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Antifertility Activity of an Oxidized Polyphenolic Acid from *Lithospermum ruderale.** (28575)

F. X. GASSNER, M. L. HOPWOOD, W. JÖCHLE†, GESTUR JOHNSON AND S. G. SUNDERWIRTH
Endocrine and Chemistry Sections, Colorado State University, Fort Collins

Since Train *et al.*(1) reported that squaws of the Shoshone tribe used a cold water extract of the roots of *L. ruderale* (lithosperm) to reduce fertility, considerable work has been published dealing with the biological effects of various extracts of lithosperm. Cranston(2) and Zahl(3) investigated the inhibitory effect of the plant on the estrous cycle of mice while Plunkett *et al.*(4,5) studied its effects on the cycling of rats. It was demonstrated that dried roots of the plant impaired development of the gonads and accessory sex organs of the immature male rat and that injection of root extracts was at least 10 times more effective than oral administration. It was also shown that similar effects could be produced in the mature animal. Noble *et al*(6), Graham *et al*(7,8), and Noble *et al*(9) conducted experiments showing *in vitro* inactivation of gonadotropin (Pregnant Mare Serum) by lithosperm extracts. Zeller *et al*(10) studied the antiovulatory action of lithosperm in hens. Breneman *et al*(11) demonstrated the inactivating effect of its extracts *in vitro* and *in vivo* on pituitary gonadotropins using the cockerel. Breneman and Carmack(12) showed that the action of glucagon was inhibited in the chicken by *L. ruderale*. Using another species of plant, *(L. officinale)*, Kemper *et al* (13) demonstrated that its extracts could reduce blood sugar levels in the rabbit. Also, the incidence of mammary tumors in mice has been reduced by lithosperm(14).

Because *L. ruderale* roots contain a very active polyphenol oxidase which rapidly oxidizes the principal polyphenol present in the roots during water extraction, a logical step was to determine if the "active principle" could possibly be an oxidized form of the main polyphenol, which we have named "lithospermic acid" (LA). The effects reported here have been produced through use of this oxidized, polymerized acid which was isolated from the roots. A similar oxidized polymer was also prepared by isolation of the pure LA and subsequent controlled enzymatic oxidation.

Materials and methods. Isolation of lithospermic acid. The dried, ground roots (100 g) were vigorously stirred for 15 minutes with 500 ml of water at 25°C maintained at a pH of 2.0 with dilute HCl to prevent enzymatic oxidation. Following filtration, the infusion was extracted in a separatory funnel using 6 x 100 ml of peroxide-free diethyl ether. The ether extracts were combined, dried over anhydrous sodium sulfate and the volume reduced by distillation. The syrupy residue was dried *in vacuo*. The crude LA (1.4 – 2.0% of the dry roots) was purified by counter current distribution during 32 transfers using diethyl ether and water. The yield of chromatographically pure product was about 50% of the crude LA. LA is a polyphenolic acid having two 1, 2, 4 tri-substituted benzene rings each containing o-dihydroxy groupings and an olefinic grouping conjugated with one of the rings. Its chemical nature has been reported(15).

* Approved by Director, Colorado Agr. Exp. Station, as Scientific Series Publication 791.
† Schering, A. G., West Berlin, Germany.

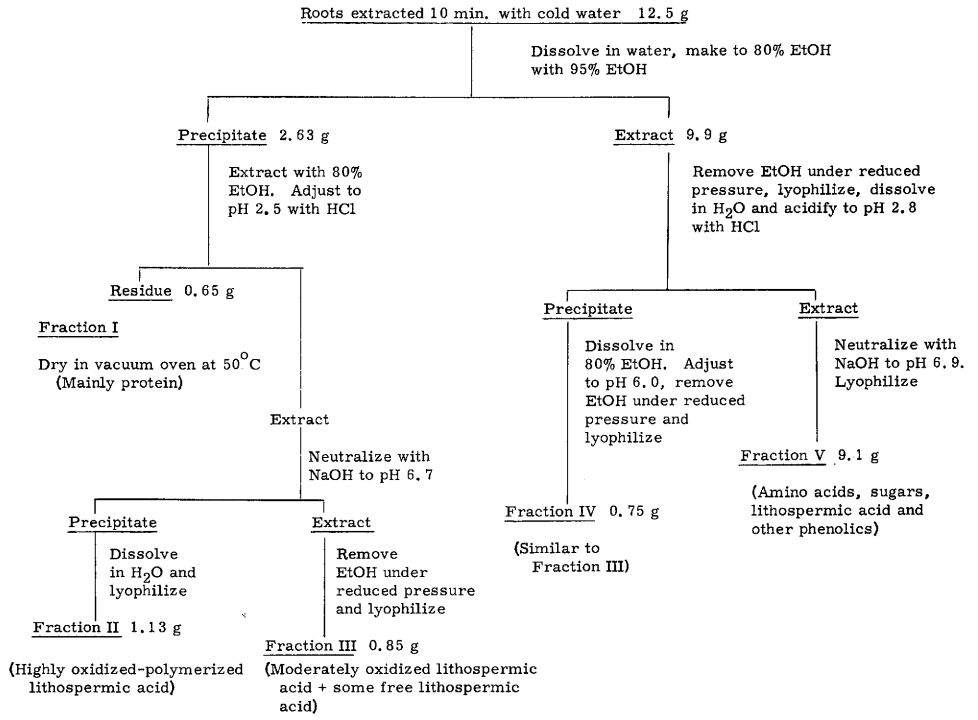

FIG. 1

Extraction and fractionation of lithosperm roots. A cold water extract was prepared using 100 g of dried ground roots and 500 ml of distilled water. After vigorous stirring for 10 minutes the mixture was filtered, the filtrate centrifuged and immediately lyophilized to yield a powder (12 – 20% of the dried roots). The powder contained LA and its polymerized form as well as proteins, amino acids, and carbohydrates. The lyophilized extract was subjected to the fractionation procedures shown in Fig. 1.

Fraction I was mainly protein. Fraction II was oxidized LA of sufficiently high molecular weight that only about 25 – 30% was dialyzable in 22 hours. Fraction III contained a trace of free LA and also some of its oxidized form which has a molecular weight that is lower than that of Fraction II. Fraction IV was similar to Fraction III. Fraction V contained most of the LA in addition to amino acids, carbohydrates, and some other phenolic compounds.

We have shown that the yield of Fraction II could be increased from 9% of the cold water extract to 24% when a 24 hour cold water extraction was employed. This increase occurred at the expense of free LA which was progressively oxidized by the natural polyphenol oxidase during the prolonged extraction. After 24 hours no free LA could be detected.

When LA was added to the first precipitate from the cold water extract and allowed to stand overnight the yield of Fraction II was greatly increased, demonstrating the conversion of this acid to its oxidized polymer.

Controlled oxidation of lithospermic acid. Using purified LA, a substance showing chromatographic characteristics similar to that of the oxidized LA in Fraction II was obtained by controlled oxidation using either tyrosinase‡ or hydrogen peroxide and peroxidase‡ by the following procedures:

A solution containing 4.0 g of LA in 180 ml of distilled water was adjusted to pH 6.8 with 10% NaOH. An aqueous solution (20 ml) containing 20 mg of tyrosinase was then added to the LA solution. Air was gently

‡ Mushroom tyrosinase and Peroxidase, Grade A, Worthington Biochemical Corp.

bubbled through the solution for 6 hours when an additional 10 mg of tyrosinase was added and the introduction of air was continued for 15 hours. The oxidized product was dried by lyophilization. This preparation was used for injection into chickens.

Oxidation by use of hydrogen peroxide and peroxidase was carried out as follows: LA (5 g) was dissolved in 230 ml of water and the pH adjusted to 6.8, and 8.5 ml of 30% H_2O_2 was added followed by 25 mg of peroxidase in 20 ml of water. After 24 hours any excess peroxide was destroyed by addition of a trace of catalase and the solution lyophilized. This product was used in subsequent chicken experiments. For injection into rats LA was reacted in similar amounts but for only 20 minutes and lyophilized (LA Oxid. A.). A portion of the LA was oxidized and injected into rats without prior lyophilization (LA Oxid. B.).

Biological assays. Female rats at 21 days of age were divided into treatment groups of 6 each and one group of 8 rats which served as the control. In the injection experiments, each rat was treated subcutaneously daily for 6 days with the lithosperm preparations described in the various Tables. On days 4, 5, and 6 the rats in the even numbered groups received, in addition, a total of 5 rat units of gonadotropin§ in 3 equally divided doses. On day 7 all rats were killed by chloroform inhalation and weighed. The uteri and ovaries were removed, trimmed free of excess tissue and weighed to the nearest 0.1 mg. Initial and final body weights as well as ovarian and uterine weights were averaged for each group and standard deviations of the means were determined.

In the experiments using gavage as a means of administration, the same procedure was used except that the lithosperm extracts were administered by stomach tube in one ml of water.

The antiovulatory action of the lithosperm fractions was determined in chickens using the procedures described by Zeller *et al*(10). The number of eggs laid in each treatment group (3-5 Hy-line hens per group) was determined for the 2 week period preceding daily injection of lithosperm preparations which were administered for one week. Eggs laid in each group were determined during the week of treatment and for 2 weeks following the last injection.

Results. The effects of controlled oxidation of LA on its biological activity in the immature female rat are shown in Table I. Unoxidized LA had no significant effect on body weight. The oxidized substance did not significantly depress body weights at 2 mg per day while at 10 mg per day significant decreases were produced. Because of the changes in body weight and their influence on the gonad and accessory sex organs, comparisons between groups for ovarian and uterine changes were made by adjusting their weights to mg per 100 g of body weight. The effect of endogenous gonadotropins was significantly depressed by both oxidized forms of LA when 10 mg per day was administered. Although at 2 mg per day ovarian weights were depressed by both oxidized forms, only LA Oxid. B. gave a significant reduction. When gonadotropin was administered, 2 mg of oxidized LA had no effect. At 10 mg per day both forms of oxidized material depressed the weight of the ovaries but only LA Oxid. B. was of significance.

To obtain further an adjustment for body weight loss, log ovary weight was divided by log body weight for individual animals. The results were similar to those discussed above.

With respect to uterine weight, endogenous gonadotropins were inactivated by oxidized LA while the pure acid had no effect. When gonadotropin was administered all the preparations caused significant increases in adjusted uterine weight.

Gavage of the lithosperm fractions did not alter body weight (Table II). Endogenous gonadotropins were not significantly inactivated by the levels of preparations administered as shown by the ovarian weights. When gonadotropin was injected, only the cold water extract and Fraction II (mainly oxidized LA) had any significant depressant influence on the ovary. Uterine weights were not significantly altered by any of the treat-

§ Gonadophysin, G. D. Searle & Co. (A preparation from sheep anterior pituitaries containing FSH and LH.)

TABLE I. Inhibition of Gonadotropin by Subcutaneous Injection of Lithospermic Acid and Oxidized Lithospermic Acid in Rats.

Group No.	Treatment	LA dose mg/rat/day	No. rats	Mean final body wt			Mean ovarian wt				Mean uterine wt			
				g	S.E. ±	Sign.†	Absolute mg	S.E. ±	mg/100 g body wt	Sign.†	Absolute mg	S.E. ±	mg/100 g body wt	Sign.†
1	Control-saline	0	8	75.2	1.7	—	21.9	1.3	29.1	—	61.4	2.1	81.6	—
2	Gonadotropin* (G)	0	6	68.5	1.1	—	48.5	6.0	70.8	—	110.6	6.6	161.4	—
3	"Lithospermic acid" (LA)	2	6	71.0	.8	NS	23.2	1.3	32.6	NS	48.2	5.1	67.9	NS
4	LA + G	2	6	73.0	1.3	"	49.2	6.0	67.4	"	153.1	6.0	209.7	.01
5	LA	10	6	73.5	1.1	"	20.2	1.6	27.5	"	62.2	3.2	84.6	NS
6	LA + G	10	6	68.0	.7	"	55.3	5.5	81.3	"	148.5	7.8	218.3	.01
7	LA oxidized-A (LA OxA)	2	6	69.3	1.4	"	18.4	1.8	26.6	"	47.4	2.9	68.3	.05
8	LA OxA + G	2	6	66.2	1.3	"	41.2	3.5	62.2	"	147.3	4.9	222.5	.01
9	LA OxA	10	6	56.2	2.1	.01	14.0	1.1	24.5	.01	35.2	1.4	62.6	.01
10	LA OxA + G	10	6	52.7	1.0	.01	26.4	2.5	50.1	NS	106.0	18.1	201.1	.05
11	LA oxidized-B (LA OxB)	2	6	70.2	2.4	NS	17.4	1.6	24.7	.01	41.7	2.9	59.4	.01
12	LA OxB + G	2	6	72.5	2.4	"	50.3	5.5	69.4	NS	150.7	8.3	207.9	.01
13	LA OxB	10	6	49.3	1.1	.01	12.2	1.2	24.7	.01	29.8	2.4	60.4	.01
14	LA OxB + G	10	6	50.3	1.2	.01	25.0	2.5	49.7	.01	107.6	12.2	213.9	.01

* Gonadophysin (Searle) 5 R. U. on days 4, 5, 6.
† Significance of odd groups compared with saline control, even groups compared with gonadotropin control.

ments although generally they were reduced. It is interesting that 2.4 mg of Fraction II and 40 mg of the cold water extract gave similar results. It therefore appears that oxidized polymerized lithospermic acid (Fraction II) was responsible for much of the activity noted in this experiment.

In testing various lithosperm fractions for antiovulatory activity it was found that cold water extracts prepared under nitrogen showed no activity, but that cold water extracts prepared under atmospheric conditions were active. This is to be expected if the activity is due to oxidized lithospermic acid. That Fraction II, lithospermic acid oxidized with tyrosinase, as well as lithospermic acid oxidized with peroxidase and H_2O_2 were antiovulatory in the hen supports this hypothesis. The percentage decrease in egg laying ability in the week following the 7th dose was used as a measure of activity. Fraction II was more active at the 50 mg/day level than the cold water extract (Table III). After dialysis of Fraction II only the retentant was antiovulatory. It appears that lithospermic acid oxidized with tyrosinase was more active than that oxidized with H_2O_2-peroxidase. In comparison to Fraction II, the retentant from the dialysis of the tyrosinase oxidized material was active while the dialysate was not. When small amounts of material were injected into the hens, there was a noticeable variation in individual response. However, at higher doses this variation tended to disappear. It was also shown that extracts of *L. officinale* species contain less activity than the *L. ruderale* extracts.

Discussion. The results indicate that a principle affecting the reproductive apparatus can be removed from the roots of *L. ruderale*. The finding that the mode of extraction can greatly influence the amount of the active component in a preparation may contribute to the varied results reported in the literature. The fraction having the highest degree of antigonadal and antiovulatory activity is composed of an oxidized-polymerized form of LA. This may result from the action of polyphenolase present in the root or from use of tyrosinase or hydrogen peroxide and peroxidase on the isolated and purified LA.

In the female rat the inactivation of endogenous as well as exogenous gonadotropins effected *in vivo* is apparently due to oxidized LA. Its mode of administration is, however, of considerable consequence. Our results indicate that the oral route is less effective than the parenteral. However, the latter procedure was accompanied by tanning of the skin and irritation caused by the oxidized preparations which complicated interpretation of the results since the unoxidized material did not show these effects.

TABLE II. Inhibition of Gonadotropin by Gavage with *L. ruderale* Extracts.

Group No.	Treatment	Dose mg/ rat/day	No. rats	Mean final body wt g	S.E. ±	Mean ovarian wt mg	S.E. ±	Sign.†	Mean uterine wt mg	S.E. ±	Sign.†
1	Control	0	11	78.3	2.0	20.6	.8		61.1	3.9	
2	Gonadotropin* (G)	0	11	79.6	1.2	50.4	3.0		120.8	6.5	
3	Cold water extract	40	8	68.6	3.0	17.4	.9	NS	45.4	3.8	NS
4	" " " + G	40	8	79.1	1.7	39.9	3.6	.05	102.1	8.6	"
5	Fraction I	2.2	8	73.8	3.1	16.6	1.5	NS	48.6	5.2	"
6	" I + G	2.2	8	81.1	3.1	45.0	5.4	"	103.2	7.3	"
7	Fraction II	2.4	8	83.2	2.2	19.9	1.7	"	53.3	2.8	"
8	" II + G	2.4	8	82.8	2.0	42.1	4.3	.05	107.5	5.2	"
9	Fraction III	3.9	8	82.2	2.4	18.4	1.1	NS	56.1	5.7	"
10	" III + G	3.9	8	79.8	1.9	46.9	4.5	"	121.9	8.1	"
11	Fraction IV	2.4	8	75.9	.7	21.0	.9	"	52.0	4.6	"
12	" IV + G	2.4	8	80.9	2.1	45.4	3.8	"	104.4	5.3	"
13	Fraction V	29	8	82.5	1.8	22.6	1.2	"	61.2	3.4	"
14	" V + G	29	8	78.8	2.0	43.6	3.8	"	116.9	5.7	"

* Gonadophysin (Searle) administered on days 4, 5, 6.
† Signficance of odd groups compared with saline control, even groups compared with gonadotropin control.

TABLE III. Egg Laying Performance of Active Lithosperm Preparations.

Substance tested	Daily dose in mg	No. birds	Pretreatment† No. eggs	Egg laying performance*: During treatment No. eggs	During treatment % reduction	Post treatment: 1st wk No. eggs	1st wk % reduction	Post treatment: 2nd wk No. eggs	2nd wk % reduction
Cold water extract	35	3	16	9	44	4	75	12	25
	45	3	12.5	8	36	3	76	8	36
	50	3	16.5	8	52	3	82	8	52
	75	3	17	9	47	0	100	1	94
	80	3	14	7	50	0	100	0	100
Fraction II	10	5	25.5	16	37	6	76	12	53
	25	3	15	10	33	1	93	3	80
	50	5	26.5	15	43	2	92	4	85
	50	5	24	13	46	0	100	8	67
Fraction II (retentant)	20	3	15	9	40	5	67	7	53
	35	3	16	12	25	1	94	3	81
LA (oxid with H_2O_2 and peroxidase)	30	3	15.5	11	29	6	61	6	61
	30	3	16	13	19	5	69	7	56
	50	5	24	12	50	0	100	12	50
LA (oxid with tyrosinase)	10	3	14	12	14	6	57	15	0
	30	3	17.5	10	43	4	77	7	60
	30	3	14.5	9	38	2	86	6	59
	30	3	17.5	7	60	1	94	9	49
	50	5	23.5	11	53	0	100	1	96
LA (oxid with tyrosinase (retentant)	7	3	16	12	25	7	56	7	56
	21	3	17.5	14	20	6	66	6	66

* Fractions producing no decrease in egg laying are not shown.
† Avg for 2 weeks preceding treatment.

The findings in the chicken corroborate those of Zeller *et al*(10) in that egg laying was inhibited. An antiovulatory component was shown to be oxidized LA whereas the unoxidized compound was ineffective. Of interest was the observation that oxidation with hydrogen peroxide and peroxidase did not give as active a product as oxidation with tyrosinase. In the latter case a lower molecular weight resulted from the procedure as revealed by paper chromatography and dialysis. The activity apparently is dependent on a fairly specific molecular size which is more readily achieved with tyrosinase. However, there is no information on the relationship of molecular weights to the potency of antifertility or antigonadal agents of plant origin.

It was also interesting that in the gavage experiment using immature female rats, a small quantity of Fraction II showed inactivation of exogenous gonadotropin as measured by ovarian weights whereas a much larger amount of the cold water extract was required. In experiments with hens, however, Fraction II and the cold water extract did not show such a large difference in potency.

Considerable emphasis has been placed on the role of quinones in the *in vitro* inactivation of protein hormones such as P. M. S.(16, 17,18) and as antifertility agents(19,20, 21,22). Preparations obtained by spontaneous oxidation occurring during water extraction of *L. ruderale* roots as well as by controlled oxidation of LA with peroxidase-peroxide or with tyrosinase were found to contain quinones as evidenced by reaction with acidified KI and starch.

Summary. A polyphenolic acid, not previously described, was isolated from the roots of *L. ruderale* and named "lithospermic acid" (LA). LA showed no biological activity in the rat or the chicken. However, when oxidized enzymically a substance was obtained which inactivated exogenous and endogenous gonadotropins in the female rat and prevented ovulation in the laying hen. In one experiment a cold water extract of the roots was partitioned into 5 fractions which were administered orally to immature female rats. Only the original extract and one fraction (II), composed of oxidized LA showed any significant inactivation of exogenous gonadotropin. In another experiment cold water extract, II, as well as enzymically oxidized lithospermic acid showed antiovulatory properties when injected into the laying hen. These experiments indicate that oxidized LA present in the roots is responsible for some of the antigonadotropic effect noted with cold water extracts of *L. ruderale.*

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