THE HISTOLOGY OF BELLADONNA ROOT

PART IV

THE DIFFERENTIAL VALUE OF THE FIBRE/VESSEL RATIO

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BELLADONNA ROOT is defined in the British Pharmacopœia, 1948, as the root, or root and rootstock, of either *Atropa Belladonna* Linn., or *Atropa acuminata* Royle ex Lindley or a mixture of both species. In the earlier parts of this work, it was noted that the xylem of *A. Belladonna* root consists in the main of cellulosic parenchyma and scattered vessel strands, whereas in *A. acuminata* the cellulosic parenchyma is largely replaced by lignified fibrous tissue. Inspection of transverse sections of the roots of the two species did not indicate any differences in the numbers of vessels per unit area, hence it seemed likely that a differential character could be based on the ratio of the numbers of fibres to the numbers of vessel elements. It was therefore decided to investigate this character on both the whole drug and the powder and to adopt the term " fibre/vessel ratio " to indicate the number of fibres associated with one vessel element. In counting the fibres, it was anticipated that the somewhat similar fibre-tracheids might cause difficulty in their discrimination, and to avoid this they were included in the fibre count if they exhibited elongated simple pits. True tracheids, due to their characteristic elliptical pits, are readily distinguished.

In Part III of this work,[1] it was shown that the vessel index provided a means of differentiating the powdered drugs, but its application to the whole drug was not investigated in detail. It appeared desirable therefore to do this also in order to compare the two characters and to assess their value when combined in a discriminant function.

MATERIALS

Specimens of the whole drug were selected from a number of parcels of commercial material obtained from reputable wholesale druggists between 1940 and 1945, and supplied as either " English Belladonna root " or " Indian Belladonna root." Ten specimens of each variety were selected, ranging in diameter from the narrowest to the widest in the material and including examples at all the stages of xylem development present. The wider of these specimens consisted of both root and rootstock, and the narrower of root only. They were numbered 1 to 10 in decreasing order of diameter, which order corresponded approximately with their age, the widest specimens of either variety being about five years old.

The powdered material consisted of six samples of commercial powder, coded AB1 to AB6, supplied as *A. Belladonna* and five coded Aa1 to Aa5, supplied as " Indian Belladonna." This was the same material as that used for the determination of the vessel index and the cork cell number reported on in Part III of this work,[1] in Table I of which fuller details appeared.

A.—The Whole Drug

Number of observations. Since the fibres are more numerous than the vessel elements, it was found convenient in practice to count the numbers of the latter associated with groups of 10 fibres. To determine the minimum number on which the ratio should be based in order to obtain good agreement between successive results, one root of each species taken at random from the commercial drug was examined and the number of vessel elements associated with a total of 400 fibres determined in each case. The range of successive results based on count of 100 fibres and 200 fibres was respectively about 8 per cent. and 4 per cent. of the mean. Subsequent work was therefore based on a minimum count of 200 fibres.

Variation in the whole drug. The 10 roots of each species were softened by soaking in dilute alcohol and cut into 5 approximately equal lengths, which were numbered 1 to 5 from base to apex or crown. The entire portion, or a longitudinal sector according to the bulk, was disintegrated separately by means of Schultz's maceration fluid, following the method described below. The fibre/vessel ratio was calculated from observations of the number of vessel elements associated with 200 fibres ; at the same time, and using the same microscopical preparations, the vessel index $135\mu^1$ (i.e. the percentage of vessel elements wider than 135μ) was also determined.

Summary of Results

The values of the fibre/vessel ratio for the whole drug are given in Table I, and of the vessel index 135μ in Table II. The ranges of the positional values of the fibre vessel ratio are : *A. Belladonna*, 0·89 to **1·79** to **4·28** to 7.69, mean 3·04, standard deviation, 1·25 ; *A. acuminata*, 2·35 to **2·20** to **8·79** to 18·18, mean 5·49, standard deviation 3·29. For the vessel index 135μ, the corresponding ranges are : *A. Belladonna*, 0 to **1·42** to **7·14** to 10·07, mean 4·28, standard deviation 2·86 ; *A. acuminata*, 6·37 to **8·58** to **22·30** to 27·74, mean 15·44, standard deviation 6·86. The ranges of either character thus show a not inconsiderable overlap so that complete differentiation of the whole drug is not possible by these means. Fisher has shown that, in cases such as this, an im-

TABLE I

FIBRE/VESSEL RATIO—VARIATION IN THE WHOLE DRUG

Species	*A. Belladonna*						*A. acuminata*					
Root No.	Position 1	2	3	4	5	Root Mean	Position 1	2	3	4	5	Root Mean
1	1·39	1·62	1·75	2·27	2·63	1·93	5·26	6·06	6·25	6·90	7·69	6·43
2	2·99	2·90	3·70	3·23	4·76	3·52	8·00	10·53	10·53	12·50	12·50	10·81
3	2·25	3·77	3·70	3·70	4·08	3·50	2·86	3·85	3·85	5·88	5·56	4·40
4	3·77	3·70	4·55	4·35	7·69	4·81	6·45	10·00	11·11	14·29	18·18	12·01
5	0·89	1·35	1·32	1·74	1·90	1·44	2·60	2·35	2·86	4·44	4·08	3·27
6	2·25	2·27	2·70	3·13	2·94	2·66	2·99	2·86	3·12	3·12	3·08	3·03
7	1·55	1·92	2·25	2·47	3·23	2·28	2·74	3·57	2·94	3·57	2·53	3·07
8	3·28	3·51	4·55	4·26	4·55	4·03	3·08	4·00	4·65	5·00	5·71	4·49
9	1·87	2·25	2·99	3·13	3·28	2·70	2·60	3·51	3·57	5·26	5·56	4·10
10	2·60	2·94	3·08	3·03	5·88	3·51	3·03	3·45	3·64	2·86	3·70	3·34
Position Mean	2·28	2·62	3·06	3·13	4·09	3·038	3·96	5·02	5·25	6·38	6·86	5·494

provement may result from the use of a discriminant function X, which is a linear compound of the available measurements, i.e. $X = \lambda_1 x_1 + \lambda_2 x_2$, the constants λ_1 and λ_2 being chosen so as to maximise the ratio of the difference between the specific means to the variance within the species.

TABLE II

135 μ VESSEL INDEX—VARIATION IN THE WHOLE DRUG

Species	*A. Belladonna*						*A. acuminata*					
Root No.	Position					Root Mean	Position					Root Mean
	1	2	3	4	5		1	2	3	4	5	
1	3·85	3·10	2·72	1·19	1·19	2·41	21·38	24·47	26·04	27·11	27·74	25·38
2	7·41	8·76	8·42	8·09	8·09	8·15	10·07	10·40	9·75	10·71	11·34	10·45
3	6·02	6·72	7·41	8·76	8·76	7·53	10·07	12·28	11·98	14·09	14·97	12·68
4	1·96	2·72	3·10	3·85	4·22	3·17	13·79	16·39	16·67	16·94	11·34	15·03
5	3·10	3·10	3·10	3·10	3·85	3·25	15·25	16·94	18·57	19·87	23·08	18·74
6	0·79	0·79	1·58	1·19	1·96	1·26	20·64	23·31	23·78	24·93	25·37	23·61
7	7·06	7·41	8·09	10·07	10·07	8·54	17·76	21·88	23·78	25·81	27·74	23·39
8	1·96	1·96	2·72	3·47	3·47	2·72	6·37	6·72	6·72	6·72	8·42	6·99
9	4·22	4·58	4·58	5·30	6·02	4·94	9·09	9·75	10·07	10·07	10·40	9·88
10	0·40	1·19	0·40	0·00	1·96	0·79	7·06	7·41	8·09	8·76	10·07	8·28
Position Mean	3·68	4·03	4·21	4·50	4·96	4·277	13·15	14·96	15·55	16·50	17·05	15·439

Accordingly, the discriminant function combining the positional fibre/vessel ratio and the vessel index values in Tables I and II was calculated. The calculation followed the method detailed by Mather[2], with the result $X = 0{\cdot}004431 x_1 + 0{\cdot}004760 x_2$, where x_1 and x_2 are the vessel index and fibre/vessel ratio values respectively. For convenience in practice, a new function $X^1 = 225{\cdot}6832X$ was employed, i.e. X^1 = vessel index + 1·074 fibre/vessel ratio. The results are given in Table III and discussed later.

B.—The Powdered Drug

Preliminary. Before the fibre/vessel ratio can be determined on the powdered drug, the method as applied to whole roots needs modifying to allow for the presence of broken elements. Accordingly, portions of two samples of commercial powdered Indian belladonna root, Aa1 and Aa2, were disintegrated and used for this preliminary work.

TABLE III

VALUES OF X^1 = VESSEL INDEX + 1·074 FIBRE/VESSEL RATIO FOR THE WHOLE DRUG

Species	*A. Belladonna*						*A. acuminata*					
Root No.	Position					Root Mean	Position					Root Mean
	1	2	3	4	5		1	2	3	4	5	
1	5·34	4·84	4·60	3·55	4·02	4·47	27·03	30·98	32·75	34·52	36·00	32·26
2	10·62	11·88	12·39	11·56	13·20	11·93	18·66	21·71	21·06	24·14	24·77	22·07
3	8·44	10·77	11·38	12·73	13·14	11·29	13·14	16·41	16·11	20·41	20·94	17·40
4	6·01	6·69	7·99	8·52	12·48	8·34	20·72	27·13	28·60	32·29	30·87	27·92
5	4·06	4·55	4·52	4·97	5·89	4·80	18·04	19·46	21·64	24·64	27·46	22·25
6	3·21	3·23	4·48	4·55	5·12	4·12	23·85	26·38	27·13	28·28	28·68	26·86
7	8·73	9·47	10·51	12·72	13·54	10·99	20·70	25·71	26·94	29·64	30·46	26·69
8	5·48	5·73	7·61	8·05	8·36	7·04	9·68	11·02	11·71	12·09	14·55	11·81
9	6·23	7·00	7·79	8·66	9·54	7·84	11·88	13·52	13·90	15·72	16·37	14·28
10	3·19	4·35	3·71	3·25	8·28	4·56	10·31	11·12	12·00	11·83	14·04	11·86
Position Mean	6·13	6·85	7·50	7·86	9·36	7·538	17·40	20·34	21·18	23·36	24·41	21·34

Three methods of assessing the numbers of whole elements equivalent to the fragmentary ones appeared worthy of trial. These were based on :—

(1) measurement of the total length of the fragments and division by the mean whole element length of the sample;

(2) counting the numbers of fragmentary elements and multiplying by a factor equivalent to the ratio of the mean fragment length to the mean whole element length;

(3) counting the fragmentary elements as whole ones according to a predetermined convention.

Method (1). The mean lengths of the broken elements based on 50 measurements were found to be :—fibre fragments—227·5μ; vessel element fragments—184·5μ. Three slides were examined for each sample, the numbers of whole elements being counted, and the lengths of the broken elements in the same fields determined with the aid of a camera lucida. Results are incorporated in Table IV.

TABLE IV

Sample	*A. acuminata.* Aa1			*A. acuminata.* Aa2		
	Fibres	Vessels	Ratio	Fibres	Vessels	Ratio
Length of fragments μ	2158	579		7020	754	
Equivalent number whole ...	9·5	3·1	3·1	30·9	4·9	6·3
Counted number whole	142	23	6·2	189	30	6·3
Total as whole	151·5	26·1	5·8	219·9	34·9	6·3

Method (2). The three slides of each sample were re-examined and the numbers of whole and broken elements counted. The mean length of the vessel elements has already been recorded for the whole drug[3], viz. 252μ, hence the factor by which the number of fragments should be multiplied to estimate the equivalent number of whole elements is 184·5/252=0·732 which can be approximated to $\frac{3}{4}$. The mean length of the fibres (including fibre-tracheids as described above) was estimated as 450μ, hence the corresponding factor is 227·5/450=$\frac{1}{2}$, approximately. Results are incorporated in Table V.

Method (3). For this method, the following convention was adopted. Broken fibres were counted as whole if they tapered towards both ends, otherwise they were ignored. Broken vessel elements were counted as

TABLE V

Sample	*A. acuminata.* Aa1			*A. acuminata.* Aa2		
	Fibres	Vessels	Ratio	Fibres	Vessels	Ratio
Number of fragments	394	105		587	125	
Equivalent number whole ...	197	79	2·5	294	94	3·1
Counted number whole	240	39	6·1	399	68	5·9
Total as whole	437	118	3·7	693	162	4·3

whole if they exhibited portions of the perforation rim at both ends ; if visible only at one end, they were counted as half ; all other fragments were ignored. The number of such vessel elements associated with 300 such fibres was determined on the three slides of each sample examined previously. The fibre/vessel ratios were :—Sample Aa1, 6·25 ; Sample Aa2, 6·67.

The relative merits of the three methods were now considered before proceeding with the examination of the powdered drug. The standard by which they may be compared is the value of the fibre/vessel ratio obtained from counts of the whole elements only. This value is reasonably consistent for the first two methods, having regard to the relatively small numbers of elements on which it is based. The results for sample Aa2 by method (1) show that this method is capable of giving consistent results, but the process of measuring the broken elements is tedious and does not recommend itself in practice owing to the time involved. Method (2) does not suffer from the latter disadvantage, but the results do not suggest that an accurate estimate of the fibre/vessel ratio could be obtained in this way. The results obtained by method (3) are consistent with those obtained by counting whole elements only and the process is simple to apply in practice. Moreover, the number of elements that can be observed in a given time is much larger than that possible by method (1), thus reducing variation due to random effects. Having regard to the above considerations, it was decided to adopt method (3) for the subsequent work.

Variation with the fineness of the portion examined. Powdering causes some destruction of individual cells and it is necessary to determine whether the action is selective on either the fibres or the vessel elements. Powder samples Aa1 and AB3 were therefore passed through a series of standard sieves and the fibre/vessel ratio determined on the portions retained by each sieve.

TABLE VI

VARIATION OF THE FIBRE/VESSEL RATIO IN PORTIONS OF THE POWDER OF VARYING FINENESS

Sieve Number		10	22	25	30	36	44	60	85
Sample	AB.3	—	5·5	5·3	4·0	3·9	4·0	4·2	3·9
Number	Aa.1	8·5	7·3	7·1	7·1	6·4	5·5	5·3	4·8

From these results it is seen that the fibre/vessel ratio decreases with increasing fineness of powder and that the decrease is not large over the 25/44 range. Portions No. 60 and 85 contained a high proportion of broken elements and counting was correspondingly difficult. In view of this and also of that fact that the No. 22/44 portion had been employed previously[1] for vessel index determination, it was decided to employ the latter portion for subsequent work on powders.

Details of the method. About 1·0 g. of the 22/44 portion of the commercial powder was boiled gently with 30 ml. of Schultz's maceration fluid for about 10 minutes or until particles ceased to float on the surface, sufficient potassium chlorate being added meanwhile to maintain a steady evolution of chlorine. The macerated material was then collected on a sintered glass filter, washed with water, transferred to a small test-tube with about 2ml. of water and disintegrated by vigorous shaking. The resulting suspension was then diluted with 3 or 4 volumes of suspending fluid containing a trace of thymol as preservative. Slides were prepared by further dilution such that each scan contained about 20 fibres and the fibre/vessel ratio calculated from observation of the number of vessel elements associated with 300 fibres. In counting, the convention for including broken elements in the count as described above (method 3) was adopted. Counting was done using a 1/6 in. objective and a ×6 eyepiece, and the whole slide covered systematically with the aid of a mechanical stage. Elements intersected by the field of view, but lying more than half within it, were included in the count, otherwise they were ignored.

Summary of Results

The values of the fibre/vessel ratio for commercial powders are given in Table VII. The ranges are :—

A. Belladonna, 2.30 to 4.55, mean 3.91.
A. acuminata, 6.54 to 12.05, mean 8.66.

These are distinct and the fibre/vessel ratio is thus a good differential character for the powdered drugs. The values for samples Aa1 and Aa2 are slightly higher than those obtained in the preliminary work, which may be accounted for by the fact that they relate to the 22/44 portions, whereas the preliminary work was done on the unsieved powders.

TABLE VII

Fibre/vessel ratio for commercial powders—22/44 portion

A. Belladonna		*A. acuminata*	
Code Number	Fibre/vessel ratio	Code Number	Fibre/vessel ratio
AB.1	4·07	Aa.1	6·54
AB.2	4·29	Aa.2	7·0
AB.3	3·91	Aa.3	8·85
AB.4	4·55	*Aa.4	12·05
AB.5	2·35	*Aa.5	8·85
AB.6	2·30		
Mean	3·91	Mean	8·66

* 44/60 portion.

Discussion of Results

The analysis of variance, Table VIII, for the fibre/vessel ratio determinations on the whole drug shows that the difference between the species is not very significant and hence the differential value of the character when applied to the whole drug is limited. The variance within the species *A. Belladonna* is contributed to almost equally by positional variation within the roots and by differences between them, while in *A. acuminata* root differences are more important. The increase in positional values from the base towards the apex of the roots is fairly uniform and the values at the mid-points approximate to the root means. The critical value for classifying whole roots is 4·27, i.e. half the sum of the specific means, and the expected proportion misclassified on the basis of a value determined at one position taken at random, as calculated from knowledge of the normal deviate, is 32 per cent. The ranges for the root means are *A. Belladonna* 1·44 to 4·8 ; *A. acuminata*, 3·03 to 12·01, but little improvement in classification results from their use. However, it may be considered that values less than about 2·0 indicate

TABLE VIII

ANALYSIS OF VARIANCE IN TABLES I AND II

Character	Species	Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio	Variance Ratio P=0·01
Fibre/vessel ratio Table I	*A. Belladonna*	Roots	9	46·97	5·22	17·7	3·1
		Positions	4	18·65	4·66	15·9	3·9
		Error	36	10·59	0·29		
	A. acuminata	Roots	9	486·34	54·04	31·4	3·1
		Positions	4	52·87	13·22	7·7	3·9
		Error	36	61·91	1·72		
		Species	1	150·82	150·82	21·8	7·0
		Error	98	677·33	6·91		
Vessel Index Table II	*A. Belladonna*	Roots	9	368·51	40·95	63·8	3·1
		Positions	4	9·38	2·35	3·7	3·9
		Error	36	23·10	0·64		
	A. acuminata	Roots	9	2126·68	236·30	99·5	3·1
		Positions	4	92·08	23·02	9·7	3·9
		Error	36	85·52	2·38		
		Species	1	3115·10	3115·10	117·1	7·0
		Error	98	2705·27	27·60		

A. Belladonna and greater than about 8·0 *A. acuminata*, intermediate values being inconclusive. In this connection, however, the fact must also be taken into account that the specimens of the whole drug were selected on a basis of diameter and development of xylem and were not taken at random. Reference to the results for commercial powders shows that the mean value of 8·66 for the *A. acuminata* material is considerably higher than that for the whole drug 5·49, indicating that specimens with high fibre/vessel ratios form the greater part of the drug from this species. Thus the misclassification rate of 32 per cent. may be unduly high when applied to specimens taken at random from the commercial drug.

The values of the vessel index for the whole drug also increase from the base towards the apex of the root, but the analysis of variance, Table VIII, shows this effect to be unimportant compared with that due to differences between the specimens. The difference between the species is highly significant, so that the vessel index is valuable for differentiating the whole drug. On the basis of a single determination, the expected misclassification rate is about 15 per cent., the critical value being 9·86 and if the root means are used this proportion is reduced to about 10 per cent. Inspection of the results in Table II does not suggest any close connection between the vessel index and the age of the specimen in *A. Belladonna* although in *A. acuminata* the values for the younger roots numbers 8, 9 and 10 are considerably lower than the mean of the other seven. The ranges for the root means, *A. Belladonna* 0·79 to 8·54, *A. acuminata* 6·99 to 25·38, are not inconsistent with those for another selection of specimens reported on in Part III of this work, namely. *A. Belladonna* 0·3 to 16·02, mean 6·9 : *A. acuminata* 5·0 to 43·58, mean 19·87. Thus values of less than about 5 may be taken as indicating *A. Belladonna* and greater than 16, *A. acuminata*.

Comparison of the mean values of the vessel index and the fibre/vessel ratio for specimens of the whole drug suggests there is little correlation between them. This is borne out by the low correlation coefficients calculated from the data in Tables I and II, which are :—*A. Belladonna* 0·6, *A. acuminata* −0·15. The two characters may thus be considered as varying independently of one another. Their combination in a linear function yields a discriminant with a critical value for classifying purposes of 14·44 and an expected misclassification rate of 12 per cent. This is a considerable improvement on the figure of 32 per cent. for the fibre/vessel ratio, but is very little superior to that of 15 per cent. when the vessel index is used alone. For this reason, use of the discriminant for other than borderline cases would not be justified.

The variation of the fibre/vessel ratio in the whole drug is not reproduced in the commercial powder owing to the thorough mixing of the elements on which it is based. Thus the values for commercial powders vary within narrow limits and, since the ranges for the two species are distinct, the fibre/vessel ratio provides a valuable character for their differentiation.

SUMMARY AND CONCLUSIONS

1. The term "fibre/vessel ratio" is adopted to signify the number of fibres (including fibre-tracheids exhibiting elongated simple pits) associated with one vessel element.

2. Consistent results are obtained when the fibre/vessel ratio is calculated from the number of vessel elements associated with not less than 200 fibres.

3. The variation in the whole drug of the fibre/vessel ratio and of the vessel index 135μ is investigated and their value as differential characters assessed.

4. Neither character is completely successful in differentiating the whole drug, but some improvement results by combining them in a linear function.

5. A method of determining the fibre/vessel ratio on the powdered drug is described.

6. The fibre/vessel ratio of commercial belladonna root powders decreases with the fineness of the portion examined, but is reasonably constant for the portion retained between a No. 22 and No. 44 sieve.

7. Fibre/vessel ratio values for commercial powders provide a means of differentiating the species from which they were prepared.

8. The ranges for the commercial powders examined are :—*A. Belladonna* 2·30 to 4·55 ; *A. acuminata* 6·54 to 12·05.

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