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Pharmacognostical authentication of heartwood of *Pterocarpus marsupium* Roxb. - An important drug used in traditional systems of medicine

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Abstract
Humans have been using medicinal plants in the treatment of various diseases since prehistoric times. Due to the presence of bioactive phytochemicals, medicinal plants have always been considered as safe and effective treatment of several diseases and ailments. In last two decades, the herbal drug market has increased manifold. Due to constantly increasing demand, there is dire need of standardization of herbal drugs in order to make them safer and more effective. *Pterocarpus marsupium* is one such large deciduous tree which contains tremendous medicinal values. The heartwood of *Pterocarpus marsupium* was studied through HPTLC fingerprinting and assorted pharmacognostical parameters such as Microscopy, Macroscopy and Physico-chemical analysis. The drug has also been put through multiple quality control parameters such as determination of heavy metals, microbial load, aflatoxins and pesticide residues for thorough assessment of quality of the drug. The data generated can be fruitful for laying down the pharmacopeial standards of heartwood of *Pterocarpus marsupium* Roxb.

Keywords: Aflatoxins, Heavy Metals, HPTLC, microbial load, microscopy

Introduction
The quest of mankind for safe and effective treatment of diseases has a very long history. In this stride, herbal materials have always remained primary source of economical remedies. *Pterocarpus marsupium* Roxb. has numerous medicinal uses and has been a part of several compound formulations in traditional systems of medicine. It is a member of the Fabaceae family and usually known as 'Bijasar'. This tree usually grows in dry mixed deciduous tropical forests and thrive in open sun light with moderate rainfall of 80 - 200 cm. It is usually found in central and peninsular India, mostly in the forest regions of Andhra Pradesh, Maharashtra, Madhya Pradesh, Chhattisgarh, some parts of Uttar Pradesh and Uttarakhand, up to an altitude of 1000 m [1]. It can tolerate high summer temperatures and grows best in fertile, clayey loam soil with good drainage. It can be identified in the forest by its upright bole, longitudinally fissured bark, imparipinnate leaf arrangement, coriaceous, dark green and shiny leaves, scented yellow flowers in large panicles, and orbicular and winged fruits with flat pods. Flowering starts in November and continues up to the month of March. The mature tree attains a height of up to 30 m and a girth up to 3 m, with clear and straight stem [2].
The heartwood of *Pterocarpus marsupium* contains several unique bioactive constituents such as pterostilbene, marsupsin, pterosupin and vijyayosin [3]. These compounds exhibit a broad range of pharmacological activities. In Unani system of medicine different parts of *P. marsupium* such as heartwood, flower and gum are commonly used for different therapeutic values. Its heartwood, popularly known as Chob-e-bijasar, is beneficial as appetizing agent (*Mushtahi*), anti-helminthic (*Dafae Kirm-e-Am'a*), blood purifier (*Muasaffi-e-Khoon*), anti-inflammatory (*Muhallil-e-Waram*), anti-bilious (*Qata-e-Safra*) and carminative (*Kaasir-e-Riyah*) [4]. National Medicinal Plant Board (NMPB) of India has estimated that the annual trade value of heartwood of *P. marsupium* is around 300–500 metric tons per year [1]. With explosive rise of plant-based pharmaceutical industries, its demand is continuously rising. Consequently, it is sometimes replaced with substitutes or adulterants which results in compromising the safety and efficacy of the herbal drug formulations. The adulterants may sometimes be potentially lethal and pose threat to human health. Therefore, in present scenario it is essential to develop pharmacopeial standards for raw drugs as well as for compound formulations.

The current study aims to resolve the issues of adulterants of *P. marsupium* and provide a precise identification of genuine plant material for traditional systems of medicine. In order to standardize the heartwood of *P. marsupium*, a number of parameters were carried out such as Microscopy, Macroscopy, Physico-chemical analysis and HPTLC fingerprinting. The quality control parameters *viz*. estimation of heavy metals, microbial load, aflatoxins and pesticide residues were also carried out as per WHO guidelines.

Materials and Methods

Sample Procurement and Identification

The heartwood samples of *Pterocarpus marsupium* Roxb. were procured from local raw drug dealer in New Delhi and substantiated by pharmacognostical methods.

Microscopy

0.5g powdered drug was boiled with chloral hydrate to remove the colouring matter. It was, then, washed with water; stained with safranin (1%, *w/v*) for 5-8 min and mounted in glycerine (60%, *v/v*). The different characters were observed under microscope [5, 6, 7].

Physicochemical analysis

The physico-chemical parameters of *Pterocarpus marsupium* Roxb. such as moisture content, water & ethanol extractive values, ash values, pH values (1% & 10% aq. solution) were carried out as per standard methods [8, 9].

High-Performance Thin-Layer Chromatography (HPTLC)

Two powdered heartwood samples of *P. marsupium* (2 g each) were extracted separately with 25 ml each of chloroform and ethanol by the process of sonication. The extracts were filtered through Whatman No.1 filter paper and concentrated up to 10 ml. The extracts so obtained were used to effectuate HPTLC fingerprinting. 10 µl of chloroform extract was applied on aluminum TLC plate pre-coated with silica gel 60 F^{254} (E. Merck) by employing CAMAG Linomat IV automatic sample applicator. The plate was developed in 10 ml solvent mixture of Toluene: ethyl acetate: formic Acid (9: 1: 0.5) up to a distance of 9 cm in twin trough glass chamber (10 x10). The plate was air-dried at room temperature and examined under UV at wavelengths 254 nm and 366 nm. Further the plate was dipped in 1% Vanillin-sulphuric acid reagent and heated at 105^{o}C till coloured bands appeared. Finally, the plate was observed under the white light. Moreover, in case of ethanol extract the process was repeated in the same manner to carry out HPTLC fingerprinting [10, 11, 12].

Quality control analysis

Assessment of the safety of herbal material contains supreme importance as the drug, which is consumed by countless people for well-being, must not become the reason for their affliction. Therefore, heartwood sample of *P. marsupium* was thoroughly assessed for various contaminants through the determination of heavy metals, microbial load, aflatoxins and pesticide residues. Estimation of microbial load was conducted as per standard method [13]. Aflatoxins and Heavy metal analysis were carried out by the respective use of HPLC (Thermo Fisher) [15] and Atomic Absorption Spectrophotometer (LAB INDIA) [13]. Pesticide residues were analyzed using Triple Quadrupole GC-MS/MS system (Thermo Fisher) equipped with mass selective detector as per standard methods [14, 15].

Results and Discussion

Macroscopic description of heartwood

Heartwood is the central tough part of stem found in large old trees. It is composed of dead cells with their walls heavily impregnated with various compounds such as tannins, resins, a large number of phenolic compounds and hence becomes unsuitable for conduction but medicinally useful. As the growth process continues the rings of sapwood covering the heartwood keeps on converting into heartwood. Pieces of heartwood showed irregular surface with strong, tough, very hard and moderately heavy structure. It has very difficult fracture to break but brittle in nature. It has no characteristics odour. It showed astringent taste. On soaking in water, it gave yellow colour solution with blue fluorescence.

Microscopic observations

Longitudinal Section (L.S.) of heartwood shows different types of cells closely arranged minute bordered pits and slit like pores, wood is composed of tracheid fibres which are thin walled and usually arranged in tangentially running bands and often associated with xylem parenchyma cells embedded with prismatic crystals of calcium oxalate. The medullary rays are uniseriate but rarely biseriate (Fig. 1a-i). Whereas, transverse section (T.S.) exhibits many alternating bands of larger and smaller polygonal cells consisting of tracheid fibres, xylem parenchyma and traversed by xylem rays, many xylem vessels distributed throughout, in singles or in groups. Vessel cells in wood are arranged in small radial groups and often blocked with tyloses impregnated with tannin. The parenchymatous cells are thin walled, pitted, partially or completely encircling the vessels. Sometime, the vessel cells show well-marked perforation rims and bordered pits. Many prismatic crystals of calcium oxalate are present in crystal fibres (Fig. 2a-f).

Powder

Powder microscopy shows different type of cells such as xylem parenchyma, medullary rays in tangential view, parenchyma embedded with prismatic crystals of calcium oxalate, group of fiber tracheids, xylem fibers, medullary rays in radial view, prismatic crystals of calcium oxalate, group of 2-3 vessel cells, thick walled xylem parenchyma cells, pitted vessel cell, group of thick walled xylem parenchyma cells (Fig. 3a-k).

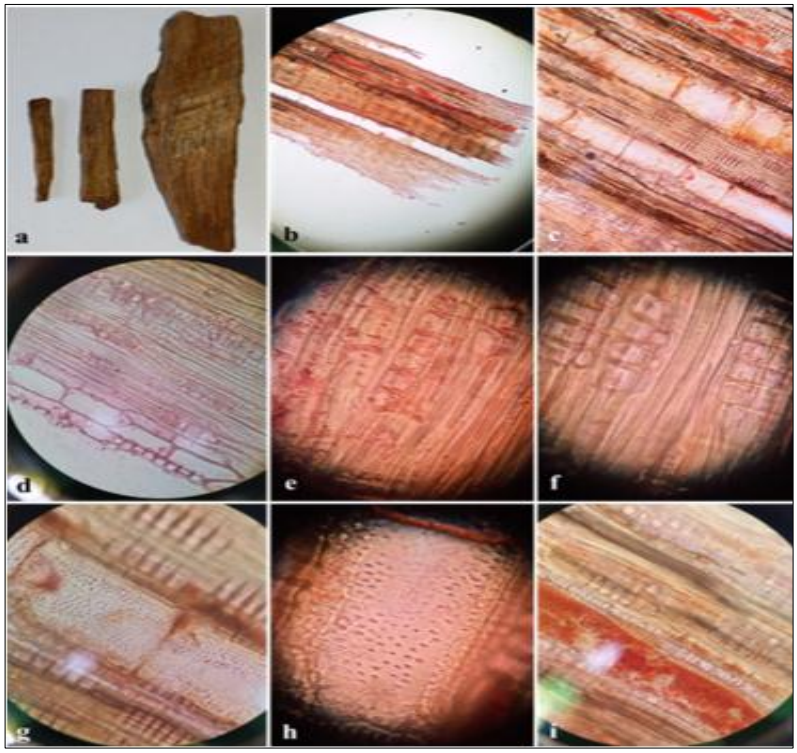

Fig 1: Longitudinal Section (L.S.) of heartwood of *Pterocarpus marsupium*. a. Dried cut pieces of heart wood; b. L.S. of heartwood, 4x; c. L.S. of heartwood, 10x; d. xylem parenchyma, 20x; e-f. prismatic crystals of calcium oxalate present in crystal fibers, 40x; g. vessels with xylem fibers arranged in tangentially running bands, 40x; h. vessel cell with minute bordered pits and slit like pores, 40x; i. medullary rays appear as narrow horizontally running bands crossing the vessels and fibers

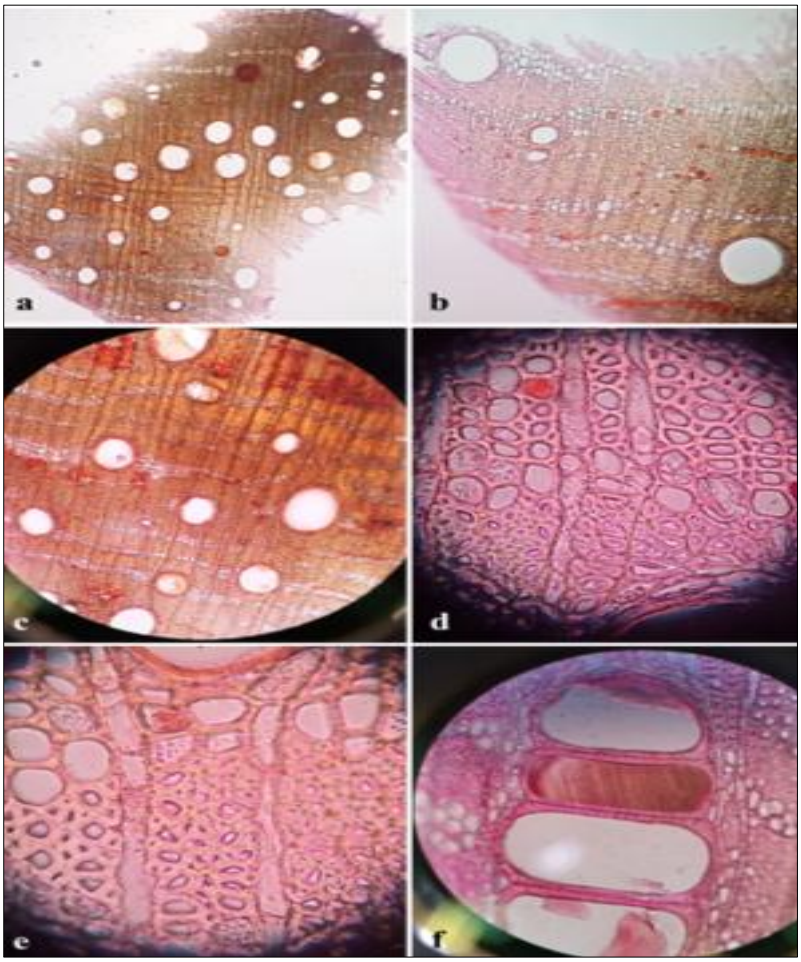

Fig 2: Transverse Section (T.S.) of heartwood of *P. marsupium.* a. T.S. of heart wood, 4x; b-c. different types of larger and smaller polygonal cells arranged in alternate bands, 10x; d. xylem parenchyma, fiber tracheids and numerous xylem vessels distributed throughout, 40x; e. vessels with well-marked perforation rims, 40x; f. vessels, 40x.

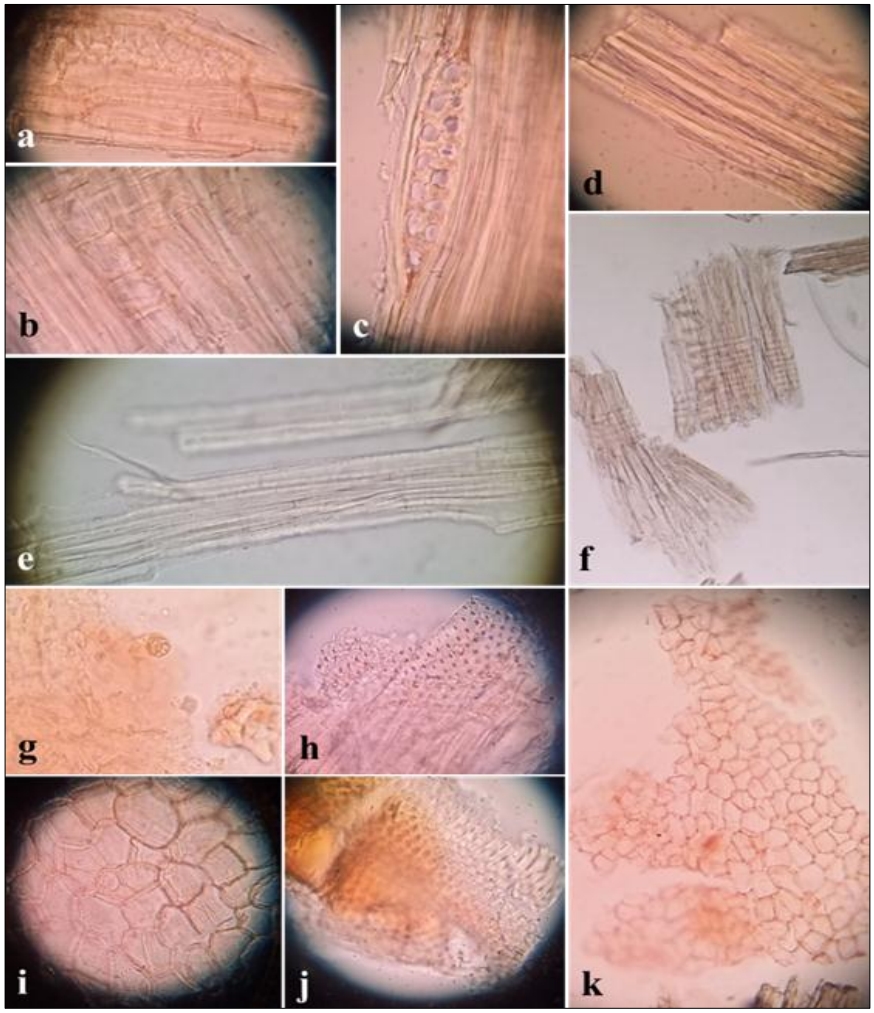

Fig 3: Powder microscopy of heartwood of *P. marsupium*. a. xylem parenchyma, 40x; b. medullary rays in tangential view, 40x; c. parenchyma embedded with prismatic crystals of calcium oxalate, 40x; d. group of fiber tracheids; e. xylem fibers, 40x; f. medullary rays in radial view, 20x; g. prismatic crystals of calcium oxalate, 40x; h. group of 2-3 vessel cells, 40; i. thick walled xylem parenchyma cells, 40x; j. pitted vessel cell, 40x; k. group of thick walled xylem parenchyma cells, 20x

Physicochemical analysis

The physicochemical data of *Pterocarpus marsupium* are given in Table I. The quantitative data indicated that the moisture content of the drug was below 10% which is optimum for minimal microbial growth and aids to longer shelf-life. The total ash content was in the range of 4 - 5% and acid insoluble ash was remained as low as around 0.10% confirming that the silicious matter was present in negligible amount in the drug samples. The water extractive values turned out to be on lower range between 10 – 11% which shows the presence of inorganic constituents and the ethanol extractive values were also a bit low and ranged between 13.42 – 14.00% revealing the extraction of polar constituents. The aqueous extract of the drug was slightly acidic in nature as pH values fall in the range of 5.45 – 6.26.

HPTLC profile

HPTLC fingerprinting is dependable and reproducible technique with versatile applications. It is convenient method for identification of crude drugs and can be used for analyzing the complex mixtures of herbal material. HPTLC fingerprints of chloroform & ethanol extracts of *P. marsupium* were observed under UV254 nm, UV 366 nm and under white light after derivatization. All the drug samples showed similar colourful bands with replicated Rf values. It indicates the consistency of the results (Fig. 4 – 5).

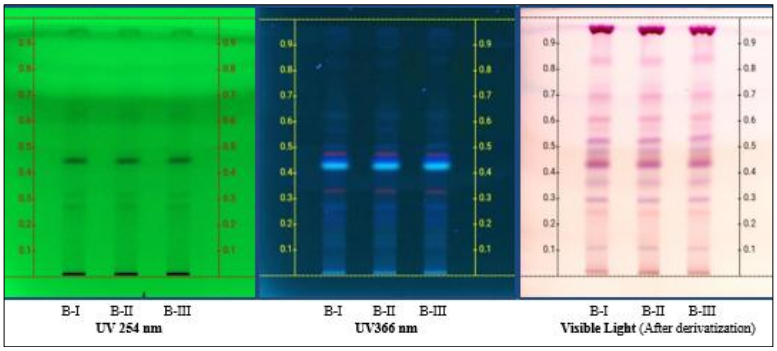

Fig 4: HPTLC of *Chloroform* extracts of *Pterocarpus marsupium*

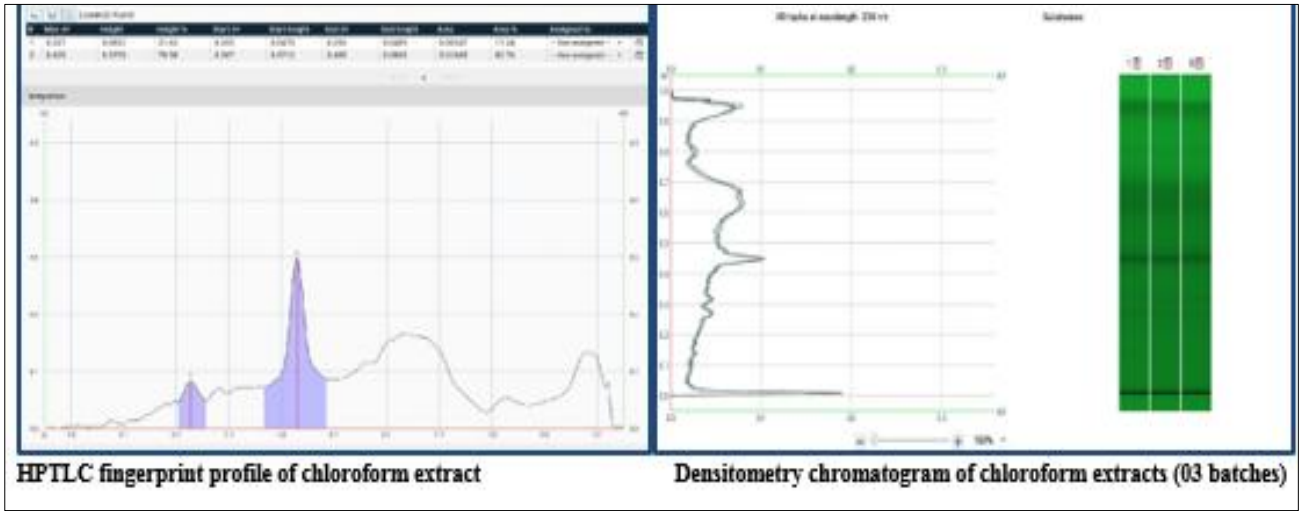

Fig 4 a): UV 254 nm

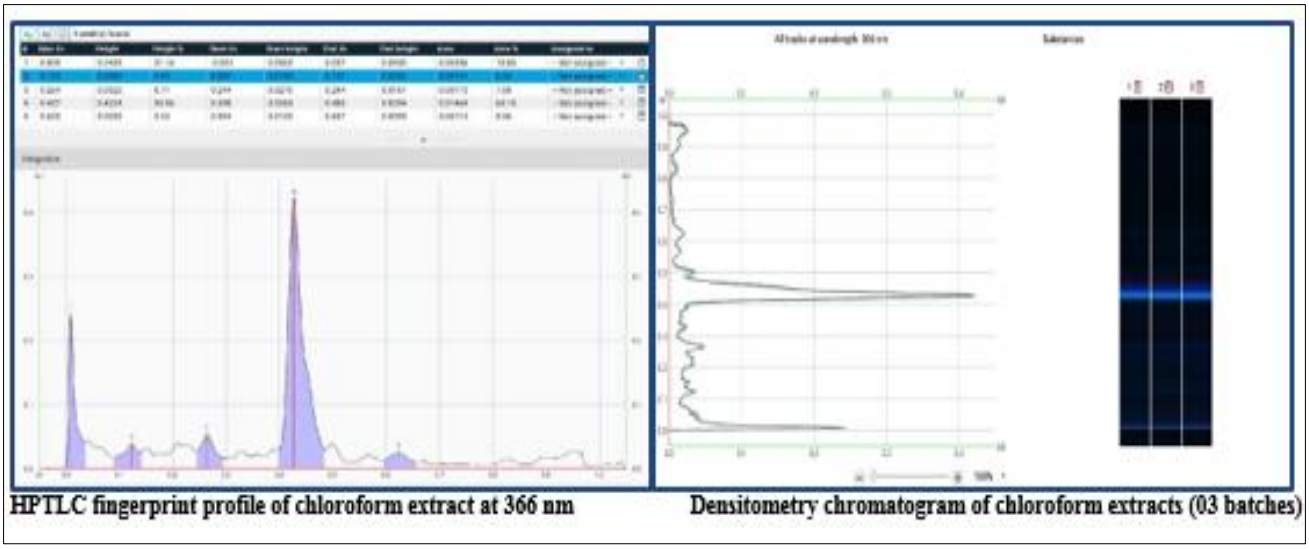

Fig 4 b): UV 366nm

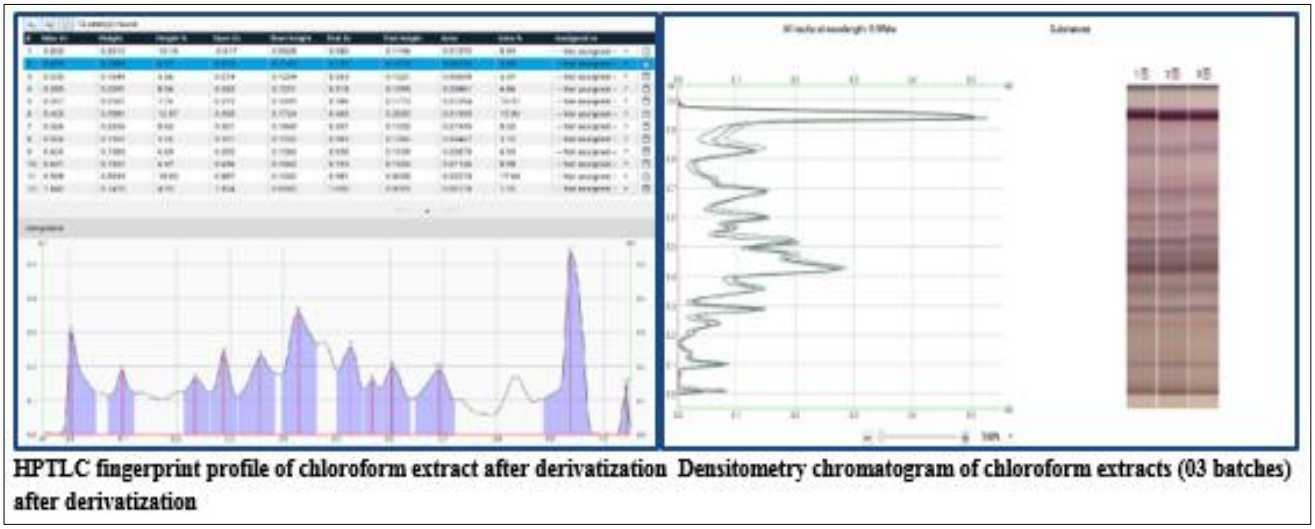

Fig 4 c): Under white light

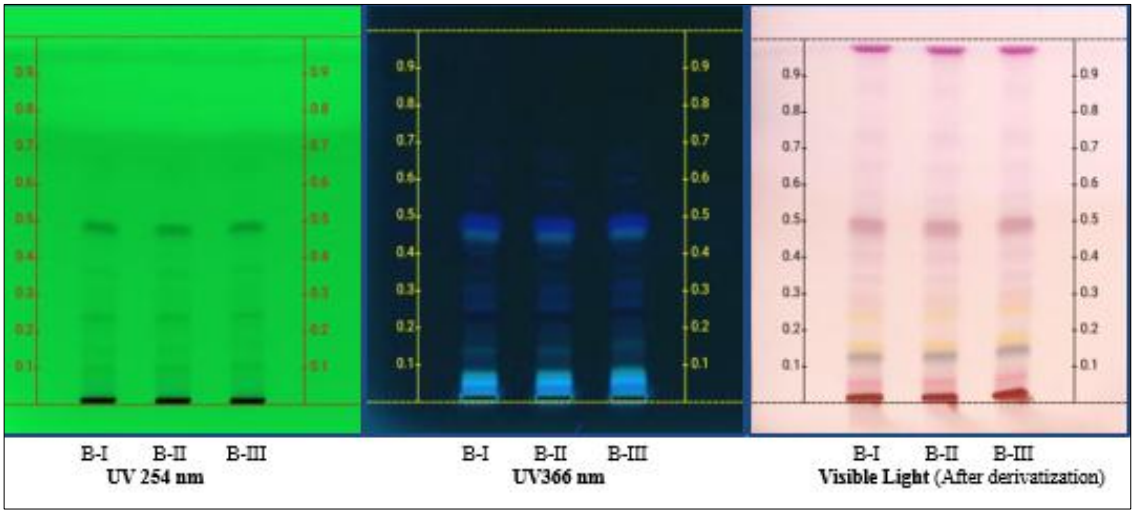

Fig 5: HPTLC of *Ethanol* extracts of *Pterocarpus marsupium*

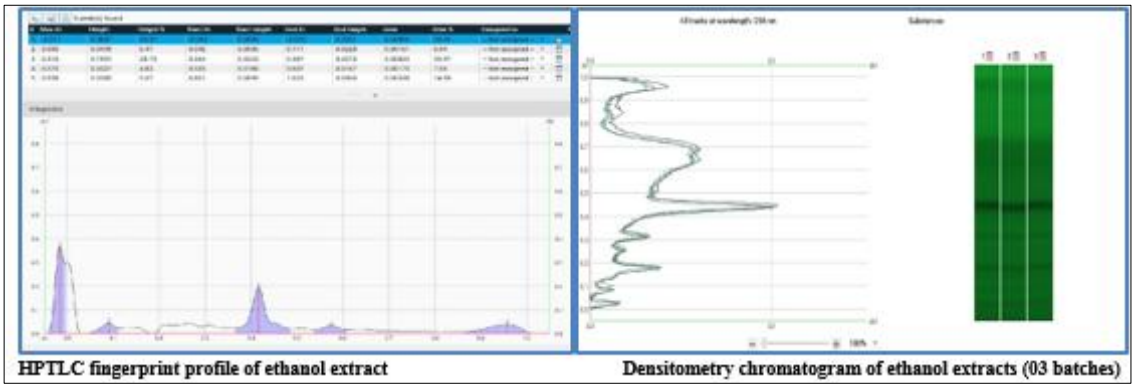

Fig 5 a): UV 254 nm

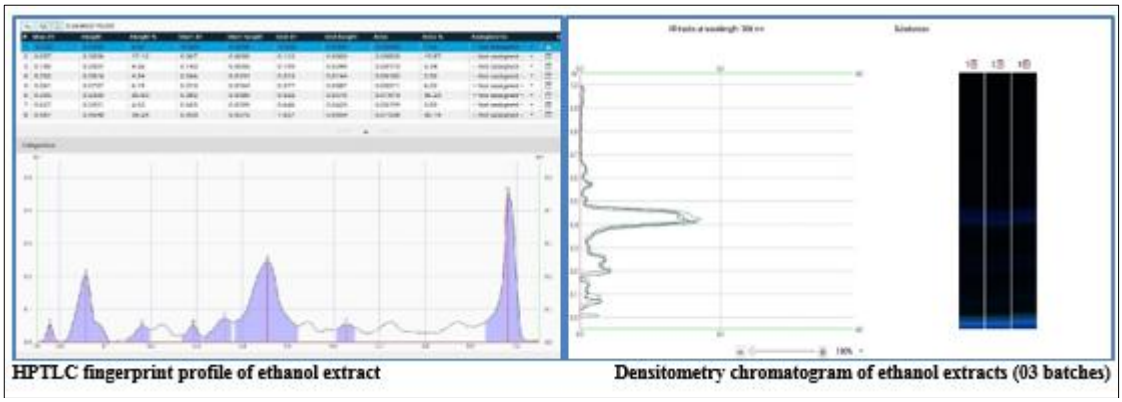

Fig 5 b): UV 366 nm

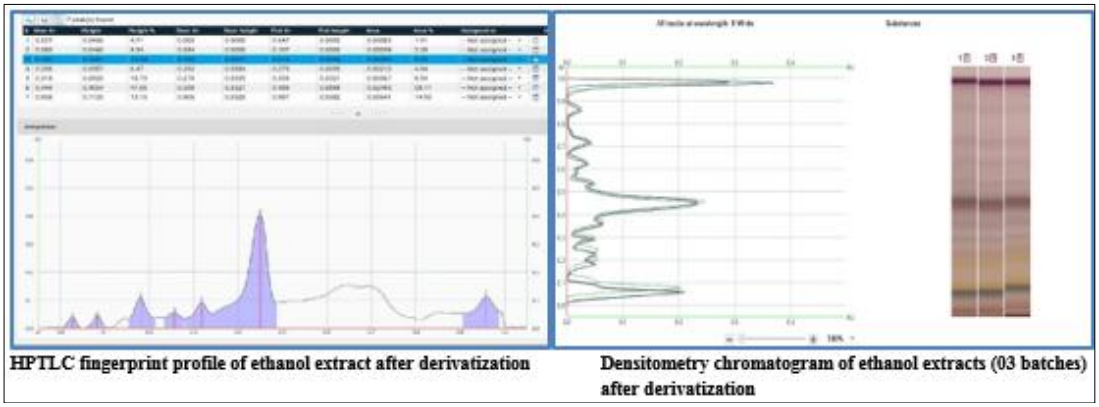

Fig 5 c): Under white light

Quality control parameters

Evaluation of harmful toxins in herbal drugs is essential to ensure its safe human consumption. Different toxic substances cause different health effects. They can sometimes develop health effects as mild as nausea to as lethal as cancer depending upon the nature of toxins and how much exposure a person gets. The results of quality control parameters such as microbial load & aflatoxins, heavy metals and pesticide residue analysis are respectively shown in Table II, III & IV. The values of all the parameters imply that the samples of *P. marsupium* were free from any hazardous or toxic substances.

Conclusion

Standardization plays a crucial role in maintaining the quality of any herbal drug. It is vital to carry out proper identification & standardization of raw drugs as well as finished product. *Pterocarpus marsupium* Roxb. was evaluated through pharmacopoeial parameters which certainly provide an assurance of quality of the drug. HPTLC fingerprinting of chloroform and ethanol extracts provides an easy method to examine the identity and purity of the drug. Values of quality control parameters *viz.* microbial load, aflatoxins, pesticide residue and heavy metals were found to be within the WHO permissible limit, indicating that the drug is free from hazardous substances and safe for human consumption. Hence, the current study ensures the authenticity, quality and efficacy of Chob-e-Bijasar (*Pterocarpus marsupium* Roxb.).

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Table 1: Physico-chemical parameters

S. No.	Parameters	Values
	Foreign Matter (%)	< 2.00
	Loss in weight on drying at 105^0 C (%)	8.40 – 9.25
	Total Ash (%)	4.60 – 5.10
	Acid insoluble ash (%)	0.10 – 0.14
	Ethanol Soluble Extractive (%)	10.20 – 11.02
	Water Soluble Extractive (%)	13.42 – 14.00
	Hexane Soluble Extractive (%)	0.90 – 1.08
	pH 1% Soln.	6.26
	pH 10% Soln.	5.45

Table 2: Microbial load

	Total aerobic bacterial count (TABC)	2.2×10^3 CFU/gm
	Total yeast and molds count (TYMC)	1.1×10^2 CFU/gm
	Enterobacteriaceae members	
	Escherichia coli	ND
	Salmonella sp.	ND
	Shigella sp.	ND
	Klebsiella sp.	ND
	Specific objectionable pathogens	
	Pseudomonas aeruginosa	ND
	Staphylococcus aureus	ND
	Candida albicans	ND
	Aflatoxin producing fungi	
	Aspergillus flavus	ND
	Aspergillus parasiticus	ND

ND – Not detected

Table 3: Heavy Metals Estimation

S. No.	Element	Values	WHO Limits for internal use
1.	Lead	< LOD	10 ppm
2.	Cadmium		0.3 ppm
3.	Arsenic		3.0 ppm
4.	Mercury		1.0 ppm

Table 4: Pesticide Residue Analysis

S. No.	Pesticide	Result (mg/Kg)	Permissible limit (mg/Kg)
	Alachlor	0.04	0.02
	Aldrin (Aldrin and dieldrin combined expressed as dieldrin)	BLQ	0.05
	Azinophos-methyl	BLQ	1.0
	Bromopropylate	BLQ	3.0
	Chlordane (cis, tans and oxychlordane)	BLQ	0.05
	Chlorfenvinphos	BLQ	0.5
	Chlorpyrifos	BLQ	0.2
	Chlorpyrifos-methyl	BLQ	0.1
	Cypermethrin (and isomers)	BLQ	1.0
	DDT (all isomers, sum of p,p'-TDE (DDD) expressed as DDT)	BLQ	1.0
	Deltamethrin	BLQ	0.5
	Diazion	BLQ	0.5
	Dichlorvos	BLQ	1.0
	Dithiocarbamates (as CS2)	BLQ	2.0
	Endosulphan (sum of isomers & Endosulphan sulphate)	BLQ	3.0
	Endrin	BLQ	0.05
	Ethion	BLQ	2.0
	Fenitrothion	BLQ	0.5
	Fenvalerate	BLQ	1.5
	Fonofos	BLQ	0.05
	Heptachlor (sum of Heptachlor & Heptachlor epoxide)	BLQ	0.05
	Hexachlorobenzene	BLQ	0.1
	Hexachlorocyclohexane isomer (other than γ)	BLQ	0.3
	Lindane (γ – Hexachlorocyclohexane)	BLQ	0.6
	Malathion	BLQ	1.0

	Methidathion	BLQ	0.2
	Parathion	BLQ	0.5
	Parathion methyl	BLQ	02
	Permethrin	BLQ	1.0
	Phosalone	BLQ	0.1
	Piperonyl butoxide	BLQ	3.0
	Pirimiphos methyl	BLQ	4.0
	Pyrethrins (sum of isomers)	BLQ	3.0
	Quintozen (sum of Quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	BLQ	1.0

***** BLQ – Below limit of quantification

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