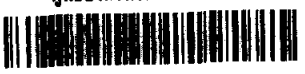

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COOPERATIVE RESEARCH PROGRAMME NO. 17

PHARMACEUTICALS

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PHARMACEUTICALS FROM *LORANTHUS PENTANDRUS* L. (KAFAK-MAMUANG)

REPORT NO. 2

EXTRACTION AND FRACTIONATION OF THE ACTIVE PRINCIPLE (S) OF *LORANTHUS PENTANDRUS* L. (KAFAK-MAMUANG) II

BY

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F O R E W O R D

This is the second report of Research Project 17/4, Pharmaceuticals from Loranthus pentandrus L. (kafak-mamuang), of Cooperative Research Programme No. 17, Pharmaceuticals. This programme, like other Cooperative Research Programmes, is a joint effort undertaken by various Thai government agencies with the Applied Scientific Research Corporation of Thailand. In this particular Research Project ASRCT has had very close cooperation with the Faculty of Pharmacy, Mahidol University. All the pharmacological work in this report was performed there.

One of the aims of this project is to isolate and identify the active principle(s) of Loranthus pentandrus. This report covers part of task 3 (4), Extraction and fractionation of the active principle. The information in this report represents only a part of the task assigned in the project outline, because it is an interim report summarizing work to date. The work has been suspended without conclusion due to the leaving for further study of Miss Sunanta Jindaprasarn and the departure of UN Associate Expert, Dr. Lars Johansson.

EXTRACTION AND FRACTIONATION OF THE ACTIVE PRINCIPLE(S) OF LORANTHUS PENTANDRUS L. (KAFAK-MAMUANG)

By Sunanta Jindaprasarn*, Lars Johansson*, and Nitasna Pichitakul*

SUMMARY

Several methods for the extraction of the leaves of L. pentandrus and for the fractionation of its extracts have been tried. It was found that boiling water is the best extraction medium and, of the methods for fractionation of the extracts so far tried, the method involving dialysis is the most promising. The active material is of a very hydrophilic nature and is hard to dissolve in other media than water. Its molecular weight is less than 6,000 and it is not a protein, peptide or amino acid, and probably not any other compound having a free NH_2 group.

INTRODUCTION

Previous works on the hypotensive activities of Loranthus pentandrus L. (kafak-mamuang) and of Viscum album L. which belongs to the same family, LORANTHACEAE, have been reviewed in an earlier report+ on this project. It was found that the hypotensive activity was most pronounced in the basic fraction from the ion-exchanger column. The aqueous extracts from leaves had a more pronounced hypotensive effect than those from the stems and this effect was not reduced by chloroform extraction. Work in this report was continued from the previous one. Several methods of extraction and fractionation were used.

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+ "Extraction and fractionation of the active principle(s) of Loranthus pentandrus L. (kafak-mamuang)" by Lars Johansson and Nitasna Pichitakul. Report No. 1 on Research Project 17/4. ASRCT unpublished report.

MATERIALS AND METHODS

(1) The leaves from the parasite Loranthus pentandrus, growing on the mango tree, Mangifera indica, were collected from Chon Buri in October 1967, dried, and minced.

(2) Tubing for dialysis, diameter 28 mm (The Scientific Instrument Centre, Ltd., London).

(3) Sephadex G-10, particle size 40-120 μ; Sephadex G-25, particle size 20-80 μ; Sephadex G-50, particle size 20-80 μ; SE-Sephadex C-25 cation exchanger, particle size 40-120 μ; DEAE-Sephadex A-25 anion exchanger, particle size 40-120 μ (Pharmacia, Uppsala, Sweden).

(4) Bio-Rex 40, cation exchanger, particle size 20-50 mesh, hydrogen form (Bio-Rad, California, U.S.A.).

(5) Neutral aluminium oxide "für die Chromatographi" (Merck AG, Darmstadt, Germany).

(6) All other chemical reagents used, such as ethanol, chloroform, dimethylsulfoxide etc., were of quality "puriss" or equivalent.

The methods used for extraction and fractionation are described in detail in EXPERIMENTAL AND RESULTS section.

EXPERIMENTAL AND RESULTS

(a) Extraction

Aqueous extract (A)

Five hundred grammes of dried and minced leaves of L. pentandrus were added to 5 litres of boiling water and allowed to boil for 5 min during continuous stirring. The extract was allowed to cool and was then filtered through cotton cloth and centrifuged. The precipitate was rejected and the centrifugate evaporated to a volume of 500 ml corresponding to a concentration of 1 g dry leaves/ml solution.

This solution is here called A_1 and its pharmacological activity is listed in Table 1 together with the results from other activity tests. (Record Book No. 118/2, p. 60.)

Dimethylsulfoxide extract (B)

Twenty grammes of dried and minced leaves of L. pentandrus were added to 200 ml of dimethylsulfoxide at room temperature and stirred for one hour. The undissolved material was filtered off and the dimethylsulfoxide solution evaporated to dryness in vacuo. The so obtained syrupy residue was dissolved in 20 ml of water and called solution B_1. (Record Book No. 118/2, p. 56.)

Ethanolic extract (C)

Twenty grammes of dried and minced leaves of L. pentandrus were added to 200 ml of 25 per cent ethanol at room temperature and stirred for one hour. The extract was filtered and the filtrate evaporated to dryness in vacuo. 100 ml water and 100 ml chloroform were added to the residue from the evaporation and the mixture shaken until equilibrium was supposed to have been reached between the two layers and the soluble substances. After centrifuging the two layers were separated and the water layer filtered. The volume was made to 20 ml by evaporation in vacuo and the activity of this solution, labelled C_1, was tested. The chloroform layer was rejected. (Record Book No. 118/2, p. 58.)

Tannin elimination (D)

Lead acetate method. The tannins were precipitated with a lead acetate solution and the lead tannate filtered off. To get rid of excess Pb^{++} hydrogen sulphide was bubbled through the solution, which afterwards was acidified and heated on water-bath for ten minutes to eliminate dissolved H_2S. The solution so obtained was filtered, neutralized, and made up to a volume suitable for the pharmacological tests. The filtrate was called D_1. (Record Book No. 118/2, p. 33.)

Cold storage precipitation method. The crude extract was evaporated to a small volume (corresponding to a concentration of about 5 g dry leaves/ml) and stored in refrigerator (+ 5°C) for two days. The precipitate formed was separated from the solution by centrifuging and the remaining clear extract diluted to a concentration corresponding to 1 g/ml and called D_2. (Record Book No. 136/4, p. 14, 16, 28.)

Removal of chloroform-soluble substances (E)

The crude extract, acidified with dilute hydrochloric acid, was shaken with an equal volume of chloroform. The layers were separated and the upper layer filtered and neutralized with sodium hydroxide. This solution was called E_1 after concentration adjustment. (Record Book No. 118/2, P. 36.)

Removal of ethyl acetate-soluble substances (F)

The crude extract was shaken with the same volume of ethyl acetate. The layers were separated and the water layer evaporated almost to dryness (vacuum) and made up with water to the suitable test concentration (1 g/ml). This solution was called F_1. (Record Book No. 118/2, p. 59.)

Removal of ether-soluble substances (Q)

A procedure similar to the one described under F was used. The remaining solution, after filtering and concentration adjustment was called Q_1 (Record Book No. 118/2, p. 56.)

Removal of proteins (G)

The proteins and peptides of the crude extract were precipitated by adding ammonium sulphate, kept in solid form in a cellophane bag which was dipped in the solution, until no more precipitate was formed. After centrifuging the extract, the precipitate was separated and redissolved in water to give solution G_1.

Absolute ethanol was added to the in vacuo concentrated (to about 3 g/ml) centrifugate to precipitate most of the amonium sulphate. The final solution was about 50 per cent ethanol : water. After the sulphate had been filtered off the ethanol was evaporated and water added. This solution was called G_2 and its concentration, as well as that of solution G_1, was adjusted to 1 g/ml. (Record Book No. 118/2, p. 49.)

Dialysis (H)

The crude extract, free from tannin, was poured into cellophane tubing and dialyzed against distilled water. The solution left inside the tubing was filtered and evaporated in vacuo to a suitable concentra-

tion (1 g/ml). This solution was called H_1 and the water solution outside the tubing, after evaporation to the same concentration, was designated H_2. (Record Book No. 136/4, p. 20, 24, 28.)

(b) Fractionation

Neutral aluminium oxide column (I)

The crude extract (10 ml) was loaded on a neutral aluminium oxide column (25 x 500 mm) and eluted with water (500 ml) and 0.5 M acetic acid (400 ml). Two fractions were obtained, the first one neutral, the second acid. Both fractions were evaporated *in vacuo* to obtain a proper concentration (1 g/ml), the acid fraction being neutralized (NaOH) before the volume adjustment. These fractions, called I_1 (water eluted) and I_2 (acid eluted), had to be filtered before the pharmacological tests as some precipitate developed during storage.

In a later experiment the procedure described above was repeated but, to avoid a high concentration of sodium acetate in fraction I_2, this was shaken several times with ether before its neutralization. A small amount of hydrochloric acid had been added to suppress the protonisation of the acetic acid. After neutralization, concentration adjustment and filtering, this fraction was called I_3. (Record Book No. 118/2, p. 34.)

Bio-Rex 40 cation exchanger column (K)

A column (25 x 500 mm) was packed with the resin in hydrogen form and washed with water until neutral reaction of the eluent. 20 ml of extract was loaded on the top of this column and 500 ml water used as the first eluent. The neutral 500 ml water solution was concentrated to 20 ml *in vacuo* and called fraction K_1. A second fraction was obtained by eluting with 300 ml, hydrochloric acid (1 M). This fraction was neutralized with sodium hydroxide before its evaporation to a small volume (about 10 ml). The solid sodium chloride which formed on standing was filtered off, and the remaining filtrate was made up to 20 ml and called fraction K_2. (Record Book No. 118/2, p. 34.)

SE-Sephadex C-25 cation exchanger column (L)

A column (25 x 500 mm) was packed with Sephadex C-25 and loaded as in K. Two fractions were collected: the first eluted with water and the second eluted with 0.5 M sodium chloride. After evaporation in vacuo to a suitable volume (conc. = 1 g/ml) the fractions were filtered and called fraction L_1 and L_2 respectively. (Record Book No. 118/2, p. 38.)

DEAE-Sephadex A-25 anion exchanger column (M)

A column and procedure similar to those described above were used. Water and 0.5 M sodium chloride were used as eluents. After volume adjustment and filtering the collected fractions were called M_1 and M_2 (water and sodium chloride eluted) respectively. (Record Book No. 118/2, p. 40.)

Gelfiltering using columns prepared from Sephadex G-10, Sephadex G-25, and Sephadex G-50 (N,O,P, respectively)

The columns and procedures were comparable to those described above and the eluents used were first water (about 500 ml) and then 1 M acetic acid (about 300 ml). The water-eluted fractions were after evaporation to a concentration corresponding to 1 g dry leaves/ml called N_1, O_1 and P_1. To the fractions eluted with acetic acid, a small amount of hydrochloric acid (about 0.5 ml 2M) was added and by shaking several times with ether most of the acetic acid was eliminated. After neutralization with sodium hydroxide the fractions were evaporated (vacuum) to a small volume (about 5 ml) and filtered. The precipitate, mainly consisting of sodium acetate and sodium chloride, was rejected and the filtrate made up to a volume corresponding to a concentration of 1 g dry leaves/ml. These fractions were called N_2, O_2, and P_2. (Record Book No. 136/4, p. 6, 17, 20, 24.)

(c) The ninhydrin test

The test for free NH_2 groups (ninhydrin test) was performed according to the method described by Fieser (1964). A modification of this method was introduced by us by adding an equal volume of n-butanol to the cooled solution after completed reaction. When the two-layer system so obtained is shaken until equilibrium any blue colour formed

will appear almost exclusively in the upper (n-butanol) layer. The reason for this change in the original procedure is that our extracts and fractions often are discoloured and the colour formed by the ninhydrin reaction is difficult to evaluate in such a solution. When the test was performed on the crude extract it was sometimes necessary to extract this solution with butanol before the reaction to get rid of the butanol-soluble coloured substances. This butanol layer was rejected after it had separately been checked for the presence of NH_2 groups by the original Fieser procedure performed in butanol.

(d) Pharmacological tests

The pharmacological tests were performed at the Faculty of Pharmacy, Mahidol University, on dogs under barbiturate anaesthesia. Blood pressure, heart rate, and the contraction force of the heart were kymographically recorded. The fractions were injected in the femoral vein together with a small amount of a saline solution. As a rule the fractions were diluted ten times before injection and doses between one and five ml of each fraction were given. The detailed procedure is described in "Class Experiments: Pharmacology" by the Faculty of Medicine; Chulalongkorn Hospital, page 87.

TABLE 1

HYPOTENSIVE EFFECT IN DOGS OF 5 ML OF A TENFOLD DILUTION OF EACH FRACTION INJECTED INTRAVENOUSLY

Sample[1]	Activity[2]	Remarks
A_1	+ +	Of 17 batches 6 showed + + +; 9, + +; and 2, +
A_1 E_1	+ +	
A_1 F_1	+ +	
A_1 Q_1	+ +	
A_1 G_1	+	This slight activity derives probably from endosed A_1 G_2

Table 1 - continued

Sample[1]	Activity[2]	Remarks
A_1 G_2	+ +	
A_1 D_1	+ +	
A_1 D_2	+ +	
A_1 E_1 G_2	+ +	Some protein was precipitated already by procedure E_1
B_1	+	
C_1	+	
D_1 I_1	+	
A_1 I_2	+ + +	This activity is probably due to high concentration of sodium acetate
A_1 I_3	+	
A_1 I_1 K_1	0	
A_1 I_1 K_2	0	
A_1 K_1	+	
A_1 K_2	+	
A_1 L_1	+	
A_1 L_2	0	
A_1 M_1	+	
A_1 M_2	+	
A_1 N_1	+	
A_1 N_2	+ +	This activity is probably due to the presence of sodium acetate
A_1 H_1	0	
A_1 H_2	+ +	Of 6 batches 2 showed + + +; 2, + +; and 2, +
A_1 H_2 O_1	+	
A_1 H_2 O_2	+	
A_1 H_2 P_1	+	
A_1 H_2 P_2	+	

Table 1 - continued

Sample[1]	Activity[2]	Remarks
Sodium acetate, 1% solution	+	Some fractions contained more than 1% sodium acetate
Synthetic γ-amino butyric acid	+ + +	Found in Viscum album (Loranthaceae) (Samuelsson 1959)

1/ The sample codings should be read from left to right and understood as follows:

The first code letter gives the starting material and the following letters indicate its subsequent working up according to the methods described under the heading of the same letter in the text above.

2/ The following activity scale was used:

Three crosses (+++) stands for a high activity (a blood pressure drop of > 36 mm Hg), two crosses means a moderate activity (a pressure drop of > 18 but < 36), one cross means a low activity (a pressure drop of > 0 but < 18), and a zero (0) means no decrease or increase in blood pressure.

DISCUSSION AND CONCLUSIONS

It is difficult to interpret the results of this series of pharmacological tests because of individual variations in the test animals. For example, in one experiment when the same samples were tested simultaneously under identical conditions on two dogs, the activity results were different. It was sometimes found that the test dog did not give a positive response to the reference substances, γ-aminobutyric acid and sodium acetate. In such cases the test results were rejected.

Under such conditions it is common to work with statistical group of test animals, but for practical and economical reasons this has not been possible in this investigation. Usually our batches were tested only on one dog each, but several individually prepared batches were tested at different times. Considerable variation between batches was seen

in the apparent activity of the preparations, as noted in the remarks column of Table 1, e.g. for preparation A_1. We believe that this spread of results is more due to the above-noted variability of the test animals than to differences between batches. We could not correct the test results by the individual response to reference drugs, probably because the reference substances differ from the active substances in the extracts. The validity of the noted activity in the various fractions (second column of Table 1) depends mainly on repetition of testing, being usually the mean activity of six or more batches.

The activities obtained by extracting with different solvents show clearly that water is the most suitable solvent. Solvents like chloroform, ether, and ethyl acetate do not dissolve or destroy the active principle(s) and can therefore be used for purification of the extract. Lead acetate solution precipitates tannins and other acid compounds without affecting the activity of the extract. Care was, of course, taken that no Pb^{++} was left in the extract when it was pharmacologically tested. It was expected that some proteins and peptides should be precipitated together with the acid components when adding Pb^{++}, but in a separate experiment the proteins were salted out more completely by adding ammonium sulphate to the extract. It was then found that a slight activity appeared in the formed, redissolved precipitate (sample G_1), an activity which we suppose mainly derives from filtrate, which could not be completely washed out from the precipitate. A result supporting this idea was found in the fact that the remaining filtrate, after the ammonium sulphate had been precipitated, showed almost the same activity as the starting material.

When the crude but filtered water extract was subjected to dialysis against distilled water the pharmacological activity was found exclusively outside the cellophane bag, i.e. the active compound(s) pass through a cellophane membrane and its (their) molecular weight consequently does not exceed 6,000. Proteins are considered to have a molecular weight exceeding 10,000 and could so be excluded as responsible for the pharmacological activity.

When performing the ninhydrin test on the dialyzate it was found that only traces of compounds containing a free NH_2 group were present. The pharmacological activity of, for example, γ-aminobutyric acid was

compared to the maximum amount of it possibly present in the dialyzate if all the colour obtained in the test emanated from this amino acid. This showed that γ -aminobutyric acid could not be responsible for the hypotensive effect and, if the slight blue colour developed in the test had anything at all to do with the activity of the dialyzate, it must have been produced by a compound with an activity at least fifty times that of γ -aminobutyric acid. Moreover, it is very improbable that the blue colour of the test was the result of the reaction between ninhydrin and a single NH_2-containing substance.

The results from the column fractionations show generally that the columns used do not give very good separations and cannot be recommended for fractionation. We found that the activity mostly followed the mono-, di- and oligo-saccharide fractions and when these fractions were evaporated they usually gave a syrupy residue from which the active compound can be separated only with difficulty. Our present investigations are therefore now mainly concerned with the fractionation of the sugars by complex-binding on a tetraborate-saturated anion exchanger column.

A surprisingly high activity was found in fraction A_1 I_2. In the "Remarks" of Table 1 it is noted that this is probably due to high concentration of sodium acetate. When comparing this statement with the reported low activity of pure 1 per cent sodium acetate solution used as a reference substance it looks like a contradiction. It must, however, be pointed out that fraction A_1 I_2 was an almost saturated solution of sodium acetate (about 30% w/v). If a 1 per cent solution gives a low activity reading, it is reasonable that a 30 per cent solution will show a high activity, provided no other effects of the high concentration become predominant. The correctness of this argument was supported when most of the acetic acid in fraction A_1 I_2 was removed with ether. The resulting fraction, A_1 I_3, had low activity. As the active material is insoluble in, and the activity uneffected by, ether (conclusions from experiment Q), it is concluded that acetic acid, or rather sodium acetate after the neutralization with sodium hydroxide, is the compound responsible for the activity of fraction A_1 I_2.

It is recommended that work on the isolation and purification of the active compound(s) should be continued. It has been found that the

extraction is best performed with boiling water and the first purification step should involve dialysis. When this report was written plans for further purification were centered on the use of some kind of complex-binding material for sugar. The results of gelfiltering experiments were not very encouraging but an experiment with Sephadex G-10 loaded with dialyzate and eluted with water and sodium chloride should be tried. Other purification methods like preparative thin-layer chromatography using impregnated plates and hydrophilic eluent systems according to Kirchner (1967) are also worthwhile trying.

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