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RESEARCH ARTICLE

Safety Profile, Standardization and HPTLC Fingerprint Analysis of *Dioscorea bulbifera* Linn

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ABSTRACT

Dioscorea bulbifera L. syn: Yam. (Family – Dioscoreaceae) is found commonly in India. Recent pharmacological findings indicate that it tubers possess significant activities like purgative, deflatulent, aphrodisiac, rejuvenating and tonic, anthelmintic and is used in haematological disorders, scrofula, syphilis, haemorrhoids, flatulence, diarrhoea, dysentery, worm infestations, general debility, diabetic disorders, polyuric and skin disorders which comply with the claims made in the traditional medicinal texts. However, no conclusive safety profile study and HPTLC finger print of its tubers has been performed yet. The present investigation deals with the safety profile of microbial load, aflatoxin and elemental analysis of the tubers and establishment of the quality parameters including physiochemical and phytochemical evaluation. The Rf values are detected at 254 nm and 366 nm by qualitative densitometric HPTLC fingerprint, can be used as identifying marker for Hydroalcoholic extract. The present studies will the information with respect to identification and authentication of crude drug. Such a study would serve as a useful gauge standardization of tubers material and ensuring the quality formulations.

Keywords: Dioscoreaceae, Microbial load, Aflatoxin, HPTLC fingerprint.

INTRODUCTION

Dioscorea bulbiferea L. Eng: Yam. (Family – Dioscoreaceae) is found commonly in India. Recent pharmacological findings indicate that it tubers possess significant activities like - purgative, deflatulent, aphrodisiac, rejuvenating and tonic, anthelmintic and is used in haematological disorders, scrofula, syphilis, haemorrhoids, flatulence, diarrhoea, dysentery, worm infestations, general debility, diabetic disorders, polyuric and skin disorders. The tubers are crushed and decoction is emulsified into oil, which is used in infected ulcers and sinus.

According to the world Health Organization [1] the safety profile of microbial load, aflatoxin, elemental analysis of medicinal plants is the first step towards the establishing the identity and degree of purity of such materials and should be carried out before any tests are undertaken.

Dioscorea bulbifera also known as Gonth, Kolkand, Varaheekand, a tribal plant belonging to the family of Dioscoreaceae. It is a climber plant with tuberous root. Dioscorea is a large genus of annual twining herbs, distributed throughout the moist tropics of world and extending into warm temperate regions. About 50 species are found in India. A large number among them occur in the wild state. Dioscorea species are distributed nearly throughout India except in the dry north-western regions. They are found growing at elevations of 8000-15000 ft. in Himalayas. In its wild state, it is extremely bitter. Under cultivation the plant loses its bitterness and is much grown for the tubers, which are roasted and eaten. The tuber is used by the tribal population of central India as a food particularly in Madhya Pradesh, Chhattisgarh, Jharkhand and Orissa. Therefore, present

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investigation of *Dioscorea bulbifera* L is taken up to establish safety profile and HPTLC fingerprint of the tubers which will help in crude drug identification as well as in standardization of the quality and purity.

MATERIALS AND METHODS

Collection and authentication of plants

Tubers of *Dioscorea bulbifera* L were collected from the Thovalai, Trichy district in May 2013. This plant are authenticated in multi centres such as Rabinat Herbarium, St. Joseph College, Trichy, St. Xavier's College, Palayamkottai and Botanical survey, CCRAS Unit, Chennai and Govt. Medical College, Palayamkottai. Herbarium and voucher sample were prepared and deposited in Department of Pharmacognosy & Phytopharmacy, Sastra University (Voucher No. DB – 0062) Thanjavur.

Microbial load

Plant material samples are obtained and thoroughly mixed to make composite sample for microbial analysis. 10 gm of appropriate plant material is weighed and 100 ml of sterile distilled water in a sterilized conical flask are placed for serial dilution. The flasks are kept in a mechanical shaker for five minutes to obtain uniform suspension of microorganisms. The dilution level is 1-10 or 10^{-1}. From this, 1 ml of dilution from 10^{-1} sample is taken out and if transferred into 9 ml, this is 10^{-2} dilution. The procedure is repeated up to 10^{-6} dilution. Transfer 1ml of serial dilution from10^{-1} to 10^{-6} into sterilized petri-plates for enumerating bacteria, fungi and severe pathogens such as *Salmonella*, *Shigella* and *E.coli*. Two replications are maintained to each dilution, in each group of microorganisms. The medium such as Nutrient Agar (NA), Potato Dextrose Agar (PDA), Salmonella Shigella Agar (SS) and Eosin Methylene Blue Agar (EMB) are added to the sterilized petri-plate with one mL sample, and rotating the plate clockwise and anti-clockwise to get an uniform distribution of microbial cells[2].

The medium is allowed to set and the plates are incubated in inverted position at 37^0C for about 1-2 days for bacteria and 3-5 days for fungi. The colonies are counted on the plates with the aid of colony counter. The numbers of colonies are observed from both the plates, which are kept for replication. The total numbers of population are enumerated individually for fungi and bacteria by taking the average of the two dilutions employed and expressed in 1 gm of plant material.

Determination of Aflatoxin B_1

Preparation of Sample

10 g plant material is mixed with 25 ml of water, 25 g diatomaceous earth and 50 ml of chloroform in a 500 ml of flask and covered with stopper. This mixture is shaken for 30 min. Then, either with filter or vacuum filter collect 50 ml portion of $CHCl_3$. It is collected by Whatman filter paper No. 41. The filtrate is allowed to proceed to column Chromatography method.

Preparation of Aflatoxin B_1 Standard

Aflatoxin B_1 is obtained using Sigma U.S.A. It is used as a standard. Aflatoxin B is mixed with Toluene – CH_3CN (2 + 98) to get a final concentration of 150 ng / ml.

Silica Gel for Column Chromatography

Merck Silica gel 60, 0.063- 0.2 mm for 10 g sample or 0.2 – 0.5 mm for 1 kg sample are selected. Silica gel is activated by drying 1 hr at 105^0C.

Silica Gel for HPTLC

Readily available fluorescent coated Merck Silica gel $60F_{254}$ plates are used for this study.

Column Chromatography

Chromatographic column of 22 x 330 mm is selected and a ball of glass wool is loosely placed in the bottom. 5 g of anhydrous Na_2SO_4 is added to give base for silica gel. $CHCl_3$ is added until the tube is ½ full and 10 g of silica gel is added. When rate of flow settling slowly, some $CHCl_3$ is drained to aid settling, leaving 5 – 7 cm above silica gel. Slowly 15 g of anhydrous Na_2SO_4 is added. 50 ml extract sample to column is added and eluted at maximum flow rate by 150 ml of Hexane, followed by 150 ml of anhydrous ether and the rest is discarded. Aflatoxin is eluted with 150 ml of CH_3OH +$CHCl_3$ (3+97). Collecting such fractions from the time of addition can be continued until flow stops. Few boiling chips are added to elute and to evaporate nearby to dryness on steam bath and quantitatively transferred residue to vial with $CHCl_3$. This fraction will be allowed to process in HPTLC Method for quantitative determination.

Determination by HPTLC Method

CAMAG HPTLC instrument is used for this experiment. Merck Silica gel $60F_{254}$ plates are placed in the applicator Linomat 5. The sampling and processing of the instrument are programmed with the help of WINCATS software in the CAMAG HPTLC system. From 5 µl to 20 µl of three replicated samples are spotted on the plate followed by Internal and External Standards. Plates are kept in the developer chamber

containing the mobile phase of 50 ml of CH_3COCH_3 + $CHCl_3$ (1 + 9). After drying, the plates are kept for scanning in 366 nm with fluorescence detection. The concentration of Aflatoxin is calibrated with help of Wincats software.

Elemental analysis using AAS

Atomic absorption is the process that occurs when a ground state atom absorbs energy in the form of light to a specific wavelength and is elevated to an excited state. The amount of light energy absorbed at this wavelength increases as the number of atoms of the selected element in the light path increases. The relationship between the amount of light absorbed and the concentration of analyses present in known standards can be used to determine unknown sample concentration by measuring the amount of light, they absorb [3].

The samples are cleaned and dried under shade. Then, the samples are dried in an oven at 40°-50° C till a constant weight is obtained. The dried samples are then, ground and powdered with agate pestle and mortar. Samples are labeled and stored in pre-cleaned polyethylene bottles for further analysis. The prepared solutions are directly subjected to flame photometry and AAS for the estimation of various elemental concentrations.

Sample Collection

The samples are cleaned and dried under shade. Then, the samples are dried in an oven at 40°-50°C till a constant weight is obtained. The dried samples are then, ground and powdered with agate pestle and mortar. Samples are labelled and stored in pre-cleaned polyethylene bottles for further analysis.

Reagents and Apparatus

All the reagents such as HNO_3 and H_2O_2 used in the study are purchased from MERCK (Analytical Grade). De-ionized water is used for all analytical work and all the glassware's, polyethylene bottles, pipette tips and others are washed with 1 % HCl, rinsed with de-ionized water before preparing standards, reagents and samples.

Sample Preparation: Ash Method

The collected medicinal plant parts are cleaned and dried under shade. The dried part is then, ground to fine powder, which is used for drying to ashing. Necessary precautions are taken at every step to avoid metallic contamination in any form. Pre-cleaned silica crucibles are kept in muffle furnace maintained at 600° C. Until the weight of the crucible reaches a constant level, the crucible is kept in the furnace. Powdered plant material (5 gm) are taken in the silica crucible and maintained in a muffle furnace at 600° C for 6 hrs. The crucible are then, taken out and cooled at room temperature by keeping it in a desiccator and then, the ash values are measured. Then, the ash is dissolved in 100 ml of 5 % HCL. The dissolved ash solutions are filtered through Whatman filter paper No.40 and are stored in tightly capped plastic bottles. The prepared solutions are directly subjected to flame photometry and AAS for the estimation of various elemental concentrations.

Digestion of Samples

A Multiwave 3000 micro oven system (from Anton paar, USA) with 16 positions for Teflon vessels with capping is being used here. The digestion vessels are provided with a controlled pressure, temperature and release valve. Before use, all Teflon vessels are soaked with 10 % HNO_3. The system is initially programmed by giving gradual rise of 20 %, 40 %, and 50 % power for 5, 15 and 20 minutes, respectively for the due warming up. The powder samples are being used without any further treatment for sample preparation. 0.2 gm of sample is weighed into the Teflon vessels followed by digestion mixture of HNO_3 and H_2O_2 in the ratio of 3:1. According to the nature of samples, the ratio is being applied.

The resulting solution after microwave digestion is filtered through Whatman 40 filter paper (if necessary) and diluted to 50 ml with de-ionized water. A sample blank containing only acid mixture is prepared at the same time. The method of standard addition is generally adapted to calibrate the instrument before going for the observation of the samples.

Determination of Metals

All the atomic measurements are carried out with PerkinElmer model 400/HGA900/AS800 coupled with Mercury Hydride System-15 (MHS-15) and Flame Photometer. The Hallow cathode lamps (HCL) for Fe, Cu, Mn, Zn, Mg, Mo etc., and Electrodeless Discharge Lamp (EDL) for Cd, Pb, Hg and As analyses are used as a light source to provide specific wavelength of the elements to be determined and high purity (99.999 %) Acetylene and Nitrous oxide are used to provide constant thermal energy for atomization process and Argon gas used for carrier gas purging purposes for Graphite furnace.

Calibration of Instruments

More than three working standard solution of elements to be determined are prepared, covering the concentration range as recommended by the manufacturer of the instrument for the elements to be determined. Before the analysis of samples, the instruments are calibrated with prepared working standard solution. The calibration curve is obtained for concentration vs. absorbance data statistically analyzed. Calibration of the instrument is repeated periodically during operations and blanks are carried with each set of 10 samples or aspirate any one of the prepared working standard for every 10 samples to check the instrument drift and to validate analytical procedures and performance. Regent blank reading will be taken and necessary correction will be made during the calculation of concentration of various elements. A reagent blank reading is taken and necessary corrections are made during the calculation of concentration of various elements [4].

HPTLC Finger Printing

A 10 x 3 cm pre-coated HPTLC Silica gel $60F_{254}$ plate is used for performing HPTLC. It is cleaned by methanol and activated at 60^0 C for 5 minutes before applying to chromatography. Using CAMAG Linomat V applicator, the samples are coated on the plate of 6 mm band.

Chromatography is performed on a 10 x 3 cm pre-coated HPTLC Silica gel $60F_{254}$ plate. The plates are washed by methanol and activated at 60^0 C for 5 minutes prior to chromatography. 20 mm/s scanning speed are maintained in a slit dimension of 5 mm x 0.45 mm. The method of trial and error is adopted to fix the mobile phase and here

10 ml of mobile phase is used per chromatography. The saturated mobile phase ascends linearly 10 cm x 10 cm twin glass chamber. CAMAG Linomat V applicator is used for spotting the mixture (HPTLC instructions manual).

RESULTS AND DISCUSSION

The moment the raw material has been brought to laboratory, it is scanned for microbial load in order to confirm the absence of pathogenic bacteria. The results of the presence/ absence of *E.coli, Salmonella sp., Shigella sp, Enterobacter sp., Total Heterotrophic Bacteria, Yeast and Mould* in DB (**Table 1**) is shown. Similarly, the hydroalcoholic extracts of DB plants have also been exposed for examination to know whether the pathogenic bacteria are present or not and the observed values are shown to DB (**Table 2**). They show that *E.coli* is present in some samples above the limit of W.H.O. standard. However, after processing for extraction, the *E.coli* cells are removed. So, it indicates that during the processing of alcoholic extraction, the cells must have been eliminated.

Table 1: Total Bacterial Count for DB

S. No	Bacterial Name	Sample Name	W.H.O. Limit	Cells in Sample/g	Interference
1	*E.coli*	*Dioscorea bulbifera* Raw Material	10^2	10 X 10^2	Within Limit
2	*Salmonella sp.*		Absence	-	Within Limit
3	*Shigella sp.*		Absence	-	Within Limit
4	*Enterobacteria sp.*		10^4	-	Within Limit
5	Total Heterotrophic Bacteria		10^7	112 X 10^4	Within Limit
6	Yeast and Mould		10^4	19 x 10^1	Within Limit

Table 2: Microbial contamination in Extract of DB

S. No	Bacterial Name	Sample Name	W.H.O. Limit	Cells in Sample/ g	Interference
1	*E.coli*	*Dioscorea bulbifera* Plant Extract	10^2	-	Within Limit
2	*Salmonella sp.*		Absence	-	Within Limit
3	*Shigella sp.*		Absence	-	Within Limit
4	*Enterobacteria sp.*		10^4	-	Within Limit
5	Total Heterotrophic Bacteria		10^7	2X 10^1	Within Limit
6	Yeast and Mould		10^4	4 x 10^2	Within Limit

Aflatoxin

The moment the hydroalcoholic extract has been brought to laboratory, in addition to microbial load, presence of Aflatoxin B1 is verified using the Standard of aflatoxin B_1. The total count of aflatoxin has also been carried out and found whatever the count present is within the W.H.O. limit. Presence of Aflotaxin in the respective plant extract has been recognized through HPTLC UV

Detector by projecting standard Aflatoxin B_1 with the plant extract has been projected together in a HPTLC plate to get bird's eye view on the quantum results of Aflatoxin B_1 in the respective plant extract (Fig 1). In order to elicit the presence, a three dimensional projection on the

quantum of absorption is presented in the form of curve to the respective plant (Fig 2). The same quantum of presence is calibrated with the standard and it is presented here in the form of standard regression curve (Plate 25, 28 & 31).

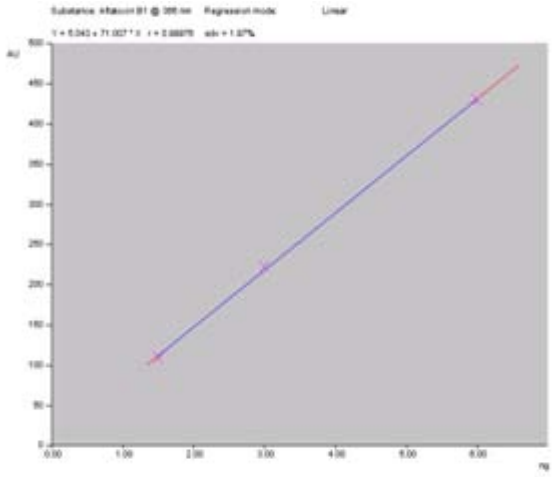

Fig 1: Standard calibration curve for DB

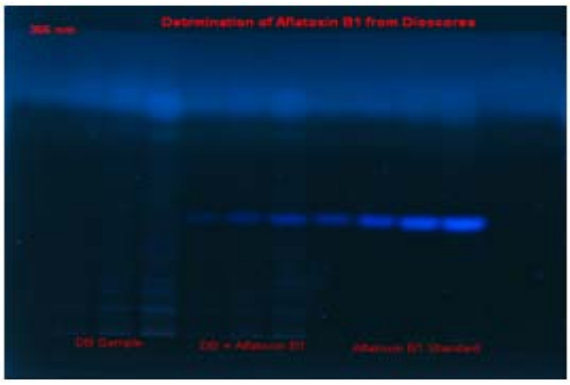

Fig 2: HPTLC plate of DB

Elemental analysis

The various mineral elements are generally being imbibed into the plants from the soil, water and atmosphere. The level of mineral elements in plant varies depending upon the environmental factors and the type of plant itself. Among plant types growing in the same environment, fungi lichen and mosses accumulate more metals than the others. For a particular species, the concentration level generally decreases in the order root >stem > leaves > fruit > seed when the source of the mineral element is only the soil. Moreover the concentration of elements also varies with the age of the plant.

High level of toxic elements occur in medicinal preparations either when they are used as active ingredients as in the case of Pb and Hg in some Chinese, Mexican and Indian medicines[5] or when the plants are grown in polluted areas fertilizers, such as near roadways, metal mining and smelting operations and when one uses fertilizer containing cadmium and organic mercury or lead based pesticides, and contaminated irrigation water[6]. Hence, analysis of various mineral/metal elements is imperative in the use of plants as drugs.

Inorganic micronutrients include Fe, Cu, Zn, Mn, Co, Mo, V, B, Cl, I. Br and Na. They are important as catalyzing metabolic reactions and in osmoregulation. They are required in optimum quantities for better growth of the plant but when supplied in excess, it is turning to be harmful. Results of the micronutrients and trace elements are given in the Table 5.6. In view of the criticism provided for the traditional drugs on the ground of metal toxicity, the extract, which is going to be tested for the drug is brought under the observation of elemental analysis. The values are very much within the limits of W.H.O. except aluminum that are also an element of useful one for the metabolism. As there is no alarming presence of heavy metals in the extracts, the extract has been taken up for further acute toxicity studies.

In the present study, the concentration of various elements in raw plants, the ashes of different plants, the aqueous extracts and in hydro-alcoholic extracts has been determined by using flame photometry in (**Table 3**) using AAS and the same is tabulated in (**Table 4**).

Table 3: Elemental Analysis using Flame Photometry

Plant	Na (mg/l)	Ca (mg/l)	K (mg/l)	Li (mg/l)
DB	1.81	79.05	160.30	0.55

Table 4: Distribution of Elements in DB

Plant Code	Fe	Cu	Mn	Ni	Zn	Co	Cr	Al
Raw plant	0.4740	0.2635	0.1220	0.0990	1.2825	0.1780	0.4435	7.3500
Ashes	0.6605	0.1620	0.2580	0.3505	0.9000	0.1490	0.3925	6.6650
HAE	0.6675	0.1320	0.1635	0.2025	0.6520	0.1695	0.3660	8.8750
Plant Code	**V**	**Mo**	**Pb**	**Cd**	**Hg**	**As**	**Se**	
Raw plant	3.4350	0.3615	0.3780	0.1660	0.0400	0.0087	0.0108	
Ashes	2.5550	0.3405	0.2835	0.1490	0.1124	0.0081	0.0122	
HAE	2.1600	0.4590	0.3790	0.1000	0.0434	0.0064	0.0079	

Heavy metals and toxic elements

The toxicity **sequence** for heavy metals varies with the taxonomical group of plants; for flowering plants the sequence observed is Hg > Pb > Cu> Cd> Cr> Ni >Zn; for algae it is Hg >Cu

>Fe >Cr >Zn >Ni >Co >Mn and for fungi the observed sequence is Ag >Hg >Cu >Cd >Cr >Ni >Co >Zn >Fe[7]. Many species belonging to Rubiaceae are aluminium accumulators. In fact, aluminium accumulators are more woody than herbaceous. Aluminium interacts with a number of other elements including calcium, fluorine, iron, magnesium, phosphorous and strontium and when ingested in excess it can reduce their absorption [8].

CONCLUSION

The raw materials and extracts of DB have been treated for the elemental analysis of Hg, Pb, Cd, and As, etc. Mineral elements are more useful to man than being harmful. Human body requires mineral elements to certain extent. At the same time, when it crosses the limit, it becomes toxic and degenerate the system. Any plant is likely to have some elements or others in low or high quantity. The quantity depends on the soil nature and the environmental conditions. Estimation microbial load and determination of aflatoxin B1 are found that they are all in safe limits within the expectation of WHO norms.

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