Detection of anti-nutritive compounds in tree fodders from India using the brine shrimp bioassay method

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Abstract

Two simple bioassays, using brine shrimp mortality and mould inhibition following thin layer chromatography (TLC/mould inhibition) were used to screen a range of tree fodders from India for anti-nutritive compounds. The two bioassays had different sensitivities to tree fodder extracts. Many extracts were toxic towards brine shrimp but had no detectable activity in the TLC/mould bioassay. There were also differences between samples of the same feed. There were no apparent relationships between total phenol contents of the extracts and their toxic activities in either bioassay. There was no consistency between the bioassays and goat keepers perceptions on the toxicity of *Acacia leucophloea* pods, but there were indications that the selective consumption of the tips of leaves of *Vitex negundo* by goats may be related to the avoidance of toxic factors. Although bioassays may prove useful in the future, clearly considerably more research will be required before they could be used with any reliability.

Introduction

Tree fodders can contain a wide range of anti-nutritive factors. Of these tannins are the most widespread, but other factors such as saponins, alkaloids, cyanogens and mimosine can occur (reviewed by Makkar, 1993). As it would take a considerable effort to screen for all possible anti-nutritive factors by conventional chemical methods, there is interest in the possible use of simple bio-assays as potential screening methods. The brine shrimp assay has been proposed as a suitable initial screening technique for feed toxins and has been shown to be sensitive to mycotoxins (Panigrahi and Dallin, 1994). TLC/fungal inhibition techniques have been used to screen plant material for anti-fungal agents (Bailey, 1973; Bailey and Burden, 1973). Wood et al (unpublished results) have previously found that the brine shrimp and TLC/mould inhibition bioassays were sensitive to an unidentified factor in the leaves of *Prunus cerasoides* and that this factor appeared to reduce the nutritive value of the leaves as judged by Nepalese farmers. In this study the approach was extended to a range of tree fodders from N W India, including some fodders which were regarded as potentially toxic to goats by local goat keepers.

Materials and methods

The samples collected in India for analysis are given in tables in 1, 2 and 3. The tables also indicate the analyses performed. "Anti-nutritional factors" corresponds to the two bioassay techniques and total phenols assay.

Preparation of extracts

500mg (± 10mg) of dried sample (ground to pass a lmm screen) was weighed into a 10ml glass beaker. 5ml of 70% aqueous acetone was added and homogenise for 1 min using an ultraturrex at medium power. The mixture was transferred to a 15ml coned centrifuge tube and centrifuge at 2000 rpm for 10 mins. The supernatant was used as the test extract.

BRINE SHRIMP ASSAY

Brine shrimp medium (BSM)

The medium described by Panigrahi and Dallin (1994) was used. This was prepared using the following compounds per litre of distilled water: sodium chloride, 30 g; calcium chloride dihydrate, 0.3 g; magnesium chloride hexahydrate, 1.5g; magnesium sulphate heptahydrate, 0.5 g; potassium chloride, 0.8g magnesium bromide hexahydrate, 0.1g; and glycine, 6.0g. The BSM was autoclaved at 121^{o}C for 15 min and stored in a brown-coloured glass bottle.

Hatching and harvesting of larvae

150 ml of BSM in a conical flask was placed in a water bath equipped with a shaker and set at 30^{o}C. A 0.5 ml scoop of brine shrimp eggs (which can be stored in a desiccator for many years) was sprinkled onto the BSM. The flask was gently shaken for oxygenation of the BSM/eggs mixture. After 15h, the contents were poured into the outer compartment of a Hykro Artemia hatcher. This is a flat-bottomed bowl with three compartments and a detachable lid with a hole that covers the central compartment. The medium between compartments is also separated by a removable double-ringed barrier that serves to stop any floating egg shells moving towards the centre whilst allowing shrimp to swim under it. Fresh BSM was poured from the central compartment until it just met the BSM from the conical flask in the outer compartment. The lid was replaced and the hatcher illuminated with an 8 w fluorescent lamp. After 4 h, the central compartment contained clean larvae (which were still at the nauplius stage of development) that had been attracted towards the light source. These larvae were harvested in a beaker and diluted with fresh BSM to achieve a concentration of 25-105 shrimps per 375 µl.

Assay procedure

Antibiotic assay discs (Whatman 6mm diameter AA grade) were mounted on dissection pins supported in a plasticine base. Various µl quantities of the test compounds in aqueous acetone were placed on the surface of each disc using a micro syringe with a bent needle tip for ease of inoculation. The solvent was allowed to evaporate, this being facilitated by placing the discs in an oven at 40 ^{o}C for 20 min. The discs were placed in the 10mm diameter wells of Falcon 3047 multiwell low evaporation lid tissue culture plates (Sterilin migration plates kept in humidity chambers may

also be used). 375µl of BSM containing shrimp was placed in each well using an automatic hand pipette. 4 replicates were used except for (IF3), (IF8), (IF25) (2 replicates) and (IF4), (IF6), (IF7) (2 replicates first assay, 4 replicates second assay) . The plates placed in an incubator maintained at 30 ^{o}C for 18 h.

The following samples were assayed more than once :

IF1, IF4, IF5, IF6, 1F10, IF12, IF21, IMy1 were assayed twice and the mean determined.

IF9 was assayed 3 times and the mean determined.

Larvae mortality scoring procedure

The numbers of dead shrimp were counted by examining under a microscope (x 10 magnification). A couple of drops of formalin were added to each well to kill the remaining live shrimp and the total number of shrimp counted. The total number of dead shrimp in each well before the addition of the formalin was expressed as a percentage of the total number of shrimp in each well. Solvent controls, blank disc controls and no disc controls were included in most batches**.**

% Mortality values from wells where the total number of shrimp fell outside the acceptable (25 – 105) range were discarded. The 50% mortality point on the graph of '%mortality' against 'extract concentration' (mg feed sample extracted/ml solution in assay well) was estimated by drawing a straight line between the average %mortality values for each concentration of extract.
Extract concentration was calculated as:
Extract conc (mg/ml) = (sample wt extracted (g) x volume extract on disk (µl)) /1.875

Results

The results are summarised in Table 4. Toxic factors were not detected in the majority of the tree fodder samples. Extracts of *Acacia leucophloea* pods (samples IF1, IF9, IF12, IF21) were toxic to brine shrimp but not to moulds under the conditions of the assays. Bordi pala (dried leaves of *Ziziphus nummularia*) gave inconsistent results with one sample (IF6) having toxic activity to both brine shrimp and moulds (DCM extract only), while the other sample (IF7) had no detectable activity in either assay. Mature *Acacia leucophloea* leaves were toxic to brine shrimp, but not mould (samples IF2, IF10, IJy7), but new leaves had not detectable toxicity (sample IMy2).

Karanj leaves (IJy6) were toxic in both assays, with the Aqueous extract giving two areas of mould inhibition in the TLC/mould assay. Fallen, dry karanj leaves (IMy5 and 6) inhibited mould growth (DCM extract only), but had no detectable toxicity to brine shrimp. Khanni (*Vitex negundo*) (samples IMy7 and IJy11) were toxic in both assays.

A wide range of tree fodders had toxic activity towards brine shrimp, but no detectable activity in the TLC/mould bioassay. These fodders included *Ziziphus mauritiana* (IF4, IJy1), *Acacia nilotica* (IMy12, 13, 14 and IJy13), *Prosopis juliflora* pods (IMy16) and *Prosopis cineraria* leaves (IMy18, but not pods IMy17). Most of the dried leaf samples of material grazed from the ground did not contain toxins. Perhaps surprisingly cactus (IMy15) had some activity in the brine shrimp assay, but not in the TLC/mould assay. As expected bamboo (IJy3) had no toxic activity with either assay.

There were no apparent relationships between total phenol contents of the samples and their activities in either bioassay. 24 samples were active in the brine shrimp assay and had detectable levels of total phenols, but there was no significant ($P>0.05$) linear correlation found between these assays. All of the samples with mould inhibition activity had detectable total phenols, but of the 10 samples with high (>50 g kg^{-1} gallic acid equivalent) levels of total phenols which were also assayed for mould inhibition, none had any detectable activity with that assay.

Discussion

There were clearly differences in the sensitivities of the two bioassays used. There were few samples where both assays detected toxic factors. There was good evidence from goat keepers that two of the species sampled could contain factors toxic to goats. Pods of *Acacia leucophloea* can be highly toxic, it is believed due to the presence of cyanogenic compounds. Investigation of one occurrence of deaths of goats implicated cyanogenic glycosides which can be detected in pods during March and April when they are fully ripe (ISGP, 1993). Goat keepers have several different ways which they use to avoid toxic pods, but there is little consistency between farmers and incidence of toxicity still occur indicating that the methods used are not completely reliable. Of the four pod samples tested, two were said by goat keepers to be toxic, and two said to be non-toxic. Neither bioassay was able to detect differences between the two types of sample, the TLC/mould assay being unable to detect any toxicity. Goat keepers also reported that all pods of *Acacia leucophloea* can become toxic after they have been lopped from the trees, while cyanogenic compounds are usually degraded quickly in lopped material. Therefore, even if the goat keepers opinions on the toxicity of the pods were correct, toxic factors could have been generated and/or lost during the transport of the samples to the laboratory. In either case it appears unlikely that either assay will be of practical help in providing advice for farmers on which pods may or may not be toxic.

Goat keepers reported that goats selected only the tips of Khanni (*Vitex negundo*) for consumption. These data indicate that leaves of *Vitex negundo* contain a toxic factor which the goats may well be trying to avoid by means of diet selection.

Conclusions

The two bioassays have different sensitivities to toxic factors, but both can be used to give semi-quantitative indicators of the presence of toxic factors which may be toxic or anti-nutritive to livestock. Toxic activity in the bioassays in the bioassays did not appear to be related to the total phenol content of the samples.

There was some consistency between the bioassay data and farmers' knowledge of feeds, but neither bioassay appeared to be reliable indicators of the presence or absence of toxic factors. There were indications of variability between samples of the same feed. Farmers are aware of such variability in some types of feed (e.g. *Acacia leucophloea* pods).

Bibliography

BAILEY, J. A. (1973). Production of antifungal compounds in cowpea (*Vigna sinensis*) and pea (*Pisum sativum*) after virus infection. *Journal of General Microbiology*, **75**: 119-123.

BAILEY, J. A. and BURDEN, R. S. (1973). Biochemical changes and phytoalexin accumulation in *Phaseolus vulgaris* following cellular browning caused by tobacco necrosis virus. *Physiological Plant Pathology*, **3**: 171-177.

ISGP (1993). Intoxication of goats by *Acacia leucophloea* pods. p38. In: *Manual for Improved Goat Production, part 3. Fodder Resource Development for Goats* Compiled by Indo-Swiss Goat Development and Fodder Production Project, Ajmer, India. Published by Dept. of Animal Husbandry, Govt of Rajasthan, Tonk Road, Jaipur and Intercooperation, PO Box 6724, CH3001 Bern, Switzerland.

MAKKAR, H. P. S. (1993). Antinutritional factors in foods for livestock.. pp 69 – 85. In: *Animal Production in Developing Countries*. Occasional Publication No 16, British Society of Animal Production, Gill M, Owen E, Pollott G E and Lawrence T L J (Eds.) 243pp.

PANIGRAHI, S. and DALLIN, S. (1994) Toxicity of the *Alternaria* spp metabolites tenuazonic acid, alternariol, altertoxin-I, and alternariol monomethyl ether to brine shrimp (*Artemia salina* L) larvae. J Sci Food Agric 66, 493 - 496.

WOOD, C. D., PANIGRAHI, S., GOODENOUGH, L., ROSSITER, J. T. and others (unpublished data). Use of bioassays to detect anti-nutritive factors in Nepalese fodder trees. (Report of project A0316, R5180).

Table 1 Samples obtained in India, February 1998

Code	Description	Analyses		
		Anti-nutritional factors	Gas production	Chemical analysis
	From Bhilwara			
IF1	A leucophloea pods, not toxic	x		
IF2	A leucophloea leaves from same branch as IF1	x		
1F3	Azadirachta indica (Neem) leaves	x	x	x
IF4	Ziziphus mauritiana leaves	x	x	x
IF5	Cotton pala	x	x	x
IF6	Ziziphus nummularia, dried leaves (bordi pala)	x	x	x
IF7	Ziziphus nummularia, dried leaves (bordi pala)	x	x	x
IF8	Azadirachta indica (Neem) leaves	x	x	x
IF9	A leucophloea pods, not toxic	x		
IF10	A leucophloea leaves from same branch as IF9	x		
IF11	as IF9 but from other branch of tree, pool with IF9 after weighing			
IF12	A leucophloea pods, said to be toxic (pods curled, reddy brown and look dry compared to non-toxic pods)	x		
IF13	barley grain, ex BAIF buck breeding programme		x	x
IF14	wheat grain, ex Patiyo Ka Khera		x	x
IF15	barley grain, ex Patiyo Ka Khera		x	x
	From Udaipur			
IF16	A leucophloea pods, not toxic[a]			
IF17	A leucophloea pods, said to be toxic (blotches on pod surface, said to due to disease, and indicate toxicity)[a]			
IF18	wheat grain + sun hemp seed "concentrate" ex Mr. Sava Roopaji Vadera, Khakad		x	x
IF19	barley grain ex Khakad		x	x
IF20	neem leaves	x	x	x
IF21	A leucophloea pods, said to be toxic (had ball-like growths, galls, on twigs, said to indicate toxicity)	x		
IF22	wheat grain, ex Kirat		x	x
IF23	dried maize, ex Gopir		x	x
IF24	dried leaves of "kadwa" from hill slopes	x	x	x
IF25	dried leaves of "kadwa" from hill slopes	x	x	x
IF26	whole weed from wheat field (Chenopodium alba)		x	x (CP)

Note a Samples attacked by mould whilst in transit to UK and not evaluated.

Table 2 Samples collected in India, May 1998

Code	Description	Analyses		
		Anti-nutritional factors	Gas production	Chemical analysis
	From Udaipur			
IMy1	*Ziziphus mauritiana* (ber) leaves	x	x	x
IMy2	*Acacia leucophloea* (arunjia) new leaves	x	x	x
IMy3	*Acacia nilotica* (babul) pods	x		
IMy4	*Derris indica* (negad) leaves	x	x	x
IMy5	Dried leaves collected underneath karanj tree, upper valley (location code 2)	x	x	x
IMy6	Dried leaves collected underneath karanj tree, lower slopes (location code 3)	x	x	x
IMy7	*Vitex negundo* (Khanni), tops of green shoots pooled from 5 trees (only part eaten by goats)	x	x	x
IMy8	Dried leaves from below tamat trees, top of hills (location code 4)	x	x	x
IMy9	Second sample as per IMy8	x	x	x
IMy10	Dried leaves from below karanj trees (mainly), but also *Vitex negundo* (khanni) and *Holorrena antidisentrica* (kadwa) trees, top of hills (location code 4)	x	x	x
IMy11	Dried leaves from below mainly *Holorrena antidisentrica* (kadwa) trees, lower slopes (location code 3)	x	x	x
IMy12	*Acacia nilotica* (babul) leaves	x		
IMy13	*Acacia nilotica* (babul) pods	x		
	From Bhilwara			
IMy14	*Acacia nilotica* (babul) pods	x		
IMy15	Cactus, *Opuntia spp.*? (thor), dried	x	x	x
IMy16	*Prosopis juliflora* pods	x	x	x
IMy17	*Prosopis cineraria* (khejri) pods	x	x	x
IMy18	*Prosopis cineraria* (khejri) leaves	x	x	x
IMy19	*Ziziphus nummularia* (bordi) fresh leaves	x	x	x

Table 3 Samples of tree leaf fodders collected by Mr Badve, Rajasthan, July 1998

Samples of fresh leaves, dried and ground to 1 mm before analysis

Code	Description	Analyses		
		Anti-nutritional factors	Gas production	Chemical analysis
IJy1	Ziziphus maritiana (ber)	x	x	x
IJy2	Mangitesa indica (mango)	x	x	x
IJy3	Bamboo	x	x	x
IJy4	Derris indica (negad)	x	x	x
IJy5	Holorrena antidisentrica (kadwa)	x	x	x
IJy6	karanj	x	x	x
IJy7	runjiya	x	x	x
IJy8	hitazi	x	x	x
IJy9	Ficus indica (pimpal)	x	x	x
IJy10	tamat	x	x	x
IJy11	khanni (all green leaves, not just shoots)	x	x	x
IJy12[a]	godla			x
IJy13	Acacia nilotica (babool)	x	x	x
IJy14	kalbi	x	x	x

Note a Samples attacked by mould whilst in transit to UK and not evaluated.

Table 4 Summary of brine shrimp and TLC/mould inhibition data, together with data on Total Phenols content

	brine shrimp assay	TLC/mould data		Total Phenols
Sample code[a]		DCM extract	Aq extract	g/kg gallic acid equiv
IF1	5.9	nd	nd	na
IF2	6	nd	nd	na
IF3	nd	nd	nd	29.2
IF4	4.4	nd	nd	14.4
IF5	nd	nd	nd	6.3
IF6	5.6	0.2	nd	41.3
IF7	nd	nd	nd	69.2
IF8	nd	nd	nd	18.7
IF9	5.9	nd	nd	na
IF10	13	nd	nd	na
IF11	nd	na	na	na
IF12	6	nd	nd	na
IF13	nd	na	na	na
IF14	nd	na	na	0
IF15	nd	na	na	1.4
IF16	nd	na	na	na
IF17	nd	na	na	na
IF18	nd	na	na	na
IF19	nd	na	na	na
IF20	6.6	nd	nd	52.2
IF21	10.3	nd	nd	na
IF22	nd	na	na	na
IF23	nd	na	na	na
IF24	nd	nd	nd	13
IF25	nd	0.57	nd	30
IF26	nd	na	na	na
IMy1	5.65	nd	nd	73.6
IMy2	nd	nd	nd	54.4
IMy3	6.8	nd	nd	95.8
IMy4	4.8	nd	nd	31.4
IMy5	nd	0.58	nd	7.6
IMy6	nd	0.25	nd	13.6
IMy7	5.4	0.38	0.4	42
IMy8	nd	nd	nd	13.1
IMy9	11	nd	nd	11.7
IMy10	nd	nd	nd	15.2
IMy11	nd	nd	nd	19.7
IMy12	8.6	nd	nd	93.1

IMy13	4.2	nd	nd		105
IMy14	4.8	nd	nd		104.1
IMy15	6.4	nd	nd		13.1
IMy16	5.4	nd	nd		18.3
IMy17	nd	nd	nd		na
IMy18	9.8	nd	nd		48.6
IMy19	4.9	na	na		94.3
IJy1	10.8	nd	nd		37
IJy2	5.2	nd	nd		63.7
IJy3	nd	nd	nd		4.2
IJy4	3	nd	nd		16.4
IJy5	nd	nd	nd		24
IJy6[b]	3.8	0.15	0.29[b]	0.26[b]	8.9
IJy7	7.3	nd	nd		86.5
IJy8	nd	nd	nd		44.2
IJy9	nd	nd	nd		9.5
IJy10	3.3	nd	nd		16
IJy11	3.7	0.41	0.52		18.3
IJy12	nd	na	na		na
IJy13	3.7	nd	nd		88.7
IJy14	7.6	nd	nd		6.3

Note a For key to sample codes see Tables 1, 2 and 3
Note b For sample IJy6 there were two spots on the TLC plates with the aqueous extract
nd = not detected
na = not analysed