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PHARMACOGNOSTICAL AND HPTLC STUDIES OF HABB-E-HINDI SUAL – A TRADITIONAL UNANI FORMULATION

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ABSTRACT
People all over the world are accepting traditional medicines as alternative treatment for chronic and common ailments. The most prominent advantage of these medicines is that they are effective with lesser side effects. Some of the countries, which were reluctant to use them because of their quality concerns, are now showing tremendous interest in their therapeutic values. As per WHO estimate, 80 % population of some Asian and African countries uses herbal medicines for their primary healthcare needs. In India alone more than 65% rural population relies on traditional system of medicine including Unani system of medicine. This rapidly growing people's faith in Unani medicines leads to the necessity of their quality check. It has been found on few occasions that these medicines are prepared in the market with spurious or low quality ingredients. To maintain the quality of the Unani medicines, their standardization is imperative. Habb-e-Hindi Sual is an important herbo-mineral Unani formulation which is used in the treatment of Shaheeqa (Pertussis) & Sual (Cough). The formulation was standardized by adopting standard procedures for physico-chemical, pharmacognostical and HPTLC studies. The other parameters like microbial load, heavy metals, aflatoxins and pesticide residues were also analyzed to ascertain the quality of the medicine. The data produced can be productive for laying down the pharmacopoeial standards and HPTLC fingerprints of Habb-e-Hindi Sual.

KEYWORDS: Microscopy, HPTLC, Aflatoxins, Microbial load, Heavy Metals.

INTRODUCTION
Pertussis (Shaheeqa), also known as whooping cough, is a highly contagious disease of the respiratory tract caused by bacteria *Bordetella pertussis* which lives in the mouth, nose and throat. In 2015, the World Health Organization reported 142,512 pertussis cases globally and estimated that there were 89,000 deaths. However, a recent publication modeling pertussis cases and deaths estimates that there were 24.1 million pertussis cases and 160,700 deaths in children younger than 5 years in 2014 worldwide.[1] Habb-e-Hindi Sual, a herbo-mineral Unani formulation, is very useful in the ailment of Shaheeqa (Pertussis). The drug is categorized under Huboob in the National Formulary of Unani Medicine Part I.[2] Habb-e-Hindi Sual is reputed for its expectorant (Munaffis-e-Balgham) and cough relieving (Musakkin-e-Sual) actions.[3]

The current study is aimed to evaluate pharmacopoeial and HPTLC data of Habb-e-Hindi Sual. The formulation was prepared according to the formula described in NFUM-I [Table I]. In order to lay down SOPs and pharmacopoeial standards, the drug was prepared on laboratory scale at DSRI, Ghaziabad. The present paper describes the salient features of preparation, microscopical characters, physico-chemical parameters, HPTLC, heavy metals estimation, aflatoxins and pesticide estimation not reported so far.

MATERIALS AND METHODS
Ingredients authentications
All the ingredients were procured from local raw drug dealer and were identified botanically using pharmacognostical methods.[4-5] The ingredients were further validated by comparing with a monograph available in UPI part II, Vol. III.[6]

Preparation of formulation
All the ingredients were taken of pharmacopoeial quality. The ingredients were cleaned and dried under shade to remove the moisture if any. The ingredients (S. No. 1-4, Table I) were crushed separately in an iron-mortar to obtain their coarse powders. The coarse powders were further ground in a mixer grinder to get

their fine forms. The fine powders were mixed together thoroughly and sieved through mesh No. 100. The ingredient no.5 was then added and the content was mixed again thoroughly. Tablets (Huboob) was prepared by mechanical process and dried under shade. The prepared tablets were stored in tightly closed glass container free from moisture and kept in a cool and dry place.

Table 1: Formulation Composition of Habb-e-Hindi Sual.

S. No.	Unani Name	Botanical/ English Name	Part used	Qty.
1.	Anardana	*Punica granatum* L.	Seed	120g
2.	Filfil Daraz	*Piper longum* L.	Fruit	60g
3.	Filfil Siyah	*Piper nigrum* L.	Fruit	30g
4.	Jawa Khar	Pearl ash	As such	15g
5.	Qand Siyah Kohna	*Saccharum officinarum* L.	Jaggery	240g

Microscopy

5g of the powdered drug was taken and stirred gently with hot water in a beaker. The mixture was centrifuged and the supernatant was discarded. The sediment was washed several times with distilled water, centrifuged again and the supernatant was decanted every time. Small quantity of the sediment was stained with iodine solution and mounted in 50% glycerin. Another small quantity was cleared with chloral hydrate solution, washed with distilled water and mounted in 50% glycerin. Various characters were observed in different mounts.[5,7]

Physico-chemical analysis

The physico-chemical parameters such as moisture content, water and ethanol extractive values, ash values, pH values were analyzed by standard methods.[8-9]

HPTLC Profile

Sample preparation

Extractions of the formulation were carried out with both polar and non-polar solvents i.e. Ethanol and Chloroform. Formulation samples (2g each) were sonicated with 20ml each of Ethanol and Chloroform separately for about 20 minutes and filtered through Whatman no.1 filter paper. The extracts were concentrated up to 10 ml under vacuum at 50^0C and used for HPTLC fingerprinting.

HPTLC-UV absorption method

HPTLC fingerprinting was performed on 10 × 10cm aluminum TLC plate pre-coated with 0.25 µm thin layer of silica gel 60 F_{254} (E. Merck). 10 µl of each extract was applied on aluminium TLC plate as 8mm band using CAMAG Linomat 5 automatic sample applicator. The plate was developed up to a distance of 9 cm in a twin trough glass chamber (10x10cm) using the solvent Toluene: Ethyl acetate: Formic acid (9: 1: 0.5) as mobile phase. The plate was dried at room temperature and visualized under UV 254nm & UV 366nm wavelengths. Later the plate was derivatized by dipping it in 1% Vanillin-Sulphuric acid reagent followed by heating at 105°C till the coloured bands appeared [10-11].

Estimation of microbial load

Microbial growth is common occurrence if any step during processing, preparation or storage of herbal medicines is compromised. Its determination signifies whether the drug contains disease causing and spoilage micro-organisms in permissible limits. The spoilage microorganisms are perilous to human health and hence must be checked before consumption of herbal medicines. The estimation of microbial load viz. Total aerobic bacterial count (TABC) and Total yeast and molds count (TYMC) were carried out as per standard methods.[12] Detection of Enterobacteriaceae members viz. *Escherichia coli*, *Salmonella sp.*, *Shigella sp.*, *Klebsiella sp.* and detection of specific objectionable pathogens *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* were all executed as per standard methods.[12]

Estimation of heavy metals

Heavy metals have relatively high densities and high atomic weights. They have toxic effects on human body if consumed even in low concentrations. Plant material sometimes may have metal contaminations depending upon the source of collection. Thus heavy metals determination is essential for meticulous assessment of quality of herbal material. The analysis of heavy metals lead, cadmium, mercury and arsenic was carried out as per standard methods.[13]

Details of the Instrument and Operating parameters

Atomic Absorption Spectrophotometer (AAS) model LABINDIA AA7000 was used for heavy metals analysis. The operating parameters:

Lead and Cadmium: Instrument technique - Flame atomization; wavelength (Lead) - 217 nm; wavelength (Cadmium) - 228.8 nm; slit width - 0. 5 mm; lamp current (Pb) - 4.0 mA; lamp current (Cd) - 3.0 mA; carrier gas and flow rate - air and acetylene, 1.1 L/min; sample flow rate - 2 ml/min. **Mercury:** Instrument technique - Cold vapour technique; wavelength - 253.7 nm; slit width - 0. 5 mm; lamp current - 3.0 mA; carrier gas and flow rate - argon, 1.1 L/min; sample flow rate - 5ml/min. **Arsenic:** Instrument technique - Cold vapour technique; wavelength - 193.7 nm; slit width - 0. 5 mm; lamp current - 6.0 mA; carrier gas and flow rate - acetylene, argon, 1.1 L/min; sample flow rate - 5ml/min. The hallow cathode lamps for Pb, Cd, Hg and As analysis were used as light source to provide specific wavelength for the elements to be detected.

Analysis of aflatoxins

Aflatoxins are mycotoxins produced by certain molds. They are highly poisonous carcinogens. Several kinds of aflatoxin occur in nature, but four aflatoxins B1, B2, G1 and G2 are particularly dangerous to humans and animals. Thus B_1, B_2, G_1 and G_2 were analyzed as per Official Analytical Methods of the American Spice Trade Association (ASTA).[14]

Details of Instrument and Operating parameters

High Performance Liquid Chromatography (Thermo Fisher) was used for the analysis of aflatoxins. **Column** - Ultra C18, 250 X 4.6 mm, 5 µm particles; **Mobile phase:** Water: Acetonitrile: Methanol (65: 22.5: 22.5); F**low rate:** 1 ml/min; **Temperature:** 35º C; **Detector:** Fluorescence detector at 360 nm; **Injection:** 20 µl (Aflatoxins mixture and sample)

Analysis of pesticide residue

The herbal material may sometimes contain high levels of pesticide residues. However, they tend to decline as the pesticide breaks down over the course of time. In addition, as the herbal ingredients are usually washed and processed before use, the residues often recede further. The analysis of pesticide residues was carried out as per AOAC, 2005[13] by employing Gas Chromatography-Mass Spectrometry (GC-MS) (Instrument-Agilent, detector-mass selective detector, column specification-DB5MS, carrier gas - helium, flow rate - 1ml/min, column length - 30 m, internal diameter - 0.25 mm, column thickness - 0.25 µm).

RESULTS AND DISCUSSION

Habb-e-Hindi Sual is blackish brown pill having soft texture, characteristic odour and sweetish-bitter taste. (Fig. I)

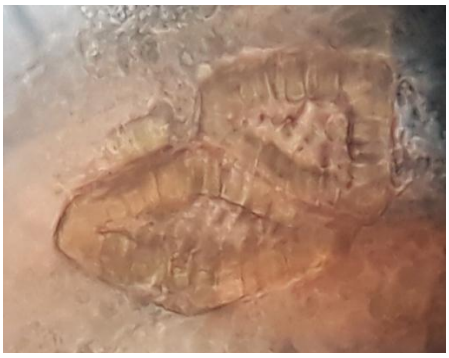
Fig. I: Habb-e-Hindi Sual.

Identification

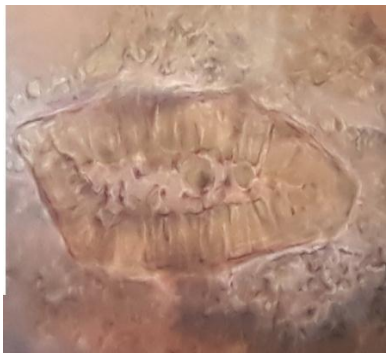
Microscopy

Following characters were observed under microscope

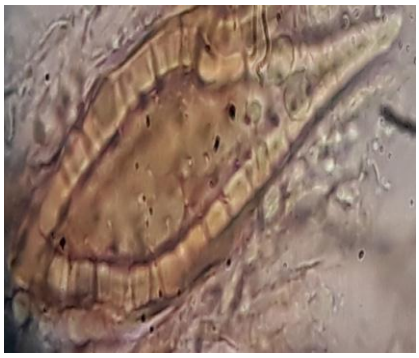
Slightly elongated oil filled cells, large endosperm cells with thick wall, spindle shaped stone cells stone cells interspread, perisperm cells filled with starch granules **(Filfil siyah)**. Elongated parenchyma cell in surface view, capsuled cells with oil droplets. spindle shaped stone cells with braod lumen (**Filfil daraz)**. Thin walled endopsermic cells, oil globules, oxallate crystals **(Anardana).** (Fig.II)



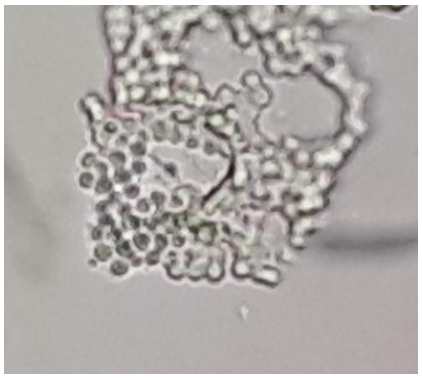
Isodimetric sclereids (Filfil Siyah)



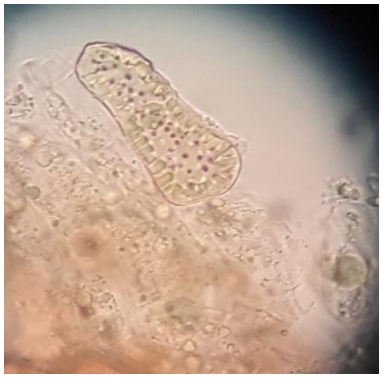
Elongated sclereids (Filfil Siyah)



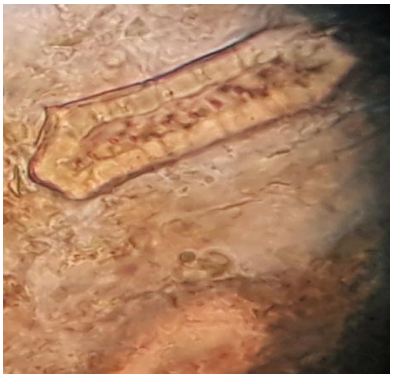
Beaker shaped stone cell (Filfil Siyah)



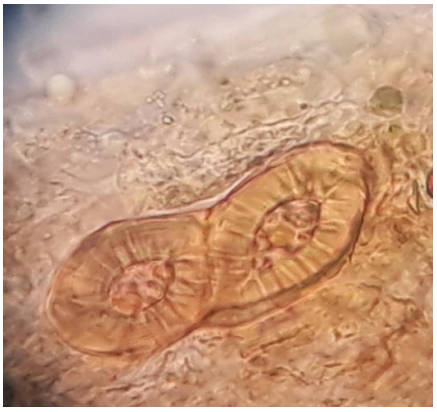
Starch grains (Filfil Siyah)



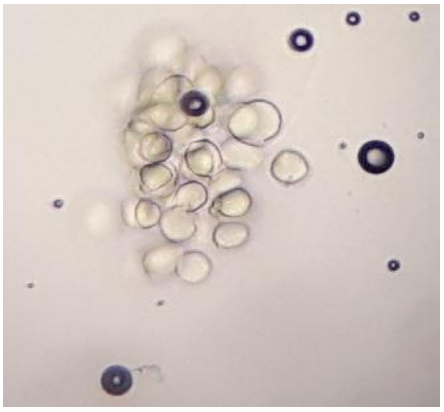
Stone cells with druse crystal and (Filfil Siyah)



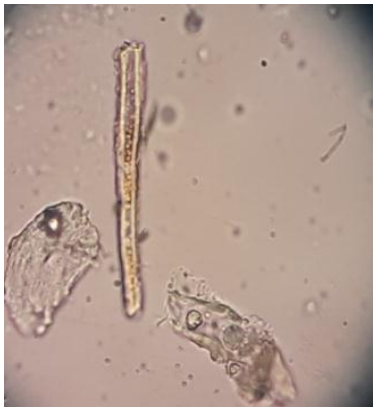
Brachysclereids (Filfil Daraz)



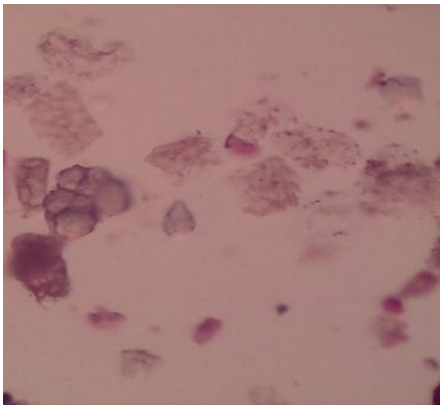
Jointed oval stone cells (Filfil Daraz)



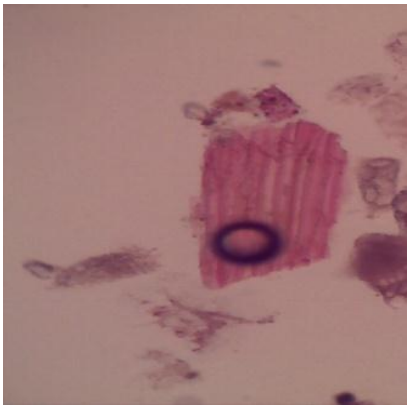
Aleuron grains (Fifil Daraz)



Oil globules and prismatic crystal (Filfil Daraz)

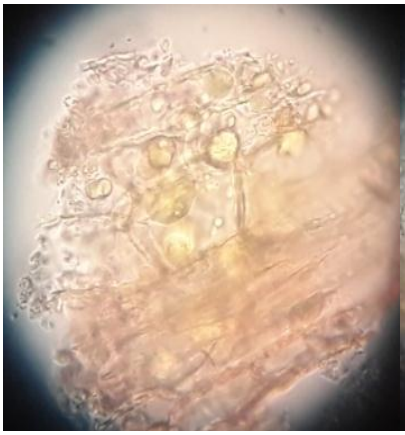


Calcium oxalate crystal (Filfil Daraz)

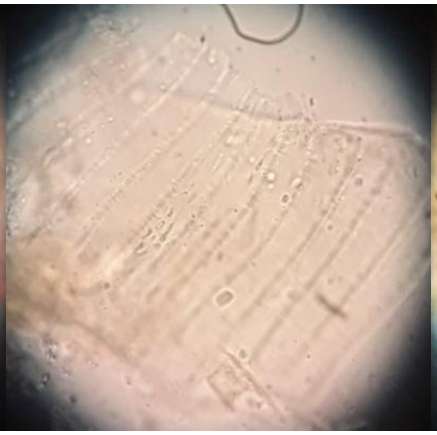


Parenchyma cells (Filfil Daraz)

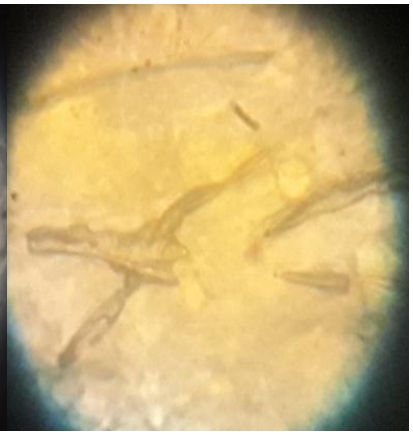
Fiber cell (Anardana)



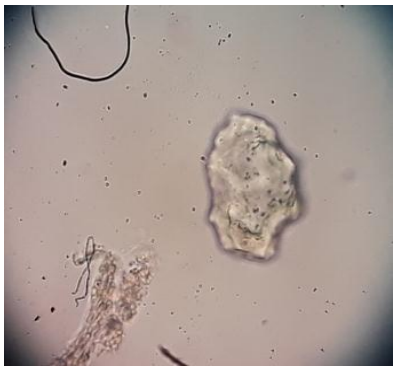
Oil globules (Anardana)



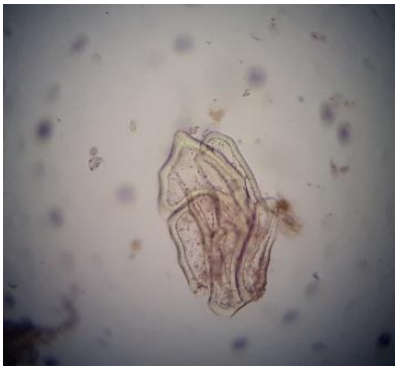
Testa cells (Anardana)



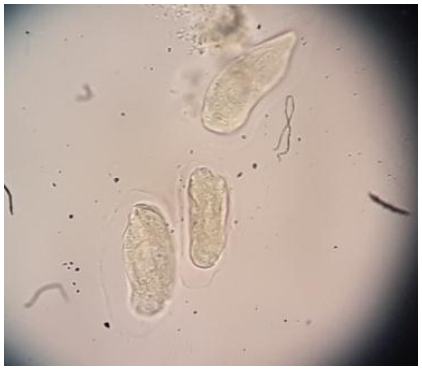
Raphide crystals (Anardana)



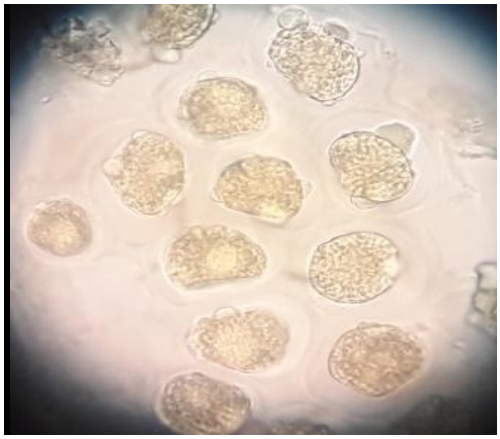
Prismatic crystal (Anardana)



Complex sclereids (Anardana)



Endospermic cells (Anardana)



Aleuron grains (Anardana)
Fig. II: Microscopy of Habb-e-Hindi Sual.

Physico-chemical parameters
The Physico-chemical data of the Habb-e-Hindi Sual (three batches) are shown in Table 2. The significantly high value of water soluble extractive (49.22 - 50.46 %) was due to the presence of Jawakhar & Qand Siyah Kohna as both the ingredients are highly soluble in water. The data with respect to the loss in weight on drying at 105^0C revealed that the moisture content of the drug is quite low. The low values of total ash (5.65 - 6.10 %) and acid insoluble ash (1.26 - 1.58 %) indicate that siliceous matter was present in the drug in negligible amount.

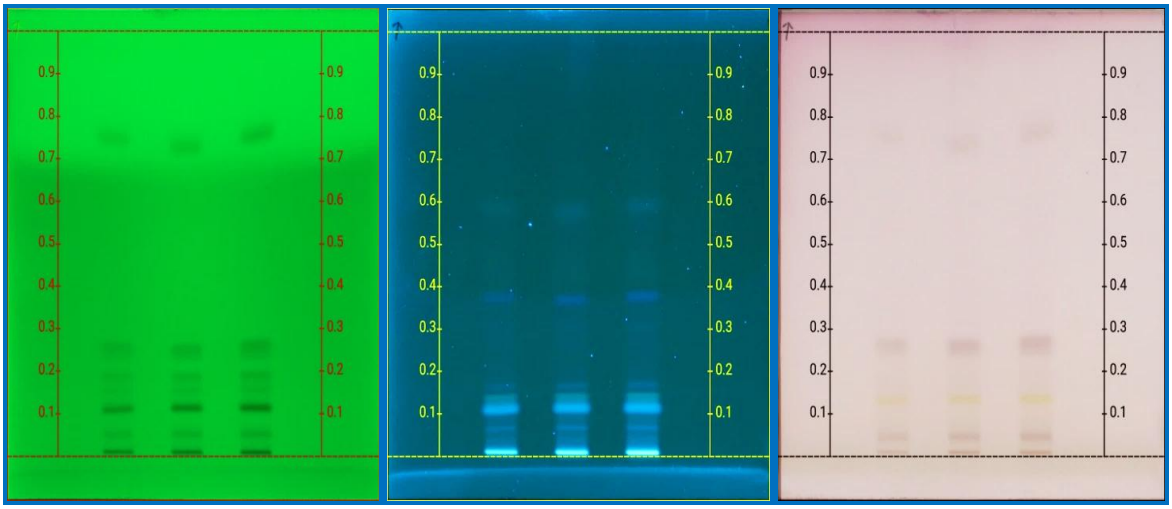
Quality control parameters
Heavy metals analysis
The results of heavy metals estimation are given in Table 3. The heavy metal content in Habb-e-Hindi Sual was found to be below detection limit which indicated that the drug was free from heavy metal contamination.

Microbial load
Determination of microbial load in traditional medicines is decided by various factors such as their origin, harvesting/ collecting methods of raw drugs, preparation procedures, packaging and storage conditions. The assessment of microbial load is done for evaluating the total aerobic bacterial count (TABC), total yeast and molds count (TYMC), count of bacteria belonging to the *Enterobacteriaceae* family and count of specific objectionable pathogens. The results of microbial load are shown in Table 4 which indicates that the drug is safe for internal use.

Aflatoxins
Aflatoxins are toxic secondary metabolites produced by a variety of fungi such as *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius*. They widely exist in nature especially in warm and humid regions and sometimes contaminate crops in the field, during harvest or processing. Their detection and control is vital to ensure the safety of consumers' health. The results of aflatoxins determination in Habb-e-Hindi Sual are given in Table 5. The results do not show any presence of the aflatoxin contents (B1, B2, G1 and G2) in the drug.

Pesticide residues
Pesticides are potentially toxic to human beings and may cause acute poisoning or long-term health effects. Their estimation in traditional medicines is essential to reduce the hazardous effects on the users. In order to estimate the pesticide residue, the drug was analyzed on GC-MS/MS. The results indicated that the drug is free from pesticide residues and safe to use. The results of pesticide residues are given in table 6.

HPTLC analysis
The HPTLC (High Performance Thin Layer Chromatography) is an advanced and convenient analytical technique to obtain reliable data for phytochemical and biomedical analysis. The HPTLC fingerprinting of both the extracts of Habb-e-Hindi Sual (three batches) were observed under UV 254nm, UV 366nm and under white light after derivatization. All the batches of Habb-e-Hindi Sual show similar colourful bands with similar R_f values. Moreover, their densitograms are almost superimposed on each other. This shows batch-to-batch consistency of the formulation. (**Fig. III a-n**).



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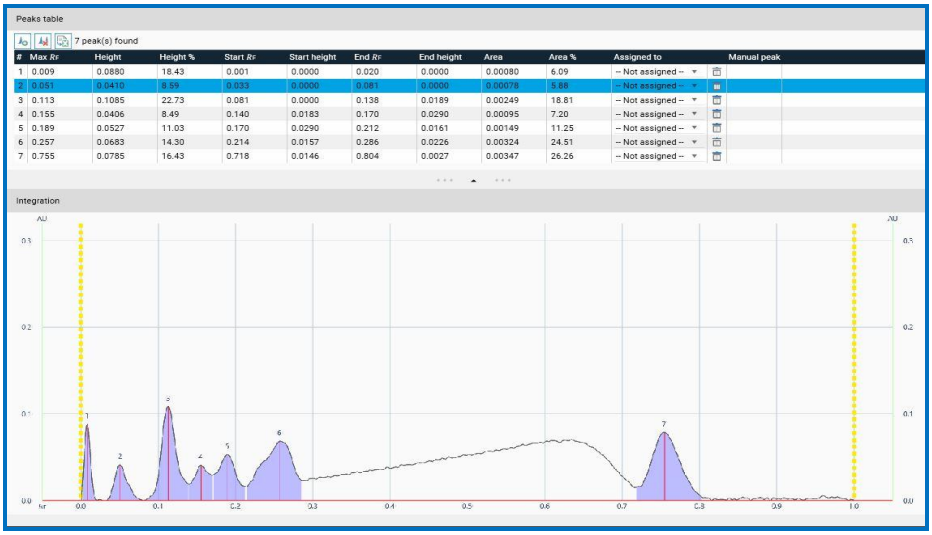
UV254 UV366 Visible light (After derivatization)

Fig. III (a): HPTLC images of chloroform extract.



#	Max Rf	Height	Height %	Start Rf	Start height	End Rf	End height	Area	Area %	Assigned to	Manual peak
1	0.009	0.0880	18.43	0.001	0.0000	0.020	0.0000	0.00080	6.09	-- Not assigned --	
2	0.051	0.0410	8.59	0.033	0.0000	0.081	0.0000	0.00078	5.88	-- Not assigned --	
3	0.113	0.1085	22.73	0.081	0.0000	0.138	0.0189	0.00249	18.81	-- Not assigned --	
4	0.155	0.0406	8.49	0.140	0.0183	0.170	0.0290	0.00095	7.20	-- Not assigned --	
5	0.189	0.0527	11.03	0.170	0.0290	0.212	0.0161	0.00149	11.25	-- Not assigned --	
6	0.257	0.0683	14.30	0.214	0.0157	0.286	0.0226	0.00324	24.51	-- Not assigned --	
7	0.755	0.0785	16.43	0.718	0.0146	0.804	0.0027	0.00347	26.26	-- Not assigned --	

Fig. III (b): HPTLC fingerprint profile of chloroform extracts at 254 nm.

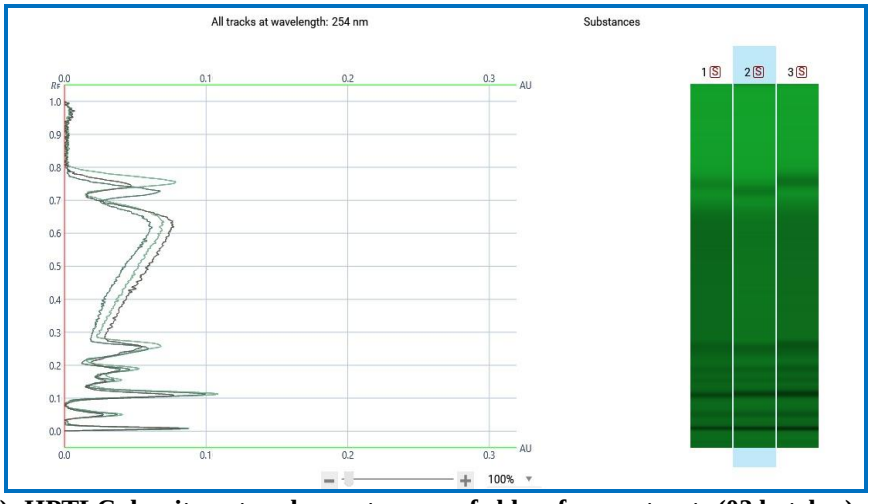


Fig. III (c): HPTLC densitometry chromatogram of chloroform extracts (03 batches) at 254 nm.

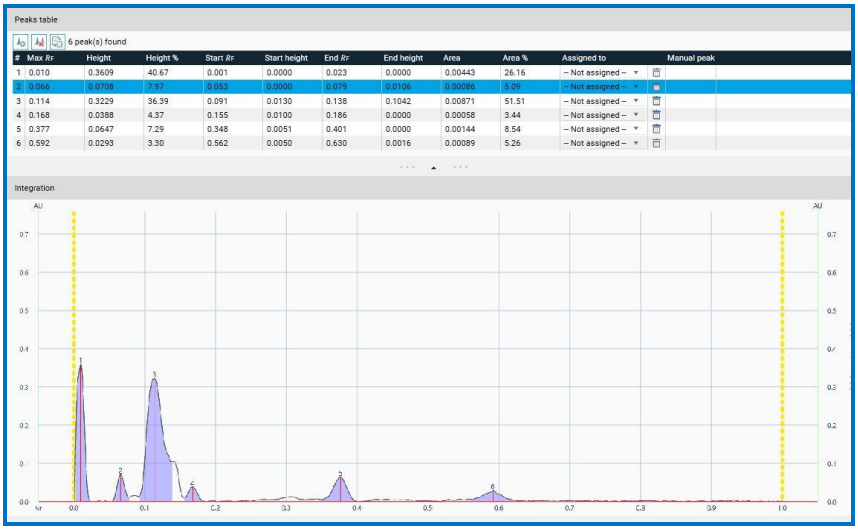


Fig. III (d): HPTLC fingerprint profile of chloroform extracts at 366 nm.

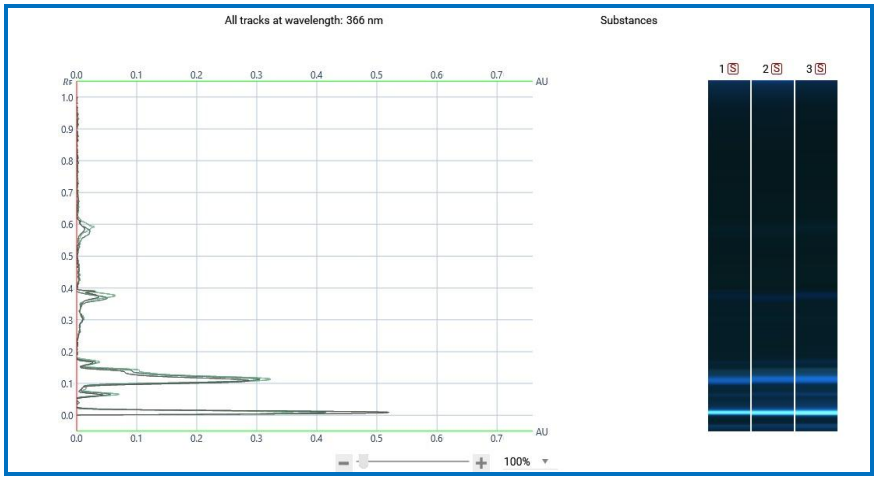


Fig. III (e) HPTLC densitometry chromatogram of chloroform extracts (03 batches) at 366 nm.

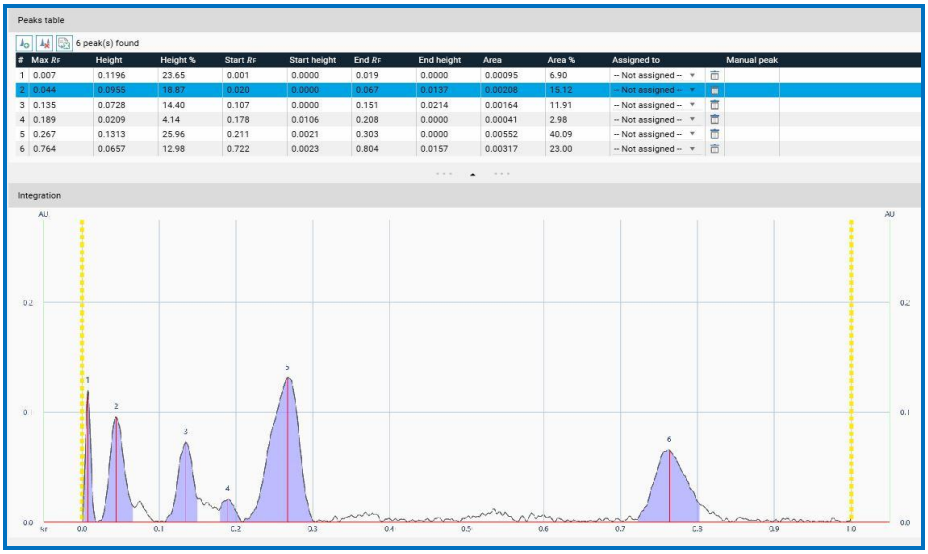


Fig. III (f) HPTLC fingerprint profile of chloroform extract under white light after derivatization.

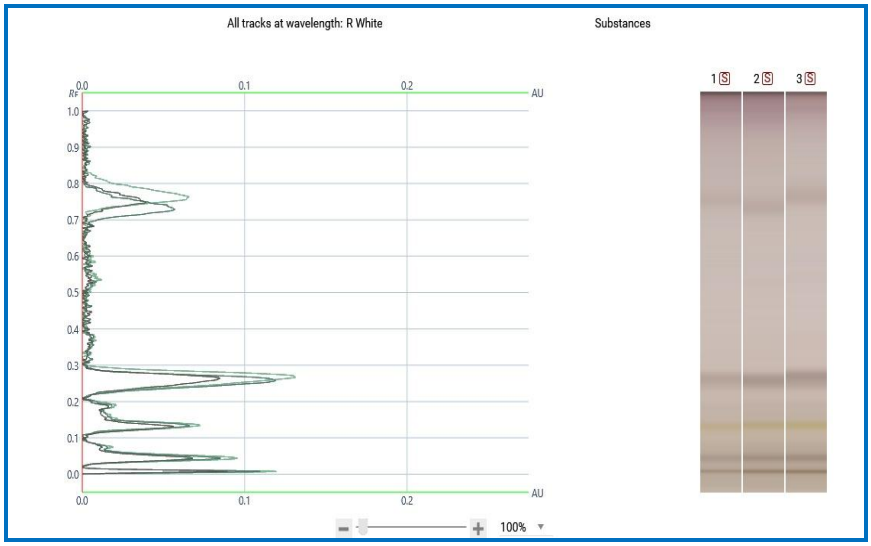
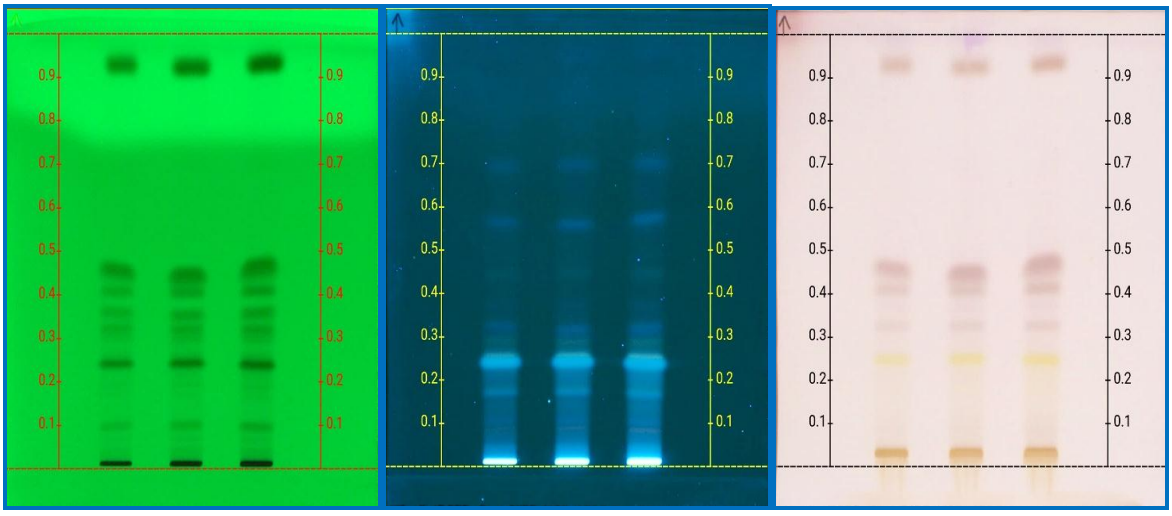


Fig. III (g): HPTLC fingerprint profile of chloroform extracts under white light after derivatization.



I II III **I II III** **I II III**

UV254 **UV366** **Visible light (After derivatization)**

Fig. III (h): HPTLC images of ethanol extract.

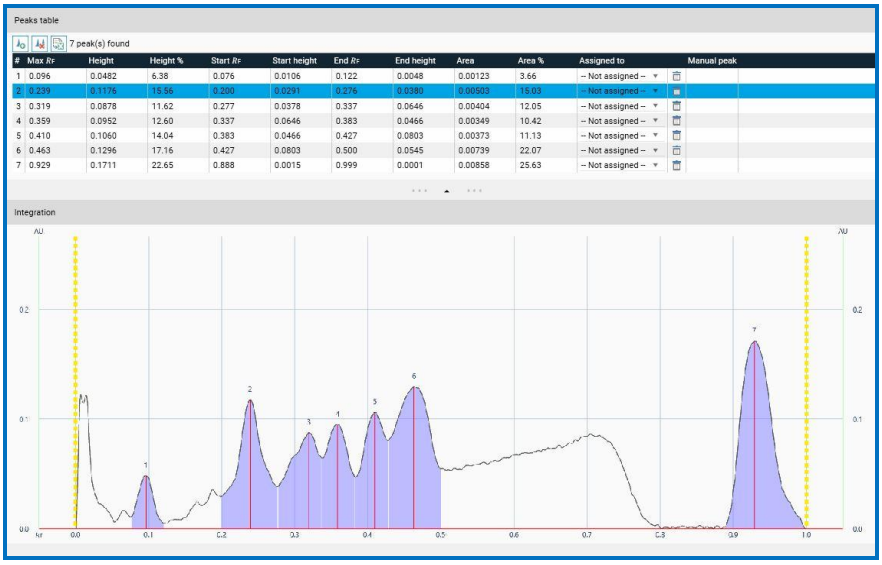


Fig. III (i): HPTLC fingerprint profile of ethanol extract at 254 nm.

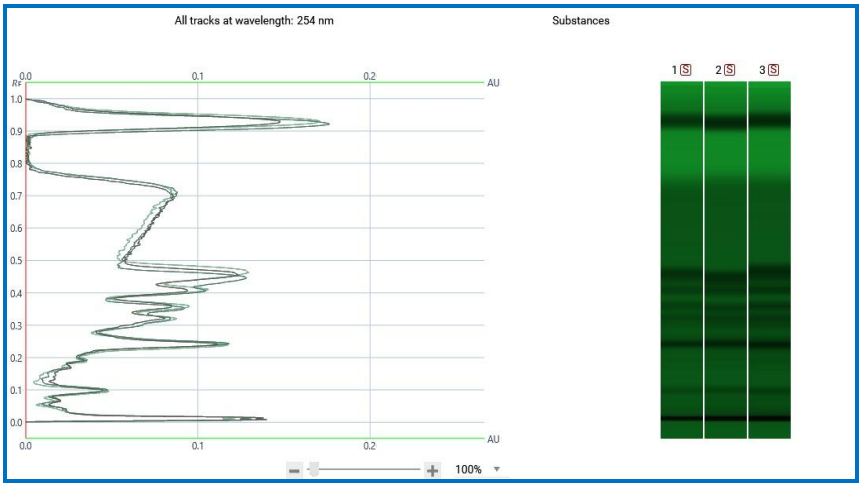


Fig. III (j): HPTLC densitometry chromatogram of chloroform extracts (03 batches) at 254 nm.

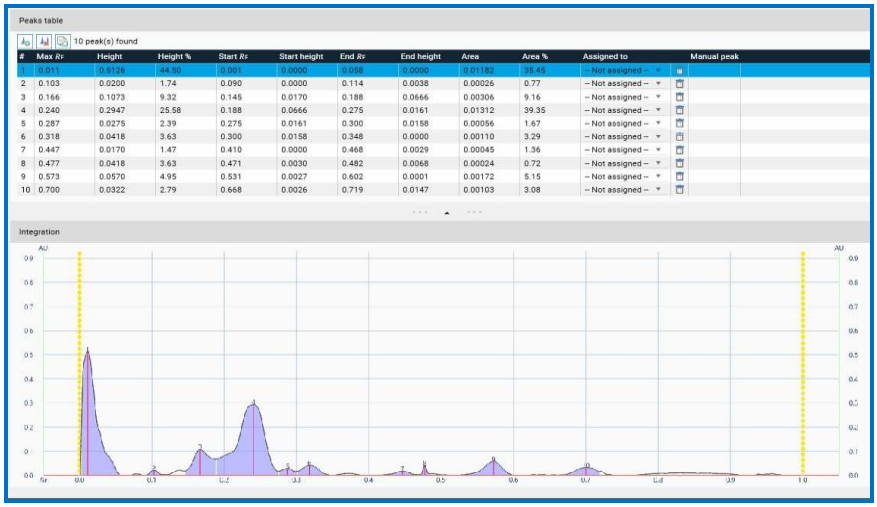


Fig. III (k): HPTLC fingerprint profile of ethanol extract at 366 nm.

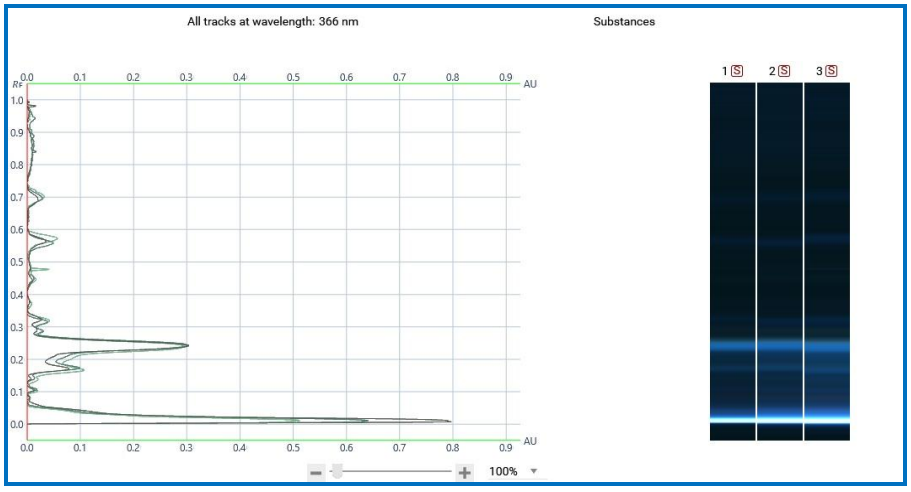


Fig. III (l) HPTLC densitometry chromatogram of ethanol extracts (03 batches) at 366 nm.

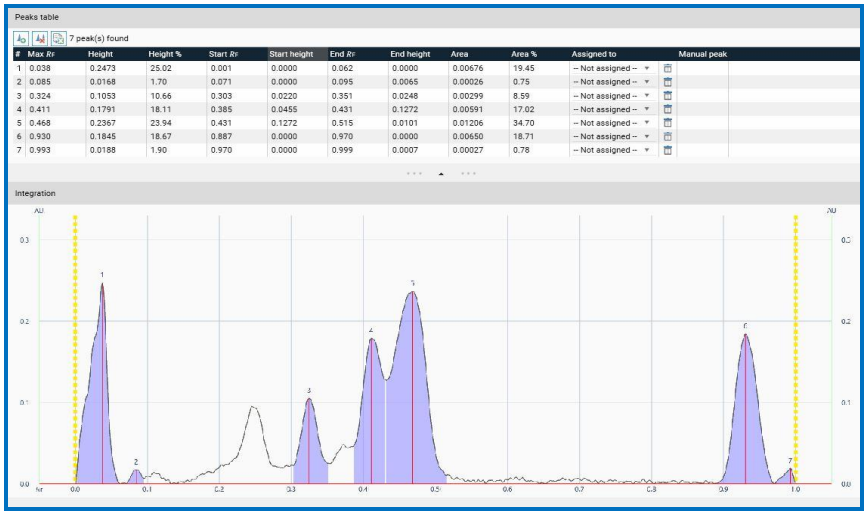


Fig. III (m): HPTLC fingerprint profile of ethanol extract under white light after derivatization.

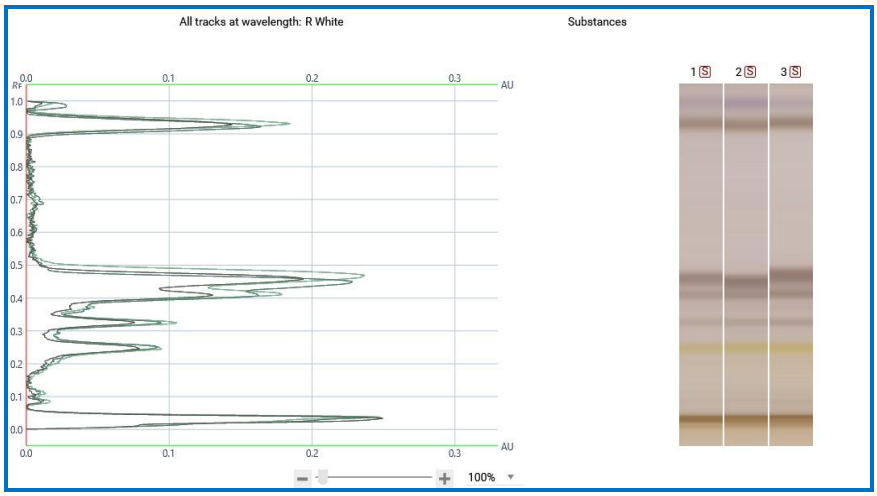


Fig. III (n): HPTLC densitometry chromatogram of ethanol extracts (03 batches) under white light after derivatization.

Table 2: Physico - Chemical parameters.

Parameters	Results
Ethanol extractive matter (w/w %)	21.25 - 22.55
Water extractive matter (w/w %)	49.22 - 50.46
Loss in weight on drying at 105^0C (w/w %)	8.20 - 8.58
Total ash (w/w %)	5.87 - 6.10
Acid insoluble ash (w/w %)	1.26 - 1.58
pH of 1% aqueous solution	4.42 - 4.90
pH of 10% aqueous solution	4.06 - 4.34
Volatile oil (v/w %)	0.92 – 1.14

Table 3: Analysis of heavy metals.

Sl. 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: Microbial load.

S. No.	Parameters	Results	WHO Permissible Limits
1.	Total aerobic bacterial Count (TABC)	6×10^2 cfu/g	10^5 cfu/g
2.	Total yeast and molds count (TYMC)	< 10 cfu/g	10^3 cfu/g
	Enterobacteriaceae members		
3.	*Escherichia coli*	Absent	NIL
4.	*Salmonella sp.*	Absent	NIL
5.	*Shigella sp.*	Absent	NIL
6.	*Klebsiella sp.*	Absent	NIL
	Specific objectionable pathogens		
7.	*Pseudomonas aeruginosa*	Absent	NIL
8.	*Staphylococcus aureus*	Absent	NIL
9.	*Candida albicans*	Absent	NIL

Table 5: Aflatoxin level.

S. No.	Parameter analyzed	Results	Detection limits
1.	B-1	Not detected	0.50 ppm
2.	B-2	Not detected	0.10 ppm
3.	G-1	Not detected	0.50 ppm
4.	G-2	Not detected	0.10 ppm

Table 6: Pesticide residues.

S. No.	Parameter analyzed	Results
1	Alachlor	< LOQ
2	Aldrin (Aldrin and dieldrin combined expressed as dieldrin)	
3	Azinophos-methyl	
4	Bromopropylate	
5	Chlordane (cis, tans and oxychlordane)	
6	Chlorfenvinphos	
7	Chlorpyrifos	
8	Chlorpyrifos-methyl	
9	Cypermethrin (and isomers)	
10	DDT (all isomers, sum of p, p'-TDE (DDD) expressed as DDT)	
11	Deltamethrin	
12	Diazion	
13	Dichlorvos	
14	Dithiocarbamates (as CS2)	
15	Endosulphan (sum of isomers & Endosulphan sulphate)	
16	Endrin	
17	Ethion	
18	Fenitrothion	
19	Fenvalerate	
20	Fonofos	
21	Heptachlor (sum of Heptachlor & Heptachlor epoxide)	
22	Hexachlorobenzene	
23	Hexachlorocyclohexane isomer (other than γ)	
24	Lindane (γ – Hexachlorocyclohexane)	
25	Malathion	
26	Methidathion	
27	Parathion	
28	Parathion methyl	
29	Permethrin	
30	Phosalone	
31	Piperonyl butoxide	
32	Pirimiphos methyl	
33	Pyrethrins (sum of isomers)	
34	Quintozen (sum of Quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	

CONCLUSION

It can be concluded that organoleptic parameters are not much reliable in identification of herbal drugs once the ingredients are powdered and mixed together for preparing compound formulation. The present study, therefore, holds high significance as the microscopic features, various Physico-chemical parameters, HPTLC profile and quality control parameters provide criteria for easy identification of the drug Habb-e-Hindi Sual and ensure the authenticity, quality and efficacy of the medicine.

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