CHEMICAL AND TOXICITY STUDIES OF *TRICHODESMA AFRICANUM* L.

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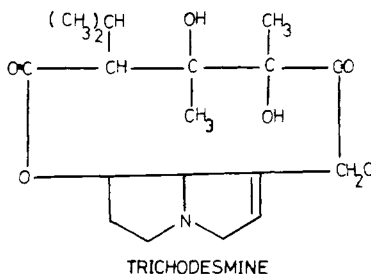
Abstract.—*Trichodesma africanum* L. (Boraginaceae), a desert plant of Africa and Asia, was found to contain β-amyrin, β-methyl oleanate, potassium nitrate and a nitrogen-containing toxin, each producing a notable toxic response when injected in laboratory mice and rats. Muscle contraction and a blood pressure reduction caused by the toxin are described. Pyrrolizidine alkaloids, *e.g.*, trichodesmine, present in other Trichodesma species were also identified in the material by tlc and spectrometric methods. These did not, however, produce the acute toxic effects observed for the nonalkaloid compounds mentioned above. β-Sitosterol and stigmasterol were identified as the principal sterols in the plant.

The causative effects of pyrrolizidine alkaloidosis (1) (Suiljuk, Winton and Picton Disease)[1] among livestock have been attributed (2–4) to the widespread occurrence of pyrrolizidines in several species of the Boraginaceae family, some of which grow in animal grazing areas of desert regions in parts of the Middle East, Africa and Asia. The above-ground parts of *Trichodesma africanum* L., when eaten by animals, have been implicated as a causative agent of this toxicity.

The alkaloid chemistry, including spectral data, and toxicity of this and other Trichodesma species have been reviewed by Culvenor (5), Bull (6), and Zalkow (7).

Asian folklore medicine records (8) the use of the leaves from this plant in the form of water decoctions and infusions for their purgative effects in humans. It is also said that when made into a paste, the roots may be applied to the swollen joints and body surfaces to reduce swelling and inflammation. The aqueous infusion of the leafy parts are also reported to relieve dysentery in children.



TRICHODESMINE

Since the literature has thus far failed to report other than the toxic alkaloid chemistry of the *Trichodesma* species, a more extensive investigation of other constituents in its above-ground parts was made. Two saponins of the olean-12-ene type, potassium nitrate and a nitrogen-containing toxin were isolated. Each was found to produce marked acute toxicities when injected intraperitoneally in mice and rats. The toxin, which is a peptide containing 11 amino acids, caused smooth muscle contraction and a significant drop in blood pressure. These effects were observed to be unlike those reported for pyrrolizidine alkaloidosis.

EXPERIMENTAL[2]

[1]Cytotoxicosis of liver and lung tissues of livestock following ingestion of plants containing pyrrolizidine alkaloids over a long period of time (8 months).

[2]Melting points (uncorrected) were performed on a Koffler hot-stage apparatus fitted with a Bausch and Lomb 100-power microscope for observation; ir spectra were taken on Perkin-Elmer model 457 grating ir spectrometer; nmr spectra were recorded with a Joelco 60 MHz spectrometer. Mass spectra were recorded with a Varian Mat CH-5 instrument. Evaporations were carried out under reduced pressure with rotavapor, R-110 (Buchi); gc was recorded on a Varian 1400.

PLANT MATERIAL.—*Trichodesma africanum* L. was collected in Egypt from the desert of Waadi Hoaf, Maadi, Cairo, during the summer of 1979. A voucher specimen was deposited in Orman's Garden Herbarium, University of Cairo. Its identification was confirmed by Professor N. El-Hadidi, Faculty of Science, University of Cairo.

EXTRACTION AND ISOLATION.—During a phytochemical screening (9) of *T. africanum* L., aqueous extracts, in particular, produced marked acute toxicity in mice and rats when injected intraperitoneally. These effects were unlike any reported thus far for the trichodesma alkaloids when similarly injected or used orally. The dried and powdered (40 mesh) above-ground parts of this species were then extracted with different solvents, as described below, in order to determine the causative agent(s) for such effects. Constituents were isolated by chromatography and identified by spectral and other methods. In addition to the alkaloids, saponins, a peptide toxin and inorganic nitrate previously mentioned, the non-toxic substances, β-sitosterol and stigmasterol, were isolated from chloroform fractions by tlc performed according to the methods of Duncan (10). These compounds were identified by the preparation of their acetates and were compared spectrometrically with authentic samples.

ALKALOIDS.—A pyrrolizidine alkaloid mixture was obtained from the ethanol and chloroform extracts by the methods of Culvenor (5) and Zalkow (7). The yield was 0.35% w/w. Six spots were obtained on silica gel G plates; a solvent system of chloroform-methanol and Dragendorff's spray reagent (90:10) were used. Column and preparative tlc chromatography of this mixture yielded principally one purified alkaloid, trichodesmine, fig. 1, as confirmed by the literature (5), (11), (12) in a yield of 14 mg/1 kg of dried plant material. The mass spectrum of this alkaloid showed a molecular ion peak at *m/e* 353. It melted at 160–161°. The ^{1}H nmr spectrum showed signals at 0.81 (3H, d), 0.96 (3H, d), 1.92–3.55 (7H, m), 3.84 (1H, q), 4.35 (1H, broad), 4.65 (2H) and 5.72 (1H) ppm.

Toxicity tests revealed that the purified pyrrolizidine alkaloid mixture in doses of 10–160 mg/kg and trichodesmine in doses of 5–50 mg/kg had no acute toxicity in a period of 72 hrs as compared with other constituents (e.g., saponins, KNO_3 and a purified toxin containing nitrogen) when similarly injected in mice and rats.[3] Five groups of mice were used with each group consisting of 3–5 mice. All animals were fasted overnight before the ip injections of alkaloids in soybean oil. Also, two groups of rats (each groups consisting of 3 rats) were injected with 80 and 160 mg/kg of the total alkaloidal fraction in soybean oil. All of the animals injected survived over the 72 hours observation time without apparent gross toxic effects. It was concluded that the alkaloid mixture was not responsible for the acute toxicity observed for the other constituents.

SAPONINS.—One kg of defatted dried powdered material was extracted by the methods of Kawai (13) and Wall (14). The acetone extract was evaporated under reduced pressure, whereupon 15 g of the residue were mixed with 60 ml of distilled water and shaken, then filtered through glass wool. The filtrate was then extracted with butanol saturated with water. The butanol extract was evaporated until dryness; 240 mg of a yellowish white powder was obtained. The residue remaining gave positive tests for saponins (15). Tlc of the residue, when developed on silica gel G plate with a solvent system of chloroform-methanol (65:35) and sprayed with sulfuric acid, revealed seven spots. Separation of the butanol extract was made on a silica gel G column with a solvent gradient of chloroform and chloroform-methanol up to (4:1) v/v. The collected fractions were purified on a preparative tlc solvent system. Two pure saponins were obtained in yields of 65 and 42 mg and named saponin A and saponin B, respectively. Saponin A was obtained as an amorphous white powder, mp 270–275°. The ir spectrum showed a peak at 3400 cm^{-1} (OH). Saponin B was also obtained as an amorphous white powder, mp 210–215°. The ir spectrum showed peaks at 3400 cm^{-1} (OH) and at 1710 cm^{-1} (C=O). The aglycone of saponin A was obtained by the method of Crowell (16) using 2% sulfuric acid in 10 ml methanol and 10 ml water and refluxing for 6 hours. Forty mg of saponin A yielded 22 mg of a white amorphous powder which was recrystallized from methanol to give white prism crystals, mp 195–198°. The ir spectrum of the aglycone of saponin A showed a peak at 3400 cm^{-1} (OH). The mass spectrum showed a molecular ion peak at *m/e* 426. Aglycone A was identified as β-amyrin when spectral comparisons were made with an authentic sample. Its mp was unchanged on admixture with the authentic material.

The sugar part of saponin A was identified by chromatography performed with Whatman paper #1 and a solvent system of *n*-butanol-pyridine-water (60:20:10), and spraying with aniline phthalate reagent. Glucose, arabinose and rhamnose were determined by comparing chromatograms with authentic sugars. These were confirmed by gc analysis. Moles of each sugar and their position/sequence in the glycoside were not quantitated.

Thirty-two mg of saponin B yielded 17 mg of white tiny crystals (mp 200–203°) from methanol after the acid hydrolysis. The ir spectrum showed peaks at 3400 cm^{-1} (OH) and at 1710 cm^{-1} (C=O). The mass spectrum showed a molecular ion peak at *m/e* 470. The aglycone of saponin B was identified as the methyl ester of oleanolic acid when spectral comparisons were made with an authentic sample. Its mp was also unchanged on admixture with an authentic sample of methyl ester of oleanolic acid. The sugar part of saponin B was found to be the same as for saponin A when similarly analyzed. Moles for each sugar and position in the glycoside were not quantitated.

The toxicity and the LD50 of the saponin mixture were determined by the Litchfield and Wilcoxon method (17). Five groups of mice, 5 mice in each group, were injected ip with doses

[3]Male mice (Crl:CD(SD)BR) weighing 30–50 g and male rats (Crl:CD-1(ICRBR) weighing from 150–500 g were obtained from the Charles River Breeding Laboratories, Wilmington, Massachusetts.

from 20–70 mg/kg. The LD50 of the mixture was 51.2 mg/kg and 95% confidence limits= 44.0–59.5 mg/kg.

Symptoms of the saponin toxicity were observed to be signs of abdominal irritation and marked depression. Some mice showed a blue color in the tail, indicating cyanosis. On necropsy, some of the animals showed internal bleeding of intestinal tissues. Pure saponin A was similarly tested in mice in doses of 10–30 mg/kg and the LD50 and its 95% confidence limits were determined to be 22.4 (16.8–29.9) mg/kg. Symptoms of saponin A toxicity were observed as abdominal pain. All mice showed marked depression and diarrhea. Respiration was shallow but rapid. The depression was progressive until all the animals became comatose with death resulting.

TRICHODESMA TOXIN.—Defatted plant material (500 g) was macerated with 1.5 liters of distilled water with the aid of gentle heating. The extract was filtered and concentrated under reduced pressure to a semisolid syrupy residue (186 g). The residue was then packed in a dialysis tube No. 6,000–8,000 and dialyzed in a jar containing distilled water. The dialyzate was rejected and the non-dialyzable portion transferred to another dialysis tube no. 12,000–14,000 and dialyzed again in a jar containing distilled water. This was repeated with an ultrafiltration apparatus[4] with a type 20UM Amicon UF membrane under pressure of nitrogen; the same results were obtained. The dialyzate, after complete dialysis, was concentrated under reduced pressure to a semisolid syrupy solution (20 g). This was then dissolved in 200 ml of distilled water. Preliminary tests showed this fraction to possess acute toxicity when injected ip into laboratory animals. To the 200 ml of the dialyzate, 750 ml of 95% ethanol were added and a flocculant precipitate appeared. A yellowish white, water soluble amorphous powder was produced (180 mg) which was odorless and tasteless and melted at 220–224°. It was insoluble in organic solvents (e.g., chloroform, acetone and methanol) and possessed uv max at 280 nm. The microanalysis[5] showed 43.37% carbon, 6.48% hydrogen and 8.31% nitrogen. Its empirical formula is suggested to be $(C_6H_{11}NO_5)_{80}$.

On acid hydrolysis of 50 ml of this substance in 6N hydrochloric acid and refluxing for 12 hours, the hydrolyzate was chromatographed on tlc of silica gel G with a solvent system of methyl ethyl ketone-pyridine-water-acetic acid (70:15:15:12) v/v. The plates were developed by spraying with 0.3% ninhydrin in butanol. From the tlc it was revealed that the hydrolyzate contained 11 amino acids (lysine, tryptophane, tyrosine, leucine, phenylalanine, serine, glutamic acid, alanine, isoleucine, proline and valine) when compared with the authentic amino acid samples. The results were confirmed by an amino acid analyzer.[6] No other residues were detected.

For preliminary testing, 13 groups of mice, each consisting of five mice, were injected ip with toxin in a series of doses from 5.0–42.5 mg/kg. Calculated by the Litchfield-Wilcoxon method, the LD50 values after 24 and 48 hours were found to be 36.1 (33.9–38.4) and 23.8 (22.2–25.7) mg/kg, respectively. The small quantity of toxin precluded further toxicity studies by oral administration at this time.

The major symptom of the acute toxicity was a severe depression in all animals after the ip injection. Respiration initially deepened and accelerated but later became progressively weaker, then irregular before finally ceasing. Weakness and twitching were followed by an inability of the test animals to stand. Death soon followed without movement.

In view of these preliminary findings, the toxin was then subjected to a series of qualitative tests involving blood pressure, hindleg blood vessel perfusion and intestinal muscle:

1) *Blood pressure:* Rats weighing from 200–300 g were prepared according to the method of Gillespie (18) using urethan and phenobarbital for sedation. Trichodesma toxin in doses from 2–12 mg/kg was given by iv injection. Each dose, repeated four times, produced a marked fall in pressure after a 5-hour period. The initial drop in blood pressure of 50 to 75 mm/Hg returned almost to the normal level in about 5 minutes, then gradually decreased over a period of 5 hours to a level of 30–40 mm/Hg from which animals at low doses recovered. Those animals given doses of 6 mg/kg and higher expired. The lethal dose varied between 6 to 12 mg/kg. When different drugs were used to interact with the toxin action, it was noted that the toxin did not antagonize epinephrine (0.2 mg) or methoxamine (0.2 mg) but appeared to be partially blocked by atropine (0.06 mg). There was no apparent change in heart rate as measured by ECG recordings.[7]

2) *Rat Hindleg Perfusion:* This test was performed by the classic methods of Burn (19). A marked vasoconstrictor action resulted with doses from 4–12 mg of toxin during which the normal perfusion rate was reduced in all experiments by 50–75%. This experiment showed a very potent vasoconstrictor action in which muscle responses failed to return to normal amplitude and tone, even following sodium nitroprusside injection (0.1 mg).

3) *Intestinal Muscle:* This test was based on the classic methods of Magnus (20). Segments of rat ileum were suspended in a bath (70 ml) containing Ringer's solution and gassed with a mixture of 95% oxygen and 5% carbon dioxide at a temperature of 37.5°. The muscle was attached to a force transducer connected to a Physiograph recorder. Two mg doses of toxin were added to the bath. In all experiments, a marked muscle contraction occurred.

From these preliminary toxicity studies, it is difficult to determine the primary mechanism of this Trichodesma toxin. Pharmacological studies are in progress to determine the actual mechanisms producing muscle contraction, vasoconstriction and reduction in blood pressures.

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[4]Amicon-52, Amicon Corp., Lexington, Massachusetts 02173.
[5]Micro-Analysis, Inc., P. O. Box 5088, Wilmington, DE 19808.
[6]Amino acid analyzer, Model D-500 DuPont, Wilmington, DE 19808.
[7]Physiograph, Narco Biosystem Inc., PMP-4B, Houston, Texas.

POTASSIUM NITRATE.—One kg of powdered above-ground parts of *Trichodesma africanum* L. was defatted with petroleum ether (bp 60–80°), then extracted with chloroform in a continuous extraction apparatus (Soxhlet). The marc was then extracted with 95% ethanol by percolation. Every 48 hours, the ethanol was changed and replaced by more 95% ethanol. This method was repeated many times until complete exhaustion. The ethanolic extracts were combined and concentrated under reduced pressure to a semisolid, sticky residue (12 g). The residue was packed on alumina G. column (60 x 1.5 cm) prepared from a slurry of alumina in benzene. Development of the column was carried out with solvents of increasing polarity, i.e., starting with benzene then chloroform-methanol (90–10) and finally with 100% methanol. A precipitate of potassium nitrate in good yields was obtained from the methanol fractions after evaporation and concentration. A white crystalline potassium nitrate was obtained after further recrystallization from absolute ethanol. Qualitative tests for its identity were carried out by the methods of Layde and Busch (21).

The inorganic nirate was injected ip in 4 groups of mice (each with three mice) in doses of from 45–360 mg/kg. The lethal dose was determined to be approximately 180 mg/kg. Symptoms of the poisoning were abdominal pain and diarrhea probably caused by the irritant action of the nitrate ion (22). These effects were accompanied by muscular weakness and loss of coordination. In some mice cyanosis occurred preceding coma and death. Similar effects were observed in rats with doses of 100 and 200 mg/kg.

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