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Phytochemical Screening and Novel Chromatographic Analysis of Unani Anti-Diabetic Combinatorial Therapy by HPTLC

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ABSTRACT

Murakkab drugs (combinatorial therapy) of Unani medicine have remained an important criterion of disease treatment since antiquity. The present study reports the phytochemical screening, chromatographic analysis of Unani combinatorial therapy or polyherbal extract by HPTLC method. The Methanolic extract prepared in two combinations coded as M.ALWC and M.WAS has shown potent antioxidant and anti-diabetic properties in STZ- induced diabetic rats. The present work was to develop a high-performance thin-layer chromatography (HPTLC) analysis technique using the solvent system developed with n-Hexane: Ethyl Acetate (7:3) v/v) on precoated aluminum silica gel plates (Merck), and densitometric determinations were done at 254 and 366 nm respectively. Various other physicochemical parameters evaluated are ash values, moisture content, extractive values, including Phytochemical screening in various extracts, thin layer chromatography (TLC), and high-performance TLC (HPTLC). A HPTLC fingerprint is the individual chromatographic track representing, as near as possible, a mixture of organic substances. By the fingerprint approach, it is possible to obtain a proper identification of the plant material, but also determine and accept the limits of the biological changes. Variations in HPTLC will track the quality of herbal formulations mainly from a quantitative perspective. A comparative account of the fingerprint TLC of any compound formulation along with its constituent ingredients will help in determining whether the genuine single drugs are mixed or not. Such studies will ensure the quality of medicine and ensure the action for which it is used.

1. INTRODUCTION:

The use of combinatorial therapy for treating disease was started centuries back by Unani physicians. It is mentioned in eminent Unani literature given by *Ali Ibne-Abbas Majoosi*, *DawoodAntaki*, *Ibn-Rushd*and *Abu SahlMaseeh*, that the treatment through Unani drugs has been rationalized in the manner that if the single drug is not adequate to treat a disease, then Murakkab drug may be prescribed.[1-4]Unani medicines are dispensed as classical, patent, and proprietary formulations well prepared using AdviaMufarradah (single drugs) and AdviaMurakkabah (compound drugs) mainly derived from plants [5]. The Unani system of medicine is a well-established scientific system of Medicine based on the teaching of Hippocrates (Buqrat-460-377BC). Galen (Jalinoos-131-210-AD), developed it into an elaborate Medical System, and, Physicians like Rhazes (850-925-AD) and Avicenna (980-1037-AD), gave a scientific basis to the system and has an impressive development record in India and abroad. India, today, is the world leader in having the largest organized setup and an integral part of the National Health Care delivery system by herbal products. Given the present trend of commercialization, the Government of India is very conscious of the quality, safety, and efficacy of Unani Drugs. Herbal products with the highest quality standards are the key driver of success. Taking this into consideration, the need to ensure the quality of medicinal plant products by using modern control techniques and applying suitable standards[6-8].

The standards of Pharmacopoeia depend upon the quantitative tests like total ash, acid-insoluble ash, water-soluble ash, alcohol-soluble extractive, water-soluble extractive, moisture content, volatile oil content, and other advanced analysis procedures.[9,10] Murakkabat (compound formulations) have a vast field, to do research work and it is the responsibility of not only of Unani experts but those having belief in traditional medicine, to come forward to welcome challenges of a modern hour and establish a new field of research not only for single drugs but also in compound drugs with new analytical techniques[11]. High-performance thin-layer chromatography (HPTLC) fingerprinting is becoming an attractive and blooming field of separation science. The preparation of highly standardized herbal products concerning chemical composition and biological activity is considered to be a valuable approach in this field. Identification of major and unique compounds in herbs is the key step to ensure quality and optimum level of active principles for their bio potency.[12] High-performance thin-layer chromatography (HPTLC) as a preferred analytical tool with

advantages of selective detection, scanning, minimum sample preparation, full optimization, hyphenation, etc to figure out information of complex mixtures of inorganic, organic, and bio-molecules [13,14].

This work aims to elucidate the phytochemical screening of various secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, and terpenoids in the Methanolic extracts of two coded unani anti-diabetic polyherbal formulations, Physico-chemical and HPTLC fingerprint profile reported here for this valuable Unani formulation may be used as a reference standard for quality control in future.

SELECTION OF DRUGS:

The selection of drugs for the study is following the ancient and authentic unani books which have given coded names with the following mentioned properties: Blood Purifier, (Mussaffi-e-Dam), Resolvent (Muhalil), Astringent (Qabiz), Carminative (Kasir-e-riyah), Stomachic (Muqawwi-e-Maida), Absorbent (Jazib-e-rutubat), Liver and Heart Tonic (Muqawwi-e-Kabid and Qalb), Detergent (Jaali), Dessicative (Mujaffif), Cooling (Mubarrid), Refrigerant (Mufarrih) and Detoxificant and Antiseptic (Dafe-e Taffun) [15-22].

2. MATERIALS AND METHODS:

2.1. Chemicals

Ethyl Acetate, n-Hexane was purchased from Merck. Methanol and ethanol of analytical reagent grade (Merck, Darmstadt, Germany) were used. All other solvents and chemicals were of the highest analytical grade.

2.2. Apparatus

DESAGA SarstedtGruppe system was used for analysis along with Automatic TLC applicator and UV visible cabinet as an imaging system, the instrument had Proquant 1.6 version as a software system for documentation, glass twin trough chamber (20 cm × 10 cm × 4 cm), aluminum TLC plate pre-coated 0.2 mm thickness with silica gel 60 F254 (Merck) were used in this study. The experiment was carried out under conditions with a temperature of (25 ± 2) °C and relative humidity of 40%.

2.3. Preparation of Formulation:

The plant material was procured from the local market and identified by Botanist, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad before carrying out the study using pharmacognostic methods. The Drugs were cleaned, washed, shade-dried, and coarsely ground. The Drugs were macerated as two different combinations ALWC and WAS, Extracts obtained were filtered using Whatman paper for phytochemical screening and Quantitative studies [23-24].

2.4. Physico-chemical Analysis

Physico-Chemical parameters such as total ash, acid insoluble ash, solubility matter in alcohol and water, loss on drying at 105^0C, were carried out as per the methods described in WHO guidelines[25, 26]. Phytochemical screening was carried out in different solvents extracts such as Ethanol, methanol, Ethyl acetate, Chloroform, Petroleum ether, acetone, and aqueous extracts as per the methods described by Trease and Evans (1989) [27].

Table No. 1: Physico-chemical parameters (Identity, Purity and Strength) of the combinatorial therapy ALWC and WAS in Mean ± S.D.:

S.No:	Parameters	Mean ± S.D.	
		ALWC	WAS
1.	Water soluble matter (% w/w)	38.39 ± 0.44	25.39±0.29
2.	Alcohol soluble matter (% w/w)	14.03± 0.31	10.32 ± 0.25
3.	Loss of wt. on drying at 105°C (% w/w)	8.36 ± 0.07	9.7 ± 0.05
4.	Total ash (% w/w)	9.86 ± 0.37	4.43 ± 0.45
5.	Acid insoluble ash (% w/w)	4.97 ± 0.21	1.38 ± 0.43
6.	pH 1% aqueous solution	5.36 ± 0.01	4.32 ± 0.02
7.	pH 10% aqueous solution	5.92 ± 0.02	4.86 ± 0.03
8.	Volatile oil	Nil	Nil

Fig: 1 Comparison of Mean of Physico-chemical parameters of ALWC and WAS:

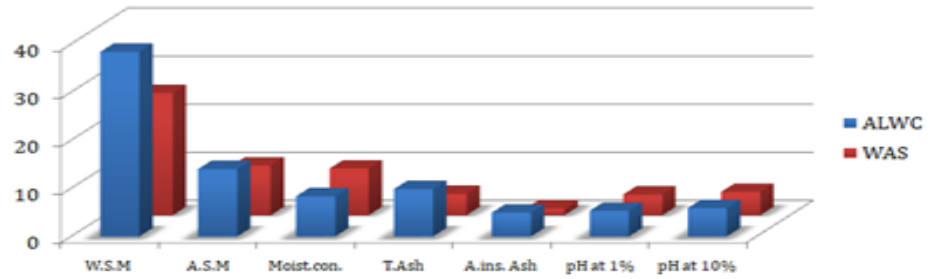

Figure showing the results of Physico-chemical parameters

Table No. 2: Phytochemical screening for the nature of compounds present in ALWC:

	Phytochemicals	**Solvents**					
		Ethanol	**Methanol**	**Chloroform**	**Aqueous**	**E.A.**	**Pet ether**
1.	**Alkaloids Test / Reagents:**						
	Dragendroff's reagent	+	+	+	+	+	+
	Hager's test	+	+	+	+	+	+
	Wagner's reagent	+	+	+	+	+	+
	Tannic acid test	+	+	+	+	+	+
	Mayer's reagent	+	+	+	+	+	+
2.	**Carbohydrates test:**						
	Fehling's test	+	+	-	+	+	+
	Molish's test	+	+	-	+	+	+
	Barfoed's test	+	+	-	+	+	+
	Benedict's test	+	+	-	+	+	+
3.	**Flavanoids (Shinoda test):**	+	+	+	+	+	+
4.	**Glycosides (NaOH Test)**	+	+	+	+	+	+
5.	**Tannins**						
	Ferric chloride test	+	+	+	+	+	+

	Gelatin test	+	+	+	+	+	+
	Lead acetate test	+	+	+	+	+	+
6.	**Proteins**						
	Warming test	+	+	-	+	+	+
	Biuret test	+	+	-	+	+	+
7.	**Steroids:**						
	Salkowski test	+	+	+	+	+	+
	Sulphur powder test	+	+	+	+	+	+
	Hosse's reaction	+	+	+	+	+	+
8.	**Saponins (Frothing with NaHCO3):**	+	+	+	+	+	+
9.	**Resinified volatile oil**	-	-	-	-	-	-
10.	**Fats and fixed oils (CuSO4):**	+	+	+	+	+	+

+ indicates presence and – indicates absence of phytochemical.

2.5. HPTLC / TLC Fingerprint Analysis:

2 g of a powdered sample of 2 combinations is taken and reflux with 50 ml of methanol separately using Soxhlet apparatus on a water bath for 30 min. The extract was filtered through Whatman No.41 filter paper. The filtrate was further concentrated up to 5 ml and used for HPTLC analysis. These solutions were used for HPTLC fingerprint analysis by applying the Methanol extract on aluminum TLC plate pre-coated with silica gel 60 F254 (E. Merck) using DESAGA SarstedtGruppe system with automatic sample applicator. The plate was developed up to a distance of 8cm in a twin trough glass chamber using the solvent system of n-Hexane: Ethyl Acetate (7:3) as mobile phase. The plate was air-dried at room temperature and observed under UV at 254nm & UV 366nm wavelengths and later on derivatized [28].

3. RESULTS AND DISCUSSION:

Data expressed in Table:1and Fig:1 shows the physicochemical parameters such as total ash of ALWC & WAS is 9.86 ± 0.37gm% and 4.43 ± 0.45gm%, acid insoluble 4.97 ± 0.21gm%

and 1.38 ± 0.43gm%, alcohol soluble matter 14.03± 0.31 gm% and 10.32 ± 0.25gm%, and water-soluble matter 38.39 ± 0.44gm% and 25.39±0.29gm% respectively; The moisture content i.e., Loss of weight on drying at 105^0C was also determined and found to be 8.36 ± 0.07gm% and 9.7 ± 0.05gm% .

In Table 2 and 3 reveals the Phytochemical screening for phytoconstituents was carried out in the various extracts. This discloses the presence of carbohydrates, alkaloids, glycosides, tannins, flavonoids, sterols, and saponins whereas chloroform extract revealed the absence of carbohydrates and proteins.

Table No. 3: Phytochemical screening for the nature of compounds present in WAS:

	Phytochemicals	**Solvents**					
		Ethanol	**Methanol**	**Chloroform**	**Aqueous**	**E.A.**	**Pet ether**
1.	**Alkaloids Test / Reagents:**						
	Dragendroff's reagent	+	+	+	+	+	+
	Hager's test	+	+	+	+	+	+
	Wagner's reagent	+	+	+	+	+	+
	Tannic acid test	+	+	+	+	+	+
	Mayer's reagent	+	+	+	+	+	+
2.	**Carbohydrates test:**						
	Fehling's test	+	+	-	+	+	+
	Molish's test	+	+	-	+	+	+
	Barfoed's test	+	+	-	+	+	+
	Benedict's test	+	+	-	+	+	+
3.	**Flavanoids (Shinoda test):**	+	+	+	+	+	+
4.	**Glycosides (NaOH**	+	+	+	+	+	+

	Test)						
5.	**Tannins**						
	Ferric chloride test	+	+	+	+	+	+
	Gelatin test	+	+	+	+	+	+
	Lead acetate test	+	+	+	+	+	+
6.	**Proteins**						
	Warming test	+	+	-	+	+	+
	Biuret test	+	+	-	+	+	+
7.	**Steroids:**						
	Salkowski test	+	+	+	+	+	+
	Sulphur powder test	+	+	+	+	+	+
	Hosse's reaction	+	+	+	+	+	+
8.	**Saponins (Frothing with NaHCO3):**	+	+	+	+	+	+
9.	**Resinified volatile oil**	-	-	-	-	-	-
10.	**Fats and fixed oils (CuSO4):**	+	+	+	+	+	+

+ indicates presence and – indicates absence of phytochemical.

HPTLC profile:

The results of the HPTLC profile are specified in Table No.4, 5, 6 and 7. HPTLC profiling is very reliable and convenient for compound formulations as plant species produce a distinct chromatogram. In Figure No.2 & 3, HPTLC photo plate scanned of M.ALWC and M.WAS was observed under UV 254nm and UV 366nm after derivatization.

In Fig.No:4 & Table: 4, The Methanolic extract of ALWC was spotted on silica gel "G" plate and developed with n-Hexane: Ethyl Acetate (7:3) as mobile phase shows two major spots under UV 366nm at R_f values 0.90 (blue) and 0.97 (blue);

In Fig.No:5 & Table:5, Methanolic extract of WAS spotted on silica gel "G" plate and developed with n-Hexane: Ethyl Acetate (7:3) as mobile phase shows three major spots in the

peak list under UV 366nm at R_f values 0.15 (blue), 0.90 (blue), and 0.97 (blue) as shown in table:5.

In Fig.No:6 & Table: 6 reveals Densitogram of methanol extract of ALWC at UV 254nm and peak list showing mainly two spots under UV 254nm at R_f values 0.90, 0.97 (both black).

In Fig.No:7 & Table: 7 elaborate Densitogram of methanol extract of WAS under UV 254nm shows two spots at R_f values 0.90, 0.97 (both black).

CONCLUSION:

It can be concluded that organoleptic parameters are not much reliable in the identification of polyherbal formulation as the ingredients are powdered and mixed for preparing compound formulation. The present study, therefore, holds high significance as the various Physico-chemical parameters, phytochemical screening, and HPTLC profile provide criteria for easy identification of the combination of drugs, and quality control analysis ensures the authenticity, quality, and efficacy of the medicine.

Figure No.2 & 3 TLC Chromatogram of Methanol Extract of ALWC and WAS at UV 366nm and 254nm:

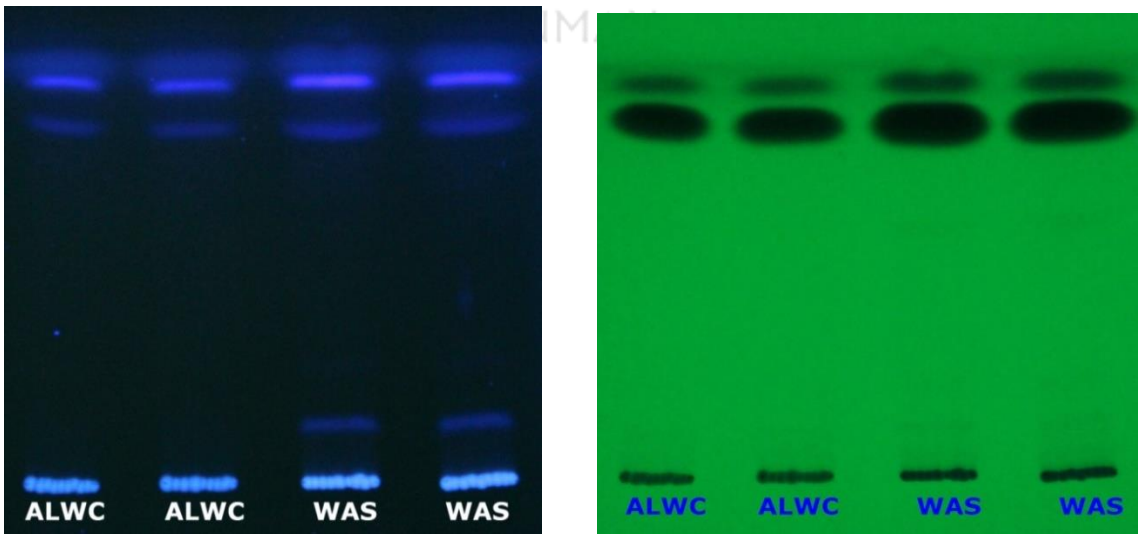

Figure No. 2: Photo plate scanned at UV 366nm **Figure No. 3: Photo plate scanned at UV 254nm**

RESULTS OF HPTLC ANALYSIS OF M.ALWC (at UV 366nm):

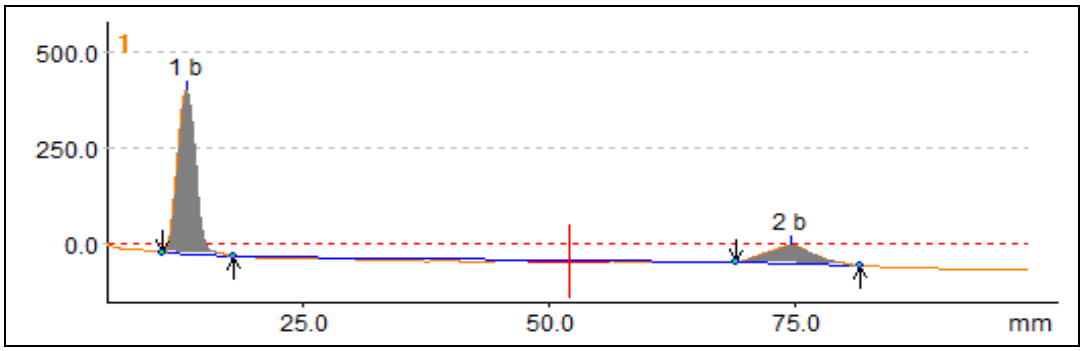

Figure No. 4: Densitogram of methanol extract of ALWC at UV 366nm

Table No. 4: Peak list of methanol extract of ALWC at UV 366nm

Peak no	Y-Pos	Area	Area %	Height	Rf value
1	13.1	871.44	76.06	421.70	0.06
2	74.7	274.25	23.94	48.05	0.98

RESULTS OF HPTLC ANALYSIS OF M.WAS (UV 366nm):

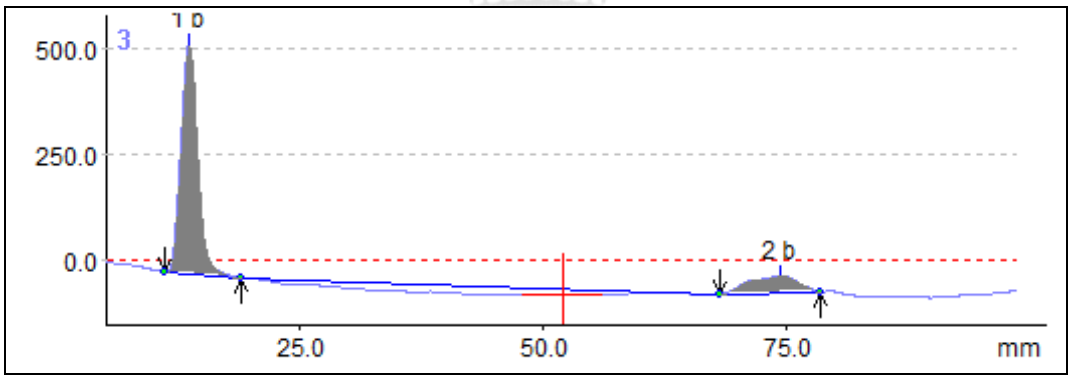

Figure No. 5: Densitogram of methanol extract of WAS at UV 366nm

Table No. 5: Peak list of methanol extract of WAS at UV 366nm

Peak no	Y-Pos	Area	Area %	Height	Rf value
1	13.5	1061.72	82.31	535.59	0.07
2	74.4	228.20	17.69	38.71	0.98

RESULTS OF HPTLC ANALYSIS OF M.ALWC at UV 254nm:

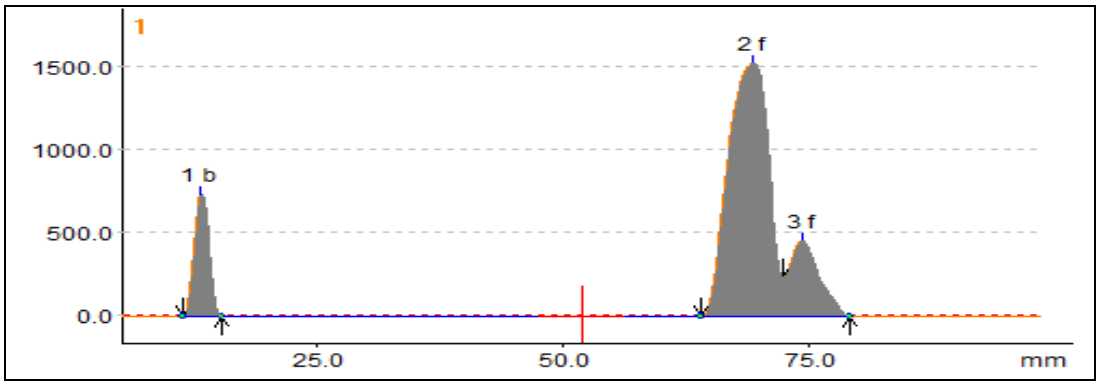

Figure No. 6: Densitogram of methanol extract of ALWC at UV 254nm

Table No. 6: Peak list of methanol extract of ALWC at UV 254nm

Peak no	Y-Pos	Area	Area %	Height	Rf value
1	13.1	1270.12	12.62	737.22	0.06
2	69.3	7201.43	71.55	1522.67	0.90
3	74.3	1592.82	15.83	458.27	0.97

RESULTS OF HPTLC ANALYSIS OF M.WAS at UV 254nm:

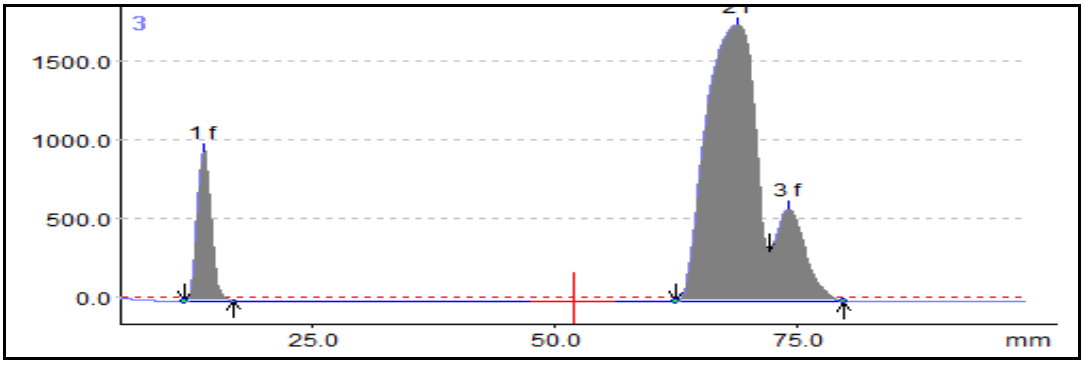

Figure No. 7: Densitogram of methanol extract of WAS at UV 254nm.

Table No. 7: Peak list of methanol extract of WAS at UV 254nm

Peak no	Y-Pos	Area	Area %	Height	Rf value
1	13.8	1923.46	13.81	1027.18	0.07
2	69.2	9665.20	69.41	1752.44	0.90
3	74.4	2336.79	16.78	631.73	0.98

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