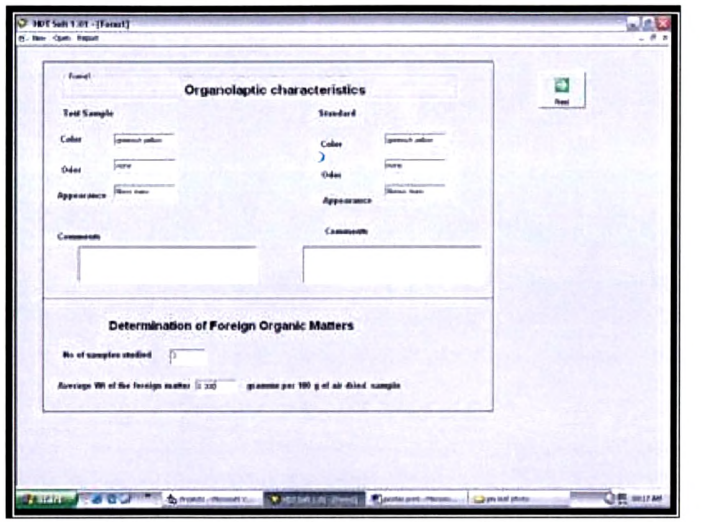
The observations of organoleptic characteristics and photomicrographs of identifying features of the powdered plant materials can be inserted in the software at the dedicated places (Fig. 3.28 (c)). The size of the microscopic features was measured and the results are expressed in the report sheet (Fig. 3.28(d)). Various physicochemical parameters determined as per WHO guidelines for standardization of the plant materials were inserted at the respective places as shown in Fig. 3.29(a) along with the summary of the result of phytochemical evaluation. The fingerprint profile developed on HPTLC in the form of chromatorgram was then inserted, as shown in Fig. 3.29(b). The result and the chromatograms along with correlative UV spectra of the compounds were inserted at the respective places. [Fig. 3.29 (c)].

The developed software, thus, will be helpful in storing and comparing the data pertaining to identity and quality of the plants. The data stored can be retrieved when necessary and the results can be seen in the form printed report.

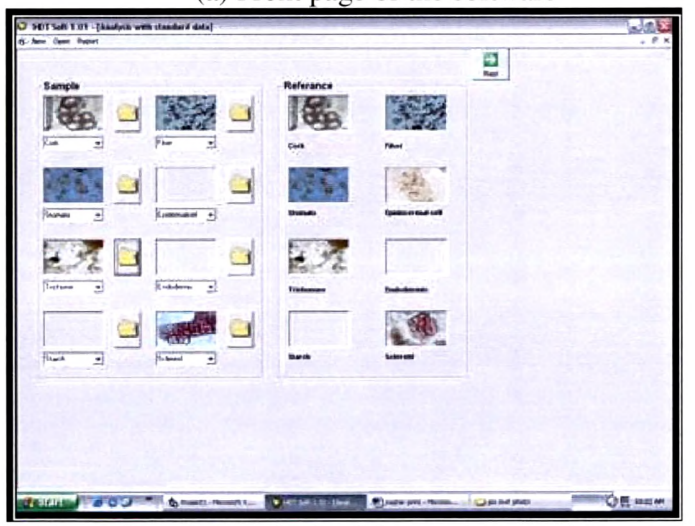
Fig: 3.28 Screenshots of the developed software:



(a) Front page of the software



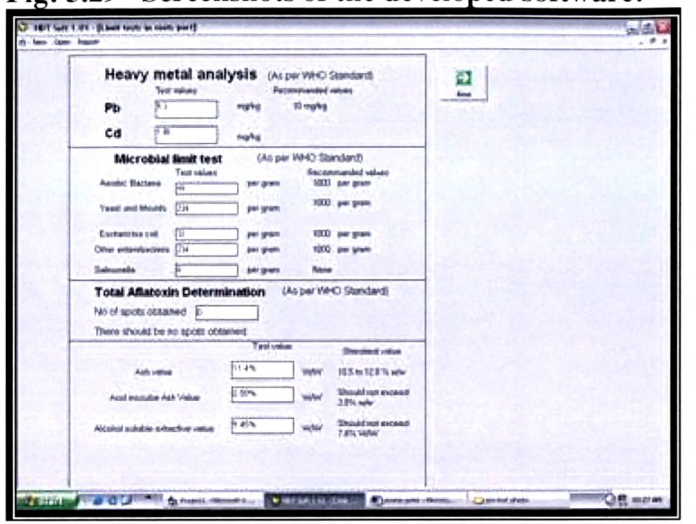
(b)Morphological Evaluation of Powdered Drug



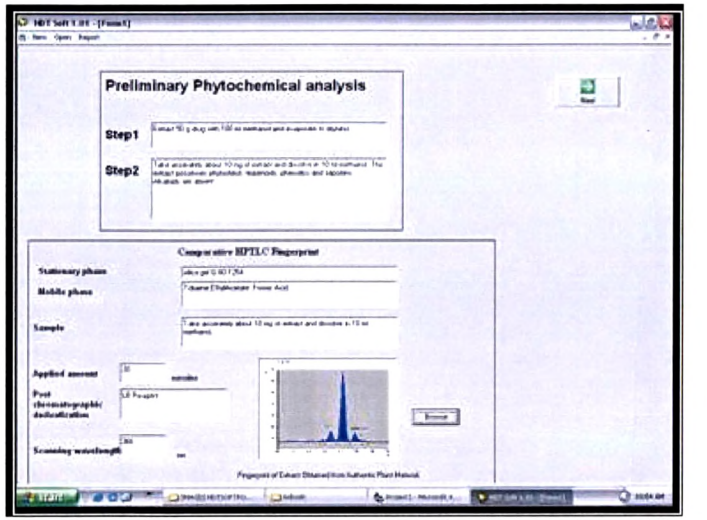
(c) Insertion of data obtained from microscopic examination

(d) Measurement of the cell sizes

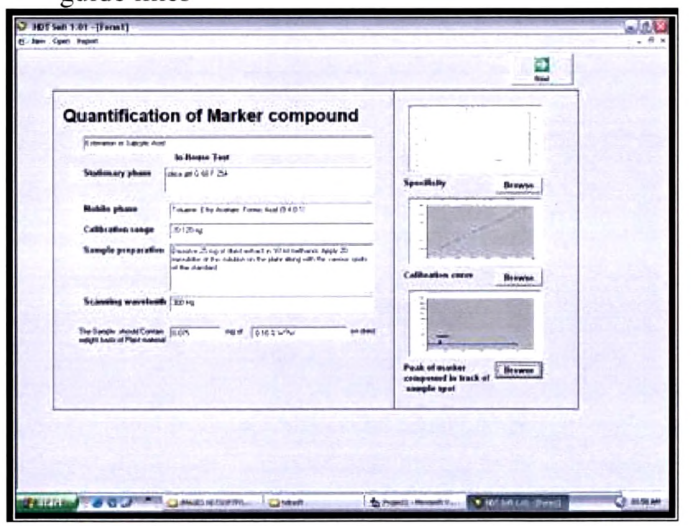
Fig: 3.29 Screenshots of the developed software:



(a) Summary of the parameters evolved as per WHO guide lines



(b) Summary of Preliminary Phytochemical screening with representative fingerprint



Summary of quantification of marker compound