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Research Article

Pharmacognostic Standardisation of *Lentinula Edodes*: A Widely Explored Medicinal Mushroom

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ABSTRACT

Lentinula edodes, the "Shiitake" mushroom is a well studied edible mushroom which has antimicrobial, antifungal, antiviral, antidiabetic and antioxidative potentiality. The present study was conducted in order to investigate various qualitative and quantitative parameters like microscopic evaluation, physicochemical constants and preliminary mycochemical analysis of the dried mushroom powder. Physicochemical constants such as percentage of loss on drying (8.9%), total ash (3.81%), swelling capacity (116.66%), water holding capacity (427.12%), water solubility index (23.85%) and methanol extractive value (11.2%) were documented. Fluorescence analysis exhibited considerable variations against treated reagents. On the other hand preliminary mycochemical tests revealed presence of glycosides, carbohydrate, terpenoids, phenol and flavonoids. Good content of phenols was also supported with HPTLC and HPLC data. The antioxidant activity was checked using DPPH radical scavenging assay (EC_{50}=1.25 mg/ml) and total antioxidant capacity test. Measurement of bioactive components confirmed presence of flavonoids, phenol, ascorbic acid, β-carotene and lycopene in descending order. Hence, the present study establishes the pharmacognostic profiles of this mushroom which will help in standardization of the mushroom powder with respect to quality, purity and identity.

Keywords: antioxidant activity, HPLC, HPTLC, mushroom**,** pharmacognosy

INTRODUCTION

After decades of serious obsession with modern medicinal system because adverse effects of synthetic drugs, peoples have started looking at the alternative healing systems. In this Centex, in recent times, mushrooms are through to possess more than 100 different medicinal function including antioxidant[1-3], anti-diabetic[4], antimicrobial[5,6], hepato-protective[7-9], antiulcer[10] and anticancer[11,12] effects because of the unique physiological and molecular structure. Mushrooms are the potential source of bioactive high- molecular-weight and low- molecular-weight compounds. Among different biologically active components a large number of mushroom polysaccharides from approximately 700 species were isolated and characterized[13]. Many of these mushroom polysaccharides were reported to have strong immunomoduletory activities[14-18].

Lentinula edodes (Shiitake) is a wood -decaying basidiomycetes, gregarious on fallen wood of a wide variety of deciduous trees. The name Shiitake means it likes to grow on wood of 'Shii' tree and 'take' means the mushroom fruit. This is an edible mushroom native to East-Asia, which is consumed and cultivated in many Asian countries. It is considered as a medicinal mushroom in some forms of traditional medicine. Shiitake's flavour is 4-10 times more intense than that of ordinary button mushroom. It is also fleshy, rich in nutrients, minerals and vitamins. It is the second most popular edible mushroom in the global market which is attributed not only to its nutritional value but also to possible potential for therapeutic applications. Several well studied preparations, extracts and active components from this mushroom showed remarkable therapeutic potentiality including antimicrobial, antiviral, cardioprotective, antifungal, antidiabetic, hemagglutinating, antioxidative, hepatoprotective, hypolipidemic, immunomodulating and antineoplastic effects[19]. Many new biological active components were isolated and characterized from this mushroom lentinan (β-D-glucans), lentinamicin, critadenine, lentin, lactin, lentysine etc[19]. This mushroom plays an important role in the health care programs in developed as well as developing countries[20,21].

Therefore, it is important to ensure quality and purity of herbal medicines in order to maximize the efficacy and minimize the adverse effects. Proper identification and quality assurance of the starting material are essential prerequisites to ascertain reproducible quality of herbal drugs. Keeping the above view in mind various macroscopic, microscopic and pharmacognostic studies on dried basidiocarps of *Lentinula edodes* were carried out in the present investigation.

MATERIALS AND METHOD

**Author for Correspondence*

Figure 1: Fruit body of *Lentinula edodes* a: Dried fruit body b: Fruit body in powder form c: Powder microscopy

Table 1: Physico-chemical parameters of dry powder from *Lentinula edodes*

Physico-chemical constants		Mean ± SD (% w/w)
Ash value	Total ash	3.81± 0.16
Moisture and volatile content	Loss on drying	8.9 ± 0.2
Hydration properties	Swelling Capacity (%)	116.66 ± 2.34
	Water Holding Capacity	427.123 ± 2.669
	Water Solubility Index	23.845 ± 0.333

Materials

Basidiocarps of *Lentinus edodes* were purchased from the markets of Imphal, Manipur, India. Basidiocarps were dried properly by a field drier at 40°C for 1 day to make them crispy. A reference specimen was deposited in Calcutta University (Accession no: AMF 1074). Dried fruit bodies were pulverized using an electric blender, sieved through 160 mesh and stored in an air tight containers.

Microscopic evaluation of powdered basidiocarps and organoleptic study

Powdered sample was hydrated and macerated with 10% KOH and mounted on glass slide with glycerol. For effective results various stains (Congo red, Melzer reagent) were used to distinguish different cellular structure. Photomicrographs were taken using compound binocular microscope having sensor aided digital camera and computer attachment. Different organoleptic characters like colour, odour, taste, nature of powdered samples were evaluated during organoleptic study.

Physico-chemical evaluation

Pharmacognostical values such as percentage of total ash value, loss on drying and methanol extractive values were performed according to WHO guidelines on quality control methods for medicinal plant materials[22]. Water holding capacity, water solubility index and water swelling capacity were evaluated as described by C. Phat (2015)[23]

Fluorescence analysis

Fluorescence analysis was done according to the methods of Kokashi et al (1958)[24]. A small quantity of dried sieved powder material was placed on grease free clean glass slide, 1-2 drops of freshly prepared reagent solutions (Hager`s, Mayer's, Dragendroff's, iodine solution, 1(N) HNO_3, 50% HNO_3, phloroglucinol, Barfoed, sodium nitroprusside, Ninhydrin, $FeCl_3$, 1(N) NaOH, acetic acid, 1(N) HCl, methanol, 1(N) NaOH in methanol) were added, mixed by gentle tilting the slides and left for 1-2 mins. Changes in colour due to addition of different reagents were recorded in normal light, as well as short (254nm) and long (365nm) UV light.

Qualitative analyses of methanol extract

The methanolic extract was subjected to various qualitative tests to detect the presence of phytoconstituents like alkaloids, carbohydrate, cardiac glycosides, flavonoids, phenol, protein, saponin, sterols and terpenoids[25].

Quantitative estimation of phytochemicals

Folin-ciocalteu reagent and sodium carbonate was used to measure the total phenolic compounds present in extract, using gallic acid as standard. The results were expressed as µg of gallic acid equivalents per mg of dry extract. Total flavonoid content was estimated using aluminium nitrate and potassium acetate. Quercetin (5-20 µg/ml) was used as a standard, expressing results as µg of quercetin equivalents per mg of dry extract. β-carotene and lycopene were estimated by measuring absorbance at 3 different wavelengths viz.; 453, 505 and 663 nm. Ascorbic acid was determined with titration against 2, 6-dichlorophenol indophenol dye in presence of oxalic acid[26].

HPTLC and HPLC profile of methanol extract

HPTLC characterization was performed as suggested by Ramalingam et al (2013)[27] with some modifications. 2 µl of the sample solution was loaded on pre-coated TLC plate (Silica gel 60 F254 sheets 20×20 cm, 0.5 mm thickness, Merck Darmstadt, Germany) using CAMAG Automatic sampler (ATS4). Plate was developed up to a distance of 80.0 mm in CAMAG twin trough glass chamber pre-saturated with the mobile phase ethyl acetate: toluene (1:1 v/v) for 30 min. The plate was fixed in scanner stage and scanning was done at 200-450 nm.

The extract was filtered through 0.2 µm syringe filter and 20 µl filtrate was loaded on the HPLC system (Agilent, USA). Separation was achieved on an Agilent Eclipse Plus C18 column (100 mm×4.6 mm, 3.5 µm) using a flow

Table 2: Fluorescence analysis of dry powder from *Lentinula edodes*

Sl No.	Reagent	Visible	UV	
			Long (365nm)	Short (254nm)
	Powder as such	Grey	Dark brown	Green
1	Hager`s	Brown	Black	Dark moss green
2	Mayer's	Brown	Black	Moss green
3	Dragendroff's	Brown	Black	Moss green
4	Iodine solution	Brown	Black	Moss green
5	1(N) HNO_3	Light brown	Black	Moss green
6	50% HNO_3	Dark brown	Black	Dark moss green
7	Phloroglucinol	Light brown	Black	Light moss green
8	Barfoed	Dark brown	Black	Moss green
9	Sodium nitroprusside	Light brown	Black	Dark moss green
10	Ninhydrin	Brown	Black	Greenish
11	$FeCl_3$	Dark brown	Black	Dark moss green
12	1(N)NaOH	Dark brown	Black	Dark moss green
13	Acetic acid	Dark brown	Black	Dark moss green
14	1(N) HCl	Yellow ochre	Black	Moss green
15	Methanol	Brown	Black	Moss green
16	1 (N) NaOH in Methanol	Brown	Black	Dark moss green

Table 3: Qualitative chemical screening of methanol extract from *Lentinula edodes*

Type of metabolites	Name of tested chemical	Reagent/Test	Nature of change	Result
Primary	Carbohydrate	Fehling's	Brick red ppt	+
Secondary	Cardiac glycoside	Keller-Killani	Reddish brown colour at the junction of the two liquid layers	++
	Flavonoid	Ferric chloride	Green coloration	+
	Phenol	Folin-ciocalteu	Bluish black colouration	+
	Steroids	Limbermann-Buchard	Changing colour of solution from red to green	+
		Salkowski	Appearance of chloroform and acid layer red and greenish yellow fluorescent respectively	+
	Terpenoids	Acetic anhydride with sulphuric acid	Reddish colour	+

rate of 0.8 ml/min at 25°C. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). A gradient program was used for elution: 0-2 min, 5% A; 2-5 min, 15% A; 5-10 min, 40% A; 10-15 min, 60% A; 15-18 min, 90% A. The absorbance of sample solution was measured at 280 nm[28].

Evaluation of Antioxidant Activity

Radical scavenging activity in extract was evaluated using purple coloured DPPH radicals based on the method by Shimada et al (1992)[29]. 2 ml reaction mixture consisted of methanol solution of DPPH (0.1 mM) and various concentrations of extract. After 30 min incubation absorbance was measured at 517 nm against blank. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison. Total antioxidant assay was carried out following methods of Prieto et al (1999)[30] with little modification. The reaction mixture consisted of 0.3 ml sample solution and 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate). Tubes were capped and incubated at 95ºC for 90 min. Samples were cooled at room temperature and absorbance was measured at 695 nm against blank. Concentrations of ascorbic acid (1-30 µg) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid.

RESULT AND DISCUSSION

According to the World Health Organization (WHO, 1998) microscopic description of a plant is the first step to establish the identity and the degree of purity of such materials and should be carried out before any tests are undertaken[22]. The purchased dried basidiocarps (Figure 1a) were powdered which showed granular buff coloured appearance (Figure 1b), tasteless in nature and possessed a sweet odour. Studies of this powder under light microscope showed that it was dimitic; with thin walled, hyaline, simple septate, generative hyphae, which had rare branching and was 4.5-5.6µm wide. Binding hyphae had firm thick wall, was hyaline, much branched and 5.5-

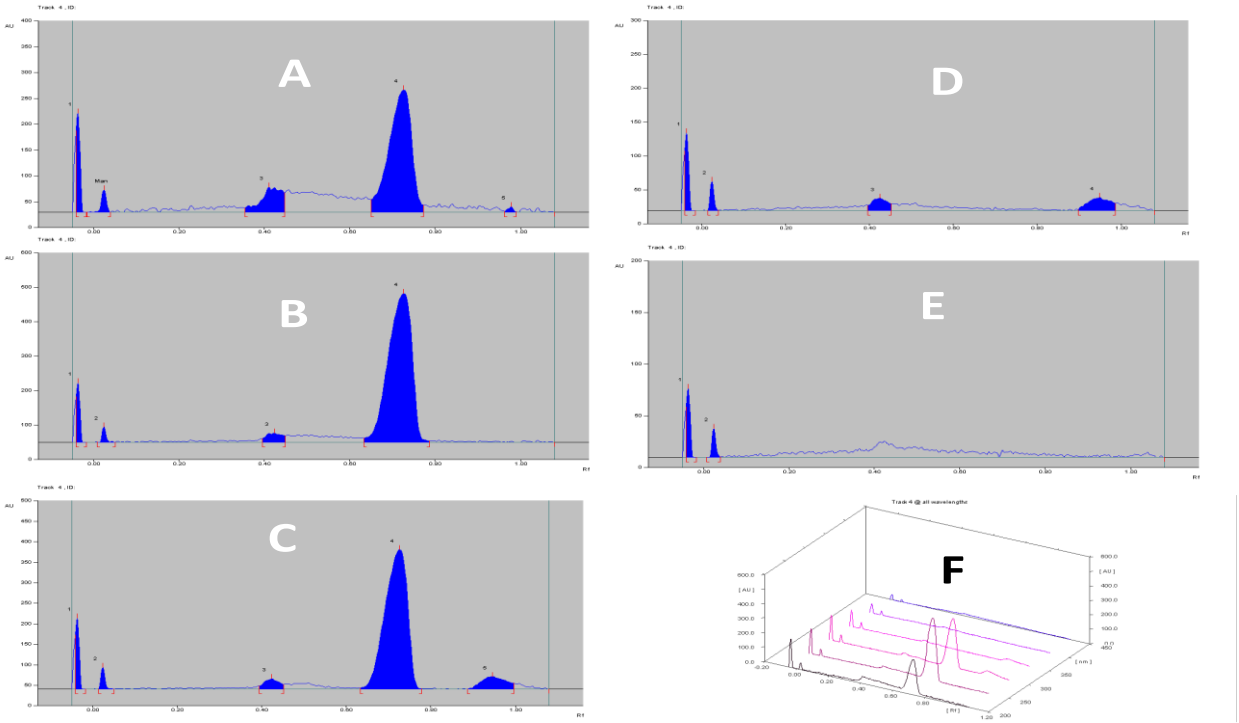

Figure 2: HPTLC chromatogram of methanol extract from *Lentinula edodes*. A: Chromatogram at 200 nm B: Chromatogram at 250 nm C: Chromatogram at 300 nm D: Chromatogram at 350 nm E: Chromatogram at 400 nm F: Chromatogram in all wavelengths ranging from 200 nm to 450 nm differing in 50 nm

Table 4: HPTLC Chromatogram at 300 nm of methanol extract from *Lentinula edodes*

Peak no	R_f	Max Height (AU)	Area (AU)
1	0.40	25.5	647.1
2	0.63	40.1	3011.3
3	0.89	30.8	1290.1

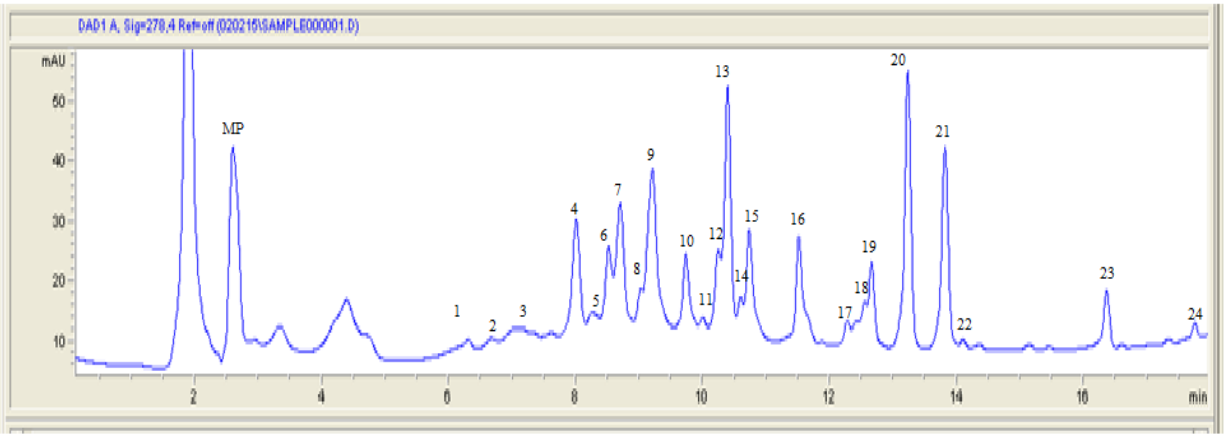

Figure 3: Enlarged HPLC chromatogram of methanol extract from *Lentinula edodes* (MP: Mobile phase)

6.7µm in width. Basidia was clavate, 4 sterigmate, and simple septa was present in the base. Spores were smooth, inamyloid, 5-6.5×3-3.5µm in size and ovoid to oblong ellipsoid in shape (Figure 1c). Mehlzer test gave negative result.

Physico-chemical parameters of the powdered drug such as ash value, loss on drying, hydration properties and solvent extractive values were determined (Table 1). Estimation of ash values is also a significant parameter for the detection of nature of material, impurities and determination of authenticity along with quality and purity of the test sample[31]. The ash value is the residue remaining after inceration, representing the inorganic salts present in the sample[31].

Loss on drying indicates the loss of volatile substances along with water. Determination of moisture content of the drugs is very important. Lesser the moisture content lesser will be chances for growth of bacteria, fungi or yeast and so lesser risks of contamination and undesirable enzymatic activity accelerating spoilage[31]. It may lead to

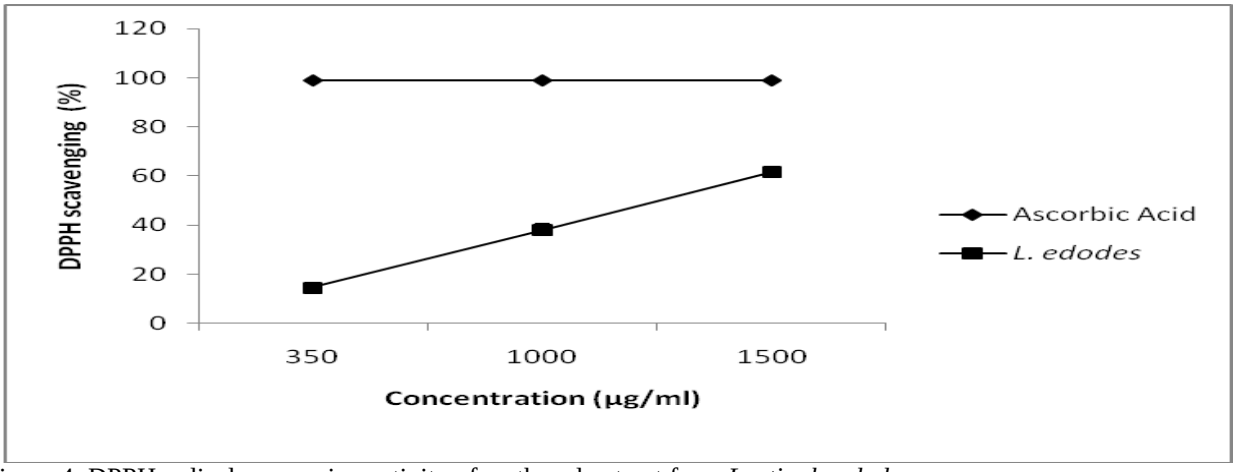

Figure 4: DPPH radical scavenging activity of methanol extract from *Lentinula edodes.*

Table 5: HPLC profile of methanol extract of *Lentinula edodes* at 278nm

Peak No.	Retention Time (min.)	Area
1	6.310	28.27
2	6.667	14.14
3	7.084	53.258
4	8.007	165.239
5	8.263	28.879
6	8.519	96.222
7	8.700	172.672
8	9.028	28.3375
9	9.207	263.605
10	9.735	87.231
11	9.998	7.482
12	10.243	70.520
13	10.395	267.158
14	10.595	17.567
15	10.730	115.97
16	11.513	157.725
17	12.278	27.088
18	12.552	71.77
19	12.659	93.013
20	13.230	336.557
21	13.816	254.812
22	14.097	8.893
23	16.365	76.284
24	17.750	16.639

breakdown of important active constituents and as a result
the drug may deteriorate quickly[32]. In *L.edodes* the range of moisture content was within the acceptable limit thus it may discourage growth of bacteria, fungi. Exractive values are often measures of amount of certain constituents that the drug contains. The methanol soluble extractive value for this mushroom was11.2± 0.4%, indicated the presence of polar active constituents which were further evaluated and presented latter on.

Absorption properties on the other hand represent the ability of the powder to associate with water which is a useful indication of whether powder can be incorporated into aqueous drug formulations. Water absorption of powder is dependent on the amount and nature of the hydrophilic constituents and to some extent on pH and nature of powder[32]. Table 1 showed that *L. edodes* showed good water holding capacity, moderate swelling capacity and low water solubility index.

The fluorescence tests of the powdered drug were carried out with different concentrated mineral acids. The colours produced by these reagents represent the presence of active constituents. It is an important pharmacognostic evaluation as it is useful in identification of authentic samples and recognizing adulterates. Various chemical constituents present in drug exhibits fluorescence in UV light. The non-fluorescent compounds often can be converted to fluorescent compounds by applying different reagents. As a result fluorescence analysis displayed an array of colours that could be employed for identification of probable classes of compounds in the mushroom[33,34]. In the present study powder drug was treated with 16 different chemical reagents which gave characteristics colour when seen under long and short wavelengths of UV light and was compared to that, observed under visible light (Table 2).

Methanolic extract was subjected for qualitative analysis of different phytochemicals using standard protocol. Chemical screening revealed the presence of glycosides, terpenoids, phenol and flavonoids, whereas carbohydrate, alkaloids, saponin and protein were not detected (Table 3).

The methanolic extract was found to contain phenol as much as 2.833 ± 0.18 µg gallic acid equivalent/mg. Total flavonoid content was determined by using quercetin as standard. The extract contained flavonoid as 3.42 ± 0.075 µg quercetin equivalent/mg. Very negligible amount of β-carotene and lycopene were found such as 0.0276 ± 0.001 µg/mg and 0.0221 ± 0.009 µg/mg of the extract respectively. Ascorbic acid was also found in minor amount (3.96 ± 0.06 µg/mg of extract).

HPTLC is an efficient and valuable technology for the preliminary separation and determination of constituents. The silica plates were dried and scanned from 200 nm to 450 nm with 50 nm intervals. At 200 nm, 250 nm, 300 nm and 350 nm, 3 (R_f 0.35, 0.65, 0.96), 2 (R_f 0.40, 0.63), 3 (R_f0.40, 0.63, 0.89) and 2 (R_f 0.40, 0.90) spots were

detected respectively (Figure 2). Whereas no spots were detected at 400nm. Maximum spots were detected in 300nm wavelength (Table 4).

HPLC chromatogram of the methanolic extract of *L.edodes* at 278nm is represented in Figure 3; consisting of 24 phenols with 2 large mobile phase (MP) peaks in the starting of the run. Their retention times and respective areas are given in Table 5.

DPPH accepts electron or hydrogen to earn stability. Antioxidants, on the other hand are capable of donating electron or hydrogen atom. A solution containing DPPH and methanol gives violet colour. But when electrons are donated to DPPH, then solution starts losing colour. This change is spectrophotometrically measured at 517nm[29]. As shown in Figure 4, at 0.35, 1.0 and 1.5 mg concentrations extract exhibited radical scavenging activity at the rate of 14.63 %, 38.02 % and 61.56 % respectively. EC_{50} value was found to be at 1.25 mg/ml.

hosphomolybdenum method is a good method for evaluation of total antioxidant capacity. The method is based on reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate /Mo (V) complex[30]. Total antioxidant capacity of all five fractions was investigated and compared against ascorbic acid. The extract presented 13.996 ± 0.285 µg AAE/ mg of extract total antioxidant activity.

CONCLUSION

So far no detailed standardized work has been reported in literature for *Lentinula edodes*. Its powder subjected for microscopic, pharmacognostical, preliminary phytochemical assay, HPLC and HPTLC were first time reported and hence adds to the existing knowledge of *L.edodes* so that it can be quite useful for identification, standardization, development and preparation of crude drug's formulation as well as inclusion in various pharmacopoeias for treating various ailments. All these data together provides relevant information which may be helpful in authentication of the crude drug and check adulteration for quality control of raw material.

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