FRACTIONATION OF EXTRACTS OF *LITHOSPERMUM ARVENSE* L. AND THEIR ACTIVITY AGAINST MOSQUITO LARVAE[1]

RICHARD V. MADRIGAL[2], F. E. KNAPP[3], ROY SIGAFUS[4] AND CECIL R. SMITH, JR.[2]

ABSTRACT. Methanol extracts of corn gromwell (*Lithospermum arvense* L.) are toxic to larvae of mosquitoes (*Aedes aegypti*). The active principle of this extract was concentrated by a fractionation sequence that included solvent partitioning, countercurrent distribution and preparative thin-layer chromatography. Fractionation at each stage was monitored by bioassays with mosquito larvae. Activity was concentrated in the hexane-soluble protion of the extract. After further fractionation by 10-stage countercurrent distribution followed by 200-tube countercurrent distribution, one form of activity was concentrated between tubes 80 and 90, as shown by treating larvae at 10 ppm. Other active components were concentrated at tubes 1 and 190.

INTRODUCTION

Several *Lithospermum* species have been used as herbal medicines in various parts of the world. Natural product chemists have given considerable attention to these species, particularly emphasizing the constituents responsible for antifertility activities. These investigations have resulted in the identification of lithospermic acid and rosmarinic acid (Kelley et al. 1975; Wagner and Wittman 1974). Recently Sosa et al. (1977) isolated and characterized a new cyanoglucoside from *L. purpureo-caerulum*.

Restrictions on the use of chemicals for insect control have stimulated investigations of the insecticidal properties of plant materials. In their studies of extracts of 36 plant species. Supavarn et al. (1974) tested extracts on larvae of *Aedes aegypti* L. mosquitoes. They found significant activity (survival rate, 22.5% or less) in 5 extracts, including those from *Lithospermum arvense* L., or corn gromwell. *L. arvense* was chosen for the present study because its crude extract appeared to contain the most toxic substances.

L. arvense is a native of Eurasia and is a member of the Boraginaceae. This weed, of minor importance, occurs in isolated areas over a wide area. It is found in Kentucky in disturbed areas, in fall-sown small grains, and in pastures and hay fields. Although designated as either an annual or a biennial in Gray's Manual of Botany (1950), the plant acts as a winter annual in Kentucky. It germinates in early to late fall and forms a low rosette. In early spring upright growth takes place and one to five stems develop which are from 20 to 70 cm in length. Small white to bluish blooms appear from April to June with seeds ripening over a corresponding period. Plants mature and die by the middle of July.

In this paper, the fractionation of the extracts of *L. arvense* are described. Each step of solvent partitioning, countercurrent distribution and chromatographic separation was followed by a bioassay of the resulting fractions for mosquito larvicidal activity.

MATERIALS AND METHODS

PLANT COLLECTION. Specimens of the above ground parts of *L. arvense* for extraction and bioassay were harvested at a stage near maximum growth. The oldest

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[2] Research Chemist, Northern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, IL 61604.

[3] Professor of Entomology, University of Kentucky, Lexington, KY 40506.

[4] Professor of Agronomy, University of Kentucky, Lexington, KY 40506.

leaves were beginning to senesce and the 1st seeds were brown to black in color. Harvest dates over a 5-year period ranged from May 8 to June 2. Plants were dried at 70° C for 24 hr and ground to pass through a 40-mesh screen.

EXTRACTIONS. The dried and finely ground whole plant material (1,364 gm) was placed in a glass percolator and covered with methanol. After overnight contact at ambient temperature, the methanol solution was drained and the solid material was covered again with fresh methanol; this procedure was repeated 5 times. Combined extracts were concentrated with a rotating evaporator to a thick syrup, which was partitioned between water and hexane (2 liters of each). The hexane solution was not washed with water, but was dried immediately over sodium sulfate. Both phases were taken to dryness with a rotating evaporator.

COUNTERCURRENT DISTRIBUTIONS. Hexane solubles (56.0 gm) were separated into a series of fractions by countercurrent distribution (CCD) (Litchfield 1972) in 10 stages with 500 ml of each phase per "tube" (1 of 10 Erlenmeyer flasks). The mobile layer was the upper phase of a solvent system prepared by mixing hexane (b.p. 68–70° C) with an equal volume of methanol-water (85:15, v/v). A rotating evaporator was used to isolate the resulting 10 fractions. CCD also was conducted with a 200-tube Craig-Post apparatus (Dutton 1954).[5] Selected fractions (13.1 gm) from 10-tube CCD were subjected to further fractionation by 200-tube CCD with the solvent system hexane-chloroform-ethanol-water (35:15:40:10, v/v/v/v) (Wetterau et al. 1964). Throughout the distribution, tubes contained 40 ml of each phase. After the 200 fundamental transfers, contents of every 10th tube were evaporated and weighed to establish a distribution curve.

CHROMATOGRAPHIC METHODS. Preparative thin-layer chromatography (TLC) of various fractions was carried out with chloroform-methanol solvent systems on 2-mm layers of Silica Gel G F-254 (Merck). The separated bands were located under ultraviolet light and were recovered from the adsorbent by conventional procedures.

BIOLOGICAL ACTIVITY. Bioassays of various fractions from *L. arvense* extract were tested against 3rd instar *A. aegypti* L. as described by Supavarn et al. (1974). Because the presently used extracts had been further purified, only 10 ppm on a dry weight basis were used in this study, instead of 100, 500 and 1,000 ppm as used in the previous study.

RESULTS AND DISCUSSION

Methanol extraction of the dried plant material (1,364 gm) provided 185.5 gm of product which, through solvent partitioning, afforded 56.3 gm of hexane-soluble and 129.2 gm of water-soluble

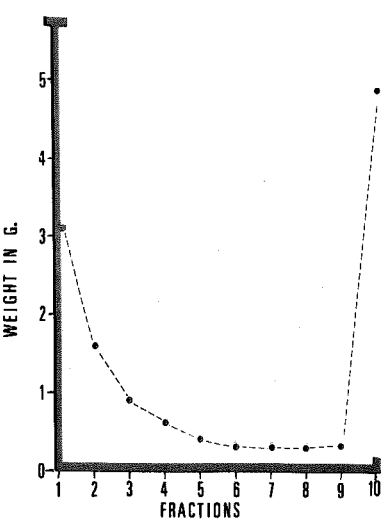

Figure 1. Ten-stage countercurrent distribution of hexane-soluble portion of gromwell extract.

[5] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

matter. According to bioassay results, the active principle was concentrated mainly in the hexane-soluble portion. Ten-stage CCD of this hexane-soluble portion (13.1 gm) produced the weight distribution indicated in Figure 1, with the active principle concentrated in fractions 3 through 8. A 200-tube CCD of combined fractions 3–8 (2.8 gm) gave the weight distribution shown in Figure 2. Bioassay of samples from every 10th tube—the same ones used for establishment of the weight distribution curve (Figure 2)—indicated that activity was concentrated around tubes 80 and 90.

A summary of selected day counts of larvae, pupae and adults is presented in Table 1. These results give a partial summary of data of the bioassay of the materials from the 200-tube CCD. In conducting these screening bioassays, counts of larvae were made at the beginning of the test and each day thereafter for 15 days or longer if significant numbers of larvae or pupae were still present. The 10 ppm concentration proved to be satisfactory in locating the presence of active ingredients.

Although larval mortality was over 50% in tube 1 and over 90% in tube 190 by day 5, these fractions were not subjected to further study because of the availability of

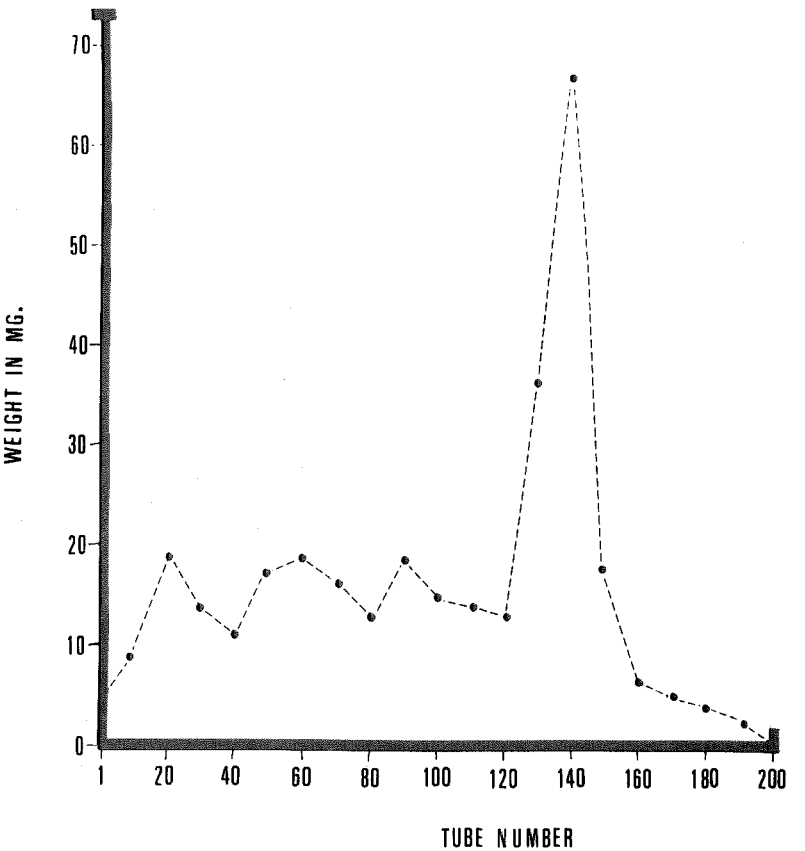

Figure 2. Further fractionation of gromwell extract by 200-tube countercurrent distribution.

Table 1. Bioassay results for gromwell extract fractions from 200-tube countercurrent distribution with *Aedes aegypti*.[a]

Tube Number	Mean numbers of surviving larvae per treatment[b]									
	Larvae				Pupae		Adults	Dead		
	Day 0	Day 1	Day 5	Day 9	Day 5	Day 9	Day 9	Day 1	Day 5	Day 9
1	10.8	10.8 a[c]	4.8 c	3.0 c-f	0 c	0.5 cd	0 f	0 c	6.0 b	7.2 b
10	11.5	11.2 a	10.0 ab	2.8 c-f	1.0 bc	5.8 a	2.5 bcd	0.2 c	0.5 cd	0.5 e
20	10.8	10.5 a	8.8 ab	1.5 d-g	1.2 abc	6.0 a	2.2 cd	0.2 c	0.8 cd	1.0e
30	10.5	10.2 a	8.2 ab	1.0 d-g	1.0 bc	4.5 ab	3.2 bc	0.2 c	1.2 cd	1.8 de
40	10.8	10.8 a	8.5 ab	1.0 d-g	1.2 abc	5.8 a	2.0 cde	0 c	1.0 cd	2.0 cde
50	10.8	10.8 a	9.2 ab	0.8 efg	1.5 ab	5.2 a	3.5 abc	0 c	0 d	1.8 de
60	10.8	10.8 a	8.2 b	1.0 d-g	2.0 ab	5.5 a	3.2 bc	0 c	0.5 cd	1.2 e
70	10.2	10.2 a	7.8 b	0.8 efg	1.8 ab	4.0 ab	4.0 ab	0 c	0.8 cd	1.5 de
80	10.0	6.5 b	1.0 d	1.0 d-g	0 c	0 d	0 f	3.5 a	9.0 a	9.0 ab
90	10.0	8.0 b	1.2 d	1.2 d-g	0 c	0 d	0 f	2.0 b	8.8 a	8.8 ab
100	11.5	11.5 a	10.0 ab	5.2 abc	0 c	2.5 bc	0 f	0 c	1.5 cd	3.8 cd
110	11.0	11.0 a	10.2 ab	3.5 dc	0 c	5.5 a	0.5 ef	0 c	0.8 cd	1.5 de
120	11.2	11.2 a	11.2a	4.2 bc	0 c	6.0 a	0.5 ef	0 c	0 d	0.5 e
130	10.5	10.5 a	10.0 ab	4.2 bc	0 c	5.2 a	0.2 f	0 c	0.5 cd	0.8 e
140	10.5	10.5 a	10.0 ab	3.5 dc	0 c	6.0 a	0 f	0 c	0.5 cd	1.0 e
150	10.8	10.8 a	9.8 ab	3.2 cde	0 c	5.0 a	1.2 def	0 c	1.0 cd	1.2 e
160	10.5	10.5 a	8.2 b	6.0 ab	0 c	0.2 cd	0 f	0 c	2.2 c	4.2 c
170	10.8	10.8 a	9.8 ab	7.0 a	0 c	1.5 cd	0 f	0 c	1.0 cd	2.2 cde
180	10.5	10.2 a	9.2 ab	7.5 a	0 c	0 c	0 f	0.2 c	1.2 cd	2.2 cde
190	10.0	10.0 a	0.5 d	0 g	0 c	0 c	0 d	0 c	9.5 a	10.0 a
Check	10.5	10.5 a	7.5 b	0.5 fg	2.5 a	4.0 ab	5.0 a	0 c	0.5 cd	1.0 e

[a] Concentration of each extract was 10 ppm on a dry weight basis.
[b] Numbers are from the average of 4 observations per treatment with approximately 10 3rd instar larvae each.
[c] Means within a column followed by the same letter are not significantly different (P=0.05) (Duncan, 1955).

the extract. Tubes 100 through 180 contained substances which delayed pupation.

Bioassays were run on each of the 4 fractionation materials but in the interest of brevity only the 200-tube CCD assay is given.

After pooling of residues from CCD tubes 81–119, further fractionation by preparative TLC (solvent system = chloroform-methanol, 75:25, v/v) yielded the following fractions in order of ascending R_f (fraction number, weight percent recovered): 1, 8%, 2, 14%, 3, 44%, 4, 34%. Fraction 4, the most active of this series according to bioassay, was further resolved by TLC (solvent system = chloroform-methanol, 95:5, v/v) into nine bands. In order of ascending R_f, these were grouped as follows (fraction, weight percent recovered): A, 23%; B, 28%; C, 16%; D, 16%; E, 17%. Fraction D was the most active of this series according to the mosquito larvae assay; some activity was observed in Fraction E as well. Fractions D and E were not obtained in sufficient quantities to facilitate further separation or characterization.

CONCLUSION

It would appear that more than one biologically active material is present in the methanol extract of *L. arvense*. This was illustrated in the 200-tube CCD fractionation where tubes 1, 80, 90 and 190 had definite larvicidal properties. A 2nd group which retarded larval growth and subsequent pupal and adult development was found in tubes 100–180.

The active components in *L. arvense* have not been identified. On the basis of solubilities, these active ingredients do not appear to be any of the compounds characterized by Kelley et al. (1975), Wagner and Wittmann (1974) or Sosa et al. (1977).

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