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Phytochemical Fingerprinting of a pharmacopoeial Unani formulation Habb e Asgandh by TLC and HPLC for Standardization

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ABSTRACT

Habb-e- Asgandh a pharmacopoeial Unani preparation used for treating joints pain was formulated and then extracted in absolute ethanol and with the 50% alcohol. After drying these extracts, HPLC & TLC fingerprints were developed. TLC studies of Ethanolic and Hydroalcoholic extract of the formulation were carried out using two mobile phases (1) Toluene, Chloroform, Acetone and Acetic acid (70:15:15+2 drops) and (2) Benzene and Ethyl acetate (90:10). Thereafter, the TLC plates were observed in daylight, UV long and short wave length, and also treated with Iodine vapours and Anisaldehyde spray. Different numbers of spots were observed and Rf values alongwith colour were documented. HPLC of Ethanolic and Hydroalcoholic extract of Habb-e- Asgnadh was also carried out at 254 nm wavelength and the chromatogram was obtained. The mobile phase and flow rate was HPLC grade methanol and 1ml/min respectively. The HPLC profile of the Ethanolic extract of the test formulation gives 21 peaks and of the Hydroalcoholic extract of the formulation depicted total 6 peaks The TLC and HPLC profile of the test formulation can be used for standardization and quality control for future batches of Habb-e- Asgnadh.

Keywords: *Habb-e- Asgandh, TLC, HPLC, Quality control, Standardization*

Introduction

There are many single or compound formulations in the Unani System of Medicine which have high success rate for treating many diseases but lacking in quality and hence acceptance among community. Therefore, quality control and standardization become unavoidable. Thus there is an urgent need for the appraisal of the plant originated drugs used in Unani System of Medicine on physicochemical parameters which make them more acceptable globally [1]. In Unani system of medicine, treatment mainly depends on herbal drugs and their formulations. But from collection to storage and from storage to processing and then production of finished goods, there are chances of deterioration in quality of herbal drugs. A Good quality drug produces maximum efficacy and safety. Therefore, all the drugs should be standardized before use to ensure the quality and hence efficacy of the drug and safety of the patient. There are many parameters which can be performed to standardize a single drug or formulation such as organoleptic characters, extractive values, ash values, solubility of the drug, pH, qualitative analysis, melting point, boiling point and chromatographic fingerprinting etc. Among these parameters chromatographic fingerprinting is a relatively new concept for the analysis of quality of herbal samples.

Chromatographic techniques are the basic, simple, quick and confirmed method of analysis for standardization and quality assurance. Chromatographic fingerprinting includes TLC, HPLC, HPTLC and GC-MS etc. originally it has been developed with the application of HPLC. Therefore, in this study a commonly used Pharmacopoeial Unani formulation Habb-e-Asgandh was selected for standardization and quality assurance on the basis of TLC and HPLC profile. Its Ethanolic and Hydro-alcoholic extracts were screened for chromatographic fingerprinting. Habb-e-Asgandh is an anti-inflammatory (Mohallil-e-Awarm) formulation and can be used to treat Arthralgia (*Waja-al-Mafasil*), Coxalgia (*Waja-al-Warik*) and other inflammatory pain conditions. Though, some standards such as microscopic characteristics, TLC in Chloroform and Methanol and few Physico-chemical parameters like Ash Values, pH, and Disintegration time etc. are mentioned in Unani Pharmacopoeia but to assure the quality at higher level chromatographic fingerprinting is essentially required.

Materials and Methods

The Unani pharmacopoeial formulation i.e Habb-e-Asgandh contains the following ingredients (table 01) [2][3].

Table 01: Composition of Habb-e-Asgandh

S. No.	Ingrdients	Scientific names	Part used	Quantity
1.	Asgandh	*Trachyspermum ammi* (L.) Sprague	Root	40 gm
2.	Ajwain Desi	*Withania somnifera* Dunal	Fruit	20 gm
3.	Bidhara	*Argyreia nervosa* (Burm. f.)	Root	40 gm
4.	Filfil Moya	*Piper longum* Linn.	Stem	20 gm
5.	Filfil Daraz	*Piper longum* Linn.	Fruit	20 gm
6.	Zanjabeel	*Zingiber officinale* Rosc.	Rhizome	40 gm
7.	Satawar	*Asparagus racemosus* Willd.	Tuberous root	40 gm
8.	Musli Siyah	*Curculigo orchioides* Gaertn.	Rhizome	20 gm
9.	Qand Surkh (Jaggery)	*Saccharum officinarum*		50 gm

Procurement of raw material: All the chemicals and reagents of analytical grade, make Thomas Fishers India Pvt. Ltd Chemicals, Mumbai, India, were purchased through the registered supplier of Aligarh Muslim University. Plant drug materials were purchased from Dawakhana Tibbiya College Aligarh Muslim University, and the local market Barahdwari, Aligarh.

Preparation of Habb-e-Asgandh

Took all the ingredients of the formulation as per given quantity and were made free from all the dirt and adulterations and dried in the hot air oven except Qand Surkh. All the drugs were powdered in an electric grinder and passed through sieve no.85. Qand Surkh were also made free from dirt and adulterants and melted in Distilled Water on fire to become semisolid. This powdered material in the requisite degree of fineness mixed and damped with a moistening agent (Semisolid mixture of Qand Surkh and appropriate quantity of Distilled Water) in order to make Lubdi (Dough). Sticks of lubdi were made by automatic stick making machine and then sticks were cut into uniform, round and small pieces by stick cutting machine. Now the pills of 650 mg were made by rolling in between index finger and thumb. The colour of pills was yellowish brown pills with characteristic taste and odour.

Extraction of formulation

30 gm powder of Habb-e-Asgandh was taken and extracted in Ethanol and distilled water in the ratio of 1:1 (i.e Hydroalcoholic-400 ml) and in absolute alcohol (400 ml) with the help of Soxhlet apparatus. The hydroalcoholic and alcoholic extraction was carried out for a period of minimum six hours. Both the extracts were filtered using Whatman filter paper No.1. The filtrate was properly dried on low temperature at water bath. These dried extracts were used for both HPLC & TLC fingerprinting.

Thin Layer Chromatography (T.L.C)

TLC was carried out on TLC pre-coated Aluminum plates, silica gel 60F 254 (layer thickness 0.25mm). Before performing TLC, the plates were heated at 110^0C in a hot air oven for 5 minutes to remove the moisture if any (activation of plates). The spots of both the alcoholic and hydro-alcoholic extracts were made on TLC plates and eluted in pre decided suitable mobile phase [(1)Toluene, Chloroform, Acetone and Acetic acid (70:15:15+2 drops) and (2) Benzene and Ethyl acetate (90:10)]. Later spots were observed in day light, ultra-violet radiation, exposure to iodine vapours and after spraying anisaldehyde. The R_f (retardation factor) values for the different spots were calculated as per formula given below.

R_f = Distance travelled by spot / Distance travelled by solvent front

High Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) analysis of alcoholic and hydroalcoholic extracts of the test drug was performed using a Shimadzu HPLC system. The system included an LC-20AD isocratic solvent pump, an SPD-20A UV/VIS detector, and a rheodyne injector with a 20 µL sample loop. A C18 column (250 × 1.6 mm, 5 µm) with HPLC-grade methanol as the mobile phase was used. The analysis was conducted at 254 nm with a flow rate of 1 mL/min for 15 minutes, and retention times of eluted peaks were recorded at ambient temperature. All chemicals used were of HPLC grade.

Results

TLC analysis of Hab-e-Asgandh

TLC of both ethanolic and hydroalcoholic extract of the test drug was carried out using different mobile phases and spraying reagents. The R_f values and color of each spots, along with mobile phase and spraying reagent are shown in table no.2, 3, 4 and 5. Out of various solvent system tried, (1)Toluene, Chloroform, Acetone and Acetic acid (70:15:15+2 drops) and (2) Benzene and Ethyl acetate (90:10) was found suitable to carry out TLC profile, because it gives maximum number of spots which were well separated to each other.

The TLC of Ethanolic extract of the formulation with mobile phase Toluene, Chloroform, Acetone and Acetic acid gives only 1 spot in daylight, 6 spots by treating with iodine vapours, 10 spots with anisaldehyde spray, while in UV light, it gives 9 spots in UV long wave length and 5 spots in UV short wave length (Table 2, Figure 1). Whereas with mobile phase Benzene and Ethyl acetate the TLC of Ethanolic extract depicted no spot in daylight, 7 spots with iodine vapours, 12 spots on spraying anisaldehyde & 8 and 5 spots by UV long and short wave length respectively (Table 3, Figure 2).

Similarly hydroalcoholic extract of the test formulation shows no spot in daylight, 3 spots each in UV short and long wave length, 11 spots in anisaldehyde spray and 7 spots on treatment with iodine vapours in iodine chamber with Toluene, Chloroform, Acetone and Acetic acid mobile phase (Table 4, Figure 3). Whereas in another mobile phase, TLC of extracts of Habb-e- Asgandh shows 5 and 3 spots in UV short and UV long wave length respectively. 5 spots shown on anisaldehyde spray and 7 spots were appeared on treatment with iodine vapours while in day light no spot was observed (Table 5, Figure 4).

Table 2: TLC of Alcoholic extract of Habb-e- Asgandh

Spray/ Light Treatment	Mobile phase: Toluene: Chloroform: Acetone: Acetic acid (70:15:15+2 drops)	
	No. of Spots	R_f values and colour of spots
Day Light	1	0.27(LG)
UV Short Wavelength	5	0.28(DP), 0.50(DP), 0.57(DP), 0.62(DP), 0.68(DP)
UV Long Wavelength	9	0.24(Y), 0.28(Y), 0.33(Y), 0.39(LB), 0.44(O), 0.53(Y), 0.64(Y), 0.71(Y), 0.77(Y),
Iodine Vapour	6	0.28(DB), 0.4(DB), 0.52(DB), 0.6(DB), 0.68(LB), 0.73(LB)
Anisaldehyde Spray	10	0.18(B), 0.22(B), 0.29(G), 0.35(GR), 0.39(DPi), 0.45(Pi), 0.52(DG), 0.6(DG), 0.69(P), 0.75(O)

Table 3: TLC of Alcoholic extract of Habb-e- Asgandh

Spray/ Light Treatment	Mobile phase: Benzene: Ethyl acetate (90:10)	
	No. of Spots	R_f values and colour of spots
Day Light	0	
UV Short Wavelength	5	0.23(P), 0.37(P), 0.44(P), 0.46(P), 0.52(P)
UV Long Wavelength	8	0.13(LY), 0.18(LY), 0.23(LY), 0.28(LY), 0.35(LY), 0.38(LY), 0.65(LY), 0.71(LY)
Iodine Vapour	7	0.20(DY), 0.25(DY), 0.31(DY), 0.36(DY), 0.43(DY), 0.49(DY), 0.68(DY)
Anisaldehyde	12	0.22(B), 0.30(Dpi), 0.33(G), 0.38(G), 0.46(DG), 0.52(DG), 0.64(Dpi), 0.70(Dpi), 0.77(O), 0.84(BL), 0.92(GR), 0.97(B)

P-purple, LY-light yellow, DY-dark yellow, B-black, GR-grey, BL-blue, O-orange, Dpi-dark pink, DG-dark green, G-green, b-black

Table 4: TLC of Hydroalcoholic extract of Habb-e- Asgandh

Spray/ Light Treatment	Mobile phase: Toluene: Chloroform: Acetone: Acetic acid (70:15:15+2 drops)	
	No. of Spots	R_f values and colour of spots
Day Light	0	
UV Short Wavelength	3	0.58(P), 0.66(P), 0.72(P)
UV Long Wavelength	3	0.49(Y), 0.59(Y), 0.76(Y)
Iodine Vapour	7	0.30(Br), 0.36(Br), 0.48(Br), 0.57(Br), 0.63(Br), 0.67(Br), 0.72(Br)
Anisaldehyde	11	0.21(GR), 0.29(GR), 0.33(GR), 0.38(GR), 0.42(GR), 0.47(Dpi), 0.53(Lpi), 0.59(G), 0.63(G), 0.68(B), 0.79(Pi)

P-purple, Y-yellow, BR-brown, Pi-pink, B-black, G-green, Lpi-light pink, Dpi- dark pink, GR-grey

Table 5: TLC of Hydroalcoholic extract of Habb-e- Asgandh

Spray/ Light Treatment	Mobile phase: Benzene: Ethyl acetate (90:10)	
	No. of Spots	R_f values and colour of spots
Day Light	0	
UV Short Wavelength	5	0.25(P), 0.31(P), 0.37(P), 0.44(LP), 0.62(LP)
UV Long Wavelength	3	0.29(Y), 0.35(Y), 0.64(Y)
Iodine Vapour	7	0.11(DB), 0.17(DB), 0.27(DB), 0.31(DB), 0.35(DB), 0.44(LB), 0.62(LB)
Anisaldehyde	5	0.22(LP), 0.26(Y), 0.33(Y), 0.37(DBL), 0.6(Dpi)

LP-light purple, P-purple, Y-yellow, LB-light brown, DB-dark brown, Dpi-dark pink, DBL-dark blue,

TLC profile of Ethanolic extract of Habbe Asgandh

Fig. 1. Toluene:Chloroform:Acetone:Acetic acid (70:15:15:Drops)

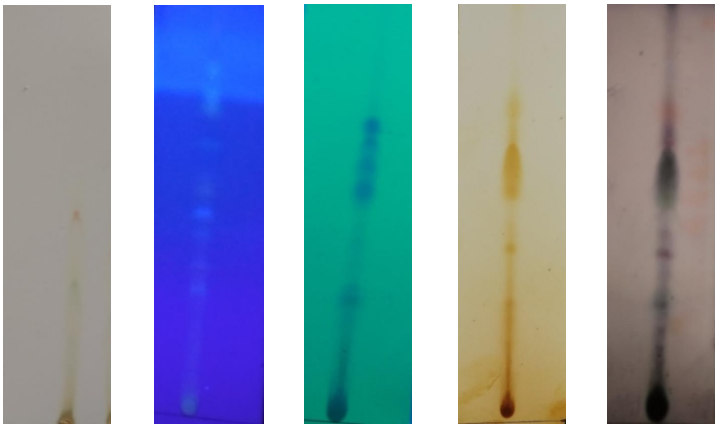
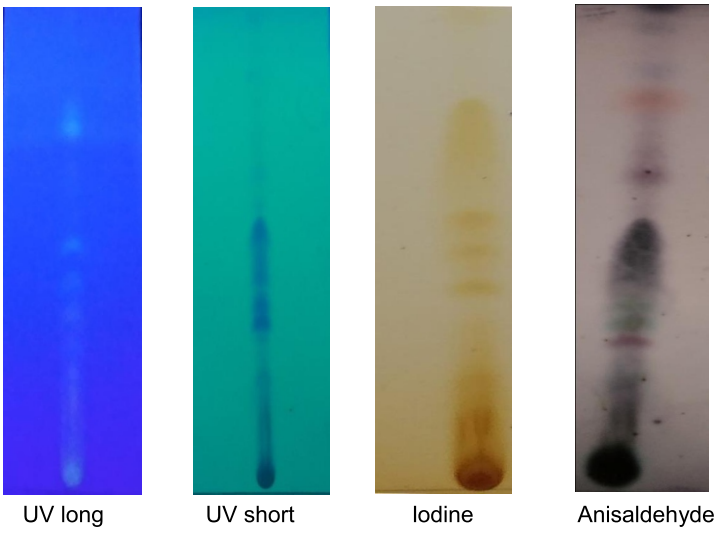


Fig. 2. Benzene:Ethyl acetate (90:10)



TLC profile of Hydroalcoholic extract of Habbe Asgandh

Fig. 3. Toluene:Chloroform:Acetone:Acetic acid(70:15:15:Drops)

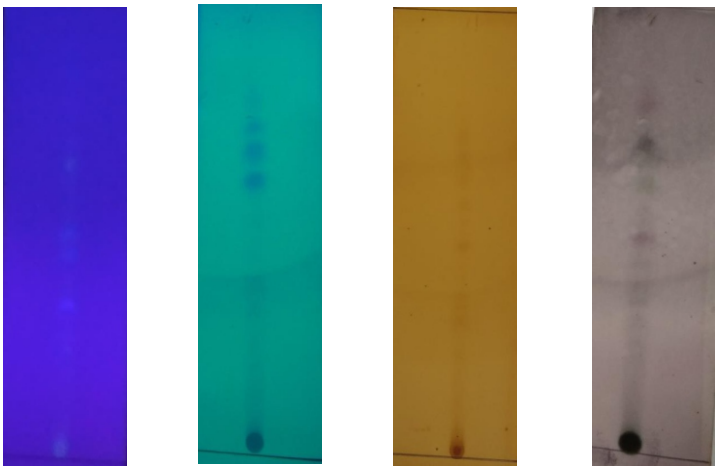
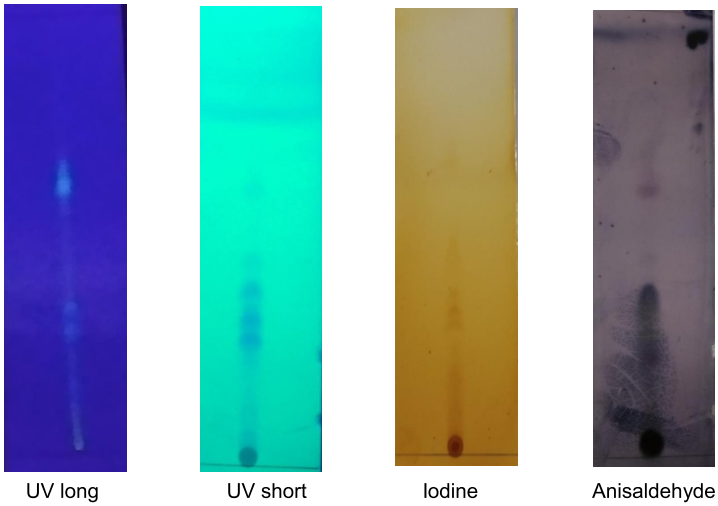


Fig. 4. Benzene:Ethyl acetate(90:10)



HPLC Analysis

Ethanolic and Hydroalcoholic extract of Habb-e- Asgandh was also analysed by HPLC to generate HPLC profile for standardization and future reference. Methanol of HPLC grade was used as mobile phase and the chromatogram was obtained.

The HPLC chromatogram of the Ethanolic extract of the test formulation shows 21 Peaks and the peak no 3 and 1 was the major peaks of the profile while peak no. 1 with retention time 2.787 minutes have maximum area percentage and thus having maximum concentration in the injected sample (Table 6, Figure 5).

Similarly, the HPLC profile of the Hydroalcoholic extract of the formulation depicted 6 Peaks and the peak no 4 is the major peak having concentration 39.944% and the retention time was found to be 3.652 minutes (Table 7, Figure 6).

Table 6: HPLC Profile of Alcoholic extract of Habb-e- Asgandh

Peak #	Ret. Time	Area	Height	Area %	Height %
1	2.787	60677413	2576892	36.157	21.894
2	3.397	7790668	712820	4.642	6.056
3	3.658	38604136	3999635	23.004	33.981
4	3.859	14037212	1346440	8.365	11.440
5	4.198	7621863	681240	4.542	5.788
6	4.669	5686263	436112	3.388	3.705
7	4.877	1893071	147672	1.128	1.255
8	5.206	1394394	100859	0.831	0.857
9	5.496	1308388	87272	0.780	0.741
10	6.234	11036079	690130	6.576	5.863
11	6.459	34975	2866	0.021	0.024
12	7.371	2885630	173032	1.720	1.470
13	7.770	9907083	613856	5.903	5.215
14	8.275	688859	32528	0.410	0.276
15	9.421	1997974	84768	1.191	0.720
16	9.866	748170	40807	0.446	0.347
17	10.410	593833	19320	0.354	0.164
18	11.405	244044	6756	0.145	0.057
19	12.267	302231	7787	0.180	0.066
20	13.534	211965	4450	0.126	0.038
21	13.961	153421	4798	0.091	0.041
Total		167817672	11770038	100.000	100.000

Table 7: HPLC Profile of Hydroalcoholic extract of Habb-e- Asgand

Peak #	Ret. Time	Area	Height	Area %	Height %
1	2.703	3240998	324074	9.291	17.402
2	2.827	4304472	298340	12.340	16.021
3	3.387	13382539	618621	38.365	33.219
4	3.652	13933195	618267	39.944	33.200
5	4.309	14201	2643	0.041	0.142
6	10.420	6722	290	0.019	0.016
Total		34882126	1862236	100.000	100.000

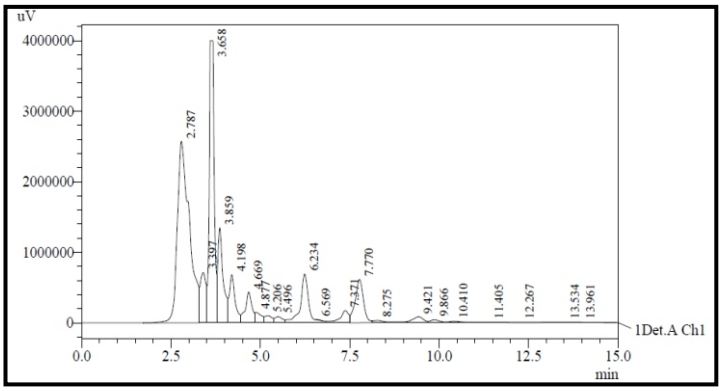


Fig 5: HPLC Chromatogram of Alcoholic extract of Habb-e- Asgandh

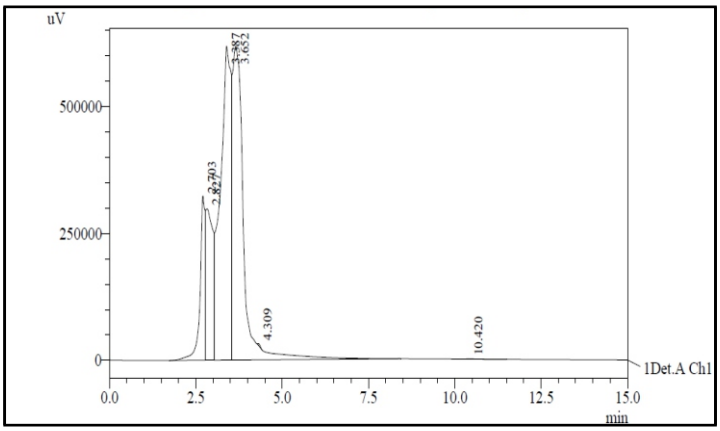


Fig 6: HPLC Chromatogram of Hydroalcoholic extract of Habb-e- Asgandh

DISCUSSION

According to World Health Organization (WHO), more than 80% of the world's population rely on traditional medicine for their primary health care needs and most of them are from plant source [4]. In Tibb-e-Unani there are many medicinal plants, mineral and animal origin drugs which have been mentioned by various authors in the classical literature to control Arthritis. But the scientific validation of their physico-chemical and pharmacological properties is the need of the day. Physico-chemical standardization of single and/or compound formulations of Unani System of Medicine is the basic need to make them acceptable globally for therapeutic uses [5].

Quality control parameters should be established for every plant, mineral and animal origin drugs and their product available in the market because, the scope for variation in different batches of medicine is enormous. The chemical contents of plant products also depend on environmental changes, places of collection, times of collection, soil conditions and environmental factors which include the rainfall, temperature and frost etc. Adding to this variability is the fact that in Unani System of Medicine several plants, minerals and animals may be used together in the same preparation. This means that there should be a quality control test and standardization for the entire preparation and for every single drug to ensure authenticity of the Unani formulation [6]. Habb-e-Asgandh is also an important drug of Unani System of Medicine having known antiarthritic activity. This preparation is frequently used by physicians for the management of Waja-ul-Mafasil. Therefore, it is necessary to standardize this formulation on scientific bases to assure its quality and make it acceptable worldwide. Thus, in the present study, Habb-e-Asgandh was analyzed by chromatographic techniques at least to set a profile for quality assurance for future reference [7].

Chromatographic fingerprinting has emerged as a crucial tool for the quality control and standardization of herbal medicines. This technique provides a comprehensive chemical profile of herbal extracts, enabling the identification and authentication of medicinal plants based on their unique chemical signatures.

The chromatographic pattern obtained reflects the presence of bioactive compounds or characteristic constituents, which are essential for ensuring batch-to-batch consistency and detecting adulteration or contamination. One of the significant advantages of chromatographic fingerprinting is its ability to differentiate between closely related species and to identify variations caused by environmental factors, harvesting conditions, and processing methods. Various chromatographic techniques, including High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Thin-Layer Chromatography (TLC), are employed for fingerprint analysis. These techniques allow for the qualitative and quantitative assessment of herbal components, aiding in regulatory compliance and quality assurance, chromatographic fingerprinting is not limited to crude plant materials; it is equally applicable to processed herbal formulations, ensuring quality control at multiple stages of production. The integration of advanced chemometric tools enhances the accuracy of this technique, enabling data interpretation through multivariate statistical analysis, chromatographic fingerprinting serves as a reliable, reproducible, and scientifically validated approach for the standardization, authentication, and safety assessment of herbal medicines, ensuring their therapeutic efficacy and consumer safety.

The present study is comprises of chromatographic fingerprinting of hydroalcoholic and alcoholic extracts of Habb-e- Asgandh by means of TLC and HPLC. Both the parameters may also be used for detecting or identifying adulteration or exhausted drug materials. If the drug substance is adulterated with any material or exhausted, then number of spots in TLC or peaks in HPLC varies from standard values. Therefore, TLC and HPLC profile was done and the data obtained may be used for standardization of Habb-e- Asgandh in future.

Thin Layer Chromatography

Thin Layer Chromatography (TLC) is a rapid and cost-effective technique for identifying and separating drug compounds. It is widely used for analyzing pure drugs, pharmaceutical

formulations, and biological samples. TLC helps in detecting adulterants, ensuring drug purity, and establishing herbal fingerprints. Advanced techniques like HPTLC enhance sensitivity and reproducibility for better quality control [9][10]. TLC studies of ethanolic and hydroalcoholic extract of the test drug has been conducted in two different solvent system as. (1) Toluene, Chloroform, Acetone and Acetic acid (70:15:15+2 drops) and (2) Benzene and Ethyl Acetate (90:10), TLC plates were later on treated by different substances (ultra violet radiation in Uv chamber, iodine vapours in iodine chamber and Anisaldehyde spray). The colour and Rf values of the spots were determined and depicted in table no.1, 2, 3 and 4. The alcoholic extract of the formulation was subjected to TLC with mobile phase Toluene, Chloroform, Acetone and Acetic acid. It gives only 1 spot in daylight, 6 spots by treating with iodine vapours, 10 spots with anisaldehyde spray, while in UV light, it gives 9 spots in UV long wave and 5 spots in UV short wave (Table no. 1, Fig.1). Whereas TLC of both extracts of the test drug with Benzene and Ethyl acetate mobile phase depicted 7 spots with iodine vapours, 12 spots on spraying anisaldehyde, 8 and 5 spots by UV long and short wave length respectively (Table no. 2, Fig.2).

Similarly TLC of hydroalcoholic extract of the test formulation in the presence of Toluene, Chloroform, Acetone and Acetic acid shows 3 spots each in UV short and long wave length, 11 spots in anisaldehyde spray and 7 spots on treatment with iodine vapours (Table no. 3, Fig.3). Whereas in another mobile phase, TLC of hydroalcoholic extracts of Habb-e- Asgandh shows 5 and 3 spots in UV short and UV long wave length respectively, 5 spots shown on anisaldehyde spray and 7 spots were appeared on treatment with iodine vapours. (Table no. 4, Fig. 4).

High Performance Liquid Chromatography

It is a technique used for separation, identification and quantification of mixture of components. It is a type of liquid chromatography and a powerful tool to establish the authenticity and quality of single and/or compound drugs [11]. HPLC profile of both alcoholic and hydroalcoholic extracts of the test drug was carried out using methanol as a mobile phase. The chromatogram was obtained at a flow rate of 1 ml/min and wave length 254 nm. The HPLC profile of the Ethanolic extract of the test formulation shows 21 Peaks and the peak no 3 and 1 was the major peaks of the profile while peak no. 1 with retention time 2.787 minutes have maximum area percentage and thus having maximum concentration in the injected sample (Table no. 6, Fig.5). Similarly the HPLC profile of the Hydroalcoholic extract of the formulation depicted 6 Peaks and the peak no 4 is the major peak having concentration 39.944% and the retention time was found to be 3.652 minutes (Table no. 7, Fig.6).

CONCLUSION

The data obtained by chromatographic fingerprinting i.e. Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) of lab samples of Habb-e-Asgandh can be used for standardization and quality control for future batches of the given formulation prepared by different Unani pharmaceutical industries. The data procured by the study may also be used to impede the adulteration and/ or exhaustion of the formulation. The Formulation may also be studied for other parameters of standardization to generate complete profile to improve quality control, so that it can be accepted globally and used safely and effectively.

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