Pharmacognostic standardization of tuber *Pueraria tuberosa*

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Abstract –

Pueraria tuberose L. Is climber woody tuberculated stem belonging to family Fabaceae, commonly known as "Vidarikand".. The tubers are globose or pot-like, about 25 centimetres (9.8 in) across and the insides are white, starchy and mildly sweet. The plant's tuber is widely used in ethanomedicine as well as in traditional systems of medicine, particularly in ayurveda. It has been used in various ayurvedic formulations as restorative tonic, antiaging, spermatogenic and immune booster and has been recommended for the treatment of cardiovascular diseases, hepatosplenomegaly, fertility disorders, menopausal syndrome, sexual debility and spermatorrhoea. The present study comprises pharmacognostical analysis and microbial load estimation, Heavy metal and aflotoxin study of the plant material.. The study will provide referential information for the correct identification of the crude drug and establish pharmacopeial standards.

Keywords- Aflatoxin, Ash value, Heavy metal and Vidarikand.

Introduction –

The subject of herbal standardization is especially wide and deep for the rationale of the research work on the herbal formulation and nutraceuticals, a profound knowledge of the important herbs found in India and widely used in the Ayurvedic formulation of outmost importance.

The more effective the natural drug more its demand and the chance of non- availability increase. To meet the growing demand, the natural drug is easily adulterated with low-grade material. Adulteration or substitution is nothing but the replacement of the original plant with another plant material or intentionally adding any foreign substance to increase the weight or potency of the product or to decrease its cost.

Pharmacognosy is the study of medicine derived from natural sources, mainly from the plant. The term pharmacognosy is derived from the Greek word 'Pharmacon' meaning drug or medicine and 'gnosis' meaning knowledge. The term was first coined by C.A. Sedler in his dissertation entitled '*Analectapharmacognostica*' in 1815. Herbal medicine as the major remedy in traditional medical system has been used in medical practice for thousands of years and have made a great contribution to maintain human health [1]. It basically deals with standardization, authentication, and study of natural drugs. This can be achieved only if the herbal product is evaluated and difficult modern technique of standardization [2].

The importance of pharmacognosy has been had been widely felt in recent time. Unlike taxonomic identification, the pharmacognostic study includes a parameter which helps in identifying adulteration in dry powder form also.

This is again necessary because once the plant is dried and made into powder form, it loses its morphological identity and easily prone to adulteration. Pharmacognostic study ensures plant identity, lays down standardization parameter which will help and prevent adulteration.

Such studies will help in authentication of the plant ensure reproducible quality of an herbal product which will lead to safety and efficacy of a natural product. Standardization of herbal formulation is necessary in order to calculate the quality of drugs, based on the concentration of active principle [3].

In general , quality control is based on three important pharmacopeias definitions-

• Identity: Is the herb the one it should be?

• Purity: Are there contaminants, e.g, in the form of other herbs which should not be there?

• Content or assay: Is the content of active constituents within the defined limits.

WHO guidelines for quality standardized herbal formulation are as follows;

1. Quality control of crude drug material, plant preparation, and herbal product.
2. Stability assessment and shelf life.
3. Safety assessment, documentation of safety based on experience or toxicological studies.
4. Assessment of efficacy by ethnomedicinal information and biological activity evaluation.

In addition, while utilizing the chemical method and other analytical tools it is mandatory to stick on to the latest validated techniques that suit the study. One needs to understand what type of raw material/formulation one is dealing with any type of preparation to be evaluated. In spite of all these factors, the microbial contamination, pesticide residue, and heavy metal analysis should be considered before drug preparation and evaluation . This is regarding the safety issue of drug .this types of analysis is required when evaluating the authenticity of botanical because these extraneous contaminations can cause an undesirable physiological effect

Aflatoxin are secondary metabolites produced by *Aspergillus flavus* and *Aspergillu sparasticus* in agriculture crops such as peanut, maize grain and cereals..Aflatoxin B_1 (AFB_1) is the most Common [4] and the widespread in the world [5,6]. These mycotoxins are recognized to be hepatotoxins and carcinogens for humans. The World Health Organization (WHO) urged that their level is reduced to as low as reasonably achievable [7].Thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography are most commonly used chromatography technique for analysis of AFB_1 .Detection of these types of metabolites in our plant, thus, beneficial for better pharmacological study.

Plant *Pueraria tuberosa (PT)* belonging to family fabaceae was selected for the pharmacognostic standardization. We have select this plant for our whole experiment on the basis of the previous study in my lab, *Pueraria tuberosa* reported as an anti-inflammatory [8], antioxidant [9], nephroprotectiv [10] and hypoglycemic [11] pharmacological activity. In Hindi is called vidarikand. we have chosen the tuberous part of the plant for pharmacological evaluation. Before the pharmacological activity study of kudzu (PT), standardization of raw materials of the plant was necessary. The crude powder of tubers, PT was used to detect the heavy metal, aflatoxin contamination, and physicochemical parameter .

Material and Methodology

Plant materials- The tubers of plant Pueraria was purchased from Ayurveda pharmacy, BHU. The collected P. tuberosa tubers washed with tap water. The tubers cut into small pieces and air dried thoroughly under at room temperature for 1 month to avoid direct loss of photo-constituents from sunlight. The dried materials were powder using ball mill and sieved up to 80 mesh .it was then homogenized to fine powder and store in airtight container for further analysis.

Standard aflatoxin$B_1$was purchased from Sigma **.** The standards of Pb, Cd, As, Hg, Zn, were purchased from Merck, Germany and utilized for the development of the respective calibration curves for these metals.

Standardization parameter – Some standardization parameters studied of our crude drug in this experimental study that physicochemical parameter (table 1), microbial load ,elemental analysis by Atomic Absorption Spectroscopy (table 2), Aflatoxin estimation with the help of HPLC and microscopy of tuber of plant *Pueraria tuberosa.*

Physicochemical property-Three samples of Tuber of plant Pueraria were subjected for determination of physicochemical parameters such as loss on drying, ash value, water soluble ash, acid insoluble ash, and foreign mater value was carried out according to the method recommended by the world health organization[7].

Determination of loss and drying-

10 g of the sample (without preliminary drying) was weighed and placed in a tared evaporating dish. It was dried at 105˚ C for 5 hours, and at 1-hour interval until difference, two successive weighings corresponded to not more than 0.25%.

Determination of water soluble ash-The ash obtained in the determination of total ash was boiled for 5 minutes with 25 ml of water. The insoluble matter was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a tarred silica crucible and ignited for 15 minutes at a temperature not exceeding 450 C. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight was considered as the water-soluble ash was calculated with reference to the air-dried drug.

(iv) Determination of Total ash-

About 2 to 3 g of sample was accurately weighed in a tarred silica crushible at a temperature not exceeding 450 C until it was free from carbon. Then it was cooled and weighed. The percentage of total ash was calculated with reference to the air- dried drug.

(v) Determination of acid insoluble ash-

The total ash obtained was boiled for 5 minutes with 25 ml of dilute hydrochloric acid; the insoluble matter obtained was collected on an ash less filter paper, washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash was calculated with reference to the air dried drug.

C. Microbial load– The pour plate method was used to cultivate serially diluted portion of the medicinal plant pueraria sample investigated. Enumeration of bacteria was carried out on nutrient ager. In here we have count the colony of heteroptrophic bacterial species Deniallia oliveri and Bacillus subtilis.

The pour plate method was used to cultivate serially diluted portion of the medicinal plant sample under investigation .Enumeration was carried out on nutrient agar (NA) for bacteria . Triplicate plates of appropriate dilutions were prepared. The NA plates were incubated at 37^0C for 24-48 h for bacterial growth. The developed microbial colony were counted and computed as colony forming unit per ml of plant material .The colonies were purified , isolated and stored for morphological and biochemical characterization . These were further identified with the aid of Bergeys Manual of determinative bacteriology .

D. Elemental analysis by Atomic Absorption Spectroscopy –Atomic absorption spectrophotometer is used in the determination of trace as well as heavy metal elements. Heavy metal analysis was performed using the Perkin Elmer AAS-200 instrument.

As per the protocol, sample digestion was carried out by multi-acid digestion system for lead (Pb), cadmium (Cd), Mercury (Hg), Arsenic (AS), zinc (Zn. The dried extracts were taken and digested by the wet digestion method. In 2gm of sample 10 mL of nitric acid was added and was heated on a hot plate at 95^{o}C for 15 minutes. After digestion of the sample, when it gets cool add 5mL of concentrated nitric acid and heated for additional 30 min at95^{o}C. The last step was repeated and the solution was reduced to about 5mL without boiling. When the sample gets cool, 2mL of deionised water and 3mL of 30% hydrogen peroxide was added. Covered the beaker and the sample was heated gently to start the peroxide reaction. If effervescence becomes excessively vigorous, sample was removed from the hot plate and 1mL of 30% hydrogen peroxide was added in increments, followed by gentle heating until the effervescence was subsides. 10 mL of deionised water and 5mL of concentrated HCL were added and the sample was heated for additional 15 min without boiling. After cooling the sample was filtered through a whatsman No. 42 filter paper and diluted upto 50mL with deionised water in volumetric flask .this sample was injected to atomic absorption spectra .

E. Determination of presence of AflatoxinB_1 in PT powder with the use of HPLC-The residue of aflatoxin B_1 was detected with the help of HPLC. High-performance liquid chromatography provides rapid and precise aflatoxinB_1 detection result within a short time.

To quantify AFB_1 content in each set of stored PT powder, HPLC (photo-diode-array (PDA) detector (Waters, Bangalore, India)) analysis was performed.The samples for HPLC were prepared by homogeneous liquid–liquid extraction (HLLE) method following **[12].** All aqueous solutions were prepared using double-distilled–deionised water prepared by a Milipore-DQ3 system (Synergy, Germany). Five gpowder well milled sample was mixed with 10 mL methanol/water (8:10) and shaken at 300 rpm for an hour on a

mechanical shaker. The sample was centrifuged at 3000 rpm for 5 min. Four milliliter supernatant was then mixed with 300 µL chloroform and 6 mL water containing 3% potassium bromide. After 5 min. centrifugation at 3000 rpm, the settled phase was collected in a screw cap vial and dried in water bath under steam of nitrogen. To this, 50 µL methanol (HPLC grade) was added and finally injected to HPLC. A calibration curve of standard solution of aflatoxin B_1 was prepared in a range of 50–500 ng/50 µL. Separation was carried out on a C-18 reverse phase column (150 mm×4.6 mm i.d×5 µm) and the mobile phase was methanol–acetonitrile–water (17:19:64 v/v) at a flow rate at 1.2 mL/min. Aflatoxin B_1 was detected at 365 nm [13].

.**F.Microscopy of tubers of *Pueraria tuberosa***-Tubers of plant PT firstly dissolved in alcohol for decreases of hardness. Use microtome for sectioning of plant tubers about 5 µm and prepared its slides. The slides were stain with sefrenine dye then used to study by with the help of a light microscope.

Statistical analysis - Statistical analysis was determined by one way ANOVA following post hoc test using dunnetts and tuckey by IBM SPSS Statistics Software. All results were expressed as mean ± SD .

RESULT -

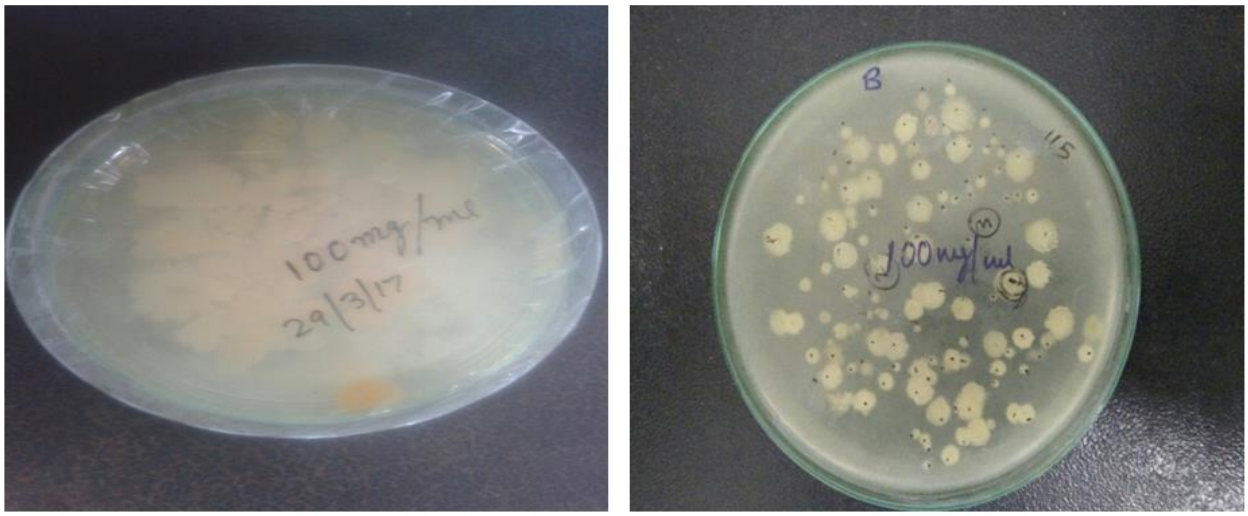
Physiochemical Property of herbal plant *Puerariatuberosa*-

The results of all above physicochemical parameters were found as per permissible limit, according to Ayurvedic Pharmacopoeia Vol. 5 page 225 (table 1).

Table 1- Physico-chemical evaluation of *Pueraria tuberosa*

S.No.	Physico – Chemical Parameters	Result	Limits of API
2	Loss of drying w/w%	3.2 ±0.8 %	Not more than 10 %
3	Water soluble Ash w/w %	24.7 ± 0.6 %	Not less than 22%
4	Acid insoluble Ash w/w %	2.28 ± 0.8 %	Not less than 1%
5	Total Ash w/w%	3.26± 0.5 %	Not more than 11%

Microbial load – The mean herterotrophic bacteria was count of the herbal sample of puerariatuberosa , under the limit 2.6x 10^3 and 1.3 x 10^3 CFU/ml of Daniellia oliveri and Bacillus subtilis respectively (fig 1) .



Deniallia Oliveri **Bacillus Subtilis**

Fig 1-Microbial load on powder of *Pueraria tuberosa*

Elemental analysis by Atomic Absorption Spectroscopy –

The level of heavy metals in the sample was found to be 1.0, 0.01, 0, 0, and 0.5458 for Pb, Cd, As, Hg and Zn respectively, as per permissible limit in Ayurveda pharmacopeia of India in vol 2 (table 2).

Table 2- Elemental analysis by Atomic Absorption Spectroscopy

Element		**Wavelength**	**Amount**
Pb	Heavy metals	283.3	1.0 ppm
Cd		228.8	0.0116 ppm
As		193.69	0 ppm

Hg		253.65	0 ppm
Zn	Trace metal	213.9	0.5458 ppm

Determination of presence of aflatoxinB_1 in PT powder with the use of HPLC –

No aflatoxinB_1 residues were found in the powder of *Pueraria tuberosa* as compared to the standard of aflatoxinB_1in HPCL chromatogram . The RT of aflatoxinB_1 was found at 4.967 min in . no aflatoxinB_1peakwas found in the chromatogram of PT sample on those respective RT in (fig 2).

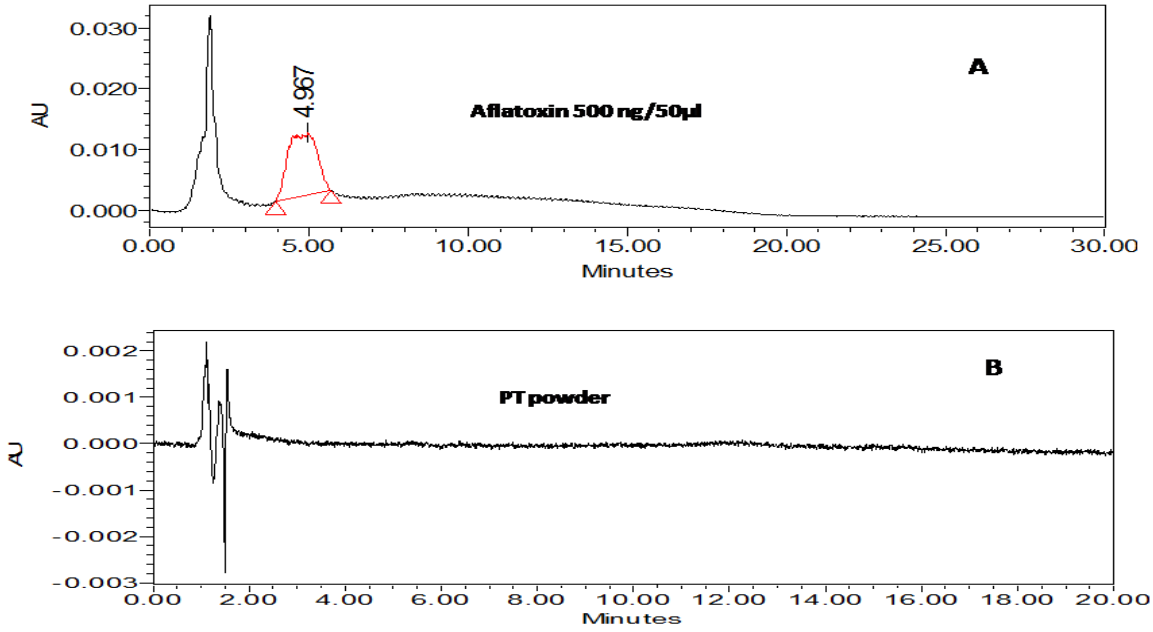


Fig 2-HPLC chromatogram of Aflatoxin B_1 standard sample (A), Powder of *pueraria* tuber sample (B).

Microscopic tuberous root T.S by safrenine staining –

Microscopic study T.S. of root tuber is slightly wavy in outline, epidermis not discernible; 3 to 4 layers of cork cells, followed by 5 to7 layers of parenchymatous cells present; cork cambium-brown in colour and 2 or 3 cells thick , endodermis well developed; pericycle fibrous followed by 2 layers of stone cells filled with sandy crystals ; phloem consists of sieve tubes, companion cells, patches of bastfibers and phloem parenchyma; xylem pentarch in young root, consist of vessels with scalariform cross perforation, tracheids, xylem fibers, and parenchyma; medullary rays broad and parenchymatous. The medullary rays and phloem cells are filled with starch grains which are polygonal, 2 to 5 µm in diameter, simple or two too many-compound, hilum usually indistinct, occasionally with a central cleft, lamellae indistinct. In macerated

preparation crystal fibers are multicellular, articulated, each cell carrying a crystal of calcium oxalate; some of the articulated fibers are swollen in the middle like a bulb pipette [fig 3].

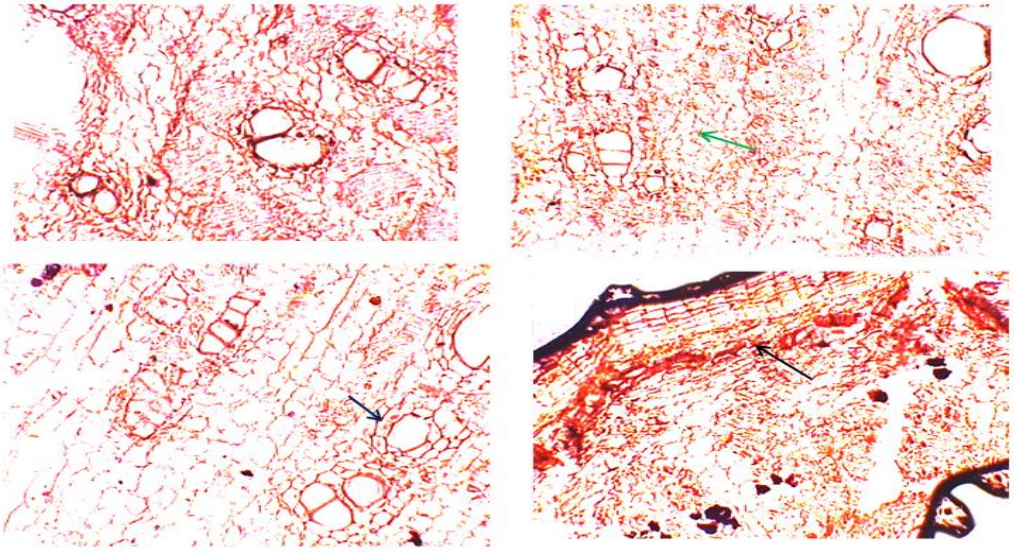


Fig 3- Cork cambium was brown in color (black arrow)Stone cells with sandy Crystals (green arrow. cell carrying a crystal of calcium oxalate (blue arrow).

Discussion -.

The powder of tuber of Pueraria tested for various standard parameters like physicochemical parameter, heavy metal analysis, aflatoxin determination and microscopy of tubers was summarised in this experimental study. The physicochemical parameter such as Ash value, water soluble, acid insoluble and loss of drying are mention in table 1. The results of all physicochemical parameters were found as per permissible limit, according to Indian Pharmacopoeia [14]. Ash value is use full in determining the purity of drug and mainly significant for the quantitative standard. Per cent weight loss on drying or moisture content was found to be 3.2 % w/w. The less value of moisture content could put a stop to bacterial, fungal or yeast growth. We have determined the in breed colony of bacterial species *Deniella oliveri* and *Bacilus subtilis*in our sample . The range of breed colony was 2.6x 103 CFU/ml . The study of microbial load are necessary for consumption of herbal drug to human beings .

Heavy metal estimation was determined by atomic absorption spectroscopy. The result of heavy metals value in ppm of Pb, Cd, As Zn and Hg, are mentioned in table 2. Those also are found in under the permissible limit according to Indian pharmacopeia of India

Preparative and analytical HPLC is widely used in pharmaceutical industry for isolating and purification herbal compound. In our experimental study aflatoxin was evaluated by HPLC. HPLC is more authentically validated, reliable technique for estimation of any bioactive compound [15,16]. Aflatoxin standard and our sample were dissolved in polar solvent then inject in HPLC. The HPLC chromatogram was run about 30 mint for aflatoxin standard and 20 mint for our drug sample.

No AFB1 residues were found to be in our crude drug sample in HPLC chromatogram B, The aflatoxin residue was found in chromatogram A, at RT about 5.46 mint. Those comparative studies were shown in fig 1. So on the basis of this result we have can say our herbal powder of *Pueraria* have no any type's harmful and carcinogenic property. In a microscopic study of tubers by safranine staining, show presence normal architecture found in root tissue such as stone cells, the crystal of calcium oxalate in inner side and cork cambium found in brown in colour The advancement of analytical techniques of standardization will serve as a rapid and specific tool in the herbal research, The purposes of the standardization are better therapeutic efficacy, safety and shelf life of herbal drugs

Conclusion - According to the experimental results, the purity of the sample was acquired as per standard Pharmacopoeia parameter, Government of India. Standardization is an important parameter for knowing the quality, purity and for sample detection. It is one of the simplest and cheapest processes for the correct identity of the materials. Physicochemical analysis of the formulation confirms the quality, and purity of plant. Heavy metal and aflatoxin estimation proved the plant do not have harmful and carcinogenic toxic property.

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