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RESEARCH ARTICLE

PHYSICO - CHEMICAL AND HPTLC STUDIES OF *LIMONIA CRENULATA* (ROOT)

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Abstract

Herbal medicines have gained popularity as potential therapeutic agents for the prevention and treatment of many diseases due to their high efficacy and low side effects. With increasing demand for safer drugs, attention has been drawn to the identity, purity and strength of the herbal medicines. Hence, there is a need for standardization and development of reliable quality protocols using modern techniques of analysis for the herbal drugs. In the present paper an attempt was made to lay down the pharmacopoeial standards of *Limonia crenulata* Roxb. (Root) based on its physico-chemical and HPTLC analysis. Determination of physico-chemical parameters such as total ash, acid insoluble ash, water and alcohol soluble extractive values and High-Performance Thin Layer Chromatography (HPTLC) studies of the chloroform extract of the plant material were carried out. The solvent system, Toluene: Ethyl acetate: Formic acid (5: 2: 0.1) efficiently resolved the components present in the crude extract. The physico-chemical and HPTLC studies on root of *Limonia crenulata* yielded a set of qualitative and quantitative parameters or standards that can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant material. These studies enable the identification of the plant material for future investigation and form an important aspect of drug studies.

Introduction:-

Herbal medicines have gained popularity as potential therapeutic agents for the prevention and treatment of many diseases due to their high efficacy and low side effects. The use of herbal medicine has increased around the world due to its presumptive efficiency, availability, and general acceptance. It is also believed by traditional medical practitioners that the phytoconstituents present in herbal medicine have better compatibility with the human system. With increasing demand for safer drugs, attention has been drawn to the identity, purity and strength of the herbal medicines. Hence, there is a need for standardization and development of reliable quality protocols using modern techniques of analysis. Analytical procedure helps in determination of the presence of phytochemicals in the plant material.

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Keeping this in view, in the present paper an attempt was made to lay down the pharmacopoeial standards of *Limonia crenulata* Roxb. (Root) based on its physico-chemical and HPTLC analysis. *L. crenulata* syn. *Niringi crenulata* is an important medicinal plant belonging to the family Rutaceae. It is commonly known as 'Magavilvam' or 'Megavilvam' in Tamil, Kattunarakam, Malanarakam, Manmatham in Malayalam and Vilvaparni, Surasi and Bilvaparni in Sanskrit (Manjula *et al.*, 2017). It is a spinous glabrous tree 8-12 m tall, bark appeared dull brown yellow, smooth, spines are sharp, leaves compound, imparipinnate, alternate rachis with oblonceolate wings. (Nadkarni 2002). It grows in tropical Africa and Asia. The plant is distributed throughout India, especially in the Western Ghats. Various parts of this plant have been employed in indigenous medicine and is used as antiepileptic, purgative, sudoferic, colic trouble and cardialgia. Leaves are given with milk to children to cure the digestive disorders and remedy for epilepsy. Fruit decoction is used as an antidote to insect poison. The bark juice is applied externally for getting speedy relief in sprain. Earlier studies showed that its methanolic extract has anthelmintic activity. Ethanolic extracts of bark and leaves showed biological activities such as anticancer, hepatoprotective, aphrodisiac and anti-inflammatory activities. The root extract is used for vomiting, dysentery and colic disorders and also to save the life of people from snake bites.(Sarada Kuppusamy *et al.*, 2014).

Fig.1:- *Limonia crenulata* Roxb.

Materials and Methods:-

Collection and authentication of plant material

The root of *L. crenulata* was collected, authenticated and supplied by the Pharmacognosy Dept., SMPG, Mettur. The plant material was dried in shade, crushed and kept in airtight bottle for experimental purpose.

Physico-chemical analysis

Physico-chemical parameters such as total ash, acid insoluble ash, water and alcohol soluble extractive values were determined by standard methods (WHO, 1998). The information collected from these tests is useful for standardization.

HPTLC Analysis

High Performance Thin Layer Chromatography (HPTLC) was carried out by the standard procedure (Wagner *et al.*, 1996). The plate was developed in a solvent system of Toluene: Ethyl acetate: Formic acid (5: 2: 0.1, v/v). The conditions applied for the HPTLC analysis are given in Table 1.

Table 1:- Chromatographic conditions.

Instrument	Camag HPTLC system
Application mode	Auto TLC sampler 4 (ATS4)
Extract used	Chloroform extract
Development chamber	Camag Twin trough chamber.
Stationary phase	Silica gel G F254
Mobile phase	Toluene: Ethyl acetate: Formic acid (5: 2: 0.1)

Chamber saturation	20 minutes
Development distance	7 cm
Visualization and Photo-documentation	Camag TLC Visualizer
Scanner	Camag TLC Scanner 4
Detection	Deuterium lamp, Mercury lamp and Tungsten lamp
Data system	Win cats software
Slit dimensions	8.00 mm × 0.40 mm
Selected Wave lengths	254 nm, 366 nm and 575 nm after derivatisation
Derivatisation reagent	Vanillin - sulphuric acid
TLC plate drying device	Camag TLC plate heater

Suitable volume of the chloroform extract of the root of *L. crenulata* were spotted as bands having width of 10 mm on precoated silica gel 60 F_{254} HPTLC plate. After the application, the plate was developed by placing vertically in a twin trough glass chamber saturated with the suitable mobile phase. Subsequent to the development, the plates were air dried and captured the images at UV 254 nm and UV 366 nm in in photo-documentation chamber. Densitometric scanning of the plate at UV 254 nm and UV 366 nm was performed with a TLC scanner 4 using winCATS software. The plate was then derivatized with the detection reagent- vanillin sulphuric acid and heated at 110°C till the colour appears. Later it was visualized under white light and scanned at 575 nm. All the chromatograms, peak numbers with its height and area, peak display and peak densitograms were documented.

Results and Discussion:-

The physico-chemical parameters were investigated and reported in Table 2.

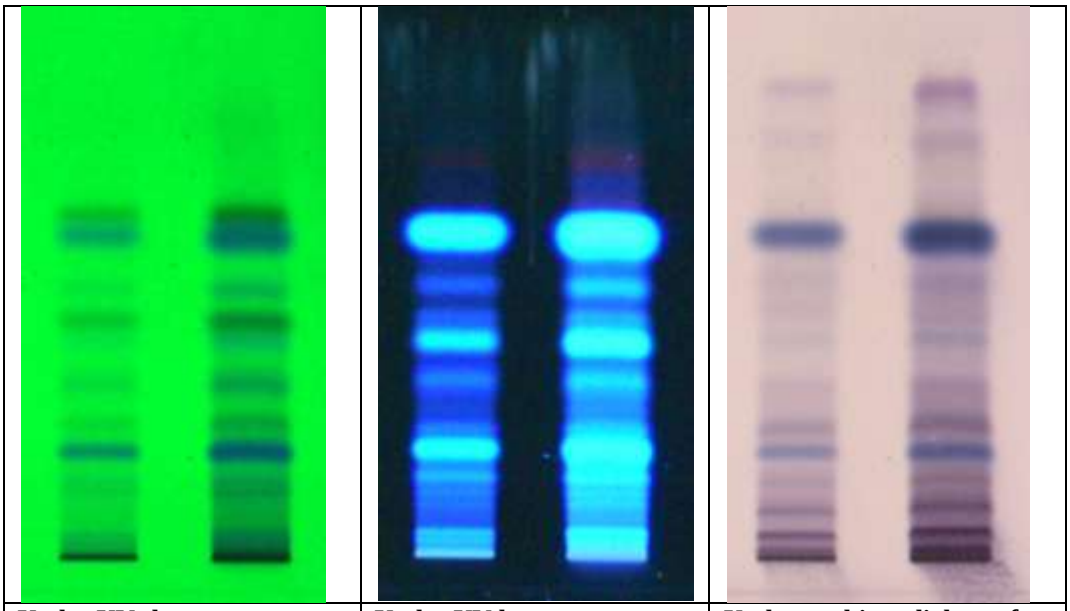
Table 2:- Physico-chemical parameters of *Limonia crenulata* Roxb. (Root).

Sl. No.	**Parameter**	**Result (%)**
1.	Foreign Matter	<2
2.	Loss on Drying at 105^0C	14.60
3.	Total Ash Content	2.64
4.	Acid Insoluble Ash	0.10
5.	Water Soluble Extractive	8.82
6.	Alcohol Soluble Extractive	6.50
7.	Volatile oil	Nil

Total ash value gives the total amount of material remaining after ignition. Total ash usually consists of carbonates, phosphates, silicates and silica which include both physiological ash-which is derived from the plant tissue itself and non physiological ash–which is the residue of sand and soil adhering to the plant surface. Acid insoluble ash measures the amount of silica present, especially as sand and siliceous earth. This value particularly indicates the contamination with siliceous material. Acid insoluble ash values were found to be low for the plant material, which might be an indication of its purity. The loss on drying of the drug was found to be 14.60 which represent the presence of moisture in the plant material. Alcohol & water soluble extractives determine the amount of active constituents extracted with alcohol and water respectively from the drug. The water soluble extractive was found to be 8.82% indicating the presence of sugar, acids and inorganic compounds in the drug. The alcohol soluble extractive value indicates the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids etc. The alcohol soluble extractive was found to be 6.50% which signifies the presence of constituents which were less soluble in alcohol.

HPTLC Analysis

Chloroform extract of root of *L. crenulata* was subjected to HPTLC analysis and the data obtained is given in Fig. 2 – 8. HPTLC chromatogram recorded at 254 nm, 366 nm and 575 nm after derivatisation with vanillin– sulphuric acid reagent are given in Fig. 2. The 3D densitometric chromatogram of the chloroform extract of the plant material at 254 nm, 366 nm and 575 nm are given in Fig.3, Fig.5 and Fig.7 respectively. The HPTLC fingerprinting profile, the R_f values and percentage area of the peaks at 254 nm, 366 nm and 575 nm are given in Fig.4, Fig.6 and Fig.8 respectively.



Under UV short	Under UV long	Under white light after derivatisation

Fig. 2:- HPTLC profile of chloroform extract of *Limonia crenulata* Roxb, Root viewed in UV short; viewed in UV long; after derivatisation using vanillin-sulphuric acid viewed in visible light; Solvent system – Toluene: Ethyl acetate: Formic acid (5: 2: 0.1).

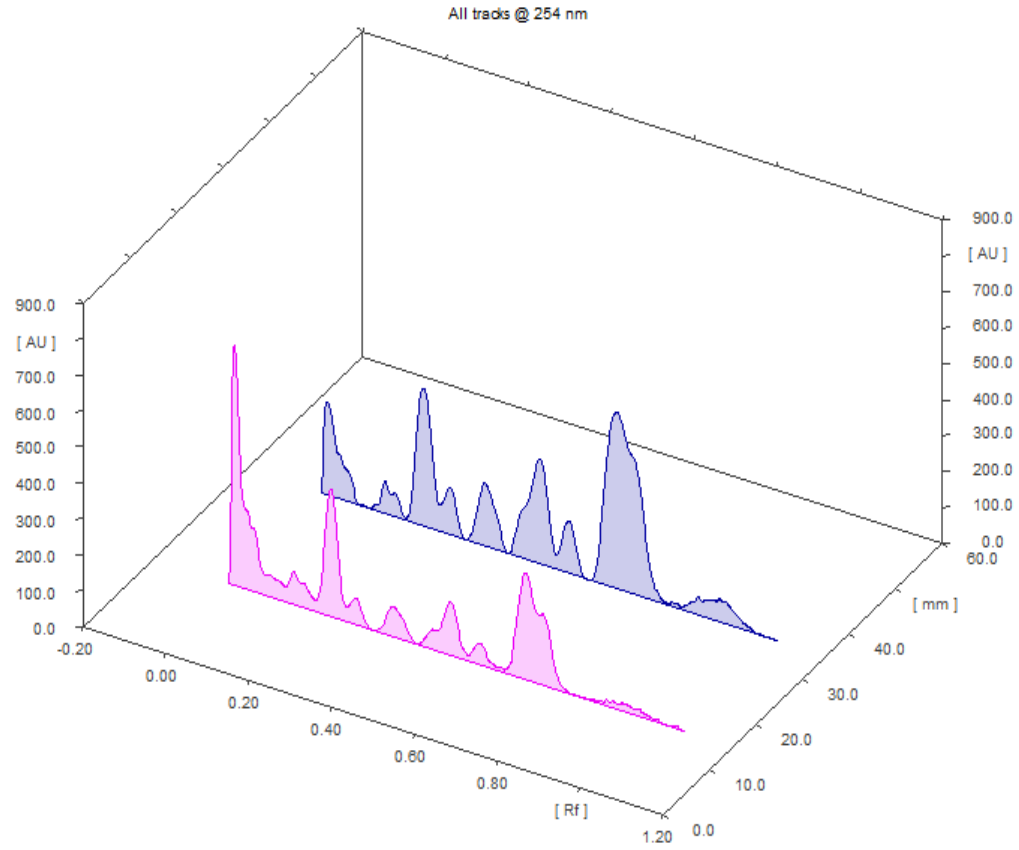
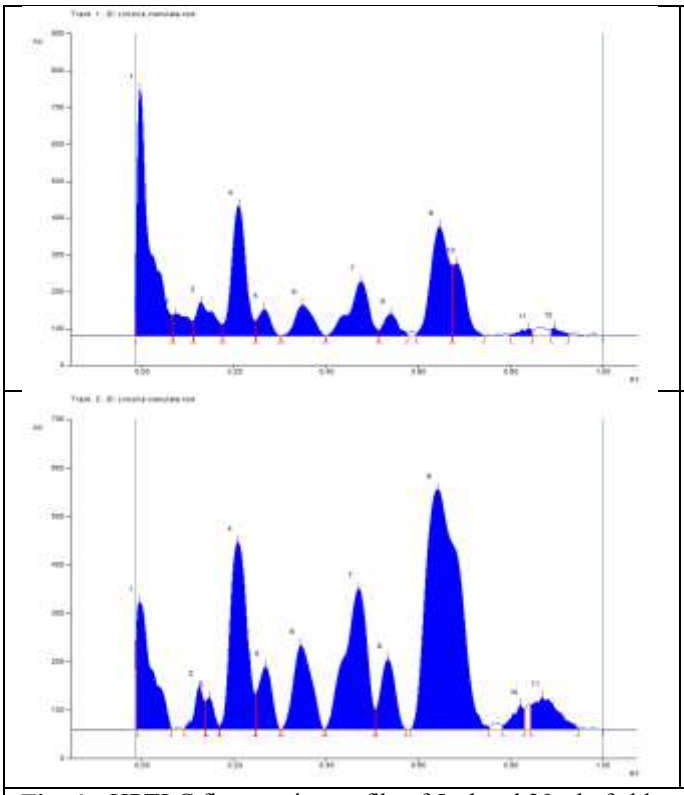


Fig. 3:- 3D densitometric chromatogram of 5 and 20 µl of chloroform extract of *Limonia crenulata* Roxb, Root at 254 nm.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	61.2 AU	-0.00 Rf	669.3 AU	32.30 %	0.07 Rf	56.2 AU	13597.5 AU	27.62 %
2	0.07 Rf	57.0 AU	0.08 Rf	68.1 AU	2.90 %	0.12 Rf	39.5 AU	1520.8 AU	3.09 %
3	0.12 Rf	40.6 AU	0.14 Rf	91.5 AU	4.41 %	0.19 Rf	31.1 AU	2565.9 AU	5.21 %
4	0.19 Rf	31.4 AU	0.23 Rf	352.8 AU	17.03 %	0.27 Rf	41.1 AU	7977.8 AU	16.21 %
5	0.27 Rf	41.9 AU	0.29 Rf	72.5 AU	3.50 %	0.33 Rf	0.0 AU	1454.2 AU	2.95 %
6	0.33 Rf	0.2 AU	0.38 Rf	83.5 AU	4.03 %	0.43 Rf	0.4 AU	2434.8 AU	4.95 %
7	0.44 Rf	0.0 AU	0.52 Rf	148.4 AU	7.16 %	0.56 Rf	14.6 AU	4750.0 AU	9.65 %
8	0.56 Rf	14.7 AU	0.59 Rf	59.2 AU	2.86 %	0.63 Rf	7.2 AU	1380.2 AU	2.80 %
9	0.65 Rf	7.4 AU	0.70 Rf	296.8 AU	14.32 %	0.73 Rf	91.4 AU	8734.8 AU	17.74 %
10	0.73 Rf	191.6 AU	0.74 Rf	196.7 AU	9.49 %	0.81 Rf	1.5 AU	4086.9 AU	8.30 %
11	0.87 Rf	3.7 AU	0.91 Rf	19.5 AU	0.94 %	0.92 Rf	15.6 AU	380.9 AU	0.77 %
12	0.96 Rf	16.6 AU	0.97 Rf	22.0 AU	1.06 %	1.01 Rf	5.8 AU	345.0 AU	0.70 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	210.2 AU	-0.00 Rf	262.5 AU	12.21 %	0.07 Rf	1.0 AU	6499.6 AU	9.16 %
2	0.10 Rf	0.8 AU	0.14 Rf	88.7 AU	4.12 %	0.15 Rf	57.4 AU	1323.2 AU	1.86 %
3	0.15 Rf	58.1 AU	0.16 Rf	64.9 AU	3.02 %	0.18 Rf	7.6 AU	825.5 AU	1.16 %
4	0.19 Rf	7.8 AU	0.23 Rf	385.8 AU	17.95 %	0.27 Rf	72.4 AU	10261.1 AU	14.46 %
5	0.27 Rf	73.0 AU	0.29 Rf	129.1 AU	6.01 %	0.33 Rf	0.2 AU	2804.1 AU	3.95 %
6	0.33 Rf	0.0 AU	0.38 Rf	173.3 AU	8.06 %	0.43 Rf	0.3 AU	5114.0 AU	7.21 %
7	0.43 Rf	0.7 AU	0.51 Rf	290.0 AU	13.49 %	0.55 Rf	40.6 AU	10490.4 AU	14.79 %
8	0.55 Rf	40.6 AU	0.58 Rf	144.3 AU	6.71 %	0.63 Rf	0.3 AU	3384.1 AU	4.77 %
9	0.63 Rf	0.2 AU	0.70 Rf	495.0 AU	23.03 %	0.82 Rf	5.7 AU	26636.4 AU	37.54 %
10	0.85 Rf	7.5 AU	0.89 Rf	49.8 AU	2.32 %	0.90 Rf	42.2 AU	922.4 AU	1.30 %
11	0.92 Rf	50.3 AU	0.94 Rf	66.1 AU	3.08 %	1.03 Rf	1.4 AU	2687.7 AU	3.79 %

Fig. 4:- HPTLC finger print profile of 5 µl and 20 µl of chloroform extract of *Limonia crenulata* Roxb, Root at 254 nm.

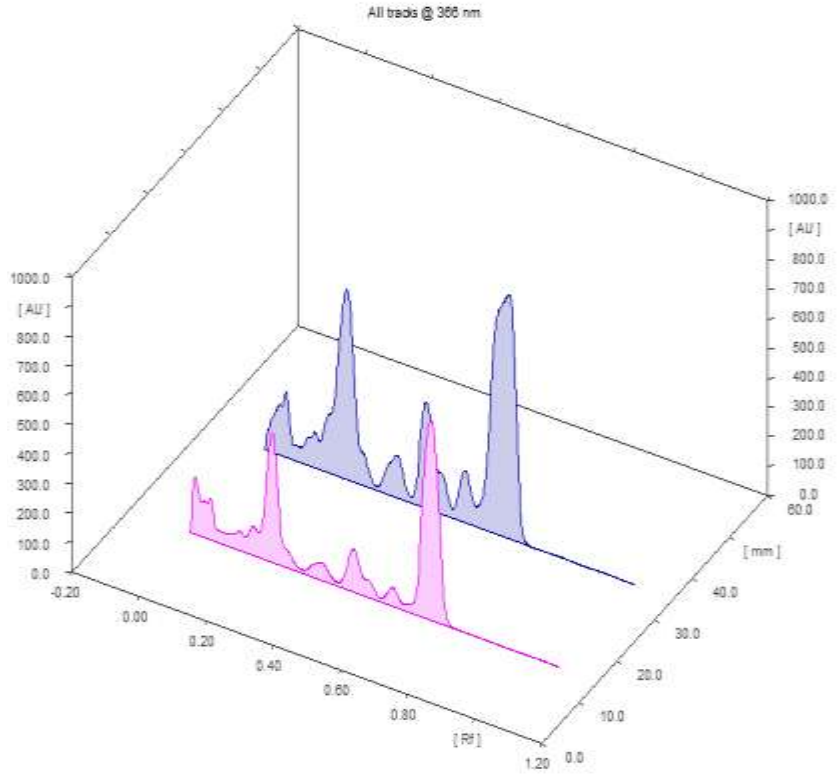
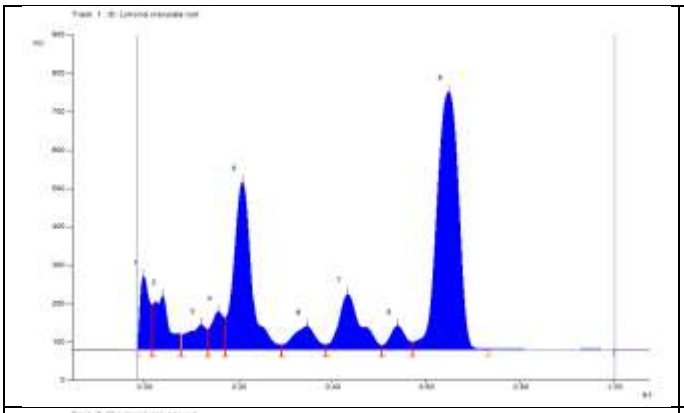
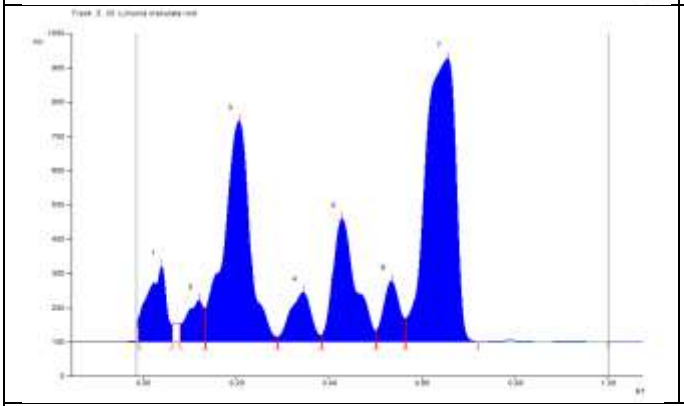


Fig. 5:- 3D densitometric chromatogram of 5 and 20 µl of chloroform extract of *Limonia crenulata* Roxb, Root at 366 nm.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	10.4 AU	0.00 Rf	191.9 AU	10.25 %	0.02 Rf	16.6 AU	2821.6 AU	5.23 %
2	0.02 Rf	116.9 AU	0.04 Rf	139.8 AU	7.46 %	0.08 Rf	40.0 AU	3371.1 AU	6.24 %
3	0.09 Rf	39.8 AU	0.13 Rf	64.9 AU	3.46 %	0.15 Rf	52.8 AU	1887.2 AU	3.49 %
4	0.15 Rf	53.4 AU	0.17 Rf	98.9 AU	5.28 %	0.19 Rf	82.1 AU	1955.4 AU	3.62 %
5	0.19 Rf	82.5 AU	0.23 Rf	436.8 AU	23.33 %	0.32 Rf	12.5 AU	12116.0 AU	22.44 %
6	0.32 Rf	12.6 AU	0.38 Rf	60.8 AU	3.25 %	0.42 Rf	12.3 AU	2195.8 AU	4.07 %
7	0.42 Rf	12.6 AU	0.47 Rf	144.6 AU	7.72 %	0.55 Rf	11.0 AU	5015.0 AU	9.29 %
8	0.55 Rf	11.0 AU	0.59 Rf	62.4 AU	3.33 %	0.62 Rf	19.3 AU	1586.8 AU	2.90 %
9	0.62 Rf	19.5 AU	0.70 Rf	672.2 AU	35.91 %	0.80 Rf	1.0 AU	23070.6 AU	42.72 %



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	68.8 AU	0.05 Rf	220.4 AU	8.84 %	0.07 Rf	50.5 AU	6545.8 AU	6.61 %
2	0.09 Rf	51.7 AU	0.13 Rf	121.3 AU	4.86 %	0.14 Rf	98.1 AU	3278.0 AU	3.31 %
3	0.15 Rf	97.8 AU	0.22 Rf	645.4 AU	25.87 %	0.31 Rf	14.2 AU	26598.3 AU	26.84 %
4	0.31 Rf	14.3 AU	0.38 Rf	144.3 AU	5.78 %	0.42 Rf	17.8 AU	4909.0 AU	4.95 %
5	0.42 Rf	18.8 AU	0.47 Rf	359.3 AU	14.40 %	0.54 Rf	32.0 AU	12956.2 AU	13.07 %
6	0.55 Rf	32.3 AU	0.58 Rf	176.9 AU	7.09 %	0.61 Rf	67.9 AU	4658.4 AU	4.70 %
7	0.62 Rf	68.4 AU	0.71 Rf	827.2 AU	33.16 %	0.79 Rf	0.1 AU	40147.4 AU	40.51 %

Fig. 6:- HPTLC finger print profile of 5 µl and 20 µl of chloroform extract of *Limonia crenulata* Roxb, Root at 366 nm.

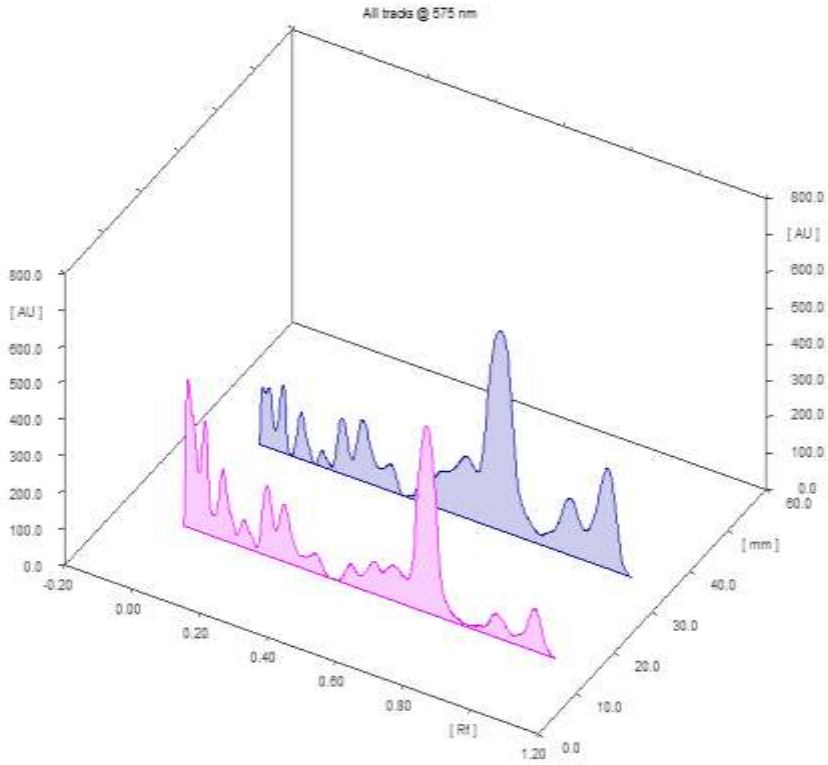
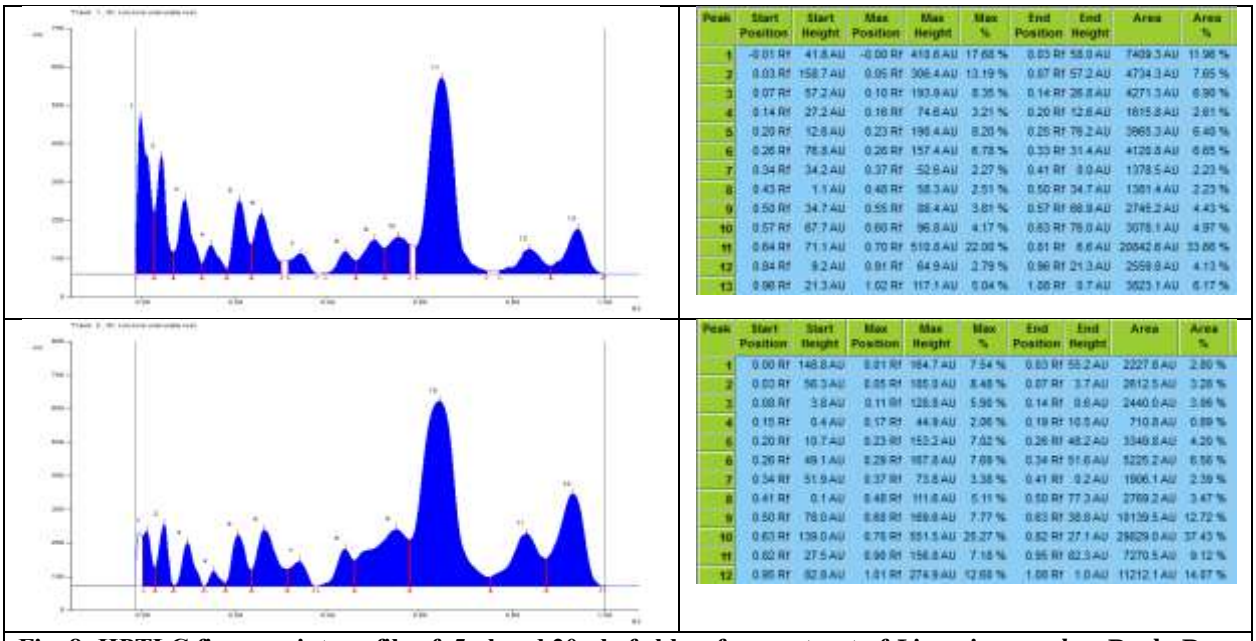


Fig. 7:- 3D densitometric chromatogram of 5 and 20 µl of chloroform extract of *Limonia crenulata* Roxb, Root at 575 nm after derivatisation.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	41.8 AU	-0.00 Rf	410.6 AU	17.68 %	0.03 Rf	58.0 AU	7409.3 AU	11.96 %
2	0.03 Rf	158.7 AU	0.05 Rf	306.4 AU	13.19 %	0.07 Rf	57.2 AU	4734.3 AU	7.65 %
3	0.07 Rf	57.2 AU	0.10 Rf	193.9 AU	8.35 %	0.14 Rf	26.8 AU	4271.3 AU	6.90 %
4	0.14 Rf	27.2 AU	0.16 Rf	74.8 AU	3.21 %	0.20 Rf	12.8 AU	1615.8 AU	2.61 %
5	0.20 Rf	12.8 AU	0.23 Rf	190.4 AU	8.20 %	0.25 Rf	78.2 AU	3965.3 AU	6.40 %
6	0.26 Rf	78.8 AU	0.26 Rf	157.4 AU	6.78 %	0.33 Rf	31.4 AU	4120.8 AU	6.65 %
7	0.34 Rf	34.2 AU	0.37 Rf	52.8 AU	2.27 %	0.41 Rf	8.0 AU	1378.5 AU	2.23 %
8	0.43 Rf	1.1 AU	0.48 Rf	58.3 AU	2.51 %	0.50 Rf	34.7 AU	1381.4 AU	2.23 %
9	0.50 Rf	34.7 AU	0.55 Rf	88.4 AU	3.81 %	0.57 Rf	68.8 AU	2748.2 AU	4.43 %
10	0.57 Rf	67.7 AU	0.60 Rf	96.8 AU	4.17 %	0.63 Rf	78.0 AU	3078.1 AU	4.97 %
11	0.64 Rf	71.1 AU	0.70 Rf	510.8 AU	22.00 %	0.81 Rf	8.6 AU	20542.6 AU	33.66 %
12	0.84 Rf	9.2 AU	0.91 Rf	64.9 AU	2.79 %	0.96 Rf	21.3 AU	2558.9 AU	4.13 %
13	0.96 Rf	21.3 AU	1.02 Rf	117.1 AU	0.04 %	1.08 Rf	8.7 AU	3623.1 AU	6.17 %

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.00 Rf	148.8 AU	0.01 Rf	164.7 AU	7.54 %	0.03 Rf	55.2 AU	2227.6 AU	2.80 %
2	0.03 Rf	56.3 AU	0.05 Rf	185.0 AU	8.48 %	0.07 Rf	3.7 AU	2812.5 AU	3.28 %
3	0.09 Rf	3.8 AU	0.11 Rf	128.5 AU	5.90 %	0.14 Rf	0.8 AU	2440.0 AU	3.06 %
4	0.15 Rf	0.4 AU	0.17 Rf	44.9 AU	2.06 %	0.19 Rf	10.5 AU	710.8 AU	0.89 %
5	0.20 Rf	10.7 AU	0.23 Rf	153.2 AU	7.02 %	0.26 Rf	48.2 AU	3348.8 AU	4.20 %
6	0.26 Rf	49.1 AU	0.29 Rf	167.8 AU	7.69 %	0.34 Rf	51.6 AU	5225.2 AU	6.56 %
7	0.34 Rf	51.9 AU	0.37 Rf	75.8 AU	3.38 %	0.41 Rf	0.2 AU	1906.1 AU	2.39 %
8	0.41 Rf	0.1 AU	0.48 Rf	111.6 AU	5.11 %	0.50 Rf	77.3 AU	2769.2 AU	3.47 %
9	0.50 Rf	76.0 AU	0.62 Rf	169.6 AU	7.77 %	0.63 Rf	36.8 AU	10139.5 AU	12.72 %
10	0.63 Rf	139.0 AU	0.76 Rf	551.5 AU	25.27 %	0.82 Rf	27.1 AU	29829.0 AU	37.43 %
11	0.82 Rf	27.5 AU	0.90 Rf	156.8 AU	7.18 %	0.95 Rf	82.3 AU	7270.5 AU	9.12 %
12	0.95 Rf	82.8 AU	1.01 Rf	274.9 AU	12.60 %	1.08 Rf	1.0 AU	11212.1 AU	14.07 %

Fig. 8: HPTLC finger print profile of 5 µl and 20 µl of chloroform extract of *Limonia crenulata* Roxb, Root at 575 nm after derivatisation

The solvent system, Toluene: Ethyl acetate: Formic acid (5: 2: 0.1) efficiently resolved the components present in the crude extract. The R_f values and colour of the prominant bands at 254 nm, 366nm and 575 nm after derivatisation are given in Table 3. These fingerprints are the specific chromatographic tracks of the chloroform extract of root of *L. crenulata* and serve a better tool for standardisation of the plant material.

Table 3:- R_f values and colour of bands obtained at different wavelengths.

Wave lengths	254 nm		366 nm		575 nm after derivatisation	
	R_f values	**Colour**	**R_f values**	**Colour**	**R_f values**	**Colour**
Limonia crenulata (Root)	0.23 0.29 0.38 0.52 0.59 0.70 0.74	Blue Light green Light green Green Light green Blue Green	0.04 0.17 0.23 0.38 0.47 0.59 0.70	Blue Dark blue Fluorescent blue Dark blue Fluorescent blue Blue Fluorescent blue	0.05 0.10 0.23 0.28 0.70 0.89 0.98	Dark purple Purple Blue Purple Dark blue Purple Purple

The bands resolved with specific R_f value and colour represent the presence of phytoconstituents in the plant material.

Conclusion:-

The physico-chemical and HPTLC studies on root of *Limonia crenulata* yielded a set of qualitative and quantitative parameters or standards that can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant material. These studies enable the identification of the plant material for future investigation and form an important aspect of drug studies. The study revealed that the chloroform extract of the root of *Limonia crenulata* contains a rich variety of phytochemicals which might be accountable for its therapeutic value and thus justifies its traditional use. In addition, the data will be useful in differentiating the plant material from adulterant/substitute.

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