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Original Article

Standardization and Development of Pharmacopoeial Standard Operating Procedures (SOPs) of Classical Unani Formulation

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ABSTRACT

Standardization of drug deals with confirmation of drug identity and determination of drug quality and purity. Unani herbal formulations are used in traditional medicine for the treatment of various diseases. Cancer is a disease which causes abnormal, uncontrolled growth of body tissue or cells, which tend to proliferate in an uncontrolled way. Spread of cancer from site of origin to other organs of the body is called metastasis. It is a hyper proliferative disorder involving, transformation, dysregulation of apoptosis, invasion and angiogenesis. The present study aimed to standardize a classical Unani formulation (CUF) described as anticancer properties. The CUF has been used for anti-cancerous activity (*Dāfi'-i-saraṭān*) in human population by Unani physicians for centuries. The standardization parameters carried out for classical Unani formulation are pharmacognostical studies, physicochemical parameters, high-performance thin layer chromatography (HPTLC), microbial load, aflatoxins, and heavy metals revealing specific identities and to evaluate Pharmacopoeial standards. Experiment and the data obtained established the Pharmacopoeial standards for this formulation for identification and quality control purpose. The CUF has been successfully standardized and standard operating procedures (SOPs) for its preparation has been laid down which may serve as a standard reference in future. The standardization data of this formulation may be used as a standard guideline for preparation of the formulation in future.

Keywords Standardization, Anti-cancer, Unani, *Saraṭān*, Physicochemical analysis, SOP, TLC.

INTRODUCTION

Nature is a reservoir of medications to cure all ailments of mankind (Kokate *et al*., 2017). Unani system of medicine is practised throughout the country for centuries. Drugs of plant, animal and mineral origin are used either as a single or in combinations. Unani medicines mostly are of plant origin drugs i.e. about 90 percent plant origin while 10 percent are derived from animal and mineral sources (Baskar *et al*., 2012; Anonymous, 2007b; Nadanakunjidam, S. and Kannabiran, 2002; Anonymous, 2012). The traditional Unani medicine plays a major role in maintaining health in developing countries (Meena *et al*., 2013). World Health Organization (WHO) has documented the Unani System of Medicine (USM) as an alternative system to provide the health care needs of human beings and is being practiced worldwide (Husain *et al*., 2010). Globally 85% of the traditional medicines are used for primary health care needs. Ethnobotanical information plays an important role in scientific research (Ignacimuthu, *et al*., 2006). Ibn e Baitar (1197-1248 AD), a great pharmacist, botanist and Unani physician compiled a book on Pharmacology after extensive field survey and research describing about 1400-1500 single drugs used in Unani Medicine for the medicinal properties (Saad and Said, 2011; Tschanz, 2003). The procedures and processes used for the manufacturing of traditional medicines are varied for complex heterogeneous mixture, it is increasingly felt to develop appropriate analytical methods and bioassay (Patil, A. *et al*., 2013).

The present classical Unani formulation are used to treat *Saraṭān* (Cancer) and known for its anti-cancerous activity from ancient time (Majoosi, 2010). The goal of standardization for present CUF is to enforce a level of consistency or uniformity to certain practices or operations within the selected environment (Grant, M., Kenton, 2019). It was realized that important Unani drugs need to be standardize and bioassay methods to be developed for correlated with nature of chemical composition.

The modern analytical technique like Thin Layer Chromatography (TLC) is an important separation technique. It is simple, quick and economical analytical technique that allow the researcher to find out how many chemical components are present in the sample. It is also used for the identification of a compounds in a mixture (Razique, A., Latif A, 2015) and used to detect purity and identity of a compound by Retention factor (R_f) value of a compound on comparison with that of R_f value of

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known reference compound, along with various detection systems like UV and derivatizations (Sasidharan *et al*., 2011). HPTLC is an advance instrumental technique for quantitative analysis over conventional TLC, it is sensitive, reproducible, versatile method of analysis. It can be used for preparative separation; for identification by R_f, UV absorbance, characterization and quantitative chromatographic detection. The HPTLC fingerprinting helps in identification characterization of drugs and their assay. Thin Layer Chromatography and HPTLC are now utilized in identification of various herbal drugs and their adulterant and the chemical constituent's analysis (Anonymous, 2007b). The present investigation is primarily done to standardize this CUF, which are being used for decades for their beneficial effects. CUF is generally being considered under the category of anti-cancer activity by Unani physicians. However, an endeavour to check the other parameters, herein made a preliminary attempt to evaluate the presence of microbial, aflatoxins and heavy metals. Organoleptic features such as appearance, colour, smell and taste play an important role in identification of the drug and these characteristics are peculiar with each drug and provide a qualitative index of identity and quality. Extractive values of a drug in specific solvent is an index of purity of a drug and plays an important role to find out adulteration, if any. The amount of drug soluble in a particular solvent is an index of its purity (Jenkins, G.L. *et al*., 2008). Ash value is a significant parameter for finding of adulteration and impurities. Loss of weight on drying indicates the amount of water and volatile substances present in a particular drug. A drug becomes ideal medium for growth of different types of bacteria and fungi if it contains moisture. The presence of bacteria and fungi affect the purity, quality and efficacy of the drug. pH helps the oral dosage forms as with increase and decrease in pH level the ability of drug to get absorbed is altered (Laurence L. Brunton, 2006). TLC analysis used to ensure the quality standards of CUF.

MATERIALS AND METHODS

The present study was conducted at National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad. The plant materials used in the study were identified and authenticated by the Botanist of NRIUMSD, Hyderabad and voucher specimen no. Halela Siyah (SMPU/CRI-Hyd13568), Aftimoon (SMPU/CRI-Hyd 13569), Bisfayej (SMPU/CRI-Hyd 13570), Ustukhudoos (SMPU/CRI-Hyd 13571), Kutki (SMPU/CRI-Hyd 13572), Ghariqoon (SMPU/CRI-Hyd 13573) and Namak Siyah (Black salt) were deposited in the herbarium.

2.1. Preparation of Unani formulation: The CUF prepared according to the composition of the formulation given by, 'Ali Ibn '*Abbās Majūsī*, in the *Kāmil al-Ṣanā'a* (Majoosi, 2010). The formulation consists of seven ingredients viz. Halela Siyah (fruit of *Terminalia chebula* Retz.), Aftimoon (whole plant of *Cuscuta reflexa* Roxb.), Bisfayej (root of *Polypodium vulgare* L.), Ustukhudoos (whole plant of *Lavandula stoechas* L.), Kutki (rhizome of *Picrorhiza kurroa* Royle ex Benth.), Ghariqoon (fungal species of *Agaricus albus* L.) and *Namak Siyah* (Black salt). All the ingredients of classical Unani formulation were weighed according to the composition. Each ingredient was powdered separately and mixed together to obtain the formulation which was further utilized for the study and three separate batches were prepared.

2.2. Powder fineness: The degree of coarseness or fineness of a powder is expressed by reference to the nominal mesh aperture size of the sieves for measuring the size of the powders for most pharmaceutical purposes and all the particles of which pass through a sieve with a nominal mesh aperture of 180 µm (Anonymous, 2007a).

Powder drug microscopy: Formulation powder was evaluated for microscopic characters, each formulation batch was separately stained with the reagents such as phloroglucinol 1% and conc. HCL, H2SO4, iodine solution, Sudan red G in acetic acid and ethanol (Kumar, Kumar and Prakash, 2011). The three batches samples are observed under microscope for their characteristic properties.

Organoleptic properties viz. appearance, form, colour, odour and taste; and microscopic study was carried out. The physico-chemical analysis was also carried out in the CUF such as total ash, acid insoluble ash, alcohol soluble extract, water soluble extract, pH of 1% and 10% aqueous solution and loss of weight on drying at 105 °C, bulk density, heavy metals, aflatoxins, microbial load carried and thin layer chromatography (TLC) was carried for separation of compound (Anonymous, 2016; Razique, A, Latif A, 2015).

2.3. Preparation of extracts of the sample for HPTLC analysis: Five grams of powdered sample was macerated in 200 ml of alcoholic solvent, separately in a stoppered conical flask and was kept for 2 h while shaking on orbital shaker. Later, the content was filtered through a Whatman filter paper No. 41 and evaporated the solution to concentrate upto 20 ml. The solution thus obtained was used for TLC. Solvent used are of HPLC grade and 5 ml glass vials are used for storage of extract.

2.4. Development of the HPTLC solvent system: Sample extract of Unani formulation was applied about 10 µl and developed by using the various suitable solvent system and recorded. Solvent system selected for developing TLC was toluene: ethyl acetate: methanol (7:2.5:0.5, v/v/v), development distance was 80 mm and detection wavelengths used are 254 nm and 366 nm. The number of spots and their R_f values were calculated (Naikodi *et al*., 2011).

2.5. Development of HPTLC technique: HPTLC was performed on 20 cm x 10 cm precoated aluminium plates of silica gel 60 F_{254} (Merck, KgaA, Germany) and plate thickness 0.2 mm, plate size 200 x 100 mm and starting distance from the edge of TLC plate 20 mm, distance from bottom 10 mm and the sample alcohol extract of 10 µl was applied as 10 mm width bands using automatic TLC applicator system of the DESAGA Sarstedt Gruppe (Germany). A linear ascending development with selected toluene: ethyl acetate: methanol (7:2.5:0.5; *v/v/v*) as mobile phase was carried out in a twin trough glass chamber previously saturated with mobile phase vapour for 20 min. at room temperature (25±2 °C), the development of solvent distance was 80 mm. After development, plates were air-dried. Scanning was performed using densitometer of DESAGA Sarstedt Gruppe (Germany) at 254 nm and 366 nm wavelength separately and operated by ProQuant 1.06 version software (Rasheed *et al*., 2012).

2.6. Determination of heavy metals: CUF was analysed for the detection of heavy metals at DSRI, Ghaziabad by Atomic Absorption Spectrophotometer method. Flame atomization technique used for detection of Lead (Pb) & Cadmium (Cd) and Hydride generator used for the detection of the elements viz, Arsenic (As) & Mercury (Hg).

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2.7. Microbial load determination: The microbial load was carried out as per the UPI/WHO guideline *(Quality control methods for medicinal plant materials World Health Organization Geneva*, 1998).

2.8. Microbial Count: The plate was counted at 24-48 hours for Soyabean casein digest agar media, HiCrome E. coli agar media and modified salmonella agar media while it was read for sabourauddextrose agar media at 48-72 hours.

2.9. Aflatoxin Solution: Aflatoxin was evaluated by the thin layer chromatography method, dissolve accurately weighed quantities of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 in a mixture of chloroform and acetonitrile (9.8:0.2) to obtain a solution having concentrations of 0.5 µg /ml each for aflatoxin B_1 and G_1 and 0.1µg per ml each for aflatoxins for B_2 and G_2 (Anonymous, 2009; Baur F.J, Ensminger LG; 1977).

RESULTS AND DISCUSSION

3.1. Microscopical observation: The powder of classical Unani formulation is dark brown in colour having initial pleasant odour and pungent taste followed by bitterness. The CUF observed under microscope shows characteristic features like cork cell, xylem fibres, pitted vessel and tracheids (Fig. 1).

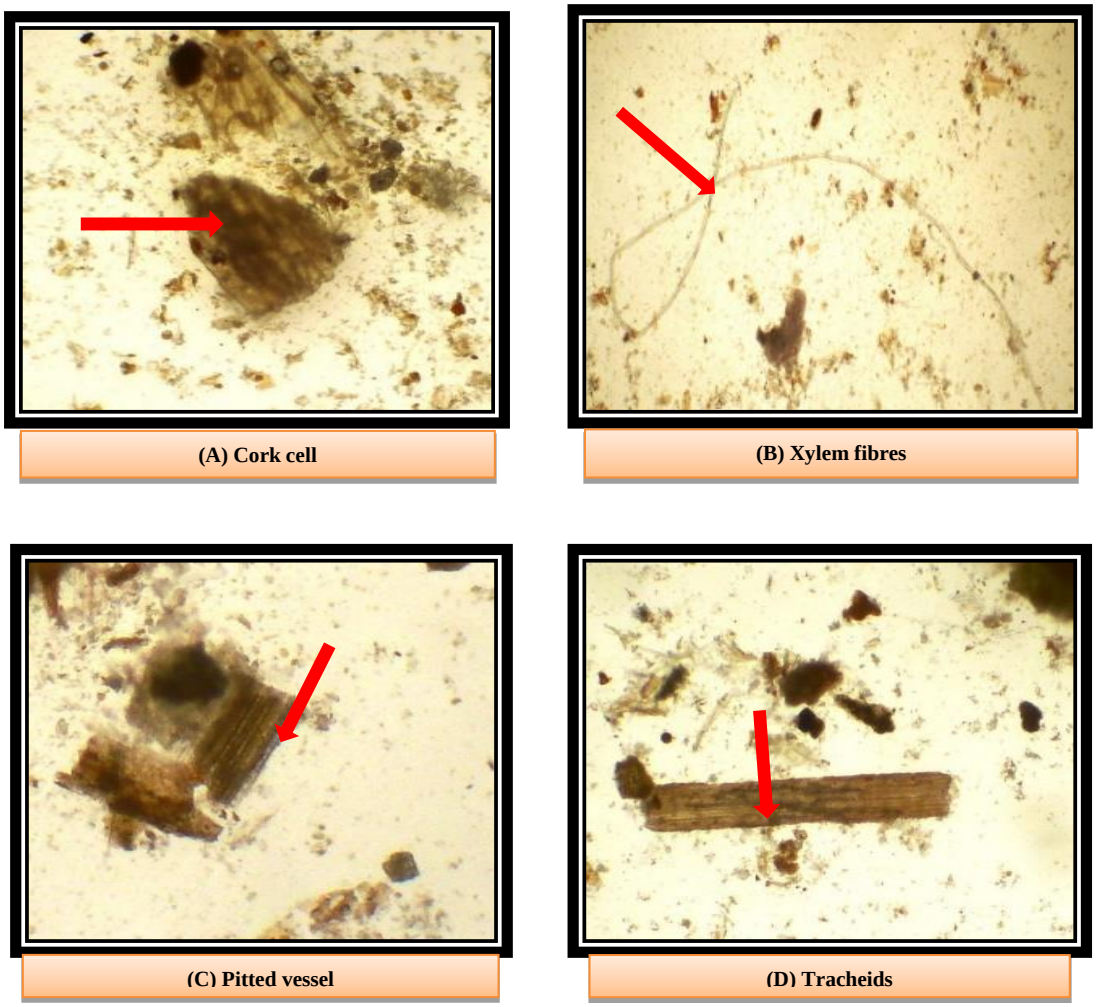

Fig. 1 Microscopical study of powder sample of formulation (A) Cork cell (B) Xylem fibres (C) Pitted vessel (D) Tracheids

3.2. Physico-chemical analysis: The mean value of total ash of classical Unani formulation was found to be 13.62 (Fig. 2) and mean value of acid insoluble ash was 2.57. The mean value of alcohol soluble extract was found to be 28.22 (Fig. 3) and mean value of water-soluble extract was 31.30 (Fig. 4). The mean of loss of weight on drying at 105 0C was found to be 5.50. The mean pH value of 1% aqueous solution was 5.30. The mean pH value of 10% aqueous solution was 5.61. The mean value of bulk density was 0.55 as obtained data tabulated in Table1.

3.3. Thin Layer Chromatography analysis: Alcoholic extract was spotted on silica gel "G" plate and developed with toluene: ethyl acetate: methanol (7:2.5:0.5, v/v/v) as mobile phase it shows ten major spots under UV 366 nm at R_f values 0.02 (blue), 0.14 (red), 0.25 (red), 0.34 (blue) 0.50 (blue), 0.57 (blue), 0.62 (green), 0.65 (blue), 0.68 (blue), 0.95 (dark blue); and under UV 254 nm shows five spots at R_f values 0.20, 0.35, 0.45, 0.54, 0.57 (All black); and under Iodine vapours shows five spots at R_f values 0.20, 0.42, 0.54, 0.58, 0.64 (All brown); under visible

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region after derivatizing with anisaldehyde sulphuric acid and heating at 105 0C shows twelve spots at R_f values 0.07 (red), 0.17 (red), 0.21 (red) , 0.24 (red), 0.31 (purple), 0.35 (purple), 0.45 (dark blue), 0.50 (blue), 0.57 (dark blue), 0.60 (blue) , 0.64 (purple), 0.68 (purple) (Fig: 5).

3.4. Heavy metals determination: The CUF was subjected to heavy metals analysis i.e. lead, cadmium, arsenic, mercury. The results showed that lead was present within the permissible limit and other elements cadmium, arsenic and mercury were found below the detection limit as given in Table 6.

3.5. Microbial load analysis: The results of the microbial load was found within the permissible limit and no significant bacterial counts were found in CUF. The total bacterial count in CUF ranges from $40x10^2$ /g to $45x10^2$ /g. other microbes such as Salmonella Spp. Escherichia. Coli and total fungal count were not present as given in the Table 7.

3.6. Estimation of Aflatoxin: The aflatoxins studies such as B_1, B_2, G_1, and G_2 were carried in classical Unani formulation and all the four aflatoxin were not detected as given in Table 8.

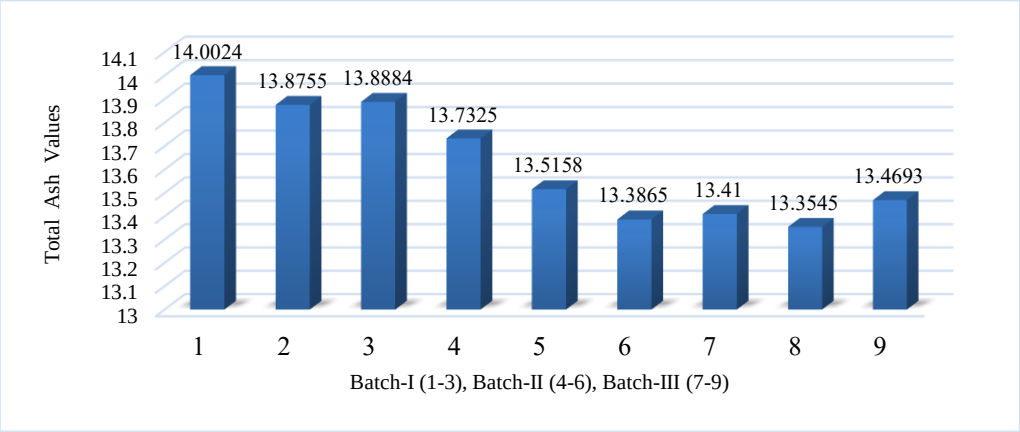

Fig. 2 Total ash (% w/w) in classical Unani formulation

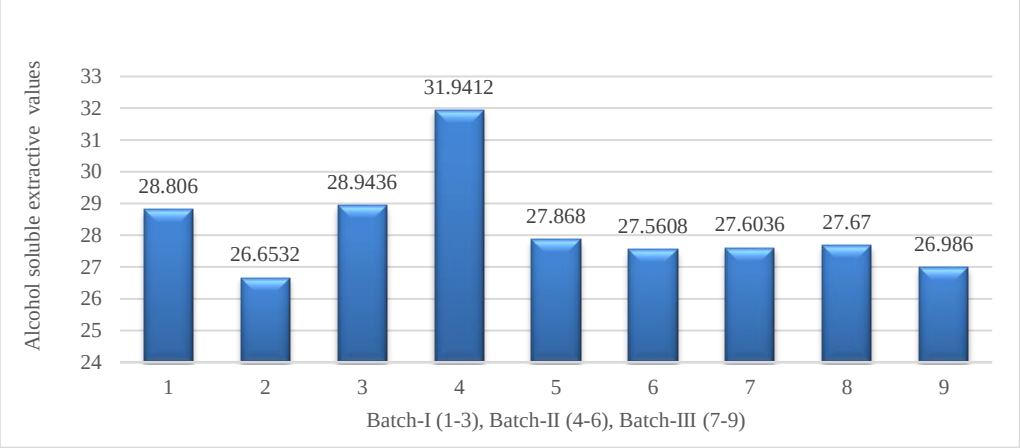

Fig. 3 Alcohol soluble extract (% w/w) of classical Unani formulation.

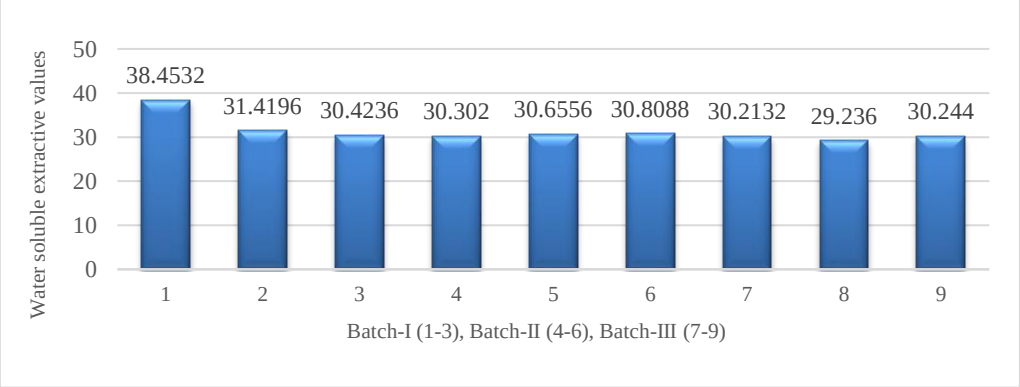

Fig.4 Water soluble extract (% w/w) of classical Unani formulation

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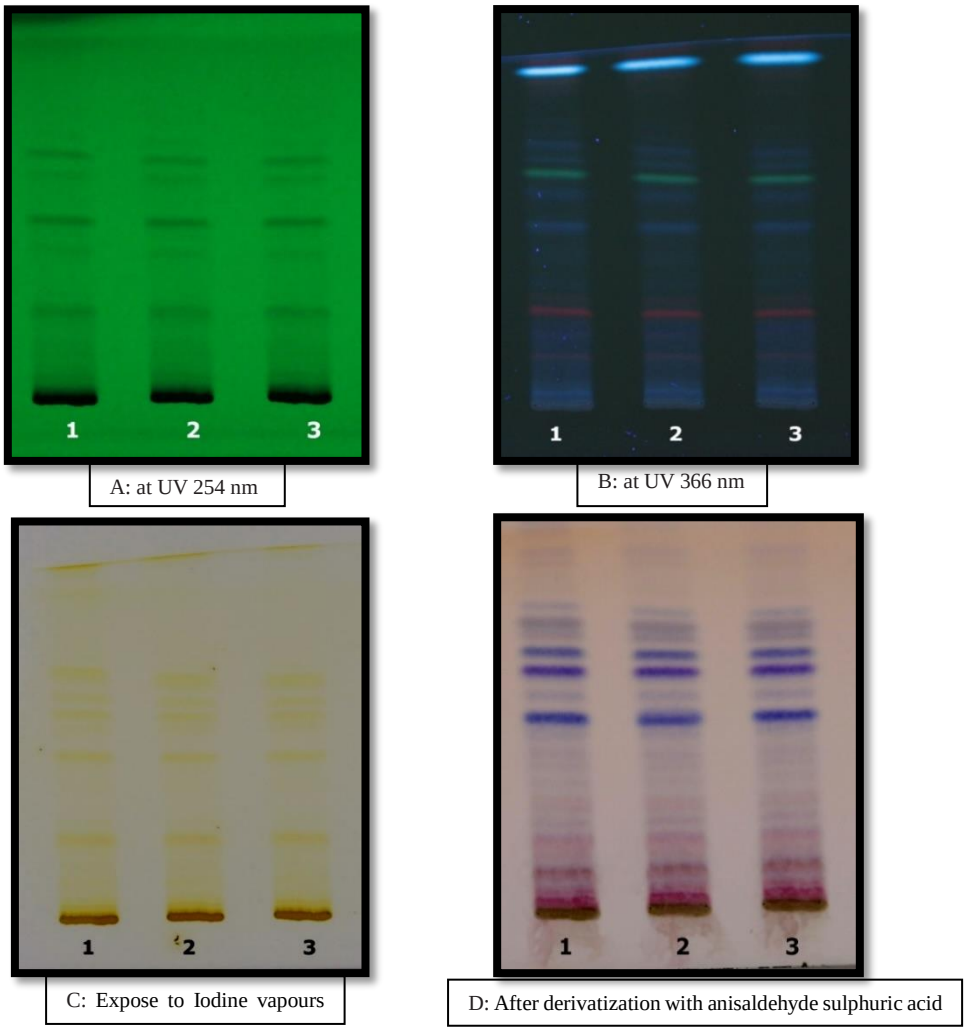

Fig. 5 TLC plate of the alcoholic extract of three batches of CUF observed in UV and after derivatization showing better separation of components (spots).

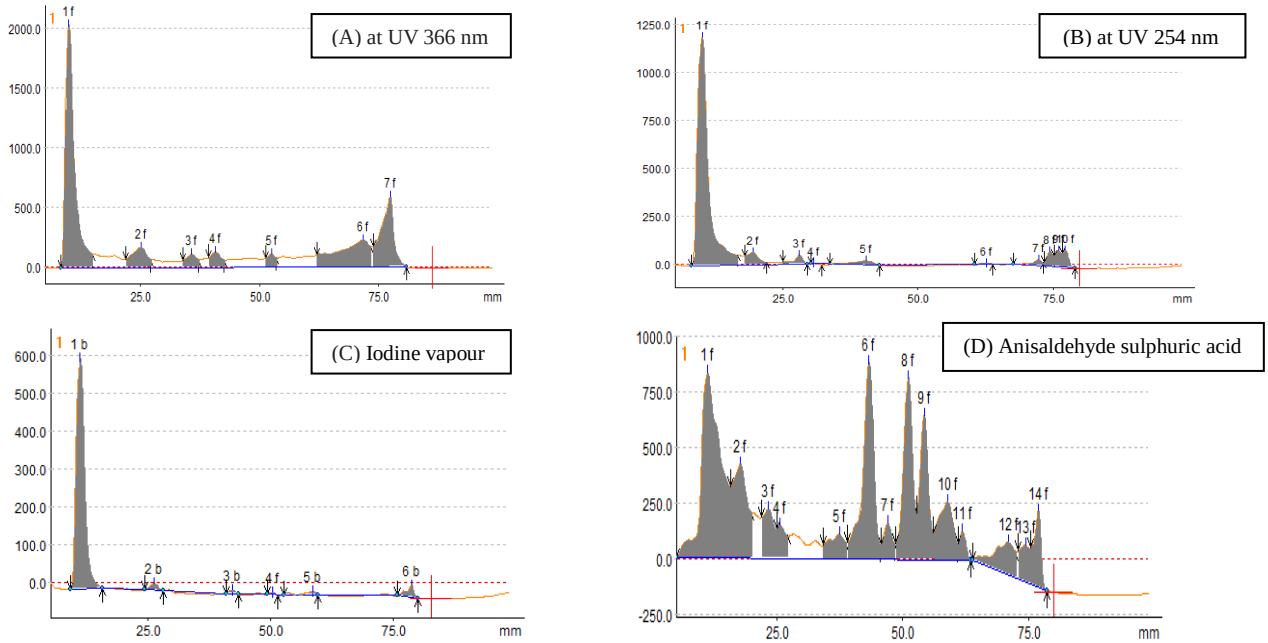

Fig. 6 HPTLC Densitogram of alcoholic extract of formulation (A) at UV 366 nm (B) at UV 254 nm (C) Iodine vapour (D) anisaldehyde sulphuric acid.

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Table 1. Physico-chemical parameters of the classical Unani formulation.

Parameter	Batch- I	Batch- II	Batch- III	Mean
Total ash (% w/w)	13.8755-14.0024	13.3865-13.7325	13.3545-13.4693	13.6261
Acid insoluble ash (% *w/w*)	2.5715-3.0966	2.6401-3.0078	2.7156-3.1499	2.5715
Alcohol soluble matter (% *w/w*)	26.6532-28.9436	27.5608-31.9412	26.986-27.67	28.2258
Water soluble matter (% *w/w*)	30.4236-38.4532	30.3020-30.8088	29.236-30.244	31.3062
Loss in wt. on drying at 105 ^{0}C (% *w/w*)	5.5872-5.902	5.2697-5.3998	5.2700-5.9017	5.5068
pH of 1% aqueous solution	5.27-5.27	5.31-5.32	5.33-5.33	5.27-5.33
pH of 10% aqueous solution	5.12-5.72	5.29-5.52	5.91-5.99	5.12-5.99
Bulk density	0.5515-0.5623	0.5506-0.5544	0.5513-0.555	0.5543

Table 2. Peak list of alcoholic extract of classical Unani formulation at UV 366 nm.

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	10.1	2796.00	78.66	1189.58	0.02
2	19.4	169.34	4.76	64.80	0.14
3	28.0	89.64	2.52	45.56	0.26
4	30.7	1.62	0.05	2.58	0.30
5	40.4	100.02	2.81	21.74	0.44
6	62.6	19.64	0.55	8.42	0.74
7	72.3	67.56	1.90	31.04	0.88
8	74.4	104.78	2.95	76.39	0.91
9	76.0	103.25	2.90	84.04	0.93
10	77.3	102.90	2.89	88.02	0.95

Table 3. Peak list of alcoholic extract of classical Unani formulation at UV 254 nm.

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	10.0	4475.57	47.67	2030.59	0.01
2	25.1	627.76	6.69	166.92	0.22
3	35.8	286.41	3.05	111.44	0.37
4	40.8	319.29	3.40	128.23	0.44
5	52.5	207.77	2.21	108.73	0.60
6	71.7	1726.41	18.39	225.57	0.87
7	77.4	1745.87	18.59	585.56	0.95

Table 4. Peak list of alcoholic extract of CUF upon exposure to iodine vapour at 580 nm.

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	11.1	1168.80	90.27	592.52	0.03
2	26.2	32.49	2.51	17.12	0.24
3	42.2	13.26	1.02	9.59	0.46
4	50.4	4.34	0.34	4.41	0.57
5	58.6	23.96	1.85	8.31	0.69
6	78.7	51.88	4.01	30.43	0.97

Table 5. Peak list of alcoholic extract of CUF upon derivatized with anisaldehyde sulphuric acid at 580 nm.

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	11.3	3611.71	25.57	814.56	0.03
2	17.8	1314.88	9.31	415.37	0.12
3	23.3	516.82	3.66	216.73	0.20
4	25.6	267.95	1.90	143.92	0.23
5	37.4	394.44	2.79	109.63	0.39
6	43.3	2194.45	15.54	868.55	0.48
7	47.0	315.47	2.23	160.87	0.53
8	51.1	1678.76	11.89	802.05	0.58
9	54.3	1235.39	8.75	635.42	0.63
10	58.9	891.43	6.31	258.36	0.69
11	61.9	183.42	1.30	127.04	0.73
12	71.0	646.20	4.58	144.86	0.86
13	74.4	316.48	2.24	163.53	0.91
14	77.0	556.44	3.94	338.45	0.94

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Table 6. Heavy metals determination

S.No.	Parameters analyzed	Results	UPI/WHO Permissible Limits
1	Lead (Pb)	0.9456 ppm	10 ppm
2	Cadmium (Cd)	Not Detected	0.3 ppm
3	Arsenic (As)	Not Detected	3.0 ppm
4	Mercury (Hg)	Not Detected	1.0 ppm

Table 7. Microbial Load Contamination

S.No.	Parameter analyzed	Results			Permissible limits as per WHO
		Sample-I	Sample-II	Sample-III	
1.	Total Bacterial Load	$45x10^2$	$40x10^2$	$43x10^2$	Not more than 10^5 / g
2.	Salmonella Spp.	Nil	Nil	Nil	Nil
3.	Escherichia. coli	Nil	Nil	Nil	Nil
4.	Total Fungal count	Nil	Nil	Nil	Not more than 10^3 / g

Table 8. Aflatoxin Contamination

S.No.	Parameter analyzed	Results			Permissible limits as per WHO
		Sample-I	Sample-II	Sample-III	
1.	B_1	Nil	Nil	Nil	Not more than 0.50 ppm
2.	B_2	Nil	Nil	Nil	Not more than 0.10 ppm
3.	G_1	Nil	Nil	Nil	Not more than 0.50 ppm
4.	G_2	Nil	Nil	Nil	Not more than 0.10 ppm

CONCLUSIONS

The CUF was successfully standardized by various parameters approved by WHO for quality control of compound formulations. The present finding for the anticancer formulation will provide fingerprint data through physicochemical parameter along with heavy metals, microbial load and aflatoxin contamination. The presented data may serve as a standard reference for identification and quality control of CUF which in turns helps to pharmacies, industries and other research organizations. The CUF may be further studied *in-vitro* and *in-vivo* for determining its property and efficacy towards anticancer activity.

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CONFLICT OF INTEREST

None

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