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PHYSICO-CHEMICAL AND MICROSCOPICAL STANDARDIZATION OF CLASSICAL UNANI FORMULATION: JAWARISH-E-FALAFALI

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ABSTRACT

Unani system of medicine has global presence as they are an important and reliable health care option for maintaining good health. The phenomenal growth in the use of Unani medicine has arisen new challenges for ensuring their safety and efficacy. The preparation of Unani medicine is usually based on traditional methods in accordance with the procedures described in classical literature which sometimes lacks scientific validation. These days in the manufacturing of poly-herbal formulations, adulteration, contamination and substitution of costly ingredients are common practices that adversely affect the quality and efficacy of formulations. Hence, it is of utmost importance to standardize the method of preparation and procedures or parameters to ensure their authenticity and therapeutic effects. Therefore, a poly-herbal Unani formulation Jawarish-e-Falafali, widely used as Digestive and Laxative, has been standardized through various phytochemical and quality control parameters. The formulation was_analysed by carrying out conventional as well as modern scientific parameters such as organoleptic evaluation (colour, odour, taste and consistency), Microscopy Physicochemical evaluation (moisture content, total ash, acid insoluble ash, pH values, water and ethanol soluble extracts, sugar content) along with the HPTLC fingerprinting. The determination of harmful contaminants such as Heavy metals, microbial loads, Aflatoxins and pesticide residue were also carried out in order to ascertain its quality assurance which consequently aid in developing the scientific standards for the formulations.

KEYWORDS: Physico-chemical evaluation, microscopy, HPTLC fingerprinting, standardization

INTRODUCTION

Approximately 75% of the world's population, living specially in the developing nations, rely on herbal medicinal products for their primary health care needs which are perceived as an integral art of their culture. World Health Organization (WHO) also endorses and boosts up the exploration of traditional drugs by virtue of their facile availability and affordability.[1] WHO has chosen India as an integrated medicine centre. The government of India has a formal structure to regulate quality, safety, efficacy, practice and documentation of herbal medicine (National Policy of Indian system of Medicine and Homeopathy- 2002).[2]

Standardization of herbal medicine is not as easy assignment as various components such as temperature, geographical locations, period and time of collection, age and part of plant collected, method of collection and various other factors affect the bio-efficacy and reproducible therapeutic effect.[3,4]

Jawarish-e-Falafali is a semisolid poly-herbal Unani formulation categorized under Majoonieth, listed in the National formulary of Unani medicine, Part 1.[5] Jawarish is a semisolid preparation where one or more drugs of plants, animals and mineral origin that are mixed in powder form are added to the base (Qiwam) made of purified honey, sugar or jaggery.

Jawarish-e-Falafali is a Hazim (Digestive) and Mulayyin (Laxative) in action[6] and in the treatment of Su'-e-Hazm (Dyspepsia), Waja' -ul-Me'da (Gastraligia), qabz (Constipation), Hurqat-e-Me'da Dauran-e-Hamal (Hyper-acidity during pregnancy).[7,8]

This classical Unani formulation is considered to be safe and non0habit forming if consumed even for a longer time. Jawarish-e-Falafali is composed of nine different ingredients which individually have magnificent medicinal properties. Ingredients like Filifil siyah is used in Ptyriasis (Bahaq) and itching (Kharaas); Filfil safaid is sued in vitiligo (Bars) and Flatulent (Nafakh-e-Shikam); Filfil Daraz is used in Gastric Debility (Zof-e-Meda) and Cough (Khansi); Zanjabeel is used stomach ache (Dard-e-Shikan) and loss of appetite (Zof-e-Ishteha); and Ood-Balsan is used in Epilepsy (Sara) and Bronchial Asthma (Zeeq-un-Nafas).[9]

The present work aims to develop quality parameters and evaluate the data to lay down pharamcopoeial standards of Jawarish-e-Falafali. The conventional parameters such as organoleptic parameters, Microscopy, Physico-chemical evaluations such as water and Alcohol insoluble extracts, Total Ash, Acid insoluble Ash, pH values, moisture content along with HPTLC fingerprinting were carried out. The WHO quality control parameters such as heavy metal estimation, aflatoxins, microbial loads and pesticide residues were also analysed in order to assess the quality of the formulation.

MATERIAL AND METHODS

Preparation of Formulation- All the ingredients were procured from local raw drug dealer and were identified botanically using pharamcognostical methods. These ingredients were further validated by comparing with the monographs available in UPI-Part 1- Vol.- I, II, IV and VII[10] and UPI-Part 2-Vol.- I.[11]

All the ingredients were taken of pharmacopeial quality. The ingredients were cleaned and dried under shade to remove moisture, if any. Ingredients no. 1-8 (Table 1) was 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. Then ingredient no. 9 was boiled in a vessel and 0.1% Citric Acid was added. It was mixed well and the qiwam of 75% (Brix) consistency was prepared. After the vessel was removed from the fire, the mixed powders of ingredients no. 1-8 were immediately added which was then followed by adding 0.1% Sodium Benzoate. It was mixed thoroughly to prepare a homogenous product. The content was allowed to cool at room temperature. The prepared drug was stored in a food grade glass/ plastic container free from moisture[12] The drug was prepared in 3 batches.

Microscopy

10g of the drug was mixed with 50ml of water by gentle warming, till the sample gets 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 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.[13,14,15]

Physico-chemical analysis

The physico-chemical parameters of Jawarish-e-Falafali such as moisture content, extractive values (solubility in water and ethanol), ash values (total ash and acid resoluble ash), pH values (1%-10% aqueous solution), bulk density and sugar content (reducing and non-reducing were assigned as per standard methods).[16,17]

High Performance Thin Layer Chromatography (HPTLC) analysis

After leaching out sugar from the drug, two samples of 2g 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 flask and used as such for HPTLC finger-printing. 10ml of ethanol extract was applied on aluminium TLC plate pre-coated with silica gel 60 F^{254} (E. Marck) 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 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, 10ml of chloroform extract was applied on a separate aluminium TLC plate pre-coated with silica gel $60F^{254}$ (E. Marck) by employing CAMAG Linomat IV automatic sample applicator. The plate was developed up to a distance of 9cm in two tough glass chambers (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.[18,19,20]

Quality control analysis

The Unani medicines are being used by a large number of people worldwide as affective and safe remedies. In order to sustain the faith of the people in Unani medicines, their quality of check is essential regardless of the form of Unani preparation. So, the different quality control parameters viz. microbial load, heavy metals, aflatoxins and pesticide residues were carried out for determination of quality of Jawrish-e-Falafali. Estimation of microbial load was conducted as per standard method. Heavy metal analysis and aflatoxins were carried out by respective use of Atomic Spectro photometer (LABINDIA) and HPLC (Thermo fisher). Pesticide medicines were analysed using GC-MS system (Agilent) equipped with mass selective detector as per standard method.[21,22,23]

RESULTS AND DISCUSSION

Macroscopic description

Jawarish-e-Falafili is a brownish-black semi-solid preparation having a pleasant smell and sweet taste with tingling sensations.

Microscopic observation

About 5g of the drug stir gently with hot water in a beaker, discard the supernatant without loss of residue. Repeat the process to get a clear supernatant, and wash the residue with distilled water. Stain some residue matter with iodine solution and mount in 50% glycerine. Clear some material in chloral hydrate solution, wash with water and mount in 50% glycerine. Observe the following characters in different mounts.

Drug consisted of fragments of medullary rays parenchyma cells, septate fibres (**Ood Balsan***)*, scalariform xylem elements, sclerenchymatous fibers with dentate walls, reticulate xylem vessel, simple starch grains with eccentric hilum (**Zanjabeel**); mesocarp layer, parenchyma cells containing fixed oil globules, endocarp layer, fibres (**Tukhm-e-Karafs**); cells of perisperm containing starch grain and oil, lignified endocarp cells, lignified sclereids (**Filfil Safed/ Filfil Siyah**); perisperm cells with angular walls filled with starch grains, lignified stone cells (**Filfil Draz**); long fibre with thick-walled and very narrow lumen, horseshoe shaped stone cell, brachysclereids (**Salikha**); starch grains with the distinct centric hilum (**Asaroon**); annular vessels (common characters of **Filfil Safed/ Filfil Siyah, Filfil Draz, Asaroon**); spiral vessel (common characters of **Tukhm-e-Karafs, Zanjabeel**); pitted vessels (common characters of **Asaroon, Ood Balsan, Filfil Draz**); prismatic crystals (common characters of **Ood Balsan, Tukhm-e-Karafs, Salikha**); rosette cluster calcium oxalate crystals (common characters of **Filfil Safed/ Filfil Siyah**) (Fig-1).

Physico-chemical analysis

Table II displays the physico-chemical information for the compound formulation Jawarish-e-Falafali. The drug's moisture content ranged between (09.04- 09.96%), which is ideal in the case of Jawarish (Majoonieth category), according to the quantitatively evaluated data. The total ash content was not more than 1.07% while acid insoluble ash was nil which indicates that the drug was free from silicious matter. While the ethanol extractive values were moderate and ranged between 51.64 - 53.78%, suggesting the extraction of polar constituents, the water extractive values turned out to be on the higher side, ranging between (68-55-

70.84%), which indicates the existence of significant quantity of sugar content. The drug's aqueous extract had a pH of 5.5- 5.64%, which indicates that it was very slightly acidic.

HPTLC profile

HPTLC finger-printing is sensitive, reliable and convenient tool for identification of crude drugs as well as complex compounds formulations as the plant species produce distinct chromatograms. HPTLC images of both the extracts of Jawarish-e-Falafali were observed under UV 254nm, UV 366nm and under visible light after derivatization. All the batches of Jawarish-e-Falafali show similar colourful bands with almost similar R_f values. Moreover, their densitograms are almost superimposed on each other. This shows batch to batch consistency of the formulation (Fig.2).

QUALITY CONTROL PARAMETERS

Microbial load

Estimation of microbial growth is very important parameter in traditional medicines. It indicates whether the drug contains disease causing and spoilage micro-organism or not and if present, they are in WHO permissible limit or not. The assessment is done for evaluating the total bacterial count, total fungal count, bacteria belonging to the Enterobacteriaceae family and count of pathogens like *E. Coli*, *Staphylococcus aureus*, *Salmonella* spp. and *Pseudomonas aeruginosa*. The results of microbial loads which are shown in table 3 are below permissible limits which indicates that the drug is safe for internal use or consumption.

Aflatoxins

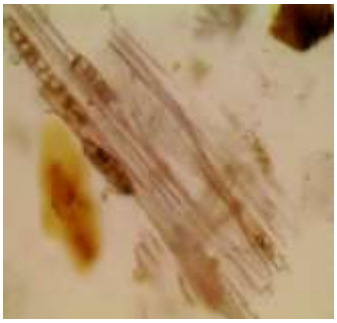
The results of aflatoxins analysis of the drug are given in table 4. Aflatoxins are toxic metabolites produced by a variety of molds such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius.* The results do not show the presence of any aflatoxins contents (B1, B2, G1, G2) in Jawarish-e-Falafali.

Pesticide residues

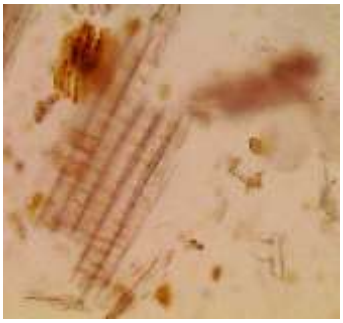
The results of pesticide residues are given in table 5. Harvest pf herbal material without the use of pesticides in very difficult due to several factors. But as per WHO guidelines, the major concern is whether the drug contains pesticide residue in permissible limit or not. In order to estimate the pesticide residue, the drug was analysed on GC-MS. The results indicated that the drug is free of pesticide residue and safe for use.

Heavy metal analysis

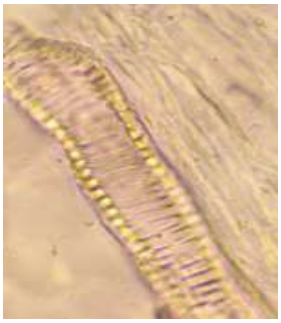
The results of heavy metals estimation are given in table 6. Heavy metals are hazardous to human health and may cause many fatal diseases. A heavy metal has relatively high density and atomic weight and is toxic or poisonous even at low concentration. The heavy metal content in Jawarish-e-Falafali was found to be below detection limit which indicated that the drug is free from heavy metals contamination and safe for use.



Medullary rays' parenchyma cells 20X **(Ood Balsan)**



Septate fibers 20X **(Ood Balsan)**

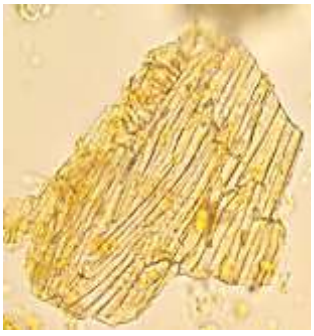


Scalariform xylem elements 40X **(Zanjabeel)**

Sclerenchymatous fibers with dentate walls 40X **(Zanjabeel)**

Reticulate xylem vessel 40X **(Zanjabeel)**

Simple starch grains with eccentric hilum 40X **(Zanjabeel)**



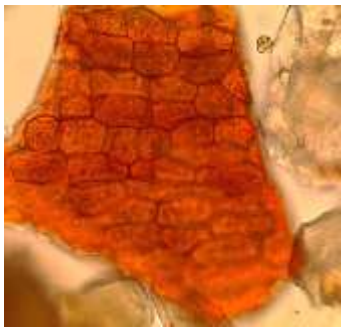
Mesocarp layer 40X **(Tukhm-e -Karafs)**

Parenchyma cells containing fixed oil globules 40X **(Tukhm-e-Karafs)**

Endocarp layer 40X **(Tukhm-e-Karafs)**

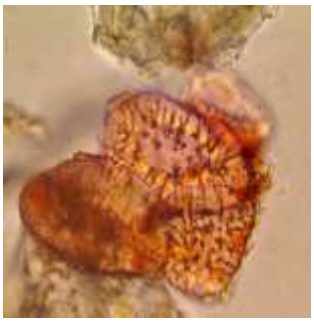
Fibres 20X **(Tukhm-e-Karafs)**

Cells of perisperm containing starch grain and oil 20X **(Filfil Safed/ Filfil Siyah)**

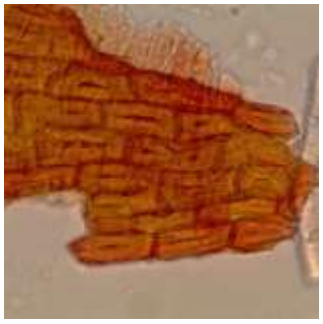


Lignified endocarp cells 20X **(Filfil Safed/ Filfil Siyah)**

Lignified sclereids 40X **(Filfil Safed/ Filfil Siyah)**



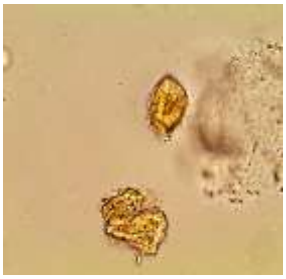
Perisperm cells with angular walls filled with starch grains 40X **(Filfil Draz)**



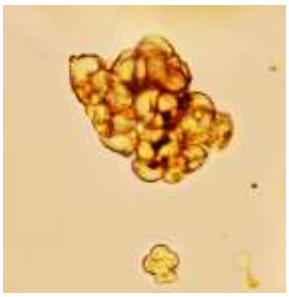
Lignified stone cells 20X **(Filfil Draz)**

Long fibre with thick-walled and very narrow lumen 20X **(Salikha)**

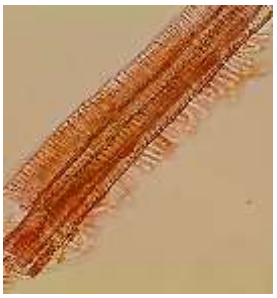
Horseshoe shaped stone cells 20X **(Salikha)**



Brachysclereids 20X **(Salikha)**



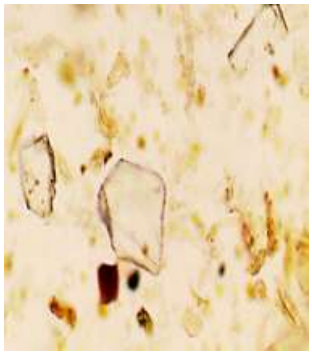
Starch grains with the distinct centric hilum 40X **(Asaroon)**



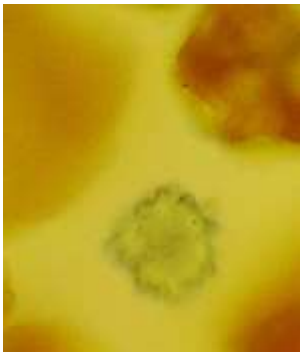
Annular vessels 20X (**Filfil Safed/Siyah, Filfil Draz, Asaroon)**

Spiral vessel 40X **(Tukhm-e-Karafs, Zanjabeel)**

Pitted vessels 40X **(Asaroon, Ood Balsan, Filfil Draz)**



Saleekha prismatic crystals 40X **(Ood Balsan, Tukhm-e-Karafs, Salikha)**



Rosette cluster calcium oxalate crystals 40X **(Filfil Safed/ Filfil Siyah)**

Figure 1. Microscopy.

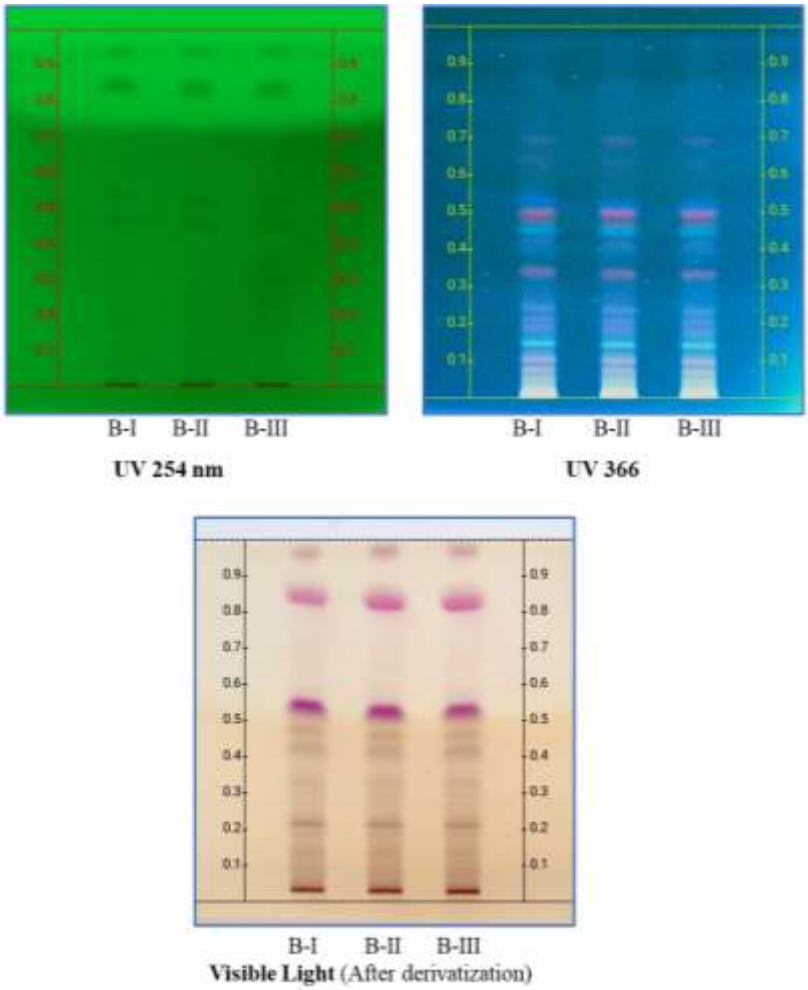
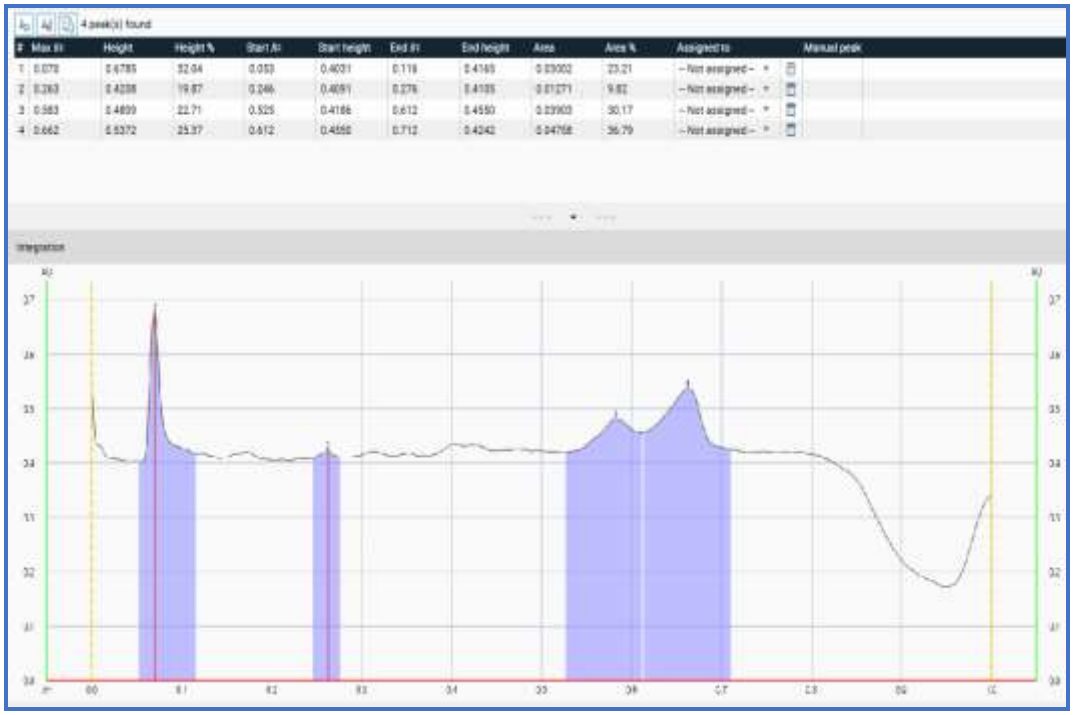
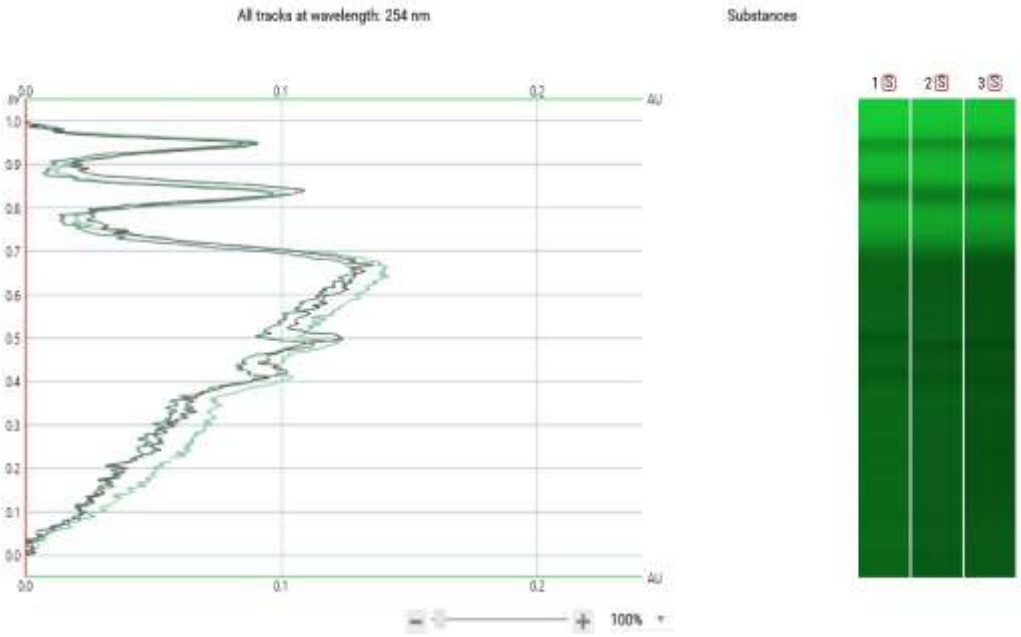


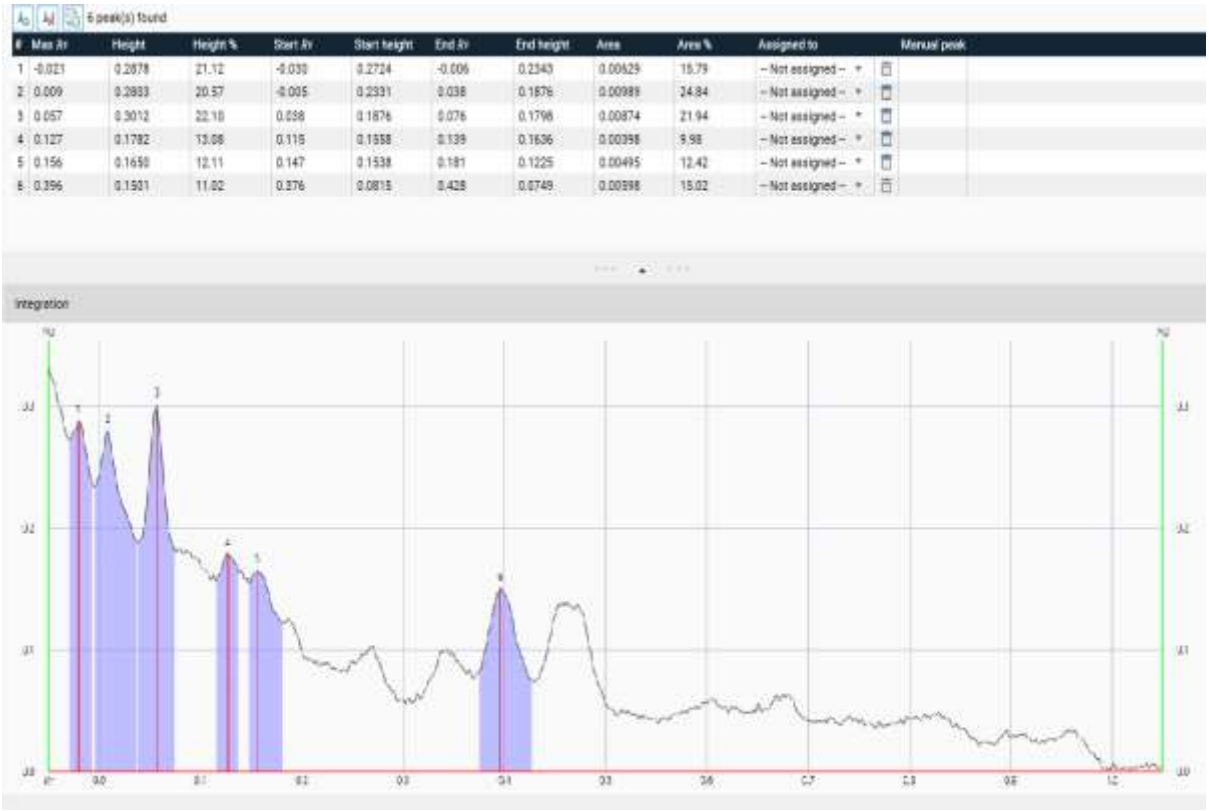
Figure 2: HPTLC of *Chloroform* extracts of Jawarishe-eFalafali.



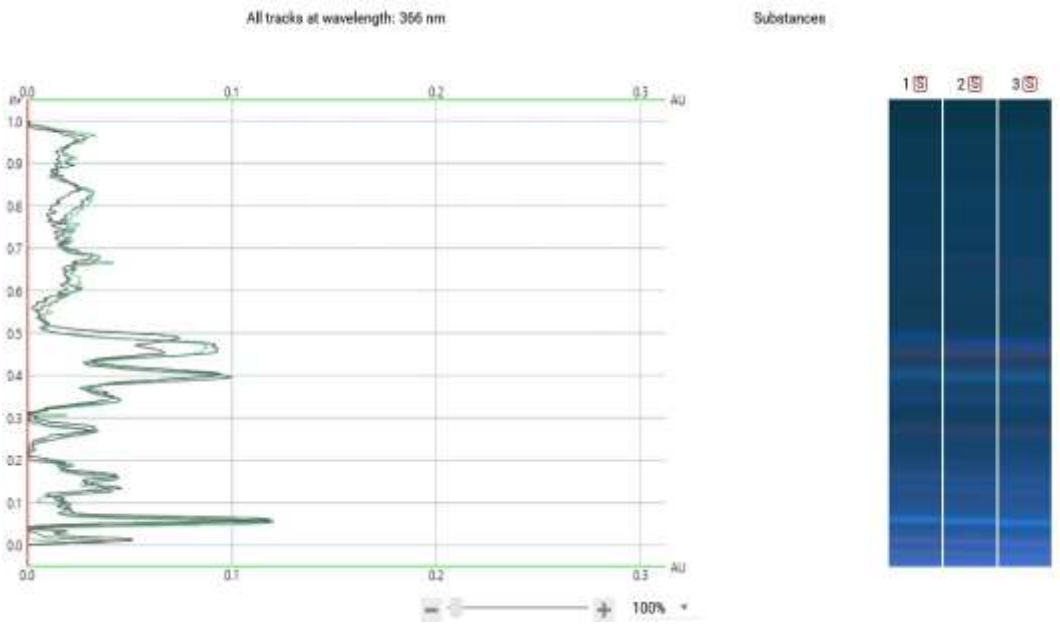
HPTLC fingerprint profile of chloroform extract of Jawarish-e-Falafali at 254 nm



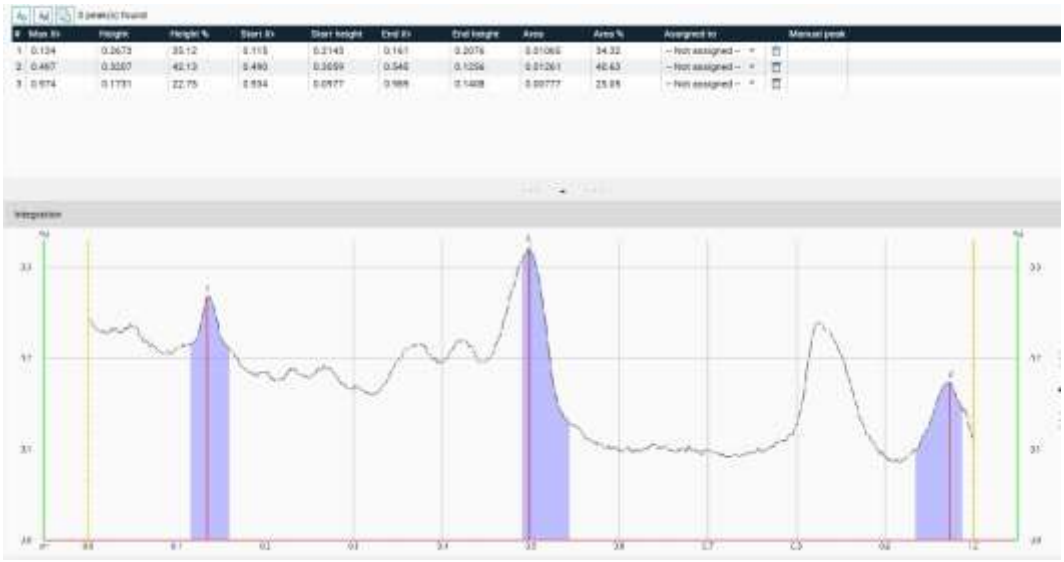
HPTLC densitometry chromatogram of chloroform extracts of Jawarish-e-Falafali (03 batches) at 254 nm



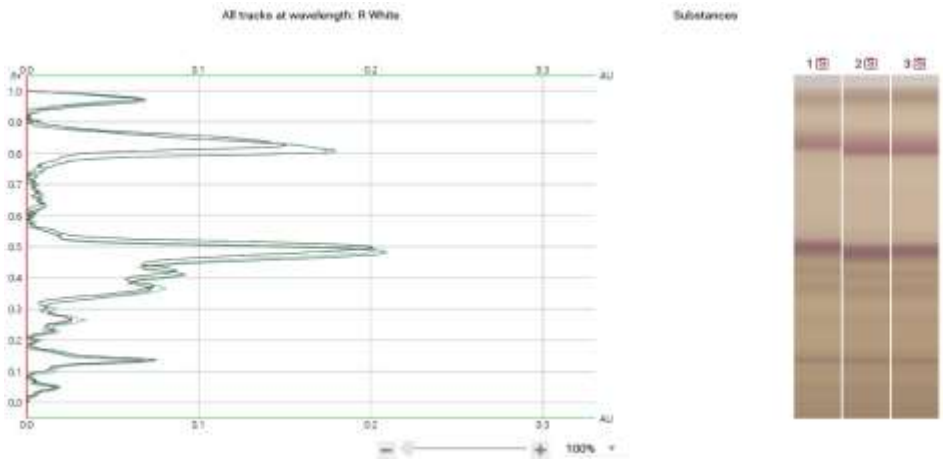
HPTLC fingerprint profile of chloroform extract of Jawarish-e-Falafali at 366 nm



HPTLC densitometry chromatogram of chloroform extracts of Jawarish-e-Falafali (03 batches) at 366nm

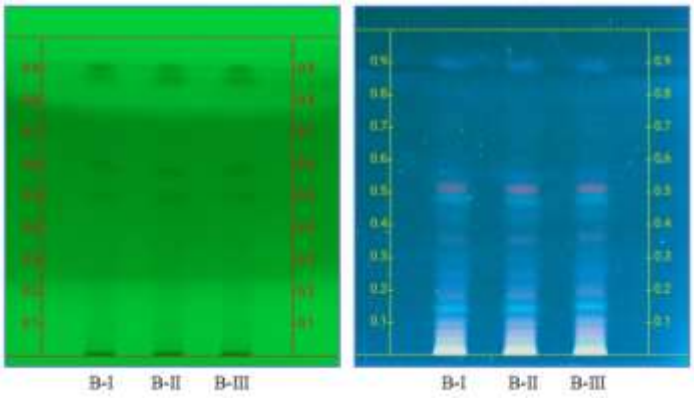


HPTLC fingerprint profile of chloroform extract of Jawarish-e-Falafali under white light after derivatization

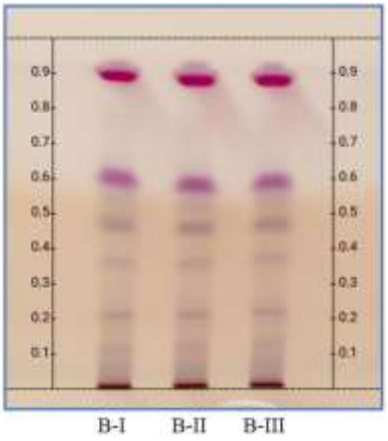


HPTLC densitometry chromatogram of chloroform extracts of Jawarish-e-Falafali (03 batches) under white light after derivatization

HPTLC of ***Ethanol*** extracts of Jawarish Falafali

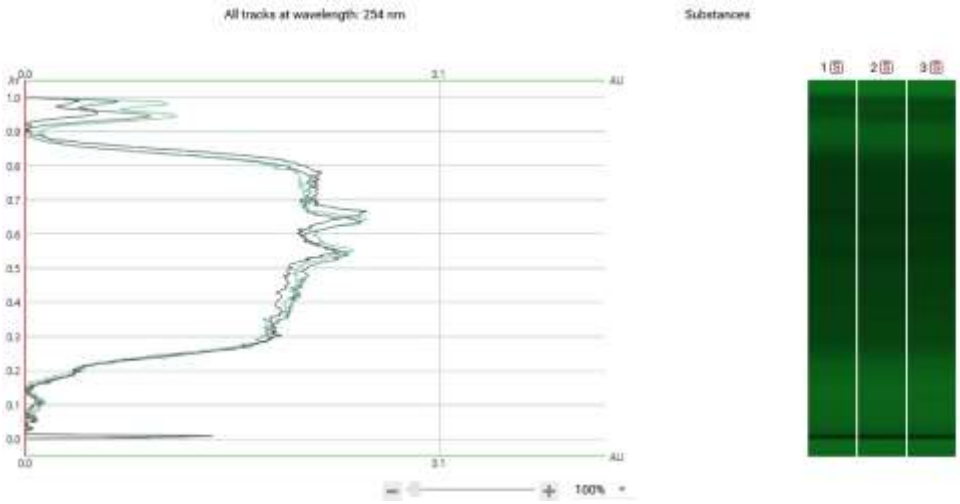


UV 254 nm **UV 366 nm**

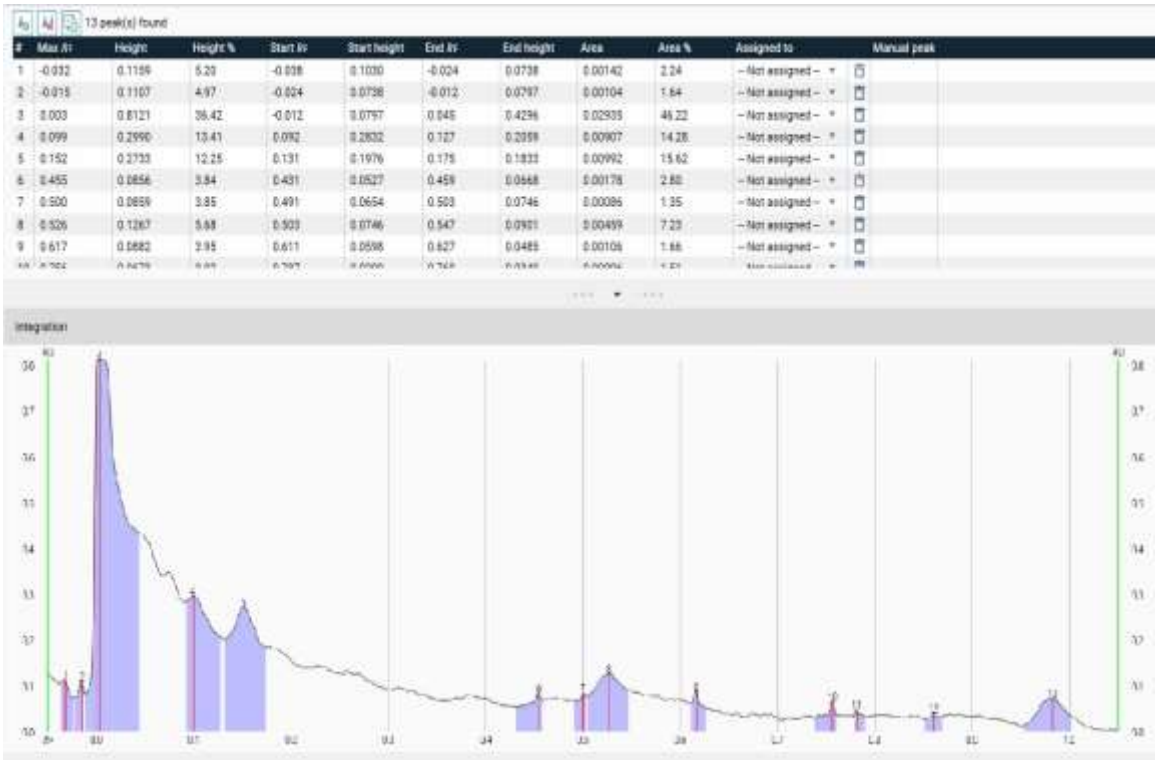


Visible Light (After derivatization)

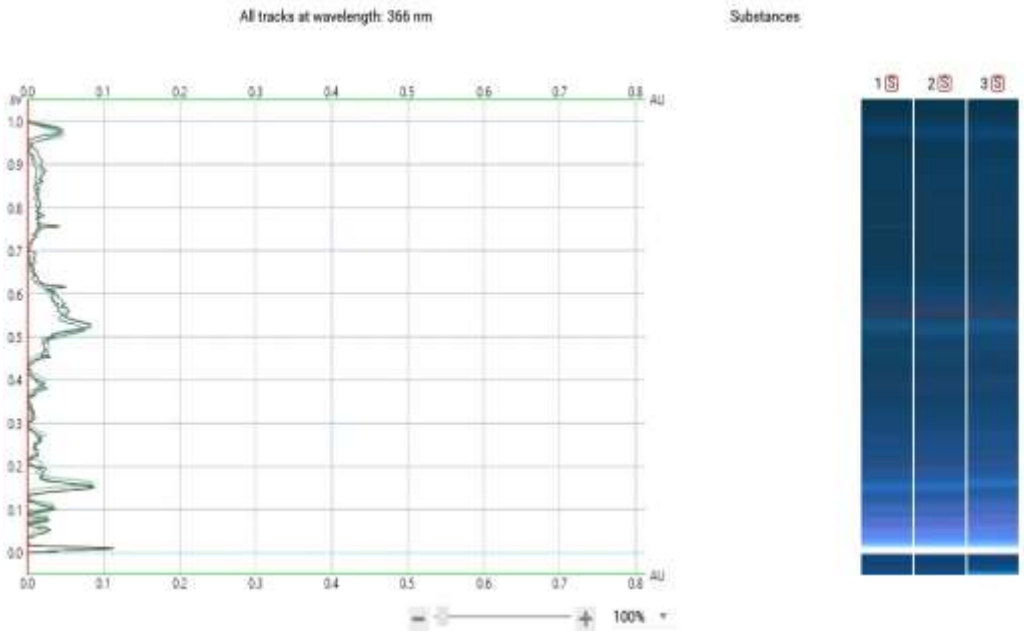
HPTLC fingerprint profile of ethanol extract of Jawarish-e-Falafali at 254 nm



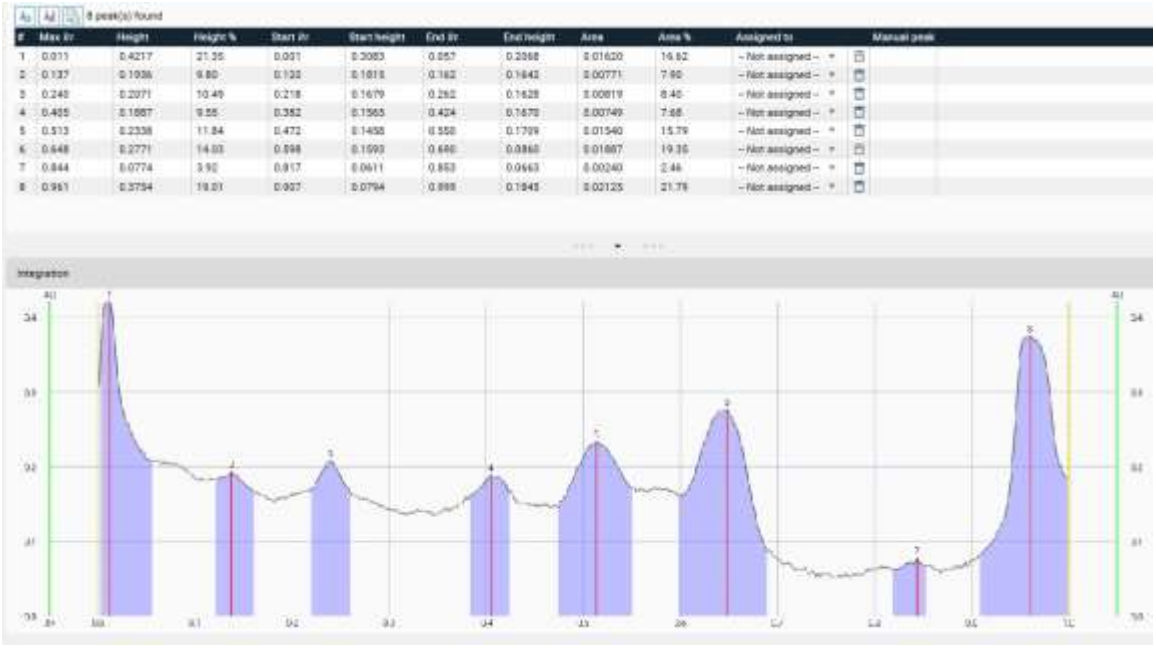
HPTLC densitometry chromatogram of ethanol extracts of Jawarish-e-Falafali (03 batches) at 254 nm



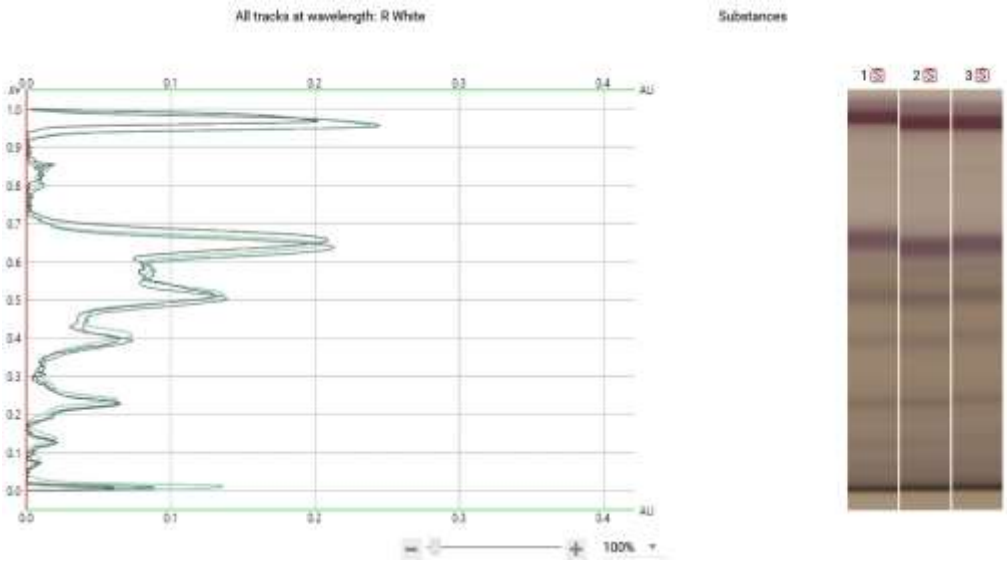
HPTLC fingerprint profile of ethanol extract of Jawarish-e-Falafali at 366 nm



HPTLC densitometry chromatogram of ethanol extracts of Jawarish-e-Falafali (03 batches) at 366 nm



HPTLC fingerprint profile of ethanol extract of Jawarish-e-Falafali under white light after derivatization



HPTLC densitometry chromatogram of ethanol extracts of Jawarish-e-Falafali (03 batches) under white light after derivatization

Table 1: ***Formulation composition.***

S. No.	Unani Name	Botanical/ English Name	Part Used
1.	Filfil Siyah	*Piper nigrum* L.	Cooked and dried unripe fruit
2.	Filfil Safaid	*Piper nigrum* L.	Ripe, Peeled fruit Seeds
3.	Filfil Daraz	*Piper longum* L.	Fruit
4.	Zanjabeel	*Zingiber officinale* Roscoe	Rhizome
5.	Ood Balsan	*Commiphora gileadensis* (L.) C. Chr.	Wood

6.	Tukhm-e-Karafs	*Apium graveolens* L.	Seed
7.	Saleekha	*Cinnamomum cassia* (L.) J.Presl.	Bark
8.	Asaroon	*Asarum europaeum* L.	Root
9.	Asl	Honey	As such

Table 2: Physico-chemical Parameters.

S. NO.	Parameters	Results
1	Water soluble extractive (%)	68.55-70.84
2	Alcohol soluble extractive (%)	51.64-53.78
3	Loss in wt. on drying at 105° C	09.04-9.96
4	Total ash (%)	0.86-1.07
5	Acid insoluble ash (%)	Nil
6	pH of 1% aqueous solution	5.50-5.64
7	pH of 10% aqueous solution	6.09-6.21
8	Reducing sugar	64.24-66.15
9	Non-reducing sugar	20.70-22.94
10	Bulk density	1.4672-1.5092

Table 3: Microbial load.

Total aerobic bacterial count (TABC)	$3.7x10^3$ CFU/gm
Total yeast and molds count (TYMC)	$4.1x10^2$ CFU/gm
Enterobacteriaceae members	
Escherichia coli	ND
Salmonella 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

Table 4: Aflatoxins.

B1	ND
B2	ND
G1	ND
G2	ND

Table 5: Heavy Metals.

Arsenic	ND
Cadmium	ND
Lead	ND
Mercury	ND

*ND – Not detected

Table 6: *Pesticide residue* (Analyzed by Thermo Fisher TSQ 9000 Triple Quadrupole GC-MS/MS system).

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 is an important aspect of any herbal formulation development. It is important to identify and record the physical, physicochemical and chemical properties of each plants material that is involved in product development of Jawarish-e-Falafali to maintain batch to batch consistency and quality of the product. The physico-chemical parameters will be helpful for fixing pharmacopoeial standards of the drug. HPTLC finger print profile of chloroform and alcohol extracts provides a suitable method for monitoring the identity and purity of the drug. Analyzed data of quality control parameters viz. microbial load, Aflatoxins, Pesticidal residue and Heavy metals were found to be within the permissible limit of WHO, indicating that the drug is free from toxic materials and can be used safely in the ailments of stomach ache, digestive issues and as laxative. The evaluated data in the above studies ensures the authenticity, quality and efficacy of the medicine Jawarish-e-Falafali.

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