Chapter 1

INTRODUCTION

Ruminant animals make a great contribution to the welfare of people through their milk, meat and other minor products and through their draught-power in developing countries. However, since the priority in land use is for production of food and plantation of crops, these ruminants are usually fed on poor quality forages, pastures and crop residues which result in low level production.

In such conditions, a significantly higher level of production will result from improvement in the efficiency of feed utilisation (Bird, 1991). The feed efficiency is closely affected by protein to energy ratio (P/E ratio), i.e. the ratio of microbial protein and bypass protein taken together, to energy absorbed, in the form of volatile fatty acids (VFA) (Veira, 1986; Leng, 1991). The P/E ratio in the nutrients absorbed by ruminants on forage-based diets is affected by the microbial growth efficiency in the rumen and by the amount of bypass protein supplement included in the diet (Leng *et al.*, 1992). On low quality feed, the P/E ratio will limit efficient feed utilisation. Under these conditions conversion of non-protein nitrogen to microbial protein will enhance the P/E ratio and as a result the efficiency of feed conversion. Meanwhile, the microbial protein synthesis is influenced by three major factors: (i) the availability of essential nutrients for microbial growth (Leng, 1991), (ii) the dilution rate of ruminal content (Harrison and McAllan, 1980; Veira, 1986) and (iii) the presence of protozoa (Rowe *et al.*, 1983; Veira, 1986; Bird, 1991).

The effect of the presence or absence of protozoa on animal production is still the subject of debate (Bird, 1991). It seems that the effects of defaunation on animal production have been shown to depend on diet and the defaunation method.

Defaunation means not only the elimination of protozoa from the rumen microbial ecosystem, but also the compensatory changes in the ecological relationship that affects the size, generic distribution and metabolic activity of the bacterial and fungal populations (Ushida *et al.*, 1991). Until *in vitro* culture techniques are able to evaluate fully the specific functions of protozoa, defaunation may provide useful information on their role. Some research workers (Bird, 1978; Rowe *et al.*, 1983; Veira, 1986; Leng *et al.*, 1992) reported that the performance of fauna-free animals is better than that of faunated animals held under laboratory and grazing conditions. Defaunation of the rumen is likely to be associated with a positive response on body weight gain when the diet does not supply sufficient protein post-ruminally to meet the animal requirements (Bird and Leng, 1983). A highly significant ($P<0.01$) increase in wool growth (37%) was obtained from a study on the effect of defaunation on sheep (Bird and Leng, 1984). Another positive production response of defaunation includes increased milk production from dairy cows (Moate, 1989).

A number of methods have been employed to obtain fauna-free ruminants such as: (1) isolation of new-born animals; (2) chemical drenching; (3) dietary manipulation and (4) breeding from ciliate-free dams. The elimination of protozoa by chemical means is potentialy the most convenient method to obtain fauna-free animals (Bird, 1989; Ushida *et al.*, 1991). However, since it is likely that chemicals are not specifically toxic to the protozoa and probably kill other microorganisms and host cells in the rumen and also because their antiprotozoal activity is inconsistent, new defaunating agents are being sought, especially from forages.

Research for antiprotozoal forages commenced with the establishment of a bioassay system to assess the effects of various additives on the viability of rumen protozoa in culture medium. A number of forages were assayed. Among those forages, *Enterolobium cyclocarpum* Griseb., commonly known as "Elephant's ear" in many areas, showed great promise as a potential rumen antiprotozoal agent as it could reduce the number of protozoa in rumen fluid to negligible numbers (Leng *et al.*, 1992; Bahaudin *et al.*, 1992). However, there is no further information available on the active agent present from this forage.

It is suspected that the active agent present in this forage is saponins. *Enterolobium cyclocarpum* is known as a source of saponins (Allen and Allen, 1981). Saponins, a class of glycosides present in a wide variety of plants are characterised by their remarkable ability to haemolyse red blood cells (Cheeke, 1983; Reichert *et al.*, 1986; Jurzysta, *et al.*, 1988). This ability to lyse unprotected lipid-based membranes suggests that saponins might be useful as antiprotozoal agents as protozoa have phospolipid based membranes unprotected by cell wall structures.

The major objectives of the present studies, therefore, were to understand the chemistry and toxicology of the natural antiprotozoal agents present in *Enterolobium cyclocarpum*. The research program consisted of two parts; (i) a preliminary study focussing on the lytic effects of *Enterolobium* extracts on sheep red blood cells, (ii) a study in which isolated rumen protozoa were used as the test organisms.

In the latter phase the *Enterolobium* extracts were subjected to fractionation by HPLC and individual fractions were examined. Examination of the fractions consisted of measurement of their biological activity on rumen protozoa and preliminary attempts at structural elucidation.

It is hoped that extension of this approach may reveal a structure/function relationship which could be exploited to permit more convenient or enhanced control of rumen protozoal populations.

Chapter 2

REVIEW OF LITERATURE

2.1. Rumen Protozoa and the Effects of Defaunation.

2.1.1. Rumen protozoa: Their nature and influence

Many and various protozoa inhabit the rumen. Protozoa can be divided into ciliates and flagellates, but most of them in the rumen are ciliate protozoa. The ciliate protozoa are present in much smaller numbers (10^5 - 10^6 per ml of rumen contents) than bacteria (10^9-10^{10} per ml) but may be equal in total mass because protozoa are larger in size (Ogimoto and Imai, 1981). The type and density of protozoa in the rumen are affected by a number of factors such as level of feeding and the roughage-to-concentrate ratio, rumen pH and physical form and particle size of the diet. Protozoa take up soluble sugar, but also ingest and digest bacteria in significant quantities (Rowe *et al.*, 1983). However, ruminants without protozoa are apparently normal and healthy, so the benefit from protozoa is questionable. Although studies on the effect of the presence or absence of protozoa on animal productivity have been undertaken, the role of protozoa in the rumen is still the subject of discussion.

The presence of ciliate protozoa in the rumen may serve an important role in ruminal fermentation. Veira (1986) observed the effects of ciliate protozoa on the ruminal ecosystem and found that protozoa contributed to the maintenance of a more stable fermentation by reducing the rate of fermentation, preventing accumulation of excessive levels of lactate, thus giving an increase in acid tolerant, lactate-utilising bacteria.

Protozoa have an important role in the degradation of plant cell wall polysaccharides. Protozoa are also quantitatively important in digestion of the major carbohydrate, protein and lipid components of feed material ingested by the ruminants (Bird, 1991). However, certain species of protozoa are preferentially retained in the rumen and only a small proportion of protozoal matter actually leaves the rumen (Leng *et al.*, 1980). Therefore, a reduction in net microbial synthesis and an increase in dietary protein degradation in the rumen is associated with an inefficiency of feed utilisation (Veira, 1986).

The absence of ciliate protozoa in the rumen increased the availability of protein for digestion by animals. Moreover, defaunation improved the growth rate by 43% without an effect on feed intake (Bird and Leng, 1978). Eliminating protozoa from the rumen of the lambs also resulted in an increase in wool growth by 37% (Bird and Leng, 1984).

Moate (1989) examined the effects of defaunation on milk yield and milk composition in grazing dairy cows. It was found that the total number of protozoa in the rumen fluid of cows treated with detergent Alkanate 3SL3 (sodium lauryl diethoxy sulphate) was reduced by 95%. The yield of milk and milk protein content was increased from 20.0 l/cow/day in control cows to 22.7 l/cow/day in treated cows. Defaunation also increased the milk protein content from 593 to 713 g/cow/day as well as the milk protein : fat ratio from 0.69 to 0.78.

2.1.2. Defaunation and its effects

A number of methods have been employed to obtained fauna-free ruminants and these can be placed in three categories: (1) isolation of new-born animals, (2) chemical drenching, and (3) dietary manipulation.

2.1.2.1. Isolation of new-born animals

Since protozoa are not present in the new-born animals, the removal of young animals from the dam and rearing in isolation will keep them free of protozoa. This method has been used to study the role of protozoa in ruminant animals (Abau Akkada and El-shazly, 1964; Eadie and Gill, 1971; Wallace *et al.*, 1987). Eadie and Gill (1971) reported that there was no significant difference of the animal performance between groups of faunated and defaunated animals. However, Ushida *et al.* (1991) reported that defaunated animals prepared by this method did not develop a "normal" microbial population in the rumen, and this could introduce a bias into the results.

2.1.2.2. Chemical drenching

Rumen protozoa are susceptible to various chemicals, especially to surface-active agents that can disrupt cell membranes. Many chemicals have been used for this purpose, such as dioctyl sodium sulphosuccinate ("Manoxol OT") (Orpin, 1977, Orpin and Letcher, 1984), nonyl phenol ethoxylate ("Terics") (Bird *et al.*, 1979) and sodium lauryl diethoxy sulphate ("Alkanate 3SL3") (Bird and Leng, 1984). However, this method has some problems for experimental design. The removal of protozoa by this method is often followed by a loss of weight after the chemical drenching due to depressed feed intake (Bird, 1989). Another factor that should be taken into account is that experimental animals are treated, then some reinoculated with protozoa for use as the control animals. This method has been criticised because there is no guarantee that the reinoculated animals are comparable to the untreated animals. After all, the chemicals used are not specifically toxic only to the protozoa but probably also to other microorganisms and the host cells in the rumen.

2.1.2.3. Dietary manipulation

Although defaunation by dietary manipulation is probably safer than by chemical drenching, this method has not been widely used. Some plants produce secondary compounds as protective agents against insect, fungal, protozoal and microbial attack. Many secondary plant compounds are toxic to the rumen microbes. Leng *et al.* (1992) surveyed tree forages with secondary plant compounds to study their potential to manipulate rumen protozoa and found that some of them indicate a great potential as defaunating agents.

2.2. Mechanism of Action of Antiprotozoal Agents

Protozoa possess a more complex genome than the prokaryotes. As a result, they contain both structural and functional features which are not shared with the prokaryotes. These features could thus constitute specific target sites for antiprotozoal action. The antiprotozoal agents should have one or more of the following attributes:

1. Selective ability to penetrate protozoa, or to exert a toxic effect from the cell surface.

2. Action directed against a unique protozoal target in the protozoa and to be active against all protozoal species and life cycle stages.

3. Where target sites are shared with other organisms (including perhaps the host animals) selectivity may result from accessibility of target sites in the protozoa which are more vulnerable than those in associated microorganisms (VanDemark and Batzing, 1987).

Generally, the mechanisms of antiprotozoal agents are related to: (1) Interference with energy metabolism; (2) Blocking cofactor synthesis; (3) Interference with protein synthesis; (4) Interference with nucleic acid metabolism and (5) Interference with membranes (Peter, 1985). In a complex ecosystem such as the rumen it is difficult to find suitably selectice agents for the specific control of protozoa in the presence of a wide range of other microorganisms. However the absence of the bacterial cell wall enhances the susceptibility of the naked membranes of protozoa to membrane active agents. For the present study, a brief discussion of points (1) to (4) is presented below followed by a more detailed examination of interactions with membrane.

2.2.1. Interference with energy metabolism

Some antiprotozoal compounds interfere with ATP production by disrupting the function of the glycerophosphate pathway which interferes with the ATP production. The specific example for this interference is shown by trypanocidal drug Suramin, the chemotherapeutic agent used against African sleeping sickness. This drug disrupts the function of the glycerophosphate pathway by interfering with the reoxidation of NADH and leading towards the production of glycerol and as a result in a reduction in ATP production (Peter, 1985).

2.2.2. Blocking cofactor synthesis

The mechanism of action of some antiprotozoal compounds results from interference with uptake or incorporation of a number of different metabolites which reduce cofactor synthesis. For example, sulfonamides act as inhibitors of dihydropteroate synthetase (DHPS) by competing with para-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate. Dihydrofolate is required in the synthesis of thymidylate particularly but also purines, methionine, thiamine, pantothenate and formyl methionyl tRNA (Bryan, 1982).

2.2.3. Interference with protein synthesis

A small group of antiprotozoal agents functions by interfering with protein synthesis. For example, chloramphenicol inhibits peptide bond formation on ribosomes by binding to the ribosome subunit (O'Leary, 1989).

2.2.4. Interference with nucleic acid metabolism

A number of agents have a marked inhibitory action on: (1) nucleotide metabolism, such as azarine which inhibits purine biosynthesis (Bryan, 1982); (2) DNA and RNA synthesis, such as chloroquine (O'Leary, 1989).

2.2.5. Interference with membranes

This kind of interference is most relevant to the present study. Some antiprotozoal compounds are known to act through interference with membrane structure. A number of studies have been conducted to remove protozoa from the rumen of sheep and cattle by using agents acting as ionophores, such as Alkanate 3SL3 and nonionic compounds, i.e. nonyl phenol ethoxylate (Teric GN9).

The cell membranes form an expandable barrier to control the entry and exit of solute molecules. These functions make the membrane vulnerable to a variety of agents (Hammond and Lambert, 1978). Essentially all membranes, whether from plants, animals, or microorganisms have a structural pattern based on a phospholipid bilayer (Fig 2.1).

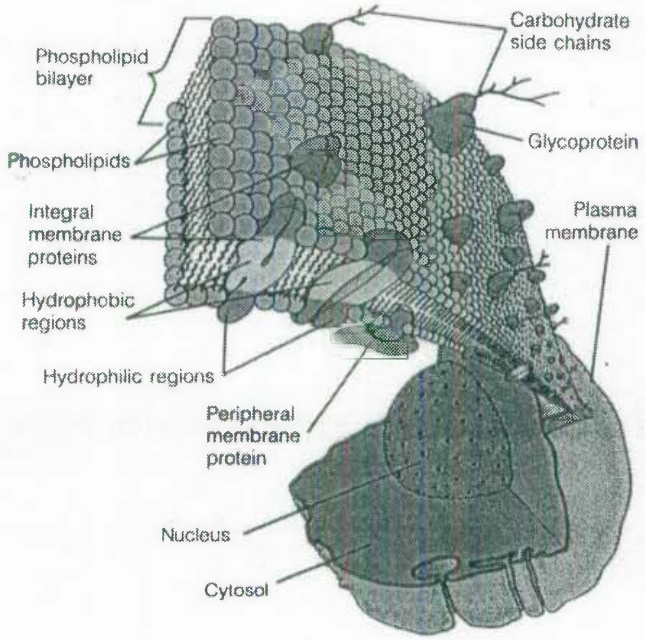


Figure 2.1. Structure of a typical cell membrane (Mathews and van Holde, 1990).

Bacterial membranes differ from eukaryote membranes in several ways notably in containing no cholesterol. Cell membranes can be disrupted by surface-active compounds. These compounds bind to membrane sterol such as cholesterol and increase the permeability of the membrane to cations (Peter, 1985). This action initiates further deterioration (Pelczar *et al.*, 1986). Some of these surface-active compounds were tested for toxicity to rumen protozoa.

2.3. Mechanism of Action of Surface-Active Agents on Protozoa

The bilipid cell membrane of protozoa can be disrupted by surface-active agents (Hart *et al.* 1988) because surface-active agents (surfactants) are amphiphilic, which means they contain separable hydrophobic and hydrophilic domain within the same molecule. Surfactants are classified into three groups according to charge: (1) anionic surfactants; (2) cationic surfactants and (3) nonionic surfactants (Hancock and Nicas, 1984).

However, not all surfactants are able to disrupt the protozoal membrane. Willard and Kodras (1967) examined more than 170 chemical agents including surface-active agents against rumen protozoa and found that only anionic surfactants showed promise as antiprotozoal agents, while both cationic and nonionic surfactants were relatively ineffective.

However, subsequent experiments conducted by Wright and Curtis (1976) found that the nonionic surfactants were also toxic to rumen protozoa, showing effects ranging from inhibition of motility to cell disintegration. "Teric GN9", nonyl phenol ethoxylate, and "Manoxol OT", dioctyl sodium sulphosuccinate, were found to have the most toxic effect on protozoa at concentrations as low as 0.01%.

Bird *et al.*, (1978) reported that Teric GN9, a nonionic surfactant given intraruminally, was an effective means of defaunating the rumen of the lambs given low-protein-high-energy diets.

An *in vitro* study on the toxicity of some surfactants against rumen protozoa by Hart *et al.* (1988) has shown that Teric GN9 killed protozoa at 250 ppm. Meanwhile, Alkanate 3SL3 (with lauryl ethyl sulphate as the active compound) an anionic surfactant, showed it self to be less toxic than Teric GN9.

However, because of their toxicity to the cell of the host animal, the commercial application of these surfactants is limited (Hart *et al.*, 1988)

2.4. Saponins as Antiprotozoal Agents

2.4.1. Chemical structure of saponins

Saponins are a type natural detergents. They are non-ionic detergents consisting of a hydrophobic portion and an uncharged hydrophilic component. Specifically saponins are made up of a hydrophobic aglycone (sapogenin) with a polymeric sugar side chain linked by a glycosidic bond to an hydroxyl group of the aglycone (Cheeke, 1983).

Monosaccharides that have been identified in the polymeric side chains are glucose, galactose, rhamnose, xylose, fucose and arabinose (Horber *et al.*, 1974; Cheeke, 1983). With the possibility of combination of numerous sapogenins with oligosaccharide side chains of various size and composition, an enormous range of saponins is possible and many variants of this general structure have been found to be present in plants. In general, saponins are classified into two groups based on the chemical nature of the sapogenins. Triterpenoid saponins include in their structure an aglycone containing 30 carbon atoms. The 30-carbon triterpenoid motif is synthesised from 5-carbon isoprenoid subunits and normally comprises a multi-ringed structure. Saponins may alternatively contain a 27-carbon steroid compound formed through a biosynthetic route which follows that of the triterpenes but undergoes the cyclisation by a different mechanism. A discussion of the nature of these aglycone types follows (section 2.4.1.1. and 2.4.1.2.).

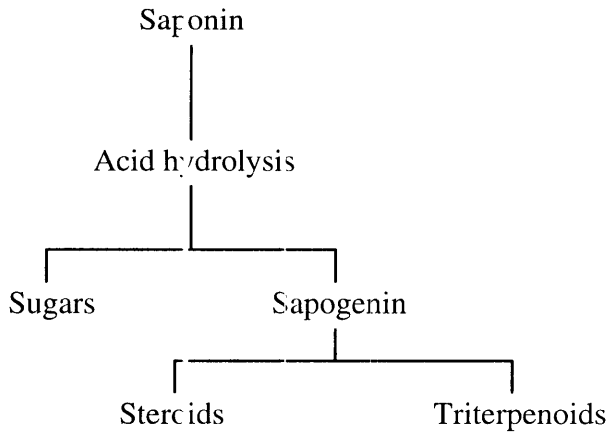


Figure 2.2. Schematic classification of saponins

2.4.1.1. Triterpenoid saponins

A large variety of cyclic triterpe ies is distributed among a wide variety of plant species. Triterpenes may be defined as C-30 compounds which are built up from six isoprene units (Fig. 2.3).

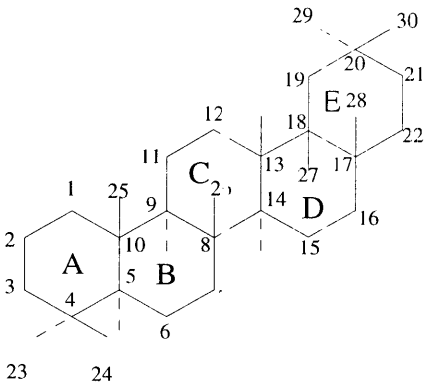
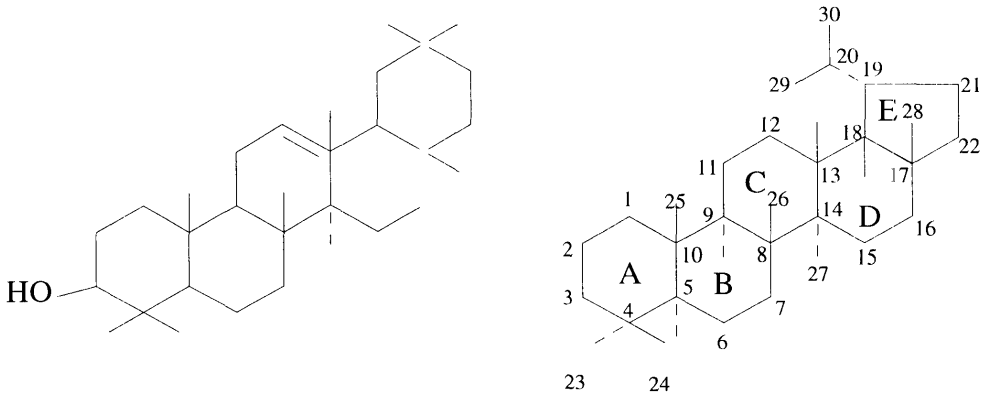


Figure 2.3. Triterpenoid sapogenin.

Studies on the mechanism of the biosynthesis have shown that triterpenes are derived from all-trans squalene by a series of combined cyclisation and rearrangement reactions to form the final product, the pen ta-cyclic triterpenes (Briggs and Brotherton, 1970).

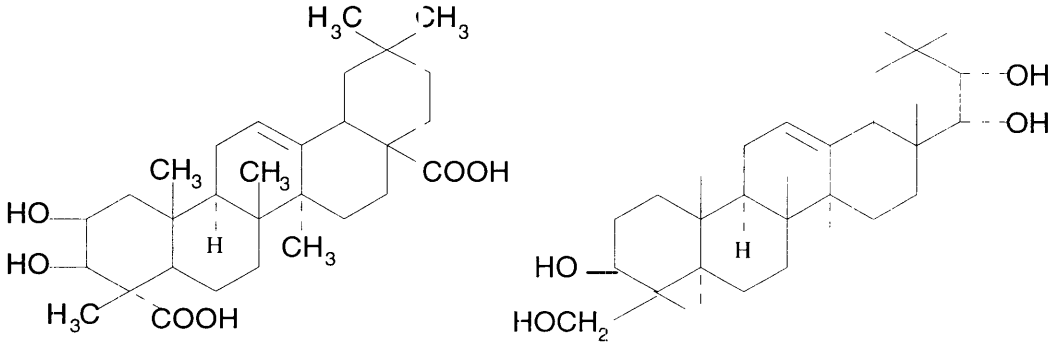
Structurally, the pentacyclic triterpenes can be divided into the hydropicine type, i.e. all five rings are of six carbons, e.g. β-amyrin, and the cyclopentanobenzophenanthrene type, i.e. the fifth (E) ring has five carbon atoms, e.g. lupane (Fig. 2.4).



ß-amyrin lupane

Figure 2.4. ß-amyrin and lupane
(Source:Devon and Scott, 1972).

Sapogenins of the ß-amyrin group were reviewed (Agarwal and Rastogi, 1974), but due to improved techniques in the isolation and structural elucidation of saponins, many new saponins have since been identified. Berrang *et al.* (1974) reported that about 30 saponins were found from the forage of DuPuits and Lahontan cultivars of *Medicago sativa.* Different cultivars of plant species may exhibit variations in the aglycone of constituent saponins. Medicagenic acid was the predominant sapogenin of the DuPuits cultivar, whereas soyasapogenol A was the prominent saponin from the Lahontan variety (Fig. 2.5).



Medicagenic acid Soyasapogenol A

Figure 2.5. Medicagenic acid and soyasapogenol A
(Source: Devon and Scott, 1972).

During the last decade, numerous new sapogenins have been isolated and identified from plant material following acid hydrolysis; for example: jujubogenin from the bark of *Zizyphus joazeiro* (Higuchi *et al.*, 1984), spergulatin-A from *Mollusco spergula* (Barua *et al.*, 1986), epikatonic acid from defatted guar meal (Curl *et al.*, 1986) and soyasaponin I, II, III and IV from soybean meal (Burrows *et al.*, 1987).

Some other triterpenoid saponins were also reported, such as saponins from *Fagonia indica* (Ansari *et al.*, 1978), *Thlandiantha hookeri* (Nie *et al*, 1989), *Albizzia anthelmitica* (Carpani *et al*, 1989), *Randia uliginosa* (Sati *et al.*, 1989), *Guaiacum officinale* (Ahmad *et al.*, 1989) and from *Cyclamen sp.* (Reznicek *et al.*, 1989).

2.4.1.2. Steroidal saponin:

The physical properties of the steroidal saponins (C-27 compounds) are virtually the same as those of the triterpenoid saponins, but they have a spiroketal or its precursor at C-22 (Heftmann, 1970) as shown in Fig. 2.6. They may differ at positions C-5 and C-25.

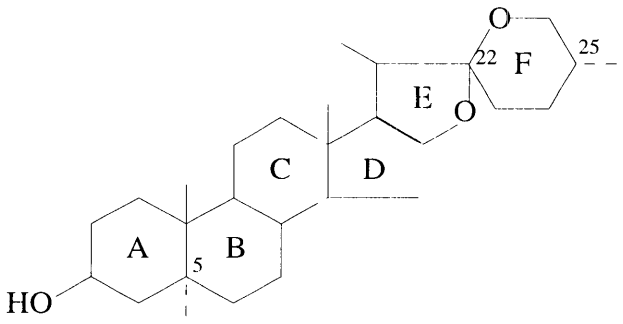


Figure 2.6. Steroidal saponin.
(Source: Heftmann, 1970).

Since nuclear magnetic resonance (NMR) spectral analysis is now being used as a routine technique in structural determination studies, the steroidal sapogenins can be further classified as spirostane, furostane, furospirostane and miscellaneous types (Agrawal *et al.*, 1985).

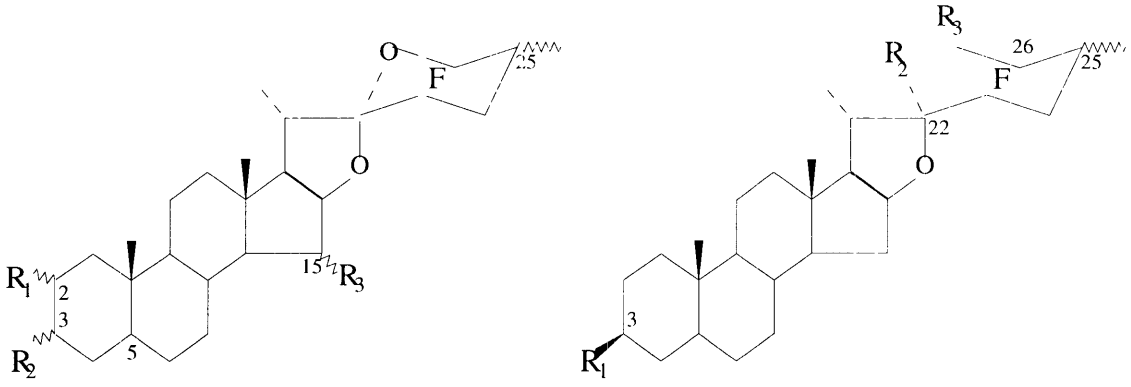


Figure 2.7. Spirostane and furostane skeleton.
(Source: Kawasaki 1978).

The spirostane type has been characterised by the presence of a spiroketal ring system and can be categorised as 5 α-spirostane, 5 ß-spirostane and 5 γ-spirostane compounds. Furostane types occurred as glycosides but not in the free form and are regarded as biogenic precursors of spirostane saponins.

In the third category, the furosp rostane type, ring F becomes a five member furan ring instead of a pyran ring as in the spirostane. The last category, the miscellaneous type, includes all compounds which are derived from the steroidal skeleton.

About 130 naturally occurring s eroidal sapogenins and saponin derivatives which were characterised up to 1983 have been reviewed by Agrawal *et al.* (1985). New steroidal sapogenins have since been found. For example, Melangosides N, O and P identified as furastanol compounds were isolated from the seeds of *Solamon melongena* (Kintia and Shvets, 1985).

Two furastanol saponins from the *Trigonella foenumgraecum* seeds were isolated and their structures elucidated (Gupta *et al.*, 1986). Steroidal saponins also have been reported in a number of *Yucca species*, but for the first time the sapogenin content of *Yucca schidigera* was reported by Kaneda *et al.* (1987). Their aglycones were identified as sarsapogenin, smilagenin, sarmogenin and neogitogenin. The structures of three new steroidal saponins, dongoside C, D and E from *Agave sisalana* were elucidated and all saponins yielded a common aglycone determined as tigogenin (Ding *et al.*, 1989).

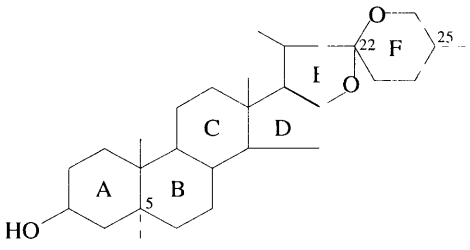


Figure 2.8. A structure of tigogenin
(Source: Ding *et al* , 1989)

2.4.2. Isolation of saponin

There were several tests such as for foaming capacity, toxicity to cold-blooded animals, bitter taste, complex formation with cholesterol and *in vitro* erythrocyte haemolytic activity (Birk and Peri, 1980; Cheeke, 1983; Agrawal *et al.*, 1985) reported to be employed for the identification and isolation of saponins. Saponins may form a complex with protein, which is insoluble in water; alternatively the water-soluble saponins may be precipitated by complexing with cholesterol or other sterols (Cheeke, 1983).

The isolation and structure elucidation of two saponins from *Fagonia indica*, a medicinal plant, have been described (Ansari *et al.*, 1987). The aerial parts of fresh plants were chopped and soaked in ethanol (EtOH), and the extract then concentrated in a cyclone evaporator. The semi solid residue was dissolved in methanol (MeOH). After precipitation, they were washed with petrol, chloroform ($CHCl_3$) and ethyl acetate (EtOAc) and dried, yielding a powder. Finally, the crude product was fractioned by flash chromatography on silica gel.

Jain (1987) has successfully isolated a steroidal saponin from the aerial part of *Agave cantala* which has anti-cancer properties. The air-dried aerial part of the plant was extracted with MeOH at room temperature. The MeOH removed and the extract was then taken up in H_2O, defatted with n-hexane and this solute extracted with n-butanol (BuOH). The n-BuOH extract was chromatographed on silica gel, using as the developing solvent $CHCl_3$ followed by $CHCl_3$-MeOH to produce a virtually pure compound.

2.4.3. Distribution of saponins in plants

Saponins are distributed in all parts of the plant, roots, stem, bark, leaves, seeds and fruits. A triterpene and saponin have been isolated from roots of *Ilex pubescens* and their structures established (Hidaka *et al.*, 1987). Some saponins were isolated from callus tissue of *Panax japonicus* (Fujioka *et al.*, 1989) and *Akebia quinata* (Ikuta and Itokawa, 1989). Two new triterpenoids were also isolated from the bark and stem of *Schefflera impressa* (Srivastava and Jain, 1989). Ahmad *et al.* (1984) reported three sapogenins isolated from the stem bark of *Guaiacum officinale*. From the methanolic extract of the leaves of *Climantis montana*, a new triterpenoid bisglycoside was isolated and its structure established (Baliaguna *et al.*, 1989).

A triterpenoid from acid hydrolysis of the ethanolic extract from the fruits of *Enterolobium contortisiliquum* (Delgado *et al.*, 1984), two saponins from the fruits of *Guaiacum officinale* (Ahmad *et al.*, 1984) and three saponins from the ethanolic extract of the fruits of *Randia uliginosa* were characterised on the basis of chemical reactions.

From the seeds of *Dodonaea viscosa* (Wagner *et al.*, 1987) and *Chenopodium quinoa* (Reichert *et al.*, 1986), the isolation and structural elucidation of saponins were reported. Amoros and Girre (1987) isolated and characterised a new saponin from the whole plant of *Anagallis arvensis*.

2.4.4. Haemolytic activity of saponins

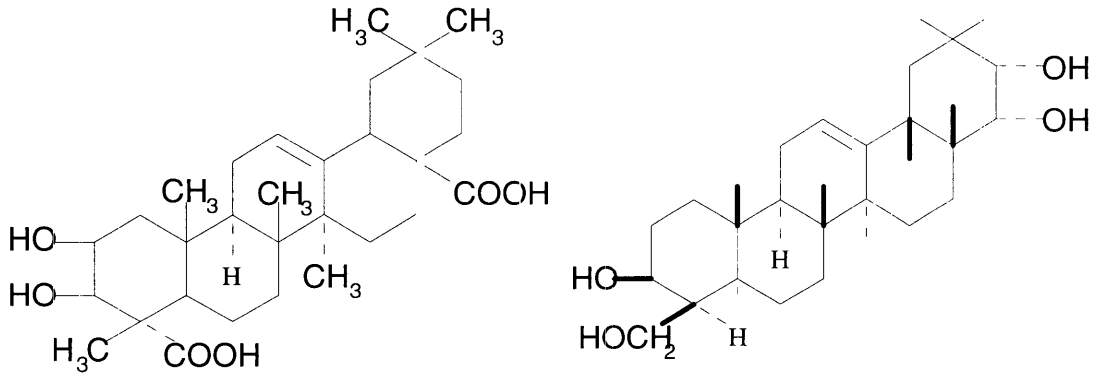
Haemolytic activity is one of a number of properties common among saponins. There are numerous assays and quantitative determinations of saponins based on these properties. However, not all saponins swell and rupture red blood cells (Jurzysta *et al.*, 1988).

Bondi *et al.* (1973) defined Haemolytic Index as a numerical ratio between the weight of reaction mixture divided by the smallest weight of saponin which still causes full haemolysis.

It has been found that the haemolytic activity of saponins may be very variable. Some factors in the extraction stage were reported to affect the haemolytic activities of saponins. Reichert *et al.* (1986) have shown that the extraction temperature, time and the storage condition before extraction, markedly affected the haemolytic activity of a crude extract of quinoa (*Chenopodium quinoa*) grain. This may be due to the enzymic degradation of the saponin molecule, which could be expected to be enhanced under some conditions of extraction and storage.

Variation in chemical structure may influence the haemolytic activity. Differences in aglycone have been shown to lead to variation in haemolytic activity. For instance, it was shown that the alfalfa saponins containing medicagenic acid and hederagin as their aglycone have a strong haemolytic activity (Birk and Peri, 1980).

However, these saponins give rise to a different haemolytic index compared to soybean saponins. This may be because of the presence of two carboxylic groups (-COOH) as their main difference (Fig. 2.9).



Medicagenic acid Soyasapogenol A

Figure 2.9. The medicagenic acid of alfalfa saponin and soybean sapogenin (Source: Birk and Peri, 1980)

However, it has been shown that blocking of the carboxylic groups in medicagenic acid by esterification results in comp.ete loss of the haemolytic activity of the saponins (Birk and Peri, 1980).

However, saponins containing i.dentical sapogenins, isolated from different plants, also showed a variation in activity. For example, neither soyasapogenol A, B, C, D, nor E as intact saponins (from alfalfa) ly se red blood cells. But soybean saponins carrying the same sapogenins have conside.able haemolytic activity (Birk and Peri, 1980). This apparent contradiction reflected that the haemolytic activities were not only determined by the sapogenins but might also be modified due to their hydrophilic side chains, or be related to their sapogenin/carbohydrate ratio. The lower sapogenin:carbohydrate ratio (1:5) in alfalfa saponins compared to 1:1 ratio in soybean saponins may explain this discrepancy (Birk and Peri, 198( ).

Takechi and Tanaka (1990) reported a study on structure-activity relationship of the saponin α-hederin by comparing its haemolytic and antifungal activities. They found that the free carboxylic acid was more important for haemolytic activity than for the antifungal activity, while the terminal rhamnose was more important for antifungal activity than for haemolytic activity. They concluded that the haemolytic centres of α-hederin were not identical to the antifungal centres.

Another study on structure-activ ty relationships of the saponins of dioscin and dioscinin conducted by Takechi *et al.* (1991) has shown that the number of sugar residues and those derivatives having branched sugar chains revealed higher haemolytic activities than those having straight chains. The presence of the 17-hydroxyl group of dioscinin derivatives appeared to reduce the biological activities of these compounds.

The sugar chains have a definite influence on the haemolytic activity, because they confer water solubility and cell permeability. Agarwal and Rastogi (1974) have shown that optimum activity required a polar grouping in ring A and moderate polarity in ring D or E. However, a marked polarity in ring D and/or E, such as a sugar or oligosaccharides substituent or a free hydroxyl group, caused inactivation.

2.5. *Enterolobium cyclocarpum* as a Potential Source of Antiprotozoal Agent

Enterolobium cyclocarpum Griseb. and *Enterolobium contortisiliquum* Morang., are the best known species of *Enterolobium*. In many areas, *Enterolobium cyclocarpum* is commonly known as "elephant's ear" which refers to the gigantic size of the trees with the ear-shape pods (Allen and A len, 1981).

Enterolobium cyclocarpum is a native of Central and Northern America but widely cultivated in the tropics. The trees can reach 15 - 30 m hight with trunk diameter of 2 m (Verdcourt, 1979). The pods and barks of *Enterolobium cyclocarpum* are used as a substitute for soap as they contain saponins (Allen and Allen, 1981).

Studies on the effects of *Enterolobium cyclocarpum* leaf on the rumen ecosystem have been reported (Bahaudin *et al*, 1992; Leng *et al.*, 1992). Bahaudin *et al.* (1992) compared the feeding level of *Enterolobium* leaf to the changes in rumen ecosystem of goats. The results showed that feeding *Enterolobium* leaf at all levels tested (0, 50, 100 and 200 g/d) stimulated the growth of rumen microbes but depressed the growth of rumen protozoa. Leng *et al.* (1992) surveyed anti protozoal properties of a number of forages *in vitro* and *in vivo*. In *in vitro studies,* all forage samples were mixed with lucerne at level 1, 10 and 100% of the lucerne. Among those forage, *Enterolobium cyclocarpum* was identified as a potent antiprotozoal agent (Table 2.1).

Table 2.1. The apparent antiprotozoal property of forages at 1% and 10% substitution rate for lucerne (Leng *et al*, 1992).

Name of plant	Forage substitution rate for lucerne leaf powder in the substrate added to incubation medium	
	1%	10%
	protozoa numbers as % of control	
Acacia deane	38	79
Acacia crassa	38	74
Acacia semilunata	72	77
Acacia spectabilis	56	73
Acacia chinchillensis	75	103
Desmanthus introtum	63	73
Centrosema pubescens	78	84
Casuarine ronophlora	79	111
Black wattle	51	8
Fern leaf	81	97
Indigofera schemperi	75	75
Macroptilium lathyroides	88	100
Leucaena leucacephala	69	99
Vigna parteri	55	86
Lotononis bainesii	75	78
Desmodium uncinatum	78	108
Aeschynomena falcata	82	99
Cassia rotundifolia	88	106
Enterolobium cyclocarpum	0	0
Enterolobium timbouva	0	0

The numbers of protozoa were reduced to negligible number when *Enterolobium cyclocarpum* leaves were given to four rumen fistulated buffaloes in Indonesia. Since the protozoa were not entirely removed, they rapidly returned to normal densities in the rumen following cessation of feeding of the leaf materials. However, there is no further information available on the active agent present in this forage.

Chapter 3

FRACTIONATION OF *ENTEROLOBIUM* LEAF EXTRACT

3.1. Introduction

The results of an investigation of tree forages for antiprotozoal activity had been reported (Leng *et al.*, 1992). Among those forages, *Enterolobium cyclocarpum* showed antiprotozoal activity (see Table 2.1). However, the nature of the active compounds present in this plant is still unknown.

In order to elucidate the chemical nature of the antiprotozoal agent, an interactive process was developed, involving fractionation of *Enterolobium* leaf extract by reverse-phase HPLC coupled to bioassays of the eluted fractions using either red blood cells or (better) rumen protozoa as the target cell. Details of this process are described in this chapter.

3.2. Materials and Methods

For this project, the *Enterolobium* leaves were obtained from Indonesia. The leaves had been air dried for 4 d followed by forced draught for 3 d and ground through a 1 mm sieve.

3.2.1. Extraction of *Enterolobium* leaf

The water soluble compounds were extracted into distilled water from the dried leaves of *Enterolobium cyclocarpum* by maceration using an Ultra Turrax Homogeniser at medium speed for 5 min. The homogenate was centrifuged on a Sorvall RC-5B

Refrigerated Centrifuge (Du Pont Instrument, USA) (5,000 x g for 15 minute, temperature less than 20°C).

The supernatant from this process was freeze-dried using a small glass laboratory apparatus cooled with liquid nitrogen and the vacuum maintained at less than 0.1 mm Hg (Torr) with a 2 stage rotary vacuum pump model E2M2 Edwards, England. Freeze-dried powder (15 g) was dissolved with 60 ml distilled water and lipids removed by extraction with chloroform ($CHCl_3$) 2 x volume solution. The organic solvent soluble fraction was discarded.

Purification of the residual aqueous solution was then commenced by partitioning between n-butanol and water (equal volumes) with the butanol layer containing the active fraction. A flow diagram for the butanol:water partitioning is shown in Fig. 3.1.

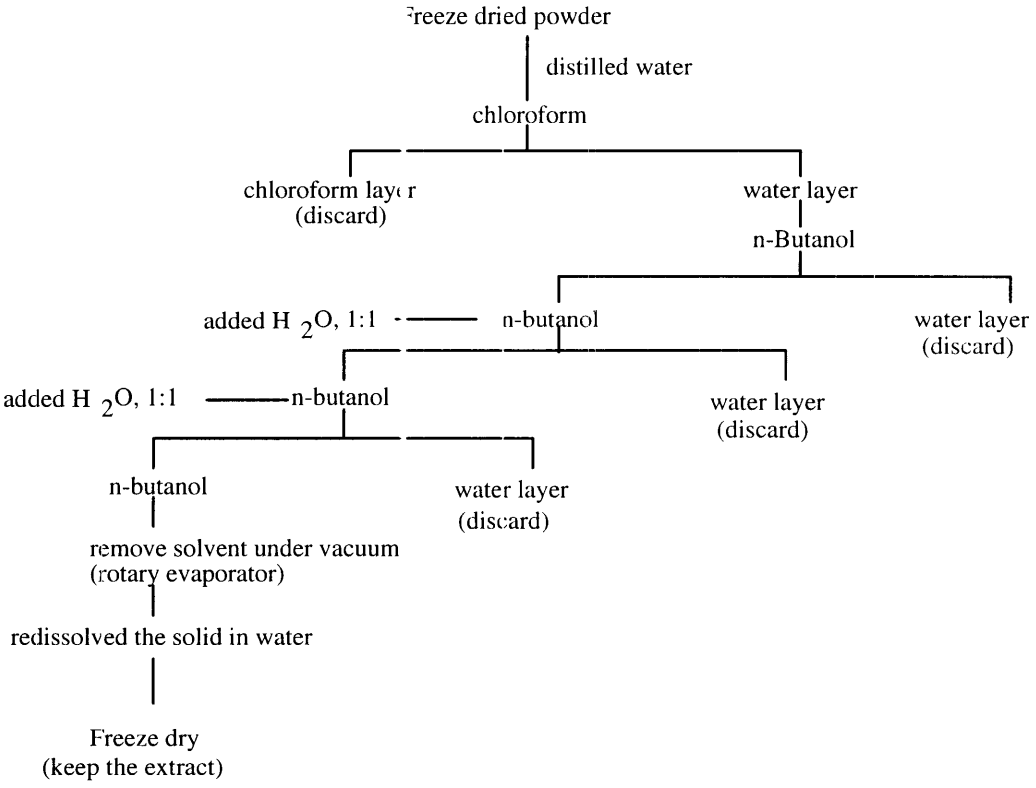


Figure 3.1. A flow diagram of the initial steps used in partitioning the crude plant extract.

3.2.2. Fractionation of *Enterolobium* leaf extract

Purification of the butanol fraction from the *Enterolobium* leaf extract was carried out using reverse-phase HPLC by Dr. David Tucker from the Department of Chemistry of The University of New England. The fractionation steps were carried out using a system consisting of an SSI 220 HPLC pump fitted with a macro pump head, an Erma CR ECR-7215 UV detector and an SSI XL injection valve. A Rainin Dynamax macro HPLC C-18 column (250 x 22 mm, 8 µm) was used. Reverse-phase HPLC on C-18 support relies on the fact that hydrophobic compounds are adsorbed strongly onto the stationary phase. Release from this binding occurs in order of increasing hydrophobicity. Also, as the amount of methanol in the eluting phase is increased the binding of hydrophobic compounds to the stationary phase is reduced. Thus hydrophilic compounds are not readily held by a C-18 column and hydrophobic compounds are released more readily as the concentration of MeOH increases in the eluent. The solvent systems used are described below.

The butanol partitioned fractions of *Enterolobium* leaf extract supplied for this project were produced from three different batches of the freeze-dried leaf extract. The purification procedures for each batch differed slightly and are detailed below.

First Batch. The freeze dried aqueous extract (0.6 g) was dissolved in 150 ml H_2O and extracted with chloroform ($CHCl_3$, 3 x 50 ml) and then butanol (n-BuOH, 3 x 50 ml). The butanol layer was evaporated to dryness to yield a brown gum (I) (Fig. 3.2).

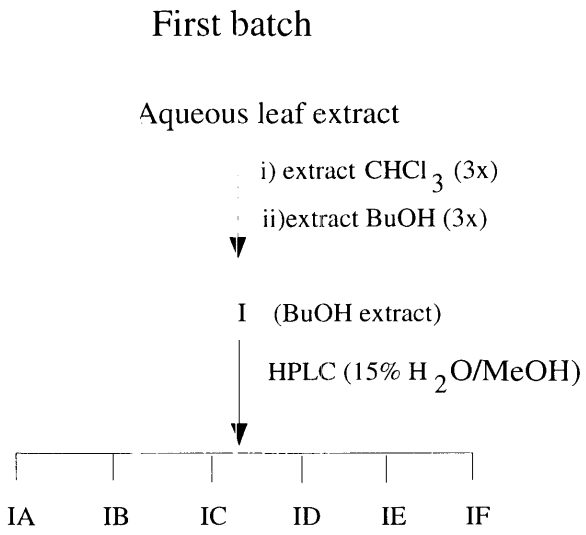


Figure 3.2. Flow diagram to summarise the purification steps of the first batch of *Enterolobium* leaf extract by reverse-phase HPLC.

This was taken up in H_2O/MeOH (15:85) and portions of this solution were subjected to reverse-phase HPLC using H_2O/MeOH (15:85) as solvent to yield 6 fractions labelled IA, IB, IC, ID, IE and IF (see Figure 3.3). These six fractions were assayed using a haemolysis system (see Chapter 4) to determine their activity towards sheep red blood cells.

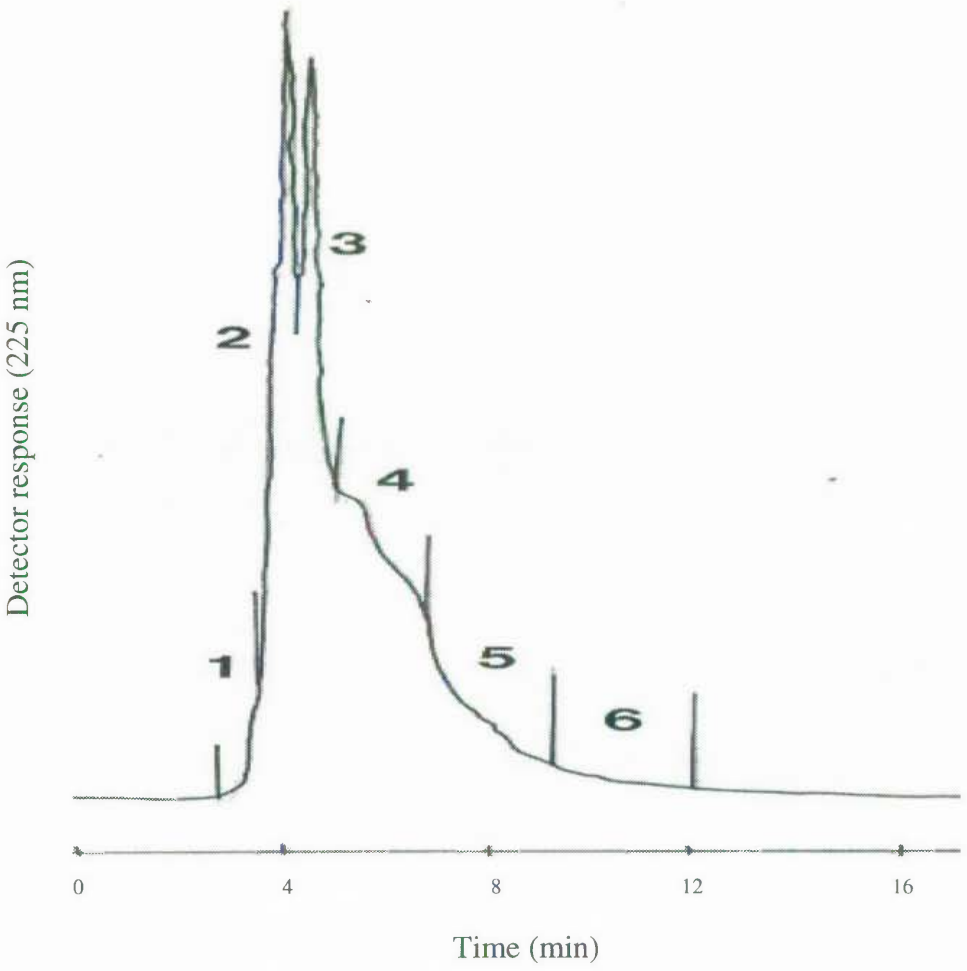


Figure 3.3. HPLC fractionation of the crude butanol extract (fraction I) on a C-18 column. The numbers indicate the individual cuts made. Each cut was recovered in solvent eluted between the vertical lines and the numbers marked within these lines designate the cuts as follows: (1) fraction IA, (2) fraction IB, (3) fraction IC, (4) fraction ID, (5) fraction IE and (6) fraction IF.

Second Batch. The above procedure was applied for the second extraction. The butanol extract (II) from the second batch was subjected to reverse-phase HPLC using H_2O/MeOH (15:85) as solvent to produce 7 fractions (IIA, IIB, IIC, IID, IIE, IIF and IIG) (Fig. 3.4).

Fractions IIC, IID and IIE which have the same retention times as fractions ID, IE and IF (first batch) by preparatory HPLC were then further purified by reverse-phase HPLC using H_2O/MeOH as the eluent but with the water content increased to 25% (v/v). This produced fractions IIC-1, IID-1 and IIE-1 (Fig.3.5).

Fraction IIC1, IIE1, the chloroform extract (fraction IIi) and water layer from butanol partitions (fraction IIii) were assayed to determine their haemolytic activity.

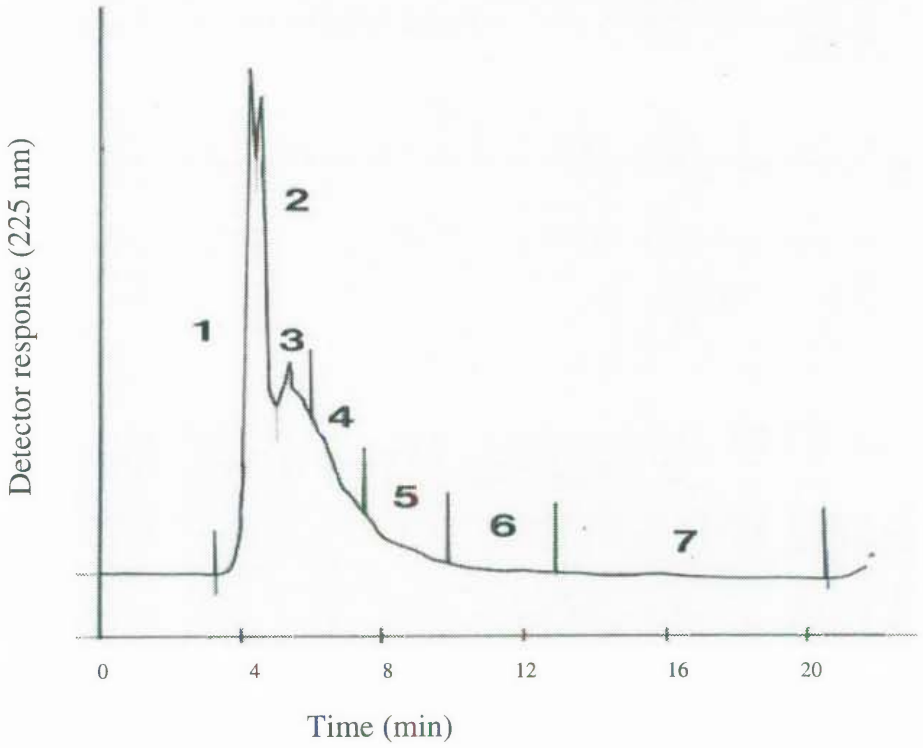


Figure 3.4. HPLC fractionation of the crude butanol extract from the second batch on a C-18 column. The numbers indicate the individual cuts made. Each cut was recovered in solvent eluted between the vertical lines and the numbers marked within these lines designate the cuts as follows: (1) fraction IIA, (2) fraction IIB, (3) fraction IIC, (4) fraction IID, (5) fraction IIE, (6) fraction IIF and (7) fraction IIG.

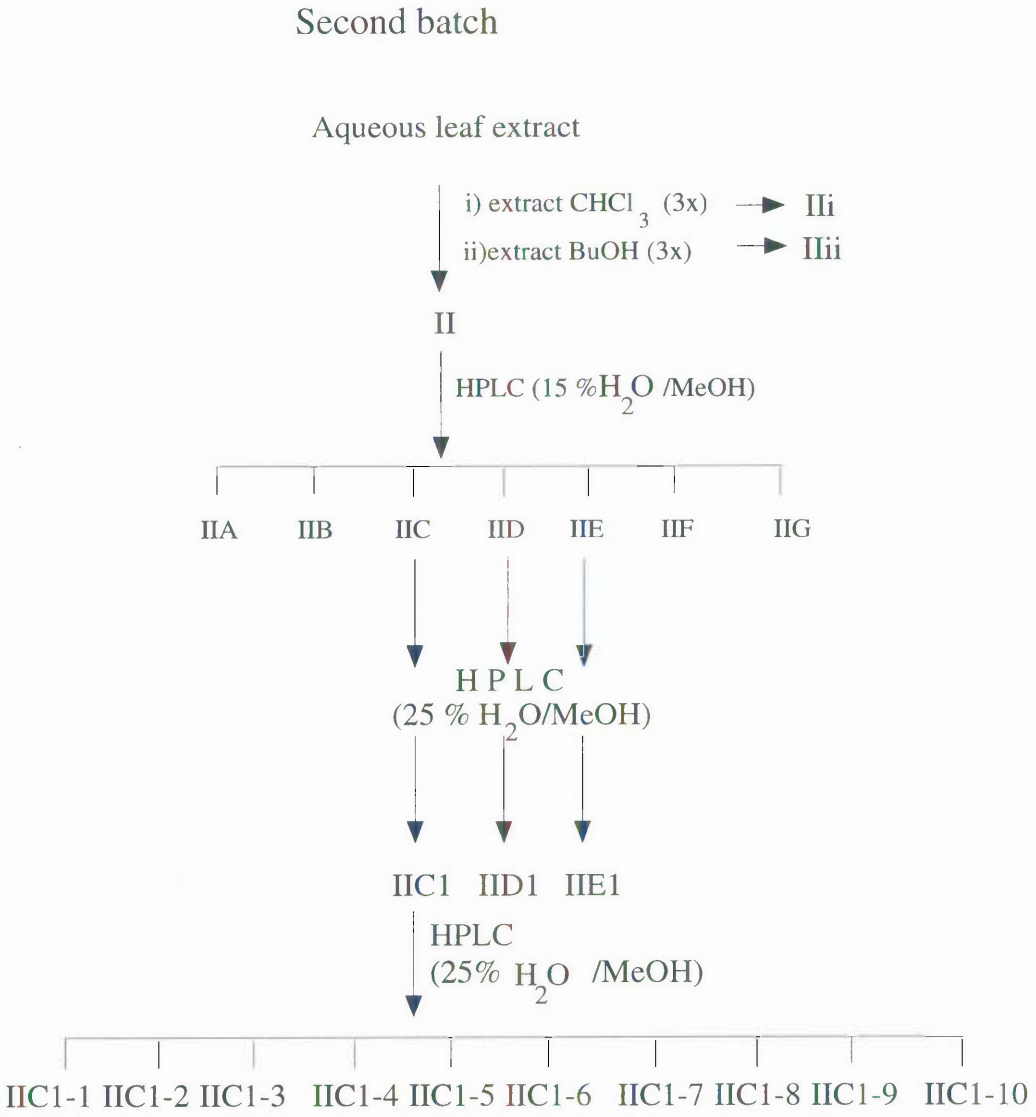


Figure 3.5. Flow diagram to summarise the purification steps of the second batch of *Enterolobium* leaf extract by reverse-phase HPLC.

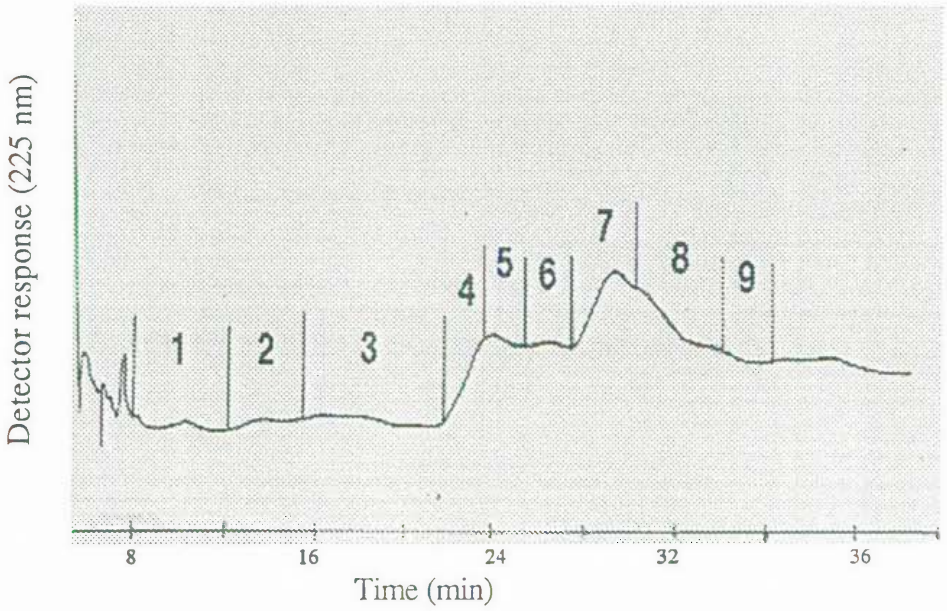


Figure 3.6. Further purification of fraction IIC1 by reverse-phase HPLC on a C-18 column. The numbers indicate the individual cuts made. Each cut was recovered in solvent eluted between the vertical lines and the numbers marked within these lines designate the cuts as follows: (1) fraction IIC1-1, (2) fraction IIC1-2, (3) fraction IIC1-3, (4) fraction IIC1-4, (5) fraction IIC1-5, (6) fraction IIC1-6, (7) fraction IIC1-7, (8) fraction IIC1-8, and (9) fraction IIC1-9.

Fraction IIC-1 was found to be the most haemolytically active fraction. This fraction was again subjected to reverse-phase HPLC using H_2O/MeOH (25:75) as solvent to yield 10 fractions (Figure 3.6). Five out of the ten fractions were subjected to *in vitro* protozoal lysis assay using sheep rumen protozoa.

Third Batch. Again, the same procedure was applied for initial extraction of the freeze dried aqueous extract. The butanol extract (fraction III) was divided into two parts. The first part (fraction III1) was subjected to reverse-phase HPLC using H_2O/MeOH (20:80) to produce 9 fractions (III1-1, III1-2, III1-3, III1-4, III1-5, III1-6, III1-7, III1-8 and III1-9). Fraction III1-3 has same retention time as fraction IIC1 (from the second batch).

Fractions III1-3 and also fraction III1-2 were then further purified by reverse-phase HPLC to produce fraction III1A and followed by further purification by reverse-phase HPLC using H_2O/MeOH (25:75) to yield 12 fractions (III1A-1, III1A-2, III1A-3, III1A-4, III1A-5, III1A-6, III1A-7, III1A-8, III1A-9, III1A-10, III1A-11 and III1A-12). Seven out of these twelve fractions were assayed to determine their protozoal lytic activity towards rumen protozoa.

The second part (III2) was dissolved in 30% H_2O/MeOH and loaded onto a Sep-Pak (Waters C-18) in three portions The Sep-Pak was washed with H_2O/MeOH (30:70) to produce three fractions (III2- , III2-2 and III2-3). The Sep-Pak was then eluted with 100 % methanol to yield three more fractions (III2-4, III2-5 and III2-6). These last three fractions were subjected to reverse-phase HPLC using H_2O/MeOH (15:85) to produce five fractions (III2-7, III2-8, III2-9, III2-10 and III2-11). After that, fraction III2-8 was loaded together with those three fractions mentioned earlier (III2-1, III2-2 and III2-3) into a reverse-phase HPLC using H_2O/MeOH (15:85) to yield 4 fractions (III2-12, III2-13, III2-14 and III2-15) (Fig. 3.7).

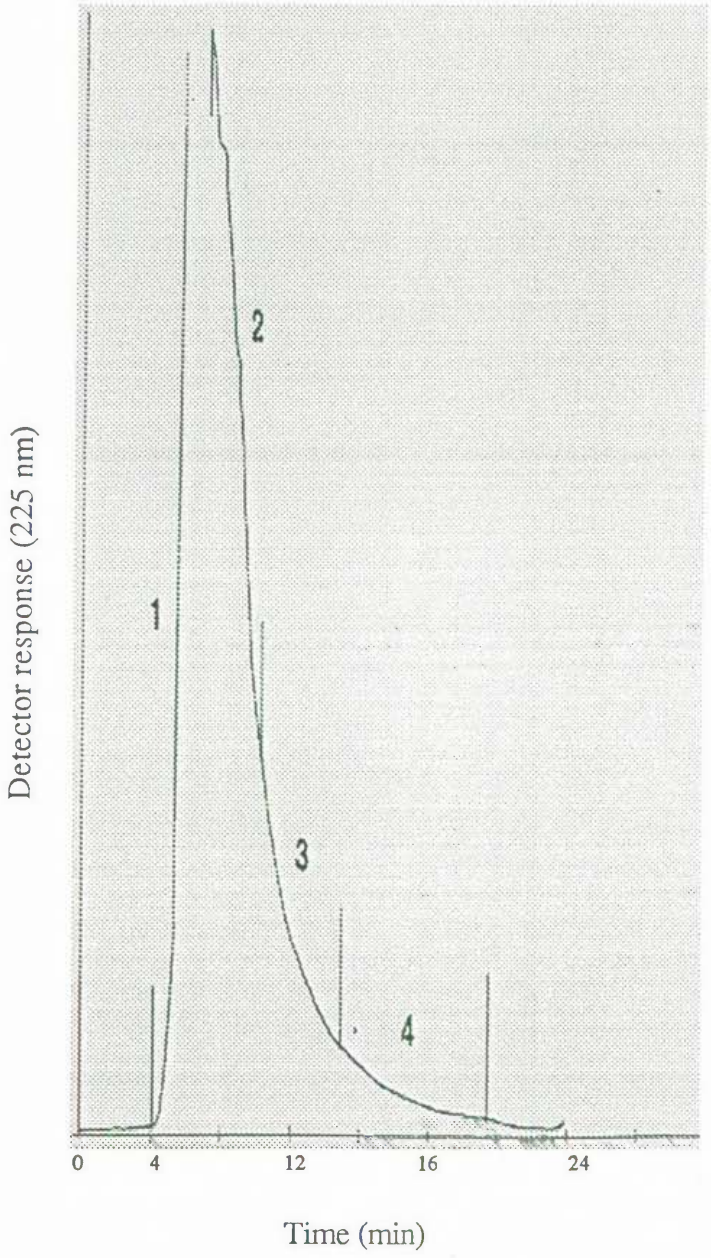


Figure 3.7. Further purification of fractions III2-1, III2-2, III2-3 and III2-8 by reverse-phase HPLC on a C-18 column. The numbers indicate the individual cuts made. Each cut was recovered in solvent eluted between the vertical lines and the numbers marked within these lines designate the cuts as follows: (1) fraction III2-12, (2) fraction III2-13, (3) fraction III2-14, and (4) fraction III2-15.

Only fractions III2-13, III2-14 and III2-15 were then further purified by reverse-phase HPLC using H_2O/MeOH (25:75) to give other seven fractions i.e. fractions III2-16, III2-17, III2-18, III2-19, III2-20, III2-21 and III2-22 respectively. The former four fractions and the six out of seven from the later factions were subjected to protozoal lysis assay under anaerobic conditions using a portable anaerobic cabinet. The flow diagram to summarise the purification steps of the *Enterolobium* leaf extract is shown in Fig. 3.9.

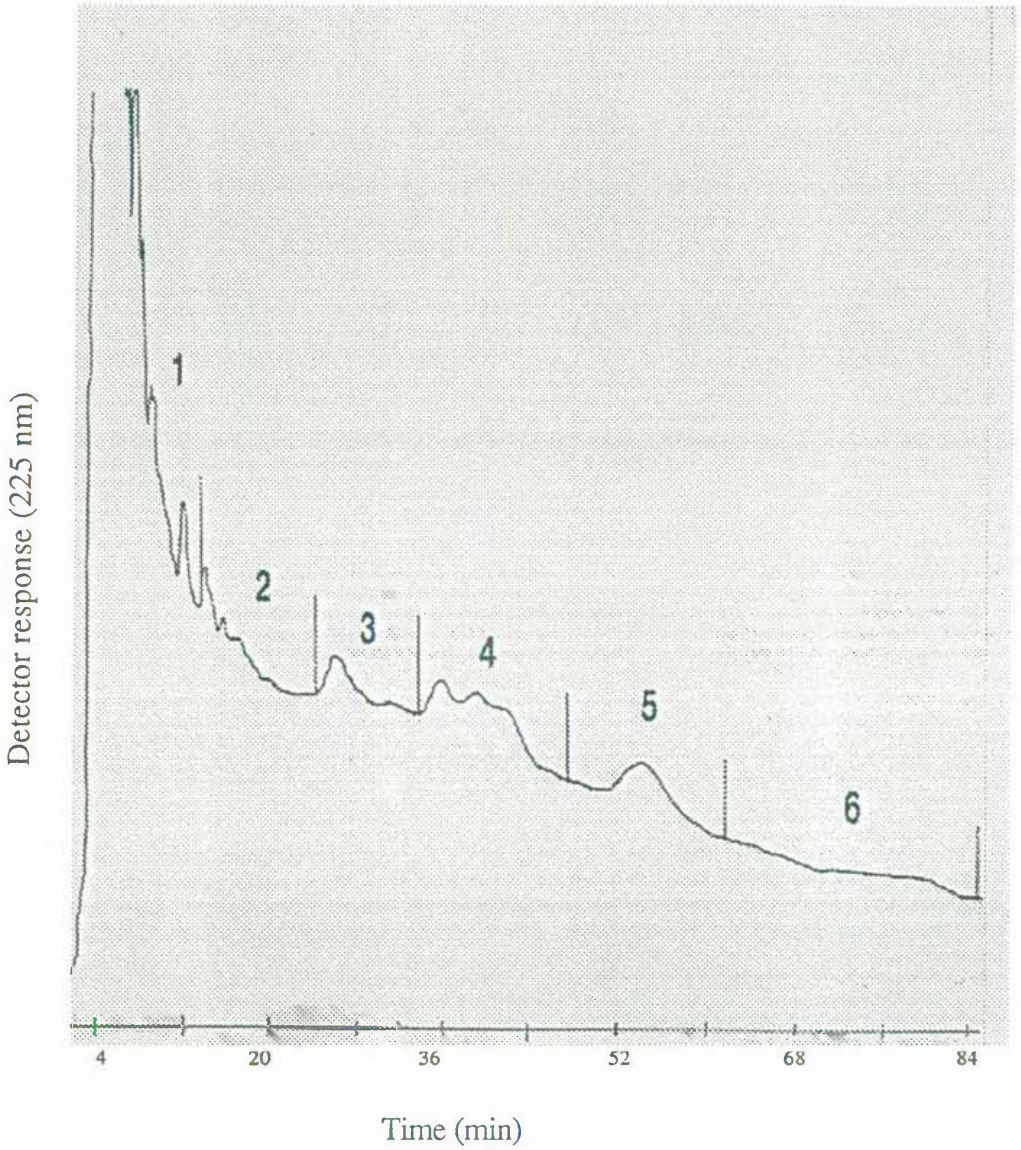


Figure 3.8. Further purification of fractions III2-13, III2-14, and III2-15 by reverse-phase HPLC on a C-18 column. The numbers indicate the individual cuts made. Each cut was recovered in solvent eluted between the vertical lines and the numbers marked within these lines designate the cuts as follows: (1) fraction III2-16, (2) fraction III2-17, (3) fraction III2-18, and (4) fraction III2-19, (5) fraction III2-20, and (6) fraction III2-21.

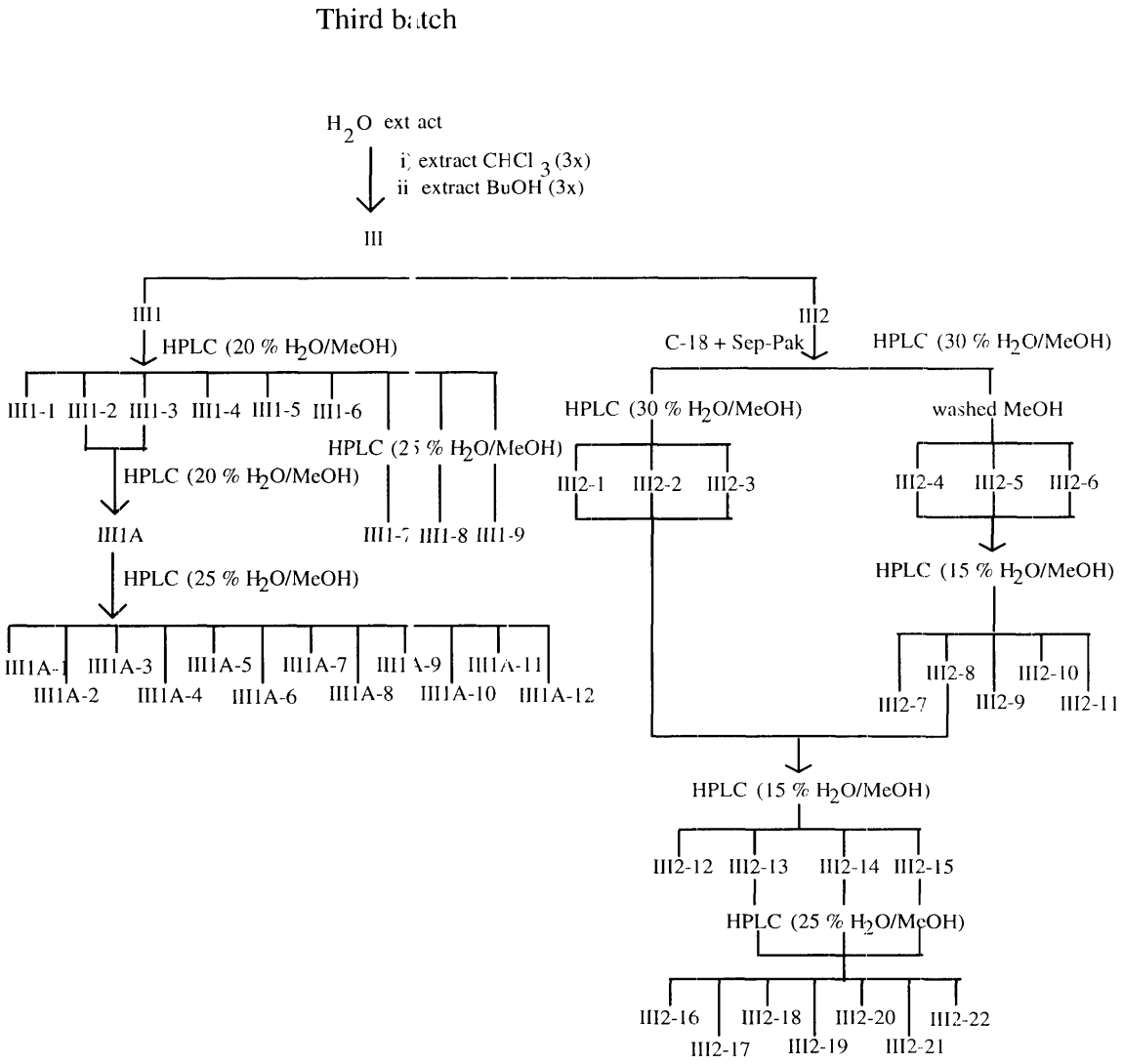


Figure 3.9. Flow diagram of the purification steps of the third batch of *Enterolobium* leaf extract by reverse-phase HPLC.

Chapter 4

HAEMOLYTIC ACTIVITY OF *ENTEROLOBIUM* FRACTIONS

4.1. Introduction

One of a wide variety of biological effects of saponins is their ability to lyse red blood cells (Birk and Peri, 1980). This property has been used diagnostically to test for the presence of saponins (Jurzysta *et al.*, 1988) and may also be employed in quantitative determination of saponin content in plants (Cheeke and Shull, 1985).

This chapter reports on a series of haemolysis assays undertaken to assay the lytic power of a fraction by determining the quantity required to lyse 50% of the red blood cells. The extent of lysis was determined spectrophotometrically and compared to a standard. Standard 100% haemolysis was determined by diluting the red cell suspension with water rather than with 0.9% saline. The relative lytic activity of *Enterolobium* fraction as compared to other lytic agents is reported.

4.2. Materials and Methods

4.2.1. Preparation of red blood cell suspension

Sheep red blood cells were prepared freshly on the day of use. Sheep blood was obtained as required from the same animal and was taken from the jugular vein using a needle and a 20 ml syringe, and transferred into a heparinised tube. After that, the red blood cells were sedimented from the heparinised blood at 3,000 x g for 15 minutes followed by washing three times with 0.9% saline. The final cell solution was resuspended in 0.9% saline to the original volume. The erythrocyte suspension was kept on ice until used.

4.2.2. Fractionation of *Enterolobium* leaf extract

Fractions from the initial purification process were supplied by Dr. David Tucker from the Department of Chemistry, the University of New England. These fractions were obtained from the fractionation of crude butanol extract (I) by reverse-phase preparative High Performance Liquid Chromatography (HPLC) (see Section 3.2.2 in Chapter 3). Fractionation of batch I of crude butanol extract produced 6 fractions (IA, IB, IC, ID, IE, and IF). Haemolytic activity of each fraction was determined and the results are reported in this chapter.

4.2.3. Standard haemolytic agents

In order to ascertain whether the antiprotozoal activity of the active compounds from *Enterolobium* was as potent as simple detergent action, the following two compounds were compared with the similar amounts of *Enterolobium* extract in the test system: (i) a commercial white saponin, produced by BDH Chemicals Ltd. Poole, England and (ii) the detergent Alkanate 3SL3 (sodium lauryl diethoxysulphate).

4.2.4. Standard system for haemolytic activity

The haemolytic activity was measured by using a reaction mixture consisting of a suspension of red blood cells and lytic agent. Release of haemoglobin into the non-sedimentable fraction of the reaction mixture is used as an indication of the lytic activity of the test mixture. Haemoglobin release into the supernatant fraction was measured spectrophotometrically, using a Varian, DMS 70 UV Visible Spectrophotometer. Measurements were made at 415 nm after incubation of washed sheep erythrocytes in the presence of the test fractions for 20 minutes at room temperature.

4.2.5. Experimental procedures

4.2.5.1. Haemolytic activity of crude butanol extract from *Enterolobium*

The aim of this assay was to determine the amount of crude butanol extract required to lyse 50% of red blood cells. The red blood cell suspension was diluted with 0.9% saline in a ratio 1:5. Diluted red blood cell suspension (500 µl) was transferred into a microfuge tube. In initial experiments, crude butanol extract was adjusted to 2 mg/ml, 5 mg/ml, 10 mg/ml 50 mg/ml and 100 mg/ml. The amount of each extract introduced into the assay system was increased incrementally from 2 to 50 µl (i.e. total *Enterolobium*-derived solid ranged between 2-5000 µg per assay).

The total volume of the assay solution was adjusted to 550 µl with 0.9% saline. After incubation for 20 minutes at room temperature, the mixture was centrifuged for 5 minutes in a micro centrifuge (Beckman Microfuge-E).

A 120 µl fraction from the supernatant was diluted to 10 ml with distilled water and the absorbance was read at 415 nm. The haemolytic activity was expressed for each analysis, as the fractional release of (non-sedimentable) haemoglobin compared with the release of haemoglobin which occurred with 100% lysis. Total lysis was achieved by transferring an equivalent quantity of erythrocytes directly into distilled water.

4.2.5.2. Haemolytic activity of purified *Enterolobium* fractions

In this assay, the haemolytic activity of the purified fractions from HPLC fractionation (series I from the first batch, Fig.3.2) was determined and compared to crude butanol extract. Even though concentration of test solids in the assay was varied because the amount of each purified fractions was very limited, the haemolytic activity was calculated on the basis of sample weight (µg) required to lyse 50% of red blood cells. The concentration of the samples was as follows: (1) fraction IA = 4 mg/ml; (2) fraction IB =10 mg/ml; (3) fraction IC =10 mg/ml; (4) fraction ID = 10 mg/ml; (5) fraction IE = 12 mg/ml; (6) fraction IF= 2 mg/ml and (7) the concentration of crude butanol extract was 50 mg/ml.

4.2.5.3. Haemolytic activity of crude butanol extract compared to detergents

A comparative study was undertaken to examine the haemolytic activity of crude butanol extract relative to white saponin and Alkanate 3SL3. All sample tests, in duplicate, were adjusted to 50 mg/ml .

4.3. Results

4.3.1. Haemolytic activity of crude butanol extract

Assay using the crude butanol extract showed that at the lower concentration (i.e. 2 mg/ml total solids) and considering that addition to the assay system was limited to 50 µl or less, the total solid added to that system was less than 100 µg which was insufficient to promote a 50% lysis of the erythrocytes.

Table 4.3.1. Haemolytic activity of crude butanol extract, in triplicate, at concentration 2 mg/ml.

Crude extract (µg)	% Lysis		
	I	II	III
0	0	0	0
10	12.0	11.5	11.4
20	14.5	13.8	16.7
30	21.0	23.9	24.1
40	23.0	23.4	23.6
50	26.3	28.8	31.6
60	28.4	31.3	34.7
70	33.0	31.5	35.8
80	36.4	35.6	39.3
90	32.9	33.8	38.3
100	35.2	33.8	41.6

However, in the higher concentrations (5 and 10 mg/ml total solids) 50% lysis was achieved with addition of about 150 µg (Table 4.3.2).

Table 4.3.2. Haemolytic activity of crude butanol extract made in two different concentrations (5 and 10 mg/ml) and absorbance was read at 415 nm.

Crude extract (µg)	% Lysis	
	I (5 mg/ml)	II (10 mg/ml)
0	0	0
25	22.7	-
50	31.6	33.3
75	34.3	-
100	48.8	35.7
125	46.8	-
150	50.3	49.9
200	50.4	55.8
250	56.8	61.7
300	-	68.9
400	-	76.0
500	-	86.6

In assays conducted using solutions with higher concentration of *Enterolobium* solids, the amount of crude extract required to produce 50% lysis was found to be greater than that suggested by the figures in Table 4.3.1 and 4.3.2. (Figure 4.3.1).

For practical convenience, a stock solution in the range 100 to 600 µg of test substance in the 550 assay mixture. Using these conditions a comparison was made between the crude *Enterolobium* extract and the commercial detergent preparations, white saponin and Alkanate 3SL3.

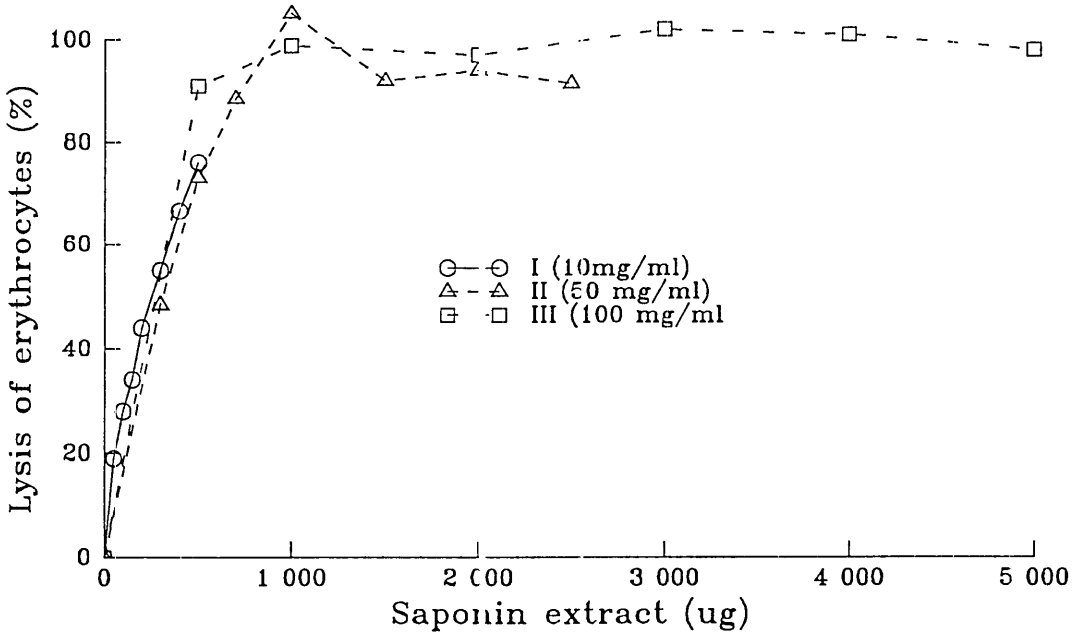


Figure 4.3.1. Haemolytic activity of crude butanol extract produced with higher concentration stock solutions (10, 50 and 100 mg/ml).

4.3.2. Haemolytic activity of purified fractions of *Enterolobium* extract.

Among the six HPLC-purified fractions of *Enterolobium* (Batch I), fraction IF was shown to be the most potent lytic agent as it produced 50% lysis on addition of about 30 µg per assay (Figure 4.3.2).

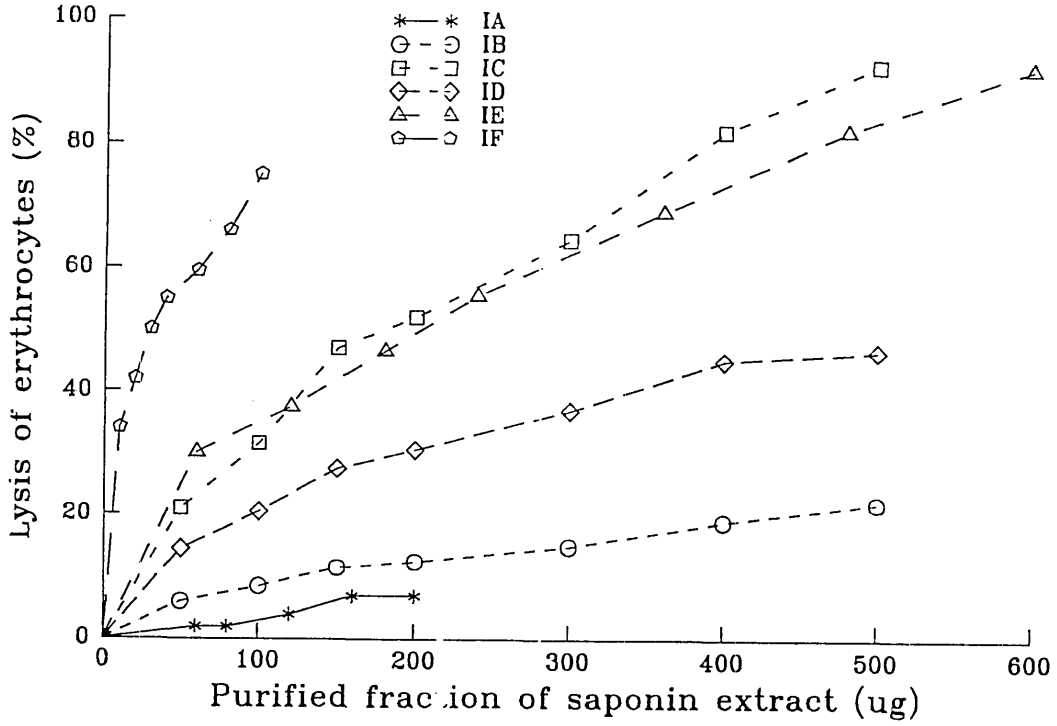


Figure 4.3.2. Haemolytic activity of the HPLC fractions from crude (butanol) fraction.

4.3.3. Haemolytic activity of crude butanol extract compared to white saponin and Alkanate 3SL3

A comparison study was made between crude butanol extract and the commercial detergent preparations to white saponin and Alkanate 3SL3 in terms of their lytic activity in the haemolysis assay.

Table 4.3.3. The haemolytic activity of the crude butanol extract relative to white saponin and Alkanate 3SL3

Amount	% Lysis		
(µg)	crude extract	white saponin	Alkanate 3SL3
100	19.0	85.2	1.0
250	57.1	91.5	32.9
500	90.1	92.1	91.2
750	93.5	103.8	94.4

4.4. Discussion

The results presented in this chapter demonstrate that crude butanol extract and the purified fractions of *Enterolobium* extract show their ability to swell and rupture erythrocytes. Some purified fractions showed stronger lytic activity to than the crude butanol extract, for example fraction IF (Figure 4.3.2) appears to be 8-9 time as active.

In a comparative study to other lytic agents, crude butanol extract was less potent to white saponin but more potent than Alkanate 3SL3. However, these results may not reflect the potential activity of individual active components in *Enterolobium* extract.

The results also showed that white saponin possesses the highest lytic activity compared to crude butanol extract and Alkanate 3SL3. However, white saponin has been claimed

to have no activity on rumen protozoa (Choo, pers. comm.,1992), and this finding has been confirmed in the present studies (data not presented). In addition, Alkanate 3SL3 showed low erythrocyte lysis activity but have been known to effectively eliminate rumen protozoa (Burggraaf, 1980). This could meant that the results obtained from the haemolytic assay might not reflect the activity obtained with rumen protozoa. Thus to study the potency of *Enterolobium* extract as an antiprotozoal agents the haemolytic assay technique was abandoned in favour of the development of an assay specifically utilizing effects on rumen protozoa.