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Phytopharmacognostic evaluation of plant *Euphorbia hirta* L.

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Abstract
The plant *Euphorbia hirta* L. belongs to family Euphorbiaceae. This plant is known for medicinal properties, such as in anti-oxidant, diarrhea, dysentery, anti-tumor, anti-bacterial etc. The present study considers the detail investigation of macroscopy, microscopy, preliminary qualititative phytochemical analysis, physicochemical evaluations, chromatography and infra red spectroscopy profiling. Microscopical investigation was adopted to identify the powder characters of whole plant include multi cellular hirsute covering trichomes, epidermal cells with sinuous wall, anomocytic stomata, spiral and retulate xylem vessels, laticiferous ducts, anther sac with pollen grains etc. Physicochemical parameters reveal the data of quality, purity while phytochemical screening reflects the presence of various secondary metabolites. Data from botanical and chemical screening in combination may be considered for further pharmacological and therapeutical evaluation of the species and will assist in standardization for quality, purity and sample identification.

Keywords: *Euphorbia hirta*, microscopy, physicochemical, phytochemical, chromatography, infra red

1. Introduction
Euphorbia hirta L. or '*Brihat Dugdhika'* is an annual herb with milky latex [1], medicinally important plant of the family Euphorbiaceae and this *Euphorbia* is the largest genus of this family with about 1600 species [2]. The abundance of the plant is noted throughout the tropical countries. This plant is wildly grown in low jungle areas, along roadside [2]. *E. hirta* is a prostate-erect herb. It has a distinct indumentum consisting of erect, yellow-brown, adpressed trichromes. It is a short-lived that germinates and flowers throughout the year and fruits in less than a month [3]. It is distributed throughout the hotter parts of India and Ceylon-Most tropical and subtropical countries [4]. *E. hirta* is commonly known as asthma weed, hairy spurge (English), Tawa-Tawa, Gatas-Gatas or Butobutonis (Philippines). It is also known as *Chamaesyce hirta* L., *E. capitata* Lam., *E. globulifera* H.B.K., *E. modiflora* Steud. and *E. obliterata* Jacq [3]. The plant is having rich contents of secondary metabolites like polyphenol, tannis, flavonoids [5]. Presence of terpenoids [6] and essential oils and their derivatives are also reported [7]. Presence of alkaloids, saponins, amino acids have been detected as minor secondary metabolites [8]. Presence of coumarinoids are reported [9]. Having a wide range of secondary metabolites the plant extracts exhibit different biological activities. Methanolic extract has shown anti-bacterial nature against dysentery exerted by Shigella species [10], while ethanolic extract has shown antibacterial action against the growth of E. coli, S. aureus [11]. Other study revealed the diuretic activity [12] of the ethanolic extract. Methanolic decoction of the leaves has shown anti-oxidant [13] and anti-tumor activities [14]. The toxicity study of the plant was carried out by a group of researchers [15] and possibility of acute and sub chronic toxicity was ruled out. The present study aimed at development of standardization and authentication parameters of this plant which is not included in API.

2. Material and methods
2.1. Material and reagents
The work has been carried out by using the chemicals, reagents and solvents of Emplura grade of Merck and aluminum supported Thin Layer Chromatography plates were purchased from E. Merck Pvt. Ltd. (Mumbai, India).

2.2. Plant materials collection and authentication
The whole herb of *Euphorbia hirta* were collected from natural habitat of Salt Lake area, Kolkata (22°31′31.8″ N: 88°21′59.4″ E), West Bengal in the month of January, 2019 and authenticated in Department of Pharmacognosy, Central Ayurveda Research Institute for Drug

Development, Kolkata, a herbarium was prepared and deposited in the Department, available for reference.

2.3. Plant sample processing
The plant materials were washed with aqueous ethanol, dried at an ambient temperature (20-23°C). A small portion of this plant sample was used for macroscopic and organoleptic studies. Rest of the plant materials were pulverized with a grinder (National SM 2000) to obtain fine powder (sieved in 60 #) used for powder microscopy, analysis of physicochemical and phytochemical features. The coarse powder (sieved in 25 #) of plant material was used for chromatographic examinations. The whole and powdered plant samples were stored at room temperature in airtight, light-resistant containers as per standard guidelines [16].

2.4. Macroscopy of plant material
The morphological and organoleptic parameters *viz.* texture, shape, size, colour, odour etc. of the whole plant material were noted mainly by naked eye observation [16, 17] and with the help of simple microscope Olympus OIC DM.

2.5. Powder microscopy of cytomorphological features
Fine dried whole plant powdered samples (~ 2 g) were separately treated with different solutions *i.e.* aqueous saturated chloral hydrate (for maceration), 50% glycerin, phloroglucinol in conc. HCl (for staining lignified tissues) and 0.02 N iodine reagent (for starch grains), mounted on slides with 50% glycerin following a standard protocol and observed under the binocular compound microscope (Olympus OIC-07964) at 10× and 40× magnifications [16,17]. The camera lucida drawing of cytomorphological features were prepared using mirror type attachment with the same microscope. Photomicrographs of different cellular structures and inclusions were taken using Magcam DC14 camera attached to an Olympus CX21i trinocular compound microscope.

2.6. Fluorescence analysis
The coarse drug powder (~ 0.5 g each) was treated with different (18 in number) reagents (5 ml each) such as, acids and alkaline solutions along with other solvents (including distilled water) inside clean test tubes, which were shaken well and allowed to stand for about 24 hours. The individual solutions were observed under normal daylight and UV (254 nm and 365 nm) light for their characteristic colors and compared with the standard colour chart [18].

2.7. Physicochemical evaluation
The physicochemical constant like ash values, loss on drying, extractive values and pH value of the plant material were determined by using finely divided powder as per standard guidelines [16]. Extractability was studied with different solvents like hexane, acetone, chloroform, ethyl acetate, methanol, ethanol, water and with equivolume aqueous ethanol. Extractions were performed by conventional cold and hot extraction method [16, 17] and microwave assisted extraction was also done.

2.8. Phytochemical
The finely powdered plant materials were subjected to soxhlet extraction for 1 h, separately with petroleum ether, chloroform, ethyl acetate, acetone, methanol, ethanol, water and with equivolume aqueous ethanol. The individual extracts were evaporated to dryness and used for screening the presence of secondary metabolites [19].

2.9. Fingerprint analysis
Given that the methanol extract of the plant materials gave the maximum extractive value, the same was used for the fingerprinting analysis. For this, the coarsely powdered plant material (1 g) was extracted with methanol (25 ml) using a Soxhlet apparatus. The extract was filtered and final volume made up to 20 ml using methanol and used for the fingerprinting analysis by High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC) and Fourier Transmission Infra Red (FTIR) spectroscopy (FTIR).

2.9.1. High Performance Thin Layer Chromatography (HPTLC)
The extract (2 µL) was applied in the form of 8 mm band, 15 mm from the bottom of a 5 × 10 cm preactivated aluminium supported precoated silica gel $60F_{254}$ plate, with the help of ATS-4 applicator attached to a CAMAG HPTLC system. The plate was developed in a pre-saturated twin trough chamber using the mobile phase as hexane: ethyl acetate: acetone: 1,4-dioxan: formic acid (4:3:2:1:0.5, v/v) to a distance of 8 cm, dried for 5 min in ambient air. Images of the developed plate were captured under 254 nm and 366 nm UV light. Densitometric scanning [20] of the developed plate at 254 nm and 366 nm were performed. An image was also captured using visible light after derivatising the plate with aqueous 20% sulphuric acid [21].

2.9.2. High Performance Liquid Chromatography (HPLC)
This was carried out with a HPLC equipment (Agilent model Infinity 1260), equipped with quaternary LC-2010 AHT VP pumps, a variable wavelength programmable UV/VIS detector, SPD-10AVP column oven and Class-VP software for analysis. The chromatographic separation was performed using a Phenomenex C_{18} (250 mm × 4.6 mm, 5 µm particle sizes) column at 25 ºC. The optimized mobile phase was found to be methanol: water (0.1% aqueous orthophosphoric acid) 85:15 (v/v) at a flow rate of 0.5 ml/min. An auto sampler with injection volume 20 µl was used for sample loading and the peaks were detected at 254 nm UV.

2.9.3. Fourier Transmission Infra-Red (FTIR) spectroscopy
A drop of the methanolic extract was placed between two potassium bromide pellets to obtain a thin layer, which was analysed with a FTIR spectrophotometer (Agilent Cary 630).

3. Results & Discussion
3.1. Morphological characters
The fresh *E. hirta* is herbaceous prostarte plant with milky latex by breakage of stem. Tap root system cylindrical with tapering ends, with thread like small rootlets, 5 to 7 cm. long, 2 to 2.5 mm in diameter, smooth surface with few minute protuberances. Stem branches soft, erect or ascending, 12 to 25 cm. high, pubescent with short curved hairs, yellowish green with reddish-purple tinge. The leaves are from 2 to 2.5 cm. long, opposite, oblong-lanceolate, shortly petiolate, margin of lamina minutely serrulate or dentate and unequal at the base, dark green in colour with reddish purple patches all over, both upper and lower surfaces are hairy. Inflorescence dense axillary or terminal cymes consisting minute flowers about 1 cm. in diameter approx (Fig. 1).

Fig 1a: Fresh plant of *Euphorbia hirta*

Fig 1b: Fragment of fresh flowering branch showing leaves minute flowers in dense axillary or terminal cymose inflorescence

Fig. 1c: Air-dried twigs of *Euphorbia hirta*

Fig 1: Morphology of *Euphorbia hirta* whole plant

3.2. Powder microscopy

Fine powder is grayish green in colour with no salient taste and odour, shows the presence of uniseriate multicellular covering trichomes with minute hairs (hirsute wall) of different shape few with irregular buldging, bulbous base originating from epidermis; epidermal cells, having straight to wavy (sinuate) wall with anomocytic stomata; oval to polygonal parenchyma with starch grains; ground tissue with thin white laticiferous ducts, anther showing wall (epidermis), pollen sac, connective tissue, pollen grains and filament; thick walled rectangular brick shaped parenchyma; portion of leaf lamina showing curved dark and light zone; reticulate and spiral xylem vessels ; prismatic crystal of calcium oxalate and simple to compound starch grains of 2-3 components (Fig. 2 & 3).

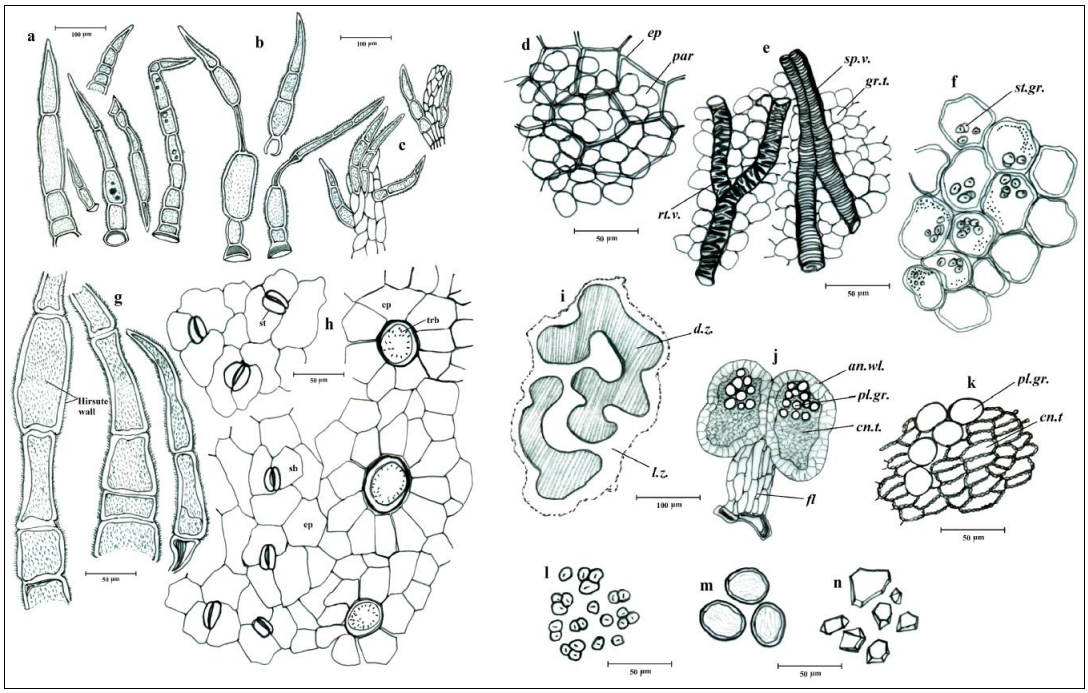
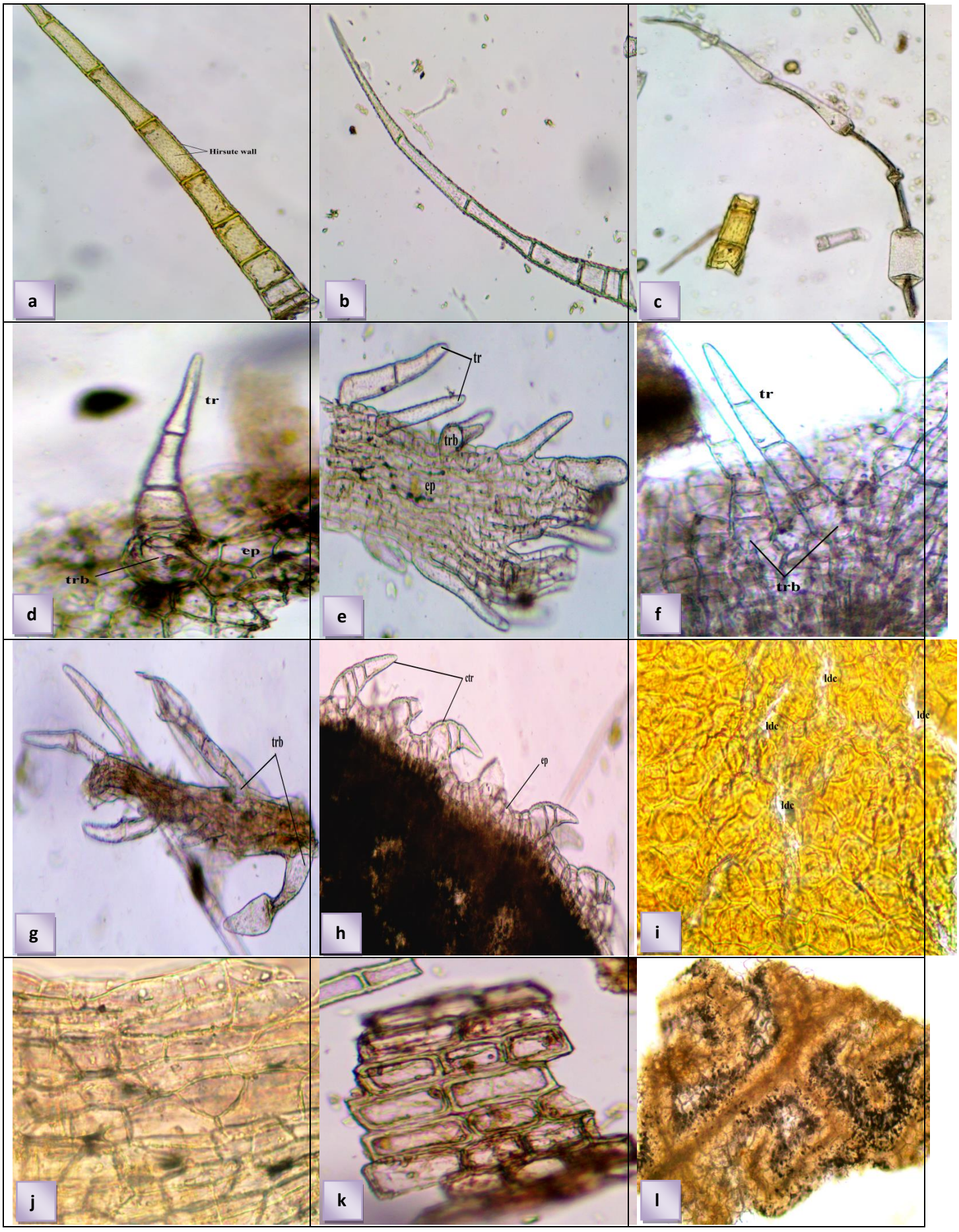


Fig 2: Powder microscopy of *Euphorbia hirta* whole plant (Camera Lucida drawings)

a,b: Multicellular covering trichomes of different size and shape; c: Epidermis with trichomes; d: Epidermal cells *(ep)* in group showing underlying palisade tissue *(pal)*; e: Fragmented ground tissue *(gr.t.)* with spiral xylem vessel *(sp.v.)* and reticutate xylem vessel *(rt.v.)*; f: Oval to polygonal parenchyma with starch grains *(st.g.)*; g: Portion of trichomes showing thick hairy or hirsute cell wall; h: Fragmented epidermis *(ep)* showing anomocytic stomata *(st)*, subsidiary cells *(sb)* and oval trichome bases *(trb)*; i: Portion of leaf lamina showing curved dark *(d.z.)* and light zone *(l.z.)*; j,k: Anther showing wall *(an.wl.)*, pollen grains *(pl.gr.)*, connective tissue *(cn.t.)*, and filament *(fl)*; l: starch grains; m: Pollen grains; n: Prismatic crystals.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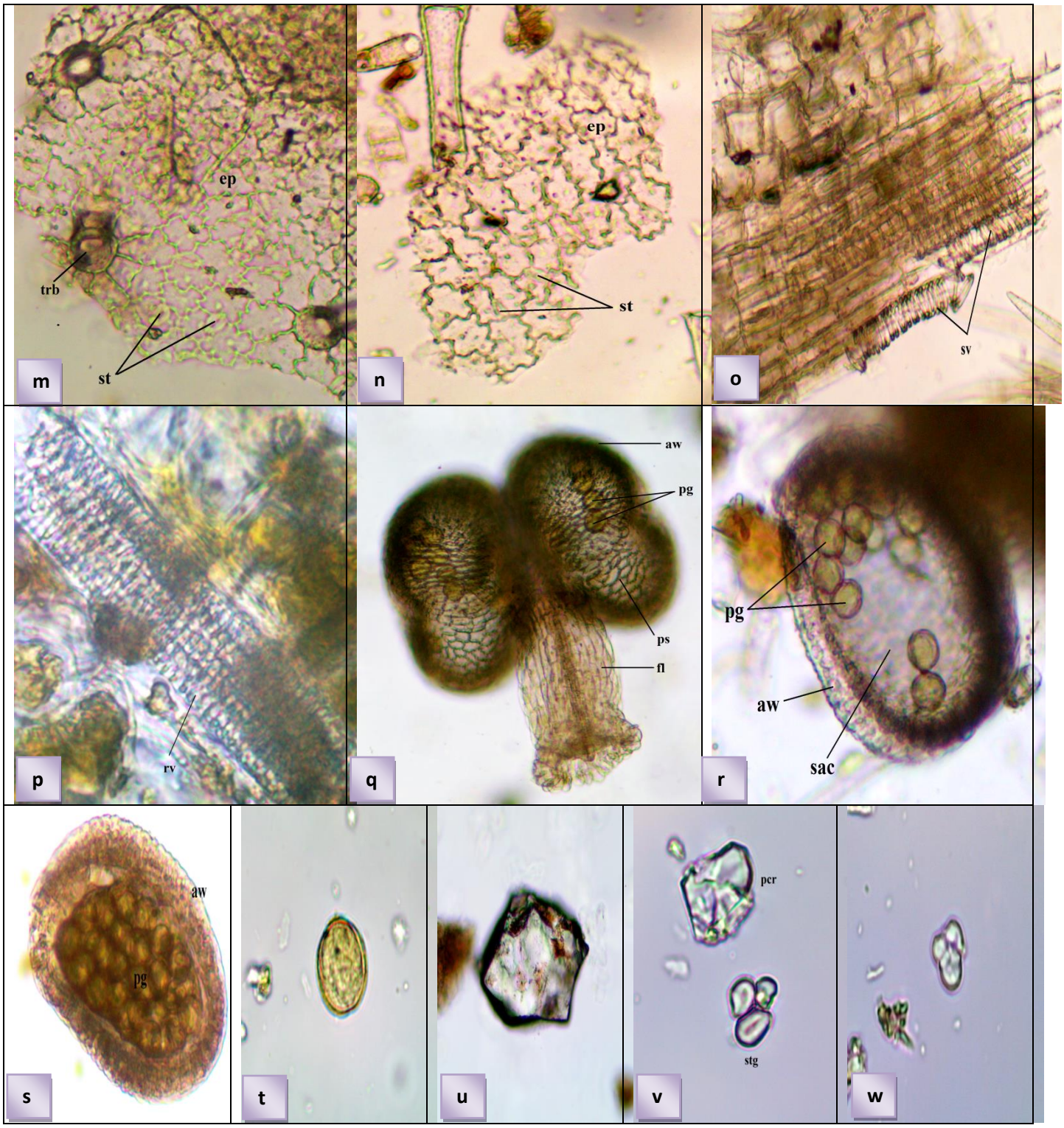


Fig 2: Photomicrographs of *Euphorbia hirta* whole plant powder

a, b: Multicellular uniseriate covering trichome with hirsute wall; c: Covering trichome with characteristic irregular buldging; d, e, f, g, h: Origin of covering trichome (ctr) with bulbous end (trb) from epidermis (ep) ; i: Ground tissue showing laticiferous duct (ldc); j: Thin walled parenchyma of anther filament; k: Thick walled rectangular brick shaped parenchyma; l: Portion of lamina showing dark and light zone m,n: Groups of epidermal cells (ep) having sinuate wall showing anomocytic stomata(st), trichome base (trb); o: Spiral vessels(sv) along with parenchymatous cells; p: Reticulate vessels(rv) in group; q,r,s: Anther showing aw: anther wall, ps: pollen sac; pg: pollen grains, fl: filament; t: Pollen grain; u: Prismatic crystal of calcium oxalate; v: Prismatic crystal (pcr) and simple starch grains in group (stg); w: Compound starch grain (3 components).

3.3. Fluorescence analysis

The fluorescence analysis of coarse dried whole plant powders treated with different reagents reveals the presence of chromophoric compounds in them (Table 1). Different shades of pink fluorescence with 50% KOH, acetic acid, chloroform, ethanol, toluene and light shades of orange and peach coloured fluorescence with 1N HCl, 1N NaOH and Ammonia respectively under 366 nm UV light. No fluorescence was observed under normal daylight and short UV (254 nm) light, indicating small amount of chromophores in the sample.

Table 1: Florescence analysis of *E. hirta* whole plant powder:

S. No.	Fluorescence Analysis Reagents	Visible/Day Light	Short UV (254 nm)	Long UV (366 nm)
1.	1N Hcl	Pink	---	Faintly fluroscent very light orange
	1N NaOH	Greenish brown	Light lemon green	Fluroscent peach
3.	1N NaOH + Methanol	Leafy green	Very faint lemon green	Fluroscent pale bluish green
4.	50% KOH	Yellowish brown	Light green	Fluroscent very light pink
5.	50% H_2SO_4	Black	Grey	Dark grey
6.	Conc. H_2SO_4	Black	Grey	Dark grey
7.	Conc. HNO_3	Yellow straw	-	Fluroscent grey
8.	Acetic acid	Light green	Very faint lemon green	Faintly fluroscent pink
9.	50% HNO_3	Light pink	-	-
10.	Iodine solution	Reddish violet	Light green with grey centre	Dark grey
11.	Distilled water	Opaque solution	-	Faintly fluroscent no colour
12.	Chloroform	Rusty green	Very light yellowish green	Faintly fluroscent pink
13.	Acetone	Green	Light green	Pink
14.	Ammonia	Reddish brown	-	Fluroscent light peach
15.	Ethanol	Light green	Faint light green	Fluroscent light pink
16.	Toluene	Yellowish green	Faint light green	Fluroscent bright pink
17.	$K_2Cr_2O_7$	Rusty brown	Lemon green	Bluish grey
18.	$FeCl_3$	Blackish brown	Light green	Dark grey

3.4. Physicochemical

Investigation of the physiochemical parameters of the plant samples showed (Table 2) its total ash value as 4.39%, water soluble and acid insoluble ash contents as 1.53% and 2.97% respectively. Very low value (2.34%) of loss on drying suggests that the plant material bears a very less amounts of water and\or volatile contents. The extractive values of different solvents for the plant samples revealed maximum and least extraction by methanol and water respectively. The extraction yields of the respective solvents were not significantly different under both cold and hot conditions while microwave assisted extraction proved low extraction efficiency. Based on the best extraction yield in methanol, the same was used for the subsequent finger printing analyses.

Table 2: Physico-chemical evaluation of *E. hirta* plant.[a]

Physicochemical Parameters	Percentage		
Loss on drying (LOD)	2.34±0.14		
Ash values			
Total ash value	4.39±0.38		
Water soluble ash value	1.53±0.53		
Acid insoluble ash value	2.97±0.26		
Sulphated Ash	2.12±0.21		
pH value (10% aq. suspension)	5.76±0.09		
Extractive values	Cold extraction	Hot extraction	Microwave assisted extraction
Hexane	11.53±0.27	11.85±0.05	4.22±0.01
Acetone	26.39±0.24	27.76±0.12	5.84±0.05
Chloroform	27.58±0.20	27.97±0.15	5.23±0.02
Ethyl acetate	31.27±0.29	29.06±0.11	8.10±0.01
Methanol	39.57±0.31	41.07±0.83	12.60±0.04
Alcohol	11.19±0.33	10.98±0.21	6.86±0.05
Water	7.23±0.21	6.54±0.19	3.12±0.05
Hydroalcoholic (1:1)	10.37±0.43	8.87±0.43	3.43±0.03

[a]Values are expressed as Mean ± S.D.

3.5. Phytochemical

Phytoconstituents are basically divided into two groups like primary and secondary metabolites, according to their functions in plant metabolism. Primary metabolites consist of common sugars, amino acids, proteins and chlorophyll while alkaloids, terpenoids, flavonoids, tannins etc. contribute as the secondary metabolites. In present study, different qualitative tests were carried out with the *E. hirta* plant samples after extraction with various solvents. The results of the phytochemical screening (Table 3) revealed the presence of alkaloids, steroids, phenolics, glycosides, but less terpenoids, flavonoids. The phenolics and glycosides were primarily present in the protic, polar solvents.

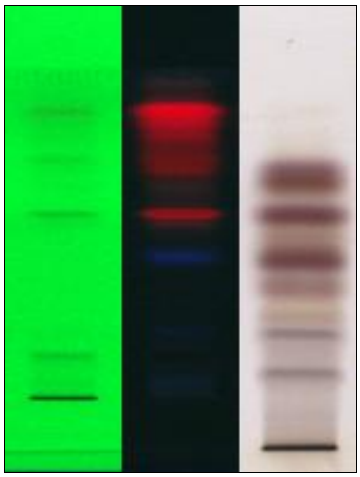
Table 3: Phytochemical screening of *E. hirta* plant

Phytochemical class	Hexane	Chloroform	Actone	Ethyl Acetate	Methanol	Ethanol	Water
Alkaloid	-	+	+	+	+	+	-
Flavonoid	-	-	-	-	-	+	-
Glycoside	-	+	+	-	+	+	+
Poly phenol	-	-	-	-	+	+	+
Phenolic		-	-	-	+	+	+
Oils	+	+	+	-	-	-	-
Steroid	+	+	+	+	+	+	-

Terpenoid	-	+	+	-	+	+	-
Fatty ester	-	-	+	+	-	-	-
Free acid	-	-	+	+	-	-	+

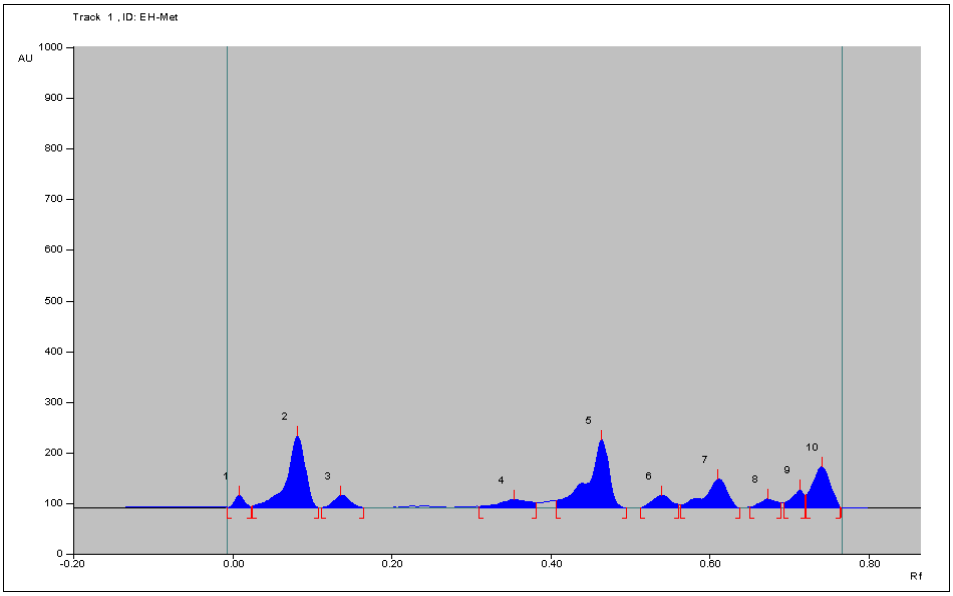
3.6. High Performance Thin Layer Chromatography (HPTLC)

The HPTLC experimental condition was optimized by using pre-activated and precoated TLC silica gel 60 F_{254} plates and different combinations of polar and apolar solvents as the mobile phases (data not shown). Best result was obtained with hexane: ethyl acetate: acetone : 1,4-dioxan : formic acid (4:3:2:1:0.5, v/v) as the mobile phase, which showed ten bands at R_f values of 0.01, 0.10, 0.16, 0.41, 0.54, 0.62, 0.71, 0.78, 0.82, 0.86, when visualized under UV at 254 nm and at 366 nm, nine bands at R_f values 0.01, 0.10, 0.16, 0.41, 0.54, 0.62, 0.71, 0.78, 0.86, were seen, while bands at R_f 0.18, 0.27, 0.29, 0.33, 0.45, 0.59, 0.68, were seen after derivatiztaion followed by exposure to visible light. Pictorial representations of the bands at different visualization are represented in Fig. 5. The relative ratios of the peaks of the HPTLC chromatograms, determined from the areas under the curves are represented in Table 4 & Table 5.

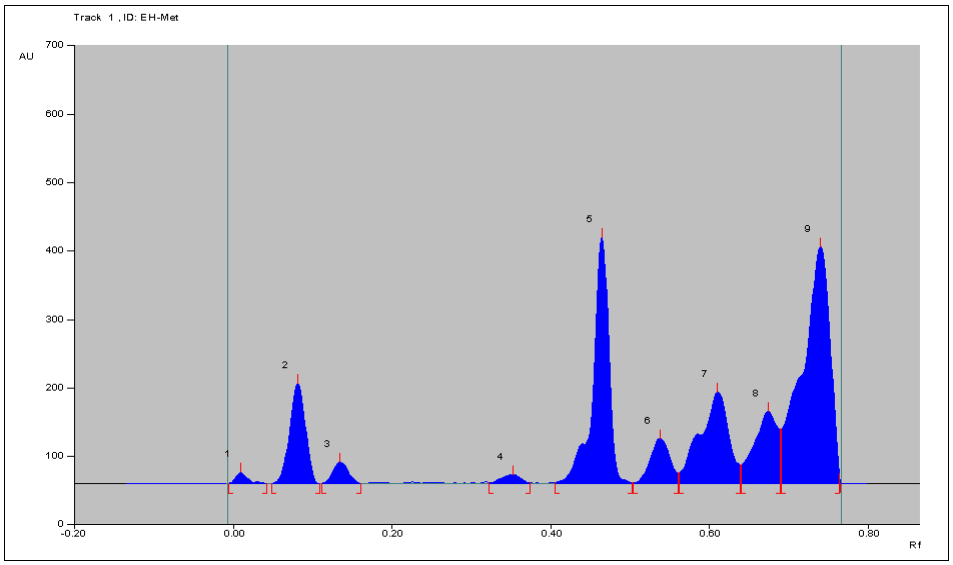


254 nm 366 nm WL

Fig 5: HPTLC profiles of *E. hirta* plant methanol extract.



(a)



(b)

Fig 6: Densitogram display of HPTLC profiles of *E. hirta* plant methanol extract (a) at 254 nm (b) at 366 nm.

Table 4: Relative ratios of the HPTLC peaks[a]

S. No.	R_f values	Relative ratio (%)
1.	0.01	02.29
2.	0.10	24.96
3.	0.16	3.85
4.	0.41	4.87
5.	0.54	26.95
6.	0.62	4.41
7.	0.71	12.06
8.	0.78	2.80
9.	0.82	4.19
10.	0.86	13.62

[a]The peaks were recorded by visualising the chromatogram spots at 254 nm.

Table 5: Relative ratios of the HPTLC peaks[a]

S. No.	R_f values	Relative ratio (%)
1.	0.01	0.74
2.	0.10	8.85
3.	0.16	1.83
4.	0.41	1.00
5.	0.54	21.85
6.	0.62	4.97
7.	0.71	14.60
8.	0.78	9.45
9.	0.86	36.71

[a]The peaks were recorded by visualising the chromatogram spots at 366 nm.

3.7. High Performance Liquid Chromatography (HPLC)

HPLC method was developed for best separation of the chemical constituents of the *E. hirta* plant methanol extract. Separated peaks were detected under UV (254 nm). The HPLC fingerprint analysis showed (Fig. 7a) ten peaks and retention times were 2.688, 3.191, 3.423, 3.472, 4.529, 5.358, 6.543, 7.606, 8.802 and 10.64 min respectively. The relative ratios of the peaks of the HPLC chromatograms, determined from the areas under the curves are represented in Table 6.

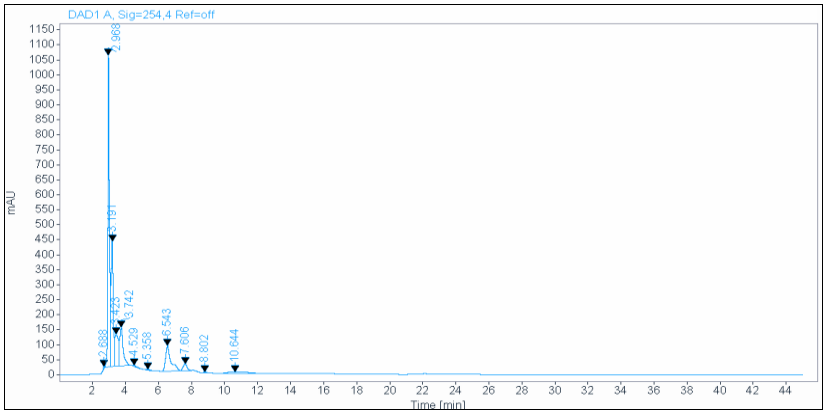
Table 6: Relative ratios of the HPLC peaks[a]

S. No.	Peak Retention time (Minute)	Realtive ratio (%)
1.	2.688	0.17
2.	3.191	37.50
3.	3.423	16.31
4.	3.742	18.81
5.	4.529	0.27
6.	5.358	0.18
7.	6.543	18.41
8.	7.606	3.59
9.	8.802	0.34
10.	10.644	4.55

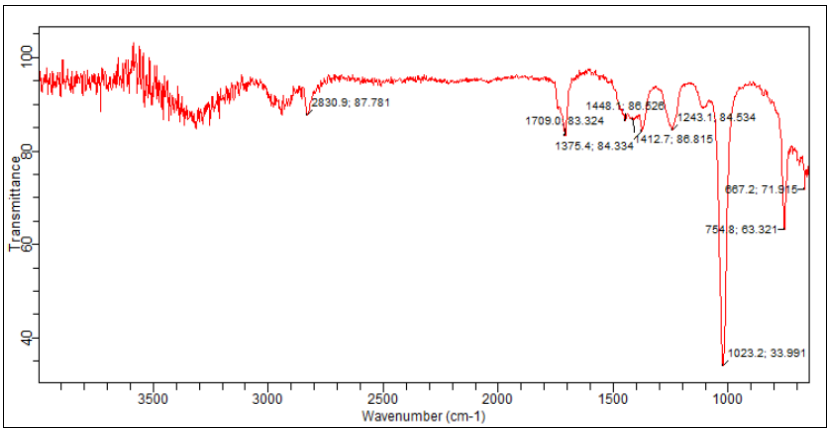
[a]The peaks were recorded by detecting the chromatogram at 254 nm.

3.8. Fourier Transmission Infra Red (FTIR) spectroscopy

The FTIR spectrum of the methanol extract using methanol is shown in (Fig. 7b). The absorption spectrum revealed distinct peaks at 1412.7, 1375.6 and 1243.1 cm^{-1} indicating the presence of compounds with C-N or C-O bonds. The intense peaks in the region of 2800-3000 cm^{-1} indicated presence of of C-H bonds suggesting presence of long-chain alkanes/alkene and their derivatives. The peak at 1709.02 cm^{-1} may account for the C=O stretch of some carbonyl containing group.



(a)



(b)

Fig 7: HPLC chromatogram (a) and FTIR spectrum (b) of *E. hirta* plant methanol extract.

4. Conclusion

The present investigations furnished a set of qualitative and quantitative phyto-pharmacognostic parameters along with HPTLC, HPLC and FTIR, fingerprinting profile of *E. hirta plant*s. These data can serve as diagnostic tools for establishment of quality standards, authentication and identification of the medicinally important plant and help in compiling of a suitable monograph of this.

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