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Pharmacognostical Characterization of an Anti-Diabetic Polyherbal Formulation

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Abstract:

Standardization of drugs and formulation is one the essential parameter in today's drug discovery. Current study includes pharmacognostic study of a polyherbal formulation which comprise of six crude powdered drugs i.e. *Acacia catechu*, *Phyllanthus embellica*, *Pterocarpus marsupium*, *Salacia reticulata*, *Tinospora cordifolia* and *Vetiveria zizanioides*, which is used locally for diabetes. Morphological, microscopical and physico-chemical studies were done to standardize the plant ingredients and also for the formulation. Current study includes lycopodium spore method, which is one of the most important methods for standardization of individual powder drug and powdered formulations. When combined with various parameters like linearity, specificity, precision, repeatability and accuracy, the method become a powerful tool to uncover and check even a very small amount of adulteration in a large extent. Mean value for the identifying characters in the mixture was near to one-sixth as compared to the drug when they were individually studied, vindicating our assumption that after mixing the ratio remained intact in the formulation. Thus, this method can be used for finding the exact ratio of drugs in any formulation in near future. All the result of the study could be useful in setting some diagnostic indices for the identification and preparation of a monograph of the drugs. The developed technique will be useful for standardization of different formulations also.

Keywords: *Salacia reticulata*, Antidiabetic formulation, Lycopodium, Morphology.

Introduction

Development of standards for the identity, quality and purity of single drugs, to start with, and of formulations, at a later stage, is the need of the time. Arrangement to evolve and lay down physical, chemical and biological tests, where necessary, to identify the drug and ascertain its quality, and to detect adulteration is an urgent necessity [1]. Lycopodium consist of the spores of the clubmoss, *Lycopodium clavatum* Linn (Family Lycopodiaceae, Phyllum: Pteridophyta); grows in the North America, Russia, Poland, India and Pakistan. The sporangial spikes are cut, dried and the spores are separated by shaking. Lycopodium is a light yellow, extremely mobile and flammable powder without odour or taste. Lycopodium spores are exceptionally uniform in size (about 25µm) and 1 mg of Lycopodium contains an average of 94,000 spores. It is possible to evaluate many powdered drugs if well-defined particles may be counted as in case of pollen grains or starch grains; or if single layered tissues or cells of the area of which may be traced at a definite magnification and the actual area calculated; or if characteristic particles of a uniform thickness; the length of which can be measured at a definite magnification and the actual length calculated. Mounts containing a definite proportion of the powder and Lycopodium are used and fields in which the number of area of the particles in the powder is determined. This method can check even minute adulteration when other

standardization parameters fail [2]. Present study includes identification, authentification, morphological and microscopical studies of all the plant ingredients of the formulation. Physical constants like Ash values which includes acid insoluble ash and water soluble ash; Extractive values i.e. Cold extractive value, Hot extractive value and Successive extractive value by different solvents; and pH value at 1% and 10% concentration were done for individual drugs and also for the formulation. Last part of the study includes Lycopodium spore method, which is one of the most important methods for standardization of individual powder drug and powdered formulations. When combined with various parameters like linearity, specificity, precision repeatability and accuracy, the method become a powerful tool to uncover and check even a negligible amount of adulteration in a large extent. This study is an attempt to study the pharmacognostical profile of all the ingredients of the Antidiabetic formulation i.e. Acacia catechu, *Phyllanthus embellica, Pterocarpus marsupium, Salacia reticulata, Tinospora cordifolia* and Vetiveria *zizanioides* and the results of the study could be useful in setting some diagnostic indices for the identification and preparation of the monographs of the plants and the formulation.

Materials

The ingredients used for the Antidiabetic formulation are *Acacia catechu* heartwood's-one part, *Phyllanthus embellica* fruit's-one part, *Pterocarpus* marsupium heartwood's-one part, *Salacia reticulate* root's-one part, *Tinospora cordifolia* stem's-one parts and *Vetiveria zizanioides* root's-one part. The ingredients were collected from different parts of Kerala and authenticated by Dr. H.B.Singh, Scientist and Head of Raw material, Herbarium and Museum, NISCAIR, New-Delhi, India with a reference no. NISCAIR/RHMD/Consult/-2009-10/1393/195. All the ingredients were powdered and made fineness of 40/60 mesh-size and then they are mixed one part each to make formulation.

Methods and Experiments

1. Morphological and Microscopical Characters

Macroscopic identity of a medicinal plant material is based on shape, size, colour, taste, surface characteristics, texture, fracture characteristic and appearance of cut surface. The size, shape and relative positions of the different cells and tissues are also determined microscopically. Microscopic examination of section aided by stains helps in distinction of anatomy in adulterants. Transverse sections of all the plant parts which are used in the formulation were cut with the help of scalpels. Sections were mounted with glycerine in the slide and care has been taken to avoid entrapment of air bubbles in the slide. Saffranin is used as a staining agent to distinguish different tissues in the transverse section of the plants. The size, shape and relative positions of the different cells and tissues are determined. The basic arrangement of tissues in each drug is fairly constants like fibres, sclereids, tracheids, vessels and cork are least affected by drying [3, 4]. Table No. 1 & 2 gives the general appearance and transverse sections of all the plant parts in the ingredient.

Fig. 1: Root of *Salacia reticulate*

Fig. 2: Wood of *Pterocarpus marsupium*

Fig. 3: Root and stem of *Tinospora cordifolia*

Fig. 4: Fruits of *Phyllanthus emblica*

Fig. 5: Roots of *Vetiveria zizanioides*

Fig. 6: Wood of *Acacia catechu*

Table 1: General appearance of the crude drugs

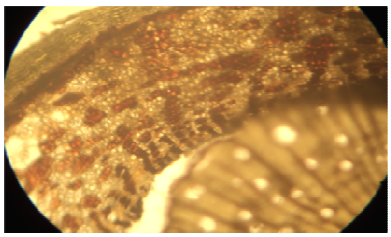

Fig. 7: T.S. of *Salacia reticulate* Root

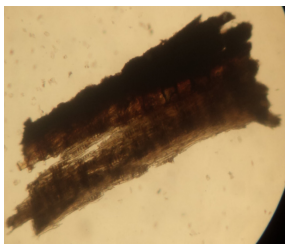

Fig.8: T.S. of *Pterocarpus marsupium* wood.

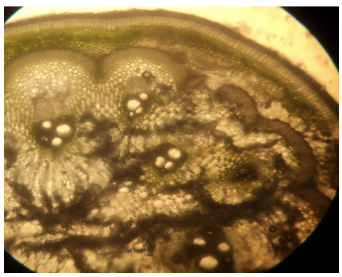

Fig. 9: T.S. of of *Tinospora cordifolia* stem

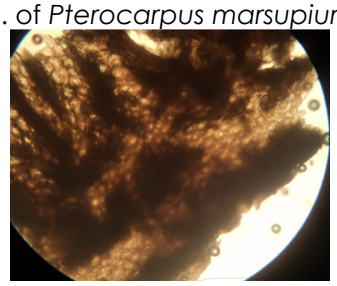

Fig. 10: T.S. of *Phyllanthus emblica* Fruits.

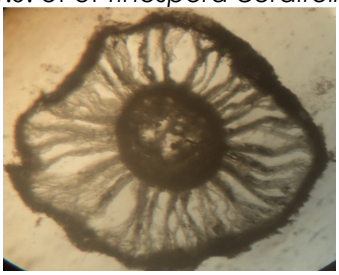

Fig. 11: T.S. of *Vetiveria zizanioides* Roots.

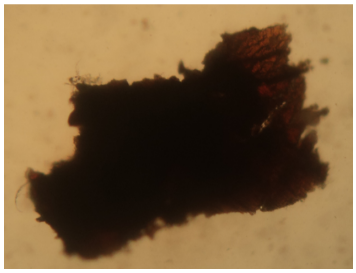

Fig. 12: T.S. of *Acacia catechu* Wood.

Table 2: Microscopy of the crude drug part

2. Ash Value

Ash Value was performed for the determination of inorganic materials, such as carbonates, silicates, oxalates and phosphates. Heating causes the loss of organic material in the form of CO_2 leaving behind the inorganic components. Ash value is an important characteristic of a drug and with the help of this parameter we can detect the extent of adulteration as well as establish the quality and purity of the drug. The acid insoluble ash consists mainly of silica and high acid insoluble ash thereby indicating the contamination with earthy materials. The water soluble ash is used to estimate the amount of inorganic elements [3, 4].

Total ash

Powdered drug (2g) is incinerated in a tarred silica crucible, spreading the material in an even layer, and ignite it by gradually increasing the heat to 500-600^{0}C until it is white, indicating absence of carbon. It is then cooled in a desiccator for 30 minutes, and weighed. Content of total ash in milligram per gm of air dried material is calculated [3, 4, 5]. (Table No. 3).

Acid insoluble ash

Total ash is boiled gently with 25 ml dilute HCL (6N) for five minutes. The insoluble matter collected on ash less filter paper washed with hot water until the filtrate is neutral and ignited at a temperature of 500-600^{0}C to a constant weight. Content of acid insoluble ash in milligram per gm of air dried material is calculated [3, 4, 5]. (Table No. 3).

Water soluble ash

Total ash is dissolved in 25 ml distilled water and boiled for five minutes, the insoluble part collected on ash less filter paper, washed with hot water, and ignited at 450^{0}C to a constant weight. By subtracting the weight of insoluble part from that of the total ash, the weight of water soluble part of ash is calculated in milligrams. Content of total ash, acid insoluble ash and water soluble ash in milligram per gm of air dried material is calculated [3,4,5]. (Table No. 3).

Ash Value							
Parameter	Drug Ingredients						
	Salacia reticulata	Vetiver zizanioides	Phyllanthus embellica	Pterocarpus marsupium	Acacia catechu	Tinospora Cordifolia	Formulation
Total Ash	5.65	4.62	11.20	0.60	5.00	10.80	5.80
Water soluble Ash	2.45	2.32	4.05	0.25	1.50	7.40	3.10
Acid Insoluble Ash	1.15	1.25	3.95	0.12	0.30	1.10	1.40

Table 3: Ash Values

3. Extractive values

The amount of an extract that a drug yields in a particular solvent is often an approximate measure of the amount of certain constituents that the drug contains. The drug should be extracted with different solvents in order of their increasing polarity to get the correct and dependable values. Generally petroleum ether, alcohol and water extractives are taken into consideration for fixing the standard of a drug.

The petroleum ether extract contains fixed oil, resins and volatile substances, but when the extract is heated at 105^{0}C until constant weight, the volatile substances are volatilized leaving only resins, colouring matter and fixed oil. Alcohol can dissolve almost all the substances, but is generally used for determining the extractive index for those drugs, which contain glycosides, resins, alkaloids etc. Water is used for the drugs containing water soluble substances as chief constituents [3, 4, 5]. The extractive values from Cold Maceration, Hot Extraction and Successive Extraction from each solvent were given in Table No. 4, 5 & 6 respectively.

Cold Maceration

The air-dried coarse powder (4g), accurately weighed in a glass stoppered conical flask was macerated with 100 ml of solvent (petroleum ether, chloroform, alcohol and water) quantitatively, and then allowed to stand for 18 hrs. It was filtered rapidly, taking precaution against loss of solvent, the filtrate evaporated to dryness in a tarred flat bottom dish on a water bath and dried at 105^{0}C for 6 hrs, and cooled in a dessicator for 30 minutes, to constant weight and weighed content of extractable matter in mg per gm of air dried material is calculated [3,4,5]. . The Extractive values by Cold Maceration are given in Table No. 4.

Cold Extraction (% Extractive value)							
	Drug Ingredients						
Solvents	Salacia reticulata	Vetiver zizanioides	Phyllanthus embellica	Pterocarpus marsupium	Acacia catechu	Tinospora Cordifolia	Formulation
Pet-Ether	0.70	0.57	6.50	0.50	4.07	0.56	2.82
Chloroform	0.95	0.57	2.42	0.20	0.30	0.72	1.02
Alcohol	0.95	0.07	8.42	0.45	4.17	0.76	3.14
Water	2.55	1.05	21.50	5.65	0.25	4.98	4.28

Table 4: Extractive Value by Cold Maceration

Hot Extraction

The air-dried coarse powder (4g), accurately weighed in a glass stoppered conical flask was macerated with 100 ml of solvent (petroleum ether, chloroform, alcohol and water) was added and weighed to obtain the total weight including the flask. After shaking well it is allowed to stand for 1 hr, which was then gently heated in a reflux condenser for 1 hr, cooled and weighed. Readjust to the original total weight with the solvent used, shake well and filter rapidly. Transfer 25 ml of the filtrate in a tarred flat bottom dish on a water bath and dried at 105^{0}C for 6 hrs, and cooled in a dessicator for 30 minutes, to constant weight and weighed content of extractable matter in mg per gm of air dried material was calculated [3,4,5]. The Extractive values by Hot Extraction are given in Table 5.

Hot Extraction (% Extractive value)							
	Drug Ingredients						
Solvents	Salacia reticulata	Vetiver zizanioides	Phyllanthus embellica	Pterocarpus marsupium	Acacia catechu	Tinospora Cordifolia	Formulation
Pet-Ether	4.95	0.62	0.50	0.83	0.75	1.80	1.92
Chloroform	4.90	1.90	0.53	1.76	1.12	2.20	1.84
Alcohol	9.60	5.23	17.46	4.81	5.35	1.20	6.87
Water	17.95	2.92	16.80	13.22	1.35	6.64	8.46

Table 5: Extractive Value by Hot Extraction

Successive Extraction

The drug collected were cleaned and crushed to a coarse powder. The drug was packed in soxhlet apparatus and extracted successively with Petroleum ether, Chloroform, Methanol and Water. Each time before extracting with next solvent the drug (marc) was removed from the soxhlet and dried at 50^{0}C in hot air oven. Extraction with each solvent was carried out about 24 hours till no residue was observed by evaporating a few drop of liquid from thimble, ensuring complete exhaustion. The various extracts were concentrated and from each solvent removed under vacuum. The drug extract obtained from each solvent was weighed and its yield in terms of air dried weight of the plant material was calculated The colour of the extract was also noted. Five different readings were taken and the average value is depicted in results [3, 4, 5]. The Extractive values by Successive Extraction are given in Table No. 6.

Successive Extraction (% Extractive value)							
	Drug Ingredients						
Solvents	Salacia reticulata	Vetiver zizanioides	Phyllanthus embellica	Pterocarpus marsupium	Acacia catechu	Tinospora Cordifolia	Formulation
Pet-Ether	3.95	0.74	0.096	0.88	0.33	1.16	1.18
Chloroform	4.05	0.70	0.08	1.43	0.30	0.74	1.12
Alcohol	7.50	4.20	3.62	6.45	2.42	4.46	4.78
Water	4.90	1.00	10.07	4.55	0.19	0.78	3.82

Table 6: Extractive Value by Successive Extraction

4. Determination of pH Value

Dissolved an accurately weighed 1 gm and 10gm of the drug in accurately measured 100 ml of distilled water, filtered and checked pH of the filtrate with a standardized glass electrode. .pH value for 1% and 10% solution of the entire drug is given in Table No. 7 [3, 4, 5].

pH Value							
	Drug Ingredients						
Conc.	Salacia reticulata	Vetiver zizanioides	Phyllanthus embellica	Pterocarpus marsupium	Acacia catechu	Tinospora Cordifolia	Formulation
1%	6	7	6	6	6	8	6
10%	6	7	6	6	6	8	6

Table 7: pH Value

5. Lycopodium Spore Method

Finding the identifying character of individual drugs

For microscopic examination invariably three slides, one in chloral hydrate, one in water and one in phloroglucinol and conc. HCl, were prepared. Specific and easily searchable characters were determined for each ingredient with the help of microscope [6,7].The **Identifying Characters** were Fibres of *Acacia catechu* (FAC, Fig.13), Sclerieds of *Phyllanthus embellica* (SPE, Fig.14), Fibrovascular tissue of *Pterocarpus marsupium* (FVPM, Fig.15), Phloem fibres of *Salacia reticulata* (PFSR, Fig.16), Crystals of Calcium oxalate in Parenchytamous cell of *Tinospora cordifolia* (PCTC, Fig.17) and Starch grains inside the fibres of *Vetiveria zizanioides* (SFVZ, Fig.18) [8]. (Table no.8).

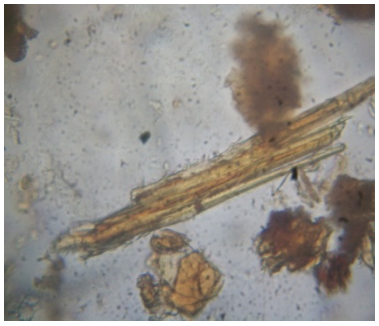

Fig. 13: Fibres of *Acacia catechu* (FAC)

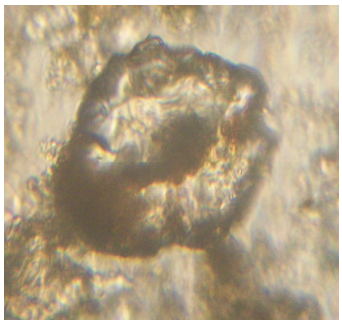

Fig. 14: Sclerieds of *Phyllanthus embellica* (SPE)

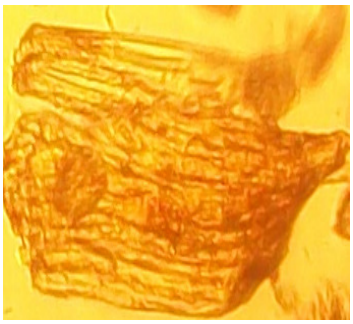

Fig. 15: Fibrovascular tissue of *Ptero -carpus marsupium* (FVPM)

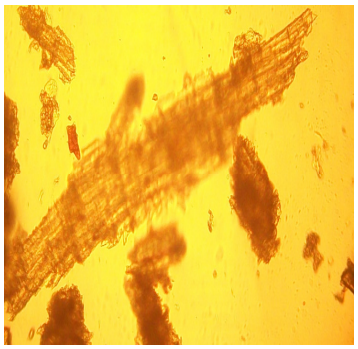

Fig. 16: Phloem fibres of *Salacia reticulata* (PFSR)

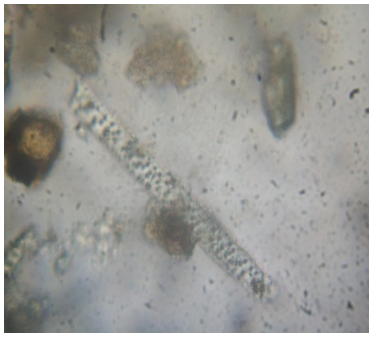

Fig. 17:Crystals of Calcium oxalate in parenchytamous cell of *Tinospora cordifolia* (PCTC)

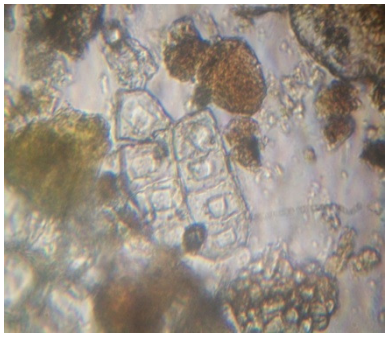

Fig. 18: Simple starch grains inside the fibres of *Vetiveria zizanioides* (SFVZ).

Table 8: Identifying Characters for Lycopodium Spore Methods

Estimation of ingredients of the formulation by Lycopodium spore method

100 mg of Salacia root powder and 50mg of Lycopodium spore was mixed using a small flexible spatula with little suspending liquid i.e. Castor oil until a smooth paste was formed. This mixture was then transferred to a stoppered tube by washing with excess of castor oil and volume of the stoppered tube was fixed (4ml). The stoppered flask was shaken thoroughly in order to obtain uniformity. One drop of this suspension was placed in slides, and spread using glass rod, cover slip was applied and was kept aside for few minutes in order to settle the fluid. The number of identifying character was counted in each of 25 different fields [6, 7]. Then 75mg, 50mg and 25mg of Salacia root powder with 50mg of Lycopodium spore were made into suspension as above and the number of identifying character was counted in each of 25 different fields by Microscope [7]. This process was repeated for each ingredient and finally with the Antidiabetic formulation.

Validation of Lycopodium Spore Method

The data obtained were validated with parameters like Linearity, Specificity, Precision, Repeatability and Accuracy.

Linearity

A series of standard curves were prepared over a range of 25mg-100mg for each ingredient (n=6). The data of amount of ingredient versus number of diagnostic characters were treated by linear least square regression analysis. The number of identifying characters varies proportionately with the quantity of powder which can be best revealed by the data obtained in the form of slope, intercept, and correlation coefficient. [7, 9, 10]. (Table No. 9)

Specificity

Specificity is an important quality criterion. Here specificity was determined by adding other ingredient to the ingredient which is being analyzed. Here analysis of a component of a mixture not interferes with other components of a mixture, which is justified by this method from the correlation coefficient value (r^2) The correlation coefficient value between different weight (i.e. 25mg, 50mg, 75mg and 100mg) of powdered ingredients were determined [7,9,10]. (Table No. 9)

Identifying character (IC)	Presence of Identifying character in different weight of powder				
	Number				r^2
	25mg	50mg	75mg	100mg	
FAC	0.44	0.72	1.28	1.8	0.98
SPE	0.52	0.88	1.36	1.72	0.99
FVPM	0.48	0.88	1.4	1.8	0.99
PFSR	0.48	0.96	1.48	1.96	0.98
PCTC	0.32	0.6	1.0	1.24	0.99
SFVZ	0.24	0.4	0.8	1.12	0.97

Table 9: Linearity and specificity with respect to Least square regression value Precision

The intraday precision was evaluated by analyzing each ingredient repeatedly for 100mg (n=6) throughout the day. Precision was measured by analysis of the method at different condition covering entire calibration range. The Precision of the method is shown by percent coefficient of variation (%CV) [7, 9, 10] (Table No. 10)

Identifying character (IC)	Intraday precision(%CV) n=5 (%CV= Stdev/Mean×100)	
	No. of LS (Lycopodium Spore)	No. of IC (Identifying character)
FAC	2.47	2.70
SPE	2.08	3.04
FVPM	3.30	3.32
PFSR	5.42	3.05
PCTC	2.94	3.20
SFVZ	3.95	4.02

Table 10: Precision in average number of identifying characters

Repeatability

The repeatability of this method was assessed by performing the experiment five times in a day (n=6 for each experiment). Standard deviation gives the value of variation in the study, ultimately the repeatability [7, 9, 10] (Table No. 11)

Accuracy

The accuracy of the method was determined by taking powdered ingredients and Lycopodium spore at ratio 100:50. Values were found to be within the limits and the individual ingredients Acacia catechu, Phyllanthus embellica, Pterocarpus marsupium, Salacia reticulate, Tinospora cordifolia & Vetiveria zizanioides are in the ratio of one part each in the formulation. [7, 9,10] (Table No.11).

Identifying Character	Lycopodium Spore with 50mg individual drug		Identifying Character for 100mg individual drug		Mean value for Mixture of 100mg
	Mean, n=25	SD, n=25	Mean, n=25	SD, n=25	
FAC	252.84	6.27	1.80	0.71	0.32
SPE	218.20	4.54	1.72	0.54	0.28
FVPM	173.96	5.75	1.80	0.58	0.40
PFSR	157.20	8.50	1.96	0.88	0.36
PCTC	186.80	5.42	1.24	0.44	0.20
SFVZ	183.40	7.22	1.12	0.40	0.16

Table 11: Accuracy and Repeatability by mean and standard deviation

Results

1. Morphological & Microscopical Characters

Detailed morphological and microscopic studies were done and photographed, giving the general appearance and internal structure of the crude drugs as shown in Table No. 1 & 2.

2. Ash Value

All the ingredients and the formulation shown total ash, acid insoluble ash and water soluble ash values within the specification limits

mentioned in Ayurvedic Pharmacopoeia and is shown in Table No. 3 [11].

3. Extractive values

The drug was extracted (Cold, Hot and Successive) with range of solvent (petroleum ether, chloroform, methanol and water) and maximum soluble **extractive value** was observed in **water and methanol** and minimum in **petroleum ether** as shown in Table No. 4, 5, & 6, indicating the presence of more polar constituents (glycosides, carbohydrates, flavonoids, proteins, alkaloids etc.). All the values are within the specification limits mentioned in Ayurvedic Pharmacopoeia [11]. .

4. Determination of pH Value

The pH value indicates the type of constituents present by describing the acidity or basicity of the constituents. The pH value of the individual ingredients ranges between 6 to 8 and that of formulation was 6 indicating moderate acidity and shown in Table no. 7. All the values within the specification limits mentioned in Ayurvedic Pharmacopoeia [11]. .

5. Lycopodium Spore Method

Finding the identifying character of individual drugs

The **Identifying Characters** were found and are shown in Table no.8.

Validation of Lycopodium Spore Method

Linearity

The number of identifying characters varies proportionately with the quantity of powder which can be best revealed by the data obtained in the form of slope, intercept, and correlation coefficient. Linearity chart showed the increase in presence of identifying character with increase in concentration of powdered drug with reference to Lycopodium which can be shown with **correlation coefficient (r^2) in the range** of **0.997 to 0.999**, which is near to an ideal condition. (Table No. 9).

Specificity

Here analysis of a component of a mixture does not interferes with other components of a mixture, which is justified by this method from the correlation coefficient value (r^2) which is in the range of **0.997 to 0.999** (Table No.9).

Precision

Precision of the method was shown in terms of percentage coefficient of variation (%CV) which was in the range of **2.70 to 4.07** (Table No. 10).

Repeatability

Standard deviation gives the value of variation in the study ultimately the repeatability. Study shows the standard deviation in the range of **0.40 to 0.88** which is very less and good repeatability value. (Table No. 11)

Accuracy

Accuracy can be assessed by comparing the mean value for the number of identifying character to the individual drug. Table shows that ratio of identifying characters in the formulation and **individual drug is exactly one-sixth**, justifying that all the ingredients are in one-one ratio. (Table No.11)

DISCUSSION & CONCLUSION

Establishing standards is an integral part of establishing the correct identity, purity and quality of crude drugs and formulations. Majority of the information on the identity, purity and quality of the plant material can be obtained from its macroscopy, microscopy and physico-chemical parameters. Our study has shown that all the ingredients have **passed the test of identity, purity and quality according to the Pharmacopoeial standards** with the help of

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morphological study, microscopical study and the quantitative microscopy i.e. Lycopodium Spore Method. Lycopodium spore method, which is one of the most important methods for standardization of individual powder drug and powdered formulations, has been combined with various parameters like **linearity, specificity, precision repeatability and accuracy** has shown that **all its ingredients when mixed where in equal ratio**. Results were precise, accurate and all the results were close to ideal condition showing the versatility of the method and the sophistication with which even if a minute adulteration is present can be checked. In addition our present result will pave the way for conducting more instrumental standardization parameters on the formulation. Further investigation into the bioactivity of the individual drug and the formulation may prove useful in the prevention of a variety of pathological condition. Isolation of the bioactive components through different chromatographical methods can lead to preclinical and clinical testing of the formulation.

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