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PHARMACOGNOSTICAL EVALUATION AND HPTLC FINGERPRINTING OF CLASSICAL UNANI FORMULATION: ITRIFAL- KISHNEEZI

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ABSTRACT

The Unani system of medicine has a long history of usage for the treatment of various diseases. In last two decades, a rapid growth has been observed in the production of Unani medicines. During & after the COVID outbreak, people realised significance of herbal medicines especially for boosting their immune system. This rise has posed novel challenges for ensuring the safe consumption of Unani medicines. Hence, adoption of judicious methodology is crucial to evaluate the quality of herbal medicines in order to maintain their efficacy. The Unani poly-herbal formulation; Itrifal- Kishneezi, widely used as carminative, stomachic and laxative, was studied through various standardization parameters. Several phytochemical test such as organoleptic evaluations (colour, odour and taste), microscopy, physico- chemical evaluations (moisture content, total ash, acid insoluble ash, pH values, water and ethanol soluble extractives, sugar content) have been carried out for its elementary assessment. Modern analytical technique HPTLC fingerprinting was also employed to achieve more authentic results. The determination of contaminants such as heavy metals, aflatoxins, pesticide residue and microbial load were also carried out to check the presence of any hazardous substance in the formulation. The data produced in this study will lead to develop pharmacopeial standards of Itrifal- Kishneezi which eventually helps to estimate the quality, safety and efficacy of the formulation.

KEYWORDS: Microscopy, physico-chemical analysis, pharmacopeial standards, HPTLC fingerprinting.

INTRODUCTION

Traditional medicines play a very important role in human life for maintaining wholesome health. Since the side effects of modern medicines are well-known, the use of traditional medicines has increased manifolds in last 2-3 decades due to people's presumption about their lesser side effects. The World Health Organization (WHO) also supports the traditional medicines and encourages the member countries to frame policies, regulatory and legal mechanisms in order to implement for health care programmes and ensuring authenticity, safety and efficacy of traditional medicines.[1] The government of India has formed structures (bodies) to regulate quality, safety, efficacy, practice and documentation of herbal medicine (National policy on Indian system of medicine and homeopathy- 2002).[2]

Itrifal- Kishneez is a semisolid poly-herbal Unani formulation categorised under Majoonieth listed in the national formulary of Unani medicine, part I.[3] Itrifal is a semisolid preparation where one or more single drugs of plant, animal or mineral origin are mixed in the powder form in the base (Qiwam) made of purified honey, sugar or jaggery. Itrifal Kishneezi is used as laxative, carminative and stomachic. It is also beneficial in Headache, Conjunctivitis, Otalgia, Flatulence, Chronic catarrh, Cage pain, Gastric debility and Piles.[4]

The present research work aims to develop quality parameters and evaluate the data to lay down pharmacopeial standards of Itrifal Kishneezi. The conventional parameters such as organoleptic parameters, microscopy and physico-chemical evaluations were carried out along with HPTLC fingerprinting. The WHO quality control parameter such as heavy metal estimation, aflatoxins, microbial load and pesticide residue were also analysed in order to determine the quality of the formulation.

MATERIALS AND METHODS

Identification of Ingredients

All the ingredients were procured from local raw drug dealers and were identified botanically using pharmacognostical methods[5,6,7] The ingredients were further validated by comparing with the monographs available in UPI, Part I, Vol. I.[8] and UPI Part II Vol. I.[9]

Preparation of Formulation

The formulation was prepared at laboratory scale where all the ingredients were cleaned and dried under shade to remove moisture. The ingredient nos. 1-6 [Table I] were crushed separately in an iron mortar to obtain their coarse powders. The coarse powders were further ground in a grinder to get their fine forms. The fine powders were mixed together thoroughly and sieved through mesh no-80 to prepare the homogenous mixture. The ingredient no.8 was heated in a vessel over low flame followed by adding the homogenous mixture of ingredients 1-6. The mixture was shallow fried slowly and kept separately. Furthermore, ingredient no.7 was heated on low flame until the boiling started. 1% citric acid was added and mixed thoroughly to prepare the syrup (qiwam) of 75% consistency. The vessel was removed from the flame and the rubbed powder of ingredient nos. 1-6 was added immediately by thorough mixing. Finally, 0.1% sodium benzoate was added as preservative and mixed again thoroughly to prepare the homogenous product. The mixture was allowed to cool at room temperature. The prepared formulation was stored in tightly closed glass container free from moisture and kept in a cool and dry place. The formulation was prepared in three batches.

Table I: Formulation composition.

S. No.	Unani Name	Botanical/ English Name	Part Used
1.	Post-e-Halela Zard	*Terminalia chebula* Retz.	Semi-ripe fruit peel
2.	Post-e-Halela Kabuli	*Terminalia chebula* Retz.	Ripe fruit peel
3.	Halela Siyah	*Terminalia chebula* Retz.	Unripe dried fruit
4.	Aamla Muqashshar	*Emblica officinalis* Gaertn.	Peeled fruit
5.	Post-e-Balela	*Terminalia belerica* Roxb.	Fruit peel
6.	Kishneez Khushk	*Coriandarum sativum* L**.**	Dried seed
7.	Asl	*Apis mellifera* L.	Honey
8.	Roghan-e-Badam	*Prunus amygdalus* (L.) Batsch.var.*dulcis*	Kernel

Microscopy

5g of the drug was mixed with 50ml of water by gentle warming, till the sample got completely dispersed in water. The mixture was stirred gently and the supernatant was discarded without loss of residue. The process was repeated to get a clear supernatant, the residue was washed with distilled water and a little residue was stained with iodine solution. A small amount of residue was treated separately with chloral hydrate solution, washed with distilled water and mounted in 50% glycerine. The various characters were observed under the digital microscope.[5,6,7]

Physico-chemical analysis

The physico-chemical parameters of Itrifal-Kishneezi such as moisture content, extractive values (solubility in water and ethanol), ash values (total ash and acid insoluble ash), pH values (1% &10% aqueous solution), bulk density and sugar content (reducing and non-reducing sugar) were analysed as per standard methods.[10,11]

High Performance Thin Layer Chromatography (HPTLC) analysis

After leaching out sugar from the drug, two samples of 2g each were extracted separately with 25ml each of ethanol and chloroform by sonicating for 30 minutes. The extracts were filtered and concentrated up to 10ml in volumetric flasks and used as such for HPTLC finger-printing. 10µl of ethanol extract was applied on aluminium TLC plate pre-coated with silica gel 60 F^{254} (E. Merck) by employing CAMAG Linomat IV automatic sample applicator. The plate was developed up to a distance of 9cm in twin trough glass chamber (10x10) using 10ml of the solvent system Toluene: ethyl acetate: formic acid (9: 1: 0.5) as mobile phase. The plate was air dried at room temperature and observed under UV at wave lengths 254nm and 366nm. Further the plate was dipped in 1% Vanillin-sulphuric acid reagent and heated at 105°C till coloured bands appeared. The plate was finally examined under visible light. Similarly, 10µl of chloroform extract was applied on a separate aluminium TLC plate pre-coated with silica gel $60F^{254}$ (E. Merck) by employing CAMAG Linomat IV automatic sample applicator. The plate was developed up to a distance of 9cm in twin tough glass chamber (10x10) using 10ml of the solvent Toluene: ethyl acetate: formic acid (9: 1: 0.5) as mobile phase. Rest of the process was repeated as carried out for ethanol extract.[12,13,14]

Quality control parameters

Although Unani medicines are popular among a large number of people in Indian subcontinent, but for more wider acceptance across the globe their exhaustive quality check is of utmost important. Due to complex nature of Unani formulations, it is difficult to establish quality control parameters. However, modern analytical techniques are helpful to get over this problem. Thus, different quality control parameters viz. microbial load, heavy metals, aflatoxins and pesticide residues were carried out for evaluation of quality of Itrifal-Kishneezi. Estimation of microbial load was conducted as per standard methods.[15] Heavy metal analysis and aflatoxins were carried out by respective use of Atomic Absorption Spectrophotometer (LABINDIA) [16] and HPLC (ThermoFisher).[17] Moreover, the pesticide residues were analysed using Triple Quadrupole GC-

MS/MS system (ThermoFisher) by adopting QuEChERS method.[15,16,17]

RESULTS AND DISCUSSION

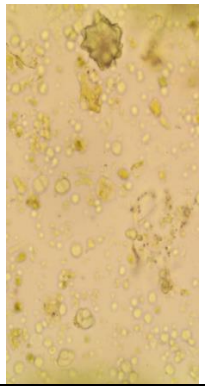
Macroscopic description

Itrifal Kishneezi is a reddish-brown semisolid preparation having aromatic smell and sweetish bitter taste.

Microscopic observation

Following characters were observed under microscope in different mounts. fragments of epicarp, rosette of calcium oxalate crystals, spiral vessel, sclerenchyma fibre, various shaped lignified sclereids with simple pits (Post-e-Halela Zard -*Terminalia chebula* Retz.); epicarp trichome hairs, pitted sclereids (Post-e-Balela- *Terminalia bellirica* Roxb.), fragment of endopsrem and endocarp (parquetry layer), group of fusiform sclereid of mesocarp (Kishneezi Khushk- C*oriandrum sativum* L.); fragment of epicarp, brachysclereid with very broad lumen and pitted walls either single or in groups (Amla Muqashshar- *Phyllanthus emblica* L.); stone cells of various types viz brachysclereid, osteosclereid, either single or in groups, starch grains, spiral vessels are common characters of Amla Muqashshar, Post-e-Halela and Post-e-Balela. (Fig. I).

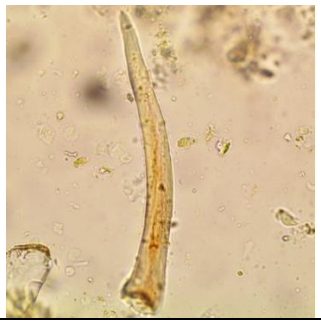
Epicarp 40x (Post-e-Halela)



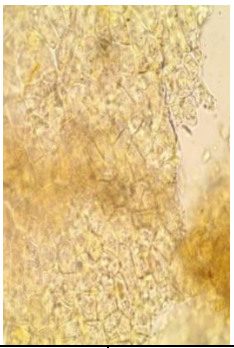
Starch grains and rosette of crystals 40x (Post-e-Halela)

Spiral vessel 40x (Post-e-Halela)

Epicarp trichome hairs 20x (Post-e-Balela)

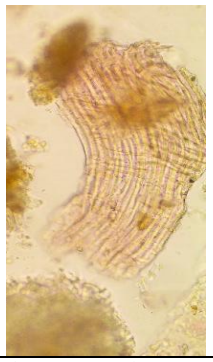


Trichome 40x (Post-e-Balela)

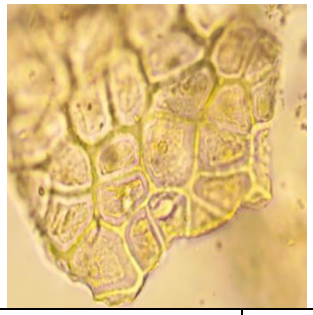


Endosperm 40x (Kishneez Kushk)

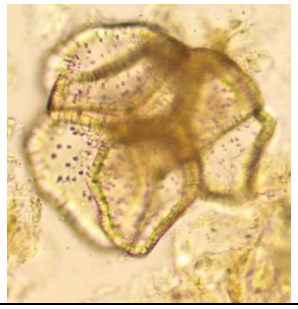
Endocarp (parquetry layer) 40x (Kishneez Kushk)



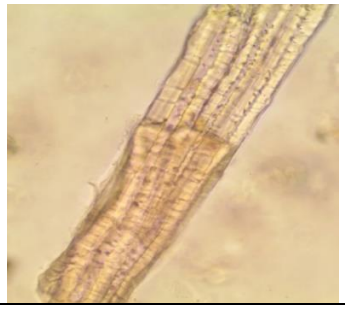
Group of fusiform sclereid of mesocarp 20x (Kishneez Kushk)



Epicarp surface 40x (Amla Muqashshar)



Brachysclereid with very broad lumen and pitted walls 40x (Amla Muqashshar)



Sclerenchyma fiber 40x (Post-e-Halela)

Sclerenchyma fiber 20x (Post-e-Balela)

Pitted sclereid 40x (Post-e-Halela or Post-e-Balela)

Osteosclereid 40x (Post-e-Balela)

Fig. I: Powder Characteristics.

Physicochemical analysis

The physico-chemical data of the drug Itrifal Kishneezi are shown in Table II. The quantitative assessment of the data exhibit that the moisture content of the drug ranged between (12.65- 14.25) which is optimal in the case Itrifals. The total ash content was not more than 1.25% while acid insoluble ash was absent which indicate that the drug was free from silicious matter. The water extractive values turned out to be on higher side, ranged between (74.82- 76.25%) which shows the presence of high amount of sugar content while the ethanol extractive values were moderate and ranged between (40.64- 42.78%) indicating the extraction of polar constituents. The aqueous extract of drug was little acidic in nature as pH values falls in the range of 4.35- 4.70.

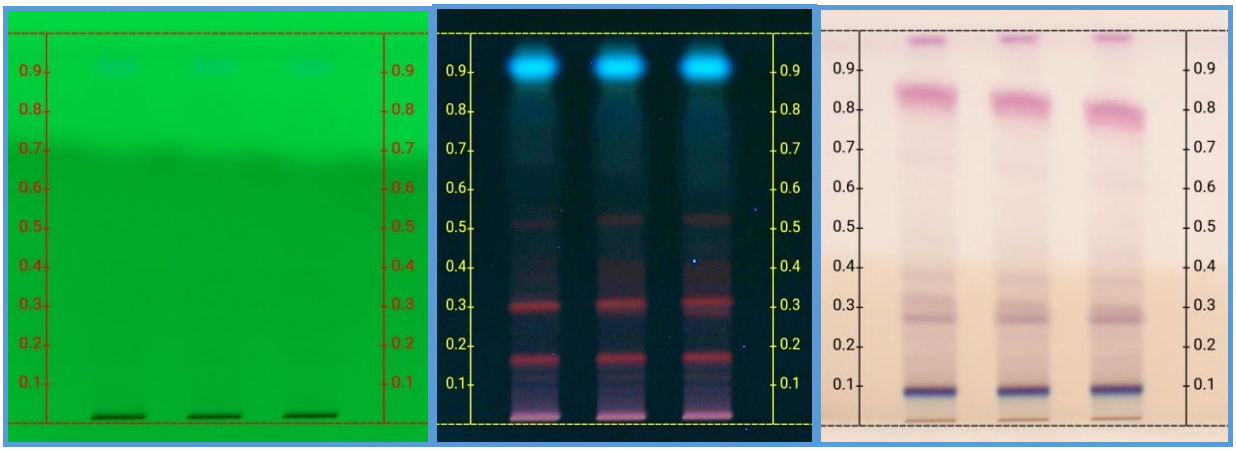
Table II: Physico-Chemical Parameters.

S. No.	Parameters	Values
1.	Ethanol soluble matter (%)	44.60 – 46.45
2.	Water soluble matter (%)	74.22 – 75.38
3.	Loss in weight on drying at 105^0C (%)	13.88 – 14.25
4.	Total ash (%)	0.72 – 0.87
5.	Acid insoluble ash (%)	Nil
6.	pH of 1% aqueous solution	4.85 – 4.92
7.	pH of 10% aqueous solution	4.10 – 4.27
8.	Bulk density	1.4292 – 1.4385
9.	Reducing sugar	61.90 – 63.38
10.	Non-reducing sugar	12.82 – 13.45

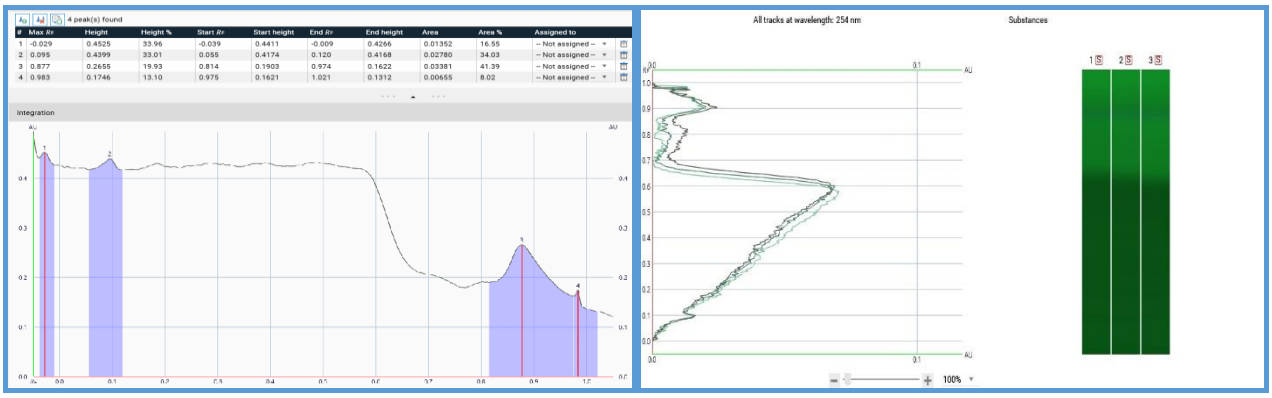
HPTLC Profile

HPTLC finger-printing is flexible, robust, and economical separation technique for identification of crude drugs and compound formulations. HPTLC fingerprints of both the extracts of Itrifal Kishneezi were observed under UV 254nm, UV 366nm and under visible light after derivatization. All the batches of Itrifal Kishneezi show similar colourful bands with almost similar R_f values. Besides, their densitograms are almost superimposed on each other. This shows batch to batch consistency of the formulation. (Fig. II: a-b)

HPTLC of **Chloroform** extracts (Fig. II: a)

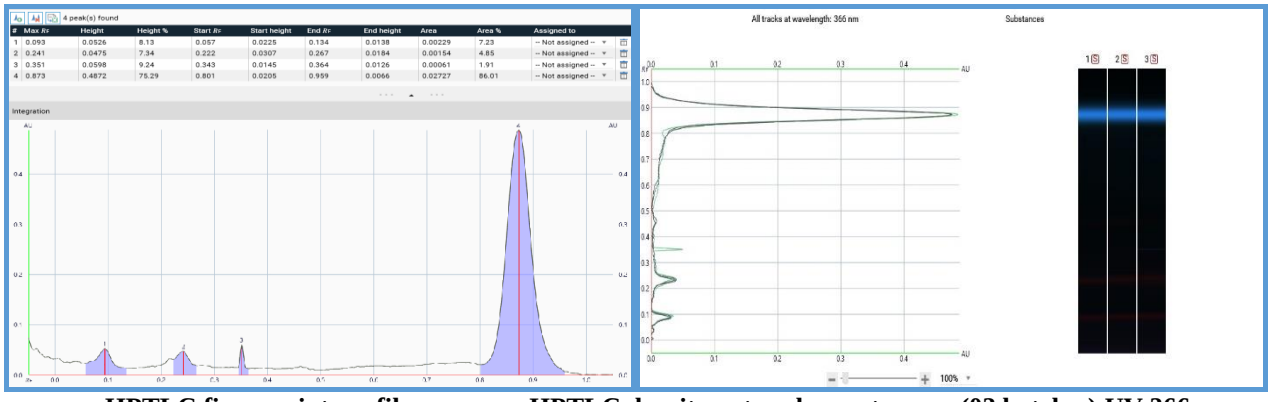


UV 254 nm **UV366 nm** **Visible Light** (After derivatization)

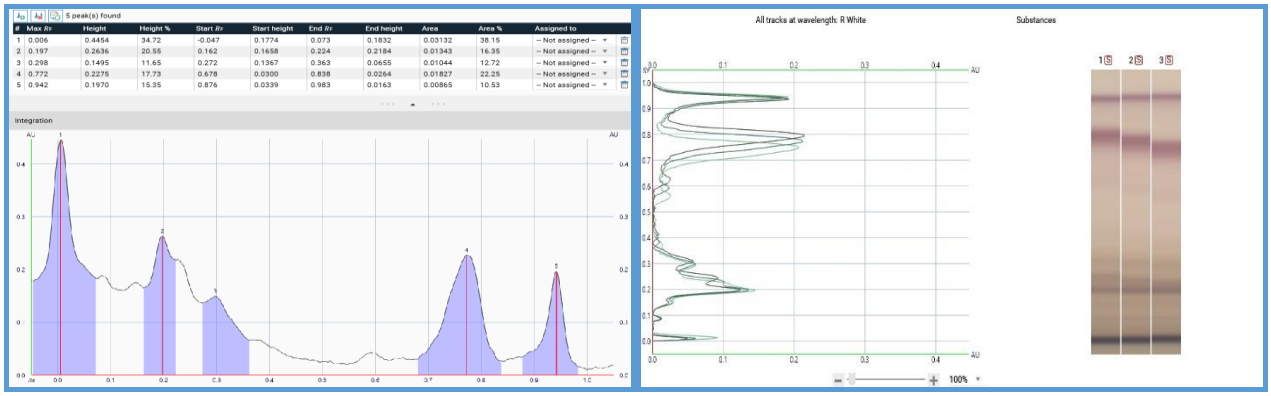


HPTLC fingerprint profile **HPTLC densitometry chromatogram (03 batches)**

UV 254 nm

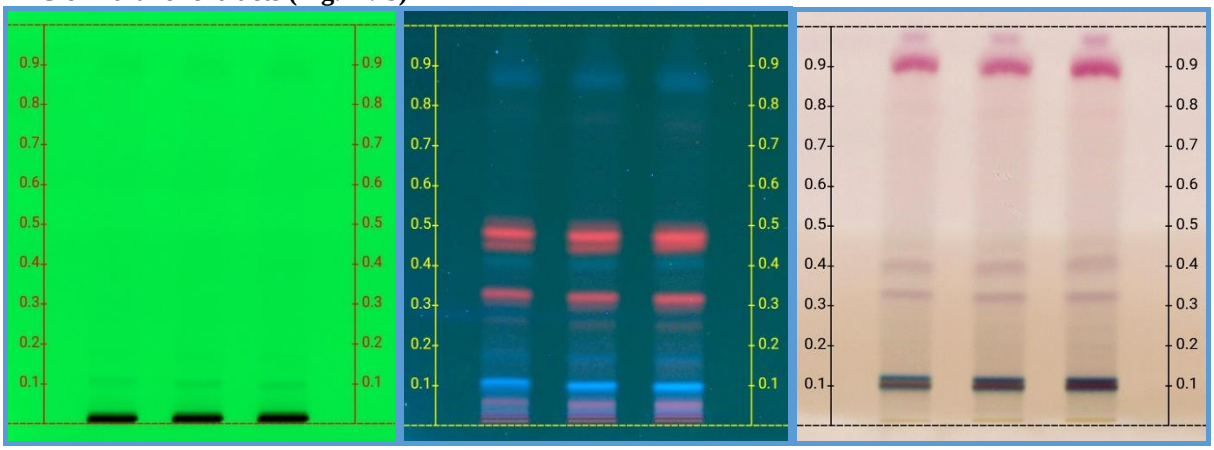


HPTLC fingerprint profile **HPTLC densitometry chromatogram (03 batches) UV 366 nm**

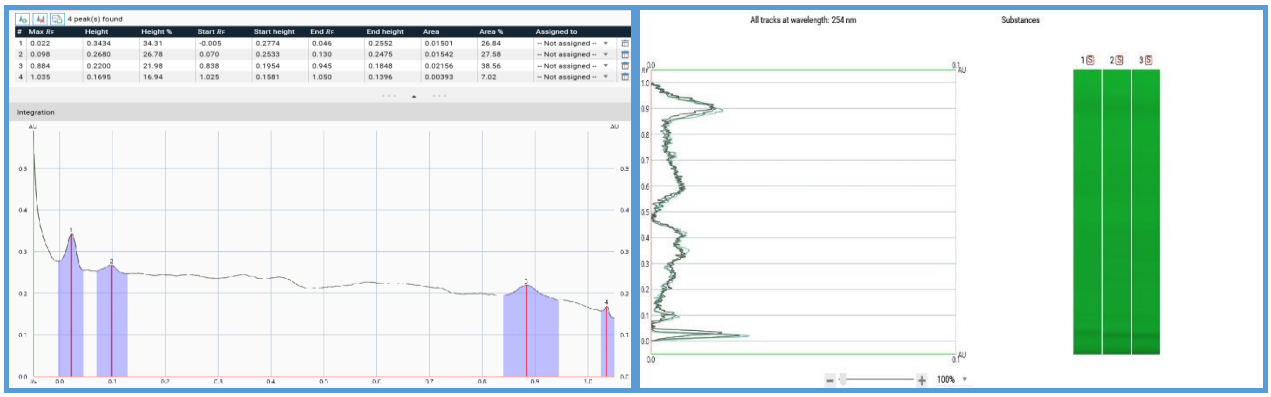


HPTLC fingerprint profile HPTLC densitometry chromatogram (03 batches) After derivatization under White Light

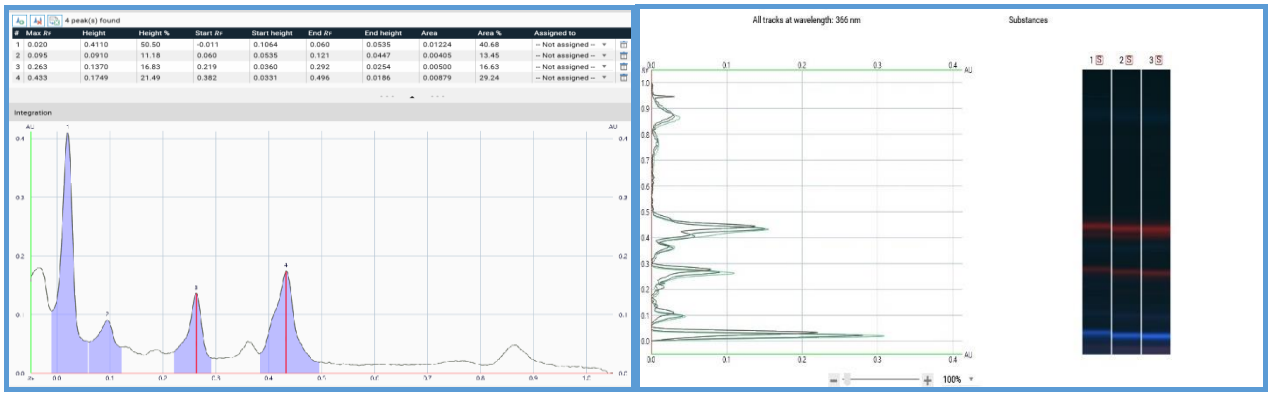
HPTLC of Ethanol extracts (Fig. II: b)



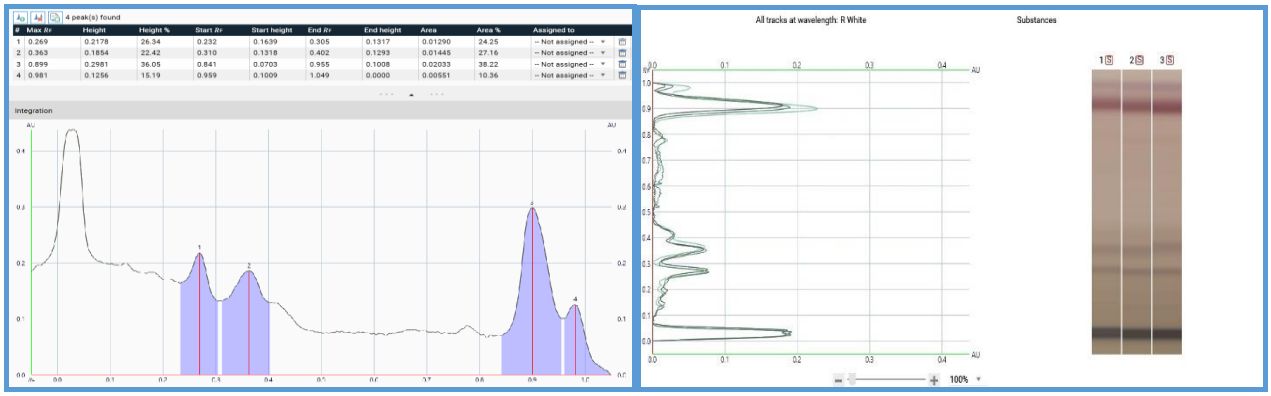
UV 254 nm **UV366 nm** **Visible Light** (After derivatization)



HPTLC fingerprint profile **HPTLC densitometry chromatogram (03 batches)** **UV 254 nm**



HPTLC fingerprint profile **HPTLC densitometry chromatogram (03 batches)** **UV 366 nm**



HPTLC fingerprint profile HPTLC densitometry chromatogram (03 batches)After derivatization under White Light

Quality control parameters

Microbial load

The microbial contamination in herbal medicines may be present due to a number of factors. The product may get it from the contaminated raw material; during unhygienic handling and processing or due to improper packaging. Estimation of microbial growth indicates whether the drug contains disease causing and spoilage microorganism in permissible limit or not. The assessment was carried out by evaluating the total aerobic bacterial count (TABC), total yeast and molds count (TYMC), bacteria belonging to the Enterobacteriaceae family and some specific objectionable pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* & *Candida albicans*. The values of microbial loads, shown in Table III, are below WHO permissible limits which indicates that the drug is safe for internal use.

Table III: Microbial Load.

S. No.	Parameters	Values
1.	Total aerobic bacterial count (TABC)	3.4×10^{3} CFU/gm
2.	Total yeast and molds count (TYMC)	3.1×10^{4} CFU/gm
	Enterobacteriaceae members	
3.	*Escherichia coli*	ND
4.	*Salmonella sp.*	ND
5.	*Shigella sp.*	ND
6.	*Klebsiella sp.*	ND
	Specific objectionable pathogens	
7.	*Pseudomonas aeruginosa*	ND
8.	*Staphylococcus aureus*	ND
9.	*Candida albicans*	ND
	Aflatoxin producing fungi	
10.	*Aspergillus flavus*	ND
11.	*Aspergillus parasiticus*	ND

*ND – Not detected

Aflatoxins

Aflatoxins are fungal toxins produced by a variety of molds such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius* that commonly contaminate crops during harvest, storage or processing. Exposure to aflatoxin can become the reason for a range of health issues from as common as abdominal pain, vomiting etc. to deadly hepatocellular carcinoma. The results of aflatoxin analysis of Itrifal Kishneezi indicate the absence of any such contamination (B1, B2, G1, G2) in the drug.

Heavy metal analysis

Heavy metals can cause chronic toxic effects to human health. They can accumulate in the body and disturb the functioning of vital organs. Their estimation in herbal drugs is imperative. The results of heavy metals estimation are given in Table IV. The heavy metal content in Itrifal kishneezi was found to be below detection limit which indicates that the drug is free from heavy metals contamination and safe for internal use.

Table IV: 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

Pesticide residues

In present times, it is very difficult to grow any crop without the use of pesticides due to a number of reasons. Pesticides have become integral part of our agriculture system. Therefore, it is crucial to check the presence of pesticide residues in herbal medicines. In order to estimate the pesticide residue, the drug was analysed on GC-MS/MS system. The results of pesticide residues are given in Table V which signify that the drug is free from any pesticide contamination.

Table V: Pesticide Residues.

S. No.	Pesticide	Result (mg/Kg)	Permissible limit (mg/Kg)
1.	Alachlor	BLQ	0.02
2.	Aldrin (Aldrin and dieldrin combined expressed as dieldrin)	BLQ	0.05
3.	Azinophos-methyl	BLQ	1.0
4.	Bromopropylate	BLQ	3.0
5.	Chlordane (cis, tans and oxychlordane)	BLQ	0.05
6.	Chlorfenvinphos	BLQ	0.5
7.	Chlorpyrifos	BLQ	0.2
8.	Chlorpyrifos-methyl	BLQ	0.1
9.	Cypermethrin (and isomers)	BLQ	1.0
10.	DDT (all isomers, sum of p, p'-TDE (DDD) expressed as DDT)	BLQ	1.0
11.	Deltamethrin	BLQ	0.5
12.	Diazion	BLQ	0.5
13.	Dichlorvos	BLQ	1.0
14.	Dithiocarbamates (as CS2)	BLQ	2.0
15.	Endosulphan (sum of isomers & Endosulphan sulphate)	BLQ	3.0
16.	Endrin	BLQ	0.05
17.	Ethion	BLQ	2.0
18.	Fenitrothion	BLQ	0.5
19.	Fenvalerate	BLQ	1.5
20.	Fonofos	BLQ	0.05
21.	Heptachlor (sum of Heptachlor & Heptachlor epoxide)	BLQ	0.05
22.	Hexachlorobenzene	BLQ	0.1
23.	Hexachlorocyclohexane isomer (other than γ)	BLQ	0.3
24.	Lindane (γ – Hexachlorocyclohexane)	BLQ	0.6
25.	Malathion	BLQ	1.0
26.	Methidathion	BLQ	0.2
27.	Parathion	BLQ	0.5
28.	Parathion methyl	BLQ	02
29.	Permethrin	BLQ	1.0
30.	Phosalone	BLQ	0.1
31.	Piperonyl butoxide	0.01	3.0
32.	Pirimiphos methyl	BLQ	4.0
33.	Pyrethrins (sum of isomers)	BLQ	3.0
34.	Quintozen (sum of Quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	BLQ	1.0

***** BLQ – Below limit of quantification

CONCLUSION

Standardization of herbal medicine provide an assurance of its quality. Therefore, evaluation of physico-chemical, microbiological and quality control parameters aids to confirm the identity and purity of the herbal medicines. Itrifal Kishneezi was evaluated through pharmacopeial parameters which certainly provides validation that the drug is safe for internal use. HPTLC fingerprinting also

contribute for maintaining its authenticity. Hence, the present study ensures the quality and efficacy of the Unani formulation Itrifal Kishneezi.

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