Chapter 5

LYTIC ACTIVITY OF *ENTEROLOBIUM* FRACTIONS ON RUMEN PROTOZOA

5.1. Introduction

Experiments presented in Chapter 4 have shown haemolytic activity of *Enterolobium* fractions on sheep red blood cells. However, this property may not reflect their ability to lyse rumen protozoa. It was found that white saponin is much more active than Alkanate 3SL3 and purified *Enterolobium* fractions. In fact, white saponin (Chapter 4) has been found to have no activity on protozoa (Choo, pers. comm., 1992), whereas Alkanate 3SL3 has been found to effectively eliminate protozoa from the rumen of lambs (Burggraaf, 1980). These findings suggest that the present study of the effects of *Enterolobium* fractions on rumen protozoa, may provide evidence for a potent, natural antiprotozoal agent.

It was considered important to develop an *in vitro* system, since an *in vivo* system would require large quantities of fractions. A modification of the *in vitro* system developed by Willard and Kodras (1967) was employed to assess antiprotozoal properties of purified fractions of *Enterolobium* leaf extract. The assessment was carried out under both aerobic and anaerobic conditions to reflect the ability of the agents to affect the cells in conditions close to nature, and also for practical advantages of the *in vitro* conditions.

5.2. Materials and Methods

5.2.1. Management of the experimental animal

1. A single fistulated sheep was used throughout this project as a source of rumen fluid. The sheep was held in an individual pen in the animal house. Basal feed consisting of 300 g lucerne and 300 g oaten chaff was given once per day, early in the morning, to the sheep. The sheep had free access to water.

5.2.2. Collection of rumen fluid

Rumen fluid samples were collected in the morning before feeding time from the fistulated sheep using a sampling probe positioned in the dorsal sac of the rumen. The probe was made of a metal cage covered with nylon gauze and connected to the cannula plug by a length of plastic tubing. A 50 ml syringe was attached to the probe. The rumen sample was placed in a flask and the lid put on to eliminate exposure to air, and held in a container filled with warmed water. The rumen fluid was kept in a water bath (Reciprocating Shaking Water Bath model RW 1812, Australia) set for 39°C until used.

In some assays the rumen protozoa were obtained by centrifuging the rumen fluid at low speed 500 x g for 2 minutes and washing with warmed Hungate's salt three times to separate protozoa from other microorganisms and feed particles.

Composition of Hungate's salt (Coleman, 1978)

NaCl	5.0 g/l
CH_3COONa	1.5 g/l
K_2HPO_4	1.0 g/l
KH_2PO_4	0.3 g/l

5.2.3. Purified fractions of *Enterolobium* leaf extract

The purified fractions examined in this experiment were supplied by Dr. David Tucker from the Department of Chemistry of the University of New England. The fractionation processes, carried out using reverse-phase HPLC, were described in Chapter 3.

5.2.4. Protozoal lytic assays under an aerobic system

A simple *in vitro* system has been developed to observe the activity of the *Enterolobium* leaf extract on rumen protozoa. In the initial method, there was no attempt to separate the protozoa from other microbes and feed particles. A known volume (100 to 300 µl) of the test compounds was added to the cell suspensions (Section 5.2.2) and the mixture volume was adjusted with a suitable saline solution to 1 ml. The mixtures were then incubated for a set time (1.5 or 3 h) in a water bath set at 39°C. After the incubation time, the motility and the degree of disintegration of the cells was observed under a microscope at 25 or 40 x magnification. This system was modified as necessary relying on the results obtained from the earlier assays. The modifications made to improve this system were as follows: (1) the separation of protozoa from other microbes and feed particles by centrifuging the rumen fluid sample at low speed (better results can be obtained when the speed is less than 500 x g), (2) collecting the rumen fluid samples in the morning 1 h before the sheep was fed, and (3) using phosphate-buffered saline (Hungate's salt) instead of 0.9% saline.

5.2.5. Observation of protozoa

Antiprotozoal activity of the *Enterolobium* leaf extract was assessed by two procedures: (1) measurement of decreased motility by subjective appraisal under the microscope and, (2) measurement of the degree of disintegration of protozoa by microscopic examination. After thoroughly mixing the sample, a subsample was quickly withdrawn

using a pasteur pipette and placed under a cover slip in the space between the grooves cut in the counting chamber (Hawkesley Crystalite Counting Chamber see Section 5.2.5.2).

5.2.5.1. Measurement of cell motility

Protozoa were examined under a microscope at 25 and 40 x power to assess motility of the cells. The chamber was kept warm until used. Protozoa which exhibited no internal or external movement of the cilia were considered dead. The motility was estimated by comparing the motile cells in a test solution to the control and graded as follows:

0 = no visible effect on protozoa

+ = up to 25% of protozoa lost motility compared to control

++ = 25% to 50% of protozoa lost motility compared to control

+++ = 50% to 75% of protozoa lost motility compared to control

++++ = 75% to 100% of protozoa lost motility compared to control

5.2.5.2. Measurement of the degree of disintegration

The degree of disintegration of the cells due to the activity of the agent under test was calculated after adding formol saline (16.2 g NaCl, 200 ml formaldehyde, and 1800 ml distilled water) to the cell solution to fix the cells without any deterioration effects. Intact or disintegrating cells were distinguished under a microscope. Calculation was made by counting the number of intact and disintegrating cells in a Hawkesley Crystalite Counting Chamber (Hawkesley. Sussex, England). The counting chamber consists of twelve divisions 0.2 mm deep with an area of 12 mm^2 per division to give a total volume 2.4 mm^3. One division consists of 16 fields. Therefore, the number of protozoa per millilitre of original solution can be calculated by counting the number of protozoa in twelve divisions multiplied by a factor 10^3 and divided by 2.4. Since

dilution is involved in fixing the cells, it should be take into account by multiplying that number with the dilution factor. Therefore, the equation would be:

$$N = n \times D \times \frac{1}{2.4} \times 10^3$$

If the number of protozoa were counted only in five divisions as mentioned in the related assays, the equation becomes:

$$N = n \times D \times 10^3$$

N = number of protozoa per 1 ml of rumen content

n = number of protozoa in twelve or five divisions of the counting chamber

D = dilution factor of the sample

The cells on the chamber were counted only in one direction, from left to right of the chamber. The cells on the left or the top line of a division were counted, while the cells on the right or at the bottom were not counted to avoid double counting (see figure 5).

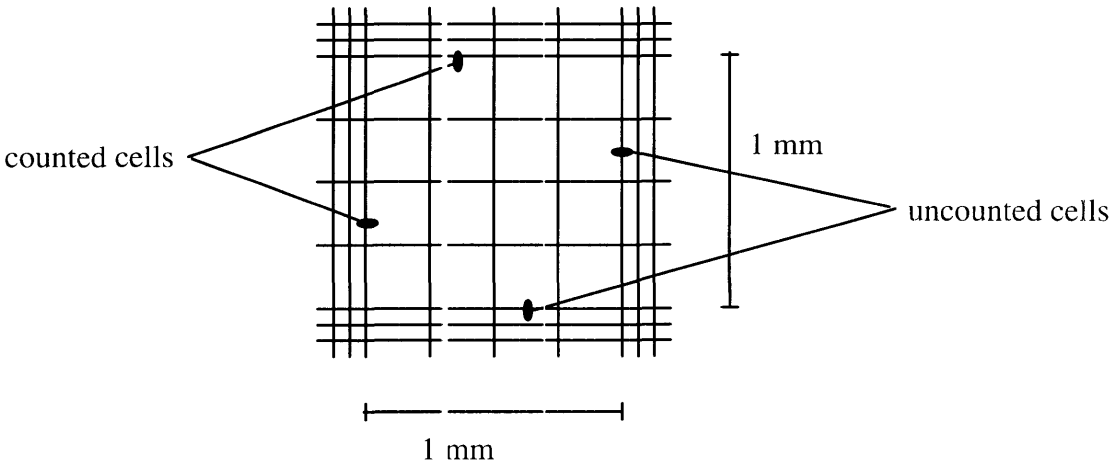


Figure 5.1. One division of the counting chamber

5.2.6. Protozoal lysis assays under an anaerobic system

A portable anaerobic cabinet (Atmosbag, 2 hands, large) was set up to provide anaerobic conditions for protozoal lysis assays. The cabinet was equipped with an air lock to control the anaerobic conditions before the materials were put in or taken out of the cabinet. A leak detector was used to monitor any leak in the cabinet. A microscope was attached to the cabinet to observe the protozoa. The cabinet was filled with nitrogen gas and hydrogen in a ratio of about 19:1. It was also equipped with catalyst to promote combination of hydrogen with any oxygen present inside the cabinet. The anaerobic condition was monitored using an anaerobic indicator. The colour of the indicator changes to pink in the presence of oxygen. If oxygen presence was indicated, the cabinet was flushed again with nitrogen and hydrogen to maintain the anaerobic condition. A thermoline hot plate, maintained at 39°C, was placed inside the cabinet as an incubator.

In order to avoid any possibilities of the rumen protozoa being exposed to air during the protozoal preparation, the rumen fluid collected from the sheep before feeding time was not centrifuged. The rumen fluid (2.5 ml) was diluted in 7.5 ml of Hungate's salt and kept in a water bath (39^{o}C) until used. Sub-samplings were made by transferring 400 µl of diluted rumen fluid into microfuge tubes. The steps of the procedure are described in Fig. 5.2.

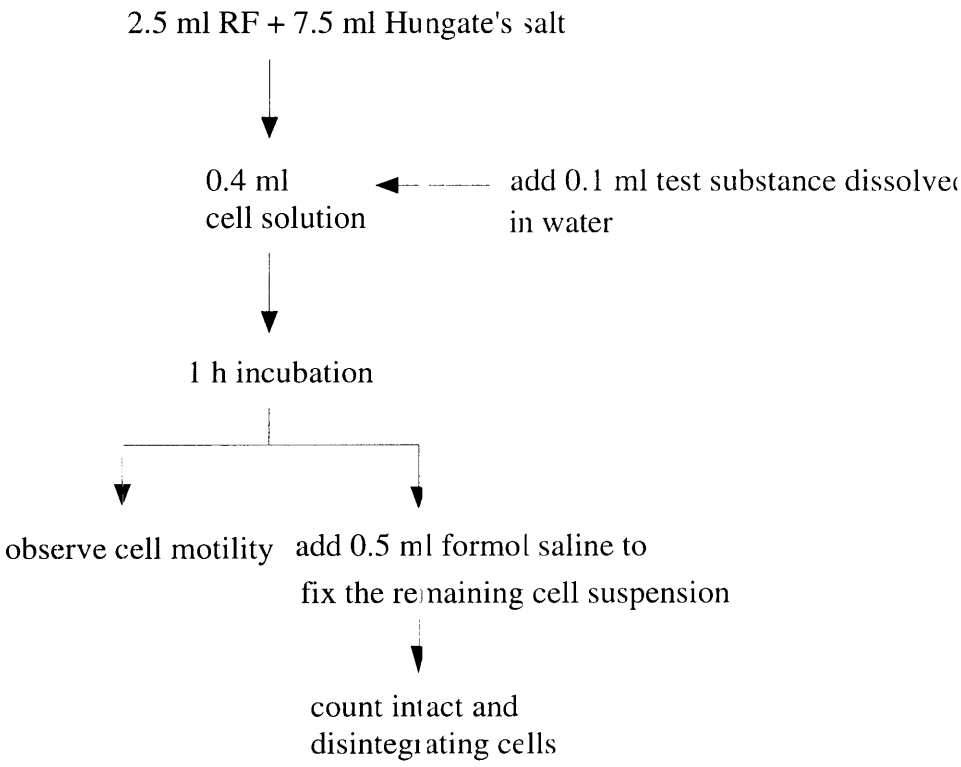


Figure 5.2. Diagram of the procedure for protozoal lysis assays under the anaerobic system

5.3. Results

5.3.1. Protozoal lytic assays under an aerobic system

Assay 5.3.1.1.

A simple *in vitro* system has been developed to observe the activity of purified fractions of *Enterolobium* leaf extract on rumen protozoa.

Eleven fractions were observed in this assay consisting of: the fraction extracted by chloroform from the crude aqueous extraction of *Enterolobium* leaf(IIi); the aqueous fraction which remained after butanol extraction (IIii); the crude aqueous extract (II); purified fractions (IIA, IIB, IIE IIF, IIG) derived from the crude aqueous extract; and fractions from further processing of fractions IIC, IID and IIE (i.e. fraction IIC1, IID1 and IIE1) (see Fig. 3.5 for fractionation scheme). All fractions were adjusted to a concentration of 1 mg/ml with distilled water and 200 µl of each fraction was added to 800 µl of rumen fluid containing protozoa. The mixtures, in duplicate, were incubated in a water bath (39°C) for 1.5 h There was no attempt to separate protozoa from other micro organisms and feed particles prior to testing. The motility and the degree of the disintegration of the cells were observed under a microscope (see section 5.2.5). The results of this assay are presented in Table 5.3.1.1.

Table 5.3.1.1. The motility and the degree of disintegration of rumen protozoa following treatment with *Enterolobium* leaf fractions *in vitro* (Assay 5.3.1.1).

Fractions	Motility[1]	Protozoal cell number[2]			
		Intact	Disintegrating	Total	% Lysis
IIi	0	131	0	131	0
IIii	0	131	17	148	11.5
II	++++	182	6	188	3.2
IIA	0	143	5	148	3.4
IIB	++	102	28	130	21.5
IIE	++++	131	34	165	20.6
IIF	+++	149	19	168	11.3
IIG	++	145	8	153	5.2
IIC1	++++	130	52	182	28.6
IID1	++++	110	49	159	30.8
IIE1	++++	142	22	164	13.4
Control	0	148	0	148	0

[1] In this assay, protozoal movement reported is relative to the control. The control appeared completely normal (100% of protozoa moving) and no disintegrating cells were observed. "0" indicates no visible effect on motility, the more "+" signs the greater the effect on motility (see section 5.2.4.1).

[2] "Protozoal cell number" is the total number of protozoa counted in five divisions of the counting chamber.

Assay 5.3.1.2

In assay 5.3.1.1, four fractions (IIB, IIE, IIC1 and IID1) derived from the parent compound (II) showed relatively high activity in terms of inhibition of cell motility and percentage of cell lysis. An assay was carried out focusing on these four fractions to study any dose-related effects In this assay, Alkanate 3SL3 was included as a comparison. The compound s a detergent proved in several studies to eliminate protozoa from the rumen.

All fractions including Alkanate 3SL3 were adjusted to concentrations of 1 mg/ml distilled water. Each test solution was tested at four concentrations and was added to the cell suspension to make a 1 ml total mixture. After that, the mixture, in duplicate, was incubated at 39°C and observed after 1.5 h and 3 h. After observing cell motility under a microscope, 0.5 ml formol saline was added to the remaining solution to fix the cells and then the number of intact or disintegrating cells was counted in twelve divisions of the counting chamber.

Results:

Table 5.3.1.2.1. Observation of cell motility (Assay 5.3.1.2)

Incubation time (h)	Fractions	Cell motility			
		100 µl	150 µl	200 µl	250 µl
1.5	IIB	0	+ +	+ +	+ +
	IIE	+ +	+ + +	+ + +	+ + + +
	IIC1	+ +	+ + +	+ + + +	+ + + +
	IID1	+ +	+ + +	+ + +	+ + + +
	Alkanate 3SL3	+ + + +	+ + + +	+ + + +	+ + + +
	Control	0	0	0	0
3	IIB	0	+ +	+ +	+ +
	IIE	+ +	+ + +	+ + +	+ + + +
	IIC1	+ +	+ + +	+ + + +	+ + + +
	IID1	+ +	+ + +	+ + +	+ + + +
	Akanate 3SL3	+ + + +	+ + + +	+ + + +	+ + + +
	Control	0	0	0	0

Table 5.3.1.2.2. Enumeration of protozoa after 3 h incubation (Assay 5.3.1.2)

Fraction	Protozoal cell number*)															
	100 µl				150 µl				200 µl				250 µl			
	Intact	Dis-integrating	Total	%lysis	Intact	Dis-integrating	Total	%lysis	Intact	Dis-integrating	Total	%lysis	Intact	Dis-integrating	Total	%lysis
IIB	580	7	587	1.2	482	62	514	12.1	231	313	544	57.5	98	359	457	78.6
IIE	434	7	441	1.6	353	163	516	31.6	62	462	524	88.2	25	492	517	95.2
IIC1	485	29	514	5.6	120	365	485	75.3	8	383	391	98.0	6	462	468	98.7
IID1	488	121	609	1.9	151	392	543	72.2	15	461	476	96.8	-	464	464	100
Alkanate	-	91	91	100	-	28	28	100	-	15	15	100	-	4	4	100

*) The number of intact and disintegrating cells was counted in twelve divisions of the counting chamber.

Assay 5.3.1.3.

The results from the previous assays showed that fraction IIC1 was relatively more active than the other fractions examined. Since fraction IIC1 was apparently still a mixture, further fractionation was carried out to yield ten more fractions (see Section 3.2.2, Chapter 3). The assay reported in Table 5.3.1.3 was carried out to examine the activity on rumen protozoa of four fractions out of ten derived from fraction IIC1. These fractions were chosen from the early, the middle and the later fractions eluted from the HPLC column. Besides these fractions, two other fractions were also examined, namely fraction III1A (a further fractionation of a pooled sample comprising fraction III1-2and fraction III1-3 from the third fractionation experiment and comparable to fraction IIC1) and fraction III1A-9 as a fraction derived from fraction III1A.

All samples were adjusted to 1 mg/ml, and 200 µl of each fraction was added into 800 µl of cell suspension. The samples were incubated in a water bath (39°C) for two different incubation times (1.5 and 3 h). The motility of the the cells was observed and the number of protozoa in twelve divisions of the counting chamber was counted.

Results:

Table 5.3.1.3. Observation of cell motility

Fractions	Motility		Protozoal cell number*)			
	1.5 h	3 h	Intact	Disintegrating	Total	% Lysis
IIC1-1	++	++--	158	80	238	33.6
IIC1-5	+++	++--	162	88	250	35.2
IIC1-6	++++	++++	108	136	283	62.2
IIC1-8	++	++	195	114	309	36.9
III1A	++++	++++	111	170	281	60.5
III1A-9	++	++	180	130	310	41.9
Control	0	0	488	0	488	0

*) The number of intact and disintegrating cells was counted in twelve divisions of the counting chamber.

Assay 5.3.1.4.

The results in assay 5.3.1.3 showed that fraction IIC1-6 and III1A were more active than the other fractions. However the percentage of cell lysis is low compared to that of the results of assay 5.3.1.2. Since these fractions were the products of a re-fractionation of the earlier products, it is necessary to know whether fractionation leads to a loss of (possible) synergistic interaction among components in the mixture. This assay was designed to examine the effect on lytic activity of the combination of two fractions compared to their activity in isolation.

Fractions IIC1-6 and III1A were adjusted to 1 mg/ml. The other sample was the combination of those two fractions in the ratio 1:1. In this case, 300 µl of each fraction were added into a final total volume of 1000 µl of the cell suspension. The samples were incubated for 1.5 h at 39°C and the protozoa in twelve divisions of the chamber were counted.

Results:

Table 5.3.1.4. Effect on lytic activity of the combination of two fractions compared to their activity in isolation.

Fraction	Motility	Protozoal cell number*)			
		Intact	Disintegrating	Total	%Lysis
IIC1-6	++++	247	115	362	31.8
III1A	++++	227	108	335	32.2
IIC1-6 + III1A	++++	250	107	357	30.0
Control	0	549	0	549	0

*) The number of intact and disintegrating cells was counted in twelve divisions of the counting chamber.

Assay 5.3.1.5.

It has been shown in the previous assay that fraction III1A and IIC1 had an ability to paralyse and to lyse the cells presumably because they are basically identical chromatographic fractions. Thus fraction III1A was subjected to further fractionation by reverse-phase HPLC to yield 12 more fractions (fractions III1A-1 to III1A-12) (see Fig. 3.6).

The activity of the first seven fractions from this separation are tested. In addition, fractions IIC1 and III1A were also included as positive controls and to check the reproducibility of the action.

All purified fractions were adjusted to 1 mg/ml and 300 µl of each fraction were added into 700 µl of the cell suspension. The samples were incubated for 1.5 h and the protozoa in five divisions of the counting chamber were counted.

Results:

Table 5.3.1.5 The activity of the first seven fractions from further separation of fraction III1A

Fractions	Motility	Protozoal cell number*)			
		Intact	Disintegrating	Total	% Lysis
IIC1	++++	88	81	169	46.3
III1A	++++	86	90	176	47.6
III1A-1	++++	115	42	157	29.9
III1A-2	++++	73	84	157	55.5
III1A-3	++++	68	81	149	58.5
III1A-4	++++	69	97	166	57.9
III1A-5	++++	53	87	140	67.7
IIIA1-6	++++	88	81	169	46.3
III1A-7	++++	113	47	160	31.1
Control	0	164	0	164	0

*) The number of intact and disintegrating cells was counted in five divisions of the counting chamber.

Assay 5.3.1.6.

In tests conducted so far, the number of protozoa in the rumen fluid has been shown to vary from time to time. As the sheep was fed with the same amount of basal feed, this variation could be attributed to collecting the rumen fluid on different occasions. This difference may affect the lytic activity of *Enterolobium* fractions. Therefore, it is necessary to calculate the number of protozoa prior to testing. For that purpose, the number of protozoa should be counted before and after the sheep was fed. Assays were undertaken in two consecutive days.

Rumen fluid was taken one hour before and every hour for five hours after feeding. Sub-samplings were made in duplicate by taking 1 ml of the rumen fluid into microfuge tubes. After that, 0.5 ml of formol saline was added to fix the cells. Protozoa were counted in twelve divisions on the counting chamber under a microscope at 40 x magnification.

Results:

Table 5.3.1.6. Protozoal cell number collected from rumen fluid

Time of collecting rumen fluid	Protozoal cell number (x 10^3)	
	Day 1	Day 2
- 1	446	649
0*)	590	696
1	391	442
2	229	374
3	222	341
4	219	269
5	154	259

*) 0 = time of feeding (08.30)

The results of this assay showed that the number of protozoa before, during and after feeding time varied remarkably with changes in the rumen volume. It also found that the rumen fluid sample taken before the sheep was fed gave cleaner rumen fluid.

Assay 5.3.1.7.

The result of assay 5.3.1.6 showed that cleaner rumen fluid could be obtained if the rumen fluid was collected before feeding time. However, the refinement of this method is necessary to separate protozoa from other feed particles.

This assay is an attempt to separate protozoa from other feed particles by centrifuging the rumen fluid at low speed.

Rumen fluid was collected (40 ml) and centrifuged at two different speeds 500 and 1000 x g for 1 minute. The supernatant was discarded and the cell suspension was washed with warmed saline (0.9 % (w/v) NaCl) twice. The suspension was then centrifuged for another 1 minute at the corresponding speed. The protozoa were resuspended in 0.9 % saline and incubated in a water bath (39°C) for three different incubation times. The cell motility was observed under a microscope.

Results:

The result of this assay showed that centrifuging the rumen fluid at 1000 or 500 x g influenced cell motility considerably, although cleaner protozoal suspensions could be obtained. Saline as the washing solution could also influence the cell motility as it was found that the pH in cell suspension was low (pH = 5.2).

Assay 5.3.1.8.

The result in assay 5.3.1.7 suggest the use of phosphate-buffered saline to improve the protozoal preparation method by stabilizing the pH level of the cell suspension.

An assay was carried out to study the effect of phosphate-buffered saline (Hungate's salt) on the cell motility compared to 0.9 % saline.

The rumen fluid was centrifuged at 500 x g or less for 1 minute. The supernatant was removed and cells were resuspended with either 0.9 % saline or Hungate's salt. The cell suspension was centrifuged and resuspended. After that, the cell suspension was incubated in a water bath for 1 h at 39 °C. The result of this assay showed that Hungate's salt had less effect on the protozoa than did the saline.

5.3.2. Protozoal lytic assays under an anaerobic system

Assay 5.3.2.1

It was found that centrifugation and washing solution affected the protozoa motility. Further observation is required to identify other factors which could also affect the protozoa motility such as the presence of oxygen.

An assay was set up to observe the effect of centrifugation and washing solution in the absence of oxygen.

Basically the same procedures were applied in this assay. However, at every stage the presence of oxygen was minimised by flushing the protozoa suspension and Hungate's salt with carbon dioxide (CO_2) gas. Unfortunately, flushing with CO_2 dropped the pH of the protozoal suspension, thus inactivating some of the protozoa. This assay was repeated by using nitrogen instead of CO_2 to preserve anaerobic conditions. This resulted in more motile cells (about 80 % of the protozoa were motile).

Above all, developing the method of protozoa separation from other micro organisms and feed particles showed that better results could be obtained if the separation was conducted under anaerobic conditions. Therefore, a portable anaerobic cabinet was set up (see section 5.2.6).

Assay 5.3.2.2

An assay was carried out under anaerobic condition to observe the lytic activity of a crude butanol extract compared to Alkanate 3SL3.

In order to avoid any possibilitie s of the rumen protozoa being exposed to air during the protozoal preparation, the rumen fluid collected from the sheep before feeding time was not centrifuged. The rumen fluid (2.5 ml) was diluted in 7.5 ml Hungate's salt and kept on a thermoline hot plate (39°C) functioning as an incubator. Sub-samplings were made by transferring 400 µl of diluted rumen fluid into microfuge tubes. Meanwhile, the crude butanol extract and Alkanate 3SLS were adjusted to three different concentrations (1, 2 and 5 mg/ml). These agents (100 µl) were added to microfuge tubes already containing the protozoal suspension. The mixtures were incubated for 1 h. After 1 h incubation, the protozoal motility was observed under a microscope, while the protozoa in the remaining solut on were fixed by adding 0.5 ml formol saline and the number of protozoa in five divis ons of the counting chamber were counted.

Result:

Table 5.3.2.2. The lytic activity of a crude butanol extract compared to Alkanate 3SL3

Lytic Agent	Concentration (mg/ml)	Motility	Protozoal cell number*)			
			Intact	Disintegrating	Total	%Lysis
Alkanate	1	+	38	4	42	9.5
	2	+	19	15	34	44.1
	5	+	8	19	27	70.4
Crude extract	1	+	15	20	35	57.1
	2	+	9	23	32	71.9
	5	+	4	29	33	87.9
Control		0	35	1	36	2.8

*) The number of intact and disintegrating cells was counted in five divisions of the counting chamber.

Assay 5.3.2.3

The new series of purified fractions were supplied in a very limited amount. Part of these fractions were subjected to an anaerobic examination to observe their lytic potency on rumen protozoa.

The same procedures as for the aerobic system were applied in this assay. All purified fractions and the crude butanol extract were adjusted to 1 mg/ml. These lytic agents (100 µl) were added to 400 µl of the cell suspension. The motility and the degree of disintegration of the protozoa were observed after 1 h incubation. Protozoa were counted in five divisions on the counting chamber under a microscope at 40 times magnification.

Table 5.3.2.3. Examination of lytic potency of the new series of purified *Enterolobium* fractions under an anaerobic condition .

Fractions	Motility	Protozoal cell number*)			
		Intact	Disintegrating	Total	% Lysis
III2-12	0	45	1	46	2
III2-13	++++	41	17	58	29
III2-14	++++	39	14	53	26
III2-15	++++	51	7	58	12
III2-16	++	38	8	46	17
III2-18	++++	41	15	56	27
III2-19	++++	46	13	59	22
III2-20	++++	44	10	54	19
III2-21	++++	47	7	54	13
III2-22	++++	41	9	50	18
Crude extract	++++	36	9	45	20
Control	0	45	1	46	2

*) The number of intact and disintegrating cells was counted in five divisions of the counting chamber.

5.4. Discussion

An *in vitro* assay using rumen protozoa was developed to study the antiprotozoal activity of the purified fractions of *Enterolobium* leaf extract under aerobic and anaerobic conditions. Motility and the degree of disintegration of the cells were observed under a microscope to assess the activity of the purified fractions. It should be noted, that the protozoal cell numbers assessed by this visual counting, are very subjective. Care has been taken to obtain a representative sample of rumen fluid by collecting the rumen fluid 1 h before the sheep was fed. The number of protozoa in the rumen fluctuates from time to time as can be seen in several assays. Especially for enumeration of protozoa, the number of protozoa in the treatment is always found less than in the control. It is probably due to some of the cells having burst completely when in contact with the agent, therefore these cells are uncountable.

The antiprotozoal activity of even fractions from the second batch were observed under an aerobic system. From examination of Table 5.3.1.1, fraction IIi ($CHCl_3$ extract from the initial extract) IIii (aqueous residue after BuOH extraction) and IIA (the initial fraction from HPLC of the BuOH extractable fraction) showed no inhibition effect on cell motility. However, the parent fraction (fraction II) showed strong inhibition of cell motility.

Based on their analogous elution times from HPLC, fraction IIE is comparable to fraction IE from the first batch (which showed relatively high lytic activity towards red blood cells). Fraction IIC and IID which are comparable to fraction ID from the first batch, were not assayed but were subjected to further fractionation to yield fraction IIC1 and IID1 respectively (see Figure 3.5). These fractions (IIC1 and IID1), fraction IIE, and also fraction IIE1 (derived from IIE) showed relatively high ability to immobilise the protozoa.

The number of protozoa were counted after fixing the cell with 0.5 ml formol saline. The average of the total protozoa counted was 158 ± 19. Administration of the fractions at 200 µg/ml produced cell lysis ranging from 0 to 30.8%. These fractions, which exhibited high immobilising activity, also produced high percentage of cell lysis (fractions IIE, IIC1, IID1 and IIE1). However other evidence showed that the parent fraction (Fraction II), which possesses high immobilising activity, did not show a high percentage of the cell lysis. Moreover, fraction IIB, which had a relatively low inhibition of cell motility, produced quite a high percentage of lysis (21.5%). Therefore, compounds responsible for cell lysis may differ from those which cause loss of motility and appear to be separated by the HPLC procedures employed.

From the second assay (Assay 5.3.1.2), attention was focussed on four fractions (IIB, IIE, IIC1 and IID1) to study any dose-related effects. All cells were immobilised by the addition of 250 µl of any fraction except fraction IIB. Alkanate 3SL3 showed very strong inhibitory action on cell motility. There was no additional effect on cell motility of increasing the incubation time from 1.5 h and 3 h. Among those fractions tested, fraction IIC1 seems to have the highest immobilising activity.

On addition of up to 150 µl of each fraction into the test system, only fraction IIC1 and IID1 produced lysis of above 50%. However, with increased addition beyond 150 µl, all fractions lysed more than 50% of the protozoa.

In the case of Alkanate 3SL3, all cells were distrupted at all levels of addition. There were no intact cells and only a few disintegrating cells were able to be counted.

In line with its high level of activity in inhibiting cell motility, fraction IIC1 also showed a high ability to lyse the cells.

Further purification of fraction IIC1 produced 10 more fractions and the antiprotozoal activity of four of them were examined. The samples were incubated in a water bath for two different incubation times (1.5 and 3 h). It can be seen in Table 5.3.1.3 that there was no difference in cell motility between incubations of 1.5 h and 3 h. Examination of the cell motility provides evidence that fractions IIC1-6 and III1A showed the highest ability to immobilise the cells. Fraction IIC1-6 was the fraction eluted in the middle of HPLC fractionation. Corresponding to their activity in cell immobilisation, fraction IIC1-6 and III1A exhibited lytic activity at almost twice that of the other fractions.

The results of examination of the effect the combination of two fractions compared to their lytic activity in isolation showed that there was no difference of their ability to inhibit cell motility, whether the fractions were present individually or in combination.

The results indicated that their activities were not affected after fractionation. It also indicated that further fractionation of fraction IIC1 to give IIC1-6 did not affect its activity as measured in this semi-quantitative measure. In terms of the HPLC separation, fraction III1A is comparable to fraction IIC1. This means that the assay would have been expected to demonstrate an increased effect after combination. This could have been expected if components in the less purified III1A fraction interacted synergistically with fraction IIC1-6 components.

Fraction III1A was subjected to further purification to yield 12 more fractions and seven of them were tested (Assay 5.3.1.5). The result showed that all fractions immobilise the cells. The average number of protozoa counted was 160 ± 11. The result of this assay also showed evidence that fraction IIC1 and III1A yield basically similar results although they were prepared from two different batches independently.

The first fraction eluted from the HPLC column showed low lytic activity compared to the later fractions. The strongest activity was shown by fraction III1A-5. This could support the assumption that the fraction eluted in the middle of the HPLC fractionation appears to have strong ability to physically distrupt the membrane of the protozoa.

Attempts have been made to improve the isolation of protozoa by centrifuging the rumen fluid to separate protozoa from other microbes and feed particles and washing the protozoa suspension using the phosphate-buffered saline (Hungate's salt). Although better protozoal suspensions can be obtained, the cell motility was still slightly affected. Since the condition of protozoa in the rumen is strictly anaerobic, an anaerobic system using a portable anaerobic cabinet was set up.

While waiting for the supply of the new purified fractions of *Enterolobium* extract from Dr. David Tucker, an assay was conducted to examine the lytic activity of crude butanol extract compared to Alkanate 3SL3 under this anaerobic system. It can be seen in Table 5.3.2.2 that crude butanol extract inhibited protozoa motility more strongly that did Alkanate 3SL3. The crude butanol extract also showed stronger lytic activity on rumen protozoa.

Although the new purified fractions were supplied in a very limited amount, some of them were examined under an anaerobic system to observe their antiprotozoal activity, whereas the remaining fractions were kept for their structural studies. The result in Table 5.3.2.3 showed that not all of the purified fractions were strong enough to immobilise the protozoa. Although almost all of the fractions showed high level activity on inhibitory cell motility, their lytic activity can not reach 50% of the cell lysis, much less potent than the previous assays. The first fractions eluted from HPLC column (i.e. III2-12 and III2-16) showed lower ability to immobilise the cells than those fractions eluted later.

Chapter 6

EXAMINATION OF THE SUGAR MOIETY OF ACTIVE FRACTIONS FROM *ENTEROLOBIUM* LEAF

6.1. Introduction

The compounds in *Enterolobium* leaf have been shown to be especially potent in lysing ruminal protozoa (Leng *et al.*, 1992). Using an erythrocyte-based assay, lytic activity present in the leaf has been fractionated and found to occur in more than one component of the extract. Thus to understand the special ability of *Enterolobium* to lyse protozoa it is important to know: (i) does one or more of the fractions possess an unusually high lytic activity? (ii) does the pronounced lytic action of the plant rely on synergistic interactions among the leaf fractions?

To explore these points it is necessary to determine the molar potency of lytic action. It is necessary to know if any of the fractions show an especially high ability to lyse protozoa when related to equivalent concentrations in molar terms.

Since no specific chemical assay is available for the *Enterolobium* saponins to determine the molar concentration in the various leaf fractions, two different approaches were possible: (i) estimation of the quantity of the aglycone, (ii) estimation of the quantity of the constituent sugars.

Although estimation of the aglycone is very direct in that there is only one aglycone per saponin molecule, the nature of the aglycone and its possible susceptibility to chemical and physical treatments involved in isolation is still unknown. For example by analogy with *Enterolobium contortisiliquum,* it might contains a lactone ring which could result in two derivatives after acid hydrolysis.

It was decided to attempt to determine the molar concentration of each active fraction on the basis of its sugar content after consideration of the ease of separation of sugar derivatives by gas liquid chromatography.

It was also possible that lytic action was regulated by the nature of the sugar moiety attached to the aglycone (Takechi and Tanaka, 1990; Takechi *et al.*, 1991). Analysis of the sugars found in each fraction could indicate a role of the sugar fragment in determination of molar lytic potency.

The ubiquitous presence of glycosidases in plant tissue raises the possibility that the active agent present in fresh leaves may be degraded by endogenous enzymes in the leaf after harvesting. Since dried leaf tissue was used in all experiments reported in this thesis, the possibility exists that the original active fraction of the leaf may have been subjected to degradation of any oligosaccharide element. Under these conditions, a family of compounds may have been generated, resulting from the degree to which the parent compound had had monosaccharides removed by the endogenous enzymes of the plant. However, sheep fed on the same dry leaf were still defaunated demonstrating that at least some active material remained (Leng *et al.*, 1992).

An analysis of the carbohydrate moiety of fractions obtained from HPLC would thus provide: (i) evidence of any partial degradation of a parent molecule; (ii) an indication that lytic potency is related to the nature of the oligosaccharide chain, and (iii) an opportunity to relate lytic activity to the molar concentration.

Gas chromatography of volatile derivatives can be confidently employed in the analysis of monosaccharides to obtain both qualitative and quantitative information on sugars present (Laine *et al.*, 1972). Good quality gas chromatographic separation of sugars can be achieved following trimethy silylation of the monosaccharides which give volatile ether derivatives.

Trimethylsilyl (TMS) derivatives and alditol acetates are the most widely used volatile forms of sugars. TMS ethers are perhaps the most generally useful derivatives (Laine *et al.*, 1972). These ethers are easily prepared and react only to a very small extent with the column support. They are easily hydrolysed, however, and adequate precaution must be taken to exclude moisture during preparation and subsequently.

According to El Khadem (1988) the complete structure of oligosaccharides is established when the following points are determined:

(i) the degree of polymerisation, i.e., the numbers of monosaccharide units present in the oligomer molecule;

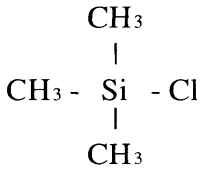
(ii) the nature of the monosaccharide monomer(s);

(iii) in the case of hetero-oligosaccharides, the monomer sequence;

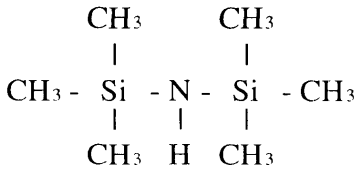
(iv) the ring size (pyranose or furanose) and the position of linkage of the different monosaccharides (1→?); and

(v) the anomeric configuration (α and β) and the conformation of the monosaccharide units.

The silylation reaction utilises the commercial mixture "Tri-Sil". Following is the theoretical background to silylation. The reagent consists of a mixture of chlorotrimethylsilane and hexamethyldisilazane dissolved in pyridine.

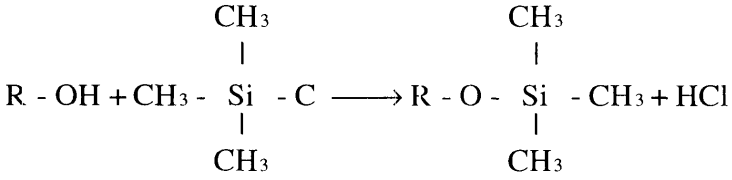


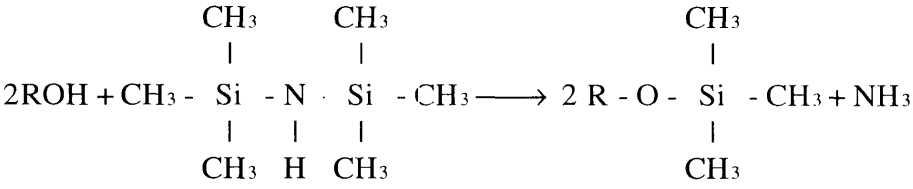
Chlorotrimethylsilane



Hexamethylsilazane

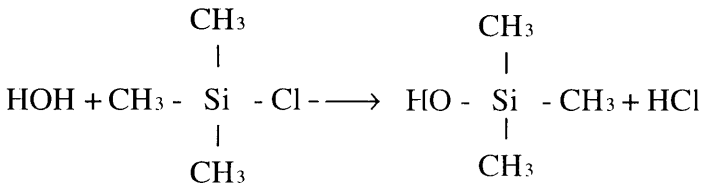
Both of these reagents react with OH groups to produce a Si-O bond and thus produce a silicone ether.

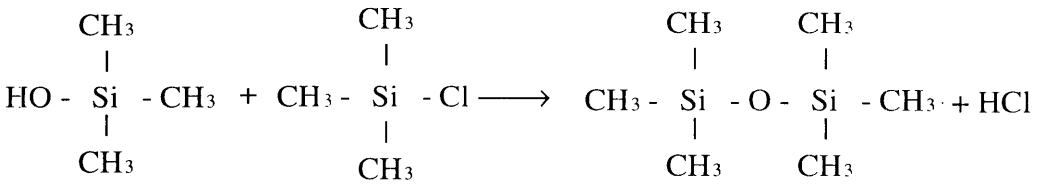




The reactions, which proceed best under basic conditions, are normally carried out in pyridine and result in precipitation of NH_4Cl which must be removed by centrifugation.

The reactivity with OH extends to reactivity with water and thus reactions involving the silylation reagents must be carried out under conditions in which they are strictly protected from water. The equations below indicate the reaction of trichloromethylsilane with water as an example.





All OH groups of sugars are readily and completely substituted, including the hemiacetal OH, which on substitution forms a full acetal and thus "locks" the derivative in either the α- or β- configuration. Thus amorphous glucose isolated by freeze-drying from aqueous solution will be present as both the α- and β- anomers in the proportions found in their former aqueous environment. Derivatization of such mixtures thus produces two peaks on the GLC trace. In the case of some sugars, more than two peaks are observed because both the pyranose and furanose rings are stabilised and each may exist as the α- and/or β- anomer

The present experimental work is focused on determining the number and variety of the monomeric units.

6.2. Materials and Methods

6.2.1. Preparation for chromatography

6.2.1.1. Acid hydrolysis

Acid hydrolysis was used to cleave the glycosidic linkages between the component sugars and between the sugar and aglycone. A known weight of each sample was dissolved in 1 ml of 1 *M* H_2SO_4 and transferred to a soda glass tube (made from a

pasteur pipette). The sample was then frozen with liquid nitrogen before evacuation of the tube, which was then sealed by fusing the glass with a bunsen burner. The sealed sample tube was then placed in a 110° C oven for 5 h. After acid hydrolysis, the sample was quantitatively transferred to a 10 ml centrifuge tube and extracted with 1 ml of diethyl ether. The extraction was repeated three times. This process was then followed by neutralisation of sulphuric acid with barium hydroxide ($Ba(OH)_2$).

6.2.1.2. Neutralisation

The sulphuric acid was neutralised with 0.17 *M* $Ba(OH)_2$ and a few drops of ammonium hydroxide (NH_4OH) added to ensure that the reaction mixture was very slightly alkaline due to a volatile base. Subsequently, the solution was centrifuged at 5000 x g for 15 minutes to separate any precipitate. The supernatant was transferred to a 50 ml round bottom flask for freeze drying. The solution was spread in a thin layer on the walls of the flask to facilitate the drying process. Because quantitative recovery of sugars from the fraction was required it was found necessary to take precautions to avoid fine particles of the dried solid being carried out of the flask in the stream of vapour. This was achieved by fixing a nylon gauze (20 µ holes) to the end of the male ground glass joint of the freeze-dried head. The gauze was attached by a thin film of a cyanoacrylate glue and did not interfere with the fit of the joint. The freeze-dried product was a fine, white fluffy powder.

6.2.1.3. Preparation of TMS derivatives

The freeze-dried sample after acid hydrolysis was treated *in situ* with 500 µl of "Tri-Sil" (Pierce Chemical Co. Rockford, Illinois, USA) reagent in the flask for freeze drying. The reagent was added in three parts, 100, 200 and 200 µl with transfer of each portion to a common vial after reaction (1 min) and before addition of the next aliquot. This precaution was designed to facilitate quantitative transfer of the sugar derivative into a 3

ml vial. The mixture was centrifuged at 5,000 x g for 15 minutes to remove ammonium chloride formed in the reaction. The supernatant was transferred into a 1.5 ml glass vial with a teflon-lined cap. The sample was then ready for injection into the gas chromatograph.

6.2.2. Chromatography

6.2.2.1. Chromatographic conditions.

All gas chromatographic analyses were performed on a Hewlett Packard Model 5890 series II Gas Chromatography equipped with dual flame ionisation detectors. The column used was a 0.54 mm x 15 m SE-30 (Alltech Associate Inc. USA). The injector-block temperature was 275° C, with temperature programming of the column oven ranging between 130° and 150° C using various programmes to optimise the separation (see below). Helium was used as carrier gas at a flow rate of 20 ml per minute, with inlet pressure 15 psi. Effluent gas from the column was "split" so that 5% of the stream was passed to the detector. A precisely known volume between 0.1-1.0 µl of the reaction mixture from the derivatization procedure was injected into the chromatography column. Quantitative analysis was performed by comparison of area responses to those of the standard sugars. Areas (mV.Sec.) for each peak were determined automatically by the integrator built into the chromatograph.

6.2.2.2. Initial separation method

An initial method was set up which achieved less than satisfactory separation of three standard sugars: glucose (Glc), galactose (Gal) and rhamnose (Rha). The temperature programming was then subjected to refinement in order to overcome problems such as over-long retention time and incomplete separations. This resulted in a standard programme that was used for all subsequent analyses.

The temperature programme was:

- column temperature at time zero (i.e. sample injection) 130° C
- column temperature at time 2.0 min 130° C
- column temperature at time 3.5 min 145° C
- column temperature at time 5.0 min 145° C
- column temperature at time 8.0 min 148° C
- column temperature at time 12.0 min 148° C
- column temperature at time 14.0 min 150° C
- column temperature at time 20.0 min 150° C

6.2.2.3. Injection technique

Due to the very small amount of sample injected each time, the precision of the injection technique needed to be checked A series of tests was carried out by injecting 0.1 µl of a standard mixture 5 times consecutively.

6.2.2.4. Analysis of standard sugars

To identify any problems with the application of the GLC programme developed (above) to identification and estimation of the likely monosaccharides from the purified *Enterolobium* fractions, a number of experiments were carried out using glucose, galactose and rhamnose as standard sugars. Each sugar was individually subjected to the same hydrolysis and recovery conditions as the *Enterolobium* fractions were. After that, the samples were individually injected into the chromatography column.

The standard sugars were prepared several times to examine the reproducibility of the derivatisation procedures. The standard mixture was then developed from these sugars by mixing them in a ratio of 1:1:1. Retention times noted for the standard mixture were used to identify peaks in the hydrolysis products of *Enterolobium* fractions. However,

when purified fractions were analysed, there were some unidentifiable peaks present in the chromatograms. An attempt to identify them was made by including two further standard sugars, i.e. xylose and arabinose using the same method. These sugars were chosen as they are commonly present in saponins.

6.2.2.5. Analysis of the purified fractions of *Enterolobium* leaf extract

In this experiment, only 9 purified fractions and a crude butanol extract (PExt) were analysed for sugar moieties. These fractions were subjected to acid hydrolysis with concentration 1 mg of sample per 1 ml of 1 *M* H_2SO_4 followed by neutralisation and silylation as described in Section 6.2.1.1. After that, 0.5 µl of each fraction was injected into the chromatography column.

6.3. Results

6.3.1. Retention time for standard sugars

Experience with repeated injections of standard sugars showed that retention times were reproducible to within ± 0.1 min of the mean retention time.

Table 6.1. The retention time of the standard sugars

Standard sugar	Retention time (minutes)		
	peak 1	peak 2	peak 3
Arabinose	3.8	4.1	---
Rhamnose	4.2	5.2	---
Xylose	4.2	5.2	6.5
Galactose	8.8	9.7	11.8
Glucose	10.9	16.7	---

This allowed unequivocal assignment of peaks to a particular component in mixtures of standard sugars. Given the limited number of monosaccharides that have been reported among saponins (see Chapter 2) it seems that one could confidently identify sugars

encountered in fractions from *Enterolobium* extracts even though some fractions of different sugar components sometimes co-eluted.

Thus peak number 2 of arabinose, number 1 of rhamnose and number 1 of xylose eluted at the same time, and peak number 2 of rhamnose and number 2 of xylose also co-eluted later. This need not constitute an obstacle to interpretation of a sugar chromatogram because second or third peaks of a given sugar are well separated from any co-eluting components, thus allowing identification and even an approach to quantitation.

6.3.2. Precision and reproducibility

The results in the following table (Table 6.2) showed a variation in total area response (i.e. the sum of the individual areas of all peaks for a given sugar - mV.Sec.) in the range 4.3 % to 14.8 %, an average of 11.4 %, although retention time for each peak was consistent. The percentage variation is the standard deviation expressed as a percentage of the mean.

The sugar derivative solutions used in the construction of Table 6.2 were prepared with varied weight of the component sugars. In the preparation of Table 6.3 care was taken to keep the weight of all three components at exactly 1 mg/ml of each sugar in the starting solution.

Table 6.2 The precision obta nable from repeated injection

Standard sugar	Retention time (min)	Area (mV.Sec.) injection					Average (mV.Sec.)	Standard deviation (mV.Sec.)	Variation*) (%)
		1	2	3	4	5			
Rhamnose	4.1	4236	4134	4039	5189	4407	4279	183	4.3
Rhamnose	5.2	2290	2413	2174	2888	2423	2437	271	11.1
Galactose	8.8	219	188	212	249	202	214	23	10.6
Galactose	9.9	707	644	689	855	693	717	80	11.2
Glucose	10.9	3530	3147	3450	4306	3453	3577	433	12.1
Galactose	11.7	3128	2732	3061	3800	3018	3148	394	12.5
Glucose	16.7	5027	4376	4985	6122	4745	4991	738	14.8
Total		19136	17635	18611	23408	18940	19546	2235	11.4

*) The variation is calculated as the standard deviation divided by the average, expressed as a percentage

In common with the procedure followed for Table 6.2, these sugars were subjected to a sham acid hydrolysis as well as procedures for neutralisation and freeze drying. Also it is probable that much of the variability seen in Table 6.2 arose because of the very low volume (1 µl) injected.

In Table 6.3 the sugar derivative solution was diluted with pyridine to one tenth of the original concentration and 1.0 ul was injected (giving the same total delivery of the derivatives to the chromatograph).

Table 6.3. Repeat test of injection precision

Standard sugar	Retention time (minutes)	Area (mV.Sec.) injection 1	2	3	4	Average (mV.Sec.)	Standard deviation (mV.Sec.)	Variation*) (%)
Rhamnose	4.2	4807	4601	4429	4471	4612	189	4.10
Rhamnose	5.2	2682	2485	2390	2393	2487	137	5.51
Galactose	8.8	279	233	223	220	239	27	11.44
Galactose	9.9	888	845	823	830	847	29	3.41
Glucose	10.9	4795	4571	4448	4478	4573	157	3.44
Galactose	11.8	4238	3995	3907	3912	4013	155	3.87
Glucose	16.8	7331	6904	6764	6787	6946	263	3.79
Total		25178	23798	23126	23236	23834	943	3.96

*) The variation is calculated as the standard deviation divided by the average, expressed as a percentage

The chromatograms used for the preparation of Table 6.3 showed some additional small peaks not seen in the chromatograms of the undiluted derivatives. These peaks appeared at 4.7 min, 5.7 min, 6.[illegible] min and 13.3 min, and the largest peak (13.3 min) was not greater than 105 mV.Sec., i.e. 0.25% of the total area under the trace. These peaks were ignored in this analysis, it is being assumed that they arose from the pyridine used to dilute the sugar-TMS derivatives.

As the volume of the standard mixture affected the results, the following test was carried out to obtain a standard curve by injecting the standard mixture with 0.2 µl increments starting from 0.1 µl.

Table 6.4. The relationship between amount of sugar-TMS injected and the detector response

Retention time (min)	Area (mV.Sec.)			
	0.1 µl injected	0.3 µl injected	0.5 µl injected	0.7 µl injected
4.1	562.89	1652.29	2433.38	3570.05
5.2	298.17	900.19	1333.05	1900.27
8.8	21.84	92.09	128.19	120.46
9.9	90.06	283.25	438.90	630.73
10.9	508.13	1533.66	2366.31	3366.47
11.7	411.79	1297.58	2061.85	2955.13
16.8	702.64	2114.01	3524.18	4971.29
Total	2644.56	7935.71	12336.46	17587.31

As can be seen on the figure below, the total area (mV.Sec.) for each injection increased linearly according to the volume injected.

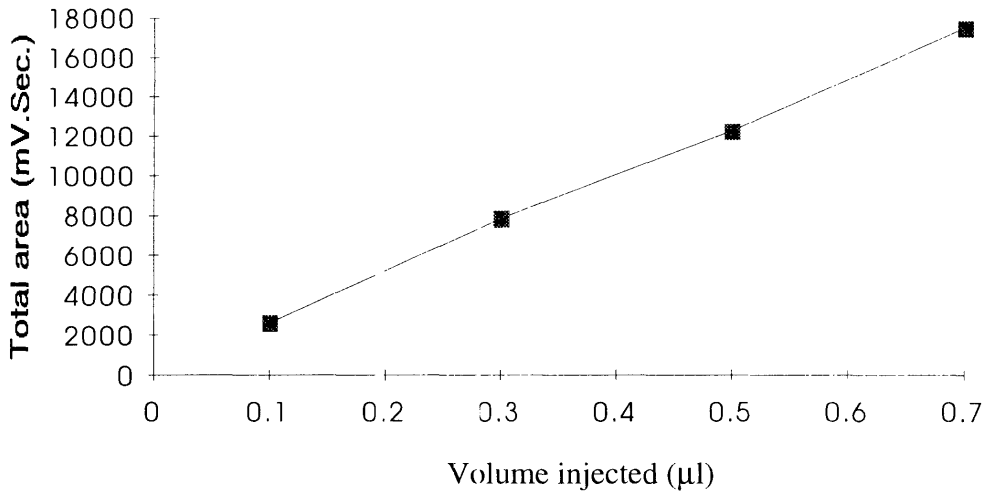


Figure 6.1. Relationship between the volume of injection and the total area

6.3.3. The composition of equilibrium mixture of standard sugars.

Because sugar analysis of *Enterolobium* fractions would be performed by GLC on freezed-dried hydrolysis products, it is expected that equilibrium mixtures of all anomeric and ring-size isomers would be present in the ratios expected in aqueous

solution. Thus standard monomeric sugars were subjected to the conditions employed for acid hydrolysis and after freeze drying were treated to form the TMS derivatives and then separated by GLC. The distribution of the various isomeric species is given in Table 6.5.

Table 6.5. The composition of equilibrium standard sugar solution as determined by gas chromatography of trimethylsilyl derivatives.

Standard sugar	The composition of equilibrium mixture of standard sugars (%)		
	peak1	peak 2	peak 3
Arabinose	35.7	64.3	---
Rhamnose	58.3	41.7	---
Xylose	11.1	36.7	52.2
Galactose	4.0	32.2	63.7
Glucose	43.1	56.9	---

6.3.4. Sensitivity of the assay procedure for various standard sugars

The following calculation was used to measure the amount of each individual sugar recovered per 1000 mV.Sec. of total area response. Suppose a mixture of a standard solution consists of rhamnose, galactose and glucose in the ratio 1:1:1 containing 0.67 mg/ml of each individual sugars

In this case,

1 µl injected ≡ 0.67 µg of each standard sugar

0.5 µl injected ≡ 0.34 µg of each standard sugar

To calculate this, rhamnose is used as an example.

0.5 µl injection of rhamnose gave total area 9162 mV.Sec. (sum of areas of peaks at 4.2 min and 5.2 min).

To produce 1000 mV.Sec requires:

$$\frac{1000}{9162} x 0.34 = 0.037 \text{ µg of rhamnose.}$$

The same calculation may be applied to the other standard sugars. Table 6.6 shows the sensitivity of the assay procedure for individual sugar for 1000 mV.Sec.

Table 6.6. The sensitivity of the assay procedure for individual sugars (i.e. mass required (µg) to yield total area response of 1000 mV.Sec.)

Standard sugar	Concentration (µg/µl)	Volume injected (µl)	Amount injected (µg)	Total area response (mV.Sec.)	Sensitivity µg/1000 mV.Sec
Arabinose	2	1	2	36258	0.055
Rhamnose	0.67	0.5	0.34	9162	0.037
Xylose	2	1	2	53547	0.037
Galactose	0.67	0.5	0.34	8699	0.039
Glucose	0.67	0.5	0.34	8506	0.040

These figures may be used to calculate the amount of sugars present in the unknown samples.

6.3.5. Chromatography of *Enterolobium* fractions

The type and amount of sugars present in the purified fractions from HPLC is shown in Table 6.7.

Table 6.7. The type and amount of sugars present in purified fractions from *Enterolobium cyclocarpum*

No	Purified Fraction	Total area response (mV.Sec.)*					The type and amount of sugar (μg)					Total
		Ara	Rha	Xyl	Gal	Glc	Ara	Rha	Xyl	Gal	Glc	(μg)
1	**PExt**	1169	2546	763	34	1673	0.06	0.09	0.03	0.00	0.07	**0.25**
2	**III1A-11**	150	201	237	0	405	0.01	0.01	0.01	0.00	0.02	**0.04**
3	**III2-12**	29	344	99	1052	667	0.00	0.01	0.00	0.04	0.03	**0.09**
4	**III2-13**	678	853	374	152	676	0.04	0.03	0.01	0.01	0.03	**0.12**
5	**III2-14**	816	1410	883	366	1479	0.04	0.05	0.03	0.01	0.06	**0.20**
6	**III2-16**	965	1108	921	324	1375	0.05	0.04	0.03	0.01	0.06	**0.20**
7	**III2-19**	786	898	518	78	889	0.04	0.03	0.02	0.00	0.04	**0.13**
8	**III2-20**	0	2389	1180	615	2746	0.00	0.09	0.04	0.02	0.11	**0.27**
9	**III2-21**	1752	2982	1906	673	2445	0.10	0.11	0.07	0.03	0.10	**0.40**
10	**III2-22**	1223	2020	658	86	1504	0.07	0.07	0.02	0.00	0.06	**0.23**

* In those cases where co-elution made it impossible to measure total areas directly, total area was calculated from a single, well separated peak.

Sugars were identified from the retention time of distinctly separated peaks. The total area response (mV.Sec.) was calculated from the area of the most clearly separated "indicator" peak and multiplied by a factor determined by reference to the data given in Table 6.5. For example, where a peak appeared at 6.5 ± 0.1 min this was attributed to xylose (see Table 6.1) and the "total area response" of xylose was calculated by multiplying the area presented by the integrator of the GLC by 100/52.2 (Table 6.5). From the "total area response" the amount of each sugar present was calculated in micrograms by using the factors determined from standard sugars and presented in Table 6.6.

The analyses presented in Table 6.7 represent sugar recoveries from the equivalent of 1 µg of the original purified fraction that was hydrolysed and converted to TMS derivatives. For these analyses the weight of each purified fraction subjected to hydrolysis and derivatisation was supplied by Dr. David Tucker.

6.4. Discussion

It was considered that the method developed early in the project for separation of standard sugar mixtures (consisting of rhamnose, galactose and glucose) may have enabled identification of the sugar moieties from *Enterolobium* saponin fractions. The method gave a consistent response with respect to retention time, sensitivity towards individual sugars (mV.Sec./ml) was, in general, uniform and a reasonable precision of replication with the standard deviation not greater than 3.96% (Table 6.3) was obtained.

As a flame ionisation detector is, in general, uniformly sensitive to the mass of components of similar chemical composition, it is expected that detector sensitivity would be the same for the series of sugars as in the analyses reported here. This is shown to be so for rhamnose, xylose, galactose and glucose which averaged 26,160 mV.Sec. per microgram of sugars (the reciprocal of the right hand column of Table 6.6).

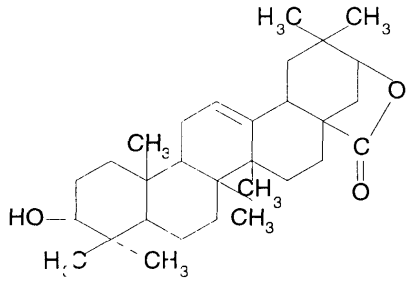
The standard deviation for this figure was ± 1000 mV.Sec. (3.8%) and with evidence that late eluting peaks were smaller than early eluting peaks. However, arabinose which was the earliest peak had only 18,000 mV.Sec. per microgram, that is 69% of the sensitivity found for the other sugars. No satisfactory explanation is available for this observation although it is possible (since all standards were subjected to sham hydrolysis, neutralisation and freeze-drying) that arabinose is subject to preferential degradation compared to the other sugars, under the conditions of exposure.

However, when additional standard sugars (xylose and arabinose) were included in the mixture using the same method, some sugar isomers were co-eluted. Due to the constraint of time, modification of the chromatographic method to overcome this problem was not undertaken. Thus, interpretation of a sugar chromatogram relied on a particular peak of a given sugar which is clearly separated under the analytical conditions employed. The peak area for a given sugar isomer is proportional to the total concentration of that sugar in the original sample (Boyer, 1986).

It has been demonstrated previously (Sweeley *et al.*, 1963; Sweeley and Walker, 1964) that aqueous solutions of aldoses known to have only two isomeric components, such as glucose and mannose, would produce 2 peaks on the chromatogram. From anomeric and ring isomerisation, a monosaccharide could produce as many as four isomers and thus give four peaks in the chromatogram (Sawardeker *et al.*, 1965). The work presented in this chapter shows that equilibrated, aqueous solutions of standard sugars, evaporated to dryness and silylated with "Tri-Sil" reagent produce two peaks or in some cases three peaks in the chromatogram depending on the particular sugar. Thus, the chromatograms indicated that xylose and galactose contain small amounts of a third component. The "third component" usually referred as "γ-anomer" are probably furanose modifications (Sweeley *et al.*, 1963; El Khadem, 1988). There is no attempt to

characterise the α-, β- and "γ-" anomers of the aqueous equilibrium mixtures at this stage.

Examination of Table 6.7 reveals that total sugar content of the fractions examined varied between 4% and 40% of the 1 µg of the original sample subjected to analysis. The nature of the aglycone for *Enterolobium cyclocarpum* has not been reported, however the aglycone for *Enterolobium contortisiliquum* has been identified as machaerinic acid lactone (Delgado *et al.*, 1984).



Machaerinic acid lactone (MAL)
$C_{29}H_{43}O_3$; mol wt = 439

Figure 6.2. The aglycone of *Enterolobium contortisiliquum* (Delgado *et al.*, 1984)

If it were assumed that the analysed fractions contained only saponins and that the aglycone was machaerinic acid lactone (MAL), then given a molecular weight of 439 for the aglycone, a saponin containing a single hexose substituent would have a molecular weight of 601. Under this condition 1 µg of such a substance would be expected to yield 0.3 µg of sugar under the conditions applying during analysis.

Calculation for a single hexose substituent (MAL-hexose) is as follows:

Molecular weight = 439 + 180 - 18 = 601

It would contain hexose =

$$\frac{180}{601} \times 1\ \mu g = 0.30\ \mu g$$

A disaccharide (hexose) substituent would produce 0.47 µg of sugar by a similar reasoning. The calculation for a disaccharide substituent (MAL-(hexose)$_2$) is as follows:

Molecular weight = 439 - (2 x 180)- (2 x 18) = 763

It would contain hexose =

$$\frac{360}{763} \times 1\ \mu g = 0.47\ \mu g$$

If the substituent sugar were a pentose, the expected total sugar yield is calculated along the same lines to be 0.26 µg for a mono substituted pentose saponin and 0.42 µg for a di substituted compound.

Therefore, if a single substituent, either hexose or pentose, were attached to the aglycone, it would be expected to reveal a content of 0.26 to 0.30 µg of total sugars on analysis. However, if there were two sugars attached to the aglycone, then it would be expected to reveal 0.42 to 0.47 µg of total sugars.

Thus the figures in the "Total" column of Table 6.7 indicate that it is unlikely that the saponin molecule is substituted with more than one sugar. Fraction III2-21 is perhaps an exception and indicates the possibility of double substitution.

All the arguments advanced above rely on the precept that the fractions from HPLC are relatively pure saponin mixtures

In terms of the type and amount of sugar present in all fractions, glucose seems predominant followed by rhamnose, arabinose and xylose, while galactose is not present in all fractions.

The crude butanol extract is likely to be a mixture of saponins with on average, one sugar attached to the aglycone. Glucose, rhamnose and arabinose are present in the ratio about 1:1:1 and no galactose could be demonstrated.

Fraction III2-12 probably contain no saponin. This assumption is also supported by the data given in Table 5.3.2.2 that this fraction has no lytic activity towards rumen protozoa.

An interesting comparison is found in the fraction III2-14 and III2-16. These two fractions have similar type and amount of sugars except for minor changes in the composition of rhamnose and arabinose. However, this difference appears to result in a significant difference in their ability to lyse protozoa (Table 5.3.2.2). Fraction III2-14 which contains more rhamnose than arabinose immobilises 100% of the protozoa with 26% lysis. However, fraction III2-16 contained more arabinose than rhamnose affects only 50% of cell motility with 17% lysis. It is, however, too early to conclude that the rhamnose content has a significant implication in the inactivation and lysis of protozoa. It can be seen in Table 6.7 that fraction III2-20 has almost double the amount of rhamnose in comparison with those mentioned above but has no arabinose, and does not produce higher percentage of lysis of protozoal cells although it inhibits cell motility.

The only *Enterolobium* fraction suspected to contains two sugars attached to the aglycone, fraction III2-21, possesses less lytic activity compared to those fractions which contain only one sugar, although it immobilises the protozoa. Therefore, the structure-activity relationship of *Enterolobium* fractions remains unclear. However, the results presented above could be interpreted to indicate the following structures based on the assumption that the HPLC fractions were, in general, mixtures of saponins (see Table 6.8).

Table 6.8. The proposed structure of the purified fractions of *Enterolobium cyclocarpum* extract.

Fractions	Proposed structure assuming the fraction is saponin only
Crude extractt	Single sugar substituent likely to be a family of saponin types containing rha, glc, ara, and xyl in the ratio of 9:7:6:3.
III1A-11	On average 1 in 7 aglycones are substituted with sugar or not a saponin structure.
III1A-12	On average 1 in 3 aglycones are substituted with sugar or not a saponin structure.
III2-13	On average 1 in 2 aglycones are substituted with sugar or not a saponin structure.
III2-14	Single sugar substituent or less than 1 likely to be a family of saponin types containing glc, rha, ara, and xyl in the ratio of 6:5:4:3.
III2-16	Single sugar substituent or less than 1 likely to be a family of saponin types containing glc, ara, rha, and xyl in the ratio of 6:5:4:3.
III2-19	On average 1 in 2 aglycones are substituted with sugar or not a saponin structure.
III2-20	Single sugar substituent likely to be a family of saponin types containing glc, rha, xyl, and gal in the ratio of 11:9:4:2.
III2-21	Double sugar substituent or less than 2 likely to be a family of saponin types containing rha, ara, glc, xyl and gal in the ratio of 11:10:10:3.
III2-22	Single sugar substituent likely to be a family of saponin types containing ara, rha, glc, and xyl in the ratio of 7:7:6:2.

Chapter 7

GENERAL DISCUSSION

7.1. Research Findings

The major objectives of these preliminary studies were to understand the chemistry and toxicology of the natural antiprotozoal agents present in *Enterolobium cyclocarpum* Griseb. This project focussed on the following points: (i) the isolation of the active agent(s) (Chapter 3), (ii) the membrane lytic effects of the agent(s) on sheep red blood cells as a convenient model system (Chapter 4), the effect on rumen protozoa particularly (Chapter 5) and, (iii) preliminary attempts at structural elucidation of the oligosaccharide component(s) by GLC analysis (Chapter 6) .

The active agents were isolated by maceration of the water-soluble compounds from the dried leaves of *Enterolobium cyclocarpum,* followed by partitioning between water and an n-butanol layer with the butanol layer containing the active fraction. A further fractionation process of the butanol extracts was undertaken using preparative HPLC and was guided by two bioassays, the haemolytic assay and the antiprotozoal assay.

A haemolytic assay using sheep red blood cells has been developed to test the presence of saponin and determine quantitatively the saponin contents in the plants. It was assumed that the active agent identified in this way was a saponin and contributed to the antiprotozoal activity of the plant (Leng *et al.*, 1992). The butanol extract and the purified fractions (fractions IA, IB, IC, ID, IE, and IF from the first batch) were subjected to this test system. Purification of the crude butanol extract by HPLC resulted in at least one fraction (IF) which markedly increased haemolytic activity on an equal weight basis. This most active fraction was more potent than Alkanate 3SL3 but not as potent as white saponin.

It is possible that certain HPLC fraction could appear more active than others because they contain a larger amount of the active principle(s). However no specific procedures are available to determine individual active components. Moreover, the possibility exists that the purified fractions were still a mixture of saponins and possibly of other compounds. The results of the comparative studies also indicated that the haemolytic activity may not reflect a parallel ability to lyse rumen protozoa. White saponin, the most potent agent observed to lyse red blood cells, has been found to have no activity on protozoa, whereas Alkanate 3SL3, the third in these comparative studies, has been found to effectively eliminate protozoa from the rumen of lambs (Burggraaf, 1980). Therefore, the antiprotozoal assay using rumen protozoa as the test organism was developed.

Taking into consideration that *in vivo* studies would require large quantities of fractions, an *in vitro* system developed by Willard and Kodras (1967) was modified and employed in these studies. The antiprotozoal assays were conducted under two different conditions, aerobic and anaerobic systems. The activity of the purified fractions was determined by observing the motility and the degree of disintegration of the protozoa under a microscope.

A number of purified fractions were subjected to the aerobic antiprotozoal system. It appears from the results of Assay 5.3.1.1 that those fractions exhibiting high immobilising activity also exhibited high lytic activity towards protozoa. However, it did not occur in all cases. The parent compound (fraction II) possesses high immobilising activity but did not produce a high percentage lysis.

Fraction IIB has a relatively low inhibition of cell motility but produces a high percentage of cell lysis (see Table 5.3.1.1). This may indicate that the compounds responsible for cell lysis differ from those which affect cell mobility.

From the results of two separate assays (Assay 5.3.1.2 and 5.3.1.3), it appears that increasing the incubation time from 1.5 to 3 h. produced no additional effect on cell motility.

A study on dose-related effects of four purified fractions (Assay 5.3.1.2) showed that all fractions lysed more than 50% of the protozoa on addition beyond 150 µl (150 µg) in a given test.

Since the active fractions detected by the previous assays were subjected to further fractionation, another assay (Assay 5.3.1.4) was conducted to observe whether synergistic interaction may have been evident among components in the original mixture. The results indicated that their activities were not affected after this fractionation.

It appears that the results are consistent within the experiment but not between the experiments (compare assays 5.3.1.3, 5.3.1.4, and 5.3.1.5). This may be because the number of protozoa fluctuated widely between assays as the result of a sequestering of protozoa on plant materials or settling of the protozoa within the rumen system (Leng *et al.*, 1980).

Attempts have been made to obtain the clean protozoa suspension separated from other microbes and feed particles. Since centrifugation of the rumen fluid sample slightly affects the motility of the cells better results could be obtained when the rumen cell suspension was prepared under anaerobic conditions.

Using a portable cabinet to provide anaerobic conditions for the protozoal toxicity test, a new series of purified fractions (fractions III2-12 to III2-21) was subjected to the system. The results (see Table 5.3.2.2) showed that not all of the purified fractions exhibited

strong ability to immobilise the cells, especially those fractions eluted early from the HPLC chromatogram, and no fraction exhibited more than 50% lysis.

In order to understand the specia ability of *Enterolobium* leaf fractions to lyse protozoa, it is necessary to determine the nolar lytic potency of lytic action. In this project, the molar concentration of each active fraction was estimated on the basis of its sugar content. A method was developed to determine the nature and quantity of the oligosaccharide portion of the lytic agent using GLC of TMS derivatives of monosaccharides liberated by acid hydrolysis. A set of standard sugars consisting of rhamnose, galactose and glucose was prepared for identification and estimation of the likely monosaccharides present in the active fraction of the *Enterolobium* leaf. However, when the purified fractions were analysed, there were some unidentifiable peaks present in the chromatogram. Therefore, two further sugars (arabinose and xylose) were included in the mixture of standard sugars using the same method. Unexpectedly, some sugar isomers co-eluted. Identification and interpretation of a sugar chromatogram was based on the well separated peaks (second or third peaks) of a given sugar under the analytical conditions employed.

The results presented in Table 6.8 showed the proposed structures of the purified fractions of *Enterolobium* extract based on the assumption that the analysed fractions contained only saponins with the aglycone machaerinic acid lactone. It appears unlikely that the saponin molecule is substituted with more than one sugar.

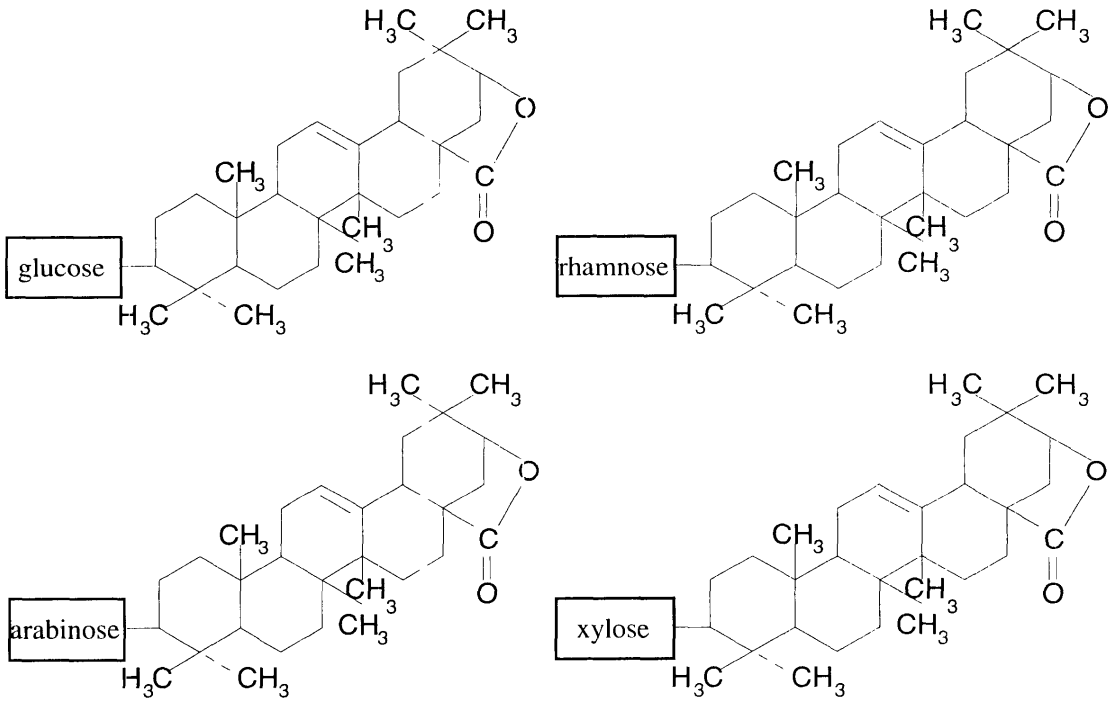


Figure 7.1. The proposed structures of saponin molecules of the *Enterolobium* fractions

The type and the amount of sugars present in the purified fractions (Table 6.7) indicated that glucose is predominant, while galactose is not present in all fractions.

On the basis of these studies the nature of the active substance(s) involved in protozoal lysis is presumed to be a family of saponin molecules probably involving molecules similar to those shown above.

7.2. Further Research

In this preliminary study, two bioassays have been employed to assess the antiprotozoal activity of the *Enterolobium* extracts. Chromatographic techniques, reverse-phase HPLC and GLC, were employed for the separation, isolation and structure determination of the active agents. In *in vitro* assessment, some of these compounds possess haemolytic activity and antiprotozoal activity. However, further investigation is required to investigate the selective antiprotozoal action and toxicities. It is also desirable to know the molar lytic activity of the agents.

In the present studies, the molar concentration of the active agents was approached by determination of the type and quantity of the sugar contents by GLC. However, when additional standard sugars (xylose and arabinose) were included in the mixture (containing rhamnose, galactose and glucose) using the same method, some sugar isomers were co-eluted. Therefore, the chromatographic method should be modified to solve this problem, probably by manipulating the temperature programme.

The other approach to determine the molar concentration of the active agents was estimation of the quantity of the aglycone. There is no information available at the moment about the nature of the aglycone of *Enterolobium cyclocarpum*.

Once the structure of the active agent identified, the structure-activity relationship can be determined. In addition, the structure of the active agent might be chemically manipulated to enhance the lytic activity of the agent.

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