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Characterization of a toxin from *Parthenium hysterophorus* and its mode of excretion in animals

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Abstract. Fractionation of methanolic extracts of air dried aerial parts of *Parthenium* resulted in the isolation of a toxic constituent which was identified as parthenin, the major sesquiterpene lactone from the weed. The LD_{50} (minimal lethal dose required to cause 50% mortality) for parthenin in rats was 42 mg/kg body weight. When [^{3}H]-parthenin was given orally or by intravenous administration, radioactivity appeared in the milk of lactating laboratory and dairy animals. Tissue distribution of radioactivity revealed that maximum label was detectable in kidneys.

Keywords. Compositae; parthenin; sesquiterpene lactone; $LD_{50;}$ toxicity; organelle drug distribution.

Introduction

Several species of the compositae family are known to be toxic when consumed by animals (Rodriguez *et al.,* 1976; Narasimhan *et al.,* 1977). Incorporation of *Parthenium hysterophorus* into the diet of live stock was found to cause chronic or acute toxicity depending upon the quantum of the weed ingested (Narasimhan *et al.,* 1980). Since no information is available in literature on the chemical constituents of *P. hysterophorus* responsible for vertebrate poisoning, studies were undertaken on the characterization of the toxic principle from the weed and fate of the administered toxin in animals. The results of these studies are presented in this communication.

Materials and methods

Materials

Diphenylamine and calf thymus DNA were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. An authentic sample of parthenin was a gift from Dr. Eloy Rodriguez, University of California, Irvine, California, USA. Parthenin was custom

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Abbreviations used: TLC, Thin layer chromatography; NMR, nuclear magnetic resonance.

labelled with tritium at Bhabha Atomic Research Center, Bombay. All other reagents and solvents used were of analytical grade available commercially.

Animals

Guinea pigs and rats were supplied by the Central Animal Facility of the Indian Institute of Science, Bangalore. A lactating cow which was on an approved nutritional regimen (Maynard *et al.,* 1978) was made available for the experiment by a livestock farm.

Isolation of the toxin

Five hundred g of air dried aerial parts of *P. hysterophorus* were ground into a fine powder and extracted continuously with 1·5 litres of methanol in a Soxhelet's apparatus for 12 h. The resulting dark brown extract was evaporated to dryness in a flash evaporator at room temperature and the residue was designated as Fraction I. This fraction was continuously refluxed with petroleum ether which constituted Fraction II. The residue from Fraction I was further extracted successively with chloroform and ethanol to obtain Fraction III and Fraction IV, respectively. Fraction III was evaporated to dryness, dissolved in hot ethanol and the volume was made upto 200 ml. After cooling, equal volumes of 4 % aqueous lead acetate solution was added to this ethanol extract, allowed the mixture to stand at room temperature for 20 min and the resultant precipitate was removed by filtration through a pad of diatomaceous earth. The clear filtrate was concentrated to half its volume to remove ethanol. The aqueous solution thus obtained was extracted with 50 ml portions of chloroform. The pooled chloroform extract was dehydrated with anhydrous sodium sulphate and evaporated to dryness *in vacuo* to obtain Fraction V. The residue was dissolved in 15 ml of a solvent consisting of benzene and acetone (2:1) and subjected to silica gel column chromatography. The column (3 ×55 cms) was equilibrated with benzene and loaded with Fraction V. After washing with 200 ml of benzene, the column was eluted with 200 ml portions of a solvent containing increasing amounts of acetone in benzene (10–100 %). The fractions (200 ml each) were evaporated to dryness and analysed by thin layer chromatography (TLC) on silica gel-G plates using benzene: acetone (4:1) or chloroform: diethylether (5:1) as solvent system. The compounds were located on TLC plates by exposing to iodine vapours or by spraying with 5% aqueous potassium permangenate. The fractions yielded, one major and a few minor compounds which were detectable on TLC plates. The fractions that contained the major compound were pooled and subjected to preparatory TLC using benzene: acetone (4:1) as the solvent system. The area corresponding to the major compound on individual TLC plates was scraped and eluted with chloroform. The pooled chloroform eluate was evaporated to dryness and the residue dissolved in a minimal amount of ethyl acetate. The compound was twice crystallized by dropwise addition of cyclohexane in cold to obtain the purified toxin (Fraction VI).

Assessment of LD_{50} of different fractions

In order to assess the lethality of different fractions obtained during purification of the toxic principle(s) from *P. hysterophorus,* 10 rats were used for each fraction. Different

doses of each fraction, ranging from 100 to 1000 mg/kg body wt. were administered intraperitoneally. Number of surviving animals in each group was recorded over a 7-day period. Animals that did not receive any test substance served as controls. The dose which brought about 50% mortality (LD_{50}) was determined by the method of Litchfield and Wilcoxin (1949) as well as by the probit method of Finney (1952).

Distribution of administered parthenin

Excretion and tissue distribution: One lactating cow and three guinea pigs respectively, were administered intravenously or intracardially with [^{3}H]-parthenin (specific activity 4·9 Ci/mmol: 500 μCi/kg body wt.). Samples of blood, milk, urine and faeces were collected at different intervals (0–72 h). To aliquots (10–50 μl) of samples other than faeces, 10 ml of scintillation fluid (methoxyethanol: toluene (1:1) containing 0·5 % PPO and 0·05 % POPOP) were added and the radioactivity was determined in a LKB, Rackbeta counter. Faecal samples (0·1–0·5 g) were homogenized in 3 ml water and passed through a 100 mesh sieve. Aliquots of the suspension (50 or 100 μl) were used to measure the radioactivity.

To follow the distribution of parthenin after oral administration, [^{3}H]-parthenin (1 mCi/kg body wt.) was given orally through an infant tube to 3 lactating guinea pigs and distribution of the label was followed as described above.

After 72 h, the animals were sacrificed by exsanguination and different organs were collected. Tissue samples (0·5–1·0 g) were homogenised in 2–5 volumes of 0·15 M NaCl. An aliquot (50–200 μl) of the homogenate was used for the determination of radioactivity.

Subcellular distribution: To elucidate the distribution of [^{3}H]-parthenin into sub-cellular fractions, 3 rats were administered intracardially with [^{3}H]-parthenin (500 μCi/kg body wt.) in phosphate buffered saline (pH 7·4). After 48 h, the animals were sacrificed by cervical dislocation, liver and kidney were collected. Mitochondrial, microsomal and cytosolic fractions were isolated by differential centrifugation (Hogeboom and Schneider, 1950; Johnson and Lardy, 1967). Nuclei were isolated as described by Wang (1967). Protein in samples was quantitated by Biuret method (Gornall *et al.,* 1949) and DNA was estimated by the procedure outlined by Burton (1956). Aliquots of each fraction (100–200 μl) were used for the determination of radioactivity.

Results

Purification of toxin from Parthenium hysterophorus

The toxicity of different fractions obtained during purification of the toxic principle from *Parthenium* weed is presented in table 1. Animals which received only water (controls) or those administered with Fraction II (petroleum ether) or Fraction IV (ethanol fraction) revealed no mortality when observed over a period of seven days. Fractions I and III (methanolic and chloroform fractions) on the other hand, were lethal to rats. Fifty per cent mortality was observed with these fractions at doses ranging from 200–300 mg/kg body wt.

Table 1. Toxicity of various fractions obtained from *P. hysterophorus* to rats*

Fraction	Intraperitoneally administered dose (mg/kg body wt.)					
	100	200	300	400	500	600
	Animals died/Tested					
I	1/10	2/10	5/10	7/10	9/10	10/10
II	0/10	0/10	0/10	0/10	0/10	0/10
III	0/10	2/10	5/10	8/10	10/10	10/10
IV	0/10	0/10	0/10	0/10	0/10	0/10
V	8/10	NT	NT	NT	NT	NT
VI	10/10	NT	NT	NT	NT	NT

NT: Not tested.
* Ten per cent stock solutions of Fractions I, II, III, IV and 2 % stock solutions of Fractions V and VI were prepared in ethanol. Aliquots of the stock solutions were diluted with water to obtain the desired doses for intraperitoneal administration. The total volume administered was 2 ml. None of the control animals (10 numbers) which were injected with equal volume of water and ethanol died during the experimental period which extended for 7-days.

As shown in table 2, LD_{50} analysis of different fractions by the method of Litchfield and Wilcoxin (1949) yielded two dose limits *viz.,* one on the higher side and the other on lower side (330 and 273; 302 and 274; 60 and 48; 42 and 32 mg/kg body wt., respectively, for Fractions I, III, V and VI). Probit method of Finney (1962) yielded LD_{50} values of 313, 278 and 51 and 47 mg/kg body wt., respectively with Fractions I, III, V and VI.

Table 2. LD_{50} assessment of different fractions from *P. hysterophorus* in rats*.

Fractions	Litchfield and Wilcoxin Method		Finney's Method
	Lower Limit	Upper Limit	
I	272·73	330·00	313
III	274·29	302·40	278
V	48·29	59·50	51
VI	31·46	42·34	47

* Dose—mg/kg body wt.
Route of administration—intraperitoneal
20 rats were used to test the lethality of the above fractions at different concentrations. (5 to 500 mg/kg body wt.)

Identification of toxin

The purified toxin (Fraction VI) was identified as the sesquiterpene lactone parthenin (figure 1) by comparing its properties with those of an authentic sample. Both isolated toxin and parthenin melted at 166°C and had ultra-violet absorption maxima at 215

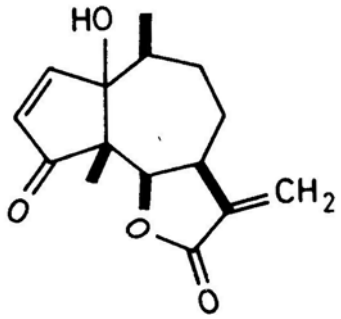

Figure 1. Structure of parthenin.

and 340 nm. The infra-red bands were at 3450,1755,1408,1592 and 1655 cm^{-1}. The nuclear magnetic resonance (NMR) spectrum of the isolated toxin (parthenin) in $CDC1_3$ is shown in figure 2. ^1H NMR (270 MHz, $CDCl_3$ with tetramethyl silane): 7·50 (*d,* H_2), 6·25 (*d,* H_3), 6·33 (*d,* H-13b), 5·61 (*d,* H-13a), 5·02 (*d,* H-6), 1·35 (*d,* C-10 Me). These values correspond to those reported for parthenin by Herz *et al.* (1962).

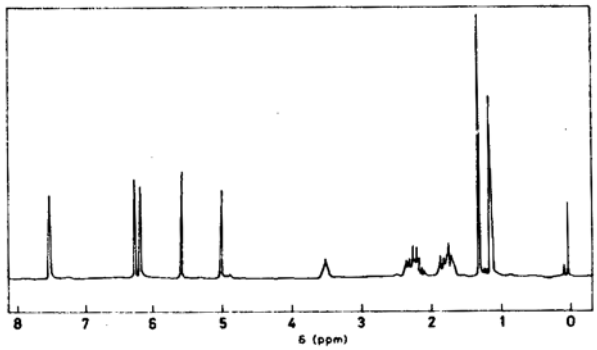

Figure 2. 270 MHz [^1H]-spectrum of parthenin in $CDCl_3$.

Distribution and excretion of parthenin

Excretion in urine: The time course of excretion of [$_3H$]-parthenin after administration to lactating guinea pigs and a cow by parentaral or oral route, is shown in figure 3. It is apparent that administration of parthenin intracardially to guinea pigs and intravenously to a cow resulted in its excretion in urine within 30 min. Urinary excretion of orally administered parthenin by guinea pigs reached a maximum after 5 h and later declined.

Excretion in faeces: No significant amount of radio-label from intravenously administered [^3H]-parthenin was detectable in the faeces of cow. Marginal amount of radio-activity could be detected in faecal samples collected after 5–7 h from guinea pigs injected intravenously with [^3H]-parthenin. Oral administration, however, resulted in excretion of the label in the faeces of guinea pigs upto 72 h with the maximum around 7 h (figure 4).

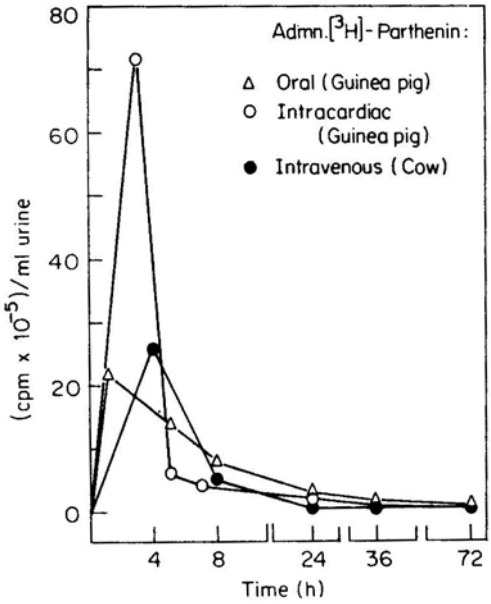

Figure 3. Urinary excretion of [^{3}H]-parthenin in experimental animals.

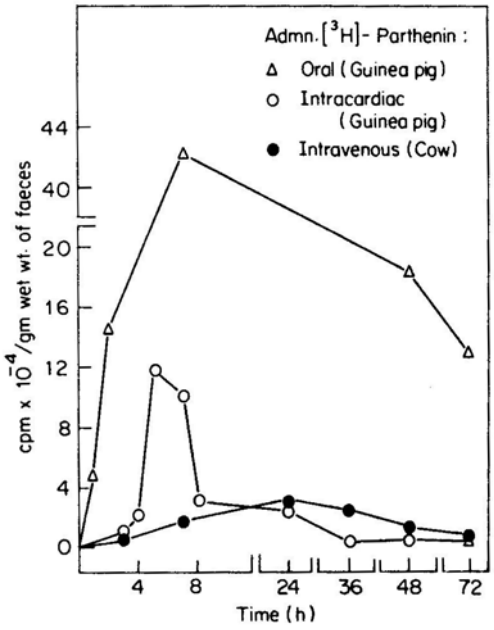

Figure 4. Excretion of [^{3}H]-parthenin in faeces of experimental animals.

Excretion in milk: Administration of labelled parthenin intracardially to guinea pigs resulted in the detection of radioactivity in milk as early as 1 h, while in a lactating cow maximum radioactivity was detectable after 5 h. Oral administration of the labelled compound to guinea pigs resulted in the detection of radioactivity in milk after 1 h and the label continued to be excreted even after 72 h (figure 5).

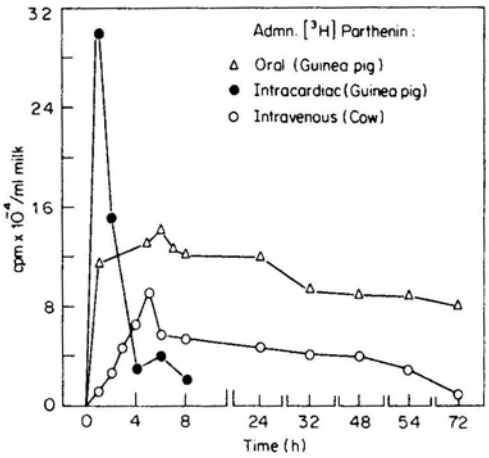

Figure 5. Excretion of [^{3}H]-parthenin into the milk of experimental animals.

Appearance of [^{3}*H*] *parthenin in blood:* The appearance of [^{3}H]-parthenin in the blood of guinea pigs administered orally with the labelled sesquiterpene lactone was detectable at the end of second hour. The maximum radio label, however, could be measured after 8 h followed by a gradual decline (figure 6).

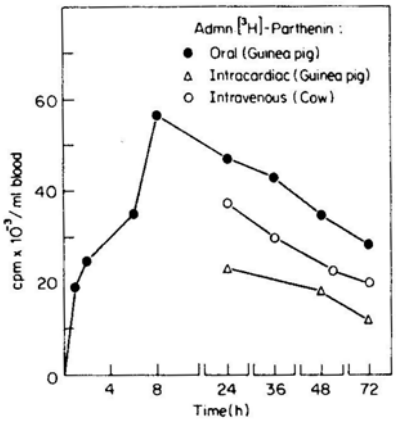

Figure 6. Blood levels of [^{3}H]-parthenin in experimental animals.

Tissue distribution of [^{3}H]-*parthenin:* Measurement of radioactivity in samples of tissues collected from guinea pigs and a cow administered orally or parentarally with [^{3}H]-parthenin after 72 h revealed that maximum radioactivity was detectable in kidney and liver (table 3). Similarly, bile collected at this time also had considerable radioactivity. The active mammary tissue too, incorporated the label in all the animals (12,000–16,000 cpm/mg protein). A comparison of the distribution of radioactivity in different internal organs revealed that spleen contained the least amount.

Table 3. Distribution of [^{3}H]-parthenin in different organs.

	cpm/mg protein		
Tissue	Guinea pig$^+$	Guinea pig^{++}	Cow^{+++}
Liver	12200	15230	18880
Kidney	24950	38870	74200
Heart	2336	18870	10200
Spleen	1092	1020	1070
Bone marrow	13165	13870	13900
Mammary gland	12240	13410	16340
Skeletal muscle	4959	7660	6700
Lungs	1764	2031	3888
Ovaries	2601	2583	4880
Intestines	9650	8914	9570
Bile†	3094	3701	3816

† cpm/ml
Route of administration: $^+$Oral; $^{++}$Intracardiac; $^{+++}$ Intravenous.

From table 4 it is apparent that mitochondrial fractions obtained from both kidney and liver of rats administered intra-cordially with [^{3}H]-parthenin had highest amount of radioactivity. Microsomal and cytosolic fractions of rat liver contained almost similar quantities of the label, while similar fractions prepared from kidneys had higher radioactivity. The per cent distribution of radioactive label in the kidney and liver of rats administered with [^{3}H]-parthenin by intracardiac route was 9·4 and 6·6, respectively.

Table 4. Subcellular distribution of [^{3}H]-parthenin in rat liver and kidney.

	cpm/mg protein	
Organelle	Liver	Kidney
Mitochondria	14673 ± 1421	22469 ± 387
Microsome	4941 ± 388	8584 ± 479
Cytosol	4852 ± 112	21532 ± 432
Nuclei*	43648 ± 2362	53426 ± 2119

* cpm/mg DNA

Discussion

Fractionation of crude methanolic extracts of *P. hysterophorus* and the evaluation of the isolated fractions for their toxicity in rats, resulted in the identification of parthenin, the major sesquiterpene lactone from the weed as the toxin (figure 1). Although parthenin constituted the major component of the terpene fraction (Fraction V), the toxicity of the minor sesquiterpene constituents of this fraction was not evaluated. However, the increase in the toxicity of parthenin (Fraction VI; LD_{50} 42·3 mg/kg body wt. (table 2) over the terpene fraction (Fraction V; LD_{50} 59·5 mg/kg body wt.) which contained 75 % as parthenin, seems to rule out the possibility of the presence in *P. hysterophorus* of toxic constituents other than parthenin.

The toxicity due to parthenin observed in the present study supports the earlier findings that sesquiterpene lactones contribute to the toxicity of several poisonous species of Compositae to livestock. Witzel *et al.* (1976) isolated a sesquiterpene lactone, helenalin from *Helinium microcephalum* and demonstrated its toxicity to sheep, mice, rats, rabbits and hamsters. Ivie *et al.* (1975a, b) and Kim *et al.* (1975) have also reported that hymenovin, hymenoxan and tenulin were toxic to animals.

The LD_{50} values for different sesquiterpene lactones range between 3 and 150 mg/kg body wt. (Ivie *et al.,* 1975a; Kim *et al.,* 1975; Kim, 1980). From the results of the present study it is apparent that the LD_{50} for parthenin was 42·3 mg/kg body wt. (table 2).

Hill and his coworkers (Hill, 1977, Hill *et al.,* 1980) observed that oral or intravenous administration of [^{3}H]-hymenoxan to rabbits resulted in maximum excretion of the label through urine and bile. Biological half life of the orally administered sesquiterpene lactone (hymenoxan) in blood was estimated to be 9 h. Parthenin also had a similar biological half life in blood (8 h, figure 6).

The rapid excretion of [^{3}H]-parthenin in the urine of all the experimental animals supports the earlier observation of Hill (1977) on the pattern of urinary excretion of hymenoxan in animals. He has also observed that accumulation of the toxin was maximal in kidneys. In the present study also, label from [^{3}H]-parthenin after administration was concentrated more in kidney and liver than in other organs (table 3). Presence of higher amounts of parthenin in these vital organs substantiates the degenerative changes in liver and kidney of *Parthenium*-fed animals observed by histopathological studies (Narasimhan *et al.,* 1980). Ishida *et al.* (1980) reported that sesquiterpene lactones were excreted as biotransformed glucuronides in both urine and bile of experimental animals. In the present study, however, no attempt was made to follow the metabolic fate of the administered parthenin.

Oral or intravenous administration of [^{3}H]-parthenin resulted in the detection of radioactivity in the milk of guinea pigs as early as 1 h, while in the cow, the excretion of the radio label into milk was observed 2 h after oral administration. The excretion of sesquiterpene lactones in the milk of cattle is well documented (Chopra *et al.,* 1965; Dupont and Adda, 1978). The current studies with [^{3}H]-parthenin do not exclude the possibility of excretion of parthenin into the milk of dairy animals that occasionally feed on *Parthenium* weed.

It is well established that parthenin is the contact allergen responsible for *Parthenium* dermatitis in humans (Lonkar *et al.,* 1974; Subba Rao *et al.,* 1978). It has been experimentally shown that in buffaloes, dermatitis could be induced by oral administra-

tion of aqueous extracts of *Parthenium* and these animals, like humans, elicit delayed hypersensitive reaction to parthenin (Subba Rao *et al.,* 1979). The results presented in this paper established that parthenin, the major sesquiterpene lactone from *P. hystertophorus* known to be a contact allergen to humans is also a potent toxin responsible for the manifestation of *Partheniosis* (Narasimhan *et al.,* 1980) in livestock fed on the weed.

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