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Isolation of a Toxic Substance from CENTAUREA REPENS

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Graduate School

ISOLATION OF A TOXIC SUBSTANCE FROM CENTAUREA REPENS

by

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A Thesis in Partial Fulfillment

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Master of Science in the Field of Chemistry

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Each person whose signature appears below certifies that he has read this thesis and that in his opinion it is adequate, in scope and quality, as a thesis for the degree of Master of Science.

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INTRODUCTION

The Compositae are the largest family of vascular plants, including about 950 genera and 20,000 species. They are known to exist over most of the earth in all habitats. Many members are noxious weeds (13). Within this family about 400 species of the genus Centaurea (star thistle) are known, mostly native to the Mediterranean region; but a few native species are also found in South America, North America, and Australia (7). Several species of this group are commonly known as knapweeds (4). The knapweeds were introduced into the United States during the nineteenth and twentieth centuries and have spread rapidly over most of the Western United States and Canada (6). They have become a serious agricultural problem in many states. Watt (20) suggests that all Centaurea species are troublesome both to man and to animals because of their spininess.

Centaurea repens (Russian knapweed), the species studied in this work, is especially noxious and in some states is considered among the worst weeds known to agriculture (16,18). Most of the references to this plant in the biological and agricultural literature are concerned with methods of eradication by physical means or chemical treatments.

It is the purpose of this study to isolate the toxic material from Russian knapweed and to make a preliminary study aimed at determining its identity.

REVIEW OF LITERATURE

Russian knapweed is a native of southern Russia, Asia Minor, and Afghanistan. It was introduced to the United States in 1910 by being brought to California in impure Turkestan alfalfa seed (18). It is now found in all the states from Washington to Michigan and south to Missouri, Texas, Utah, Colorado, and California (19). According to Jepson (11), it grows locally around Arlington in western Riverside County.

The name "knapweed" refers to the heavy gray hair or nap on the stems. This nap is especially noticeable on the young plants (18). The plants when mature measure from one to three feet tall and grow from creeping perennial rootstocks (11). The mature leaves are small, 1¼"-3" long (11) and narrow with smooth surfaces and edges and without a pronounced mid-rib (18). Numerous lilac-colored flowers on long stems appear in small, almost-spherical heads about 3/8" and ½" in diameter. The absence of thorns and the size of its flower heads distinguish it from similar thistles (Fig.1) (18). Russian knapweed flourishes from May to September (9).

Russian knapweed is referred to in the literature by several different names. They are listed here for clarification:

Centaurea repens (Linnaeus) = Centaurea picris (Pallas) (19)
Turkestan thistle (11)
Hardheads (20)
Mountain bluet (15)

When reviewing the literature, it was noted that the common names are sometimes used for other species.

Figure 1--A.--The stem, leaves and heads of the mature Russian knapweed.

B.--A leaf of a young plant.

C.--Seeds of the Russian knapweed.

Work done by Fletcher and Renney (6) showed that Centaurea repens contains a plant growth inhibitor which may partially account for the rapid growth of knapweeds and their tendency to exclude other plants. According to Rogers (18), Russian knapweeds make so dense a stand that no other species can compete with them. Fletcher and Renney took ether extracts of Centaurea repens and separated the components by paper chromotography. The inhibitor was located by germinating a seed directly on the chromatogram. The ultraviolet spectrum, as well as positive tests with Salkowski and Ehrlich reagents, indicated a compound with an indole nucleus. No further positive characterization was carried out.

Generally, Centaurea species possess spines and because of these are likely to cause distress to both men and animals(20). Rogers (18) states that the rough leaves and stiff stems of Centaurea repens are almost inedible when dry. According to Muenscher (17), Centaurea species (knapweed, star thistle) are not toxic to animals but possess spines that produce mechanical injuries in and around the mouth and around the eyes. Swelling and infection may result in the inability to see and lead to starvation. However, it should be pointed out again that Centaurea repens is described as having no thorns (18), unlike many other Centaurea species (9,18).

It would appear, then, that any animal death resulting from consumption of Russian knapweed would not be attributable to mechanical injury.

The toxicity of Russian knapweed seems to be established in the literature, but no reference to the isolation or nature of the toxic material was found.

Hurst (10) states that Centaurea picris is toxic to sheep. Watt (20) confirms this, indicating that a 600-gram dose seems to be more than enough to cause death in a sheep. Among the symptoms, which appear rapidly, are apathy, groaning, labored respiration, weak and quickened pulse, and fever. Watt also points out that the species is poisonous to cattle. In Russia, Centaurea repens has been used experimentally to kill horses. Khalimbekov (12) has reported that 7% of the weed in hay, consumed over a period varying from three to sixty days, produced 75-90% fatalities. Three-and-a-half kilograms eaten with oats in 48 hours was a fatal dosage in several cases. Russian knapweed has also been known to be fatally-toxic to horses in Colorado (personal communication).

Several workers have carried out partial analyses of Centaurea repens. As mentioned earlier, Fletcher and Renney (6) found a growth-inhibiting substance, evidently having an indole structure. In Russia, seeds of Centaurea repens were found to contain 0.05% alkaloid, 13% oil, and 0.25% neutral substances (1). Gol'dberg and Allakheurdibekov report the following partial analysis: alkaloid 0.033 to 0.037%, resinous substances 6.8%, tannins 3.03%, no glucoside, no fatty ester (8).

Correlation of these analyses with the known effects on animals suggested that the alkaloids may be responsible for the toxicity of the plant.

agents, they were each tested using known alkaloid compounds. Tests for all reagents with each compound were positive.

Since no alkaloids were present, the $CHCl_3$ extract was tested on mice to determine its toxicity. Several milligrams of this water-insoluble plant extract were dissolved in absolute ethanol and then diluted to obtain a 30% alcohol suspension. A 0.25-ml dose was injected into the abdominal cavity of a 30-g female white mouse. Within 5 minutes all activity ceased, and the mouse was quite unresponsive to external stimuli. After 10 minutes the rate of respiration had slowed to about half the rate of several mice which had not been injected. Besides being slowed, the normal steady breathing was altered to very sharp gasps, sometimes at irregular intervals. The irregularity became more pronounced in the few minutes before death. Forty-seven minutes elapsed from injection to respiratory failure.

To establish the effect of alcohol alone, two mice were given 0.25 ml of 30% ethanol and 0.25 ml of 50% ethanol respectively. Although their activity was reduced after about 15 minutes, no change in their breathing rate or pattern was detectable. Both were apparently back to normal activity within the hour.

A second test of the toxicity of the chloroform extract was attempted by mixing it with a mouse's regular food. A small pellet 1/8" x 3/8" was made of 2 parts food to 1 part plant extract. Voluntary feeding on only about 1/3 of this pellet produced a definite reduction in activity and its normal responsiveness to external mechanical stimuli. Breathing was slowed and very jerky after 1½ hours, and it remained that way for another 1½ hours. No further food was eaten, and activity gradually returned to normal about 3½ hours after the feeding began.

Tests with mice on the ammoniated fraction from the chloroform extraction showed no effect whatever when doses of 0.5 ml saturated aqueous solution were administered by injection.

Further isolation was carried out by thin-layer chromatography. Diagnostic tests were carried out with layers of Silica Gel G, (Merck & Co.) 0.25 mm thick. Thirty grams of Silica Gel G was mixed with 58 grams of water and the resulting slurry applied with a Brinkmann spreader. The plates were allowed to air dry at room temperature overnight and then activated by heating for at least one hour at 105°C. Samples of various sizes were applied with a syringe at several positions across an 8" x 8" plate to determine the optimum separation capabilities of the silica gel. Development was carried out in a sealed tank, which was saturated by suspending solvent-soaked filter paper around the sides. The plates were placed sloping in the tank with the silica-gel layer toward the filter paper to afford maximum saturation.

Ten developing solvents were tested to check the mobility and separation characteristics of the plant mixture. These solvents were benzene, chloroform, ether, ethanol, methanol, n-propanol, benzene-methanol (19:1), benzene-ethyl acetate (19:1), chloroform-methanol (4:1), and chloroform-methanol (19:1). Of the tested developers, pure ethanol gave the most satisfactory separation, showing three bands (A, B, and C) with Rf values of 0.0, 0.24, and 0.90 respectively. All three were essentially immobile in benzene and other non-polar solvents, while all showed Rf values above 0.9 in methanol. A 19:1 mixture of benzene and methanol did not produce any appreciable separation, since all migrated in the methanol to above Rf 8.0. Poor separation or streaking occured in all other developers.

After ethanol development Band C showed light greenish-yellow in daylight and fluoresced brilliant yellow under ultraviolet excitation. Band B was pale yellow in daylight and invisible in U. V. Its position was detectable, however, by a slight U. V. quenching effect that it had on the weak, deep-purple fluorescense of the silica-gel layer. Band A was barely perceptible as faint yellow in daylight and fluoresced weakly yellow upon exposure to U. V. radiation. From appearance in daylight, and U. V. light and subsequent charring with concentrated H_2SO_4, Band C was the main constituent in the mixture.

Following development of the chromatograms with ethanol, diagnostic tests for steroids were carried out. Reagents for detection were prepared according to Stahl (2).

Test 1: The TLC plate was sprayed with a 30% (v/v) solution of o-phosphoric acid until the silica gel was saturated. Heating of the plate at 110°C. for 10 - 15 minutes produced a marked intensification of the band at Rf 0.90 when viewed continually under U. V. The other bands showed no change during this treatment.

Test 2: A second plate was sprayed lightly with 30% H_3PO_4, followed by a freshly-prepared 1.5%-alcoholic solution of phosphomolybdic acid. Subsequent heating produced dark blue coloration in Band C only.

Test 3: Antimony trichloride-glacial acetic acid (1:1, w/w) solution was sprayed onto a third plate. Band C changed from yellow to reddish-brown after heating for 5 minutes at 95°C. Other bands were not affected by this reagent.

Test 4: One gram of vanillin was dissolved in 100 ml of 50% aqueous o-phosphoric acid. Light spraying and heating at 120°C. for 20 minutes produced a violet color in Band C.

These four tests are listed by Stahl as suitable for the detection of steroids (2). Phosphomolybdic acid is listed as specifically detecting hydroxysteroids (3). A further test, suggested for identification of higher alcohols and ketones, is listed by Stahl (2). Three grams of vanillin and 0.5 ml of sulfuric acid were dissolved in 100ml of ethanol. A rich violet-color reaction was evident with Band C only.

Although no specific chemical test for the steroid nucleus could be found in the literature, it is tentatively assumed that Band C contained a compound of steroid structure on the basis of the selective isolation procedures used and the positive results of the spot tests used.

Preparatory thin-layer chromatography was then carried out on about half of the chloroform extract. Layers of Silica Gel G 1 mm thick were applied, using a slurry of the same proportion as for the thinner layers. To prevent "running" of the layer edges immediately following application, the slurry was allowed to partially set for one minute before spreading. The edges of the silica gel were dressed so that the layer was of uniform thickness across its entire width. Overload edge effects were thus prevented during development. The prepared plates were air dried overnight and activated for several hours at 105°C. as before.

The chloroform solution of toxic plant extract was applied to the plates with a syringe while at about 50°C. This elevated temperature was high enough to evaporate the chloroform before it spread widely, but not so quickly as to stop it from penetrating down through the solid layer. All plates were developed over an 8-cm path in a saturated ethanol atmosphere, dried at 35°C to remove the solvent, and then redeveloped for 15 cm in the same solvent. Double development gave a noticeably-better separation.

After drying, the silica gel containing Band C was scraped from the TLC plates, combined, and exhaustively extracted with ethanol. Following evaporation of the solvent, the residue was crystallized from ethyl ether. A greenish, sticky-but-crystalline mass appeared. Traces of solvent were removed by vacuum evaporation for 12 hours. An infrared spectrogram of this material was obtained (Fig. 2a). In making this and other I. R. traces, a small quantity of the crystalline sample was dissolved in ethyl ether and mixed with spectrophotometric-grade potassium bromide. All traces of solvent were removed by continual pumping from a vacuum dessicator for 1 hour. The remaining solid was ground in a mortar and pressed into a clear pellet.

At the same time the chloroform was removed from the remaining portion of the toxic plant extract which was not subjected to TLC. To be sure of complete removal, it was pumped continually from a vacuum dessicator for about 12 hours. A highly viscous, dark brown, waxy material remained. This material was extracted with ethyl ether. Removal of the solvent over a period of 48 hours yielded a crop of sticky greenish crystals similar to those from Band C of the TLC separation. Infrared analysis (Fig. 2b) showed this material to be essentially the same as Band C.

An ethanol extract of the material in Band C was subjected to gas chromatography to check on the number of constituents in it. The separation was achieved with a four-foot column of 3.8% SE-30 on diatomaceous earth. The hydrogen-flame detector revealed 6 peaks at a column temperature of 220°C. Attempts to separate the mixture on a preparatory scale, using 20-foot columns of 30% SE-30 (Silicone Gum Rubber-Methyl) and 30% PDEAS (Phenyl Diethanolamine Succinate) and

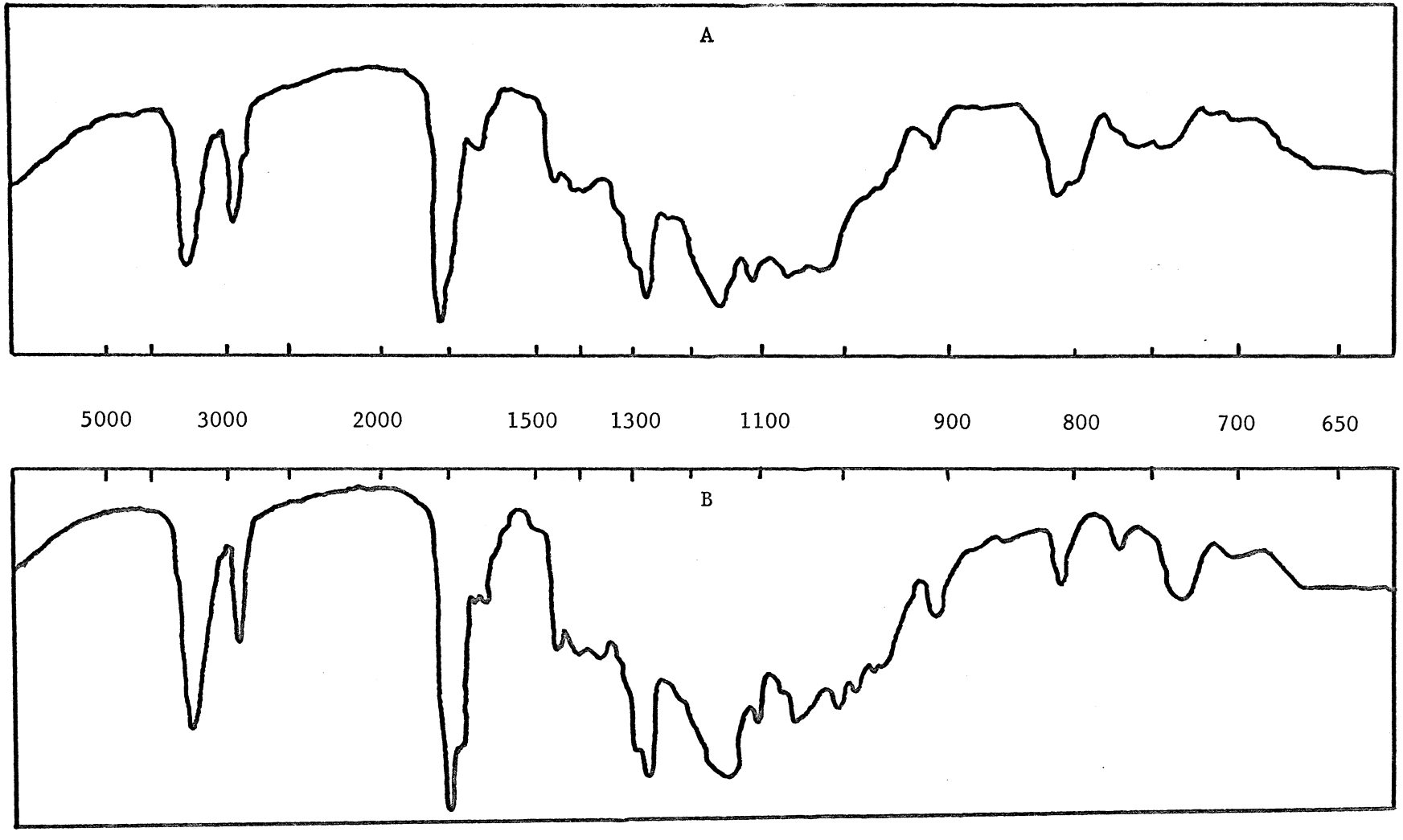

Figure 2 -- A. -- Infrared spectrum of the toxic steroid band from TLC of $CHCl_3$ residue.

B. -- Infrared spectrum of the crystalline ether extract from $CHCl_3$ residue.

(Wavenumbers given in cm^{-1})

10-foot columns of QF-1 (Silicone-Fluoro) and FFAP on Chromasorb-W, all proved unsuccessful. No other columns were available.

To achieve further separation by TLC, the crystalline material from the previous ether extract was again extracted with ether. Although this material had been soluble in ether one week earlier, only about half of it would now dissolve, leaving a brown precipitate which was partially soluble in chloroform. The chloroform-insoluble part was taken up in ethanol. Tests showed only the ether extract contained the poisonous material. Mice suffered acute breathing difficulty and frequent muscular spasms as before, followed by death. Apparently decomposition of the concentrate had taken place, however, since larger doses had to be given to produce death.

To separate the toxic ether extract by TLC, a mixture of 1,2-dichloroethane and methanol (10:1) was used for development. Three bands were visible at Rf values of 0.0, 0.42, and 1.0 respectively. All three were extracted with methanol for 30 minutes in a Soxhlet apparatus. The band at Rf 0.42 was found to contain the toxic material. TLC purity tests showed 3 compounds closely oriented. Redevelopment of this band in a solution of 1,2-dichloroethane and methanol (55:6) gave a satisfactory separation. The band at Rf 0.54 showed positive reactions in the steroid tests listed earlier. Removal from the plate and injection of this compound into mice proved it toxic, with the same symptoms as before. Its infrared spectrum is shown in Fig. 3. No further tests were carried out since all of the available sample was used up.

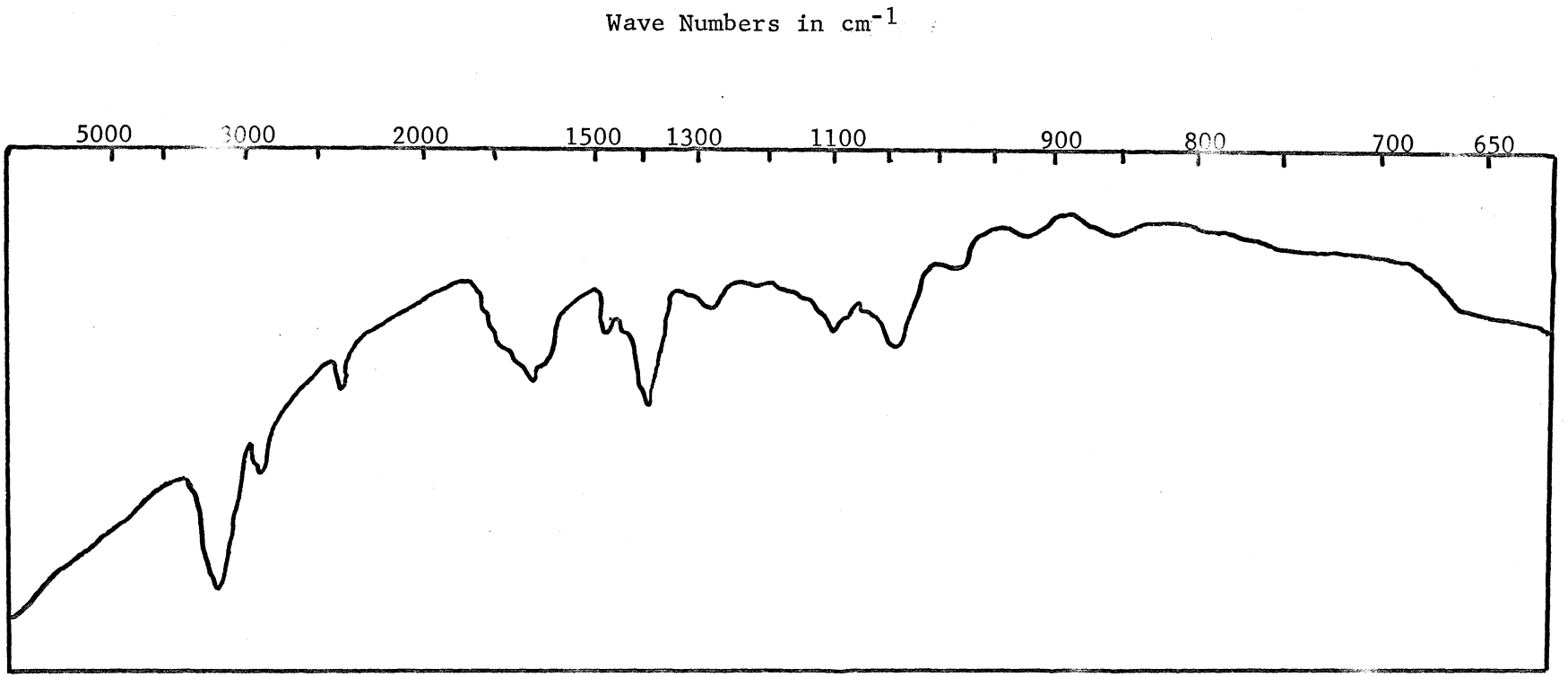

Figure -- 3. -- Infrared spectrum of the toxic compound isolated from Centaurea repens.

DISCUSSION OF RESULTS

The ether extract which was subjected to TLC contained several compounds which were of very similar polarity. During exploratory tests for a suitable solvent, it was found that a change of as little as 0.2% in the concentration of the polar component caused a large change in the mobility and separability of the compounds in the mixture.

Difficulty with separation and the reproducibility of Rf values was also encountered after the same solvent had been used for development of several plates. This was, in part, probably due to changes in concentration resulting from the volatility difference between the methanol and dichloroethane. These changes were even more pronounced when using developing tanks of large capacity, without vapor-tight seals, over a period of several days.

Apparent decomposition of the concentrate throughout the extraction period of seven weeks was evidenced by the necessary mass increase in fatal dosage. This effect would be expected if several physiologically active substances were present and were becoming separated by successive isolation procedures. However, all extracts were tested on mice, some in very large doses; and only the extract containing the isolated compound ever produced any observable physiological effect. Following the final two TLC separations, it was not possible to elute the sample from the silica gel at room temperature, even though a variety of techniques and solvents was employed. Close examination showed a gel-like formation

which had not occurred with more-crude extracts of the sample. Raising the temperature broke down this gel and allowed the extraction of the sample. During the earlier test, TLC-separations elution had been possible by suction filtration at room temperature. Tests showed these fractions toxic, indicating that the active compound had not lodged on the silica gel.

Because of the above-mentioned decomposition and separation problems, coupled with the knowledge of the apparent steroid structure of the poison, future isolations may best be carried out by other methods. For example, precipitation of the steroids as their digitonides could be effected immediately from the ether extract.

SUMMARY AND CONCLUSION

Russian knapweed is a noxious weed both because of its plant-growth inhibiting ability and its toxic effect upon animals. The toxic principle was isolated by solvent extraction followed by thin-layer chromatography. The active compound was monitored throughout the extraction procedure by noting its physiological effects upon mice. Breathing became difficult, slowed, and came in gasps. Muscular spasms were frequent and intense. All except the small doses were fatal.

Several chemical tests showed that the poison was a steroid-type compound.

Analysis of the infrared spectrum in Figure 3 yields further indirect evidence that the toxic material is of steroid structure. Comparison with the spectra of several known steroid compounds shows characteristic absorption bands occurring at 1040-1050 and 1120 cm^{-1}. The band around 1400 cm^{-1} is characteristic of absorption resulting from bending of the angular methyl groups located between the six-membered rings.

The broad band between 1650-1750 cm^{-1} may be accounted for by C = O and C = C bond stretching. Inter-molecular H-bonding is evidenced by the strong absorption band at 3380 cm^{-1}. Lack of strong absorption at 3630 cm^{-1} suggests the absence of O - H groups. However, since the final compound was not of high purity, high resolution of the true spectrum was not possible. Consequently, any structural assignments made on the basis of the I. R. spectrum available must be understood as temporary.

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ABSTRACT

A poisonous steroid-type compound was isolated from Centaurea repens. After extraction with absolute ethanol, removal of the solvent, addition of 2% HCl to precipitate the pigments, and filtering, the pH was adjusted to 9 with aqueous ammonia. This solution was exhaustively extracted with chloroform, evaporated to dryness, and extracted with ethyl ether.

This fraction was chromatographed on silica-gel plates, using 10:1 1,2-dichloroethane-methanol as the developer. The band at Rf 4.2 was removed and found toxic to mice. Labored, slow, gasping respiration and frequent involuntary muscular contractions became apparent within a few minutes following the intraperitoneal injection into each animal. Death resulted after sufficient dosages.

Further separation was achieved by subjecting the Rf 0.42 toxic band to a second development in 55:6 1,2-dichloroethane-methanol. A compound appeared at Rf 0.54 which gave positive color tests for steroid structure with o-phosphoric acid, and 1:50:50 (w/v/v) vanillin-phosphoric acid-water reagents.