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VALIDATED MODIFIED *LYCOPODIUM* SPORE METHOD DEVELOPMENT FOR STANDARDISATION OF INGREDIENTS OF AN AYURVEDIC POWDERED FORMULATION SHATAVARYADI CHURNA

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Abstract

Validated modified lycopodium spore method has been developed for simple and rapid quantification of herbal powdered drugs. Lycopodium spore method was performed on ingredients of Shatavaryadi churna, an ayurvedic formulation used as immunomodulator, galactagogue, aphrodisiac and rejuvenator. Estimation of diagnostic characters of each ingredient of Shatavaryadi churna individually was carried out. Microscopic determination, counting of identifying number, measurement of area, length and breadth of identifying characters were performed using Leica DMLS-2 microscope. The method was validated for intraday precision, linearity, specificity, repeatability, accuracy and system suitability, respectively. The method is simple, precise, sensitive, and accurate, and can be used for routine standardisation of raw materials of herbal drugs. This method gives the ratio of individual ingredients in the powdered drug so that any adulteration of genuine drug with its adulterant can be found out. The method shows very good linearity value between 0.988-0.999 for number of identifying character and area of identifying character. Percentage purity of the sample drug can be determined by using the linear equation of standard genuine drug.

Keywords: Shatavaryadi Churna, Lycopodium spore, Ayurvedic formulation, Leica microscope, Standardisation

Introduction

Lycopodium spore is oval-triangular to nearly circular and trilete. Its arms of laesura is straight, the entire inner margin 15–18 µm long, extending to spore margin. Its proximal face laevigate and distal face regulate with spaces between rugulae, appearing as narrow, elongated, and slit-like openings. Its wall is 2-3 µm thick, with size 30-34 µm (Graham, 1998). These characteristics lead to uniform identity and size of Lycopodium spore. In the present study, it is selected as control to monitor the variations to develop the method.

Admixture of exhausted drugs is one of major menace faced by the herbal industry. Low cost substitutes can pass the chemical test because of the presence of active ingredients. The number of particular characteristic particles per unit weight is also constant as is useful in assessing the quality and quantity of each ingredient in the sample. In the case of powdered drugs the standard methods like ash value, extractive value, solid content, total alkaloid content and volatile oil content can pass through the standard tests with the help of adulterated or substituted drugs. So, in order to provide a support to this standardisation a symbiotic method is required to detect the adulterated and substituted drugs. Modified Lycopodium spore method can fill this gap, and there is a need of the method to support the chemical method of standardisation.

Modified Lycopodium spore method is an important analytical technique for powdered drug, especially when other methods of evaluation of crude drugs fail as accurate measures of quality (Kumar and Jha, 2011). Modified Lycopodium spore method has been developed for simple and rapid quantification of dried powder of plants and its parts. Lycopodium spore method was performed on each ingredient of an ayurvedic formulation Shatavaryadi churna which contains powder of *Asparagus racemosus* Willd. tubers-1part, *Tribulus terrestris* Linn. fruits-1part, *Mucuna pruriens* Linn. seeds-1part, *Withania somnifera* Dunal. roots-1part and *Chlorophytum tuberosum* Baker bulbs-1part. (Pathak, 1999). Generally, Ayurvedic practice involves the use of medications that typically contain herbs, metals, minerals, or other materials (Chopra & Doiphode, 2002; Thatte et al., 1993). Shatavaryadi churna is a traditional Ayurvedic powdered formulation used for centuries with claimed efficacy and safety in immunomodulator, galactagogue, aphrodisiac and rejuvenator activities (Pathak, 1999).

Materials and Methods

The ingredients were collected from Chhattisgarh and purchased from local market of New Delhi and authenticated by Dr. E. Roshini Nayar, Principal Scientist, National Bureau of Plant Genetic Resources, Indian Council of Agriculture Research, Pusa Campus, New Delhi. The well known identifying characters were determined for each ingredient with the help of Leica DMLS-2 microscope, i.e., Starch grains of *Asparagus racemosus* Willd.

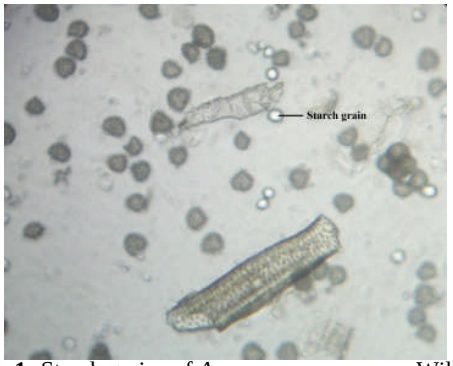

Figure 1: Starch grains of *Asparagus racemosus* Willd. Tuber.

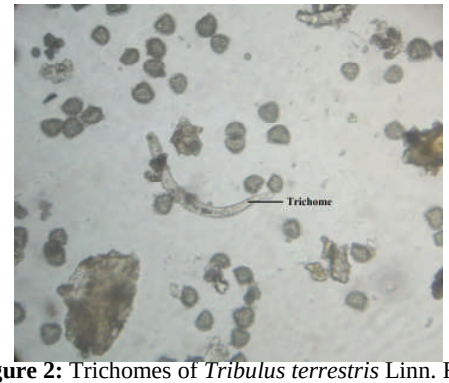

Figure 2: Trichomes of *Tribulus terrestris* Linn. Fruit.

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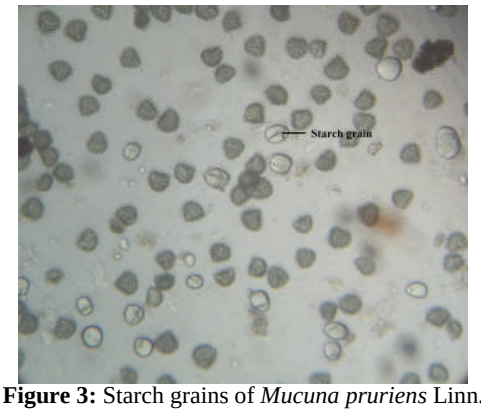

Figure 3: Starch grains of *Mucuna pruriens* Linn. Seed.

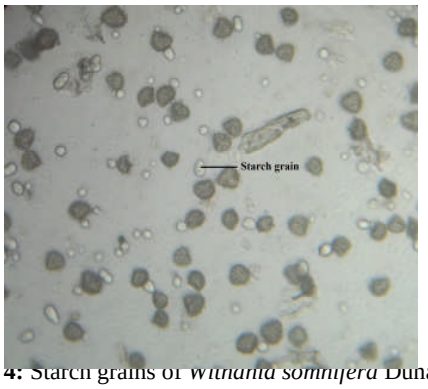

Figure 4: Starch grains of *Withania somnifera* Dunal. Root.

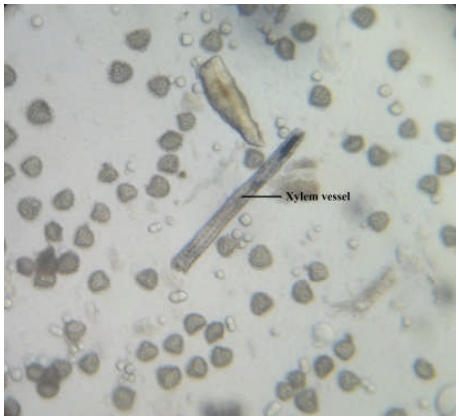

Figure 5: Xylem vessel of *Chlorophytum tuberosum* Baker.

Lycopodium spore method

25mg of drug powder and 50mg of Lycopodium spore were mixed using a small flexible spatula with little suspending liquid (i.e. Corn oil). This mixture was incorporated with sufficient quantity of corn oil until a smooth paste was formed. The mixture was then transferred to a stopper tube by washing with excess of corn oil and volume of the stopper tube was fixed (4ml). The stopper tube was oscillated gently in order to obtain uniformity. One drop of this suspension was placed in microscopic slide, and spread using glass rod. Cover slip was applied and was kept aside in a plane surface for few minutes in order to settle the fluid (Wallis, 1953). The number, area, length and breadth of identifying character were counted in each of 25 different fields. Then 50mg, 75mg and 100mg of drug powder with 50mg of Lycopodium spore were made into suspension as above and the number, area, length and breadth of identifying character were counted in each of the 25 different fields using Leica DMLS-2 and also with the aid of Leica QWIN software. This process was repeated for each ingredient. (Table 1)

Leica DMLS-2 Instrumentation

Lycopodium spore method was performed using Leica DMLS-2 microscope with the aid of Leica Qwin software at following specific instrumental conditions:

Windows display:	1.24×768 pixel.
Objective lens:	×4
Calibration value (4*x*):	1pixel=0.863µm.
Exposure:	106.0 milliseconds.
Gain:	1
Color saturation:	1.5
Captured image:	1499×1299
Color depth:	8 bit/channel.
Image type:	Colour.

Accuracy (European Medicines Agency, 1995)

The accuracy of the method was determined by taking powered ingredients and Lycopodium spore at ratio 25:50, 50:50, 75:50 and 100:50 respectively and determined by average and standard deviation. (Table 3)

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Specificity (Ermer, 2001)

Specificity is an important quality criterion. Here analysis of each component of Shatavaryadi churna was done by correlation coefficient value to determine the specificity of the method. (Table 1)

Table 1: Identifying characters, linearity and specificity with respect to least square regression value

Name of identifying characters	Average number of Identifying Character (n=25)					Average area of Identifying Character (n=25) (in µm^2)				
	Amount of drug sample taken					Amount of drug sample taken				
	25mg	50mg	75mg	100mg	r^2	25mg	50mg	75mg	100mg	r^2
Starch grains of *Asparagus racemosus* Willd.	20.16	40.80	57.92	82.48	0.995	2577.69	5246.82	7554.38	10415.23	0.998
Trichomes of *Tribulus terrestris* Linn	3.00	5.96	9.80	11.88	0.988	4069.19	8100.43	11032.57	16219.61	0.988
Starch grains of *Mucuna pruriens* Linn.	16.16	32.52	46.32	64.04	0.997	10559.96	21372.91	32055.40	42807.09	0.994
Starch grains of *Withania somnifera* Dunal.	18.48	35.08	52.64	74.36	0.995	8081.39	16192.87	24185.70	32005.16	0.999
Xylem vessel of *Chlorophytum tuberosum* Baker	1.52	3.32	4.48	6.08	0.993	10480.64	19911.47	31710.67	42136.26	0.998

Validation of the method

Linearity **(Ermer, 2001)**

A series of standard curves were prepared over a range of 25mg-100mg for each ingredient (n=25). The data of amount of ingredient versus number and area of diagnostic characters were treated with 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 correlation coefficient. The correlation coefficient value between different weight (i.e.-25mg, 50mg, 75mg and 100mg) of powdered ingredients were determined. (Table 1)

Precision (Ermer, 2001)

The intraday precision was evaluated by analysing each ingredient repeatedly for 25mg, 50mg, 75mg and 100mg (n=25). Precision was measured by analysis of the method at different conditions covering entire calibration range. The Precision of the method in terms of intraday variation i.e. % Co-efficient of variation (% CV) was determined at different times in a day. (Table 2)

Table 2: Results for determination of precision and repeatability in average number of identifying characters

Name of identifying characters	Number/ Area (in µm^2)/ Length(in µm)/ Breadth(in µm)	Intraday precision(%CV) n=5 (5 different times in a day) (%CV= Standard deviation/Average×100)			
		25mg	50mg	75mg	100mg
Starch grains of *Asparagus racemosus* Willd.	Number	3.01	3.02	2.45	1.42
	Area	1.06	3.17	3.59	3.60
	Length	1.81	3.46	1.68	2.39
	Breadth	1.71	2.02	3.02	0.68
Trichomes of *Tribulus terrestris* Linn.	Number	4.71	5.51	2.50	3.49
	Area	0.69	0.96	2.45	3.88
	Length	3.64	6.96	9.19	4.01
	Breadth	9.24	7.38	5.43	3.81
Starch grains of *Mucuna pruriens* Linn.	Number	2.03	1.28	2.97	1.70
	Area	1.92	3.47	3.05	2.07
	Length	3.47	0.33	2.21	1.00
	Breadth	2.25	1.67	1.65	2.10
Starch grains of *Withania somnifera* Dunal.	Number	1.23	3.06	2.98	0.61
	Area	0.76	2.90	2.47	1.53
	Length	1.56	2.35	3.33	3.75
	Breadth	1.04	2.05	1.50	2.70
Xylem vessel of *Chlorophytum tuberosum* Baker	Number	7.21	5.39	7.47	4.41
	Area	1.92	2.63	1.55	3.10
	Length	9.74	3.49	6.21	7.74
	Breadth	9.16	7.99	8.80	7.26

Repeatability (European Medicines Agency, 1995)

The repeatability of this method was assessed by performing the experiment at different times in a day. The repeatability of this method was determined by % CV by performing the experiment in different times in a day (n=5 for each experiment). (Table 2)

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System suitability (European Medicines Agency, 1995)

System-suitability of the method was determined by analysing the sample 5 times in a day (n=5). System-suitability tests are composed of a system's precision measurement and system's power of resolution measurement to check the performance of the system on a given day with respect to standard deviation. (Table 3)

Results

Average number and area (n=25) of identifying characters with respect to average number of Lycopodium Spore in ingredients of Shatavaryadi Churna were determined and this shows the linear relationship. Average length and breadth were also determined for all the identifying characters. Average length and width were found in the range of 16.34-16.62µm and 9.67-9.99 µm; 89.27-91.3µm and 26.56-30.83µm; 34.38-35.04µm and 25.39-25.97µm; 27.64-28.08µm and 20.19-20.43µm; 173.94-176.55µm and 65.44-67.35µm for starch grains of *Asparagus racemosus* Willd., trichomes of *Tribulus terrestris* Linn., starch grains of *Mucuna pruriens* Linn., starch grains of *Withania somnifera* Dunal., and Xylem vessel of *Chlorophytum tuberosum* Baker respectively. This shows a relatively constant value. (Table 1) Average number and area of *Lycopodium* spore were found between the range of 142.40-144.60 and 98998.69-100503.62µm^2 respectively.

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 correlation coefficient. Linearity was found to be in the range of 0.988-0.997 for number of identifying characters and 0.988-0.999 for area of identifying characters by correlation coefficient. (Table 1)

Precision

The Precision of the method in terms of intraday variation (%CV) was determined at five different times in a day.
Precision was found between the range of 0.61-7.47 for number, 0.69-3.88 for area, 0.33-9.74 for length and 0.68-9.24 for breadth of identifying characters by %CV. For Lycopodium spore it was found to be 0.26-1.59 and 0.49-2.09 for number and area respectively. (Table 2)

Table 3: Results for studies of accuracy by Average and standard deviation

Name of identifying characters	**Number /Area (in µm^2)**	**Average(Avg)/Standard deviation(SD) (n=5) (5 different times in a day)**							
		25mg		**50mg**		**75mg**		**100mg**	
		Avg	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Starch grains of *Asparagus racemosus* Willd.	Number	20.16	0.61	40.8	1.23	57.92	2.52	82.48	1.17
	Area	2577.69	27.42	5246.82	166.43	7554.38	270.84	10415.23	375.33
	Length	16.34	0.30	16.62	0.57	16.51	0.28	16.48	0.39
	Breadth	9.99	0.17	9.67	0.20	9.78	0.30	9.98	0.07
Trichomes of *Tribulus terrestris* Linn.	Number	3.00	0.14	5.96	0.33	9.80	0.24	11.88	0.41
	Area	4069.13	28.09	8100.43	78.03	11032.57	270.03	16219.61	629.35
	Length	89.98	3.27	92.00	6.36	90.24	8.30	82.87	3.32
	Breadth	30.83	2.85	26.56	1.96	27.21	1.48	28.18	1.07
Starch grains of *Mucuna pruriens* Linn.	Number	16.16	0.33	32.52	1.16	46.32	1.38	64.04	1.09
	Area	10559.96	203.09	21372.91	741.51	30055.40	917.79	42807.09	885.88
	Length	34.3	1.19	34.44	0.11	34.66	0.77	35.03	0.35
	Breadth	25.40	0.57	25.97	0.43	25.75	0.42	25.64	0.54
Starch grains of *Withania somnifera* Dunal.	Number	18.48	0.23	35.08	1.07	52.64	1.57	74.36	0.46
	Area	8081.39	61.52	16192.87	469.26	24185.70	596.56	32005.16	489.27
	Length	28.08	0.44	27.64	0.65	27.80	0.93	28.02	1.05
	Breadth	20.19	0.21	20.38	0.66	20.23	0.30	20.43	0.55
Xylem vessel of *Chlorophytum tuberosum* Baker	Number	1.52	0.11	3.32	0.18	4.48	0.33	6.08	0.27
	Area	10480.64	200.97	19911.47	523.80	31710.67	492.89	42136.26	1306.17
	Length	176.55	17.20	175.32	6.12	173.94	10.80	174.19	13.49
	Breadth	67.35	6.17	66.88	5.34	67.26	5.92	65.44	4.75

Accuracy

The accuracy of the method was determined by taking four different proportions of powered ingredients, i.e., 25mg, 50mg, 75mg and 100mg by keeping the amount of Lycopodium spore constant, i.e. 50 mg. Results for studies of accuracy by standard deviation were found to be

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in the range of 0.11-2.52 (average 1.52-82.48) for number, 27.42-1306.17 (average 2577.69-42807.09) for area, 0.11-17.20 (average 16.34-176.55) for length, and 0.07-6.17 (average 9.67-67.35) for breadth of identifying characters. Lycopodium spore was found to be 0.37-3.34 (average 142.40-144.60) and 493.31-2078.55µm^2 (average 98998.69-100503.60µm^2) for number and area respectively. (Table 3)

Specificity

Specificity was found to be in the range of 0.988-0.997 for number of identifying characters and 0.988-0.999 for area of identifying characters determined by correlation coefficient. (Table 1)

Repeatability

The repeatability of this method was assessed by performing the experiment five different times in a day and found to be good repeatability value with only variation in the range of 0.61-7.47 for number, 0.69-3.88 for area, 0.33-9.74 for length and 0.68-9.24 for breadth of identifying characters by %CV. For Lycopodium spore, it was found to be 0.26-1.59 and 0.49-2.09 for number and area respectively. (Table 2)

System suitability

Suitability of the system with respect to standard deviation was found to be range of 0.11-2.52 (average 1.52-82.48) for number, 27.42-1306.17 (average 2577.69-42807.09) for area, 0.11-17.20 (average 16.34-176.55) for length and 0.07-6.17 (average 9.67-67.35) for breadth of identifying characters. For Lycopodium spore, it was found to be 0.37-3.34 (average 142.40-144.60) and 493.31-2078.55µm^2 (average 98998.69-100503.60µm^2) for number and area respectively and showed good system suitability. (Table 3)

Discussion and Conclusion

The number and area of selected identifying character of each ingredient are directly proportional to the concentration of the prepared suspension keeping the quantity of Lycopodium spore constant. By using the linear equation of the particular standard powdered crude drug, percentage purity of the sample powdered crude drug can be determined. Identifying characters which cannot be broken down into further parts (i.e. starch grains) is more reliable as compared to breakable characteristics (i.e. trichome, xylem vessel) for all four parameters i.e. number, area, length and breadth. The analytical method developed in this study is specific for powdered crude drug having specific identifying characters, which can be used to find out the percentage purity of the powdered crude drug. After validation with the various items, the developed method can be applicable for the quality assurance or quality control of the specific powdered crude drugs. The proposed modified Lycopodium spore method was found to be simple, precise, accurate, specific, suitable and cost effective for standardisation of powdered crude drug. It can be used for routine standardisation of the powdered crude drug as well as powdered crude dug formulations (i.e. churna).

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