ANTIBIOTIC PRINCIPLE FROM *MORINGAE PTERYGOSPERMA*

Part I. Purification

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SUMMARY

1. Various methods of extraction of the antibiotic principle from the roots of *Moringa pterygosperma* have been investigated. Chromatographic method for its purification, studies on the adsorption and elution of the antibiotic on acid washed activated charcoal preparations are reported.

2. The remarkable occurrence of free sulphur as an impurity in crude Pterygospermin preparations is reported.

3. Two fractions showing a potency of 1 in 600,000 when assayed against *Micrococcus pyogenes* var. *aureus* have been obtained by careful fractionation in high vacuum.

The introduction of specific chemical compounds of vegetable, microbial or synthetic origin for systemic treatment of infectious diseases is relatively recent, but since the discovery of the striking therapeutic properties of such drugs as Salvarsan and more recently of sulphonamides and penicillin, greater effort is directed to the study of synthetic compounds and microbial metabolites for possible anti-bacterial drugs than those of the higher plants, which in fact constituted the main therapeutic material in earlier medicine. However, the possibility of naturally occurring compounds in higher plants showing pronounced antibacterial activity has been amply demonstrated by many workers in this field.[1] That many of the common Indian medicinal plants would constitute a fertile scurce of such substances has been shown in some of the earlier contributions from these Laboratories.[2] While it is planned to explore these resources in greater detail, it appeared expedient to undertake the purification and characterisation of the active principle in the crude preparation designated previously as "Pterygospermin,"[3] a reddish brown oil, isolated from the roots of the common Indian Drumstick tree (*Moringæ pterygosperma*), in view of its low toxicity even in an impure state, and many other desirable properties. In the present communication some of the experiments carried out on the purification of the active principle are described.

EXPERIMENTAL

Materials and Method.—The roots used in our experiments were collected in all seasons round about Bangalore.

The moisture content of the roots was determined by the method of azeotropic distillation according to A.O.A.C., using toluene.

The activity was assayed by the serial dilution method, using nutrient broth at pH 6·8 containing 1% phosphate buffer. A standard strain of *Micrococcus pyogenes* var. *aureus* was used throughout these experiments.

The *crude pterygospermin* was prepared as previously described[3] by the cold extraction of the roots with absolute alcohol, adsorption of the active principle on charcoal followed by elution with petroleum ether.

RESULTS

The Variation of Antibiotic Potency with the Age of the Plant and Season of Collection of Roots.—The results obtained on the variation of the antibiotic potency with the age and season are illustrated in Table I. In general, it is found that younger plants give cleaner products of higher antibiotic potency. Roots gathered in winter months (November-February) gave slightly more active preparations than those of other seasons.

TABLE I

Variation of Activity and Yield with Season of Collection and Age of the Plant

Age of the plant years	Season of collection	Wt. of roots taken lb.	Moisture content average %	Wt. of crude Pterygo-spermin mg.	Activity*	Colour of the product
3	June—July	12	84	1·170	1/200,000	Pale yellow
4—5	Nov.—Jan.	15	do	1·50	1/210,000	do
10–15	do	9	do	0·850	1/180,000	Reddish brown
do	June—July	12·5	do	1·120	1/160,000	do

* Minimum inhibitary concentration.

Effect of Drying on the Antibiotic Potency.—Due to high moisture content of the roots and consequent dilution of the alcohol used for extraction, experiments were carried out to ascertain the effect of drying the roots on the antibiotic activity. Continuous blowing of warm air at 37° in a chamber containing chopped roots, resulted in gradual disappearance of antibiotic potency. Drying in an evacuated vessel over anhydrous calcium

chloride for two or three days at room temperature also gave a product with low activity.

Direct Extraction of the Active Principle with Fat Solvents:—

Fresh chopped roots were extracted with benzene, the entrained water being continuously removed and the solvent returned to the extractor. After all the moisture had been removed, the solvent was separated, the roots were extracted a second time and from the combined extract, a pale yellow semi-solid (56·5 mg.) was obtained by evaporation of the solvent in vacuum. This product assayed to 1 in 80,000. Extraction with toluene, accomplished in a shorter time gave also a similar product but slightly less active. Other solvents like petroleum ether, chloroform, etc., were not satisfactory.

The total recovery of the product of comparable activity to that of crude "Pterygospermin" assaying 1/200,000 is therefore 0·240 mg. per lb. of the fresh roots which is in fact nearly $2\frac{1}{2}$ times the yield obtainable by the older process. However, it was found expedient to adopt the alcohol extraction method using the counter-current extraction principle for the preparation of crude pterygospermin. This preparation has the following characteristics:—

(*a*) Activity in 1/200,000; (*b*) N = 4·86% and S = 10·15%; (*c*) Its ultraviolet absorption in the region 2,800–2,200; and (*d*) D_4^{25}, 1·0103; n_D^{25}, 1·5283.

This preparation constituted the starting material for further purification described below.

Purification Experiments:—

(*a*) *Separation of Elemental Sulphur.*—It was observed that pale yellow needles separate when crude pterygospermin preparations were kept *in vacuo* over phosphorous pentoxide in refrigerator for a week or ten days. Sufficient quantity of the material was collected from several lots by centrifugation and washing with cold petrol. This substance was found to melt at 114° C. and was sparingly soluble in the usual organic solvents but dissolved readily in carbon disulphide. This was identified as pure sulphur, by analysis, mixed melting point, solubility tests and crystallographic data. This remarkable occurrence of free sulphur could possibly be explained as a result of oxidation of hydrogen sulphide found in considerable quantities in the original alcoholic extract of the roots. Variation in the quantities of sulphur deposited was encountered (from about a few mg. to about 100 mg. from 1·2 gm. of crude pterygospermin). The removal of sulphur showed slight

but distinct increase in the antibiotic titre of the preparation. Thus there appears to be no reason to suppose that this deposition of sulphur was due to chemical alteration of the active principle. Sulphur was without any effect on the antibiotic activity of product in high dilutions.

(*b*) *Adsorption Chromatography.*—Magnesia, acid washed alumina, sugar, tricalcium phosphate and silica gel were the absorbents used. The experiments were carried out in pyrex glass tubes with a column of 20 cm. × 1·2 cm. of the adsorbent. The crude pterygospermin dissolved in purified dry petroleum ether (b.p. 30–60°) was run down the column and the filtrate was collected into number of fractions and assayed for antibiotic activity. Development of the chromatogram and elution were carried out using petroleum ether-acetone mixture and pure acetone. Under these conditions no adsorption of the activity was observed with sugar. Table II shows the adsorption characteristics of crude pterygospermin with tricalcium phosphate and acid washed alumina.

TABLE II

Adsorbent	Substance taken	Solvent petrol	Unabsorbed fraction		Adsorbed fraction	
			Weight	Activity	Weight	Activity
1 Tricalcium phosphate	240 mg.	25 c.c.	60 mg.	1/300,000	(*a*) 100 mg. (*b*) 65 mg.	1/180,000 do
2 Acid washed alumina	429·4 mg.	42 c.c.	212·8 mg.	1/280,000	(*a*) 109 mg. (*b*) 71·4 mg.	1/290,000 1/40,000

By rechromatography of the highly active fractions no great increase in the activity was observed. The active principle was only very slightly adsorbed on silica gel and partial destruction of activity was observed when ordinary alumina and magnesia were used.

(*c*) *Adsorption on Charcoal.*—The adsorption of active principle was tried using various commercial preparations of activated charcoal as well as some prepared in these laboratories from paddy husk. However, most of the commercial varieties gave highly alkaline reaction (9·8–10·0) at which the active principle is unstable. Acid washed charcoal, prepared by repeatedly treating with 5% acetic acid followed by washing with distilled water until free from acid and then with absolute alcohol was used after drying at 120°. 30 c.c. of 1% alcoholic solution of crude pterygospermin was shaken at room temperature (26° C.) with 3 gm. of the activated acid washed, granular charcoal (found most suitable after a number of trials) and 3 c.c. portions removed at stated intervals and the activity was assayed (*vide* Table III and Graph I).

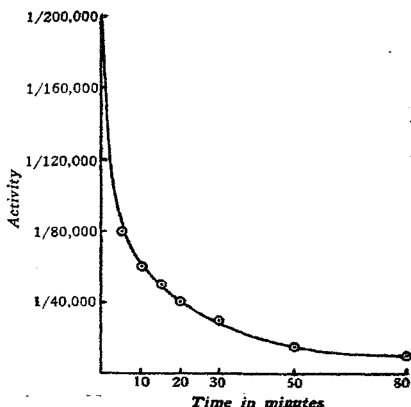

FIG. 1. Adsorption on acid washed activated charcoal (*Vide Table III*)

TABLE III

Adsorption of Activity on Acid Washed Active Charcoal with Time

Time in minutes	Activity in dilution 1 in						
	10,000	20,000	40,000	60,000	80,000	100,000	180,000
0	–	–	–	–	–	–	–
5	–	–	–	–	–	+++	+++
10	–	–	–	–	++	+++	+++
15	–	–	–	±	+++	+++	+++
20	–	–	±	+++	+++	+++	+++
30	–	–	+	+++	+++	+++	+++
50	–	±	++	+++	+++	+++	+++
80	–	±	+++	+++	+++	+++	+++

– clear; +++ normal growth; ++ slight growth; + just visible growth; ± doubtful growth.

The amount of activity that was adsorbed on charcoal after making appropriate correction for the activity removed for assay is given in Table IV and Graph II which is a straight line illustrates the course of the adsorption.

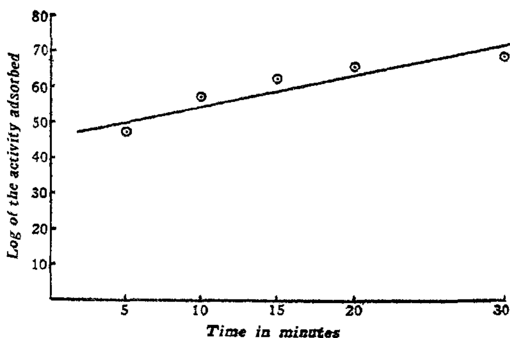

FIG. 2. (*Vide Table IV*)

It appears therefore most likely that the product contains only one active component, a view which receives confirmation from a study of the results of the single dimension paper partition chromatography of the substance. The results of the latter experiments will be published shortly.

TABLE IV

Adsorption of the Antibiotic

Time	Antibiotic adsorbed in dilution units $\times 10^4$ (U)	Log U
0	..	..
5	300	2·4771
10	378	2·5778
15	419·8	2·6222
20	455·6	2·6580
30	487·3	2·6875
50	509	2·7067

(*d*) *Elution of the Active Principle from Charcoal Adsorbates.*—Even though nearly 50% of the activity was adsorbed within 5 min. of shaking, it was not found possible to obtain a homogeneous product by elution with different solvents. A typical experiment carried out for eluting the active principle is given below. 40 c.c. of 1% alcoholic solution of crude pterygospermin was shaken with 4 gm. of acid washed charcoal as described above for 80 min. at room temperature (26° C.). The charcoal was filtered, washed twice with 1 c.c. of alcohol. After drying in a desiccator it was shaken with 40 c.c. of petroleum ether (30–40° C.) (a solvent which proved quite efficient for the extraction of crude pterygosperin when norit was used for adsorption from plant extracts) and 3 c.c. portions removed at stated intervals. The

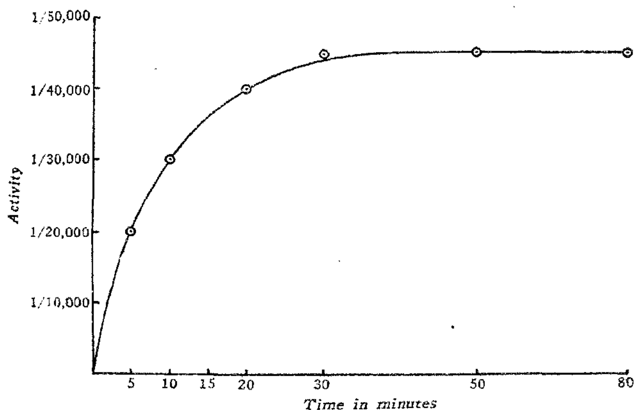

FIG. 3. Elution with Petrol Ether (30–60°)
(*Vide Table V*)

results are tabulated below (Table V). It has not been found to elute the active material in a state of enhanced purity by methods till now studied by us.

(*e*) *Distillation of Crude Pterygospermin.*—The behaviour of crude pterygospermin on fractionation in the high vacuum is shown in Table VI. Though much of the material remained undistilled, as a resinous mass two fractions (4 and 5) gave high antibiotic titre. No apparent sign of decomposition was observed during distillation although prolonged fractionation

TABLE V

Elution of Antibiotic Substance from the Charcoal Adsorbate

Time in minutes	Activity in dilutions					
	1/10,000	1/20,000	1/40,000	1/60,000	1/80,000	1/100,000
5	–	–	±	+++	+++	+++
10	–	–	±	+++	+++	+++
15	–	–	±	+++	+++	+++
20	–	–	–	±	+++	+++
30	–	–	–	±	+++	+++
50	–	–	–	±	+++	+++
80	–	–	–	±	+++	+++

TABLE VI

Fractionation of Crude Pterygospermin at 10^{-3} *mm.*

3·8 gm. of Crude oil (35 lb. of roots) — fractionated at 10^{-3} mm.

Fraction minutes	*Temp. of bath in t °C.	Yield in mg.	Microanalysis†				Activity against	
			C	H	N%	S	*S. aureus*	*E. coli*
1	36–40	98·6					Inactive	Inactive
2	60–4	47·0					do	do
3	94–5	58·4					do	do
4	125–8	75·8	69·85	7·03	6·40 6·44	12·61	1 in 2×10^5	1 in 2×10^5
5	140–1	212·2	65·00	5·29	7·17 6·94	20·46	1 in 4×10^5 to 5×10^5	1 in 4×10^5 to 5×10^5
6	Residue	3·0 g.					Inactive	Inactive

* Fractions 1 to 5 are colourless mobile liquids.

† By Dr. Weler, Oxford.

greatly lowered the activity of the fractions. These two fractions were colourless oils of characteristic odour, easily soluble in all organic solvents but only sparingly soluble in water.

They were optically inactive and showed no fluorescence in ultra-violet light. They did not give any reactions of thiol. Detailed analysis indicated that they are still heterogeneous.

However, as will be presented in a forthcoming publication, a homogeneous product (distilling at 45° C., bath temp. at 10^{-4} mm.) could be obtained by molecular distillation after the removal of sterols by digitonin, which has been identified as benzyl iso-thiocyanate. The evidence that this product arises as a result of decomposition of the antibiotic will also be discussed later.

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