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Drug standardization research, physicochemical analysis, HPTLC fingerprinting, quality and safety studies of the polyherbal Unani formulation Habb-e-Pachlona

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ABSTRACT

Background: Drug standardization research, Physicochemical, HPTLC fingerprinting, quality, safety studies, QC and QA remain a big challenging task for ASU (*Ayurvedic*, *Shiddha*, and *Unani*) formulated drugs. There needs to be more than the QC, QA, and PV scientific screening parameters to validate, authenticate and differentiate hazardous and toxic substances, adulterants in formulated ASU polyherbal drugs. Habb-e-Pachlona is one of the polyherbal Unani classical formulated drug used to treat various human ailments. **Objective:** This study aims to evaluate the DSR, HPTLC fingerprint, Safety, QC and QA profiling studies of the HEP. The quality, safety and toxicity activities of the HEP and extracts of HEP were also studied. **Material and Methods:** The physicochemical, phytochemical, and drug-standardization research, along with safety studies such as heavy metal, aflatoxin, and pesticide residue analyses of the coarse powder of the formulated polyherbal HEP pill, were carried out using standard methods. HPTLC of the extracts of HEP were performed using standard methods. The quality and safety effects of the tested HEP drug samples of different microbes, hazardous metaxies and toxins were also investigated. **Results:** The physicochemical and DSR, HPTLC fingerprint, toxicity, and QC and QA properties of HEP have shown that all the parameters were within the permissible limits. The HPTLC fingerprint profile of the various extracts of HEP exhibited various peaks under UV 254 nm, under UV 366 nm, and under the V-S reagent, visible light region. The HPTLC fingerprint and GC-MS analysis confirmed and illustrated the presence of various bioactive compounds and hazardous adulteration in the test sample. The tested HEP drug samples showed significant quality, safety and toxicity studies against certain pathogenic organisms and promising antimicrobial load activity. **Conclusion**: The drug standardization research and quality control findings revealed that the test HEP drug was free from adulterations. The physicochemical constants and HPTLC profiles of HEP can be essential data in the future. This polyherbal classical Unani-formulated drug may potent, treat and cures in poor digestion, gastric debility, dyspepsia, anorexia, flatulence, and hiccough/hiccup diseases.

Keywords: DSR, HPTLC profiles, HEP drug, Quality, Safety activities.

INTRODUCTION

Standardization of ASU (*Ayurvedic, Shiddha* and *Unani*) herbal drugs is not an easy challenge as various factors influence the bio efficacy and reproducible therapeutic effects. In order to obtain assured quality based herbal products, care through pharmaco-vigilance should be taken right from the proper identification of plants, season and area of collection, grading, drying, extraction, purification process and rationalizing the combination in the case of poly-herbal drugs [7-15, 36]. The subject of standardization of herbal drugs is massively wide and deep. There are many seemingly contradictory theories on the subject of herbal medicines and its relationship with human physiology and mental function [19-21,23-25,28]. For the purpose of drug standardization research work of herbal formulated products, a complete profound knowledge is of utmost important [1-4,7-13]. Historically, herbal medicines have played a significant role in the management of both minor and major medical illness [22]. The quality assurance and quality control of herbal crude drugs and formulated products are important in justifying their acceptability in modern system of medicine. Hence it is required to conduct the research on drugs standardization to provide effective, curable and safe drugs to the needy mass suffering from various ailments [1-4,9-17,23-25]. Total 19 Raw drugs, single ingredients used in preparation of classical Unani compound formulation mentioned in Table-1 respectively. Storage of HEP: Stored in cool and dry place in tightly closed containers, protected from light and moisture. Important therapeutic wise and Action wise indication of HEP classical compound formulated drug: Useful therapeutics potential in Zof-e-Hazm (Poor digestion / Dyspepsia), Zof-e-Ishteha (Anorexia), Nafakh-e-Shikam (Flatulence), Fuwaq (Hiccough / Hiccup), Zuf-e-Meda (Gastric debility). In Action wise uses Hazim (Digestive), Kaasir-e-Riyah (Carminative), Mushtahi / Mushahhi (Appetizing / Appetizer), Muqawwi-e-Meda (Stomachic).

Dose: 500 mg to 1g, 2 pills (each pill - 500mg), twice a day after meals. As per guidelines of Anonymous, NFUM -I,1984:1.47; Hakim Kabiruddin.2010; part-II;1.49:232 [29,52].

MATERIAL AND METHODS

Ingredients used for preparation: The formulated drug was prepared in different batches at Laboratory scale as per the ingredient's compositions and guidelines of Anonymous, NFUM -I,1984:1.47; Hakim Kabiruddin.2010; part-II;1.49:232 [29,52]. Detail of polyherbal classical Unani formulated drug HEP Formula composition with their required ingredients has shown in Table 1, respectively. The required quantities of all the necessary ingredients of Pharmacopoeial quality have been gathered. Initially, we cleaned and dried all the required ingredients. Next, we took the required amount of ingredients numbered 1 to 14, made them into powder, sieved through an 80-mesh sieve, and kept them separately. We then ground the required amount of ingredients numbered 15 to 17 using a kharal, made fine powders, and kept them separately. We prepared extracts of ingredient number 18 (Aab-e-Lemu) and kept them separately. We added the required quantity of powders from ingredients numbered 1 to 17, mixed thoroughly, and kept them separately. Then, we added the juice of ingredient number 18 (Aab-e-Lemu) to the mixed powdered ingredients, mixed thoroughly, and kept them separately for fermentation. We also prepared extracts of ingredient number 19 (Aab-e-Aamla Taza) and kept them separately. After filtering the fermentation and soaking the fermented ingredients with ingredient number 19 (Aab-e-Aamla Taza), we dried them. Once dried, we added the juice of ingredient number 18 (Aab-e-Lemu) to the fermented dried powders and prepared the lubdi mass. We rolled the lubdi mass, shaped it into sticks of the required size and thickness, and cut it into pieces using a knife. We manually rounded the cut pieces and shaped them into Huboob or Habb (Pills) of the required size and weight. (Anonymous, NFUM-I, 1984:1.47; Hakim Kabiruddin, 2010, part-II, 1.49:232.) [29,52].

Pharmacopoeial standard parameters:

The usage of ASU herbal drugs and products along with higher safety margins, WHO has taken necessary steps to ensure quality assurance and quality control parameters with the modern techniques and application of suitable standards, Pharmacopoeial research studies such as organoleptic characters, microscopical, macroscopical and physicochemical, HPLC finger printing profiling, quality control and quality assurance parameters were carried out [9-17,19-21,22-27,30-37,39-44].

1. Organoleptic Evaluation: Organoleptic evaluation refers to evaluation of formulation by colour, odour, taste, texture etc., using the sensory organs of our body. The organoleptic characters of the drugs samples were carried out based on the method described [32,39,47-51].

2. Physico-chemical analysis: If the moisture or water content has found high side in the tested drug sample. It can easily be deteriorated due to fungus. The ash content indicates the total amount of inorganic material or heavy metals impurities after complete incineration and the acid insoluble ash is an indicative of silicate impurities might be due to improper washing of the drug, in ash values, acid insoluble ash %. The alcohol and water-soluble extractive matter (ASEM%, WSEM%), Successive extractive matter (ASSEM%, CSSEM%) indicate and exposed the amount of bioactive chemical constituents' concentration in a given amount of particular tested drug when extracted with respective solvent, solubility in water and alcohol. Some of the useful tools in standardization of ASU herbal products such as moisture content or LOD of the powdered / semi solid tested drug sample at 105ºC, pH values and bulk density and estimation of sugar etc., are useful tools were studies as per standard methods [1-6,9-18, 21-27,30-34,36-38,40-44].

3. Estimation of microbial load: The microbial load viz. total bacterial count (TBC), total fungal count (TFC), *Enterobacteriaceae*, *Escherichia coli*, *Salmonella* spp and *Staphylococcus aurous* were estimated as per standard method. microbial load of HEP Batches samples were deducted and found to be Table -6, respectively [1-6,9-17,21,23-27,30-38,40-44].

4. Estimation of heavy metals: The method used for the analysis estimation of heavy metals like lead, cadmium, mercury and arsenic as per Guidelines of WHO. of HEP Batches samples. Heavy metals samples were estimated and deducted by Atomic Absorption Spectroscopy with Graphite furnace instrument and shown to be in Table - 7, respectively [1-18,21,23-27,31-34, 36-38,40-44].

5. Analysis of Aflatoxins: Aflatoxins B1, B2, G1 and G2 were analyzed as per Official Analytical Methods of the AYUSH/WHO/AOAC. Aflatoxins were estimated by Kobra cell techniques using CAMAG or Anchrom HPTLC instruments as per the standard methods. Aflatoxins samples were deducted and found to be Table - 8 respectively [1-6,9-18,21-27,30-44,45-51].

6. Analysis of pesticide residue: The method used for the analysis of pesticide residues was as per AOAC [40]. Pesticide residues were analyzed by Gas Chromatography Mass Spectra (GC-MS). pesticide residue samples were deducted, found and shown in to the Table-9 respectively [1-18,22,23-27,30-34,36-38,40-44,47-51].

A graphical workflow summarizing the preparation, extraction, physicochemical analysis, pharmacognostic evaluation, HPTLC profiling, GC–MS/LC–MS analysis, microbial load testing, and toxicity investigations of Habb-e-Pachlona is presented in Figure 1.

RESULTS AND DISCUSSION

Quality assurance and quality control parameters: With the use of ASU herbal products and higher safety margins, WHO has taken necessary steps to ensure quality assurance and quality control parameters through modern techniques and the application of suitable standards. The microbial load and heavy metal parameters of the tested powder drug were carried out in accordance with WHO guidelines [12,31,39,42-44]. Heavy metals were analyzed using Atomic Absorption Spectroscopy. Aflatoxins were estimated using Kobra cell techniques with CAMAG HPTLC instruments from Switzerland following the ASTA 27 method, and pesticide residues were analyzed using GC-MS from Thermo Scientific, Model -TSQ9000 instruments equipped with a Mass selective detector following the AYUH/WHO/AOAC methods [12,31-51].

Physicochemical Standardization: Organoleptic evaluation of the whole plant of HEP Batches samples revealed that it was brown, tasteless, and had a characteristic odour (Table 1, entries 1-3). The morphological characteristics of all 19 ingredients used in HEP Batches samples were found to be consistent with those mentioned in authenticated standard botanical literature. Foreign substances such as other plants, mould, insects, excrement, sand, stones, chemical residues, etc. are prohibited in herbal medicines and must be within permissible limits according to API/UPI-AYUSH/WHO Standard guidelines. Bulk Density, gm/ml-0.464, 0.465,0.465, The LOD / moisture content in any herbal drug / products is recommended to be up to 5 to10%, thus preventing spoilage. The Loss of weight on drying (LOD) at 105 °C in HEP Batches - B-1, B-2 and B-3 were found to be 4.06, 4.08 and 4.12%. The ash value is an important parameter for identifying adulterants in an herb. The higher ash value shows the presence of inorganic substances in the tested plant material. The total ash and acid-insoluble ash % values of HEP Batches samples were found to be 10.64,10.66,10.68% and 0.558, 0.560, 0.562% respectively. The extractive values %, (ASEM%, WSEM%) of ethanol, and water were found to be 18.86, 18.88, 18.89% and 38.74, 38.76 and 38.75%, Alcohol and Chloroform soluble successive extractive values %, (ASSEM%, CSSEM%)-27.80, 27.82, 27.83% and -9.98, 9.98, 9.99% respectively. Such results indicate that most of the phytoconstituents of HEP Batches samples are soluble in ethanol, water and chloroform solvents. The pH (1%

and 10% aqueous solution) of the test samples were found to be - 4.69,4.71, 4.71 and - 4.38, 4.49, 4.50, In Analyzed and tested parameters of HEP B-1, B-2, B-3 batches Average values, Standard deviation (SD) and Relative Standard deviation (RSD, should be NMT-5.0%) values performed complies as per standard permissible Limits shown in given Table-2 (Table 2, entry 4 -13) [1-4,7-13,17-23,26-39,40-46]. The acidic nature of the test drug shows its good absorption through the mucous membrane of the stomach. Toxic and hazardous contaminants of heavy metals, Microbial load, Aflatoxins and Pesticide Residues in natural medicinal plants based polyherbal products may cause serious health issues in humans [1-6,9-17,21-27,30-34,45-51]. The lead, cadmium, arsenic, and mercury, Microbial load, Aflatoxins and Pesticide Residues analyzed parameters were found to be below the permitted limits according to HEP Batches samples heavy metal, Microbial load, Aflatoxins and Pesticide Residues estimations and analysis findings shown in (Tables - 6,7,8 and 9) respectively. The entire HEP Batches samples plant's physicochemical constants were all within acceptable limits according to the Indian Ayurvedic Pharmacopoeia and Indian Unani Pharmacopoeia basis [1-17,21-27,30-44,47-51].

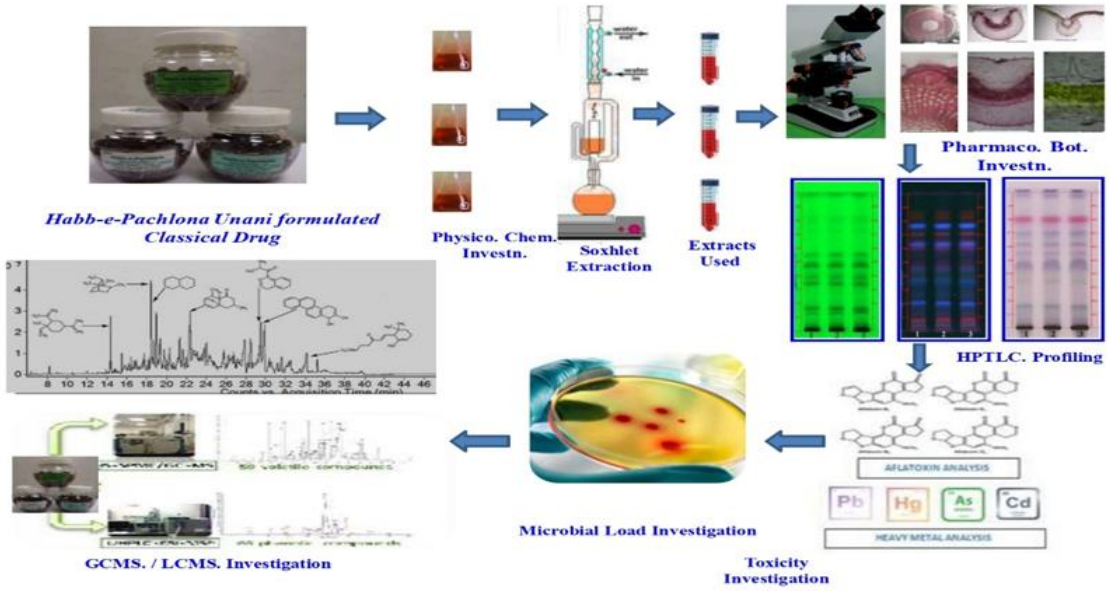


Figure 1: Graphical Illustration

Table 1: HEP raw drugs has been composed of the following ingredients used in compound formulation preparation

S. No.	Unani Name	Botanical/English Name	Part used	Qty.	Reference
1.	Nankhwah	*Trachyspermum ammi* Linn.	Fruit	125g	Anonymous, UPI-I, Vol.-VI
2.	Badiyan	*Foeniculum vulgare* Mill	Fruit	125g	Anonymous, UPI-I, Vol.-I
3.	Kishneex Khushk	*Coriandrum* sativum Linn.	Fruit	62.5g	Anonymous, UPI-I, Vol.-I
4.	Zeera Safaid	*Cuminum cyminum* Linn.	Fruit	20g	Anonymous, API-I, Vol.-I
5.	Zeera Siyah	*Carum carvi* Linn.	Fruit	20g	Anonymous, UPI-I, Vol.-I
6.	Waj-e-Turki	*Acorus calamus* Linn.	Rhizome	20g	Anonymous, UPI-I, Vol.-V
7.	Zanjabeel	*Zingiber officinale* Rosc.	Rhizome	20g	Anonymous, UPI-I, Vol.-I
8.	Filfil Daraz	*Piper longum* Linn.	Fruit	20g	Anonymous, API-I, Vol.-IV
9.	Filfil Siyah	*Piper nigrum* Linn.	Fruit	20g	Anonymous, UPI-I, Vol.-IV
10.	Aamla	*Emblica officinalis* Gaertn.	Pericarp	20gm	Anonymous, UPI-I, Vol.-I
11.	Post-e-Balela	*Terminalia belerica* Roxb.	Pericarp	20g	Anonymous, UPI-I, Vol.-I
12.	Post-e-Halela Zard	*Terminalia chebula* Retz.	Pericarp	20g	Anonymous, UPI-I, Vol.-I
13.	Zarambad	*Curcuma zedoaria Rosc. / Curcuma zeodaria* Linn.	Rhizome	12.5g	Anonymous, API-I, Vol.-IV
14.	Pudina	*Mentha viridis* Linn.	Aerial portion	12.5g	Anonymous, UPI-I, Vol.-V
15.	Namak-e-Sang	Rock Salt	Mineral drug	5g	IH
16.	Namak Siyah	Black Salt	Mineral drug	5g	IH
17.	Namak-e-Sambhar	Salt from Sambhar Lake	Mineral drug	5g	IH
18.	Aab-e-Lemu Kaghzi	*Citrus limon* (L) Burm.f.	Juice	Q. S.	Anonymous, API-I, Vol.-IV
19.	Aab-e-Aamla Taza	*Emblica officinalis* Gaertn.	Juice	Q. S.	Anonymous, API-I, Vol.-I; UPI-I, Vol.-I

Table 2: Physicochemical parameters analyzed of HEP Batches

S. No.	Parameters	Resulted Values					
		HEP-B-1	HEP-B-II	HEP-B-III	Average Values	Std. Deviation, (SD) Values	Related Std. Deviation, (RSD) Values, (Should be NMT-5.0%)
1.	Colour	Brown	Brown	Brown	--	--	--
2.	Odour	characteristic	characteristic	characteristic	--	--	--
3.	Taste	sour and salty	sour and salty	sour and salty	--	--	--
4.	Bulk Density, gm/ml	0.464	0.465	0.465	0.464	0.001	0.216%
5.	Loss in weight on drying at 105^0 C (%), w/w	4.06%	4.08%	4.12%	4.08%	0.0167	0.410%
6.	Total Ash (%), w/w	10.64%	10.66%	10.68%	10.66%	0.064	0.549%
7.	Acid insoluble ash (%), w/w	0.558%	0.560%	0.562%	0.560%	0.002	0.357%
8.	Ethanol Soluble Extractive Matter, (%), w/v	18.86%	18.88%	18.89%	18.87%	0.0174	0.092%
9.	Water Soluble Extractive Matter, (%), w/v	38.74%	38.76%	38.75%	38.74%	0.05	0.129%
10.	ASSEM, (%), w/v	27.80%	27.82%	27.83%	27.81%	0.0347	0.0125%
11.	CSSEM, (%), w/v	9.98%	9.98%	9.99%	9.98%	0.0316	0.317%
12.	pH (1% aqueous solution)	4.69	4.71	4.71	4.70	0.0317	0.673%
13.	pH (10% aqueous solution)	4.38	4.49	4.50	4.45	0.0671	1.507%

Table 3: Summary of results of phytochemical screening of HEP Course powder extracts

Phytochemical Test	Positives Performing Result	Observed Result		
		Water Extract (100%)	Water and Ethanol Extract (50:50)	Ethanol Extract (100%)
Polyphenols: Ferric chloride test	dark green and bluish-black colour	++ve	++ve	+ve
Flavonoids: Lead acetate test Shinoda's test	 yellow precipitate, red colour	++ve, ++ve	++ve, ++ve	+ve, +ve
Cardiac Glycosides Keller-Killani test Kedde's test Liebermann Burchard test	 brown ring between layers disappearing violet colour colors ranging from blue to green, violet and red colour	 ++ve, ++ve, ++ve	 +ve, +ve, ++ve	 -ve, -ve, ++ve
Tannins: Gelatin Test	 white colour precipitate	++ve	++ve	+ve
Alkaloids: Mayer's test Wagner's test Dragendorff's Test Hager's Test	 creamy white/yellow precipitate, brown/reddish precipitate, red/reddish-brown precipitate, creamy white/yellow precipitate	 ++ve, ++ve, ++ve, ++ve	 ++ve, ++ve, ++ve, ++ve	 +ve, +ve, +ve, +ve
Saponins: Foam test	foam persists for 10 min	+ve	-ve	-ve
Steroids and Triterpenoids: Salkowski test Liebermann Burchard test*	 appearance of performing colour, colours ranging from blue to green, violet and red	 -ve, ++ve	 -ve, +ve	 -ve, -ve
Test for Quinones:	appearance of red colour indicates the presence of quinone	-ve	-ve	-ve
Test for Carbohydrates: Fehling's test Tollen's test Benedict's test	 appearance of performing colour, reddish violet or purple colour ring at the intersection of two liquids shows	 ++ve, ++ve, ++ve	 ++ve, ++ve, ++ve	 +ve, +ve, +ve
Proteins / amino acids: Millon's test Biuret test	appearance of performing colour, appearance of purple colour indicates	++ve, ++ve	++ve, ++ve	+ve, +ve
Starch	Potassium iodide solution	-ve	-ve	-ve

Where: (+ indicates present, ++ indicates adequately present, - absent)

Table 4: R_f values of Chloroform extract: (By HPTLC)

Solvent System	R_f Values		
	UV Light at 254nm.	**UV Light at 366nm.**	**Visible Light, V-S reagent**
Toluene : Ethyl acetate (8 : 2)	0.76 (Green)	0.88 (Violet)	0.82, (Grey)
	0.68 (Green)	0.77 (Brown)	0.71 (Grey)
	0.52 (Dark green)	0.72 (Blue)	0.65 (Violet)
	0.43 (Dark green)	0.68 (Orang Red)	0.56 (Violet)
	0.33 (Dark green)	0.62 (Brown)	0.27 (Yellow)
	0.28 (Dark green)	0.57 (Blue)	0.22 (Grey)
	0.25 (Green)	0.52 (Violet)	0.13 (Grey)
	0.19 (Green)	0.44 (Grey)	
	0.14 (Dark Green)	0.39 (Blue)	
	0.09 (Dark Green)	0.31 (Blue)	
		0.25 (Violet)	
		0.17 (Blue)	
		0.15 (Blue)	

Table 5: R_f values of Ethanol extract: (By HPTLC)

Solvent System	R_f Values		
	UV Light at 254nm.	**UV Light at 366nm.**	**Visible Light, V-S reagent**
Toluene : Ethyl acetate (8 : 2)	0.80 (Dark green)	0.85 (Brown)	0.80 (Orang Red)
	0.71 (Green)	0.82 (Brown)	0.70 (Grey)
	0.55 (Dark green)	0.80 (Brown)	0.65 (Violet)
	0.48 (Dark green)	0.77 (Violet)	0.62(Grey)
	0.39 (Dark green)	0.70 (Dark Orang Red)	0.46 (Grey)
	0.35 (Dark green)	0.66 (Brown)	0.35 (Yellow)
	0.22 (Green)	0.64 (Violet)	0.16 (Greenish blue)
	0.16 (Dark green)	0.62 (Blue)	
	0.12 (Dark green)	0.59 (Orang Red)	
		0.57 (Violet)	
		0.49 (Brown)	
		0.45 (Violet)	
		0.36 (Blue)	
		0.27 (Blue)	
		0.20 (Violet)	
		0.12 (Brown)	

Table 6: Analysis of Microbial load

S. No.	Parameter Analyzed	Results	AYUSH/ WHO Limit
1	Total Bacterial Count	4×10^2 cfu/gm	10^5cfu/gm
2	Total Fungal Count	1×10^2 cfu/gm	10^3cfu/gm
3	*Escherichia coli*	Absent	Absent
4	*Salmonella typhai Spp.*	Absent	Absent
5	*Staphylococcus aurous*	Absent	Absent

Table 7: Estimation of Heavy Metals

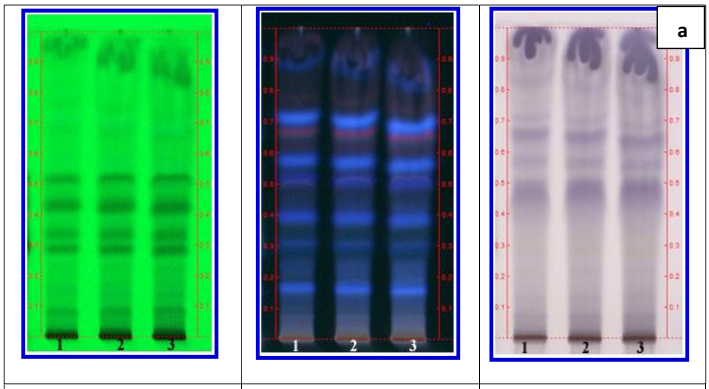
S. No.	Parameter Analyzed	Results	AYUSH/ WHO Limit
1	Lead	Not detected	10ppm
2	Cadmium	0.001ppm	0.3ppm
3	Mercury	Not detected	1.0ppm
4	Arsenic	0.024	3.0ppm

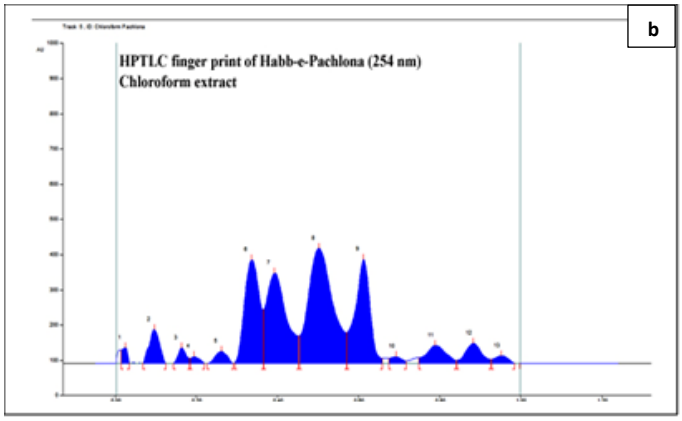
Table 8: Estimation of Aflatoxins

S. No.	Parameter Analyzed	Results	AYUSH/ WHO Limit
1	Aflatoxine, B1	Not detected	0.5ppm
2	Aflatoxine, B2	Not detected	0.1ppm
3	Aflatoxine G1	Not detected	0.5ppm
4	Aflatoxine G2	Not detected	0.1ppm

Table 9: Estimation of Pesticide Residues

S. No.	Parameter Analyzed	Results	AYUSH / WHO Limit (mg/kg)
1	DDT (all isomers, sum of ρ, ρ'-DDT, α, ρ' DDT, ρ, ρ'-DDE and ρ, ρ'-TDE (DDD expressed as DDT)	Not detected	1.0
2	HCH (sum of all isomers)	Not detected	0.3
3	Endosulphan (all isomers)	Not detected	3.0
4	Azinphos-methyl	Not detected	1.0
5	Alachlor	Not detected	0.02
6	Aldrin (Aldrin and dieldrin combined expressed as dieldrin)	Not detected	0.05
7	Chlordane (cis& tans)	Not detected	0.05
8	Chlorfenvinphos	Not detected	0.5
9	Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	Not detected	0.05
10	Endrin	Not detected	0.05
11	Ethion	Not detected	2.0
12	Chlorpyrifos	Not detected	0.2
13	Chlorpyrifos-methyl	Not detected	0.1
14	Parathion methyl	Not detected	0.2
15	Malathion	Not detected	1.0
16	Parathion	Not detected	0.5
17	Diazinon	Not detected	0.5
18	Dichlorvos	Not detected	1.0
19	Methidathion	Not detected	0.2
20	Phosalone	Not detected	0.1
21	Fenvalerate	Not detected	1.5
22	Cypermethrin (including other mixtures of constituent isomers sum of isomers)	Not detected	1.0
23	Fenitrothion	Not detected	0.5
24	Deltamethrin	Not detected	0.5
25	Permethrin (sum of isomers)	Not detected	1.0
26	Pirimiphos methyl	Not detected	4,0





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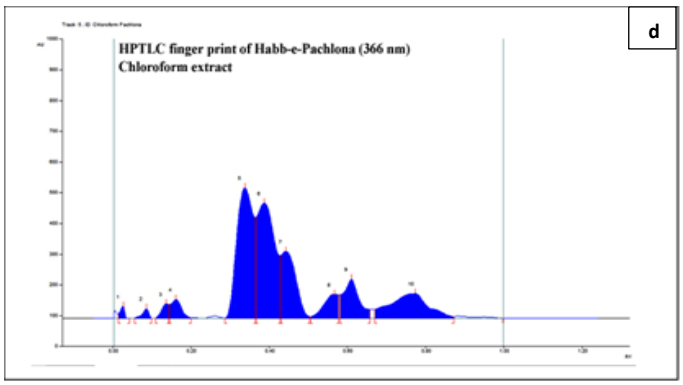
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	13.7 AU	0.03 Rf	38.5 AU	2.60 %	0.04 Rf	0.0 AU	317.8 AU	0.71 %
2	0.06 Rf	0.5 AU	0.09 Rf	31.7 AU	2.14 %	0.10 Rf	0.3 AU	362.3 AU	0.81 %
3	0.11 Rf	0.4 AU	0.14 Rf	46.8 AU	3.15 %	0.14 Rf	45.6 AU	606.8 AU	1.35 %
4	0.15 Rf	45.0 AU	0.16 Rf	61.2 AU	4.13 %	0.20 Rf	1.4 AU	1204.4 AU	2.68 %
5	0.29 Rf	0.3 AU	0.34 Rf	425.2 AU	28.67 %	0.37 Rf	28.1 AU	13023.4 AU	28.97 %
6	0.37 Rf	329.4 AU	0.39 Rf	375.2 AU	25.30 %	0.43 Rf	04.4 AU	12135.9 AU	26.99 %
7	0.43 Rf	205.0 AU	0.44 Rf	217.8 AU	14.68 %	0.50 Rf	5.2 AU	5851.7 AU	13.02 %
8	0.51 Rf	5.3 AU	0.57 Rf	78.6 AU	5.30 %	0.58 Rf	75.6 AU	2128.5 AU	4.73 %
9	0.58 Rf	76.8 AU	0.61 Rf	127.6 AU	8.60 %	0.66 Rf	29.4 AU	3642.4 AU	8.10 %
10	0.67 Rf	29.6 AU	0.77 Rf	80.6 AU	5.44 %	0.87 Rf	5.6 AU	5687.8 AU	12.65 %

HPTLC finger print and Rf values of HEP- Chloroform extract (254nm)



e

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	36.7 AU	0.03 Rf	44.4 AU	2.85 %	0.04 Rf	2.1 AU	472.7 AU	1.00 %
2	0.07 Rf	1.6 AU	0.10 Rf	96.5 AU	6.19 %	0.13 Rf	0.0 AU	1760.9 AU	3.74 %
3	0.14 Rf	0.3 AU	0.16 Rf	44.1 AU	2.83 %	0.18 Rf	13.9 AU	624.1 AU	1.32 %
4	0.19 Rf	14.2 AU	0.20 Rf	19.0 AU	1.22 %	0.22 Rf	0.5 AU	287.6 AU	0.61 %
5	0.23 Rf	0.1 AU	0.26 Rf	34.0 AU	2.18 %	0.29 Rf	0.0 AU	710.7 AU	1.51 %
6	0.30 Rf	0.9 AU	0.34 Rf	294.4 AU	18.88 %	0.37 Rf	51.5 AU	7840.4 AU	16.64 %
7	0.37 Rf	153.5 AU	0.39 Rf	257.0 AU	16.48 %	0.45 Rf	77.7 AU	9033.2 AU	19.17 %
8	0.45 Rf	78.1 AU	0.50 Rf	326.9 AU	20.95 %	0.57 Rf	87.3 AU	14253.5 AU	30.25 %
9	0.57 Rf	87.9 AU	0.61 Rf	294.7 AU	18.89 %	0.66 Rf	14.9 AU	7804.5 AU	16.57 %
10	0.68 Rf	14.8 AU	0.69 Rf	18.8 AU	1.20 %	0.72 Rf	7.1 AU	390.6 AU	0.83 %
11	0.75 Rf	16.5 AU	0.79 Rf	50.9 AU	3.26 %	0.84 Rf	8.3 AU	1777.5 AU	3.77 %
12	0.84 Rf	8.6 AU	0.88 Rf	57.5 AU	3.69 %	0.93 Rf	11.0 AU	1640.4 AU	3.48 %
13	0.93 Rf	11.4 AU	0.95 Rf	21.7 AU	1.39 %	0.99 Rf	0.4 AU	515.1 AU	1.09 %

HPTLC finger print and Rf values of HEP- Chloroform extract (366nm)

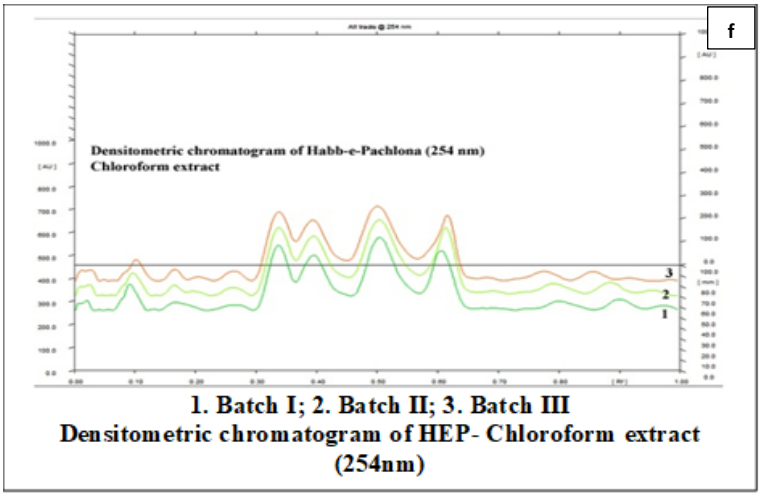
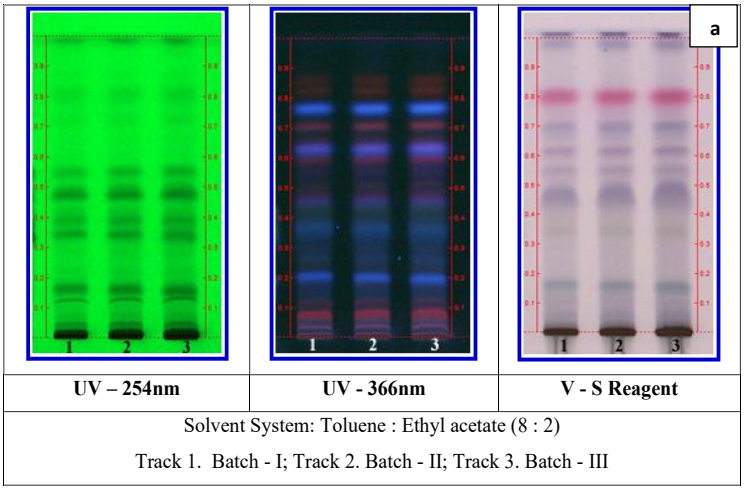
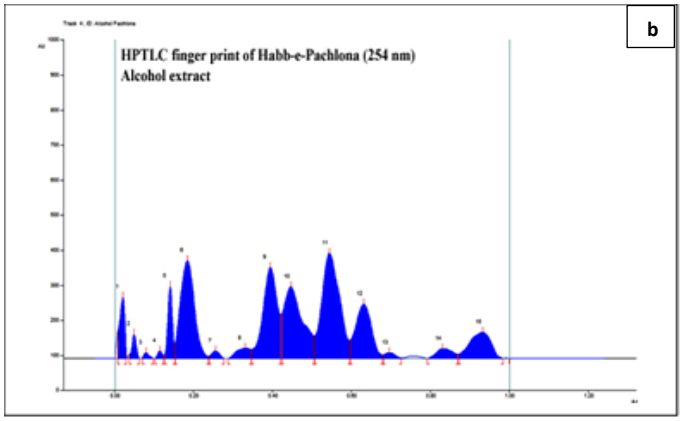


Figure 2: (a) HPTLC finger print of HEP (chloroform extract); **(b)** HPTLC chromatogram of HEP at 254 nm; **(c)** Rf values and peak table of HEP at 254 nm; **(d)** HPTLC chromatogram of HEP at 366 nm; **(e)** Rf values and peak table of HEP at 366 nm; **(f)** Densitometric overlay chromatogram of HEP at 254 nm (Batch I–III)

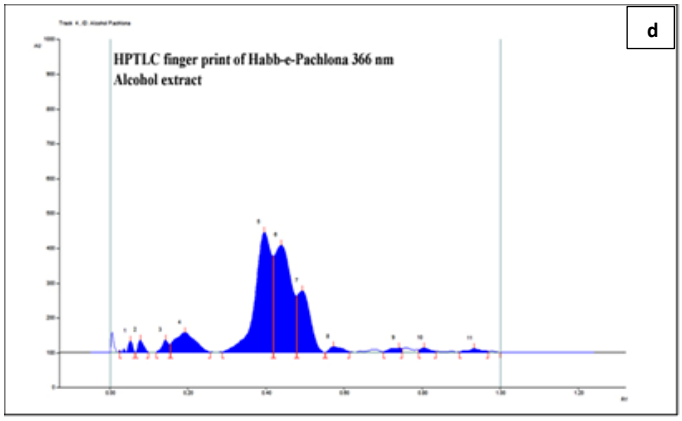




c

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	79.9 AU	0.02 Rf	174.7 AU	9.40 %	0.03 Rf	14.9 AU	1641.8 AU	3.68 %
2	0.04 Rf	13.1 AU	0.05 Rf	69.3 AU	3.73 %	0.06 Rf	1.4 AU	544.1 AU	1.22 %
3	0.07 Rf	1.7 AU	0.08 Rf	16.5 AU	0.89 %	0.10 Rf	0.2 AU	155.1 AU	0.35 %
4	0.10 Rf	0.6 AU	0.12 Rf	21.7 AU	1.16 %	0.13 Rf	8.4 AU	190.2 AU	0.43 %
5	0.13 Rf	9.6 AU	0.14 Rf	206.0 AU	11.08 %	0.15 Rf	41.0 AU	1743.3 AU	3.91 %
6	0.15 Rf	45.8 AU	0.19 Rf	279.0 AU	15.00 %	0.24 Rf	6.4 AU	7088.2 AU	15.89 %
7	0.24 Rf	6.9 AU	0.26 Rf	21.6 AU	1.16 %	0.28 Rf	0.5 AU	313.4 AU	0.70 %
8	0.29 Rf	0.2 AU	0.33 Rf	29.8 AU	1.60 %	0.35 Rf	26.0 AU	757.9 AU	1.70 %
9	0.35 Rf	26.0 AU	0.40 Rf	259.9 AU	13.98 %	0.42 Rf	25.8 AU	6760.2 AU	15.15 %
10	0.42 Rf	127.2 AU	0.45 Rf	204.4 AU	10.99 %	0.51 Rf	65.2 AU	7011.9 AU	15.72 %
11	0.51 Rf	65.5 AU	0.55 Rf	301.0 AU	16.19 %	0.60 Rf	52.4 AU	9764.9 AU	21.89 %
12	0.60 Rf	53.3 AU	0.63 Rf	155.2 AU	8.35 %	0.68 Rf	10.0 AU	4523.6 AU	10.14 %
13	0.68 Rf	10.1 AU	0.70 Rf	17.0 AU	0.91 %	0.73 Rf	1.7 AU	314.4 AU	0.70 %
14	0.79 Rf	0.0 AU	0.83 Rf	28.3 AU	1.52 %	0.87 Rf	12.0 AU	857.3 AU	1.92 %
15	0.87 Rf	12.3 AU	0.93 Rf	74.9 AU	4.03 %	0.99 Rf	0.1 AU	2942.7 AU	6.60 %

HPTLC finger print and Rf values of HEP - Ethanol extract (254nm)



e

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.03 Rf	5.8 AU	0.05 Rf	32.6 AU	3.12 %	0.06 Rf	0.8 AU	291.8 AU	1.03 %
2	0.07 Rf	0.4 AU	0.08 Rf	35.2 AU	3.38 %	0.10 Rf	0.1 AU	357.9 AU	1.26 %
3	0.12 Rf	0.4 AU	0.14 Rf	35.6 AU	3.41 %	0.15 Rf	23.4 AU	436.4 AU	1.54 %
4	0.16 Rf	24.6 AU	0.19 Rf	56.7 AU	5.44 %	0.26 Rf	0.7 AU	2085.3 AU	7.34 %
5	0.29 Rf	1.6 AU	0.40 Rf	345.4 AU	33.11 %	0.42 Rf	76.2 AU	10496.6 AU	36.96 %
6	0.42 Rf	276.8 AU	0.44 Rf	308.1 AU	29.53 %	0.48 Rf	62.2 AU	9424.0 AU	33.18 %
7	0.48 Rf	162.9 AU	0.49 Rf	176.4 AU	16.91 %	0.55 Rf	0.1 AU	4114.2 AU	14.49 %
8	0.55 Rf	0.3 AU	0.57 Rf	16.9 AU	1.62 %	0.61 Rf	2.4 AU	388.4 AU	1.37 %
9	0.70 Rf	1.9 AU	0.74 Rf	12.5 AU	1.20 %	0.75 Rf	11.2 AU	288.4 AU	1.02 %
10	0.79 Rf	7.5 AU	0.81 Rf	12.9 AU	1.24 %	0.84 Rf	2.8 AU	237.0 AU	0.83 %
11	0.90 Rf	1.5 AU	0.93 Rf	11.1 AU	1.06 %	0.97 Rf	2.7 AU	282.9 AU	1.00 %

HPTLC finger print and Rf values of HEP - Ethanol extract (366nm)

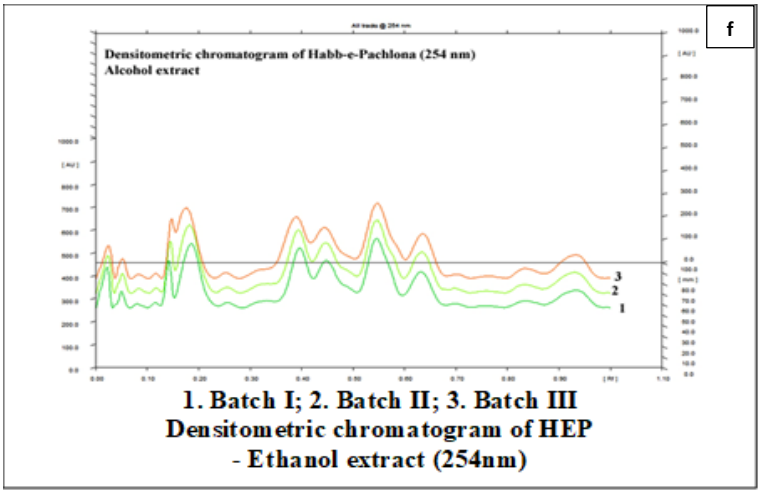


Figure 3: (a) HPTLC finger print of HEP (ethanol extract); **(b)** HPTLC chromatogram of HEP at 254 nm; **(c)** Rf values and peak table of HEP at 254 nm; **(d)** HPTLC chromatogram of HEP at 366 nm; **(e)** Rf values and peak table of HEP at 366 nm; **(f)** Densitometric overlay chromatogram of HEP at 254 nm (Batch I–III)

Phytochemical screening:

The preliminary phytochemical screening of HEP was carried in, aqueous (100%), hydro-alcoholic (50:50) and alcoholic (100%) extracts of HEP to check qualitatively different groups of active phytochemical constituents of compounds and secondary metabolites presents, which showed the presence of Alkaloids, Triterpenoids, Glycoside, Flavonoids, Carbohydrates, Polyphenols, Proteins, Amino acids, and Tannins which has presented in Table- 3 respectively [4-6,13].

High Performance Thin Layer Chromatography (HPTLC) Fingerprinting:

The investigation and deduction of polyherbal HEP Drugs samples by HPTLC have emphasized and exposed the bioactive phytochemical constituents and concentrations of secondary metabolites. Alkaloids were confirmed in the UV region at 366nm with variable appearances of Orange Red and Brown colours. Flavonoids were confirmed in the UV region at 254nm, 366nm, and 320 to 370nm with strong appearances of green and blue colours. Terpenoids, Diterpenes & Triterpenes were confirmed in the UV region at 366 nm and 270-290nm with variable violet and blue colours appearances. Phenolic Glycosides, Phenolic acids, and Polyphenolic Compounds etc. were confirmed in the UV region at 366nm, 270-300nm, and 280 to 360nm with strong and moderate violet and blue colours appearances in separated spots. Tannins were confirmed in 254nm, 366nm with strong and moderate greenish and blue colours appearances in separated spots of bands. Details are clearly mentioned in Table-4 and 5, Figures - 2a and 3a. [4-6,12-13,31-51].

HPTLC fingerprinting profiles: Samples of 2g of HEP, B-1, B-2, B-3 batches were extracted with 20ml of chloroform and alcohol separately and refluxed on a water bath for 30 minutes. The extracts were then filtered and concentrated to 5ml before carrying out the HPTLC. The chloroform extracts were applied to the TLC plate. Developed the plate used Toluene : Ethyl acetate (8 : 2) as mobile phase. After development allowed the plate to dried in air and examined under UV (254nm), it shown major 10 spots at R_f 0.76, 0.68 (Green), 0.52, 0.43, 0.33, 0.28 (Dark green), 0.25, 0.19 (Green), 0.14 and 0.09 (Dark green). Under UV (366nm), it shown major 13 spots at R_f 0.88 (Violet), 0.77 (Brown), 0.72 (Blue), 0.68 (Pink), 0.62 (Brown), 0.57 (Blue), 0.52 (Violet), 0.44 (Grey), 0.39, 0.31 (Blue), 0.25 (Violet), 0.17 and 0.14 (Blue). Dipped the plate in vanillin-sulphuric acid reagent followed by heating at 110° about 5 min and observed under visible light, the plate shown major 7 spots at R_f 0.82, 0.71 (Grey), 0.67, 0.56 (Violet), 0.27 (Yellow), 0.22 and 0.13 (Grey). HPTLC Fingerprinting findings shown in (Table - 4, Figures -2 a, b, c, d, e and f)

Applied the alcohol extracts on TLC plate. Developed the plate used Toluene: Ethyl acetate (8 : 2) as mobile phase. After developments allowed the plate to dried in air and examined under UV (254nm), it shown major 9 spots at R_f 0.80 (Dark green), 0.71 (Green), 0.55, 0.48, 0.39, 0.35 (Dark green), 0.22 (Green), 0.16 and 0.12 (Dark green). Under UV (366nm), it shown 15 major spots at R_f 0.85, 0.82 (Brown), 0.77 (Violet), 0.70 (Dark orange Red), 0.66 (Brown), 0.64 (Violet), 0.62 (Blue), 0.59 (Orange Red), 0.57 (Violet), 0.49 (Brown), 0.45 (Violet), 0.36, 0.27 (Blue), 0.20 (Violet) and 0.12 (Brown). Dip the plate in vanillin-sulphuric acid reagent followed by heating at 110° about 5 min and observed under visible light, the plate shown 7 major spots at R_f 0.80 (Orang Red), 0.70 (Grey), 0.62 (Violet), 0.65, 0.46 (Grey), 0.35 (Yellow) and 0.16 (Greenish blue). HPTLC Fingerprinting findings shown in (Table- 5, Figures - 3 a, b, c, d, e and f)

In polyherbal HEP compound formulated drugs, separations: Bioactive secondary metabolites, phytochemical constituents of polyphenolic acids, and flavonoids compounds have been shown in HPTLC separated spots chromatograms appearance, particularly in the near 254nm UV region to 360nm UV region, showing weak blue fluorescence color. When using ferric chloride and anisaldehyde as common derivatizing reagents, blue and violet brown separated color spots are shown for polyphenolic acids compounds separation. In particular, in the near 256nm UV region to 374nm UV region, yellow-green-blue fluorescence color is shown, and when using natural (UV) or AlCl3 as common derivatizing reagents, yellow-green fluorescence separated color spots are shown for flavonoids compounds separation.

Comparative analysis with established reference standards facilitated the tentative identification of key marker compounds in Ayurveda, Siddha, and Unani (ASU) medicine. The chemical nature of these bioactive phytochemical constituents was inferred based on Rf values, spectral characteristics, and prior literature reports. Shown in separated Table 4 and 5, Figures 2a and 3a, figures and Table 4, Figures 2a, b, c, d, e, and f. The presence of flavonoids, alkaloids, phenolic acids, and triterpenoids compounds suggests the potential therapeutic efficacy of ASU drug samples in traditional medicine, supporting its pharmacological applications in Ayurveda, Siddha, and Unani (ASU) medicine.

- This study of HEP polyherbal drugs provides data that will improve drug standardization research, development of pharmacopeial monographs, novel drug discovery and development, guide the sustainable propagation, and cultivation of new crops with rich therapeutic effects for advanced research purposes.

- Based on previous research, further exploration of necessary additional research is required. This includes in-vivo studies, clinical trials, secondary metabolites, and active phytochemical constituents found in various parts of the HEP polyherbal drug, such as aerial flowers, leaves, branches, roots, stems, and bark parts. This research expands the current understanding of the mechanisms of action that underlie the observed bioactivities from a human health and wellness perspective.

- Exploring advanced research from the perspective of drug standardization involves investigating and evaluating research data related to the exploration of important plant parts. This requires the use of sophisticated instruments such as GC-MS, LC-MS, Proton NMR, C13 NMR, FT-IR, XRD, and SEM-EDX to properly profile the structural and functional groups, as well as identify and confirm novel bioactive phytochemical constituents and compounds.

- Future studies should explore these HEP polyherbal drugs to uncover potential bioactive compounds for novel drug development and health benefits.

CONCLUSION

Standardization is an essential part of evaluating and validating scientific standards to ensure the quality of polyherbal formulations. To maintain batch-to-batch uniformity, consistency, and quality of the drug, each plant material used in the preparation of 'Habb-e-Pachlona' was identified and evaluated for their Pharmacopoeial standards. The HPTLC fingerprint profile of chloroform and alcohol extracts provided a suitable method for monitoring the identity, purity, and standardization of the drug. It explored and emphasized the presence and confirmation of active phytochemical constituents and secondary metabolites such as alkaloids, triterpenoids, glycosides, flavonoids, carbohydrates, polyphenols, proteins, amino acids, and tannins. These constituents were separated out in various UV-254nm, UV-366nm, and visible regions, showing colored bands and spots. In the research studies conducted, various quality standard parameters, including quality, safety, and toxicity activities such as heavy metals, aflatoxins, pesticide residues, and microbial load, were found to be within the permissible limits of AYUSH/WHO standard guidelines. Physico-chemical, HPTLC fingerprinting, and WHO parameters were analyzed and can be used as reference standards for the drug HEP. It can be concluded from these studies that the formulated HEP is safe, free from any toxic or hazardous substances, and is an economical

drug. The efficacy of the drug can be utilized as a traditional alternative medicine, supported by clinical studies conducted on patients suffering from poor digestion, gastric debility, dyspepsia, anorexia, flatulence, and hiccups. The classical Unani formulated HEP drug has been used since ancient times, as mentioned in classical Unani texts and NFUM Pharmacopeial literature. This information can be helpful as a reference for the development of Pharmacopoeial standard monographs, DSR, QC, QA, and PV aspects. Further advanced research studies on the isolation and characterization, structural detection using GC-MS, LC-MS, XRD, SEM-EDX, and other sophisticated instrumental techniques of these investigated drugs can still be carried out for the purpose of exact confirmation, advanced research investigations into isolated novel bioactive phytochemicals, constituents, secondary metabolites compounds, and novel drug discovery confirmation. Studies on the classical Unani formulated drug HEP can also be conducted to discover potential bioactive compounds that can be explored for the development of novel drugs and health benefits.

Limitations and Future Remarks of the Study

The present study's Drug Standardization, Physicochemical, HPTLC Finger printing profiling Toxicity profiles show the reconfirmation and presence of DSR, QC, QA of Unani classical formulation HEP. In the future, investigated data may be used in to incorporate of Drug Standardization Research, Pharmacopeial monographs development profiling and confirm these investigated resulted data.

Ethical approval

As the work is purely an in-vitro study, ethical clearance is not required.

Author contributions

Dr PKS - Work designed, carried out Instrumental, Chemistry part research data interpretation works and Manuscript written and supervised. Dr K A H - Unani expert, and revised manuscript review. Mr S K, Dr ASK and Dr RPM - Carried out Chemistry, HPTLC Fingerprinting, Toxicity Microbiological and Analytical data analysis studies designed and revised manuscript review research work.

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Conflict of interest

The authors declared no conflict of interest.

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