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ORIGINAL ARTICLE

Standardization of Unani Antidiabetic Tablet - *Qurse Tabasheer*

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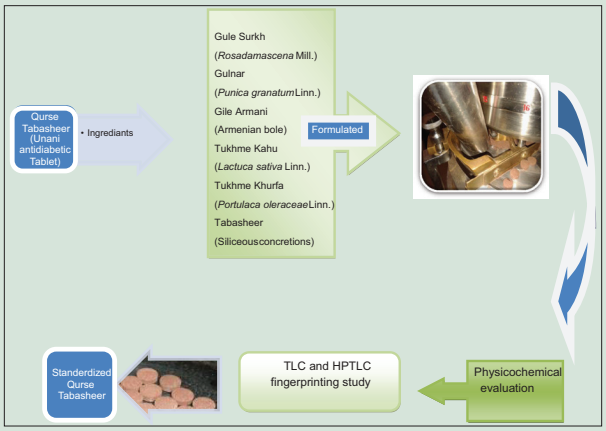
ABSTRACT

Background: Quality control of Unani polyherbal formulations is the need of the day for better acceptance of Unani medicine. *Qurse Tabasheer* (QT) is a Unani polyherbal formulation containing six ingredients, Tabasheer (Siliceous concretions) (*Bambosa arundinaceae* Retz.), Gule Surkh (*Rosa damascena* Mill. flower), Gulnar (*Punica granatum* Linn. flower), Tukhme kahu (*Lactuca sativa* Linn. seed), Tukhme khurfa (*Portulaca oleraceae* Linn. seed), and Gile Armani (bole) widely used in treatment of diabetes. The present study was taken up to scientifically evaluate the various physicochemical parameters to standardize the formulation. **Objective:** To evaluate various physicochemical parameters including ash values, moisture content, extractive values, thin layer chromatography (TLC) and high-performance TLC (HPTLC), friability, disintegration, uniformity, and weight variation for standardization of QT. **Materials and Methods:** Ingredients were identified by the experts. The method mentioned in national formulary of Unani Medicine with modification was followed for preparation of the tablets. Physicochemical standards were established for ideal batch of tablets on the basis of set parameters regarding friability, hardness, and disintegration. Various parameters such as organoleptic characters, extractive values for the extract and HPTLC fingerprinting postcompression were carried out for evaluation of QT. **Results:** Parameters for loss of weight on drying, pH, ash values, extractive values documented. Qualitative chemical tests indicated the presence of alkaloid, glycoside, tannins, and steroids. TLC and HPTLC fingerprinting studies showing the presence of major peaks were documented. Friability, hardness, and disintegration time of ideal batch was 0.09 ± 0.0057, 4.03 ± 0.087, and 25.57 ± 0.4860 min, respectively, and it was found to be within the set limit. Weight variation was <5%. Total fungal and bacterial counts were found to be within the limit. **Conclusion:** Standards were established for poly herbal formulation QT, which may be used as reference for preparation and standardization of QT.

Key words: Physicochemical, *Qurse Tabasheer,* standardization, tablet, Unani

SUMMARY

- In this work Standardization of anti-diabetic tablet *Qurse Tabasheer* with diverse ingredients including herbal and mineral origin drugs has been attempted with identification of its ingredients, formulation, physicochemical evaluation and HPTLC finger printing, which may help in preparing consistent and better efficacious formulations.



Abbreviations Used: QT: Qurse Tabasheer, TLC: thin layer chromatography, HPTLC: high-performance thin layer chromatography, WHO: World health organization, FRLHT: Foundation for Revitalization of Local Health Traditions, Fe_2O_3: Iron oxide, Sio_2: Silica $CaCo_3$: Calcium carbonate, Tio_2: Titanium Oxide, NIUM: National Institute of Unani Medicine, #: Mesh size, LOD: Loss of weight on drying, USP: United state Pharmacopeia, UV: Ultra Violet, λ: Lambda, θ: theta, CFU: Colony-forming unit

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INTRODUCTION

Like other contemporary traditional medicine pharmacy, Unani pharmacy is also facing, several issues related to quality control of formulations such as improper use and un-sustained availability of original raw material, in efficient processing techniques leading to poor quality product, poor quality control procedure, lack of implementation of current good manufacturing practices, difficulty in maintaining batch to batch consistency etc. To overcome this, it is necessary to carry out standardization work. WHO has emphasized the need to ensure quality using modern analytical techniques and setting up physicochemical standards.

Out of several oral unit dosage forms, *Qurs* (tablet) is one of the most suitable/practical dosage forms due to its easy portability for prolong use, stability and accuracy of dose, etc. Therefore, in the present study, physiochemical parameters for *Qurse Tabasheer* (QT) were investigated. Formulation selected for study is being commonly used and manufactured by the Unani pharmacies. It contains six ingredients, Tabasheer (Siliceous concretions) (*Bambosa arundinaceae* Retz.), Gule Surkh (*Rosa damascena Mill.* flower), Gulnar (*Punica granatum* Linn. flower), Tukhme kahu (*Lactuca satisva* Linn. seeds), Tukhme khurfa (*Portulaca oleraceae* Linn. *seeds*), and Gile

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Armani (*Armenian bole*).[1,2] This particular formulation is mentioned in *Bayaaze Kabeer* and *Kitabul Murakkabat Al Maroof Makhzanul Murakkabat*. It is used in the treatment of *Dhayabitus* (diabetes), *Hummae Hadda* (acute fever), and *Is'hal* (diarrhoea).[1,3] Moreover, this formulation has been reported for its pharmacological activity as a hypoglycaemic[4] and there is a need to develop its quality control standards.

MATERIALS AND METHODS

Collection and identification of drugs

Gulnar, Gule Surkh, Tukhme Khurfa, and *Tukhme Kahu* were procured from A.B. General Store, Avenue Road; Bengaluru and identified by expert at FRLHT (Foundation for Revitalization of Local Health Traditions) Bengaluru. *Tabasheer* was procured from a raw drug dealer "Herbo World Associates," New Delhi, and identified by expert. Different samples of *Gile Armani* were collected from crude drug local market of Bengaluru, Delhi, and Malegaon (MS) and for its identification, X-ray diffraction was conducted at Department of Material Engineering, Indian Institute of Sciences Bengaluru out of which one sample was selected which was looking like of natural combination containing Fe_2O_3 Hematite; Silica (Sio_2)-Quarts alpha; $CaCo_3$ - Calcite form and Tio_2 - Titanium Oxide, Anatase) and its constituents resembled Red Ochre (*Gairika*/*Geru*) as per its constituent mention in Ayurvedic Pharmacopeia.[5] The sample of *Gile Armani* was identified as *Geru* which is a genuine substitute of *Gile Armani* in Unani text as literature also reveals that *Gile Armani* (Armenian bole) is generally unavailable,[6,7] this sample of *Gile Armani* was taken for study as ingredient of the formulation as available market sample. The drug samples were submitted in NIUM Drug Museum and voucher specimen No. 22/IS/Res/2014 was collected for future reference.

Method of preparation of *Qurse Tabasheer*

The method mentioned in National Formulary of Unani Medicine was followed for the preparation of *Qurse Tabahseer* with modifications.[8] Eighteen different batches were prepared (trial and error) by varying the following parameters: (a) Mesh size of powder (#80, 100 and 120), (b) concentration of binder (10, 15 and 20% of gum acacia), (c) duration of drying of granules (30 and 60 min at 60°C temperature),[9] and (d) postcompression drying (30 min at 60°C).

One percentage liquid paraffin as lubricant and 1% magnesium carbonates as glidant were added slowly in dried granules.[10] Oscillating granulation machine GMP model (Cemach machinery Ltd., Ahmadabad SN. 1417) was used for granulation, Hot air oven (Labline Mod. No. HO 6.7) was used for drying and Tablet compression was done using multi-station rotary presses (tableting machine) GMP model (Cemach machinery Ltd., Mod. No. CM-D-20).

The batch with powder #100 mesh size, binder 20% of total wt. of powder (16% in the formulation), duration of drying of granules 60 min and post compression drying for 30 min at 60°C was selected as the final batch for physiochemical standardization on the basis of set parameter, i.e. minimum friability (<1%),[11] hardness near to standard value (4 kg)[12] and disintegration time <30 min.[11] Pre-compression parameters including bulk density, tapped density, compressibility index, Hausner's ratio,[13] angle of repose[14] of final batch was also done.

Physiochemical parameters

Organoleptic properties

Appearance, color, smell, and taste were evaluated.[15]

Friability test

Friability test apparatus Roche's friabilator (Labinda mod. no. 1020) was used for determination of friability of tablet. This device subjected the tablet to the combined effect of abrasion and shock in a public chamber and dropping the tablets at a height of 6 inches in each revolution. Weighed tablets were placed in friabilator revolving at 25 rpm for 100 revolutions. Tablet was de-dusted using a soft muslin cloth and weighed.[16]

$F = (W_1 - W_2/W_1) \times 100$ (W_1 = Initial weight of tablets, W_2 = Final weight of tablets)

Tablet hardness test

Randomly three tablets were pickup and they were individually tested for the hardness by Monsanto hardness tester (Shital scientific industries Sr. no. 11012010) in terms of kg/cm.[16]

Disintegration test

Disintegration testing apparatus (Thermonik: Mod. no. TD 20S) was used for determination of disintegration time.[11]

Uniformity of diameter

Diameter of three randomly selected tablets was measured individually using a Vernier Caliper (UTTAR, IME type 6 inch/15 cm) and expressed in mm.[17]

Extractive value (Soxhlet apparatus)

Successive extractive value

The coarse powder of *QT* was extracted successively using soxhlet apparatus with different solvent, in increasing order of polarity, petroleum ether → benzene → chloroform → ethanol. 10 g powdered drug was taken and subjected to successive extraction with each solvent for 6 h. The extracts were filtered using filter paper (Whatman No. 1) and dried on water bath. The extractive values were determined with reference to the weight of the drug taken (w/w). The procedure was repeated 3 times to calculate mean extractive values.[18-20]

Non successive extractive value

The coarse powder of *QT* was extracted separately in different solvent (water, ethyl alcohol and petroleum ether) using soxhlet apparatus. 10 g powdered drug was taken and subjected to separate extraction with each solvent. The extracts were filtered using filter paper (Whatman No. 1) and evaporate on water bath. Extractive values were determined with reference to drug taken (w/w).[20]

Extractive value (Cold maceration)

Determination of alcohol and water-soluble extractive was done as per protocol for testing of Ayurvedic, Siddha and Unani Medicines.[21]

Ash value

Total ash and water soluble ash were done by method mentioned in protocol for testing.[21] Acid insoluble ash and sulphated ash were done by method mention in UPI;[22]

Loss of weight on drying at 105°C

Loss of weight on drying (LOD) at 105°C was done by method mention in UPI.[22]

pH value

pH value of 1% solution and pH value of 10% solution was determined as per the method mentioned in physiochemical standardization of Unani Medicine part IV.[15]

Weight variation

Twenty tablets were selected randomly from selected batch and weighed individually. Average weight was calculated, and individual weights were

compared to average weight. If not more than 2 tablets are outside the percentage limit, tablets meet the USP test (USP weight variation test).[11]

High-performance thin layer chromatography analysis

Preparation of extract of the drug for high-performance thin layer chromatography analysis

Successive extracts (Petroleum ether, chloroform, ethanol, methanol and water) obtained by soxhlet extraction were dried and diluted with small amount of methanol, filtered by Whatman no. 41. Filter paper. The solution so obtained was used as sample for high-performance thin layer chromatography (HPTLC) analysis. "CAMAG thin layer chromatography (TLC) scanner 3" system was used for analysis along with automatic TLC applicator and UV visible cabinet as imaging system, the instrument had "win CATS-version 1.3.3" software for documentation.[9]

Optimization of thin layer chromatography system

TLC procedure was optimized using various solvents. The solvent system toluene: ethyl acetate: formic acid (5:4:1) which gave good resolution was selected. The samples were spotted on Merck Silica gel 60 F 254 plates (10 cm × 10 cm) using a linomat. The chamber was saturated for 10 min, and the plates were developed up to migration distance of 93 mm. Scanning wavelengths: UV 366 nm, 254 nm and 280 nm were used for visualization.

Development of high performance thin layer chromatography technique

After developing, TLC plates were dried completely and scanned with the CAMAG TLC scanner 3' at 200–400 nm. λ max was found to be 280 nm. The plates were again scanned at 366, 280 and 254 nm. The number of peaks were noted and tabulated to be used as finger print.

Qualitative test for chemical constituent

The extracts were tested for tannin,[23] terpenoide.[23] Glycoside, alkaloids (Dragendroff's test), protein (Millon's test), carbohydrates (Fehling's test), steroids (Salkowaski reaction), and resins using the methods mentioned in Physiochemical Standardization of Unani formulation Part I.[24] Test for saponins,[23] flavonoids, phenols (Ferric chloride test),[23] and test for reducing sugar were also performed.[23]

Total fungal and specific pathogen like *Escherichia coli*, *Salmonella* spp., *S. aureus*, and *Pseudomonas aeruginosa* tests were done at Bengaluru test house Bengaluru by method mention in the Ayurvedic pharmacopeia of India. Part II, Vol. 2nd ed. 1st.[25]

Figure 1: *Qurse Tabasheer*

RESULTS

Organoleptic properties: Appearance: Circular uncoated tablet (slightly biconvex); Colour: Dark Brown; Smell: Rosy; Taste: Clayey, astringent and slightly bitter; Texture: Hard and smooth [Figure 1].

The mean values of precompression parameters of granules of selected batch: Bulk density (g/ml): 0.5084 ± 0.0, tapped density (g/ml): 0.5884 ± 0.006669, compressibility index (%): 13.56 ± 0.9786 and Hausner's ratio: 1.157 ± 0.01311 and angle of repose (θ): 29.98°C.

The mean value of friability (%), hardness (kg/cm), disintegration time (minutes) and diameter (mm) of QT. Were determined and the values are depicted in [Table 1].

The mean percentages of the successive and nonsuccessive extractive values were calculated, and the results are depicted in [Table 2]. The values of the alcohol and water-soluble content, total ash, water soluble ash, acid insoluble ash, sulfated ash, LOD at 105°C, pH at 1% and 10% solution were determined, and the values in mean percentage are depicted in [Table 3].

The mean value of weight of randomly selected 20 tablets was found to be 793.7 ± 4.755 mg. The deviation of individual tablet weight from the average weight of 20 tablets was found within the percentage limit of 5% of mean weight.

TLC analysis was carried out using toluene: ethyl acetate: formic acid (5:4:1) as mobile phase. Numbers of peaks in all the extracts are shown in [Table 4]. The TLC analysis of chloroform extract of the tablets showed

Table 1: Postcompression parameter of *Qurse Tabasheer*

Parameters	Mean±SEM
Friability (%)	0.09±0.0057
Hardness (kg/cm)	4.03±0.087
Disintegration time (min)	
Aqueous media	25.57±0.486
Simulated gastric fluid (water with 0.1 M hydrochloric acid)	24.72±0.1881
Uniformity of diameter (mm)	13±00

SEM: Standard error of mean

Table 2: Extractive values of *Qurse Tabasheer*

Solvents	Mean±SEM (%)	
	Successive extractive values	Nonsuccessive extractive values
Petroleum ether	7.380±0.2884	7.69±0.3011
Benzene	1.000±0.02082	-
Chloroform	0.4267±0.05239	-
Ethyl alcohol	10.93±0.3187	13.48±0.3398
Water	-	27.67±0.5783

SEM: Standard error of mean

Table 3: Physiochemical parameters of *Qurse Tabasheer*

Physiochemical parameters	Mean±SEM
Alcohol soluble matter (%)	9.180±0.6350
Water soluble matter (%)	17.08±0.6021
Total ash (%)	26.50±0.07638
Water soluble ash (%)	0.8667±0.07265
Acid insoluble ash (%)	21.28±0.3632
Sulfated ash (%)	25.85±0.2754
Loss of weight on drying (105°) (%)	6.027±0.1641
pH value at (%)	
1	5.450±0.08021
10	4.727±0.02404

Table 4: High-performance thin layer chromatography peaks for all extract

Extract	Number of peaks at λ max 280 nm	Number of peaks at 254 nm	Number of peaks at 366 nm
Petroleum ether	7	6	5
Chloroform	13	14	12
Ethanol	10	11	11
Methanol	15	14	5
Water	12	11	10

Mobile phase - Toluene: ethyl acetate: formic acid (5:4:1)

Table 5: Peak list of chloroform extract of *Qurse Tabasheer* at UV 254 nm

Peak number	Area (AU)	Area (%)	Height (AU)	Rf
1	26,883.4	16.87	737.0	0.07
2	11,531.4	7.24	394.9	0.13
3	8143.5	5.11	341.0	0.17
4	11,785.7	7.40	327.8	0.22
5	16,633.1	10.44	327.8	0.31
6	15,711.4	9.86	321.3	0.36
7	15,826.9	9.93	402.2	0.43
8	21,378.7	13.42	427.7	0.53
9	3350.4	2.10	97.6	0.62
10	3116.8	1.96	81.8	0.68
11	5313.9	3.33	99.1	0.78
12	6868.1	4.31	148.8	0.88
13	6355.0	3.99	160.3	0.93
14	6452.0	4.05	148.3	1.02

Table 6: Peak list of chloroform extract of *Qurse Tabasheer* at UV 280 nm

Peak number	Area (AU)	Area (%)	Height (AU)	Rf
1	30,865.8	15.42	766.9	0.07
2	24,694.0	12.34	472.6	0.13
3	13,344.7	6.67	390.4	0.22
4	21,294.0	10.64	399.3	0.31
5	20,016.6	10.00	402.5	0.36
6	19,073.1	9.53	452.4	0.43
7	31,854.6	15.92	599.2	0.53
8	3880.2	1.94	120.2	0.62
9	3950.5	1.97	100.4	0.68
10	7607.0	3.80	129.6	0.78
11	13,169.7	6.58	277.2	0.89
12	5560.7	2.78	164.7	0.95
13	4814.1	2.41	134.3	1.02

Table 7: Peak list of chloroform extract of *Qurse Tabasheer* at UV 366 nm

Peak number	Area (AU)	Area (%)	Height (AU)	Rf
1	25,338.1	17.81	771.0	0.07
2	16,502.7	11.60	524.0	0.13
3	11,278.0	7.93	448.6	0.17
4	15,568.6	10.94	433.2	0.21
5	34,559.1	24.29	382.5	0.30
6	15,244.1	10.72	362.1	0.43
7	13,461.8	9.46	233.8	0.53
8	3102.5	2.18	88.5	0.62
9	2161.2	1.52	61.9	0.67
10	2534.4	1.78	64.7	0.76
11	1268.1	0.89	41.5	0.80
12	1243.3	0.87	27.4	0.99

good separation and hence was selected for HPTLC fingerprinting study. The peak details of the same are shown in Tables 5-7. The photo and the chromatograms are depicted in [Figure 2-5].

Preliminary phytochemical studies of QT showed the presence of glycoside, tannin, terpenoid, saponins, flavonoids, alkaloids, phenols, steroids, protein, and carbohydrates whereas steroid, reducing sugar, and resins were absent.

Total fungal and total bacterial count/g were within limit and specific pathogen such as *E. coli*, *Salmonella* spp., *S. aureus*, *P. aeruginosa* were found to be absent [Table 8].

DISCUSSION

Selected formulation QT has got very important indication in diabetes and is also a commonly marketed formulation for diabetes, but its standardization has not been reported, hence in the present work, an attempt has been made. Appearance, color, and texture play an important role such as in quick identification. Appearance, color, smell, taste, and texture of tablets prepared as per the standard protocol were found acceptable. The presence of a particular smell/odor could be characteristics of a drug and indicates quality and identity of particular drug. QT shows dark brown color, and a particular taste and smell, dominant smell is of Gulab (*Gule surkh*) and Anar (*Gulnar*) flower. *Gulab* (Rose) smell was dominating over *Anar* (Pomegranate). Slight bitterness in taste might be due of *Tukhme Kahu* Seeds (presence of bitter principles lectucin, lectopicrin, and lactucic acid)[26] and slightly due to *Tukhme khurfa*. The tablet was of hard and smooth texture.

Friability was found to be within acceptable limit, i.e. 0.5–1%. It is an important parameter to measure the strength of tablets. Hardness was found to be 4.03 ± 0.087 kg/cm. It was within acceptable limit of 4 kg. It is essential to hold up mechanical distress during manufacturing, packaging, storage and transportation.[12] Hardness is one of the issue in Unani tablets and special attention was given to improve hardness of QT, hardness of 4 kg was achieved by binder 20% of total wt. of powder (16% in the formulation).

Disintegration time in aqueous media and simulated gastric fluid (water with 0.1 M hydrochloric acid) were found to be 25.57 ± 0.486 min and 24.72 ± 0.1881 min. This test represents breakdown of tablets into smaller particles and shows that QT disintegrate within permissible limit (maximum 30 min)[11] when placed in liquid medium in the experimental circumstance.

The mean percentage of the successive extractive values was found maximum in alcohol (10.93 ± 0.32). The mean percentage of the nonsuccessive extractive values was found maximum in water (27.67 ± 0.5783) followed by ethyl alcohol (13.48 ± 0.3398). Extractive value of a drug in specific solvent is an index of purity of a drug and plays a major role to determine adulteration.[27,28]

Ash value is an important parameter for detection of adulteration and impurities. The total ash value and sulfated ash was found to be high which may be attributed to the presence of large amount of silica and other inorganic constituent in two drugs, i.e. *Tabasheer* and *Gile Armani*.

Moisture content or LOD was found to be 6.027 ± 0.1641. LOD indicates the amount of water and volatile substances present in a particular drug. If any drug has more moisture level, it becomes ideal medium for growth of different types of bacteria and fungi affecting the purity, quality and efficacy of a drug.[25]

The pH of 1% and 10% solution was found to be 5.450 ± 0.08021 and 4.727 ± 0.02404, respectively. Acidic pH indicates better absorption from stomach.[29]

Qualitative chemical test indicated the presence of for glycosides, tannin, terpenoid, saponins, flavonoids, alkaloids, phenols, steroids, protein, and carbohydrates. Several reports suggest role of Phenols as antioxidants in

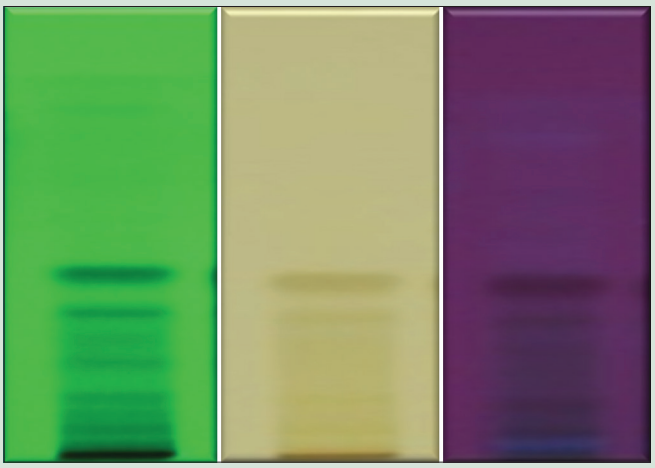

Figure 2: High-performance thin layer chromatography photograph chromplate at 254 nm, 280 nm and 366 nm From L → R, in extract of Chloroform in toluene: ethyl acetate: formic acid (5:4:1) mobile phase

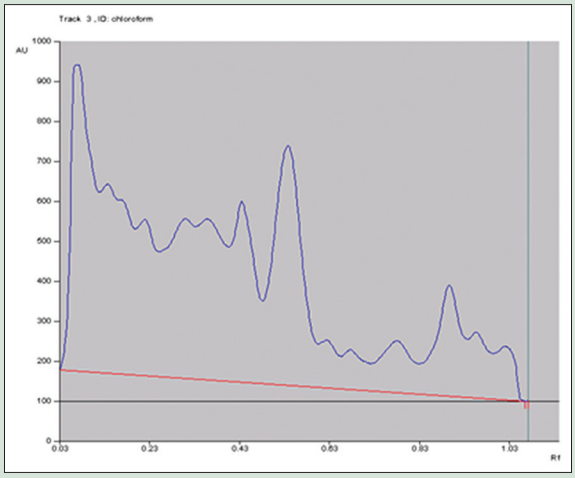

Figure 4: High-performance thin layer chromatography densitogram of *Qurse Tabasheer* at 280 nm chloroform extract

Table 8: Estimation for microbial contamination and specific pathogens

Parameters	Results	Limits (as per Ayurvedic Pharmacopoeia of India part II)
Total fungus count/g	190 CFU	1000 CFU
Total bacterial count/g	210 CFU	100,000 CFU
Escherichia coli/10 g	Absent	Absent
Salmonella spp./10 g	Absent	Absent
Staphylococcus aureus/10 g	Absent	Absent
Pseudomonas aeruginosa/10 g	Absent	Absent

diabetes. Hence, the anti-diabetic activity of QT may be attributed to phenols, tannins, and others.

TLC analysis is one of the best methods for characterization and standardization of herbal drugs. The number of spots and Rf value of each spot in a particular mobile phase is an index of identity, purity, and quality of a drug and plays a major role to determine adulteration in drug. Keeping this point in mind, TLC study was done for all the extracts and maximum peaks were seen in Chloroform and Methanol extract [Table 4].

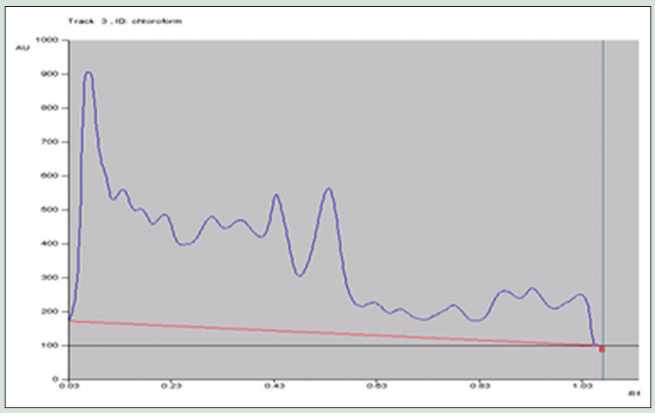

Figure 3: High-performance thin layer chromatography densitogram of *Qurse Tabasheer* at 254 nm chloroform extract

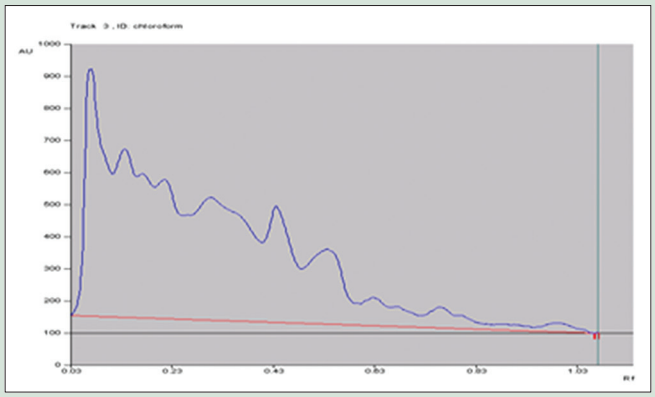

Figure 5: High-performance thin layer chromatography densitogram of *Qurse Tabasheer* at 366 nm chloroform extract

HPTLC fingerprinting is a suitable for rapid and simple authentication and comparison of different herbal formulations. The unique characteristic finger print in terms number of peaks, their Rf values and area under the curve can play a role in monitoring the quality, consistency, and stability of the product. We have reported the HPTLC data for QT for the 1st time in this study. The peak detail of chloroform extract at 254, 280, and 366 nm are reported which will be useful in standardization of the product.

Total fungal and total bacterial count/g was found to be 190 CFU and 2100 CFU, respectively which is under permissible limit.[25] Specific pathogens such as *E. coli*, *Salmonella* spp., *S. aureus*, *P. aeruginosa* were found to be absent.

Thus, it may be concluded that ash values, extractive values and HPTLC analysis at 280, 254, and 366 nm could be used as important parameters for standardization of the formulation. QT has not been standardized using these parameters in any previous work, so present work may act as a reference for its future evaluation.

There are some exceptions and lack of corrections which should be taken care of in future work including work on various pharmaceutical procedures such as further reduction of disintegration time, detailed Assay, test for heavy/toxic metal, pesticide residue (Organochlorine and organophosphorus pesticides, and pyrethroids), test for aflatoxins (B1, B2, G1, and G2). Metallic content of this formulation can vary due to the use of clay that is obtained from various sources like from iron or lead ore etc. Though this formulation is not purely herbal, it contains

mineral/clay, so it can be mentioned under herbo mineral category. Heavy metal content guidelines are for formulation of herbal and/or animal origin.[30] A new amended guideline is needed by experts for herbo-mineral category of formulation, as they are studied as drugs intended to be used in humans. In the present formulation, preservatives have not been used and moisture level noted by LOD was 6.027 ± 0.1641, this procedure may also include volatile content of the drug.[28] Further development in the formulation due to spore formation, condition of storage and packaging needs further evaluation with accelerated stability and sophisticated pharmaceutical study.

CONCLUSION

Data for standardization of *Qurs Tabasheer* were developed and can be used as standards for future evaluation and reference.

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Conflicts of interest

There are no conflicts of interest.

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