# Isolation and Characterization of a Hypoglycemic Agent from *Xanthium strumarium*

FLOYD P. KUPIECKI*[x], C. DWAYNE OGZEWALLA‡, and FRED M. SCHELL‡§

**Abstract** □ A compound isolated from seeds of *Xanthium strumarium* exhibited potent hypoglycemic activity in the rat. Partial characterization of the crystalline compound showed that it contains only carbon, hydrogen, oxygen, and sulfur and that the sulfur appears to be present as the sulfonate group. The agent differs from all compounds previously isolated from *X. strumarium*.

**Keyphrases** □ *Xanthium strumarium* L. (Compositae)—isolation and characterization of a hypoglycemic agent □ Hypoglycemic activity—isolation and characterization of crystalline compound from *Xanthium strumarium*

Biological studies have been reported on extracts or compounds isolated from *Xanthium strumarium* L. (Compositae), the common cocklebur. An attempt to verify arthralgic activity in *Xanthium* resulted in isolation of a depressor substance which proved to be identical with choline (1). A tincture of seed stimulated respiration in the frog at low doses and depressed respiration at high doses (2). Leaves of the plant yielded xanthinin (3), a plant growth-regulating compound isolated earlier from *X. pennsylvanicum* (4). An investigation into the cause of the often reported toxicity of cocklebur led to the conclusion that hydroquinone is responsible for the toxic effects produced by ingestion of various species of *Xanthium* (5). This paper describes the isolation and characterization, from seed of *X. strumarium*[1], of a new crystalline compound that lowers blood sugar in the rat.

## EXPERIMENTAL

**Partial Purification**—An extract was prepared by boiling 300 g of coarsely ground seed in 1300 ml of water in an open beaker for 30 min. After cooling and centrifuging, the volume was adjusted to 1000 ml. Extracts prepared in this way contained 23–40 mg solids/ml and were fractionated through the following four steps before chromatography[2].

1. The solution was concentrated to 185 mg/ml and 2 volumes of acetone were added with stirring at room temperature. After 0.5–1 hr, the solids were removed by centrifugation and discarded. A 3rd volume of acetone was added and the precipitate was removed as described and discarded. The solution was concentrated to remove acetone and brought back to a solids concentration of 185 mg/ml. Absolute ethanol was added to a final concentration of 95% during 10–15 min while the solution was stirred and maintained between 65 and 75°. After it cooled to room temperature, the flask was placed in ice and held at 0° for 24–36 hr. The loose precipitate was harvested on a sintered-glass funnel and dissolved in water to a concentration of 100 mg/ml for Step 2.

2. Absolute ethanol was added to a final concentration of 90%, the flask was cooled, and the precipitate was harvested as in Step 1.

[1] Identified by Dr. C. D. Ogzewalla, University of Cincinnati, Cincinnati, OH 45221. Specimens are deposited at the Herbarium, University of Cincinnati, Cincinnati, OH 45221

[2] Amberlite XAD-2 resin.

**Table I**—Comparative Hypoglycemic Activity of Crude Extract and Material Obtained in Step 1 of Purification

| Fraction Injected | Dose, mg/kg | Blood Glucose, mg% | Percent Lowering | *p* (*versus* Control) |
|---|---|---|---|---|
| Saline (control) | — | 63[a] | — | --- |
| Crude extract | 15 | 49 | 22 | <0.05 |
| Crude extract | 30 | 41 | 35 | <0.01 |
| Purified, Step 1 | 12 | 29 | 54 | <0.01 |

[a] Each number is the mean of five rats dosed by intraperitoneal injection and bled 3 hr later. The standard error of a treatment mean is 3.25.

3. The solids from Step 2 were dissolved in water to a concentration of 50 mg/ml. Acetone (4 volumes) was added in 5–10 min while the solution was stirred in a 55° bath. The solution was removed from the bath and, after 1 hr, the precipitate was removed by centrifugation and discarded. The solution was stored at from −55 to −70° for 24–48 hr and the precipitate was harvested on a sintered-glass funnel at that temperature and dried.

4. The solids from Step 3 were dissolved in water to a concentration of 85 mg/ml. Absolute ethanol (6 volumes) was added in 10–15 min while the solution was stirred in a bath held at 75–80°. The solution was removed from the bath, allowed to reach room temperature, and placed in a freezer at from −15 to −20° for 24–48 hr. The precipitate was harvested as in Step 3.

**Chromatography on a Resin[2] Column**—In a typical procedure, 100 mg of material from Step 4 was fractionated on a 10-ml column in a 10-ml pipet at an elution rate of 1% column volume/min. Water was passed through the column until 2.4 column volumes were collected. This was followed by 1.2 column volumes of 50% methanol.

**Crystallization**—*Method 1*—The material that came off the resin with 50% methanol was dried and dissolved in 50% ethanol at a concentration of 50 mg/ml. The solution was stored in ice for 2–4 days and the fine colorless needles were harvested by filtration. Recrystallization was accomplished from 50% ethanol in the same way.

*Method 2*—The solids from Step 4 were dissolved in acetic acid at a concentration of 50 mg/ml. One volume of *n*-butanol and 2 volumes of ethyl acetate were added and the solution was stored in ice overnight. The precipitate was removed by centrifugation at 0° and discarded. The remaining solids were recovered by evaporation under vacuum and crystallized from 50% ethanol as in Method 1.

Progress in purification was followed by assaying for hypoglycemic activity and by TLC on precoated silica gel plates in three solvent systems: System 1, *n*-butanol–acetic acid–ether–water (45:30:15:5); System 2, *n*-butanol–methanol–water–acetic acid (80:10:10:1); and System 3, *n*-butanol–methanol–water (80:10:10). Routinely plates were developed once in System 1 and twice in Systems 2 and 3. The hypoglycemic component appeared as a red-violet spot at $R_f$ 0.60, 0.45, and 0.14 in Systems 1, 2, and 3, respectively, when plates were sprayed with vanillin in phosphoric acid and heated 5 min at 140°.

**Chemical Methods**—Since the crystalline product yielded a residue of 17–20% when analyzed for carbon, hydrogen, and nitrogen, it appeared to be a salt. Therefore, it was passed through a resin column[3] in the $H^+$ form at 4° (100–200 mg/5 ml column)

[3] Amberlite IR-120.

**Table II**—Comparative Activity of Crude, Partly Purified, and Crystalline Hypoglycemic Agent

| Fraction Injected | Dose, mg/kg | Blood Glucose, mg% | Percent Lowering | *p* (*versus* Control) |
|---|---|---|---|---|
| Saline (control) | — | 70[a] | — | — |
| Crude extract | 30 | 42 | 40 | <0.01 |
| Purified, Step 3 | 8 | 23 | 67 | <0.01 |
| Purified, Step 3 | 12 | 19 | 73 | <0.01 |
| Purified, Step 4 | 6 | 17 | 76 | <0.01 |
| Purified, Step 4 | 12 | 9 | 87 | <0.01 |
| Purified on resin[b] | 5 | 21 | 70 | <0.01 |
| Crystalline | 1.25 | 33 | 53 | <0.01 |
| Crystalline | 2.50 | 14 | 80 | <0.01 |

[a] Each number is the mean of five rats dosed by intraperitoneal injection and bled 3 hr later. The standard error of a treatment mean is 2.08. [b] Amberlite XAD-2.

and titrated with potassium hydroxide. Titration showed two strong and two weak acid groups per molecular weight of approximately 885. Other aliquots were passed through the column, collected in dilute ammonium hydroxide, and dried for analyses. The results for the ammonium salt showed: C, 42.82; H, 6.8; N, 4.74; O, 37.29; and S, 7.15; therefore, a formula of $C_{32}H_{52}O_{21}S_2$ for the free acid was indicated. The two sulfur atoms and the two strong acid groups appear to be sulfonates, since elemental analyses showed that the sulfur was not lost when the compound was warmed with 0.1 *N* $Ba(OH)_2$ at 37° for 4 days. The IR spectrum (mineral oil) of the crystalline compound showed hydroxyl at 3440, carbonyl at 1720, and $SO_3H$ at 1240 $cm^{-1}$. Peaks at 1040 and 1000 $cm^{-1}$ could be attributed to $SO_3H$ and/or alcohol C—O. Other peaks occurred at 1625, 1560, 1375, 1165, 1120, 925, 900, and 810 $cm^{-1}$. The mass spectrum, after trimethylsilylation of the compound, showed a fragment at 1042 which, by use of the deuterated silyl reagent, was found to contain six trimethylsilyl groups. This indicates a fragment with a mass of 610. Further structure studies were complicated by the fact that the crystalline compound, when passed through the resin[3], decomposed spontaneously to yield six red-violet spots on TLC in System 3 unless it was collected in alkaline solution.

**Assay for Hypoglycemic Activity**—Male rats[4], weighing 130–150 g, were injected intraperitoneally or subcutaneously with the agent in saline after an overnight fast. Immediately after treatment, they were injected subcutaneously with 1 g/kg of glucose in 1 ml of saline. They were bled from the abdominal aorta after cyclopentobarbital[5] anesthesia. Blood glucose was measured using an automated analyzer by a modification of the ferricyanide procedure of Hoffman (6).

## RESULTS AND DISCUSSION

Tables I and II show the comparative hypoglycemic activity of the crude extract and the products of the several steps of purification to crystallinity. The results show a good dose–response relationship at each step of purification. The time course of hypoglycemia from 1 to 7 hr is shown in Table III. There is no lowering of blood glucose after 1 hr. After 2 hr the lowering of glucose is significant, but maximal lowering occurs at 3 hr and persists at this level through Hours 5 and 7. To eliminate the possibility that the drug acts directly on the liver when injected into the peritoneal cavity, the activity of subcutaneously and intraperitoneally injected doses was compared. The two routes of injection produced equal effects on blood glucose (Table IV). The crude material, partly purified fractions, and crystalline compound had only weak hypoglycemic activity when given orally at 3–5 times the injected doses.

The demonstrated presence of only carbon, hydrogen, sulfur, and oxygen in the molecule eliminates the various classes of compound containing nitrogen. The possibilities are narrowed further by absence of peaks in the UV spectrum (2 mg/10 ml water). The presence of sulfate ester or sulfonate is shown by titration, and the results of alkaline hydrolysis suggest the latter. The following reagents for specific classes of compounds or functional groups (7) repeatedly failed to react with the purified compound on TLC under conditions that worked with standards: antimony trichloride for steroids and glycosides, basic lead acetate for uronic acids and flavonoids, *p*-toluenesulfonic acid for steroids and flavonoids, benzidine–sodium metaperiodate and silver nitrate–sodium hydroxide for sugars and sugar alcohols, and 2,4-dinitrophenylhydrazine for aldehydes and ketones.

Partial characterization of the compound demonstrates that the hypoglycemic activity is not due to compounds isolated previously from cocklebur (1–5). Further attempts at identification of the agent are underway.

[4] Upj:TUC(SD) spf.
[5] Cyclopal.

**Table III**—Time Course of Activity of Partly Purified Hypoglycemic Agent

| Hours | Blood Glucose | | Percent Lowering | *p* (*versus* Control) |
|---|---|---|---|---|
| | Control | Treated | | |
| 1 | 61[a] | 63[a] | 0 | NS |
| 2 | 60 | 38 | 37 | <0.01 |
| 3 | 63 | 21 | 67 | <0.01 |
| 5 | 65 | 21 | 68 | |
| 3 | 77 | 24 | 69 | <0.01 |
| 5 | 72 | 29 | 60 | <0.01 |
| 7 | 76 | 31 | 59 | <0.01 |

[a] Each number is the mean of five rats dosed by intraperitoneal injection. The standard error for 1-, 2-, 3-, and 5-hr groups is 2.48, 1.19, 3.23, and 2.88, respectively; for 3-, 5-, and 7-hr groups, it is 1.91, 2.25, and 1.73, respectively. Each treated animal was dosed with 6 mg/kg of material purified through Step 4.

**Table IV**—Comparison of Routes of Injection on Activity of Partly Purified Hypoglycemic Agent

| Experiment Number | Route | Blood Glucose | | Percent Lowering | *p* (*versus* Control) |
|---|---|---|---|---|---|
| | | Control | Treated | | |
| 1 | Intraperitoneal | 70[a] | 21[a] | 70 | <0.01 |
| | Subcutaneous | 61 | 22 | 64 | <0.01 |
| 2 | Subcutaneous | 57 | 15 | 74 | <0.01 |

[a] Each number is the mean of five rats. The standard error for lines 1, 2, and 3 is 2.66, 4.07, and 1.98, respectively. Each treated animal was dosed with 5 mg/kg of material purified through the resin step.

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§ Present address: Department of Chemistry, University of Tennessee, Knoxville, TN 37916

ˣ To whom inquiries should be directed.