Original Article

Standardization of the Unani drug – *Myristica fragrans* Houtt. (Javetri) – with modern analytical techniques

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Abstract

Today, there is a tremendous demand of herbal medicine in the global market and the scarcity of data regarding the parameters and methods employed for assessing the quality of medicines. Aril (Mace) of *Myristica fragrans* Houtt., known as "Javetri," belonging to the Myristicaceae family, plays a foremost role in the Unani system of medicine. It contains Myristicin, an active principle of drug isolated by column chromatography, and its structure was established by spectroscopic methods. Different solvent drug extracts posses pharmacological properties like hypocholesteremic, anti-inflammatory, anti-diarrheal, chemopreventive action, etc. and hence there is a great need to determine the amount of myristicin present in the different extracts. The proposed method employed the High Performance Thin Layer Chromatography (HPTLC) DESAGA Sarstedt Gruppe and pre-coated aluminum sheets of silica gel developed with 100% chloroform to quantitatively determine the myristicin concentrations present in various extracts that are responsible for their different pharmacological actions. An attempt was made through instrumental analysis for quantitative estimations that are widely accepted for the quality assessment of herbal drugs such as TLC and HPTLC studies, etc. Physicochemical parameters, microbial load, aflatoxin and heavy metals and fluorescence studies were also carried out to lay down the standard for genuine drug. HPTLC studies were carried out in petroleum ether, chloroform, ethyl acetate, ethanol and methanol extracts and detected at 254 nm. Estimated high amount of myristicin in the petroleum ether extract w.r.t. the other extracts was confirmed by spectroscopy. The present paper describes the isolation, characterization and quantification of myristicin along with chemical standardization in order to develop standard parameters for the genuine drug.

Key words: HPTLC, myristicin, physicochemical parameters, quantification, TLC

INTRODUCTION

Aril of *Myristica fragrans* Houtt. is known as Javetri in the Unani system of medicine, which belongs to the family Myristicaceae. A well known drug since ancient times, it constitutes the outermost third integument of the seed, covering its basal part by scarlet or pale yellow ribbon-like lobes, and is strongly aromatic in nature. It is known differently in various languages such as Arabic: Jouzbuwwa, Jouz-ul-teeb and Jainsiban; Italian: Moscatero; Kashmiri: Zafal; Latin: Merisiniae; Marathi: Jaiphal; Malayalam: Jatika; Oriya: Jaipholo; and Urdu: Jaiphal.[1-15]

The plant has been successfully cultivated in Madras and Southern India, Nilgiri hills, Coimbatore, Salem, Ramanathapuram, Tirunelveli, Kanya Kumari and Madurai districts, Malabar Coast, Assam and in other states.[10,13] *Myristica fragrans* Houtt. requires a hot and moist climate with a rainfall of 150–300 cm per annum. It is cultivated in the hotter parts of India[16] and grows best at low elevation in alluvium formed of deep friable loam, with good drainage that is well sheltered from high winds; it does not thrive above an altitude of 750 m.[13] Its therapeutic uses are in cardiac diseases (Amraz-e-Qalb), indigestion (Sue Hazim) and sexual debility (Zofe Bah). *Myristica fragrans* (nutmeg) was profoundly used as

a carminative, stimulant, flavoring agent and also in the treatment of rheumatism. In eastern countries, it is used as a drug more than a condiment (something used to enhance the flavor of food). Mace has a bitter pungent taste and, therefore, is useful in bronchitis, thirst and improves appetite (Ayurveda). Oil of maces is employed for flavouring food products and liquors, used for scenting soaps, tobacco and dental creams and also in perfumery.

Oil of mace and nutmeg is useful in sprains, rheumatism and paralysis.[17] Myristicin, one of the major essential oils of nutmeg, was found to possess extraordinarily potent hepatoprotective activity.[18] The petroleum ether extract showed activities similar to non-steroidal anti-inflammatory drugs and also anti-diarrheal activity.[19] The chloroform extract also inhibited the carrageenan-induced edema in rats.[20] Hydroalcoholic extract shows antidiabetic and antihyperlipidemic effects of *Myristica fragrans*.[21] The methanol extract showed a lasting anti-inflammatory activity, and results suggest that the anti-inflammatory action of mace is due to the myristicin that it contains. The methanolic extracts shows antiplaque action against *Streptococcus mutants*. It also reduced the acetic acid-induced vascular permeability in mice.[22] Myristicin is a phenylpropene, a natural organic compound present in small amounts in the essential oil of nutmeg and, to a lesser extent, in other spices such as parsley and dill. Myristicin is a naturally occurring insecticide and acaricide with possible neurotoxic effects on neuroblastoma cells.[23]

Chemical constituents

The main chemical constituents of *Myristica fragrans* are myristicin, myristic acid, elemicin, saffrole, eugenol, palmitic, oleic, lauric and other acid, protein and starch[24], as shown in Figure 1.

Mass spectrum of myristicin shows a mol. wt. of 192 and m/z 192 (100%); 165 (23%); 161 (14%); 131 (13%); 119 (15%); 91 (25%); 65 (16%) and 39 (13%). Separation and isolation of myristicin from the drug, characterization by the spectroscopic method and quantification by the reported method is an easy and accurate analytical technique that is cost effective. Because of the broad spectrum of biological activities of the drug, we have attempted to isolate and characterize (by means of UV spectra) myristicin with reference compound and also estimate the amount of myristicin by the HPTLC analytical technique in different drug extracts, which is responsible for the pharmacological properties of the drug. Thus, quantification with HPTLC can provide results that are either superior or comparable with other analytical methods such as HPLC, etc.

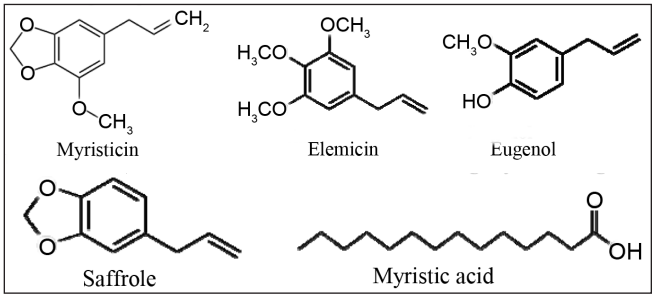

Figure 1: Sturcutures of main chemical constituents of *Myristica fragrans* Houtt.

MATERIALS AND METHODS

Collection of material, extraction, isolation and quantification

Myristica fragrans Houtt. was procured from Vinayak Dawasas Ayurvedic Chaudapudi, Herbal store at Borabanda, Hyderabad, India. The sample was identified with the help of a botanist at the Central Research Institute of Unani Medicine, Hyderabad, before the study was carried out. The present study of herbal drug includes parameters such as morphology, physicochemical parameters, TLC and HPTLC fingerprints, quantification of myristicin in different extracts and powdered study safety evaluation, etc. HPTLC and UV spectra were carried out by the HPTLC DESAGA Sarstedt Gruppe system along with the Automatic TLC applicator and UV visible cabinet as the imaging system; the instrument had Proquant 1.6 version as software system for documentation. All the solvents used were of HPLC grade. Physicochemical parameters were determined according to the methods described in Anonymous (2009).[25] Fluorescence analysis was carried out as per the method described by Trease and Evans (1972).[26] Heavy metals, aflatoxins and microbial load were analyzed according to the methods given in the WHO guidelines, 1998.[27]

Preparation of extracts of the drug for TLC analysis

Five grams of the powdered drug was macerated in 100 ml of specific selected different solvents, i.e. acetone, chloroform and petroleum ether separately in a stoppered conical flask and was kept for 2 h while shaking at regular intervals. Later, the contents were filtered through a whattmann No. 41 filter paper and evaporated the solution to 20 ml. The solution thus obtained was used as sample for the TLC. Sample of

Table 1: TLC profile of different solvent extracts of Myristica fragrans and their Rf values

Extract	Solvent system	Spray/Treatment	No. of spots	Rf values
Pet. ether	Pet. ether (40-60%)	5% alcoholic H_2SO_4 heated at 105^{0}C for few min.	7	0.17, 0.23, 0.35, 0.43, 0.50, 0.79, 0.91
Pet. ether	Benzene chloroform (4:1)	-do-	5	0.18, 0.30, 0.54, 0.79, 0.91
Chloroform	Benzene chloroform (4:1)	-do-	6	0.19, 0.33, 0.54, 0.79, 0.91, 0.95
Chloroform	Chloroform Methanol (97:3)	-do-	4	0.09, 0.38, 0.70, 0.90
Acetone	Benzene:Methanol (85:5)	-do-	7	0.25, 0.32, 0.68, 0.77, 0.85, 0.91, 0.97
Acetone	Toluene:Ethyl formate: formic acid (5:4:1)	-do-	9	0.37, 0.49, 0.52, 0.56, 0.66, 0.69, 0.70, 0.84, 0.98

the specific solvent drug extract of 20 µl was applied and developed using the various suitable solvent systems, and recorded as tabulated in Table 1. Also, the number of spots and Rf values were recorded.

Extraction and isolation of Myristicin

Myristicin, the active principle of the drug, was isolated using column chromatography and was characterized and confirmed spectroscopically for its structure. Spectroscopic data of the isolated compound were generated with the help of the Indian Institute of Chemical Technology, Tarnaka, Hyderabad, and was confirmed with the reference pure compound.

HPTLC studies of the drug extracts with quantitative estimation of Myristicin in the drug

One gram of the powdered drug was macerated in 20 ml of the different solvents, i.e. petroleum ether, chloroform, ethyl acetate, ethyl alcohol and methanol separately in a stoppered conical flask and was kept for 2 h while shaking at regular intervals. Later, the contents were filtered through a whattmann No. 41 filter paper and evaporated the solution to 10 ml. The solution thus obtained was used as sample for the determination of components.

The solvent extracts along with the myristicin were applied as a 10-mm band of about 1 µg with the help of an automatic TLC applicator system of the DESAGA Sarstedt Gruppe on pre-coated aluminum sheets of Silica Gel 60 F_{254} Merck (Darmstadt, *Germany*). The plate was developed with 100% chloroform in the twin-through TLC chamber to the maximum height of the plate so that components were separated on the polar phase of the silica gel and the mobile phase of the solvent system.

Development of the HPTLC technique

After developing, the TLC plate was dried completely and detected under a UV cabinet for detection of spots and photographed at 254 nm. Further, the plate was scanned with the Densitometer CD60 of DESAGA Sarstedt Gruppe system at the 254 nm UV region. A densitogram was obtained in which peaks appeared corresponding to spots being detected in the densitometer while scanning as shown in Figure 2(a-f). The peak area under the curves corresponds to the concentration of the component in the sample to the amount of solution applied on the plate. Overall, overlapping of the chromatogram of myristicin and extracts were shown in Figure 2g.

UV spectrum

With the help of the HPTLC software system, i.e. Proquant 1.6 version, the UV spectrum was taken out under the UV range to get the absorption bands in the spectrum for the peaks obtained in the densitogram with respect to their component positions, i.e. myristicin, as shown in Figure 2h.

RESULTS AND DISCUSSION

Organoleptic characters

The crude drug consists of the dried aril of *Myristica fragrans* Houtt. of the Myristicaceae family, Figure 3 a.

Morphology of mace

Macroscopic

The drug consists of reddish pieces, about 2–4 cm in size, which are the blades of the arils. They are flat, smooth, irregularly slit, slightly flexible or brittle and somewhat translucent. They are rich in oil and therefore exude a reddish or orange oily color when pressed. It bears some odor and taste as that of nutmegs, as shown in the macroscopic photograph in Figure 3a.

Microscopic

The cross-section of the aril shows somewhat leaf-like structures. It is bounded by a single-layered epidermis on either side, while the rest of the area is occupied by simple, thick-walled cells with no intercellular space, and oil cavities are in abundance. The epidermal cells (with no intercellular space) are about 25 x 40–25 x 45 in size, while the thick-walled cells measure 28 x 30.5-35 x 35 in size.

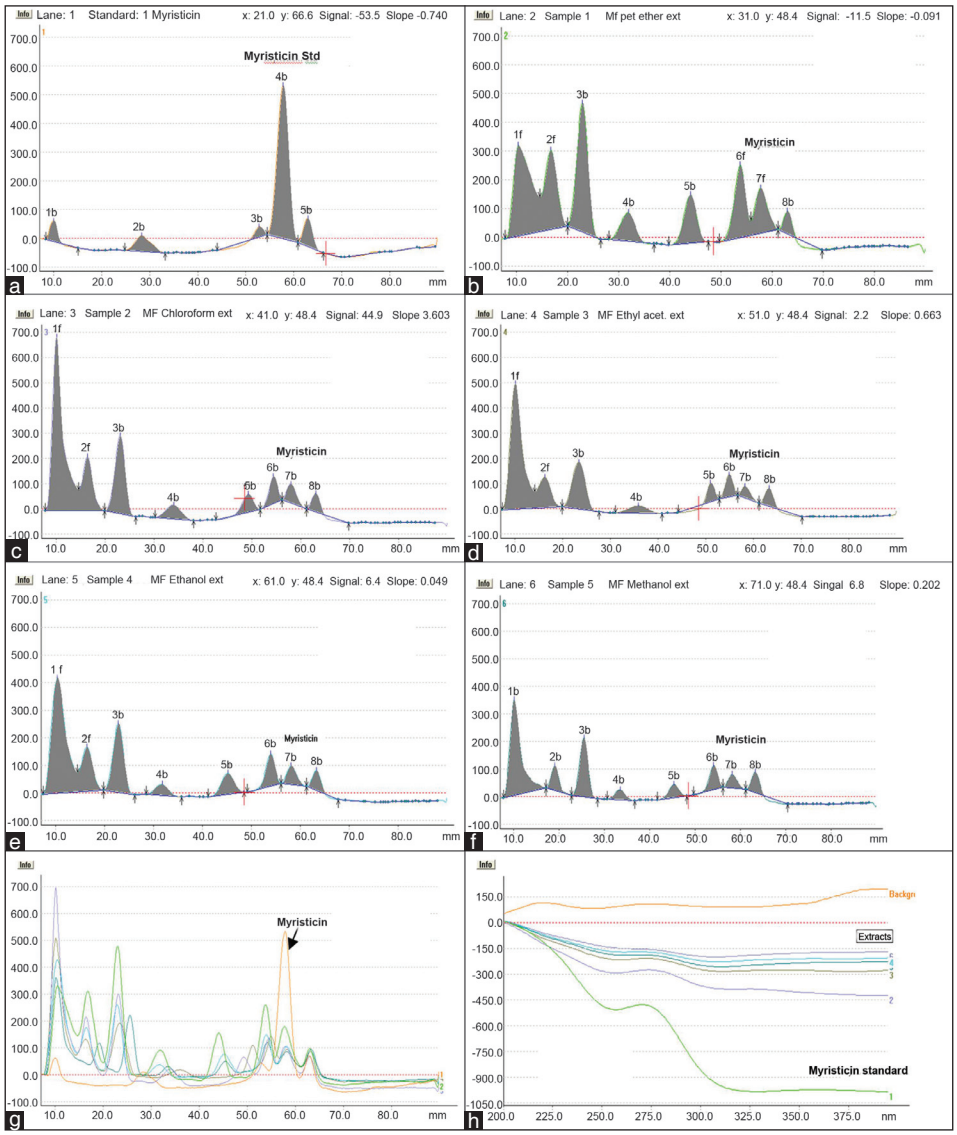

Figure 2: (a–f) Densitograms of the myristicin and petroleum ether extract, chloroform extract, ethyl acetate extract, ethanol extract and methanol extract of *Myristica fragrans* at 254 nm. (g) Overlapping densitograms of all the extracts. (h) UV spectra of all the extracts showing a band at 254 nm in all extracts in comparison with Myristicin

Powder analysis

The powder is fine, homogeneous and yellowish-brown with a strong aroma and slightly bitter taste. The powder when seen under the microscope shows abundance of thick-walled cells. Starch and aleuronic grains are absent.

Chromatographic profile

The TLC profile of petroleum ether, chloroform and acetone along with the solvent system and Rf values are recorded in Table 1. The HPTLC profile for *Myristica fragrans* in the different solvent extracts with the Rf values are tabulated in Table 2 and the developed chromatogram is shown in Figure 3b.

HPTLC studies of petroleum ether, chloroform, ethyl acetate, ethyl alcohol and methyl alcohol extracts of the drug in comparision with the myristicin standard revealed eight peaks, in which the seventh peak is of myristicin in comparison with the standard peak of the myristicin in the densitogram, which is obtained at Rf 0.82, as depicted in Table 2. The corresponding Rf values identical to myristicin at 254 nm for petroleum ether, chloroform, ethyl acetate, ethyl alcohol and methyl alcohol are 0.82, 0.82, 0.83, 0.82 and 0.83, corresponding to the spot of myristicin.

The mount of myristicin obtained in the different solvent extracts with respect to the amount of the sample applied is tabulated in Table 3. Results from the HPTLC data of myristicin along with the different solvent extracts with respect to the concentration present in the drug extract is tabulated in Table 4. Graph for the quantification is shown in Figure 3c–d.

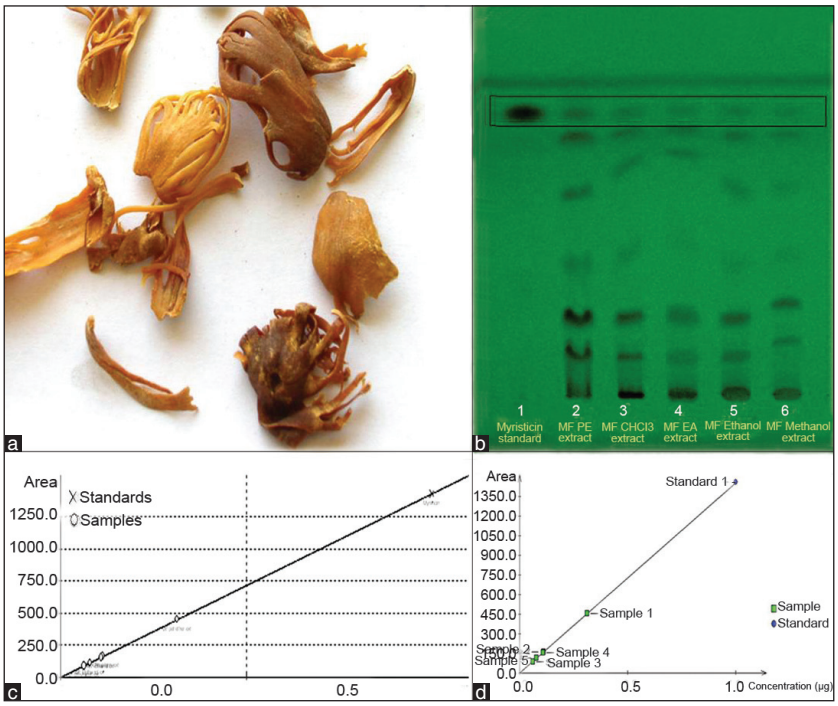

Figure 3: (a–d) TLC-developed chromatogram with myristicin spot shown in rectangular box. Concentration of myristicin with respect to the sample applied (a) Macroscopic feature of *Myristica fragrans* Houtt, (b) Finger print TLC chromatogram of *Myristica fragrans* Houtt, (c) Concentration of Myristicin present in the different solvent extracts of *Myristica fragrans* (d) Comparative graph between concentration and the amount of sample applied with respect to standard myristicin

Table 2: HPTLC data for different solvent extracts with Rf values and position of spot in chromatogram

Peaks	Myristicin		Pet. Ether extract		Chloroform extract		Ethyl acetate extract		Ethanol extract		Methanol extract	
	Rf values	Y-pos.	Rf values	Y-pos.	Rf values	Y-pos.	Rf values	Y-pos.	Rf values	Y-pos.	Rf values	Y-pos.
1	0.05	10.0	0.05	10.4	0.05	10.1	0.05	10.1	0.05	10.4	0.05	10.1
2	-	-	0.16	16.7	0.15	16.4	0.15	16.3	0.15	16.4	0.20	19.1
3	0.34	28.3	0.26	22.9	0.26	23.1	0.26	23.4	0.25	22.8	0.30	25.5
4	-	-	0.40	31.9	0.44	34.0	0.47	35.9	0.40	31.8	0.43	33.5
5	-	-	0.60	44.0	0.68	49.2	0.71	51.0	0.62	45.2	0.62	45.3
6	0.74	52.8	0.75	53.7	0.76	54.4	0.77	54.9	0.76	53.9	0.76	54.0
7	0.82	57.8	0.82	57.7	0.82	58.0	0.83	58.2	0.82	58.1	0.83	58.2
8	0.90	63.0	0.90	63.0	0.90	63.1	0.91	63.3	0.91	63.3	0.91	63.3

Table 3: HPTLC profile of myristicin along with different solvent extracts w.r.t concentration obtained

Sample	Name	Y-Pos (mm)	Area	Area	Concentration (µg)
Standard 1	Myristicin	57.8	1426.090	524.054	1.0
Sample 1	MF pet ether ext	57.7	447.095	157.282	0.3
Sample 2	MF Chloroform ext	58.0	161.759	74.463	0.1
Sample 3	MF Ethyl acet. ext	58.2	90.062	43.878	0.1
Sample 4	MF Ethanol ext	58.1	155.839	68.045	0.1
Sample 5	MF Methanol ext	58.2	113.038	49.563	0.1

Table 4: Amount of myristicin obtained in different solvent extracts

Sample Name	Extract	Component	Type	Amount of substance (µg)
Sample 1	MF pet ether ext	Myristicin	Probe 1	313.51 (0.03)
Sample 2	MF Chloroform ext	Myristicin	Probe 2	113.43 (0.01)
Sample 3	MF Ethyl acet. ext	Myristicin	Probe 3	63.15 (0.01)
Sample 4	MF Ethanol ext	Myristicin	Probe 4	109.28 (0.01)
Sample 5	MF Methanol ext	Myristicin	Probe 5	79.26 (0.01)

Figures given in parenthesis are in percentage.

Physicochemical studies

Physicochemical parameters of *Myristica fragrans* such as total ash 2.31gm%, acid-insoluble ash 0.11 gm%, water-soluble ash 0.98% and loss on weight drying at 105°C 17.837 gm% are summarized in Table 5, and successive extractive values were carried as petroleum ether (60–80%) extract 20.66 gm%, chloroform 5.13 gm% and alcohol 10.20 gm%.

Table 5: Physico-Chemical parameters values as obtained for *Myristica fragrans*

Parameter	Values (gm%)
Total ash	2.31
Acid insoluble ash	0.11
Water soluble ash	0.98
Loss on drying at 105° C	17.837
Successive extractive values	
Pet. ether (60-80%)	20.66
Chloroform	5.13
Alcohol	10.20

Other parameters include microbial load, aflatoxin and heavy metal contamination, in which the total bacterial load was found to be 4 x 10^4/g and the total fungal count was found to be 2 x 10^2/g, which were below the permissible limit of WHO, as tabulated in Table 6. No aflatoxins such as B1, B2, G1 and G2 and heavy metals were detected in the drug, as shown in Table 6, and fluorescence behavior along with powdered study of drug with chemical reagents in ordinary light and UV light were carried out as shown in Tables 7 and 8 to lay down the standard for the genuine drug.

UV spectrum for the myristicin spots obtained in the densitogram was carried out corresponding to their positions. The bands resulting in the spectrum for the spots corresponding to Rf values identical to myristicin at 254 nm for petroleum ether, chloroform, ethyl acetate, ethyl alcohol and methyl alcohol are 0.82, 0.82, 0.83, 0.82 and 0.83, as shown in Figure 2h.

Table 6: Microbial, Aflatoxin and Heavy metal quantitative analysis of the drug

Safety evaluation	Parameters	Result found	Permissible limit
Microbial contamination	Total Bacterial Load	4 x 104 /g	Not more than 105/g
	Salmonella Spp.	Nil	Nil
	Escherichia. Coli	Nil	Nil
	Total Fungal count	2x102/g	Not more than 103/g
Aflatoxin contamination	B1	Nil	Not more than 0.50 ppm
	B2	Nil	Not more than 0.10 ppm
	G1	Nil	Not more than 0.50 ppm
	G2	Nil	Not more than 0.10 ppm
Heavy metal contamination	Arsenic	Nil	Not more than 3.0 ppm
	Cadmium	Nil	Not more than 0.3 ppm
	Lead	Nil	Not more than 10.0 ppm
	Mercury	Nil	Not more than 1.0 ppm

Table 7: Reaction of chemicals with crude powdered drug

Parameter	Observation
Powder triturated with water	Emulsion formed
Powder shaked with water	Froth little
Powder treated with 5% ferric chloride	No change
Powder treated with 66% sulphuric acid	Coffee colour appears
Powder treated with 5% sodium hydroxide	Brick red colour
Powder pressed between 2 folds of filter paper	Oily stain appears

Table 8: Fluorescence analysis of powdered drug

Treatment	Colour in ordinary day light	UV light
Powder as such	Reddish brown	Dark chocolate
Powder treated with 1 N NaOH in methanol	Reddish brown	Chocolate brown
Powder treated with 1 N NaOH in water	Reddish brown	Chocolate brown
Powder treated with 1N HCl	Yellowish brown	Dark brown
Powder treated with 50% oHNO3 (aq)	Chocolate brown	Dark brown
Powder treated with 50% sulphuric acid (aq.)	Yellowish brown	Dark chocolate
Powder treated with 1 N NaOH in methanol	Black red	Dark chocolate
Powder treated with in HCl	Yellowish brown	Dark chocolate
Powder treated with in NaOH in water	Reddish brown	Dark chocolate

The amount of myristicin was found to be higher in the petroleum ether extract with 313.51 µg (0.03 %) with respect to the drug taken for the extract. Hence, this proposed method of HPTLC is very easy and cost-effective for the isolation and quantification of myristicin, which is the chief principle in the drug responsible for its pharmacological properties.

CONCLUSION

The drug under study was subjected for physicochemical analysis, which is supportive in establishing the standard along with the other parameters such as macroscopic, microscopic and fluorescence behavior, as reported. In the present investigation, microbial load, aflatoxins and heavy metal contamination resulted within the permissible limits of the WHO guidelines. The modern technique of HPTLC analysis was employed with respect to standardization and to separate the compounds that can be isolated for further studies. HPTLC studies were correlated with UV spectrum data, illustrating the number of individual components present in it and also thoroughly studied in the different solvent extracts for determining the amount of myristicin, an active principle of the drug present in different extracts of the drug. The amount of myristicin was found to be higher in the petroleum ether extract with 313.51 µg (0.03%) with respect to the amount of drug taken for the extract in comparison with other drug extracts. On the basis of these data, the drug was brought up in determining and ascertaining its quality and standardization of drug. The isolation, characterization and quantification of myristicin with the reference sample along with standardization were successfully carried to develop standard parameters for the genuine drug, which can be useful for future pharmacological studies.

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