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Isolation and Characterization of Leaf Extract of *Derris trifoliata*

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Abstract: In the present work, phytochemical analysis of leaf extract of mangrove *Derris trifoliata* was carried out. Leaf material of the plant was successively extracted with methanol. For further characterization, the leaf extracts were subjected to thin layer chromatography and high performance thin layer chromatography. HPTLC is a high-throughput and the most sensitive method of detecting the fragments of the compound. Eventhough many ready-to-use commercial kits are available, the R_f values are identified accurately for individual peaks. This yields the complete phytochemical characterization of the methanol extracts of *Derris trifoliata*. Three different compounds were isolated from the methanol extracts of *Derris trifoliata*.

Introduction:

The chemical constituents of the medicinal plants, particularly the secondary metabolites have pronounced pharmacological actions on animal system and organs. Several bioactive compounds were isolated from the plant sources such as digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine, quercetin ¹ etc. which have different pharmacological properties. Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts ². Phytochemical constituents are the basic source for the establishment of several pharmaceutical industries. The constituents present in the plant play a significant role in the identification of crude drugs. Phytochemical screening is very important in identifying new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc³.

Derris trifoliata is one of the most commonly used insecticides. The leaves contain the chemical compound rotenone, a poison that kills a wide range of creatures from insects to earthworms and fish ^{4,5}. This poison is used in catching the fishes. The leaves of *Derris trifoliata* were proved to have many therapeutic uses including stimulant, antispasmodic, counter-irritant, rheumatism, chronic paralysis, and dysmenorrhea. The decoction of roots is used against fever and internally against sores. The roots or stem are used as laxative, carminative and anti-arthritis agent ⁶.

Materials and Methods:

Collection of *Derris trifoliata*:

Derris trifoliata leaves were collected at random from Rameshwaram Mangrove forests (Tamilnadu), India.

Preparation of the Plant Materials

The leaves were dried in the shade for 7 days at room temperature ($28 \pm 2^\circ\text{C}$) and ground to a fine powder using Grinder IKA®-WERKE, IKA MF10 Machine and sieved through a $0.25 \mu\text{m}$ mesh. The powder samples were kept at room temperature in a covered glass containers to protect them from humidity and light prior to extraction.

Extraction of *Derris trifoliata* Leaves Extracts

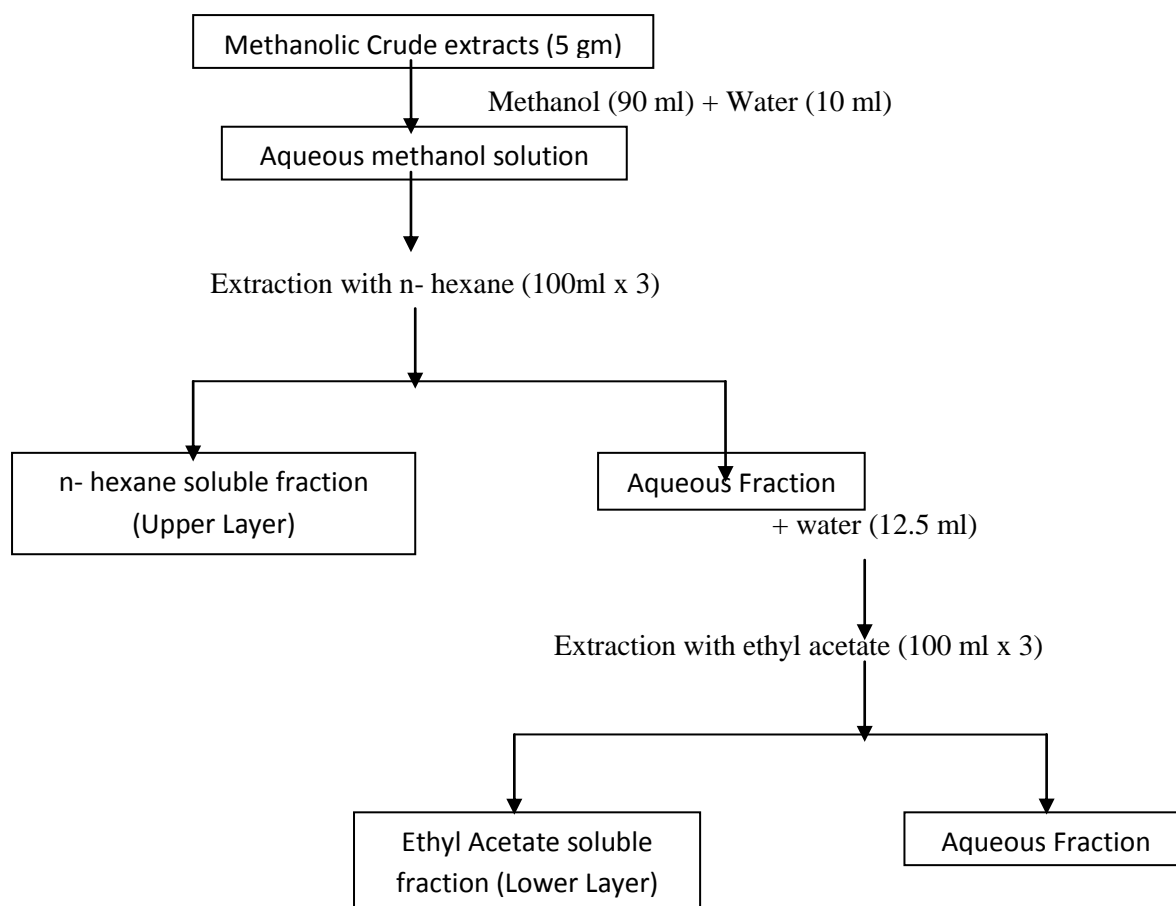
50g dried powder leaves were exhaustively extracted by macerated in 2.0 L methanol solvent for 2 days at room temperature ($28 \pm 2^\circ\text{C}$). The solvent-containing extract was then decanted and filtered by vacuum filtration (GAST, DOA-P504-BN, USA). The extraction of the ground leaves were further repeated (twice) with methanol (1.5 L each time). The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure at 40°C using a rotary evaporator (Heidolph-instruments, Rotavapor, Germany) to give concentrated crude methanolic extracts, dried in oven at 50°C to give dark green extracts. The weights of all the extracts were measured after solvent evaporation and then kept into a glass container prior to use.

Phytochemical Screening of Mangrove isolates:

Column Chromatography

Solvent-solvent partitioning (Figure- 1) was done by using the protocol designed by Kupchan and Tsou^{7,8}. The crude extract (5 gm) was triturated with 90% methanol. The prepared solution was then fractionated successively using solvents of increasing polarity, such as hexane and ethyl acetate. Both the fractions were evaporated to dryness by using rotary evaporator at low temperature of 39°C and kept in air tight containers for further analysis.

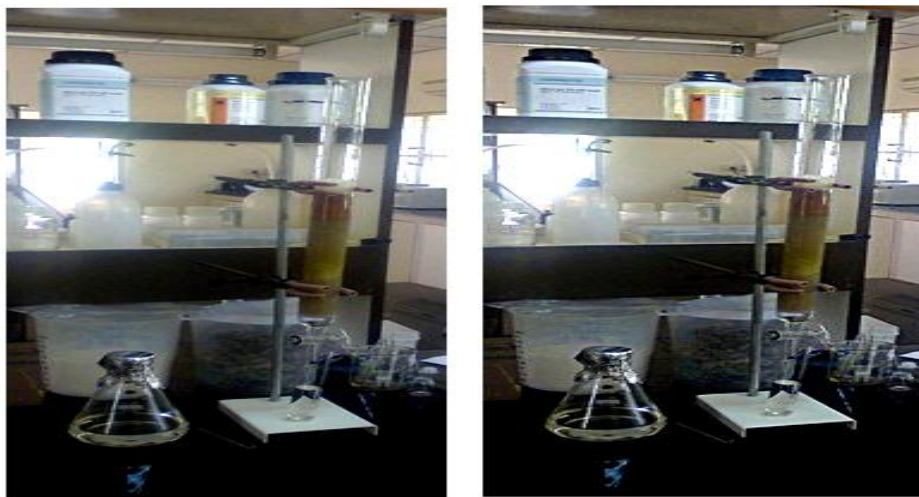
Figure 1: Schematic representation of modified Kupchan Partitioning of methanolic extract of *Derris trifoliata*



Isolation and Identification of compounds

The hexane- soluble and ethyl acetate soluble mixtures were fractionated using column chromatography (Fig 2). 30 gm of crude was mixed with 60 gms of Silica gel (60-120 mesh) to make the adhesive mixture. A column of diameter 2.4 cm and column bed height of 20 cm was packed with the adhesive mixture mixed with hexane. The column was eluted with increasing solvent polarity from hexane to ethyl acetate.

Figure 2: Isolation of Derris trifoliata leaf extracts using Column Chromatography



Thin Layer Chromatography

For extraction, isolation and identification of active ingredients such as alkaloids, phenolics, terpenoids etc. solvent extraction procedure of Harborn⁹ was adopted. Extracts obtained as above were concentrated to 1ml and 20µl loaded on TLC plates (Silica gel G 0.2 ml) and developed by the following solvents: Hexane: Ethyl acetate (1:1). The spot was observed on the TLC plates observed on the TLC plates and Rf value was calculated by using the following formula :

$$R_f = \text{distance traveled by center of component} / \text{distance traveled by solvent front}$$

Rf value signifies the retention factor i.e., more the molecular weight the more will be the distance traveled by the isolates.

Detection of compounds by HPLTC

Following extraction, Derris trifoliata standard and extracted samples were processed on the automated HPTLC system (CAMAG, Muttenz, Switzerland) according to the instructions of the manufacturer. The sample was sonicated to ensure complete solubility of extract, as well as filtration or centrifugation to remove undissolved excipients¹⁰⁻¹³. Ready-to-use silica coated plates (Manufacturer: E.MERCK KGaA) were activated by blowing hot air for 5-10 min and placed in the automatic sample applicator. Precoated TLC Silica Gel 60 F 254 (Merck TLC Plates) plates were used as the stationary phase. Plates were developed with methanol and dried in oven at 120°C for 20 minutes in order to remove contamination¹⁰⁻²⁰.

The HPTLC was programmed to automatically spray 5-10 µl of each sample in band form using specialized Hamilton syringe on one-side of the TLC plate in individual tracks. The TLC plate was developed in Hexane : Ethyl acetate : Formic acid : Acetic acid (ratio 60 : 40 : 10 : 10) (v/v) solvent system^{21,22}. The plate was developed in the automated developing chamber (CAMAG) until the solvent front reached the maximum distance (80 mm distance in a typical 20 x 10 cm plate).

The developed plate was dried with a plate drier and subjected to UV analysis (wavelength: 200-600 nm) in the dedicated UV detector. All tracks in the plate were scanned at user-defined wavelength (254nm, 366 nm and visible light) and individual Rf values of peaks were obtained.

Results

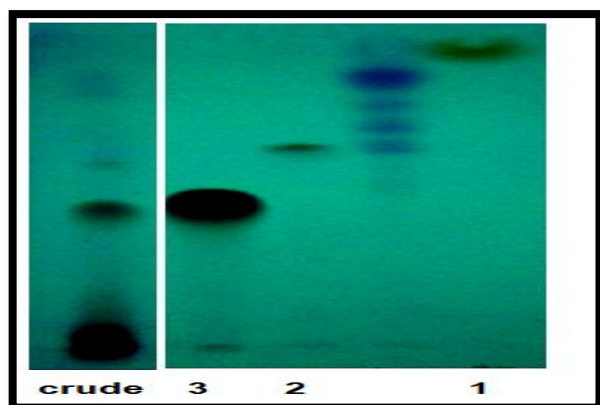
Chemical Characterization of Mangrove isolates

Semi quantitative estimation and identification of active principles of the crude leaf extracts of *Derris trifoliata* were performed by TLC method (Table- 1). In the present study TL separation of methanolic extract of the plant material present three different compounds as revealed by fluorescents spots when visualized under UV light (Figure- 3).

Table 1: Isolation of compounds from *Derris trifoliata* using Column Chromatography

S.No	Number of Fractions	% of Solvent	Volume of Solvent (ml)	TLC Spot	Mobile phase
1	1-20	100% Hexane	500		H:Etoac(7:3)
2	21-26	97%Hex: 3% Etoac	200	Yellow gel liquid	H:Etoac(7:3)
3	27-36	95%Hex :5% Etoac	250		H:Etoac(7:3)
4	36-41	90%Hex:10% Etoac	300		H:Etoac(7:3)
5	41-51	87%Hex:13% Etoac	500	Brown powder	H:Etoac(6:4)
5	42-50	85%Hex:15% Etoac	500	White powder	H:Etoac(6:4)
6	51-56	80%Hex :20%Etoac	250		
7	57-61	70%Hex:30% Etoac	250		
8	62-70	100% Etoac	100		

Figure 3: Photograph of TLC plate under UV light, solvent – hexane: ethyl acetate (1:1)



Three different compounds were isolated. The compound 1 Yellow Gel was collected from the fractions 21-26. Compound 2 White powder was collected from the fractions 42-50 and the compound 3 Brown powder was collected from the fractions 41-51 (Table 1). The R_f value of the three different spots were determined (Table-2). To further characterize the components of *Derris trifoliata*, the three different compounds were subjected to HPTLC analysis in different solvent. Based on preliminary experiments, it was found that the metabolites exhibited different R_f values (Figure- 8, 9, 10). HPTLC based separation of *Derris trifoliata* extracts was done using Hexane : Ethyl acetate : Formic acid : Acetic acid (ratio 60 : 40 : 10 : 10) solvent system. (Figure- 4, 5 & 6) shows the spectral scanning curve of the metabolites of *Derris trifoliata*. The plates were heated to remove the mobile phase the zones with the fluorescence were viewed under Duterium (D_2) and tungsten (W) lamps. The chromatogram was scanned using CAMAG TLC Scanner at 298 nm^{9-12,20}.

Table 2: Thin Layer Chromatography of leaf extracts and their R_f values.

Solvent : Hexane: Ethyl acetate (1:1)		
Material: Methanolic extract of <i>Derris trifoliata</i>		
Compound	R_f value	Iodine Vapour
1	2.296	Dark Green
2	1.566	Brown
3	1.185	Dark Brown

Figure 4A & 4B: Shows the spectral scanning of compound 1 of *Derris trifoliata* with absorbance (AU) plotted against wavelength.

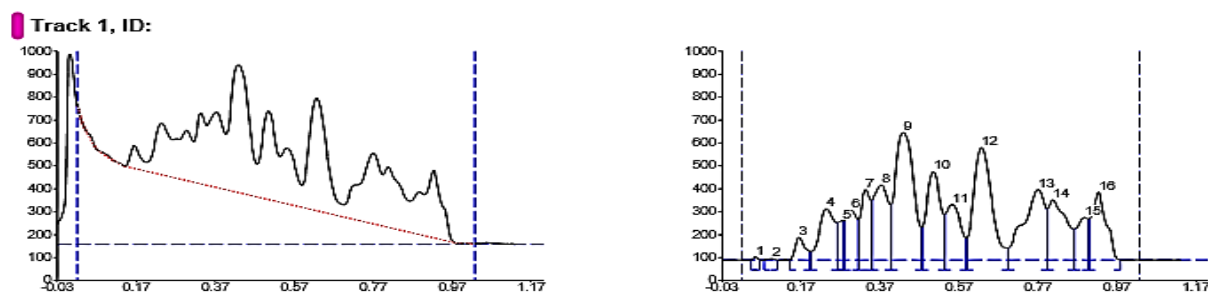


Figure 5A & 5B: Shows the spectral scanning of compound 2 of *Derris trifoliata* with absorbance (AU) plotted against wavelength.

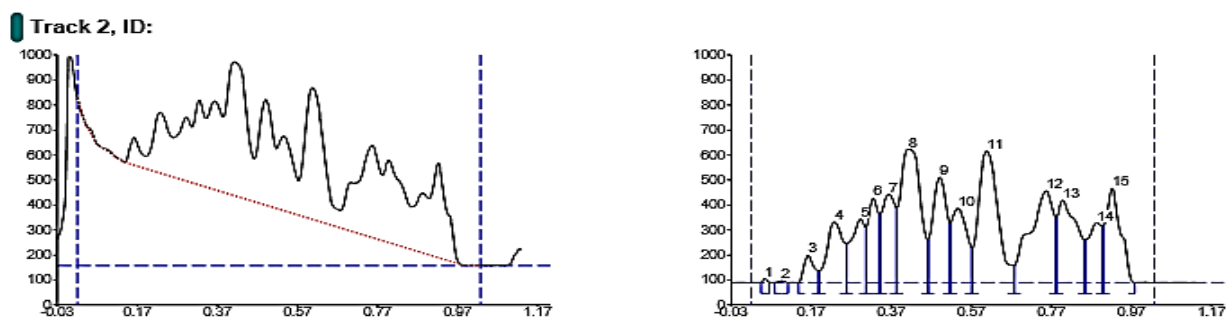


Figure 6A & 6B: Shows the spectral scanning of compound 3 of *Derris trifoliata* with absorbance (AU) plotted against wavelength.

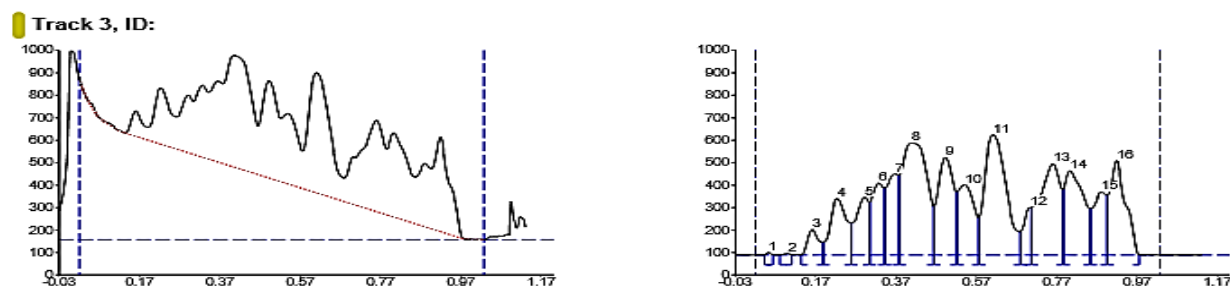


Figure 7: HPTLC profile of *Derris trifoliata* extract (a) at 254 nm (b) at 254 nm (c) at visible light.

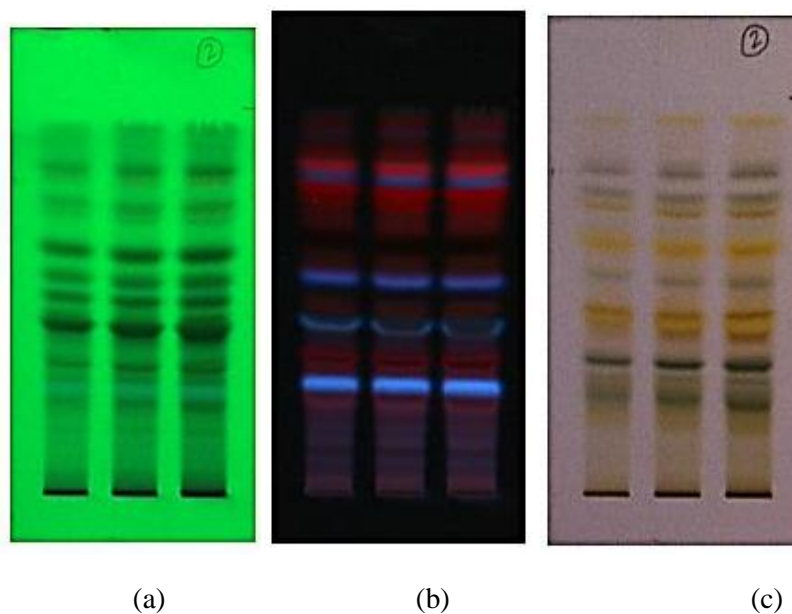


Figure 8: R_f values of individual peaks of compound 1 of Derris trifoliata

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.1	0.06	15.1	0.37	0.07	0.5	128.1	0.10	unknown *
2	0.08	0.5	0.10	6.7	0.16	0.11	0.1	91.6	0.07	unknown *
3	0.15	0.2	0.17	99.8	2.43	0.20	38.2	2143.5	1.61	unknown *
4	0.20	38.4	0.24	224.4	5.47	0.27	163.3	7483.7	5.63	unknown *
5	0.27	163.8	0.28	172.2	4.19	0.28	172.0	2033.3	1.53	unknown *
6	0.28	172.4	0.30	219.5	5.35	0.32	175.4	5303.9	3.99	unknown *
7	0.32	176.6	0.34	308.3	7.51	0.35	259.9	6307.8	4.74	unknown *
8	0.35	261.7	0.38	328.2	7.99	0.40	241.3	10512.1	7.90	unknown *
9	0.40	243.5	0.43	556.3	13.55	0.48	142.5	22107.5	16.62	unknown *
10	0.48	143.3	0.51	385.9	9.40	0.54	198.6	11779.9	8.86	unknown *
11	0.54	199.3	0.56	243.1	5.92	0.59	96.3	7828.4	5.89	unknown *
12	0.59	96.6	0.63	491.7	11.98	0.70	53.1	19234.5	14.46	unknown *
13	0.70	53.5	0.78	308.1	7.50	0.80	223.1	14216.2	10.69	unknown *
14	0.80	224.1	0.81	263.2	6.41	0.87	135.8	10082.4	7.58	unknown *
15	0.87	136.0	0.89	186.8	4.55	0.90	179.0	4485.9	3.37	unknown *
16	0.90	179.1	0.93	296.2	7.21	0.98	0.6	9263.4	6.96	unknown *

Figure 9: R_f values of individual peaks of compound 2 of Derris trifoliata

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.7	0.06	17.2	0.39	0.07	1.1	152.3	0.10	unknown *
2	0.08	1.6	0.10	7.8	0.18	0.12	0.0	109.9	0.07	unknown *
3	0.14	0.4	0.17	110.8	2.50	0.19	48.7	2441.9	1.58	unknown *
4	0.19	48.9	0.23	243.4	5.49	0.26	156.4	8224.2	5.33	unknown *
5	0.26	156.5	0.30	255.0	5.76	0.31	222.4	7531.8	4.88	unknown *
6	0.31	224.5	0.33	338.4	7.64	0.35	273.6	7384.2	4.78	unknown *
7	0.35	274.3	0.37	354.9	8.01	0.39	300.5	10023.8	6.49	unknown *
8	0.39	300.5	0.42	534.8	12.07	0.47	170.8	23037.2	14.92	unknown *
9	0.47	171.8	0.50	423.1	9.55	0.52	238.6	13030.9	8.44	unknown *
10	0.52	239.9	0.54	298.3	6.73	0.58	137.7	9633.0	6.24	unknown *
11	0.58	138.1	0.61	527.9	11.92	0.68	68.8	21719.7	14.07	unknown *
12	0.68	69.2	0.76	367.8	8.30	0.79	261.9	18492.7	11.98	unknown *
13	0.79	262.0	0.80	330.7	7.47	0.86	170.9	13756.3	8.91	unknown *
14	0.86	171.1	0.89	240.3	5.42	0.90	226.1	6788.9	4.40	unknown *
15	0.90	226.1	0.93	378.9	8.55	0.98	0.3	12028.4	7.79	unknown *

Figure 10: R_f values of individual peaks of compound 3 of Derris trifoliata

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.0	0.06	13.9	0.29	0.07	0.0	109.2	0.06	unknown *
2	0.08	0.0	0.10	7.5	0.16	0.12	0.1	90.3	0.05	unknown *
3	0.14	0.0	0.17	115.0	2.39	0.19	57.9	2694.3	1.58	unknown *
4	0.19	57.9	0.23	251.7	5.24	0.26	142.6	8551.0	5.03	unknown *
5	0.26	142.7	0.30	256.8	5.34	0.31	235.4	7091.2	4.17	unknown *
6	0.31	236.5	0.33	322.2	6.70	0.35	298.0	7953.4	4.67	unknown *
7	0.35	298.3	0.37	361.7	7.52	0.38	355.4	8832.7	5.19	unknown *
8	0.38	355.8	0.41	498.9	10.38	0.47	216.3	26626.0	15.65	unknown *
9	0.47	216.8	0.50	434.3	9.03	0.53	282.0	14616.5	8.59	unknown *
10	0.53	282.6	0.55	313.4	6.52	0.58	167.1	10613.1	6.24	unknown *
11	0.58	169.0	0.62	535.4	11.14	0.68	103.2	24570.5	14.44	unknown *
12	0.68	103.7	0.71	212.0	4.41	0.71	210.6	3295.0	1.94	unknown *
13	0.71	210.9	0.77	406.9	8.46	0.79	292.4	18306.7	10.76	unknown *
14	0.79	294.5	0.81	375.1	7.80	0.86	207.8	15213.6	8.94	unknown *
15	0.86	207.8	0.89	282.1	5.87	0.90	266.3	7568.0	4.45	unknown *
16	0.90	266.4	0.92	420.7	8.75	0.98	0.4	14008.1	8.23	unknown *

Discussion

Isolation of Compounds

The plant leaves were extracted with methanol and then the phytochemical screening analysis was conducted. The methanolic leaf extracts were fractionated with column chromatography by using increasing polarity of Hexane to ethyl acetate solvent system. The thin layer chromatography of methanolic extract of Derris trifoliata leaves furnished three compounds with R_f value 2.296, 1.566, 1.185 in hexane: ethyl acetate (1:1)

Characterization of Compounds

Compound 1 was obtained as white crystalline solid. The TLC of the compound showed no fluorescence under UV light but showed a spot when put in iodine vapor. Compound 2 was obtained as grey powder. The TLC of the compound showed a single spot in iodine vapor. Compound 3 was obtained as light yellow gel. The TLC of the last compound showed a spot in iodine vapor.

For reliable psychiatric correlation, there needs to be well-standardized methods. Although ready-to-use cassettes are available, it has limited utility. Because it is relatively expensive and has limited sensitivity threshold, and gives only qualitative and not absolute quantification. Most of the commercial kits clearly state that the test provides only a preliminary result and more specific alternative testing method should be used to confirm the immunoassay result²³. This could be by either HPTLC or GC/MS or HPLC^{24,25}. In routine TLC testing, the detection is only by spray method and the R_f value is not accurately recorded²⁶. However, UV based scanning after developing HPTLC plate not only provides opportunity for scanning at specific wavelengths but could also be useful for quantitation.

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