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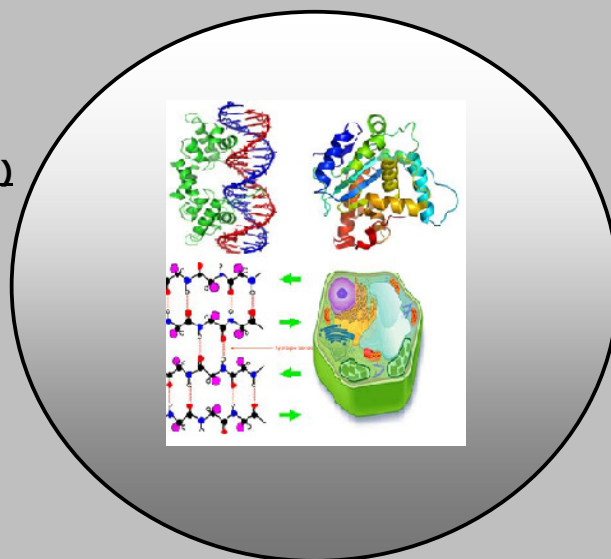
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Bioactive and toxic products found in *Euphorbia* sp

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ABSTRACT

Euphorbia species contain methyl esters and derivatives as well as diterpene polyesters and other terpene compounds. The oil consists mainly of sesquiterpenes, and a small percentage of monoterpenes and aliphatic compounds. Eleinol (57.5%) was found to be the major constituent among the 24 compounds identified in Euphorbia teheranica Boiss. Other constituents found in Euphorbia species include ingenol 3-angelate kaempferol, scopoletin, kaempferol 3-O-glucopyranoside, quercetin, vanillic acid. Domestic sheep and goats can consume considerable amounts of leafy spurge and are used to help control it, but even these species may suffer a toxic response at high levels of intake.
We conducted bioassay-guided fractionations of leafy spurge in an attempt to isolate toxic and aversive compounds. These bioassay-guided fractionations led to identification of ingenol and one of its diesters as two toxic compounds in leafy spurge that are potentially aversive to cattle. In an animal study, mice with chronic oral treatment of a polysaccharide extract from Euphorbia kansui had elevated enzymatic activities of superoxide dismutase and glutathione peroxidase accompanied by a corresponding decrease in malondialdehyde after strenuous exercise
Keywords—Euphorbia species, leafy spurge,polysaccharide, toxic compound, Cattl

INTRODUCTION

Euphorbia is reported as having anti-tumor properties. Of 12 terpene compounds derived from the roots of *Euphorbia kansui* eight of these compounds showed significant inhibition of cellular proliferation at low concentrations in Xenopus embryo cells. Four of the terpenes inhibited cellular proliferation only at higher concentrations. It was also found that most of the diterpene compounds that inhibited cellular proliferation also inhibited topoisomerase II activity. Another species, *Euphorbia tirucalli* L., significantly enhanced survival and concurrently reduced tumor growth in the peritoneal cavity in mice.

It is thought that the modulatory effect of *Euphorbia tirucalli* L. on myelopoietic response and on the levels of prostaglandin E (2) may be related to its antitumor activity as a possible mechanism for the regulation of granulocyte and macrophage production and expression of functional activities. Jolkinolide B, a constituent of *Euphorbia fischeriana*, significantly decreased in the proliferation of three cancer cell lines, possibly by arresting the cell cycle in the G1 phase and subsequently inducing apoptosis it has been reported that *Euphorbia helioscopia* has antitussive properties and might be useful in patients with chronic bronchitis. The exact mechanism of action is not known. Leafy spurge (*Euphorbia esula* L.) is a serious invasive weed on lands with both native and introduced vegetation on the northern Great Plains of North America. Although domestic sheep and goats usually graze this perennial forb, it appears to be aversive and toxic to cattle, and is a serious threat to cattle production (Johnston and Peake, 1960; Hein and Miller, 1992; Heemstra et al., 1999). Domestic sheep may also experience toxicity from leafy spurge ingestion at high levels of intake, after ingestion of particular plants or plants growing at certain locations (Johnston and Peake, 1960; Kronberg andWalker, 1999). Little is known about which species of wildlife eat or avoid leafy spurge, but it is generally considered to have a negative impact on wildlife habitat (Belcher and Wilson, 1989). With cattle, the predominant species of grazing livestock in North America, their avoidance of leafy spurge may be one important reason why this introduced plant from Eurasia has spread across two million acres of range and pasture lands in the continental United States. It is considered the worst noxious weed on these lands, with an economic impact in the millions of dollars annually (Leitch et al., 1996). Leafy spurge has high protein and low fiber levels during its early growth stages (Fox et al., 1991). However, it is not utilized as feed by cattle because it contains aversion-inducing chemicals that cause negative feedback after they ingest small amounts of it. Thus, they learn to avoid it (Kronberg et al., 1993). Extracts of leafy spurge were shown to cause skin irritation and weak tumor promotion, but not solitary carcinogenic activity (Seip and Hecker, 1982). Ingenol (Seip and Hecker, 1982) and several diterpene derivatives such as 3-*O*-(2*E*,4*Z*)-decadienoylingenol (*Euphorbia* factor E1), 3- *O*-(3,4,6)-decatrienoylingenol, and 3-*O*-(3,4,6,8)-dodecatetraenoylingenol were identified and shown to mediate irritant activity (Hecker, 1978). Ingenol and ingenol esters appear to induce toxicity because of their ability to mimic the function of diacylglycerol and activate protein kinase C (Hasler et al., 1992; Winkler et al., 1993). Protein kinase C is a phosphorylating enzyme that mediates cellular signal transduction for a large group of hormones and cellular effectors (Winkler et al., 1993). Other diterpenes, such as jatrophane and lathyrane derivatives, were also found in leafy spurge (Manners and Davis, 1987; Onwukaeme and Rowan, 1992).

Leafy spurge has been studied chemically and ecologically, but has had little evaluation of its aversion-inducing capacity for cattle or other ruminants. Ingenols are suspected as aversion-inducing compounds in leafy spurge, and ingenol 3- monobenzoate was found to be aversive to rats (Kronberg et al., 1995). Our study focused on the identification of aversive toxins in leafy spurge to further understand their impact on cattle. Bioassay-guided fractionations led to the identification of two compounds—ingenol and its ester—that are likely responsible for some of the aversion that cattle develop to leafy spurge.

Plant Material. Air-dried spurge was used throughout the study. Aerial parts of *Euphorbia esula* in the seed-ripening growth stage were collected in June 1995 near Veblen in northeastern South Dakota. Plants were air-dried and ground through a 2-mm screen. Ground plant material (3.6 kg) was extracted with cold 90% methanol (6 £ 6 leters). The aqueous methanol extract was concentrated under reduced pressure at 40±C and partitioned with petroleum ether (40–60±C, 6 £ 1 liters), chloroform (6 £ 1 liter) and acetone (6 £ 1 liter). A thick viscous layer, formed at the interface of the chloroform and aqueous phases, was collected separately and designated as the middle layer.

Evaluation of Middle Layer and Petroleum Ether Extracts for Aversive Response from Cattle
Three groups of yearling cattle (5/group) were trained for a one-week period to eat for 30 min in the morning from feed boxes within individual pens. They were held off feed, but not water, overnight during this period and allowed ad libitum intake of grass hay as a group only after the morning training period. A test period immediately followed the training period. At the same time as the training period, the cattle were given oat silage as a novel feed in feed boxes within individual pens for 30 min. After consuming about 700 g of the novel feed, one group was treated with the petroleum ether extract and the other with the middle layer residue at a dosage of 0.059 g/kg body wt and 0.176 g/kg body wt, respectively (materials orally dosed in gelatin capsules), and the control group was given empty capsules. On the following day at the same time, the cattle were again offered the same novel feed for 30 min. The amount of feed consumed by each animal was recorded.

Novel feed consumption on both days of the test period was compared using a repeated-measures design and PROC GLM (SAS, 1991) to determine if the cattle had an aversive response to the novel feed on the second day. Treatment group was the between-animal effect, and postgavage response was the within-animal effect. When appropriate, individual means were compared with the protected LSD procedure (SAS, 1988).

Screening of Aversive/Toxic Effect of Petroleum Ether Fractions on Rats and Brine Shrimp
The middle layer did not induce aversions in cattle, but the petroleum ether extract induced strong aversions, so this extract was investigated as follows. The petroleum ether extract (20 g) was subjected to fractionation on silica gel (G60, 300 g) using flash column chromatography (5 £ 50 cm) and developed with 100%

Table 1. Toxic and aversive effects of fractions from leafy spurge (*euphorbia esula* l.)

Fractions	Solvent	%	Aversive effect[a,b]	Toxic effect[b,c]
1—4	Pet.ether–ethyl acetate	8:2	—	—
5, 6	Pet.ether–ethyl acetate	75:25	—	—
7, 8	Pet.ether–ethyl acetate	65:35	—	+ + + +
9, 10	Pet.ether–ethyl acetate	50:50	+	+ + + +
11	Pet.ether–ethyl acetate	25:75	+	+ + + +
12	Pet.ether–ethyl acetate	25:75	+ + + +	+ + + +
13	Ethyl acetate	100	+ + + +	+ + + +
14, 15	Methanol	100	—	—

[a] Aversive effect measured as reduction of novel fluid intake by rats.
[b] + = 25% aversive/cytotoxic activity; + + ++ = 100% aversive/cytotoxic activity.
[c] Toxic effect measured as percentage of brine shrimp killed.

petroleum ether with a gradient increase in polarity with ethyl acetate (Table 1). One-liter fractions were collected, concentrated with a rotary evaporator, and evaporated to dryness under nitrogen. Fifteen fractions were collected. Aversive activity of fractions 1–15 was tested with rats (dosage 30 mg/rat) to measure their response to each fraction and compare them among fractions (Kronberg et al., 1995). Data on their aversive activity for rats was coupled with data from the brine shrimp toxicity assays to determine if these were consistent with cytotoxic effects of compounds in leafy spurge. For the brine shrimp toxicity assays, 0.25 mg of each extract was added to 8-ml vials (*N* D 4) and dissolved in 20 ^{7}l of DMSO (dimethyl sulfoxide) and mixed. The brine shrimp assays were conducted as outlined by Anderson et al. (1991).

Isolation and Identification of Aversive/Toxic Compounds

Combined fractions 12 and 13 (1.21 g) were subjected to a second step of purification using flash column chromatography (5 liters, 1:1, petroleum ether–ethyl acetate) and afforded 10 fractions (500 ml each) of which fractions 8 (32.5 mg) and 9 (25.6 mg) were finally purified with normal-phase HPLC using Econosil, 5^7(4:5 £ 250 mm) and Econosil 10^7(22 £ 250 mm) columns. HPLC columns were developed with a gradient system using hexane and a gradual increase in percentage of isopropanol. Compounds 8.5 (3.0 mg) and 9.4 (2.0 mg) were isolated from fractions 8 and 9, respectively. Structural elucidation was achieved using spectroscopic techniques. Nuclear magnetic resonance experiments were conducted with a Bruker DRX 400 spectrometer. Mass spectra were recorded using EI and CI techniques with a Finnigan 995633. Infrared spectra were recorded on a Bio-Rad FTS-60A infrared spectrophotometer.

Cytotoxicity Assays

Evaluation of the isolated compounds for cytotoxicity was made with bovine lymphosarcoma cells (CRL-8037 BL-3, American Type Culture Collection). The culture medium was Eagle's MEM with 0.1 mM nonessential amino acids and Earle's BSS (90%) and fetal bovine serum (10%). Two milligrams of each of the isolated compounds from leafy spurge was dissolved in 400 ^{7}l dimethyl sulfoxide (DMSO). Either zero, 1, 5, 10, 25, and 50^{7}l ofDMSOalone (control) or 0, 1, 5, 10, 25, and 50 ^{7}l of DMSO plus one of the isolated compounds were incubated with 2ml of the cells for 18 hr at 37±C. Aliquots of cells were withdrawn after 18 hr and stained using a two-step acridine orange (AO) technique: 200 ^{7}l of cell suspension (1–2 £ 106/ml) treated for 30 sec with 200 ^{7}l of a buffer containing 0.1% Triton and 0.08 N HCl followed by staining with AO staining buffer (6 ^{7}g AO/ml).

The metachromatic AO-stained cells were measured for green flourescence (510– 530 nm band-pass filter) for amounts of DNA and red fluorescence (630 nm longpass filter) for amounts of RNA.

Evaluation of Ingenol and Ingenol 3-Benzoate for Aversive Response from Cattle

Seip and Hecker (1982) found 99 mg of ingenols in 20 kg of air-dried leafy spurge. Therefore, we assumed that 0.0005% of air-dried leafy spurge was ingenol. Previous work (Kronberg et al., unpublished data) indicated that 0.3% of body weight is an aversive dosage of air-dried leafy spurge for cattle, so treated calves were dosed with 0.0148 mg of ingenol or ingenol 3-

benzoate/kg body wt. Ingenol and ingenol 3-benzoate were purchased from LC Laboratories (Woburn, Massachusetts, USA). Ingenol, the parent compound, has weak affinity for protein kinase C while ingenol 3-benzoate has considerable affinity for protein kinase C and, therefore, likely has greater toxicity to cattle. Ingenol 3-benzoate is similar to ingenol esters found in leafy spurge. The gavage solution was made by initially solubilizing 25 mg of each ingenol with 5 ml of ethanol. Fifty milliliters of deionized water was then added to each solution, and these mixtures were gavaged. Control animals received an equivalent amount of ethanol and water. Calves were trained and managed as described for the initial cattle trial. They were given 907 g of millet as a novel feed for 15 min in the first test period for ingenol, and 907 g of sugar beet pulp pellets for 15 min in the second test period for ingenol 3-benzoate. After consuming some of the novel feed, calves were gavaged with either ingenol or ingenol 3-benzoate in the ethanol–water solution. On the morning of the following day, they were offered novel feed again to determine if they formed an aversion to it. One calf (I4) was gavaged with ingenol a second time shortly after it ate a significant amount of novel feed, indicating that ingenol had not elicited an aversion. Because of the small amounts of ingenol and ingenol 3-benzoate available, the number of calves was limited, as was the number of days that they were treated with the compounds. Novel feed consumption on both days of the test periodwas compared using a repeated-measures design and PROCGLM (SAS, 1991) to determine if the cattle had an aversive response to the novel feed on the second day. Treatment group was the between-animal effect, and postgavage response was the within-animal effect. When appropriate, individual means were compared with the protected LSD procedure (SAS, 1988). *Specroscopic Data*

Compound 8.5. EI-MS *m/z* 348, 330 (MC-18), 313, 295 (313-18), 246, 236, 214 (100%) and 197. IR (CHCl3) 3450, 2930, 1685. UV abs (MeOH) 214 nm. 1H NMR (MeOH): $\pm$ 6.10 (1H, s, H-1), $\pm$ 3.65 (1H, s, H-3), $\pm$ 3.85 (1H, br s, H-5), $\pm$ 6.10 (1H, m, H–7), $\pm$ 4.10 (1H, m, H–8), $\pm$ 2.35 (1H, s, H–11), $\pm$ 2.25 (1H, m, H–12a), $\pm$ 1.65 (1H, m, 12b), $\pm$ 0.93 (1H, m, H–13), $\pm$ 0.92 (1H, m, H–14), $\pm$ 1.30 (1H, s, H–16), $\pm$ 1.35 (1H, s, H–17), $\pm$ 1.7 (1H, m, H–19), $\pm$ 4.10 (1H, d, H–20a, *J* D 12:5) and $\pm$ 4.25 (1H, d, H–20b, *J* D 12:5). 13C NMR (MeOH) $\pm$ 12.68 (C- 1), $\pm$ 139.42 (C-2), $\pm$ 79.44 (C-3), $\pm$ 84.47 (C-4), $\pm$ 73.25 (C-5), $\pm$ 143.25 (C-6), $\pm$ 127.56 (C-7), $\pm$ 42.97 (C-8), $\pm$ 206.59 (C-9), $\pm$ 42.67 (C-10), $\pm$ 23.14 (C-11), $\pm$ 30.49 (C-12), $\pm$ 22.62 (C-13), $\pm$ 22.25 (C-14), $\pm$ 37.97 (C-15), $\pm$ 15.29 (C-16), $\pm$ 15.40 (C-17), $\pm$ 17.05 (C-18), $\pm$ 28.25 (C-19), $\pm$ 62.98 (20).

Compound 9.4. EI-MS *m/z* 552, 538, 496, 382, 368, (100%), 348, 330, (348- 18), 313, 295 (313-18), 246, 236, 214, 183, and 43 and 197. IR (CHCl3) 3450, 2930, 1720, 1685. UV abs (MeOH) 214 nm.

RESULTS AND DISCUSSION

Initial screening for aversive activity showed the petroleum ether and chloroform extracts and the middle layer to be aversive to rats. Further screening of the middle layer and petroleum ether extracts on cattle showed no aversive response from cattle to the middle layer, but strong aversion to the petroleum ether extract ($P < 0:01$; Figure 1).

Chromatographic separation guided by rat aversion trials and brine shrimp assays showed fractions 12 and 13 (Table 1) to have the greatest aversive and toxic effects. Further separation of the combined fractions 12 and 13 by column chromatography provided 10 fractions. Fractions 8 and 9 of the second separation constituted more than 80% of the combined original fractions 12 and 13. Preparative HPLC using normal-phase gradient analysis was conducted and monitored at a wavelength of 220 nm. All fractions were collected. HPLC purifications afforded compounds 8.5 and 9.4 as the major compounds in fractions 8 and 9.

Detailed 1D NMR (HC, 13C, and DEPT) and 2D NMR (GCOSY, HMBC, HMQC, and NOESY) experiments were conducted for compounds 8.5 and 9.4.
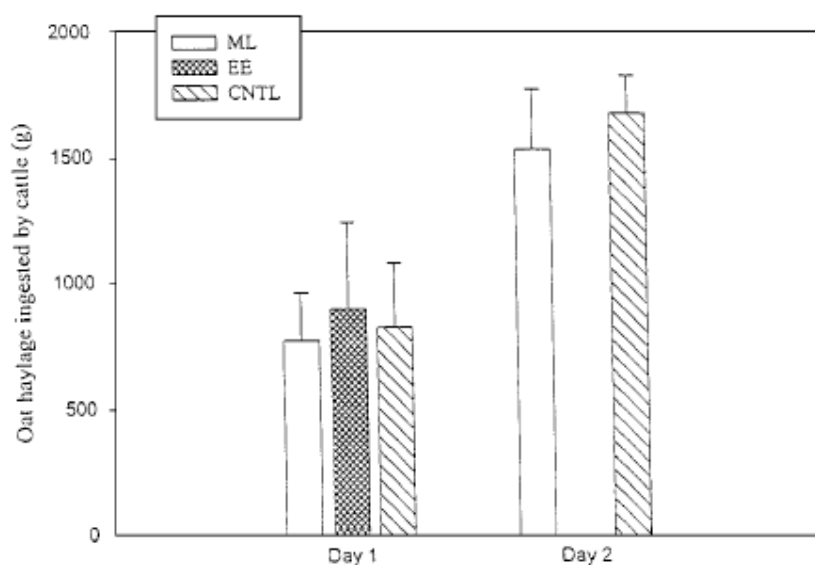

Fig. 1. Ingestion by cattle of a novel-tasting feed before and after receiving different portions of the original methanol extract of leafy spurge in gelatin capsules.

The portions were a thick viscous layer at the interface between the chloroform and aqueous layers named the middle layer (ML) and the petroleum ether extract (pEE). A third group of control cattle (CNTL) received an empty gelatin capsule

Mass spectral analysis of the two compounds showed evidence of a diterpenoid structure with molecular ion peaks at $m=z$ 331 and 348. Mass spectral analysis showed a molecular ion peak for compound 8.5 at $m=z$ 348 and fragments of $m=z$ 313 and 295, which are characteristic of ingenol (Seip and Hecker, 1982). Compound 9.4 showed a molecular ion peak at $m=z$ 330 and fragments of 313, 295, 183 and 43, which are characteristic of the ingenane skeleton and fragments of dodecanoate and acetate ($m=z$ 183 and 43, respectively). The latter fatty acids were shown clearly from the $m=z$ fragments of 43 and 183 corresponding to acetyl and dodecayl radicals, respectively. Figure 2 illustrates the structures of compounds 8.5 and 9.4.

HC and 13C NMR data corresponded well with published data for the diterpene ingenol (Seip and Hecker, 1982). 1H-1H connectivities were confirmed from COSY and NOESY experiments. DEPT experiments assigned carbon shifts for all protonated carbons. A heteronuclear multiple bond correlation (HMBC) assigned shifts at ± 139.42 and 143.25 to quaternary carbons C-2 and C-6, respectively. NMR, MS, and IR data are in full agreement with published literature (Seip and Hecker, 1982). Therefore, compound 8.5 is identified as ingenol. NMR data for compound 9.4 could not be completed with this experiment,
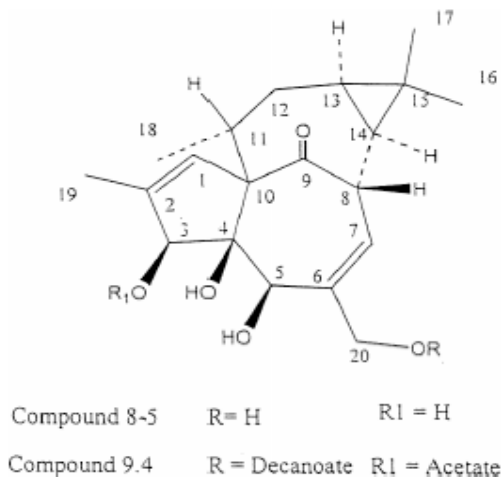

Compound 8-5   R= H   R1 = H

Compound 9.4   R = Decanoate   R1 = Acetate

Fig. 2. Aversive diterpene ingenol (compound 8.5) and diester derivative (compound 9.4).
But the identity of it was established from mass spectral analysis.

Moreover, alkaline hydrolysis of compound 9.4 followed by comparison of the diterpene part showed a complete match with ingenol in TLC and HPLC analysis. Therefore, compound 9.4 was tentatively identified as ingenol-20-dodecanoate, 3-acetate by comparison with published data (Seip and Hecker, 1982). Further NMR analyses are in progress to establish the positions of the diester derivatives of ingenol.

Compounds 8.5 and 9.4 showed toxicity to lymphosarcoma cells at 10^{-1}l or more of DMSO plus a compound (Figures 3 and 4). The cell assay showed little difference in the cytotoxicity of compound 8.5 (ingenol, the parent diterpene alcohol) and compound 9.4 (ingenol diester derivative). Ingenol and its esters vary considerably in their biological activity (Hirata, 1975; Hirota et al., 1980). Ingenol has lower activity than some of its esters, but will still bind to protein kinase C and activate the enzyme (Hasler et al., 1992). Ingenol has been shown to be active in different cell systems with effects similar to those of the phorbol esters in respect to morphological change, cell to cell communication, epidermal growth factor binding, arachidonic acid metabolite release and ornithine decarboxylase activity (Hasler et al., 1992). For the first trial with ingenol, change in intake of novel feed by treated and control cattle from the first to second days was similar (P D 0:41), and the ingenol treatment elicited an aversion in only one animal (Figure 5).

This calf decreased intake of the novel feed by 83% from the first to second days. One calf that did not show an aversion from the initial ingenol treatment was dosed again and avoided
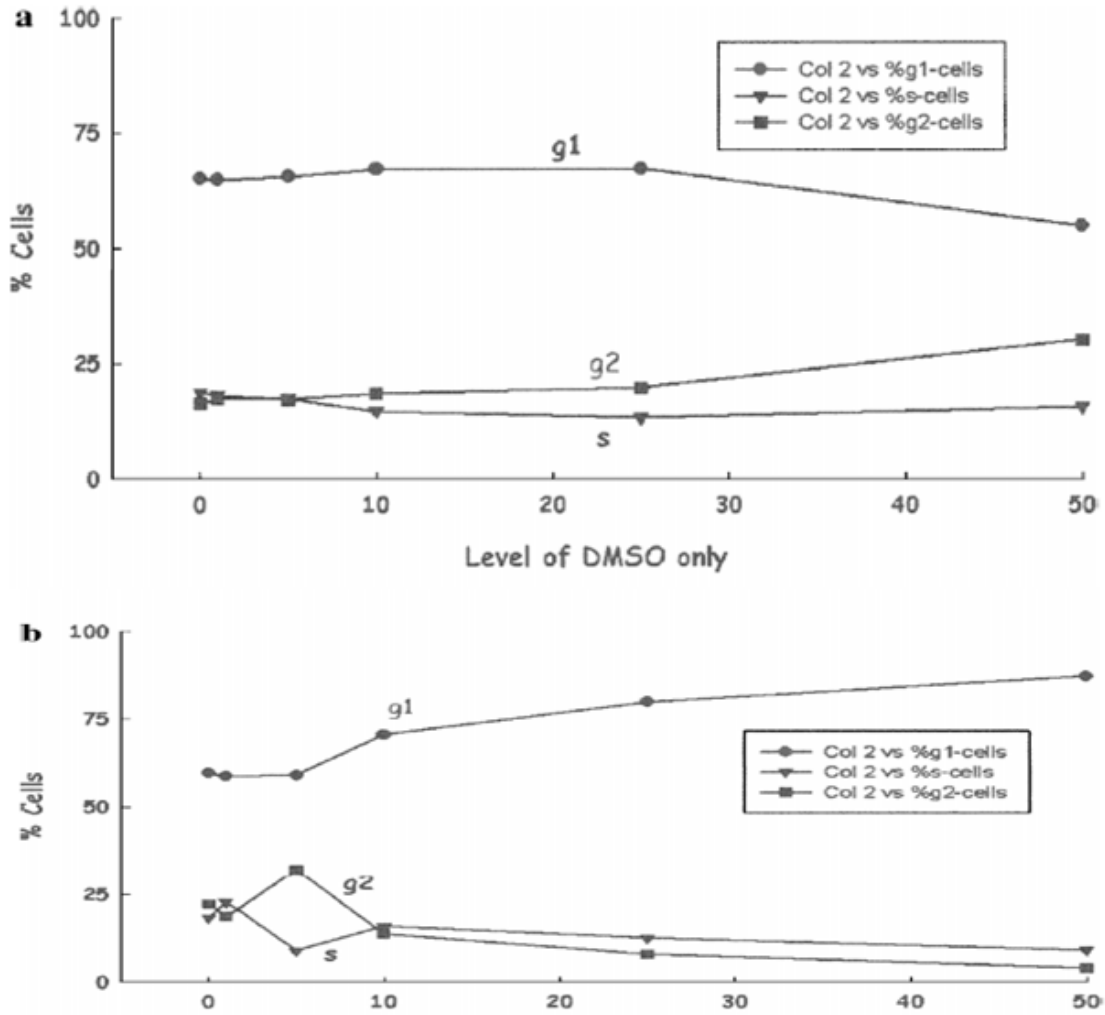

Fig. 3. Cell cycle analysis based on percent of bovine lymphosarcoma cells in each of three phases of the cell cycle: lag phase (g1), exponential phase (g2), and stationary phase (s). Effect on cell cycle when different amounts ofDMSOwas added to the lag phase of growing cells (a) and when different amounts of compound 8.5 dissolved in DMSO was added to the lag phase of growing cells (b).

the novel feed after a second treatment with ingenol. These results are consistent with work indicating that ingenol, the parent alcohol, is less toxic than some of its esters (Hasler et al., 1992). However, we recently determined that the air-dried leafy spurge from our South Dakota site contained at least 0.0074% ingenol. This
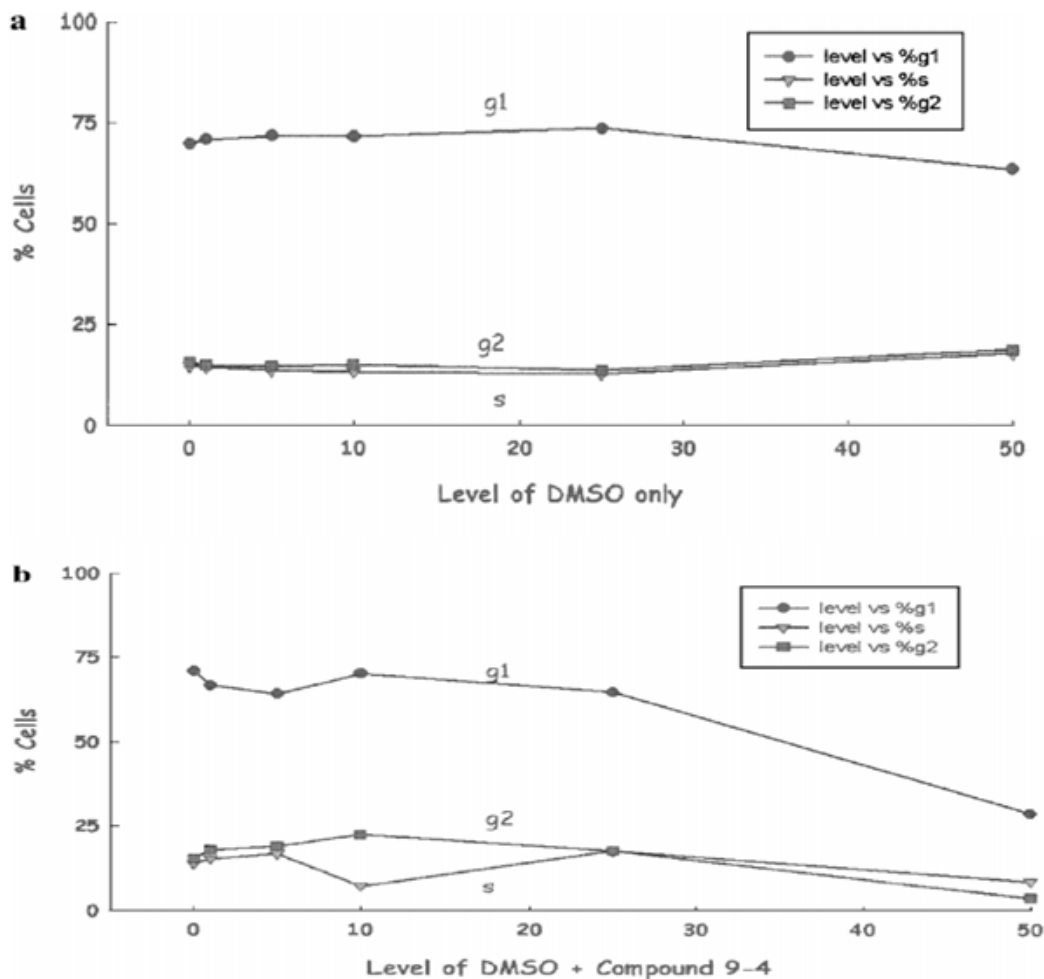

Fig. 4. Cell cycle analysis based on percent of bovine lymphosarcoma cells in each of three phases of the cell cycle: lag phase (g1), exponential phase (g2), and stationary phase (s). Effect on cell cycle when different amounts of the DMSO was added to the lag phase of growing cells (a) and when different amounts of compound 9.4 dissolved in DMSO was added to the log phase of growing cells (b).

level is considerably higher than our assumption of 0.0005% of air-dried leafy spurge that we used when determining our dosage rate for both aversion trials. Ingenol may have elicited more aversion had we used a higher dosage based on this level of ingenol in leafy spurge.
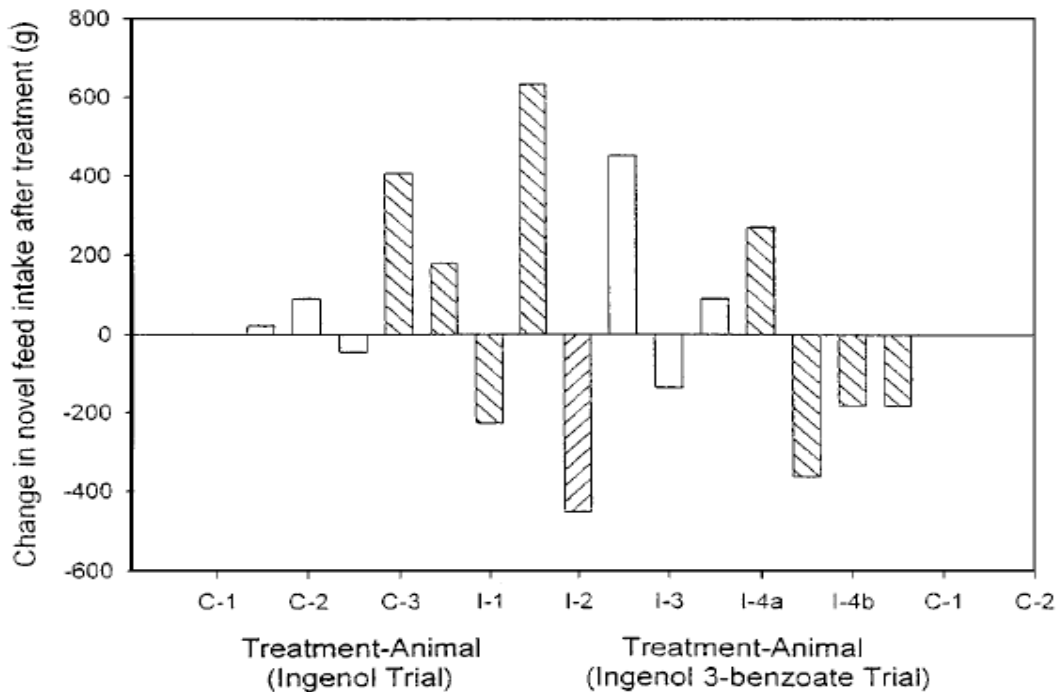

Fig. 5. Change in intake of a novel feed by cattle after gavage with ingenol (or ingenol 3-benzoate) in ethanol and water or only ethanol and water (control). Animals with "I" before their number were gavaged with ingenol or ingenol 3-benzoate (separate trials for each ingenol). Animals with "C" before their number were gavaged with only ethanol and water. Animal 940 did not show an aversion to the novel feed after the first time it was gavaged with ingenol (intake indicated at I-4a), so it was dosed again and its intake following the second gavage of ingenol is indicated by I-4b.

The esters are more lipophilic than the parent ingenol and, therefore, more likely to be absorbed through the intestinal mucosa. Ingenol 3-benzoate is similar to other ingenol esters that are in leafy spurge or possibly created by microbial metabolism of ingenols in the rumen of cattle. Hubert et al. (unpublished data) observed that extracts of 6-hr-long fermentations of leafy spurge and cattle ruminal digesta are more toxic to brine shrimp than are extracts of mixtures of leafy spurge and cattle ruminal digesta are more toxic to brine shrimp than are extracts of mixtures of leafy spurge and cattle ruminal digesta that were not allowed to ferment.

One dose of ingenol 3-benzoate elicited an aversion in three of four calves tested (novel feed intakes decreased 89, 33, and 80% from the first to second day for these three calves). However, therewas no significant difference (P D $0{:}30$) between the control and treated cattle with respect to their change in novel feed intake from the first to second day (Figure 5). Kornberg and Walker (unpublished data) observed that air-dried leafy spurge did not elicit a feed aversion in one of five yearling cattle gavaged with air-dried leafy spurge after ingesting a novel feed. If some cattle do not experience aversions to leafy spurge after one exposure, then it is logical that

not all cattle will experience an aversion from a single dose of ingenol that is similar to that in leafy spurge. The response of cattle to ingenol 3-benzoate was similar to that observed with rats (Kronberg et al., 1995). Rats formed an aversion to a novel fluid when they received a gastric infusion of ingenol 3-monobenzoate that followed their initial drink of the novel fluid (Kronberg et al., 1995).

This study helps define toxic and aversive compounds in leafy spurge that are probably, at least in part, responsible for the toxic and aversive effect that leafy spurge has on cattle. In view of the widespread distribution of leafy spurge on the

Northern Great Plains and its negative impact on cattle production in the region, understanding the chemical interaction between cattle and leafy spurge is important. Furthermore, given that sheep and goats will ingest substantial amounts of leafy spurge and that cattle may ingest small amounts, it is of considerable importance

to elucidate the overall environmental risk factors for the potential cumulative toxicity of these cocarcinogenic compounds. Several reports have suggested that direct or indirect exposure of people and livestock to such plants or plant parts may lead to a potential risk of cancer by conditional carcinogenesis (Upadhyay et al., 1978; Hecker, 1981, 1987a,b; Zayed et al., 1998; Vogg et al., 1999). This clearly indicates the necessity to investigate the overall toxicity of leafy spurge on livestock, especially in areas highly infested with this noxius weed.

ACKNOWLEDGMENTS

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