Standardization and Phytochemical Screening of a Unani Compound Formulation UNIM 041 (Mushil Drug) along with Modern Analytical Technique

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Abstract

Herbal medicine has seen quite phenomenal growth in the recent years. India has a wealth of flora with hundreds of plants possessing medicinal or curative properties. Despite this wealth, India has a small share in medicinal plants trade in the world market. This dismal condition is attributable to several factors including non-identification of bio-active molecules, lack of uniformity in extraction and formulation processes, quality control, standardization of drugs etc. There is a great need for the standardization of drugs, development of standard operating procedures and scientifically validated analytical methods to provide evidence. The current formulation under study UNIM 041 is a Unani compound drug (Mushil) prescribed to the patients by the Unani Physicians in order to prepare the waste products for excretion which occurs by the expelling process and to bring the body in equilibrium. There are different types of Munzij and Mushil drugs which are administered to the patients depending on the humour involved in that particular disorder for the patient of the bars (vitiligo). Therefore, UNIM 041 has been taken up in this study to carry out standardization.

Key Words: UNIM 041, Standardization, Physico-chemical analysis, TLC.

Introduction

In Unani system of medicine the Munzij-e-Balgham (maturative for phlem) and Mushil (expulsion by purgation) therapy forms a unique and specific line of treatment. The Munzij and Mushil (MM) therapy is for humoral derangement of the body. This therapy is useful in chronic and established diseases. According to drug Soorate Naueya (structural property), the drugs pass so many substances through intestine. So some drugs help to excrete Phlegmatic matter, some bile matter and some Phlegmatic matter. Mus-hil (Purgative) increases frequency of stool. These act in several ways either by squeeze or by increasing peristaltic movement or the other way. These also pass balghem, sauda and safra and so they are also called as Mus-hil balghem and Mus-hil sauda, Mus-hil safra. The Mushil-e-balghem (Phlegmagogue and Phlegm purg) drugs due to their particular structural property excrete Phlegm through intestine (Hifzul Kabir, 2002).

Munzij brings about the correction of the abnormal humour at the level of Hazm-e-Uzvi (digestion at the tissue level), thus facilitating the nutrient to assemble and prepare the waste products for excretion which occurs by the expelling process of Mushil therapy. There are different types of Munzij and Mushil drugs which are administered to the patients depending on the humour involved in that particular disorder here for patient of the bars (vitiligo). It is given as per the mode of administration of Munzij-e-Balgham (maturative for phlem) and Mushil

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(purgative/expulsive) (Anonymous, 1986). *Bars* (Vitiligo) is a chronic disease and usually caused by excessive accumulation of *balgham-e-ghaleez* (thick phlegm). Therefore, all the *Unani* physicians are of the opinion that its treatment should be started with *Tanqiyah-e-Badan* (removal of morbid material from the body) through *Munzij* (concoctive) and *Mushil e-balgham* (laxative to phlegm) (Alam et al., 2014). Munzij-e-Balgham was given in cases of Bars as it was supposed that Balgham (Phlegm) plays an important role in the causation of the disease.

The current formulation under study UNIM 041 is a Unani compound drug (Mushil) prescribed to the patients according to the Unani classical method by the Unani Physicians in order to prepare waste products for excretion which occurs by the expelling process and to bring the body in equilibrium. Hence UNIM 041 has been taken up to carry out the UPLC analysis with fingerprinting profiling which may help in the quality control and authentication of the formulation and also in batch to batch analysis for consistency. This method is also extended to quantitative estimation and identification of compounds. The formulation under study is a Unani coded compound formulation i.e., UNIM 041. It is rough or coarse powder. The formulation consists of 1. Zanjabeel (*Zingiber officinale* Rosc) 2. Sana (*Cassia angustifolia* Linn.) and 3. Turbud (*Operculina turpethum* (L.) Silva Manso.). WHO has emphasized the need to ensure quality control of herbal drugs by using modern techniques (Imam, et al., 2009); (Rasheed, et al., 2010a; 2010b; 2010c;2010d; 2011; 2012,2013; 2014a; 2014b) and (Naikodi et al., 2011) and applying suitable parameters and standards. It was subjected to the analysis of physico-chemical parameters, microbial load, aflatoxin and thin layer chromatographic studies (Anonymous, 2009). The present paper describes the salient features of UNIM 041 in terms of phytochemical screening and drug standardization.

Material and Methods

Samples of UNIM 041 and its ingredients Zanjabeel, Sana and Turbud were procured from the pharmacy, Central Research Institute of Unani medicine, Hyderabad and reference standards viz., Sennoside A, Sennoside B and 6-gingerol were procured from Sigma Aldrich, Bangalore, India (Figure 1). Syringe filter PTFE membrane of 0.22 µm pore size, dia. 25mm (Axiva). All reagents used were of HPLC grade. HPLC-grade methanol, acetonitrile, water, formic acid, orthophosphoric acid (Fischer scientific, India) and triflouroacetic acid (Loba Chemie, India) were used.

Physico-Chemical Parameters

The Physico-Chemical parameters of compound formulation UNIM 041 was studied in terms of total ash, acid insoluble ash, alcohol soluble matter and water soluble matter, microbial load and aflatoxins as per the methods described in

Anonymous, 2009. Thin layer chromatography was carried out in the methanolic extract of UNIM 041 and its ingredients. Phyto-chemical screening was carried out in different solvents extracts such as methanol and aqueous as per the methods described by Evans, 2002. Microbial load, fungal count and Aflatoxins contamination were analyzed as per the methods described in WHO guidelines (Anonymous, 1998).

Thin Layer Chromatography Profile

Preparation of Extract of the Sample Drug

Five grams of fine powder of UNIM 041was dissolved in 100 ml of aqueous and methanol separately in a Stoppered conical flask and was kept for 2 hours and in the meantime shaking of the flask continued at regular intervals. Later the contents were filtered through whattmann No. 41 paper and evaporate the solution to 20 ml. Zanjabeel, Sana and Turbud powder was taken and prepared similar to UNIM 041. The resultant extracts were used to carry out TLC.

Development and Determination of the Solvent System

The samples were spotted as 5mm band on Precoated Aluminium Sheets of Silica Gel 60 F254 (Merck). After trying with various solvent system with variable volume ratios, the suitable solvent system as stated in Table 2 was selected in its proportional ratio and developed in the Twin through TLC chamber to the maximum height of the plate so that components are separated on the polar phase of silica gel and mobile phase of solvent system.

Detection System

After developing, the TLC plate was dried completely and detected under the UV and also exposed to iodine vapours for detection of spots and photographed as shown in Figure 2.

Preparation of Sample Solutions

Initially fine powder formulation UNIM 041 and its ingredients viz., Zanjabeel, Sana and Turbud was prepared. Accurately weighed 2gms each and transferred to a 100 ml beaker and extracted with 20ml methanol by Ultrasonic extraction method with the help of Ultrasonicator for 15 minutes. Sample extract was filtered through syringe filter PTFE membrane of 0.22 µm pore size and stored. Thus obtained solution was used for further analysis.

Accurately weighed 2gms each and transferred to a 100 ml beaker and extracted with 20ml each of water and 50% hydroalcoholic (50:50) for aqueous and hydroalcoholic (50:50) extracts by Ultrasonic extraction method with the help of

Ultrasonicator for 15 minutes. Sample extract was filtered through syringe filter PTFE membrane of 0.22 µm pore size and stored. Thus obtained solution was used for further analysis.

UPLC analysis

The study describes the UPLC analysis which was carried out on Waters Acquity UPLC H-Class Bio system with the BEH C18 reversed-phase analytical column (2.1 × 100 mm, 1.7 µm) at a flow rate of 0.2 mL/min. The detection wavelength was set at 280 nm. The injection volume was 2 µL, and the column temperature was maintained at 40°C. The mobile phase consisted of the solvent A (0.1%, v/v solution of formic acid in water) and solvent B (0.1%, v/v solution of formic acid in methanol) filtered through a 0.22 µm membrane filter using the gradient elution as 0 min 0% B, 0.20 min 15% B, 8 min 25% B, 24 min 30% B, 32 min 50% B, 50 min 90% B and 55 min 90% B, and 60 min 15% B. The data were collected and analyzed using Waters Acquity UPLC Empower 3 Software.

UPLC Analysis of Different Solvent Extracts of Aqueous, Hydroalcoholic (50:50) and Methanolic Extracts of UNIM 041

Aqueous, hydroalcoholic (50:50) and methanol extracts of UNIM 041 were subjected to reversed phase UPLC-PDA on Waters Acquity BEH C18 column under the optimized chromatographic conditions and the assay for the 6-gingerol, Sennoside A and Sennoside B were carried out.

Batch to Batch Analysis for UNIM 041 Formulations

The assay for the twelve batches of UNIM 041 formulation was subjected to methanolic extraction through ultrasonication method separately. The extract was filtered through 0.22µm PTFE syringe filter; the solution obtained was used for analysis.

Results and Discussion

Standardization and Quality Control Analysis

The Physico-chemical parameters were analyzed in the formulation as per the Unani pharmacopoeial methods as shown in Table 1. The parameters values for Zanjabeel as total ash was found to be 4.32-5.21 gm%, and acid insoluble ash 0.61-0.69 gm%, alcohol soluble extract 3.46-3.57 gm% and water soluble extract 14.79-15.48 gm% whereas for Sana as total ash was found to be 10.23-10.43 gm%, and acid insoluble ash 0.45-0.48 gm%, alcohol soluble extract 14.61-15.06 gm% and water soluble extract 40.38-41.26 gm% whereas for Turbud as total ash was found to be 4.60-4.68 gm%, and acid insoluble ash 1.00-1.20 gm%,

alcohol soluble extract 11.77-13.24 gm% and water soluble extract 9.50-10.10 gm% and in the UNIM 041 as total ash was found to be 7.52-8.90 gm%, and acid insoluble ash 4.39- 4.99 gm%, alcohol soluble extract 10.57-12.80 gm% and water soluble extract 25.96-28.45 gm%.

TLC Analysis

The methanolic extracts of formulation and its ingredients were subjected to TLC on Silica gel 'G' plate using Toluene: Ethyl acetate: methanol (7:2:1) and detected using the UV visible chamber which clearly showed various spots at UV 366nm and under iodine vapours. The formulation UNIM 041 under UV 366 nm detection shows nine spots at Rf values 0.18 (pink), 0.25 (blue), 0.32 (pink), 0.40 (blue), 0.54 (Fluorescent blue), 0.64 (light blue), 0.76 (yellow), 0.90 (red) and 0.94 (red). Up on exposure to Iodine vapour shows three spots at Rf values 0.61, 0.79 and 0.90 (All brown). Zanjabeel extract under UV 366 nm detection shows one spot at Rf value 0.78 (Green). Upon exposure to Iodine vapour shows one spot at Rf value 0.79 (brown).

Sana extract under UV 366 nm detection shows nine spots at Rf values 0.03 (blue), 0.06 (blue), 0.18 (red), 0.25 (red), 0.32 (red), 0.67 (red), 0.76 (red), 0.90 (red) and 0.94 (red). Upon exposure to Iodine vapour shows three spots at Rf values 0.06, 0.60 and 0.89 (all brown). Turbud extract under UV 366 nm detection shows two spots at Rf values 0.37 (light blue) and 0.88 (light blue). Upon exposure to Iodine vapour shows two spots at Rf values 0.61 and 0.79 (all brown). The data are presented in Table 1. Thus established a TLC profile which helps in the quality control analysis of formulation as a reference.

Phyto-chemical screening was carried out as qualitative test for the phytoconstituents presence. It was observed that alkaloids, carbohydrates, fixed oil, glycosides, phenols, proteins, steroids, saponins, tannins and flavonoids are shown to be positive and negative for starch as shown in Table 2. The results of total microbial load and total fungal count studies were found to be within the permissible limits and the other parameters were found to be absent in the formulation which are given in Table 3.

Ultra-Performance Liquid Chromatography-Photo Diode Array Detector Analysis

The UPLC analytical method was successfully used to simultaneously determine three components in UNIM 041 samples. Standard mixture of Sennoside B, Sennoside A and 6-gingerol were subjected to UPLC-PDA analysis. The corresponding chromatogram at 280nm is shown in Figure 3a. In the UPLC chromatogram of standard mixture, the reference compound peaks detected at retention times t_R 25.707 minutes for Sennoside B, 31.158 minutes for Sennoside A and t_R 40.078 minutes for 6- gingerol. Their corresponding UV absorbance

max at 267.5, 363.4nm for Sennoside B, 267.5, 340.4 nm for Sennoside A, and 281.0 nm for 6- gingerol. The methanolic extract of UNIM 041 formulation was subjected to UPLC-PDA analysis. The corresponding chromatogram at 280nm is shown in Figure 3b and the peak list with retention time is given in Table 4. In the chromatogram the peaks detected at retention times t_R 25.391 minutes for Sennoside B, 31. 014 minutes for Sennoside A and t_R 40. 025 minutes for 6- gingerol. Their corresponding UV absorbance max at 267.5, 363.4nm for Sennoside B, 267.5, 340.4 nm for Sennoside A, and 281.0 nm for 6- gingerol.

Different Solvent Extract Assay for the UNIM 041

Aqueous, hydroalcoholic (50:50) and methanol extracts of UNIM 041 were subjected to reversed phase UPLC-PDA on Waters Acquity BEH C18 column. The UPLC analysis under the optimized chromatographic conditions the peaks for the marker compounds of Sennoside B, Sennoside A and 6- gingerol were detected and identified with the UV data. The amount of Sennoside B, Sennoside A and 6- gingerol present in three different extracts in mg/g was estimated. In aqueous extract of UNIM 041, the amount of sennoside A and 6 Gingerol were found as 0.0486 and 0.0837 mg/g respectively; whereas in the hydroalcoholic extract, the amount of the Sennoside B, Sennoside A and 6-gingerol were found as 0.5333, 0.2598 and 0.3781 mg/g respectively and in the methanolic extract of UNIM 041 formulations, the amount of Sennoside B, Sennoside A and 6-gingerol were found as 0.7449, 0.4480 and 0.6322 mg/g respectively. It was found that the content of Sennoside B, Sennoside A and 6- gingerol is more in methanol extract as compared to that of aqueous and hydro alcoholic extract of UNIM 041.

Batch to Batch Analysis of UNIM 041

The assay for the twelve batches of UNIM 041 formulation was subjected to methanolic extraction through ultrasonication method separately. Under the optimized chromatographic conditions the results obtained are as shown in Figure 4. The corresponding peaks Sennoside B, Sennoside A and 6-gingerol in formulation were identified in the chromatogram by injecting the standard marker compounds. Identification was done with respect to retention times of sennoside B, Sennoside A and 6-gingerof and UV spectra were recorded by PDA detector. The assay of 12 different batches of UNIM 041 for the three components was identified by comparing retention times and UV spectra with authentic standards in the methanolic extract of UNIM 041

The assay for quantification of Sennoside B, Sennoside A and 6-gingerol in various batches for UNIM 041 formulations were found in the range 0.09% -0.12%, 0.046%-0.050% and 0.06%-0.07% respectively expressed in mg/g and the graphical representation is shown in Figure 4. It can be seen that the

measured amount agree with the actual values.

Conclusion

The Unani formulation UNIM 041 (Mushil drug) under study was subjected to Physico-chemical analysis which is helpful in establishing the standard along with the other parameters such as phyto-chemical screening and TLC analysis. The safety evaluation for aflatoxins contamination analysis was done and found absent; microbial load was found within the permissible limits of WHO guidelines. Modern analytical technique of UPLC analysis was employed with respect to separate compounds and generate fingerprint pattern for the formulation. The reverse phase UPLC-PDA method was successfully applied to determine the three active compounds in twelve different batches of UNIM 041 for the quantitative estimation of Sennoside B, Sennoside A and 6-gingerol. The assay for quantification of Sennoside B, Sennoside A and 6-gingerol in various batches for UNIM 041 formulations were found in the range of 0.09 %-0.12%, 0.046%-0.050% and 0.06%-0.07%, respectively expressed in mg/g. Thus the method could be used for batch to batch quality control analysis development as well as for quality assurance of UNIM 041. The different solvent extracts viz. UPLC analysis found that the amount of Sennoside B, Sennoside A and 6- gingerol is more in methanol extract as compared to that of aqueous hydro alcoholic extract of UNIM 041. Thus, the formulation UNIM 041 was successfully standardized along with physico-chemical parameters, TLC, UPLC-PDA analysis, batch to batch assay and different solvent extract assay.

Table 1 : Physico-Chemical Parameters of the Compound Formulation UNIM 041 and Ingredients.

S.No.	Name	Total Ash (gm%)	Acid insol. Ash (gm%)	Alc. Sol. Ext (gm%)	Wat. Sol. Ext (gm%)	TLC of methanolic extract on Silica gel 'G' plate using Toluene: Ethyl acetate: methanol (7:2:1)
1	**Zanjabeel**	4.32-5.21	0.61-0.69	3.46-3.57	14.79-15.48	Shows under UV (366 nm) one spot at R_f value 0.78 (Green). Upon exposure to Iodine vapour shows one spot at R_f. 0.79 (brown).

S.No.	Name	Total Ash (gm%)	Acid insol. Ash (gm%)	Alc. Sol. Ext (gm%)	Wat. Sol. Ext (gm%)	TLC of methanolic extract on Silica gel 'G' plate using Toluene: Ethyl acetate: methanol (7:2:1)
2	**Sana**	10.23-10.43	0.45-0.48	14.61-15.06	40.38-41.26	Shows under UV (366 nm) nine spots at R_f Values 0.03 (blue), 0.06 (blue), 0.18 (red), 0.25 (red), 0.32 (red), 0.67 (red), 0.76 (red), 0.90 (red) and 0.94 (red). Upon exposure to Iodine vapour shows three spots at R_f Values 0.06, 0.60, 0.89 (all brown).
3	**Turbud**	4.60-4.68	1.00-1.20	11.77-13.24	9.50-10.10	shows under UV (366 nm) two spots at R_f values 0.37 (light blue) and 0.88 (light blue). Upon exposure to Iodine vapour shows two spots at R_f values 0.61, 0.79 (all brown).
4	**UNIM 041**	7.52-8.90	4.39-4.99	10.57-12.80	25.96-28.45	shows under UV (366 nm) nine spots at R_f values 0.18 (pink), 0.25 (blue), 0.32 (pink), 0.40 (blue), 0.54 (Fluorescent blue), 0.64 (light blue), 0.76 (yellow), 0.90 (red) and 0.94 (red). On exposure to Iodine vapour shows three spots at R_f values 0.61, 0.79 and 0.90 (All brown).

Table 2 : Phytochemical Screening in Methanol and Aqueous Extracts of UNIM 041.

S. No.	Phytoconstituent	Methanol ext.	Aqueous ext.
1.	Alkaloid	+	+
2.	Carbohydrates	+	+
3.	Fixed oil /Resinified volatile oils	+	+
4.	Glycosides	+	+

S. No.	Phytoconstituent	Methanol ext.	Aqueous ext.
5.	Phenols	+	+
6.	Saponins	+	+
7.	Proteins	+	+
8.	Steroids	+	+
9.	Tannins	+	+
10.	Flavonoids	+	+
11.	Starch	-	-

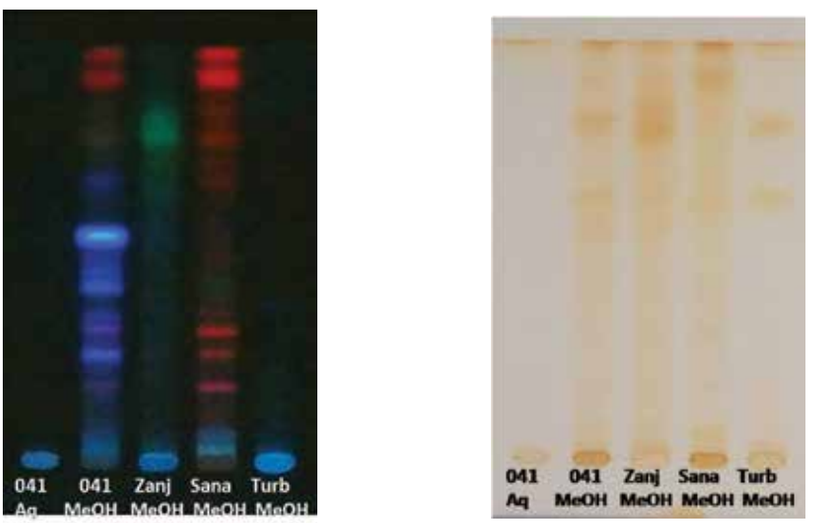
Table 3 : Aflatoxins, Microbial and Fungal Contamination in the UNIM 041.

Aflatoxin Contamination (Permissible limits as per WHO)					
S. No.	**Name**	**B1 (NMT 0.50 ppm)**	**B2 (NMT 0.10 ppm)**	**G1 (NMT 0.50 ppm)**	**G2 (NMT 0.10 ppm)**
1	Zanjabeel	Nil	Nil	Nil	Nil
2	Sana	Nil	Nil	Nil	Nil
3	Turbud	Nil	Nil	Nil	Nil
4	UNIM 041	Nil	Nil	Nil	Nil
Microbial and fungal Contamination (Permissible limits as per WHO)					
S. No.	**Name**	**Total Microbial Load (NMT 105/g)**	**Salmonella Spp (Nil)**	**Escherichia Coli (Nil)**	**Total Fungal count (NMT 103/g)**
1	Zanjabeel	27 x 102	Nil	Nil	15 x 102
2	Sana	12x103	Nil	Nil	1x10
3	Turbud	Nil	Nil	Nil	Nil
4	UNIM 041	81 x 103	Nil	Nil	21 x 102

Table 4 : Peak List and Retention Time of Unim 041 Under Optimized Chromatographic Condition.

S. No.	Name	Retention Time	Area	% Area	Height	% Height
1	Sennoside B	25.391	104198	12.24	9115	6.12
2	Sennoside A	31.014	329278	38.67	62168	41.75
3	6-gingerol	40.025	418089	49.10	77610	52.12

Fig. 1 Photograph of formulation UNIM 041 and its ingredients used



At UV 366nm

Exposed to Iodine vapours

Fig. 2 TLC plate of UNIM 041 formulation 1.Aq:Aqueous extract 2.MeOH:Methanolic extract 3.Zanj:Zanjabeel 4.Sana 5.Turb:Turbud *(Mobile Phase: Toluene: Ethyl acetate: methanol (7:2:1))*

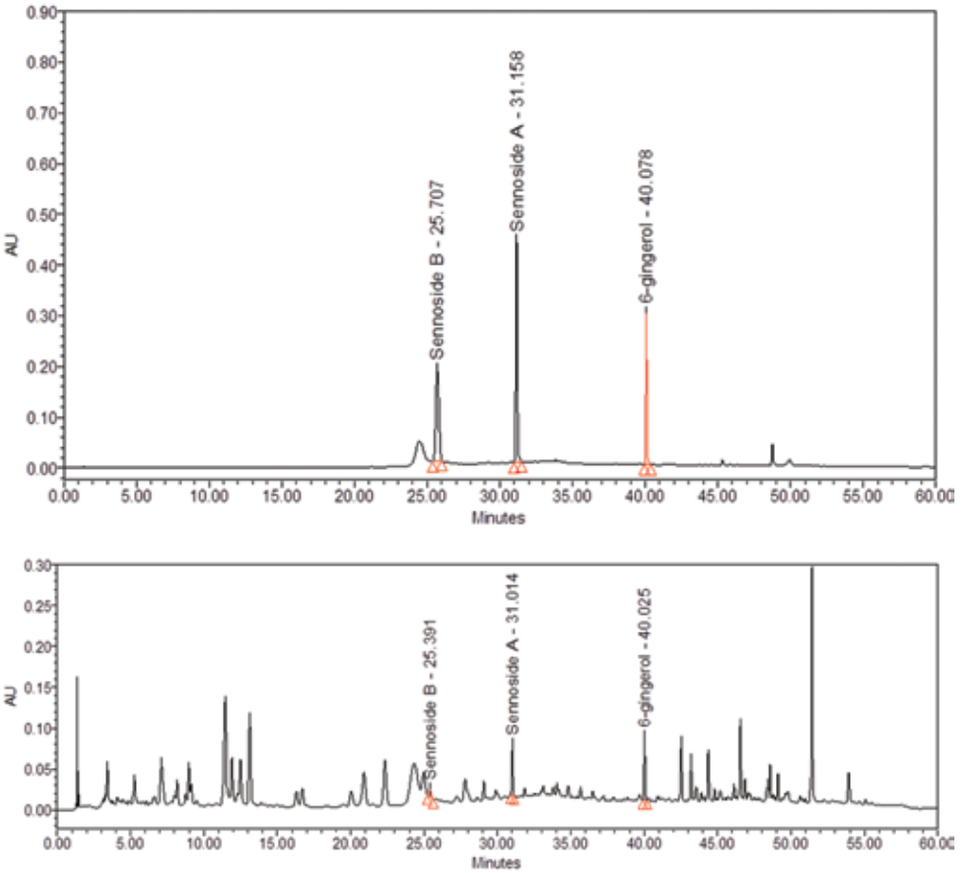
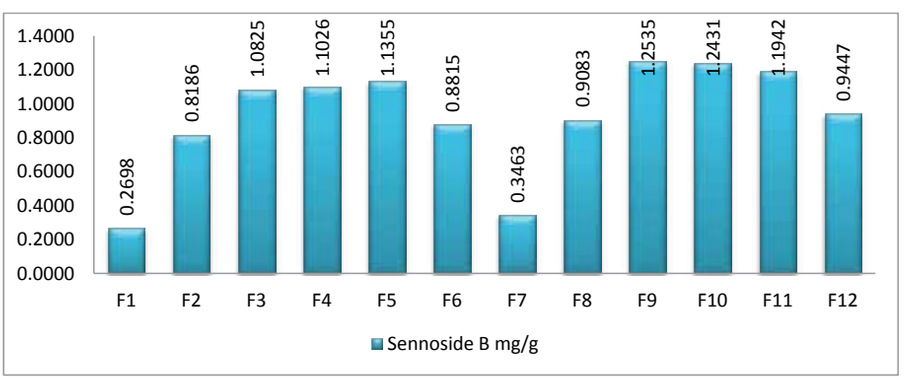
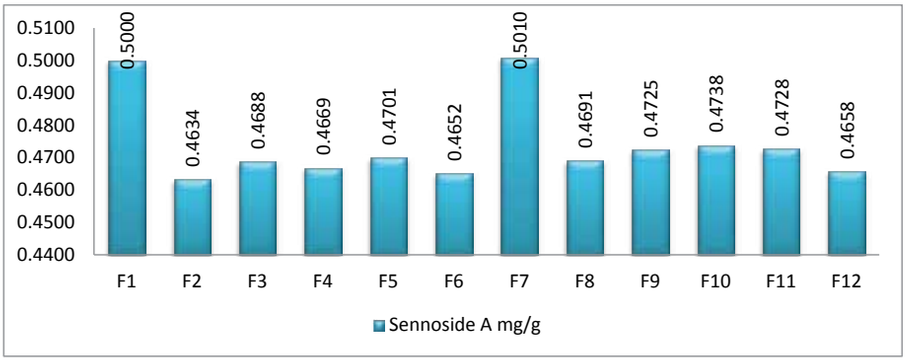


Fig. 3 UPLC chromatogram of a) standards of Sennoside B, Sennoside A, 6-gingerol. (b) UNIM 041 formulation.





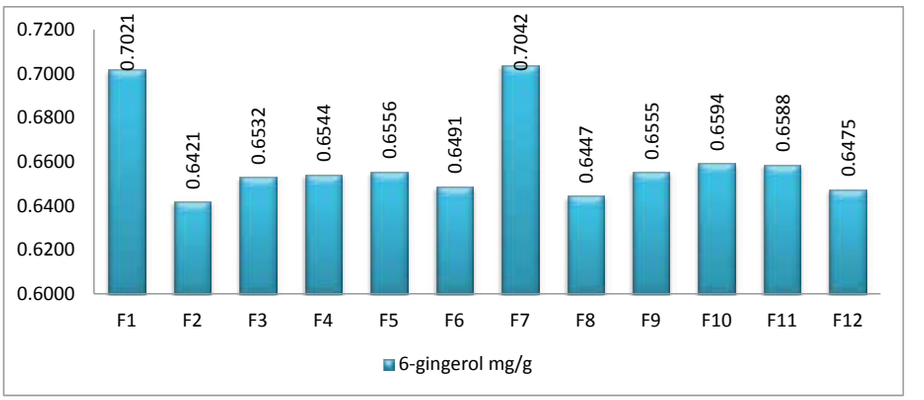


Fig. 4 Assay of 12 batches UNIM 041

Acknowledgement

The authors would like to record their gratitude to the Director General, CCRUM, New Delhi, for providing necessary facilities to carry out this study. The authors also thank the associated staff of Central Research Institute of Unani Medicine, Hyderabad, for their constant support and encouragement throughout the study.

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सारांश

यूनानी कंपाउंड फॉर्मूलेशन यूनिम-041 (मुसहिल औषधि) का आधुनिक विश्लेषणात्मक तकनीक द्वारा मानकीकरण एवं पादप-रसायनिक जाँच

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पिछले कुछ वर्षों से हर्बल औषधियों में काफी अभूतपूर्व वृद्धि देखी गई है। भारत एक वनस्पति औषधियों का भंडार है जिनमें सैकड़ों पौधों में औषधीय तथा रोग निवारक गुण पाए जाते हैं। इस विशाल भंडार के बावजूद, विश्व बाजार में औषधीय पादप व्यापार में भारत का छोटा सा स्थान है। यह निराशजनक स्थिति कई कारकों के कारण है जिनमें जैविक–सक्रिय अणुओं की पहचान न होना, निष्कर्षण और निर्माण प्रक्रियाओं में एकरूपता की कमी, गुणवत्ता नियंत्रण एवं औषधियों का मानकीकरण आदि शामिल है। आज के युग में औषधियों को गुणवत्ता प्रदान करने के लिए इनका मानकीकरण करना, मानक संचालन प्रक्रियाओं को विकसित करना एवं वैज्ञानिक तरीके से आधुनिक विधियों द्वारा विश्लेषण करना अति आवश्यक है। वर्तमान अध्ययन के अन्तर्गत यह देखा गया है कि औषधि यूनिम–041 एक यूनानी मिश्रित औषधि है जिसे यूनानी चिकित्सक रोगियों के उत्सर्जन एवं निष्कासन प्रक्रिया द्वारा शरीर को संतुलन में लाने के लिए उपयोग करते हैं। यूनानी पद्धति में अलग–अलग तरीके की मुंज़िज एवं मुस्हिल औषधियाँ पाई जाती हैं जिन्हें रोगियों की मनोवृत्ति के आधार पर एक विशेष रोग जैसे विटिलिगो आदि में दी जाती है। चूँकि औषधि यूनिम–041 विटिलिगो बीमारी में बहुत ही उपयोगी पाई गई है। अतः यूनिम–041 को मानकीकरण अध्ययन करने के लिए चुना गया है।