THE TOXICITY OF *SENECIO INAEQUIDENS* DC.

by

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SUMMARY

THE TOXICITY OF *SENECIO INAEQUIDENS* DC.

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This study was designed to confirm the toxicity of a plant implicated in an outbreak in Frankfort, Free State Province, Republic of South Africa. Cows died acutely after being introduced into a camp, where an abundant, green shrublet was noted to be heavily grazed. The plant was subsequently identified as *Senecio inaequidens* DC. (Asteraceae) by the South African National Biodiversity Institute (SANBI). Extraction and chemical analyses for pyrrolizidine alkaloids (PA's) in *Senecio inaequidens* revealed the presence of four different compounds, namely retrorsine and senecionine (known to be hepatotoxic) and two unidentified compounds. The total alkaloid content (free bases plus *N*-oxides) in the dried, milled *S. inaequidens* plant material was 0.18%.

Four (4) male Sprague Dawley rats, aged 8-9 weeks were dosed per os. Each rat received a different dose; ranging from 0.049 mg/g b.w. to 0.25 mg/g b.w.; of the crude *Senecio inaequidens* extract. No clinical signs were observed in the rat receiving the lowest dose. Rats receiving higher doses showed depression, an unsteady gait, pilo-erection and jaundice, which was particularly noticeable in the ears.

Clinical chemistry evaluation revealed an increase in the activities of ALP, AST (except Rat 1) and GGT in all animals. Total serum bilirubin, creatinine and urea concentrations were also elevated. All rats had low serum globulin concentrations with an A/G ratio above 1.88 (except Rat 1). Post mortem examination of the rats revealed marked hepatic lesions. Histopathological changes were characterized by necrosis (variable in extent) of the centrilobular and midzonal hepatocytes, sparing the portal hepatocytes, with extensive haemorrhage and congestion. Proliferation of the bile ducts, fibrosis and oedema was also present. Ultrastructural changes in affected rats were characterized by margination of chromatin, the presence of numerous autolysosomes in necrotic hepatocytes, intramitochondrial woolly inclusions and changes in the endoplasmic reticulum.

A sheep was also dosed in an attempt to reproduce the intoxication. The sheep did not exhibit clinical signs, clinical chemistry aberrations or macroscopic lesions. Histopathological and ultrastructural changes, although milder, were similar to those displayed by the rats.

Pyrrolizidine alkaloids were extracted from the liver and kidneys of the rats and sheep. Retrorsine was also detected in the lungs, urine and bile of the sheep.

Chapter 1

INTRODUCTION

Southern Africa has a huge diversity of flora, which includes a large variety of poisonous plants. Approximately 600 indigenous poisonous plants are known to occur in this region (Kellerman, Coetzer, Naudé and Botha, 2005).

According to Kellerman, Naudé and Fourie (1996) the five most important plant poisonings affecting cattle in South Africa are: cardiac glycoside-containing plants, seneciosis, gifblaar (*Dichapetalum cymosum)* poisoning, *Lantana* poisoning and gousiekte (caused by *Pavetta* species, *Pachystigma* species and *Fadogia homblei*). The most important plant poisonings of sheep are geeldikkop (*Tribulus terrestris)*, cardiac glycoside-containing plants, vermeersiekte (*Geigeria* spp.), seneciosis and gousiekte. In South Africa alone, losses attributed to plant poisoning are estimated to amount to R 150 million annually (Kellerman *et al*. 1996).

Senecio species (Asteraceae) are, as mentioned above, one of the most important plant poisonings in South Africa. Certain *Senecio* species contain toxic pyrrolizidine alkaloids (PA's) (Bicchi, D´Amato and Cappelletti, 1985; Cheeke, 1998; Elliot, Bain and Latimer, 2005), which induce acute or chronic hepatotoxicity in livestock and man, as well as other toxicological effects.

During September 2004 a private practitioner from Frankfort (Free State Province, Republic of South Africa) reported mortalities in cattle. Cows died acutely after being moved to a camp with a marshy area where a small green shrublet grew abundantly. It

was noted that this shrublet was heavily grazed. The plant was collected and submitted for botanical identification. The plant was later identified as *Senecio inaequidens* DC. by the South African National Biodiversity Institute (SANBI).

Necropsy examinations indicated severe hepatic necrosis, multiple haemorrhages and icterus in the longer surviving cases. Histologically, the liver revealed diffuse centrilobular to submassive necrosis and haemorrhage.

Although all *Senecio* species must be regarded as potentially toxic if ingested (Kellerman *et al*., 2005), there are no reports, as far as could be ascertained, of poisoning induced by *S. inaequidens* in South Africa. Thus, the toxicity of *S. inaequidens* required confirmation.

OBJECTIVES

The aims of this study were:

- to extract pyrrolizidine alkaloids (PA's) from *Senecio inaequidens* DC. plant material and to compare the PA concentrations with those of other *Senecio* species;
- to confirm the toxicity of *S. inaequidens* in rats and sheep and to isolate the parent PA's and respective *N*-oxides from organs/tissues and body fluids after dosing.

Chapter 2

LITERATURE REVIEW

2.1 INTRODUCTION

Several *Senecio* species occur world-wide. According to Cheeke (1998) and Braun, Linggi and Pospischill (1999) there are over 1200 *Senecio* species. Habermehel, Martz, Tokarnia, Dobereiner and Mendez (1988) mentioned the existence of more than 1450 species, while Stegelmeier, Edgar, Colegate, Gardner, Schoch, Coulombe and Molineux (1999) stated that about 3000 *Senecio* species are distributed throughout the world. There is an increasing interest in this genus due to the economic losses associated with mortalities amongst livestock and potential human poisoning.

Seneciosis caused by *Senecio jacobaea* was described in Canada as far back as 1882 (Drewes and Kaye, 1985), but according to Cheeke (1998) livestock producers had suspected the plant to be toxic long before this date. Livestock poisoning was recognized in two major outbreaks in the period from 1860 to 1900 in Nova Scotia (Pictou disease) and New Zealand (Winton disease). From the time since Gilbruth, cited by Watt and Breyer-Brandwijk (1962), demonstrated in 1903 that *Senecio jacobaea* caused Winton disease, many *Senecio* species have since been incriminated as causing diseases, which were assigned a variety of common names (Rose, 1972). In the United States of America (USA) the condition known as walking disease in horses was attributed to the consumption of *Senecio riddelli* (Cheeke, 1998). Chase (1904) (Fig. 2.1) reported a disease caused by *Senecio burchelli* in the Molteno district (Eastern Cape Province, South Africa), which was known as Molteno cattle disease. Other common names referring to seneciosis in South Africa are

Molteno straining disease in cattle and stomach staggers, grass staggers, ragwort poisoning and 'dunsiekte' in horses (Watt and Breyer-Brandwijk, 1962). The disease is also known as 'suiljuk' in central Asia (Rose, 1972).

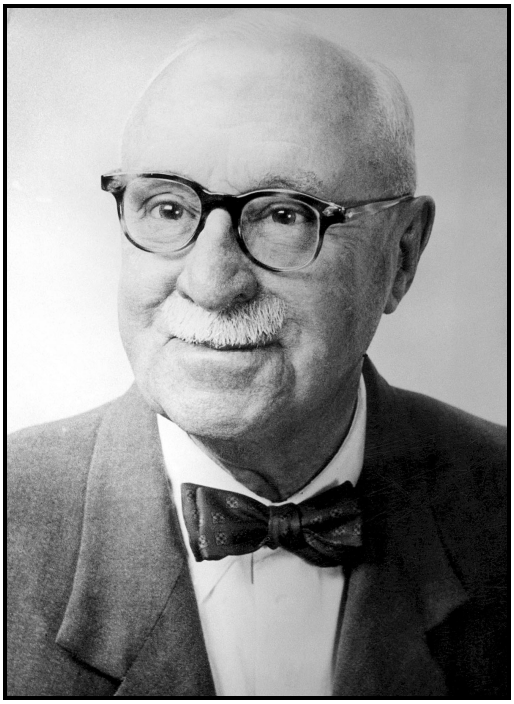

Fig. 2.1 W. H. Chase ESQ., CBE., F.R.C.V.S., Principal Veterinary Officer.

Seneciosis has also been described in species other than horses and cattle. It has been reported to occur in pigs, chickens, sheep, goats, quails and doves and poisoning also occurs in man (Hill, 1960).

Senecio species are usually unpalatable and not readily eaten by livestock. Animals may ingest *Senecio* plant material when other forage is scarce (Pearson, 1990) or when the stand of the plant is so dense that it cannot be avoided or differentiated from edible forage. Young plants may also be cropped with grass and poisoning has been described as a result of contamination of hay and silage (Pearson, 1990; Cheeke, 1998; Elliot *et al.*, 2005).

The content of the pyrrolizidine alkaloids (PA's) in *Senecio* species varies enormously. According to Kellerman *et al*., (2005), this variation can depend on season, meteorological conditions, growth stage and location of the plant. The concentration of PA's is higher in flowers compared to the leaves and stems (Craigmill, 1981; Kellerman *et al*., 2005). On the other hand, *Senecio* species are reported to be more toxic in the pre-flowering stage (Watt and Breyer-Brandwijk, 1962).

The susceptibility of animal species to the toxic effects of S*enecio* also varies. The toxic dose of dried *Senecio* as a percentage of body weight is estimated to be 5% for horses and cattle; 125% to 400% for goats and over 150% for sheep (Pearson, 1990). The disease can be acute or chronic depending on the toxicity and amount of *Senecio* plants ingested and the duration of exposure. Larger quantities ingested over a short period may cause acute toxicity and animals may die within a few days. Chronic disease is seen when animals are exposed over a prolonged period to multiple small doses, or when they have survived a single large dose (Kellerman *et al*., 2005).

2.2 DESCRIPTION AND IDENTIFICATION

2.2.1 General description

The genus *Senecio* belongs to the family Asteraceae (=Compositae), tribe Senecioneae. Members of the genus vary remarkably in growth form and foliage. They are herbs, sub-shrubs or shrubs, occasionally succulent or climbing. The leaves are alternate, sometimes radical with the margins of the blades entire or lobed. The capitula (flower heads) can be either heterogamous with marginal fertile female florets and bisexual disc florets, or homogamous. A calyculus, usually of short basal bracts, is present. The receptacles are flat or more or less convex, without scales, pitted or shortly fimbriate (Hyde and Wursten, 2006). Although the species of *Senecio* are morphologically extremely variable, common characteristics include the presence of a single whorl of bracts forming a cup under the head, and the cypselas (which have a pappus of many fine bristles) are produced in disc and ray florets (Stegelmeier *et al*.,

1999). The name '*Senecio*' is derived from '*senex*' and means 'old man' probably referring to the white pappus (Fig. 2.3, F) (EPPO, 2006).

2.2.2 Poisonous species of South Africa

According to Kellerman *et al*. (2005), toxic S*enecio* species belong to the 'Paucifolii' group. They are perennial herbs, with annual, unbranched erect stems which give rise to much-branched inflorescences. The rootstocks form shoots in early spring and the aerial parts die off in winter. Both the stems and leaves are almost glabrous. The leaves, light-blue to greyish-green, are arranged alternately with the leaf base folding around the stem. Short prickly teeth are present in the margins of the leaves at about 3 mm intervals. In South Africa, *S. latifolius* and *S. retrorsus* (Fig. 2.2) are regularly implicated as the cause of intoxication in livestock (Kellerman *et al*., 2005).

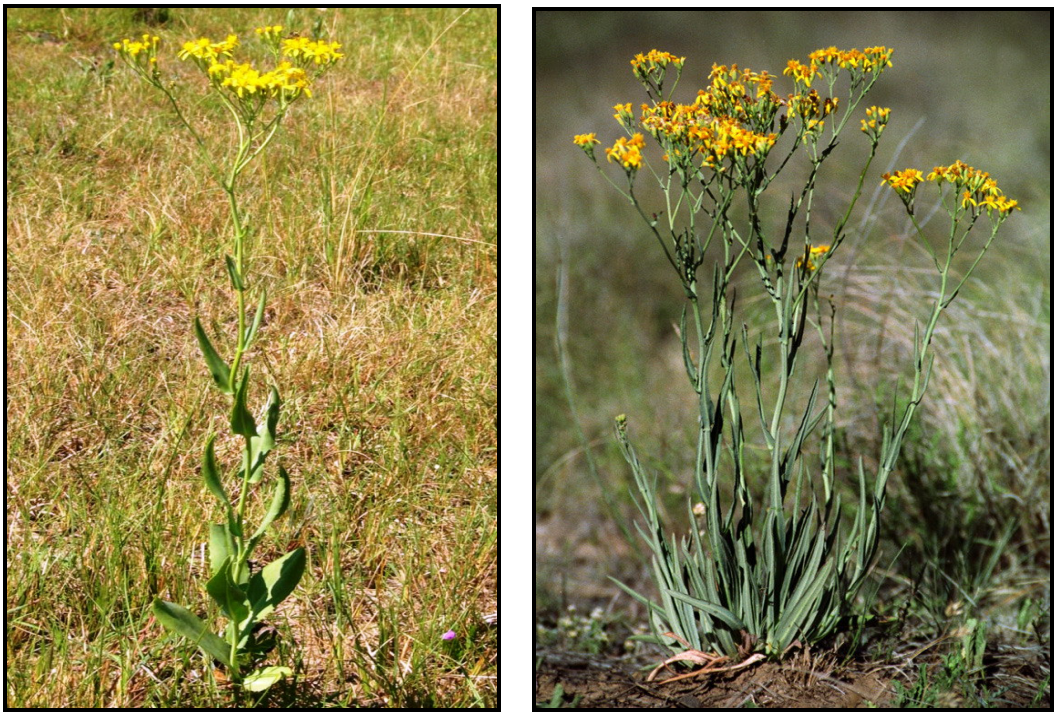

Fig. 2.2 *Senecio latifolius* DC. (left) and *S. retrorsus* DC. (right).

2.2.3 *Senecio inaequidens* DC.

Synonym: *Senecio burchelii* DC.

Senecio inaequidens is known by the common names 'narrow leaved-ragwort' and 'South African ragwort' in English, in French as 'séneçon du Cap', in Italian as 'senecione sudafricano' and in German as 'schmalblättriges Greiskraut', 'schmalblättriges Kreuzkraut' and 'südafrikanisches Greiskraut' (EPPO, 2006). 'Boton de oro' and 'senecio amarillo' are common names used in Spain and Argentina (Rzedowski, Vibrans and Rzedowski, 2003). In South Africa, common names such as 'canary weed' (English); 'geelopslag' and 'geelgifbossie' (Afrikaans), are used to identify *S. inaequidens* (Van Wyk and Malan, 1997; Powrie, 2004).

Senecio inaequidens (Fig. 2.3, as described by Rzedowski *et al*. (2003), EPPO (2006) and Elizabeth Retief (SANBI, personal communication, 2006) is a perennial herbaceous or woody shrub, which is up to 100 cm tall, spherically shaped, rising from a shallow taproot. The erect, leafy stems, which rise from the woody base, are numerously branched and glabrous, but sometimes sparsely hairy. The leaves are alternate, usually sessile, occasionally petiolate, with the blade bright green, simple and slightly thickened, usually with the base clasping the stems. The basal leaves are sessile, and have linear to elliptic-lanceolate blades with acute apices; the size of the blades is variable, from 3 to 14 cm long and 0.3 to 1 cm wide. The name '*inaequidens*' means 'irregular teeth' in Latin and refers to the margins of the leaf blade, which are irregularly-toothed. The upper leaves are shortly petiolate, subsessile or sessile and occasionally pinnately-lobed. The inflorescence is an open, terminal or axillary, corymbose panicle ranging from 80 to 100 per plant. Radiate capitula, 18–25 mm in diameter, with about 20 involucral bracts are characteristic of the species. The bracts are narrowly ovate with acute apices, more or less glabrous, keeled, (4–) 5 (–7) mm long and resinous. The calyculus bracts, 8 to 12, have acute apices, are more or less glabrous and dark tipped. The ray florets, 7–13, are female, with bright yellow ligules, which become revolute. *Senecio inaequidens* has numerous perfect disc florets, with bright yellow corolla tubes and lobes with a median resinous line. A cypsela (fruit) is

2.0-2.5 mm long, cylindrical, pubescent between ribs with a white pappus, 2 to 3 times as long as the cypsela and readily detached. The flowering time is mainly in spring to autumn but occurs all year long.

Senecio inaequidens occurs in different habitats, from plains to mountains and from coastal areas up to 2850 m altitude. It colonizes open and disturbed lands, such as wastelands, fallow lands, railway tracks, roadsides, trampled areas, burnt land and pastures. The plant occurs in rocky soil as well as in drained sandy or stony soil, or sandy loam soil. However, the species is also found in natural environments such as dunes and cliffs in littoral areas. The plant is resistant to cold and tolerates dry and flooded conditions. Vineyards, where zero tillage is practised, are favourable for the plant, but it is never found in annual crops (Michez, 1980).

Fig. 2.3 *Senecio inaequidens* DC. A. individual plant; B. upper leaves; C. inflorescences; D. flower heads - top view; E. flower heads - lateral view; F. flower head showing achenes with white pappus (Photo's taken by Pedro Tenorio; in Rzedowski *et al*., 2003, reproduced with permission).

2.3 DISTRIBUTION OF POISONOUS SENECIO SPECIES IN SOUTHERN AFRICA

Of the thousands of *Senecio* species that occur world-wide, many contain toxic PA's, but only a few have been incriminated in livestock and human poisoning.

In South Africa, over 250 *Senecio* species are known to occur (Kellerman *et al*, 2005; Drewes and Kaye, 1985). *Senecio latifolius* (Mpumalanga and KwaZulu-Natal) and *S. retrorsus* (Eastern Cape) cause the most losses (Kellerman *et al*., 2005). According to Drewes and Kaye (1985), these two species plus *S. sceleratus* (in the former North-eastern Transvaal) and *S. isatideus* (widespread throughout South Africa) are responsible for the most fatal cases of *Senecio* poisoning in livestock.

In Zimbabwe, *S. sceleratus* is predominant in the Eastern Districts (Shone and Drummond, 1965). According to Watt and Breyer-Brandwijk (1962), *S. latifolius* was associated with livestock poisoning in Mashonaland. The same authors also report the occurrence of *S. retrorsus* in Zambia.

Although *Senecio* species are known to occur in Mozambique, no reports of poisoning have been documented.

2.3.1 Distribution of *Senecio inaequidens* DC.

Senecio inaequidens originates from South Africa and is also found in Mozambique, Namibia, Lesotho and Swaziland (EPPO, 2006). In South Africa the plant occurs in all the provinces, namely Limpopo, North West, Gauteng, Mpumalanga, Free State, KwaZulu-Natal, Northern Cape, Eastern Cape and Western Cape. In Mozambique the plant has been collected in Guijá (Gaza Province), Inhaca Island, Polana and between Quinta da Pedra and Salamanga in Maputo Province (Hannelie Snyman and Elizabeth Retief, SANBI, personal communication, 2006). Data from the Herbarium of the Botanical Department of the University Eduardo Mondlane, Maputo, which dates back to 1969 refer to the occurrence of the plant in Namaacha district (Maputo) and in Caniçado (Gaza). Fig. 2.4 shows the distribution of *S. inaequidens* in southern Africa.

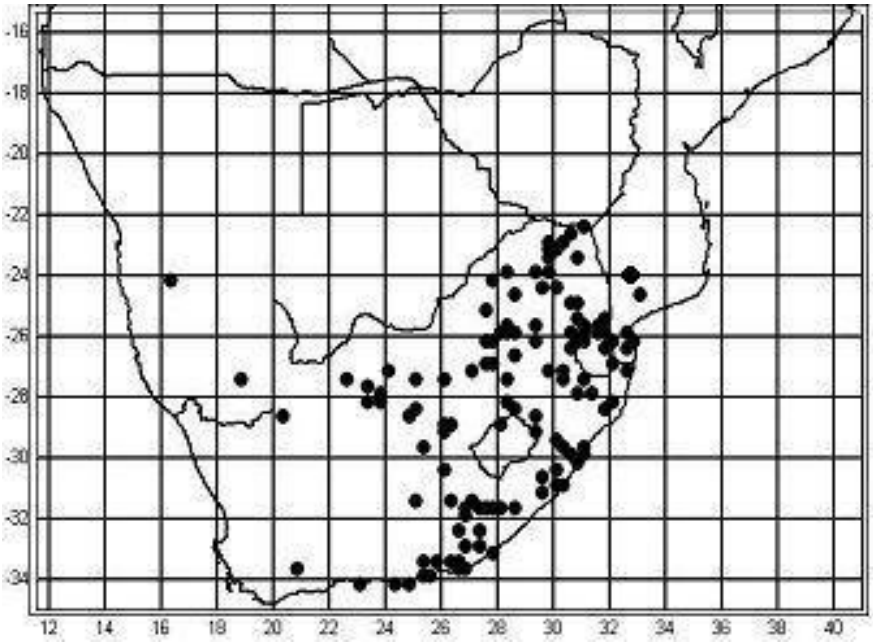

Fig. 2.4 Distribution of *S. inaequidens* in southern Africa (courtesy of Hannelie Snyman, SANBI).

The plant was introduced into Europe through seeds present in imported sheep wool at the end of the nineteenth century (Sans, Garcia-Serrano and Afán, 2004). In France it was introduced in 1936 and is now widely distributed (Michez, 1994). *Senecio inaequidens* is considered a successful invader in the western Mediterranean Basin (Guillerm, le Floc'h, Maillet and Boulet, 1990) and is now widely distributed in many countries such as Austria, Belgium, the Czech Republic, Denmark, Finland, Germany, Italy, Netherlands, Norway, Spain, Sweden, Switzerland and the United Kingdom. It has also been recorded in Central America (Mexico), South America (Argentina, Brazil and Colombia) and Australia (EPPO, 2006).

2.4 POISONOUS PRINCIPLE(S)

2.4.1 Chemical structure

Senecio species contain pyrrolizidine alkaloids (PA's) which are common chemical compounds of a variety of genera. About 3% of the world's flowering plants contain PA's (Duringer, Buhler and Craig, 2004).

Pyrrolizidine alkaloids are colourless, chemically stable compounds, with variable solubility in water. Their salts, however, are very water soluble. The common characteristic of PA's is the pyrrolizidine nucleus consisting of two fused five-membered rings joined by a single nitrogen atom at position 4 to form a heterocyclic nucleus (Stewart and Steenkamp, 2001).

Toxic PA's are esters of necine ring structures, which are also known as amino alcohols (Wachenheim, Blythe and Craig, 1992a). Hepatotoxic PA's are esters of unsaturated necine bases. Pyrrolizidine alkaloids with saturated necine structures are usually not hepatotoxic (Lin, Zhou, Zhao, Wang and But, 1998). Most of the hepatotoxic PA's are esters of the bases retronecine and heliotridine (Kedzierski and Buhler, 1986; Cheeke, 1988; Cheeke, 1998, Lin *et al.*, 1998). However, a few are esters of the amino alcohol otonecine (EHC 80, 1988; Lin *et al.,* 1998). Of the toxic PA's, three major chemical configurations are distinguished, namely cyclic diesters, noncyclic (open) diesters and monoesters (Cheeke, 1998).

In nature, PA's frequently coexist with *N*-oxides, but they can be, in some instances, predominantly in the *N*-oxide form (Mattocks, 1986; Coulombe, 2003). The *N*-oxides are also toxic (Cheeke, 1998).

The content of PA's in several plant species has been extensively studied. Smith and Culvenor (1981) and Robins (1981) give a comprehensive list of plants containing PA's and the respective PA constituents. In the genus *Senecio* the occurrence of the

PA's senecionine, retrorsine and integerrimine is well known (Habermehel *et al.*, 1988). *Senecio latifolius,* the most important *Senecio* species responsible for poisoning of livestock in South Africa, contains the alkaloids retrorsine, seneciphylline and platyphylline; *S. retrorsus* and *S. isatideus* contain retrorsine and *S. sceleratus* contains sceleratine, retrorsine and chlorodeoxysceleratine (Smith and Culvenor, 1981; Mattocks, 1986). According to Röder, Wiedenfeld and Stengl (1981), *S. inaequidens* contains senecionine and retrorsine, although Bicchi *et al.,* (1985) also identified senecivernine, integerrimine and a retrorsine analogue in this species (Fig. 2.5).

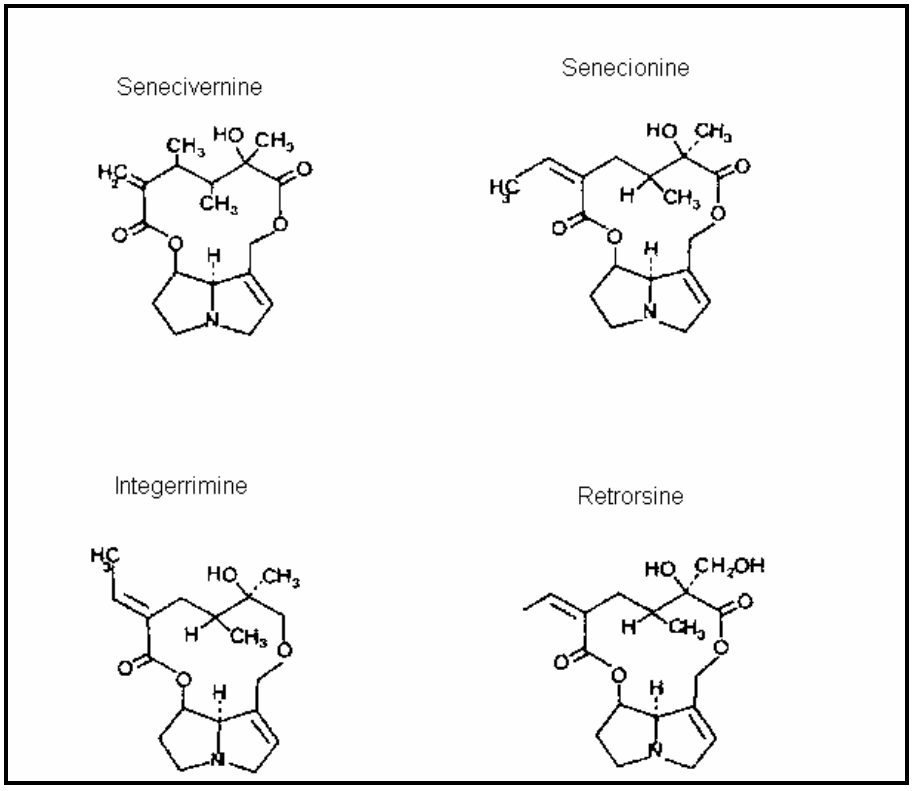

Fig. 2.5 Chemical structures of PA's identified in *Senecio inaequidens* (Bicchi *et al.*, 1985)

2.4.2 Extraction and isolation

Pyrrolizidine alkaloids are extracted from plants and other biological materials for chemical and analytical investigations owing to their potential hepatotoxic, carcinogenic, mutagenic and teratogenic properties. They are also prospective anti-

tumor agents (Parker, Verma, Tomer, Reed and Buhler, 1990). In plants it is usual to have more than one basic alkaloid, frequently accompanied by *N*-oxides, which may or may not be those of the corresponding free base. To simplify separations, *N*-oxides are often reduced to bases during extraction of alkaloids from plant material (Mattocks, 1986).

Procedures to obtain PA's from plants often involve extraction with polar solvents such as methanol. The solvent is evaporated and the alkaloids dissolved in dilute aqueous acid. Chlorophyll and waxes are removed by extraction with ether or petroleum (Mattocks, 1986) or hexane as described by Holstege, Seiber and Galey (1995). *N*-oxides are reduced to bases by adding zinc powder to the acidic solution, which is then filtered. The solution is made alkaline by adding ammonia and the alkaloids are extracted using an organic, non-polar solvent (Mattocks, 1986). Alternative methods of PA extraction have been described (Mattocks, 1986; Parker *et al.*, 1990; Holstege *et al.*, 1995 and Rösemann, 2007).

Pyrrolizidine alkaloids can also be recovered from materials other than plants. The methods described for plant material can be used to extract alkaloids from animal tissues and biological fluids such as liver, rumen contents, plasma, bile and urine. Pyrrolizidine alkaloids have also been isolated from milk (Dickinson, Cooke, King and Mohamed, 1976) and honey (Deinzer, Thomson, Burgett and Isaacson, 1977). As PA's may be rapidly metabolized in animals to products that bind strongly to tissues (forming stable pyrrolic thioethers) or are promptly excreted, the amount of PA's recoverable from tissues after ingestion may be very small after a few hours (Mattocks, 1986). A simple procedure has been developed to recover the nucleus of the sulphur bound pyrrolic metabolites in tissues or body fluids, involving the breakdown of the thioether by silver nitrate and the subsequent reaction of the pyrrolic moiety with alcohol. Pyrrolic ethers are extracted and identified by analytical techniques such as thin layer chromatography, capillary gas chromatography and mass spectrometry (Mattocks and Jukes, 1990; Mattocks and Jukes, 1992). These methods have been utilized to identify pyrrole metabolites in preserved liver samples

of yaks to confirm a diagnosis of PA poisoning (Winter, Seawright, Mattocks, Jukes, Tshwang and Gurung, 1990). When applied to blood samples, as described by Seawright, Hardlicka, Wright, Kerr, Mattocks and Jukes (1991), this method can be advantageous to monitor animals previously exposed to PA's.

2.4.3 Chemical analysis

Analytical techniques such as gas chromatography (GC), thin-layer chromatography (TLC), supercritical fluid chromatography (SFC) and high-performance liquid chromatography (HPLC) have been used to detect PA's and their metabolites. These techniques are sensitive, but lack specificity (Parker *et al*., 1990). Mixtures of PA's from plant extracts have been separated using predominantly HPLC techniques (Mattocks, 1986). The use of off-line or on-line mass spectral analysis (MS), usually by GC/MS, brings more specificity to the analysis. GC/MS has the disadvantage that thermally labile compounds such as *N*-oxides of the alkaloids may undergo decomposition (Parker *et al*., 1990). HPLC has advantages over GC as less clean-up and preparation of samples is required. HPLC/MS is more convenient for the analysis of liquid biological samples such as blood and urine (Lin *et al*., 1998). Bicchi *et al.* (1985) identified five PA's from *Senecio inaequidens* using capillary gas chromatography (CGC) and capillary gas chromatography-mass spectrometry. Parker *et al*. (1990) described the use of thermospray liquid chromatography/mass spectrometry (TSP LC/MS) with ammonium acetate as a mobile phase to separate PA's and *N*-oxides in *Senecio* extracts. Lin and co-workers (1998) determined hepatotoxic PA's and distinguished them from the non-toxic PA's by using either in-source collision-induced dissociation high performance liquid chromatography mass spectrometry (CID-HPLC/MS) or HPLC/MS/MS (CID in the collision cell).

The Ehrlich reaction is a colorimetric method used to detect and measure unsaturated PA's and their *N*-oxides. A basic alkaloid is oxidized to its *N*-oxide by hydrogen peroxide (for the estimation of *N*-oxides alone, this step is omitted), and then converted to a pyrrolic derivative by acetic anhydride. The pyrrolic derivative reacts

with the Ehrlich reagent (4-dimethylaminobenzaldehyde) containing boron trifluoride to give a strong colour. The Ehrlich reaction is also suitable to detect pyrrolic metabolites formed *in vivo*. Tissue samples containing PA metabolites take a mauve or magenta colour when heated with the reagent (Mattocks, 1986). Mattocks and Jukes (1987), described an improved field test to qualitatively detect unsaturated PA's and their *N*-oxides in fresh and dried plant material and plant based foodstuffs.

2.5 OTHER PA CONTAINING PLANTS

Plants are known to be the only natural source of toxic PA's (EHC 80, 1988). Over 350 PA's, the majority being toxic, have been isolated from more than 6000 plant species (Coulombe, 2003). Most of them belong to the families Boraginaceae, Asteraceae and Fabaceae (genus *Crotalaria*) (Smith and Culvenor, 1981; Knight and Walter, 2001; Coulombe, 2003). Important species in the Boraginaceae family include *Amsinckia intermedia, Symphytum officinale, Heliotropium europaeum, Cynoglossum officinale, Echium plantagineum* and *Borago officinalis* (Cheeke, 1998). The number of species investigated in each genus for the presence of PA's is very small compared to the total number of species in the genera concerned. Thus, Smith and Culvenor (1981) recommended that all species in the Boraginaceae family and the genera *Crotalaria*, *Eupatorium* and *Senecio* should be regarded as potentially hepatotoxic.

Echium plantagineum and *Heliotropium europaeum* are the two most important plants in the Boraginaceae family that cause livestock toxicoses (Cheeke, 1988). *Crotalaria* poisoning occurs when livestock graze on infested pasture or when the seeds of the plant contaminate grain intended for animals.

Crotalaria seed contamination of grain is a concern for human health, particularly in developing countries (Cheeke, 1998). Several of the known PA-containing plants have been or are used as food or phyto-medicines for humans (Coulombe, 2003).

Plant species of the families Apocynaceae, Ranunculaceae and Scrophulariaceae have also been identified as possessing toxic PA's (EHC 80, 1988). *Festuca arundinacea* (Poaceae) contains amino pyrrolizidines which are not hepatotoxic, but may play a role in other toxic effects (Mattocks, 1986).

2.6 TOXICOKINETICS

2.6.1 Absorption and distribution

Following the ingestion of plant material containing PA's, a series of events must occur before intoxication results. These include the release of the PA's from the plant matrix, transport to the liver via the portal circulation, bioactivation to pyrrolic metabolites and reaction of the latter with nucleophilic cell structures (Wachenheim, Blythe and Craig, 1992b). Some of the alkaloids that are not converted to toxic metabolites may be converted to harmless compounds, which are eliminated from the body or excreted unchanged (Mattocks, 1986).

Pyrrolizidine alkaloids are mainly absorbed in the small intestine (Stewart and Steenkamp, 2001; Elliot *et al.*, 2005). Very small quantities of pyrrolizidine alkaloids are percutaneously absorbed when applied to intact skin (EMEA, 2000).

The toxicokinetics of PA's are alkaloid specific (Stegelmeier *et al.*, 1999). Studies of tissue distribution in male rats of a radio-labeled compound {[^{3}H]synthanecine A bis(N-ethylcarbamate)} inducing PA-like hepatotoxicity revealed that the highest concentrations of radioactivity were detected in the liver (where the compound is metabolized) and in the lungs, kidneys and spleen (Mattocks, 1986). ^{14}C-senecionine and ^{14}C-seneciphylline show very similar patterns of distribution when administered to lactating mice (EMEA, 2000). Hepatotoxic PA's reach high liver pyrrole concentrations (peaking at about two hours) after a single PA dose. Non-hepatotoxic PA's result in lower pyrrole production and these are rapidly removed from the liver (Stegelmeier *et al.*, 1999).

2.6.2 Metabolism

Three major routes of PA metabolism have been described (Fig. 2.6), namely:

- hydrolysis of the ester groups by esterases and excretion of the acid and amino alcohol products (which are not toxic),
- *N*-oxidation and excretion of water-soluble *N*-oxides and
- dehydrogenation to pyrrolic derivatives.

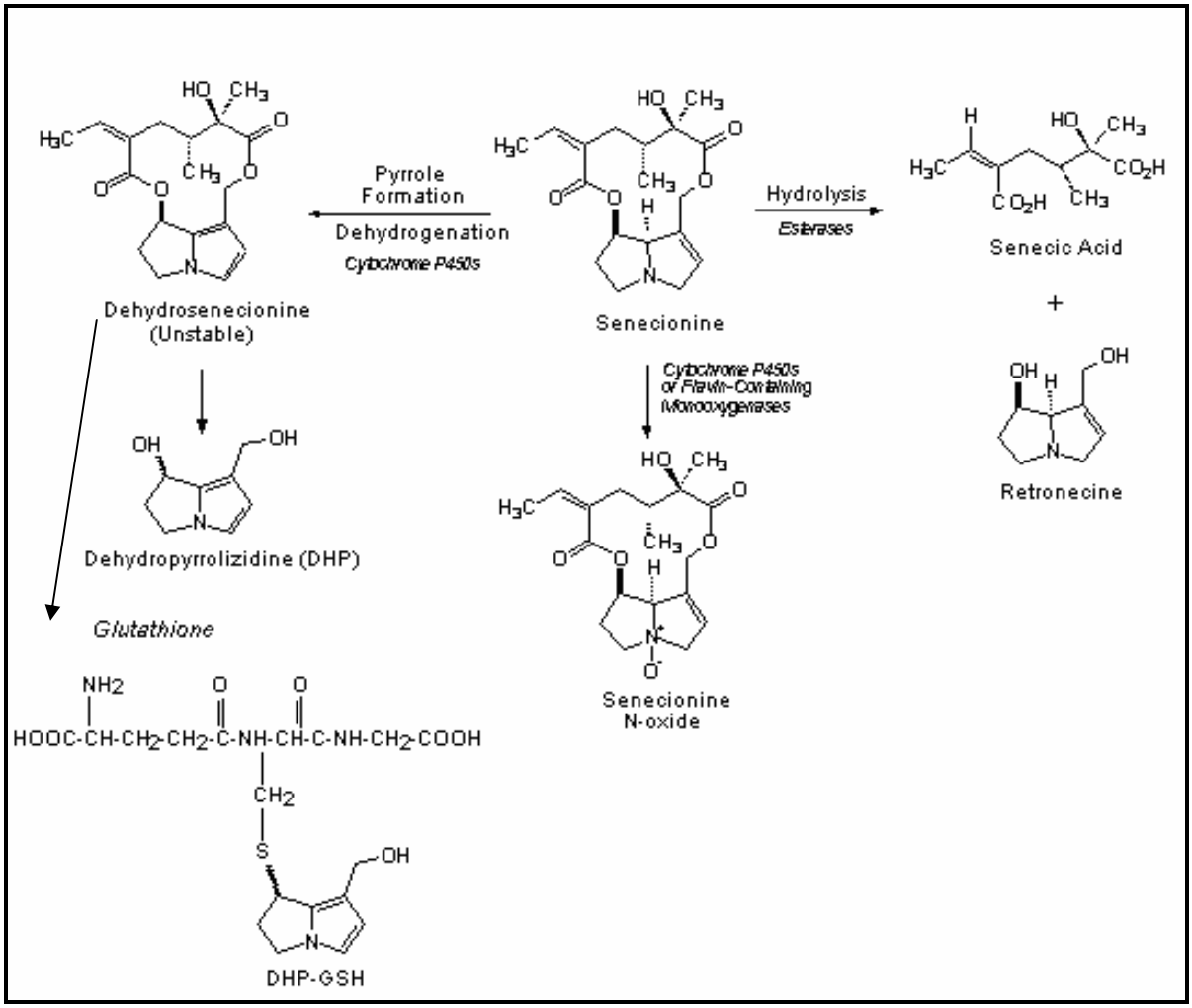

Fig. 2.6 Schematic diagram of the major pathways of hepatic metabolism of pyrrolizidine alkaloids. GSH = Glutathione (Duringer *et al.*, 2004). Reproduced with permission from Morrie Craig.

The first two are considered detoxification pathways. The latter is responsible for much of the toxicity of PA's (Mattocks, 1986; Kedzierski and Buhler, 1986; Cheeke, 1998; Duringer *et al*., 2004).

Pyrrolic derivatives and *N*-oxides are produced by enzymes located in the liver microsomes. Pyrrolizidine alkaloids can be converted by cytochrome P450s to the reactive and highly toxic dehydropyrrolizidine (DHP) or oxidized to *N*-oxides. Flavin-containing monooxygenase (FMO) enzymes can also convert PA's to *N*-oxides (Duringer *et al*., 2004). *N*-oxides of PA's are water soluble and readily excreted. In animals their main metabolic route is reduction to the corresponding bases, which may be reabsorbed and metabolized again (Mattocks, 1986). According to Schoental (1960), *N*-oxides and basic alkaloids are interconvertible, the reactions being dependent on the red-ox potential of the biological systems.

Dehydropyrrolizidine alkaloids may alkylate cellular nucleophiles including proteins and nucleic acids, thus remaining in tissues long after exposure, causing cellular damage. Alternatively, they may react with soluble nucleophiles such as glutathione (GSH) to form a conjugate which is subsequently excreted (Stegelmeier *et al*., 1999; Duringer *et al*., 2004). The conjugate DHP-GSH may alternatively serve as a transport vehicle to other organs, such as the lungs, where toxicosis may occur (Duringer *et al*., 2004).

It appears that *N*-oxide and pyrrolic metabolite formation follow parallel pathways which are not competitive. High *N*-oxide formation does not necessarily mean low pyrrole production (Mattocks and Bird, 1983). Hepatotoxicity is increased when factors tend to maximize pyrrole production and minimize other pathways. On the other hand, toxicity is more related to the level of pyrroles bound to the tissue than to the rate of pyrrolic metabolites converted from PA's (Mattocks, 1986).

Pyrrolizidine alkaloid metabolism is self limiting and substantially affects hepatic enzyme activity. Pyrroles inhibit (after 1 hour) the enzymes involved in their own production (Shull, Buckmaster and Cheeke, 1976a).

Factors such as gender, age and pretreatment of animals may affect the formation of the pyrroles in the liver. In male rats, liver pyrrole production is higher than in females after comparable doses of the PA's, retrorsine and monocrotaline, were administered (Mattocks, 1986). On the other hand, Williams, Reed, Kedzierski, Dannan, Guengerich and Buhler (1989), demonstrated that in male rats the *N*-oxidation pathway in the metabolism of senecionine is higher than in females, which means that females may have comparatively high pyrrolic metabolite levels. Very young rats also have high liver pyrrole concentrations compared to adults, with the levels falling two to three weeks later. These differences may be linked to metabolic rates (Mattocks, 1986).

Some pretreatments may enhance or inhibit hepatic bioactivation of PA's (Mattocks, 1986). Pretreatment with phenobarbitone increases the rate of pyrrole production in rats, while pretreatment with SKF 525A, for example, decreases microsomal enzyme activity (Mattocks, 1972). According to White, Mattocks and Butler (1973) it appears that animals which normally have high liver pyrrole production become less susceptible to toxic effects when pretreated with phenobarbitone, while in animals whose alkaloid metabolism is slow, the susceptibility increases with phenobarbitone pretreatment.

The rate of hepatic pyrrole production varies significantly between different animal species. According to Shull, Buckmaster and Cheeke (1976b), with a few exceptions (e.g. rabbit and chicken), it appears that a direct relationship between the rate of pyrrole production *in vitro* and susceptibility to PA's exists. On the other hand, Huan, Miranda, Buhler and Cheeke (1998) are of the opinion that there is not a strong correlation between pyrrole production and susceptibility to PA's.

Animals with high hepatic glutathione content might be expected to be more resistant to PA toxicity as more pyrroles may be conjugated with glutathione and eliminated (Stegelmeier *et al*., 1999). Pretreatments that enhance detoxification pathways can probably increase the animal's resistance to PA toxicity.

2.6.3 Excretion

The excretion of unchanged alkaloids and most related metabolites is very rapid. Within a few hours after ingestion a relatively small proportion of the dose remains in the body, mainly in the form of metabolites bound to cellular constituents. After the first day it is probable that no unchanged alkaloid will remain in the body (EHC 80, 1988). Studies with radioactive PA's and analogues in mice showed that most of the dosed compound is rapidly eliminated via the urine and faeces. Very little is eliminated via the expired air and in the milk of lactating mice (Eastman *et. al.* cited by Mattocks, 1986). Radioactive senecionine administered to male rats was excreted mainly in the form of *N*-oxide via bile and urine and only a small amount of the parent compound was excreted unchanged (Estep, Lamé and Segall, 1990). Biliary excretion is also important in the elimination of retrorsine metabolites, but not of the parent compounds (White, 1977). Dickinson *et al.* (1976) demonstrated that PA's were present in milk from cows dosed with dried tansy ragwort. Calves fed milk obtained from those cows remained unaffected. Johnson (1976) did not detect histological lesions in calves fed milk from cows dosed with *S. jacobaea*, but clinical chemistry aberrations suggested the presence of hepatic lesions. Eggs from hens fed up to 4% of *Senecio vernalis* in their diet did not have detectable residues of free PA’s (Eröksüz, Eröksüz, Özer, Yaman, Tosun, Kizilay and Tamer, 2003).

2.7 MECHANISM OF ACTION

2.7.1 Relationship between structure, metabolism and toxicity

The toxicity of the PA's is influenced by their chemical structure. For hepatotoxity to occur, three factors are essential:

- there must be a 1-2 double bond in the necine base;
- the hydroxyl group must be esterified at one or more positions and
- a branched carbon chain must be present in at least one of the ester side chains (Stewart and Steenkamp, 2001).

The more complex the esters of the PA's, the more toxic they are. Thus, cyclic diesters are the most toxic followed by non-cyclic diesters and the least toxic are the monoesters (Cheeke, 1998). Diesters of heliotridine and retronecine are approximately four times as toxic as the respective monoesters and heliotridine esters are 2 - 4 times as toxic as retronecine esters (Culvenor, Edgar, Jago, Qurteridge, Peterson and Smith, 1976).

Cytotoxicity is associated with the highly reactive pyrrolic derivatives formed by the hepatic microsomal enzyme system. The degree to which PA's are metabolized to pyrroles and the extent to which these pyrroles can bind to cellular structures determines their toxicity. Microsomal metabolic rates of formation of *N*-oxides and pyrroles depend on the alkaloid structure. Conformations favouring dehydrogenation rather than *N*-oxidation and resistance to hydrolysis lead to increased pyrrole production. Lipophilic characteristics allowing access to microsomal enzymes is also important. Macrocyclic diesters, for example, are more prone to microsomal dehydrogenation (Mattocks, 1981; Mattocks and Bird, 1983).

Pyrrolizidine alkaloids are capable of DNA cross-linkage. Those PA's that are potent DNA cross-linkers are also potent cytotoxic agents. Senecionine, seneciphylline, retrorsine and riddelliine, members of the macrocyclic α,β-unsaturated ester group, are potent DNA cross-linkers and consequently potent cytotoxic inducers. On the

other hand, monocrotaline, a macrocyclic diester which does not have an α,β-unsaturated ester group, and open diesters, are less potent DNA cross-linkers. Pyrrolizidine alkaloids have the ability to inhibit mitosis and induce megalocytosis (Kim, Stermitz, Molineux, Wilson, Taylor and Coulombe, 1993). Biological activity of the different PA's is not determined by the structural features *per se*, but is related to the pyrrolic metabolite formation. For instance, dehydromonocrotaline is biologically very active, while the parent compound shows little activity (Kim *et al*, 1993).

2.8 TOXICITY

Pyrrolizidine alkaloids which are esters of unsaturated necines can cause tissue damage. This is primarily seen in the liver of affected animals although the lungs and other tissues may also be affected. Poisoning may be either acute or chronic. In the former, the animals die between one day and about a week after ingestion of the alkaloid, whereas chronic intoxication is seen in animals that survive sub-lethal doses or have ingested multiple small doses of the PA's (Mattocks, 1986). Age also plays an important role in the toxicity of PA's. Young rats are more susceptible to acute and chronic effects of hepatotoxic PA's (Mattocks and White, 1973).

The differences in PA metabolism and the nature of toxicity are related to the reactivity of the primary toxic metabolite, the dehydroalkaloid (Cooper and Huxtable, 1999). The number of the reactive groups (one or two) in the pyrrolic metabolite affects its toxicity. Those pyrroles that are bifunctional (two reactive ester or hydroxyl groups) can bind more strongly to target molecules (EHC 80, 1988).

Primary hepatotoxic alkaloids, such as retrorsine or seneciphylline, have dehydroalkaloid intermediates. These are much more reactive than the metabolites of monocrotaline and trichodesmine, which are reported to cause pneumo- and neurotoxicity, respectively. This means that more stable dehydroalkaloids are capable of reaching organs other than the liver and induce toxicity (Cooper and Huxtable, 1999).

2.8.1 Acute hepatotoxicity

There is strong evidence that a relationship exists between acute hepatotoxicity and the amount of pyrrolic metabolites found in the liver of animals exposed to PA's (Mattocks, 1972). The liver, where pyrrolic metabolites are formed, is the only organ exposed to relatively high concentrations (EHC 80, 1988). Different PA's can produce similar hepatotoxic effects, but the degree of toxicity varies widely. Highly reactive short-lived pyrroles act primarily on the cellular molecules. Acute toxicity is associated with severe haemorrhagic necrosis that occurs a few days after a single dose has been consumed (Mattocks, 1986).

2.8.2 Chronic hepatotoxicity

Chronic liver lesions developing either as a result of a single sub-lethal dose or multiple small doses are similar. Post-necrotic fibrosis and progressive enlargement of parenchymal cells are features of chronic alterations (Mattocks, 1986). Weeks later, numerous giant cells or megalocytes are present. Megalocytes (with large and abnormal shaped nuclei) are the result of persistent inhibition of mitosis and failure of a stimulus for cell division (Mattocks, 1986; Kim *et al.*, 1993). Proliferation of bile ducts is another common feature of chronic hepatotoxicity, along with varying degrees of fibrosis and thickening of central veins (Mattocks, 1986).

2.8.3 Pneumotoxicity

Extrahepatic toxicity is ascribed to the stability of the dehydroalkaloid produced in the liver. Those pyrroles that are more stable have longer half-lives and are able to reach other organs such as the lungs and induce toxicity (Cooper and Huxtable, 1999). Monocrotaline, an 11-membered macrocyclic diester, whose bioactivation produces much more stable DHP’s compared to 12-membered macrocyclic diesters, such as retrorsine, is known to cause lung damage (Mattocks, 1986; Huxtable, Yan, Wild, Maxwell and Cooper, 1996). Some PA's may reach the lungs unchanged and are

activated by MFO within endothelial cells or Type II pneumocytes, producing toxic pyrroles (Huxtable, 1990, cited by Stegelmeier *et al*., 1999).
Structural requirements for pneumotoxicity are the same as those for hepatotoxicity. Pyrrolizidine alkaloids that are hepatotoxic should also be pneumotoxic when administered in higher doses. Chronic lung lesions may not be observed in such cases because peracute or acute death may occur (Culvenor *et al.* 1976). Acute pneumotoxicity is characterized by alveolar oedema and haemorrhage, while chronic lesions are marked by progressive proliferation of alveolar walls, arteritis and hypertension (Mattocks, 1986).

2.8.4 Other toxic effects

Chronic cardiac damage may develop secondary to lung toxicity. Elevation of pulmonary arterial pressure leads to *cor pulmonale* with characteristic right ventricular hypertrophy (Mattocks, 1986; EHC 80, 1988; Coulombe, 2003).

Hepatic encephalopathy is attributed to chronic hepatotoxicity. As a result of liver failure, ammonia, mercaptans and other substances accumulate in the blood and reach the brain where they induce lesions. The resulting degeneration of the white matter of the brain and spinal cord is referred to as *status spongiosus* (Kellerman *et al*., 2005).

Trichodesmine, a PA with a structure closely related to monocrotaline, is much more resistant to hydrolysis and therefore more toxic than the latter and induces neurotoxicity. This is ascribed to the highly lipophilic character of the dehydroalkaloid metabolite, which may allow the compound to penetrate the brain more readily (Huxtable *et al*., 1996).

In a study with riddelliine gavaged to rats, Chan (1993), in addition to the lesions in the liver, also found lesions in the heart, spleen, kidneys and pancreas.

According to Mattocks (1986), some hepatotoxic PA's are potent teratogens in rats. Dehydroheliotridine, for example, causes delayed ossification, cleft palate, distorted bones and feet defects. Senecionine and senecionine *N*-oxide extracted from *S. vulgaris* have antifertility and teratogenic effects in rats (Tu, Konno, Soejarto, Waller, Bingel, Molineux, Edgar, Cordell and Fong, 1988). On the other hand, tansy ragwort (*S. jacobaea*) consumed by cattle at nearly lethal amounts did not cause direct reproductive or teratogenic effects under field conditions (Johnson and Smart, 1983).

Carcinogenic effects in rats have been demonstrated for many hepatotoxic PA's. Tumours develop in organs such as the liver, lung, kidney, gastrointestinal tract, brain, spinal cord and pancreas (Culvenor, 1983). Rats treated with dehydroretronecine and monocrotaline developed rhabdomyosarcomas, hepatocellular carcinomas, pulmonary adenomas and myelogenous leukemias (Allen, Hsu and Carstens, 1975).

Some PA’s possess mutagenic activity, which might be related to the necine base of the PA. Pyrrolizidine alkaloids of the otonecine and heliotridine base types produce mutagenic responses, while PA's of the retronecine base type produce negative responses in the *Salmonella* and mammalian microsome tests. Pyrrolizidine alkaloids of *S. jacobaea*, which are predominantly of the retronecine base type, produce negative mutagenic responses (White, Krumperman, Cheeke, Deinzer and Buhler, 1984).

2.9 SPECIES AFFECTED

2.9.1 Animals

2.9.1.1 Livestock

Field and experimental cases of livestock poisoning with PA-containing plants, particularly *Senecio* species, have been reported worldwide in a variety of animals. Horses and cattle are the most susceptible species (Kellerman *et al*., 2005), followed by pigs and chickens and the least susceptible species are goats, sheep, and turkeys (Craigmill, 1981). However, according to Stegelmeier *et al*. (1999) and Knight and Walter (2001), pigs are the most susceptible animal species. Hooper and Scanlan (1977) reported a severe and fatal disease of pigs and chickens dosed with ground *Crotalaria retusa* seeds, which contain the PA monocrotaline. Pyrrolizidine alkaloid poisoning has also been reported in yaks (Winter *et al*., 1990).

According to Craigmill (1981), young animals of all susceptible species are more sensitive to PA intoxication than are adults. Juvenile cattle, newly introduced to infested pastures, are more prone to acute poisoning than adult animals (Kellerman *et al*., 2005). In contrast to this, Barros, Driemer, Pilati, Barros and Castilhos (1992), investigated a seneciosis outbreak and reported that, with few exceptions, affected cattle were two years and older.

Feeding trials conducted in cattle (Johnson and Molineux, 1984; Johnson, Molineux and Stuart, 1985; Molineux, Johnson, Olsen and Baker, 1991), horses (Mendel, Witt, Gitchell, Gribble, Rogers, Segall and Knight, 1988), sheep (Araya, Hernandez, Espinoza and Cubillos, 1983) and goats (Goeger, Cheeke, Schmitz and Buhler, 1982) involving different *Senecio* species, demonstrated that the time/dose-response is an important feature in PA intoxication. High doses of PA's over a short period will produce toxicity more rapidly, while low doses over an extended period will produce delayed toxicity (Craigmill, 1981; Kellerman *et al*., 2005). For PA toxicosis to develop

there is a threshold level that must be exceeded (Johnson and Molineux, 1984). The estimated toxic dose of dried *Senecio* as a percentage of body weight ranges from 5-10% for cattle and horses and more than 100% for sheep and goats (Craigmill, 1981).

It is reported that sheep are resistant to PA intoxication (Ilha, Loretti, Barros and Barros, 2001; Duringer *et al*., 2004). Resistance is attributed primarily to ruminal detoxification due to bacterial activity (Wachenheim *et al*., 1992a; Wachenheim *et al*., 1992b; Craig, Latham, Blythe, Schmotzer and O'Connor, 1992; Blythe and Craig, 1994; Duringer *et al*, 2004). Conversely, other researchers attribute the resistance of sheep to decreased hepatic microsomal enzyme bioactivation (Swick, Cheeke, Ramsdell and Buhler, 1983; Cheeke, 1988). Resistance varies between individuals and also from time to time in the same animal (Lanigan, 1970). Nevertheless, in South Africa, acute and chronic seneciosis of sheep is regularly reported and is of great economic importance in certain areas (Kellerman *et al*., 2005).

2.9.1.2 Laboratory animals

A number of laboratory animal species have been used in PA toxicity studies. Mattocks (1986) gives a comprehensive list of experimental studies and toxic effects of PA's in laboratory animals such as rats, hamsters, guinea-pigs, gerbils, mice, rabbits, quail, chickens, turkeys and monkeys.

Pyrrolizidine alkaloid intoxication in laboratory animals may be peracute, acute or chronic. Acute and chronic toxicity are attributed to the bioactivation of PA's in the liver cells, causing hepatotoxicity. Peracute toxicity is mainly ascribed to a pharmacological effect resulting from exceptionally large doses of PA's, which are rapidly absorbed, causing sudden death preceded by convulsions and/or coma (Mattocks, 1986).

There are species differences in susceptibility to PA's. White *et al*. (1973) compared the acute toxicity of retrorsine administered by intraperitoneal injection to rats, mice, hamsters, guinea-pigs, fowl and quail. The male rat was the most susceptible species (LD_{50} = 34 mg/kg) followed by the mouse, hamster and fowl. Quail and guinea-pigs

were the most resistant species with LD_{50} of 279 and over 800 mg/kg, respectively. Cheeke and Pierson-Goeger (1983) investigated the toxicity of *Senecio jacobaea* to herbivorous laboratory animals, chickens and turkey poults. Chickens and turkey poults suffered chronic *S. jacobaea* poisoning, whereas gerbils, hamsters and guinea-pigs were resistant to chronic toxicity.

Susceptibility to PA's is a complex interaction between the animal and the alkaloid. Guinea-pigs, for example, are resistant to toxicity induced by most PA's, but are very sensitive to jacobine (Stegelmeier *et al.*, 1999).

2.9.2 Humans

People are exposed to PA's in a variety of ways. Sources of poisoning are food contaminated with PA's, plants eaten deliberately and the use of traditional remedies and herbal teas (Stewart and Steenkamp, 2001; Coulombe, 2003). Outbreaks of human poisoning occur when food or grain is accidentally contaminated with plant parts or seeds of PA-containing plants (Stegelmeier *et al.*, 1999) and have been reported mainly from developing countries (Coulombe, 2003). Epidemics have occurred in Afghanistan, the Caribbean, India, Iraq, Nigeria, South Africa, Sri Lanka and Tadjikistan (Mattocks, 1986; Coulombe, 2003). The South African outbreak numbered about eighty cases of which most were fatal. It occurred over a ten year period and was the result of contamination of wheat flour with *Senecio* flower heads and seeds (Rose, 1972; Coulombe, 2003).

Several PA-containing plants are consumed as food by people (Mattocks, 1986). About fifteen *Senecio* species, including *S. inaequidens* and *S. burchellii*, are consumed in South Africa as a type of "spinach" (Rose, 1972). Pyrrolizidine alkaloids can also enter the human food chain through residues in milk of animals that have ingested PA-containing plants, honey from bees foraging in fields of PA-containing plants and eggs laid by hens that have consumed contaminated rations (Coulombe, 2003).

Traditional remedies and herbal teas prepared from PA-containing plants are another source of human poisoning. This accounts for a regular occurrence of cases and is not only restricted to developing countries where modern medical facilities are scarce, but also occurs in industrialized countries as there is a growing tendency in these countries towards therapeutic use of natural products (Mattocks, 1986; Coulombe, 2003). In South Africa (and most African countries) herbal remedies are an integral part of traditional culture, with 60-80% of the population relying on these remedies (Zuckerman, Steenkamp and Stewart, 2002). Rose (1972) lists several *Senecio* species occurring in South Africa, which are used as food and medicine. Steenkamp, Stewart and Zuckerman (2000) reported 20 cases of liver disease in children admitted to two hospitals in South Africa following the administration of traditional remedies. González, Villamil and Uribe (1997) presented a historical review of veno-occlusive disease in Colombia as a result of *Senecio formosus* used as a remedy. In the West Indies, medicines prepared from *Crotalaria fulva* were associated with veno-occlusive disease. Several other PA-containing plants such as *Symphytum officinale* (comfrey), *Tussilago farfara* and *Petasites officinalis* are used for medicinal proposes and are also associated with veno-occlusive disease (Coulombe, 2003).

In humans, as in animals, PA's induce acute, sub-acute and chronic hepatotoxicity (Mattocks, 1986; Steenkamp *et al*., 2000). The acute to subacute syndromes manifest as veno-occlusive liver disease and patients exhibit nausea, acute epigastric pain, acute abdominal distention (ascites), dilated veins on the abdominal wall and hepatomegaly (Mattocks, 1986; Steenkamp *et al*., 2000). Survivors of acute to subacute veno-occlusive disease or people ingesting small amounts of PA's over a long period may develop chronic disease characterized by fibrosis of the liver which leads to cirrhosis. Megalocytosis, a characteristic feature in affected animals, is not observed in human subjects (Mattocks, 1986).

Children and adolescents are often the most severely affected, probably because they are especially susceptible to the effects of PA's and partly because they receive large doses of traditional remedies or herbal teas (Mattocks, 1986). Steenkamp, Stewart,

van der Merwe, Zuckerman and Crowther (2001) suggested that chronic low dose treatment with traditional remedies may lead to teratogenic and/or carcinogenic effects.

2.10 CLINICAL SIGNS IN LIVESTOCK

Clinical signs of PA toxicosis in livestock result from loss of liver function (Cheeke, 1998). Poisoning can be acute or chronic and depends on the toxicity, the amount of PA-containing plants ingested and the duration of exposure (Kellerman *et al.*, 2005).

2.10.1 Acute toxicity

Large quantities of S*enecio* species ingested over a short period of time induce acute poisoning and result in animals dying within a few days after exposure (Kellerman *et al.*, 2005). Animals exhibit signs of acute liver failure, which include anorexia, depression, icterus and sometimes ascites (Stegelmeier *et al.*, 1999).

Acutely affected cattle are anorexic, may display abdominal pain and sometimes diarrhoea (Kellerman *et al.*, 2005). Nervous signs characterized by incoordination of the hind limbs, circling and apparent blindness may be present in acutely affected cattle and in these cases death is usually preceded by tremors (Barros *et al*. 1992). The affected animals are often in a good condition (Barros *et al*. 1992; Noble, Crossley, Hill, Pierce, McKenzie, Debritz and Morley, 1994).

Acutely affected sheep may die within a few days after ingestion of large amounts of *Senecio* plants without any specific clinical signs. Lambs are particularly susceptible and may die within 24 hours of being introduced onto an infested pasture (Kellerman *et al.*, 2005).

2.10.2 Chronic toxicity

Survival after a single large dose or multiple lower doses of a PA-containing plant ingested over a long period may cause chronic disease (Seawright *et al*., 1991; Stegelmeier *et al*., 1999; Kellerman *et al*., 2005). Often animals show no clinical signs during the latent period, which can last months or even years after PA ingestion (Molineux, Johnson and Stuart, 1988; Stegelmeier *et al*., 1999).

Clinical signs generally appear abruptly (Elliot *et al*., 2005). Stresses associated with gestation and parturition may precipitate the onset of symptoms (Johnson and Smart, 1983; Molineux *et al*., 1988). The most common clinical signs are weight loss, slight to moderate icterus and abnormal behaviour (Pearson, 1990; Knight and Walter, 2001; Elliot *et al*., 2005). Photosensitization of the unpigmented areas of the skin is sometimes observed (Pearson, 1990; Knight and Walter, 2001).

In cattle, clinical signs appear earlier in the course of the disease and are of short duration (Kellerman *et al*., 2005). The disease is characterized by a rough hair coat, loss of weight, intermittent or severe diarrhoea and tenesmus (Johnson and Molineux, 1984; Barros *et al*., 1992; Kellerman *et al*., 2005). Tenesmus may even lead to rectal prolapse (Wiltjer and Walker, 1974; Barros *et al*., 1992; Kellerman *et al*., 2005). Animals may be depressed, the head slightly lowered with “staring” eyes, or continuous walking may occur (Johnson and Molineux, 1984; Molineux *et al*., 1988). In some animals an unpleasant sweetish-sour odour may emanate from the skin and breath (Barros *et al*., 1992). Later in the course of the disease, emaciation, unthriftiness and dehydration are prominent signs and some animals exhibit nervous signs before they succumb (Kellerman *et al*., 2005). The latter are characterized by abnormal behaviour, ataxia, apparent blindness, bellowing and terminal recumbency with muscle tremors (Finn and Tennant, 1974; Kellerman *et al*., 2005).

Chronic seneciosis in sheep is characterized by weight loss, listlessness and emaciation (Seaman, 1987; Kellerman *et al*., 2005). Icterus and photosensitization are

rare findings (Seaman, 1987) and, according to Kellerman *et al.* (2005), nervous signs are never seen. However, Ilha *et al.* (2001) described nervous signs in sheep poisoned with *Senecio brasiliensis*. They observed depression, unsteady gait, walking in a straight line, apparent blindness and due to weakness the animals assume a wide-base stance. Aggressive behaviour is sometimes seen (Ilha *et al*., 2001).

According to Kellerman *et al.* (2005) the course of the disease in horses is generally prolonged. One of the first signs of poisoning is yawning. Unthriftiness, a staring coat and weight loss are prominent symptoms. Horses may show variable degrees of lethargy and poor performance (Lessard, Wilson, Olander, Rogers and Mendel, 1986). Pyrrolizidine alkaloid poisoned horses may develop photosensitization of the unpigmented skin, haemoglobinuria, ataxia and abnormal behaviour such as licking inanimate objects (Knight, Kimberling, Stermitz and Roby, 1984). Abortions may also occur (Lessard *et al*., 1986; Pearson, 1990). In South Africa chronic seneciosis in horses is also referred to as ‘dunsiekte’ (emaciation) or stomach staggers. These are very apt descriptions of the disease as the animal loses weight, stands with its head lowered or supporting it against a wall. The horse walks aimlessly, bumps against objects and injuries occur. The animal may stagger about like a drunken person and may have violent bouts of colic. Some horses may develop diarrhoea. At this stage the animal either dies or becomes frenzied before terminal recumbency sets in and the animal expires (Kellerman *et al*., 2005).

2.11 CLINICAL PATHOLOGY

A number of clinical chemistry parameters have been used to aid in the diagnosis of PA poisoning in animals. In general, gamma glutamyltransferase (GGT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities as well as the concentrations of bile acids, bilirubin and serum protein are useful in assessing liver damage due to PA intoxication (Cheeke, 1998; Elliot *et al*., 2005).

Pyrrolizidine alkaloid-induced hepatocyte damage results in elevated activities of certain enzymes, which are then detectable in the serum (Pearson, 1990). According to Craig, Pearson, Meyer and Schmitz (1991), glutamate dehydrogenase (GLDH) is the first enzyme to increase in most affected animals, followed by increases in ALP and GGT. Dehydrogenases may return to normal by the time clinical signs associated with liver disease are apparent (Pearson, 1990). Johnson *et al*. (1985) found an early increase in the activity of AST in poisoned calves, which decreased during the course of the disease. Increased GGT activity is reported to be a good indicator of early liver damage and of sub-clinical liver disease in animals exposed to PA's (Lessard *et al*., 1986; Curran, Sutherland and Peet, 1996). Craig *et al*. (1991) suggested combining ALP and GGT analysis with liver biopsies as the first screening tests in suspected cases of PA intoxication.

Pyrrolizidine alkaloid intoxicated animals may have increased bile acid and bilirubin concentrations and decreased serum albumin concentrations (Mendel *et al*., 1988; Braun *et al*., 1999). An increased bromosulphophthalein (BSP) half-life is also sometimes used to assess liver function (Lanigan and Peterson, 1979; Mendel *et al*., 1988; Araya and Fuentealba, 1990). However, early stages of PA-induced liver damage are not diagnosed by the BSP clearance test (Mendel *et al*., 1988).

Haematological evaluation of PA poisoned cattle revealed anaemia (Noble *et al*. 1994; Cheeke, 1998) and leucocytosis has also been reported (Braun *et al*., 1999; Elliot *et al*., 2005).

2.12 PATHOLOGY

The liver is primarily affected by PA-containing *Senecio* species in southern Africa. As a result of liver damage, the brain and gastrointestinal tract may be indirectly affected (Kellerman *et al.,* 2005). Some *Senecio* species and other PA-containing plants may also affect the lungs and kidneys (Kellerman *et al.,* 2005).

2.12.1 Macroscopic lesions

The carcass of acutely affected animals may exhibit icterus, effusion into the body cavities and visceral oedema (Stegelmeier *et al*., 1999; Kellerman *et al*., 2005). Oedema is pronounced in the abomasal folds, omentum and large intestinal walls of affected cattle (Johnson and Molineux, 1984; Molineux *et al*., 1991). Haemorrhages occur in serosal, visceral and subcutaneous tissue (Molineux *et al*., 1991; Kellerman *et al*., 2005). The liver of acutely affected animals is typically swollen, with rounded edges and a mottled surface (Johnson and Molineux, 1984; Johnson *et al*., 1985; Molineux *et al*., 1991; Kellerman *et al*., 2005). Johnson *et al*. (1985) reported that the liver colour varies from yellow-green to tan with reddish-brown patches. According to Kellerman *et al*. (2005), liver colour may vary in different animals from dark red to bluish-red or even orange-brown in other animals. Appreciable amounts of blood may ooze from the engorged livers when sectioned (Kellerman *et al*., 2005). Usually the gall bladder wall is oedematous and the gall bladder is enlarged with excess bile, which may be blood-tinged (Molineux *et al*., 1991; Kellerman *et al*., 2005). Rats dosed with PA's exhibit bloodstained ascites and congested livers with a rough surface and massive haemorrhagic necrosis (Mattocks, 1972).

Gross findings in chronic affected livestock are indicative of liver failure. Animals present with icterus, ascites, hydrothorax and hydropericardium (Kellerman *et al*., 2005). In cattle, oedema of the abomasal folds and other digestive tract tissues is accentuated and may be severe in the perirectal area (Kellerman *et al*., 2005). The liver of chronically affected animals is often reduced in size, cirrhotic and due to the increased consistency, difficult to cut (Molineux *et al*., 1988; Barros *et al*. 1992; Noble

et al., 1994; Kellerman *et al*., 2005). The liver may be pale or brownish-grey in colour with few to numerous nodules that can be seen through the capsule and on the cut surface (Barros *et al*. 1992; Noble *et al*., 1994; Kellerman *et al*., 2005). The gall bladder is usually distended and oedematous (Barros *et al*., 1992). Some *Senecio*-poisoned animals may present with photodermatitis (Noble *et al*., 1994).

Horses may present with a distended stomach and oedema of the caecum and colon (Kellerman *et al*., 2005). Rats dosed with the PA retrorsine also exhibit ascites and small livers, which are sometimes covered by numerous nodules (Mattocks, 1972; Mattocks and White, 1973).

2.12.2 Histopathology

As described by several authors (Johnson and Molineux, 1984; Molineux *et al*., 1991; Craig *et al*., 1991; Barros *et al*., 1992; Kellerman *et al*., 2005), the principal microscopic lesions of *Senecio*-poisoned animals are present in the liver. Characteristic features of acutely affected animals are centrilobular necrosis of the hepatocytes, which may extend to the midzonal area with haemorrhaging in the affected areas (Kellerman *et al*., 2005). Hepatocytes spared from necrosis are usually swollen (Johnson and Molineux, 1984; Kellerman *et al*., 2005). There is proliferation of fibroblasts with collagen deposition in the centrilobular and periportal areas and oedema of the portal triads (Molineux *et al*., 1991; Kellerman *et al*., 2005). Bile duct proliferation and focal accumulation of inflammatory cells are common features in affected animals (Molineux *et al*., 1991).

Histopathological changes of chronic affected animals are dominated by diffuse fibrosis and severe proliferation of bile ducts (Molineux *et al*., 1988; Barros *et al*., 1992). Fibrosis is prominent around the portal triads, but may extend into the lobules, forming isolated groups of hepatocytes (Barros *et al*., 1992; Kellerman *et al*., 2005). Veno-occlusive lesions, characterized by fibrosis around the sublobular and

centrilobular veins, megalocytosis (cells 10-30 times their normal size) and kariomegaly of hepatocytes are characteristic of PA poisoning (Molineux *et al*., 1988;

Cheeke, 1998; Kellerman *et al*., 2005). Later in the course of the disease, multiple foci of nodular regeneration of parenchymal cells can be seen (Seaman, 1987; Noble *et al*., 1994; Kellerman *et al*., 2005). Animals showing nervous aberrations before death may have spongiform degeneration of the white matter of the brain and spinal cord (Noble *et al*., 1994; Kellerman *et al*., 2005). The *status spongiosus* is particularly evident in the midbrain, brain stem, cerebellar peduncles and at white-grey matter junctions of the brain and brain stem (Barrros *et al*., 1992; Kellerman *et al*., 2005). Sheep may have severe spongy lesions without showing central nervous signs (Kellerman *et al*., 2005). Johnson *et al.* (1985) and Barros *et al*. (1992) also refer to sporadic occurrence of tubular nephrosis in affected cattle. According to Seaman (1987), megalocytosis of the tubular epithelium of the kidney is frequently encountered in PA intoxicated sheep.

2.12.3 Electron Microscopy (EM)

The first changes observed in the hepatocytes and Kupffer cells of rats receiving the PA lasiocarpine or *Crotalaria* extracts were the separation of the fibrillar and granular components of the nucleoli. Large cells with large nuclei and enlarged Golgi and endoplasmic reticulum were seen within 2-3 days of exposure to bioactive PA's and on Day 5 the cells of the luminal surface of the central veins were enlarged (EHC 80, 1988; Shah, Patel and Sehgal, 2004). Necrosis of the centrilobular hepatocytes occurs at this stage. Megalocytic hepatocytes which appear weeks later in the course of the disease may contain acidophilic vacuoles which in fact are cytoplasmic invaginations into the nucleus (EHC 80, 1988; Craig *et al*., 1991; Barros *et al.,* 1992; Ilha *et al*., 2001). The enlarged nucleus has an "open-faced" appearance with margination of chromatin at the periphery of the nucleus and particularly noticeable nucleoli (Molineux *et al*., 1988; Barros *et al.,* 1992). The cytoplasm may show vesicles of smooth endoplasmic reticulum with mitochondria of various shapes and sizes (EHC

80, 1988). According to Ilha *et al*. (2001) the hepatocytes of affected sheep may present with various degrees of degeneration characterized by numerous lipid droplets and electro-dense lysosomes containing lipofuscin, along with discrete dilatation of the rough endoplasmic reticulum and in some areas, moderate hyperplasia of the smooth endoplasmic reticulum. In areas where hepatic degeneration is accentuated, Kupffer cells exhibit numerous lysosomes containing lipofuscin.

2.13 DIAGNOSIS

Pyrrolizidine alkaloid poisoning can readily be diagnosed in animals that die acutely whilst grazing in a field infested with PA-containing plants by linking the presence of the plant and whether the plant has been eaten, to clinical and histopathological evidence (Molineux *et al*. 1988). In chronic affected animals, signs appear weeks to months after the plants have been ingested, rendering the diagnosis much more difficult (Molineux *et al*., 1988; Stegelmeier *et al*., 1999; Kellerman *et al.,* 2005).

Determination of liver enzyme activity plus microscopic examination of a liver biopsy and/or necropsy material exhibiting the characteristic histopathological features of PA poisoning are useful in arriving at a definitive diagnosis (Craigmill, 1981; Pearson, 1990; Craig *et al.,* 1991). However, in chronic affected animals, determination of liver enzyme activities is of limited diagnostic value as there is a low rate of cell death and enzyme release (Lanigan and Peterson, 1979).

Analytical methods, such as GC, TLC, CID-HPLC/MS/MS and LC-MS/MS, used to determine PA's in different matrices such as plants, animal tissues and biological fluids, are unequivocal diagnostic aids (Holstege *et al*., 1995; Lin *et al*., 1998; Rösemann, 2007). Steenkamp *et al*. (2000), described a colourimetric screening test used to detect PA's in urine of acutely affected human patients. The Ehrlich reaction is also used as a diagnostic aid to detect unsaturated PA's and their metabolites in tissues (Mattocks, 1986).

2.14 DIFFERENTIAL DIAGNOSIS

In southern Africa acute seneciosis may be confused with acute poisoning caused by *Crotalaria* species (which also contain PA's), *Cestrum* species and *Pteronia pallens*, which may all induce similar histopathological lesions (Kellerman *et al*., 2005). Acute and chronic aflatoxicosis and Rift Valley fever in cattle should also be ruled out (Pearson, 1990; Kellerman *et al*., 2005). Wasting diseases such as gastrointestinal parasites, liver flukes and Johne's disease may have a similar clinical presentation to those of chronic PA poisoned cattle (Pearson, 1990).

2.15 TREATMENT

There are no specific antidotes for PA-induced liver disease (Craigmill, 1981). If severe liver fibrosis has occurred, attempts to treat the animals are fruitless since regeneration is impossible at this stage (Pearson, 1990). If the degree of fibrosis is moderate and exposure to PA-containing plants is prevented, the animal may recover due to the liver's great regenerative capacity (Craigmill, 1981). In those cases, treatment is symptomatic and should include a low protein, high energy diet (Pearson, 1990).

2.16 CONTROL AND/OR AVOIDANCE

Prevention of PA poisoning in farm animals may be achieved by appropriate pasture and livestock management (Cheeke, 1998; Kellerman *et al*., 2005). Susceptible animals should be kept away from contaminated pastures or feed (Pearson, 1990). In South Africa, it is believed that livestock that are reared in infested fields learn to avoid *Senecio* plants (Kellerman *et al*., 2005).

The larvae of the cinnabar moth (*Tyria jacobaeae*), which feeds only on PA-containing plants and the flea beetle (*Longitarsus jacobaeae*) have been shown to be very successful in controlling *S. jacobaea* in North America (Cheeke, 1998). Due to the lower susceptibility of sheep to PA's, this species is sometimes used to graze on

Senecio-infested pastures to control the weed (Ilha *et al.,* 2001). Herbicide spraying is an alternative method to control *Senecio* weeds, but in very extensive areas it is economically impractical (Cheeke, 1998).

Attempts have been made to protect animals against the toxicity of PA's by manipulating dietary and nutritional factors. Miranda, Reed, Cheeke and Buhler (1981) demonstrated that butylated hydroxyanisole (BHA), an antioxidant used in foods, reduces the conversion of monocrotaline to the toxic pyrroles, thus having a protective action in mice. The use of synthetic antioxidants and sulphur amino acids to protect animals against PA toxicity did not have any beneficial effects in cattle (Cheeke, Schmitz, Lassen and Pearson, 1985).

Chapter 3

EXTRACTION OF PLANT MATERIAL AND ISOLATION OF PYRROLIZIDINE ALKALOIDS

3.1 INTRODUCTION

Senecio species may contain pyrrolizidine alkaloids (PA's) that are mainly hepatotoxic. There is a great variation in the composition, concentration and pattern of distribution of PA's within the same plant (between the leaves, stems and inflorescences) and between plants. In *Senecio* species, PA's are synthesized in the roots from senecionine *N*-oxide and transformed in the aerial parts into the species-specific PA's (Toppel, Witte, Riebesehl, von Borstel and Hartmann, 1987; Hartmann, Ehmke, Eilert and Borstel, 1989; Hartmann and Dierich, 1998).

In this study, the PA's of *Senecio inaequidens* DC., a plant incriminated in a suspected outbreak where cows died, were identified. Extractions and chemical analyses were conducted to determine the concentration of the PA's in the different plant parts (*viz*. leaves, stems and flowers/seeds). Qualitative and quantitative PA compositions were determined to estimate the toxicity of *S. inaequidens*. For comparison, analysis of *S. inaequidens*, collected at two other localities in South Africa was also performed. In addition, the PA's contained in three other *Senecio* species, amongst them *S. latifolius* and *S. retrorsus* (the two species most often associated with livestock poisoning in South Africa) were also identified.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Plant material was collected during September 2004, when the outbreak occurred, on the farm Makoupan (27° 19' S; 28° 32' E) near Frankfort, Free State Province, Republic of South Africa. Additional plant material was collected, where the outbreak occurred, during November 2004 for a confirmatory dosing trial. The plant was identified as *Senecio inaequidens* DC. by the South African National Biodiversity Institute (SANBI). A voucher specimen has been retained at the Section of Pharmacology and Toxicology, Faculty of Veterinary Science, University of Pretoria. Botanically verified (by SANBI) preserved plant specimens of *S. inaequidens*, collected during December 2003 near Ermelo (26° 48' S; 29° 48' E), Mpumalanga Province and collected during April 2005 near Queenstown, Eastern Cape Province (grid reference unknown) were available at the Section of Pharmacology and Toxicology, Faculty of Veterinary Science, Onderstepoort. *Senecio retrorsus* was collected during October 2005 on the farm Spes Bona (31° 33' S; 26° 48' E) near Molteno, Eastern Cape Province. The towns where the plant material was collected are indicated on the map (Fig. 3.1). In addition, dried, milled *S. latifolius* and *S. consanguineus* plant material stored frozen at the Toxicology Division, Onderstepoort Veterinary Institute were obtained.

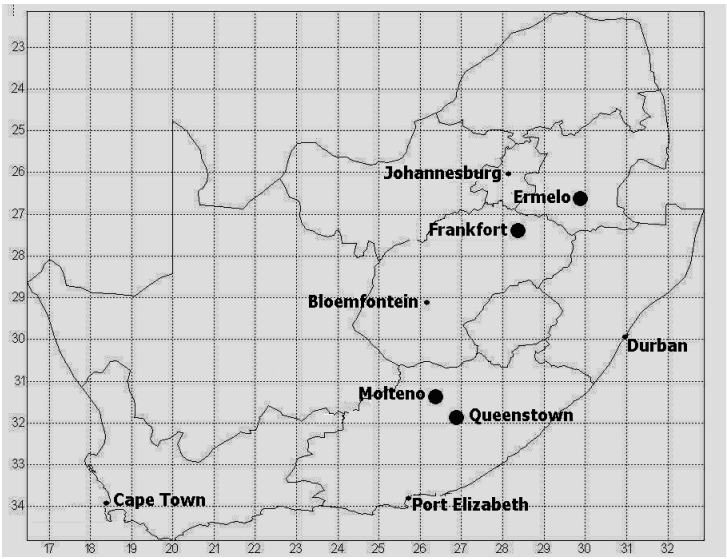

Fig. 3.1 Localities where *S. inaequidens* plant material was collected.

3.2.2 Sample preparation

Senecio inaequidens and *S. retrorsus* plant material was air dried and the different plant parts (leaves, seeds/flowers and stems) were separated and milled prior to extraction. The dried, milled *S. consanguineus* and *S. latifolius* plant material was used as is. Dried, milled *S. inaequidens* collected later for the dosing trial was also used.

The mass of samples used for PA extraction and isolation ranged from 0.21 g (seeds/flowers of *S. inaequidens* from Ermelo - the only available plant material) to a maximum of 5 g.

3.2.3 Chemical extraction

The extraction procedures followed the method described by Rösemann (2007). The chemicals and reagents used for the extractions were bought from Merck (Darmstadt, Germany), except the zinc powder, which was obtained from Analar (The British Drug Houses Ltd, South Africa). A 0.6 M H_2SO_4 solution was prepared at the Pharmaceutical Analytical Laboratory, Faculty of Veterinary Science, Onderstepoort.

The quantity of reagents was adjusted to the mass of sample to be extracted. In general, 5 g milled plant material was homogenized for 10 min (using a Heidolph Diax 600 apparatus) with 20 ml ethanol plus 2 ml of deionized water and then shaken mechanically (Labotec® 202, S. A.) for about 2 h. The layers were allowed to separate and the sample was centrifuged (Allegra ™ X-22R, Beckman-Coulter) for 3 min at 2500 rpm. The clear solution was divided into two equal fractions, marked A and B, and evaporated at 38°C in a Büchi Rotavapor R-200 with a Büchi Heating Bath B-490 (Labotec®, S.A). The extracts were reconstituted in 2 ml 0.6 M H_2SO_4. The crude extract was dewaxed and chlorophyll was removed with 10 ml hexane. The *N*-oxides in sub-samples marked A were reduced to basic alkaloids by adding 500 mg zinc powder and left overnight. Samples A and B were alkalinized (pH > 9) by adding approximately 0.5 ml of a 25% ammonia solution. The alkaloids were extracted 3

times with 3 ml ethyl acetate, the latter evaporated at 38°C in a Turbo Vap® LV Evaporator, Zymark. The extracted alkaloids were stored at -20°C until analysis.

3.2.4 Pyrrolizidine alkaloid analysis

Pyrrolizidine alkaloid analysis was performed at AMPATH Laboratories, Pretoria, South Africa, using the stored extracts. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC/MS) were used. Quantification was achieved with a retrorsine calibration curve; therefore, only retrorsine is reported as µg/g retrorsine. The quantities of the other PA's are reported as µg/g retrorsine equivalents.

3.2.4.1 LC-MS/MS

Liquid chromatography-mass spectrometry was performed with a Waters 2795 gradient system, equipped with a Micromass Ultima MS/MS with ESI+Mode. Separation was achieved using a Luna C18 250 mm x 2.0 mm x 5 µm column from Phenomenex (Torrance, CA, USA). The following conditions were used: buffer 0.02 M ammonium acetate, pH 3.84; organic phase 80% acetonitrile, 20% methanol. Gradient 0-5 min., 2% organic phase increasing to 90% at 20 min. and held for 5 min.

3.2.4.2. GC-MS

The instrument used for GC-MS was a Hewlett Packard (HP) 5973 GC-MS with EI+mode (electron impact positive mode) with a CPsil 5CB (Crompack) 25 m x 0.32 mm x 0.25 µm column installed. The detector temperature of the MS was set at 230°C, Aux at 280°C; the oven set at 50°C for 0-5 min. to 200°C; increasing 30°C/min. to 290°C and held for 6 min. Runtime = 25 min. The spectral library used was Wiley version 6.

3.3 RESULTS

LC-MS/MS and GC-MS analyses revealed the presence of four different PA's in *S. inaequidens* plant material, namely:

- retrorsine, molecular mass (MM) 352, confirmed on GC-MS with reference standards;
- senecionine, MM 336, confirmed on GC-MS by library references and
- two unidentified compounds, with MM of 338 and 368 respectively, assumed to be PA's given the presence of the fractions 94, 120 and 138, which were also present in the known PA's, retrorsine and senecionine (Tables 3.1 and 3.2).

Figures 3.2 and 3.3 show the spectra of the retrorsine standard and retrorsine as contained in *S. inaequidens*, respectively.

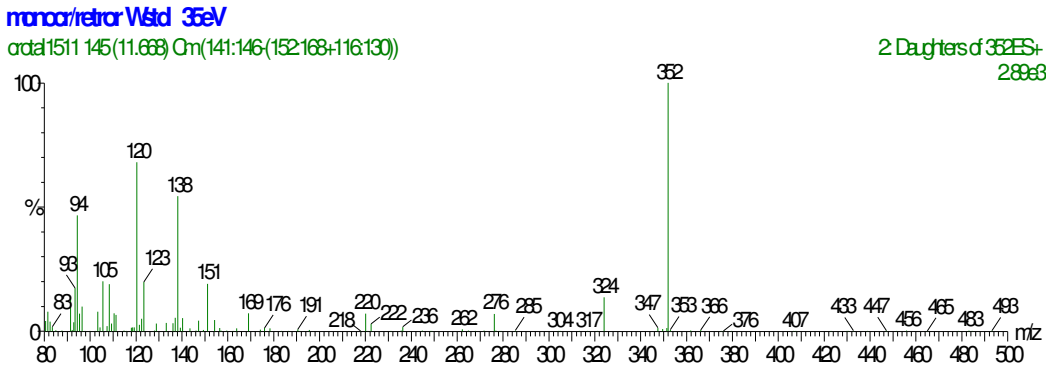

Fig. 3.2 MS spectrum of retrorsine standard (11.6 min.).

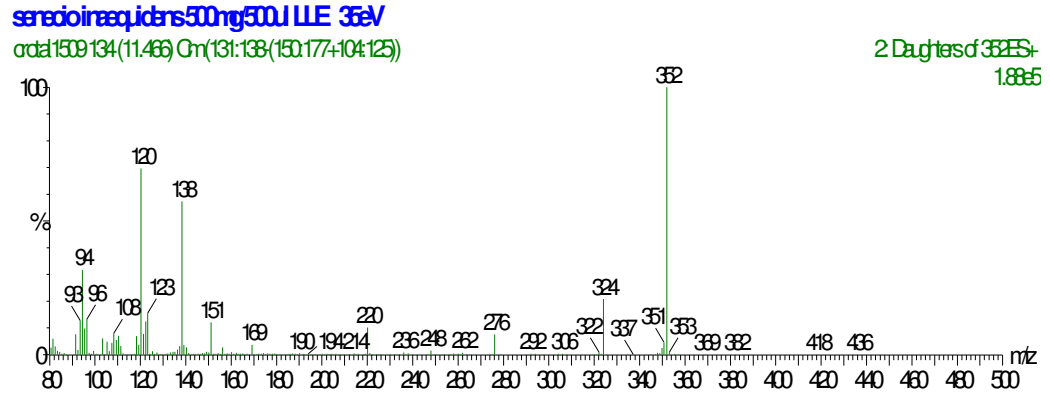

Fig.3.3 MS spectrum of retrorsine in *S. inaequidens* (11.5 min.).

The major PA constituent of the dried, milled *S. inaequidens* plant material was retrorsine, with *N*-oxide:free base ratio of 4.12:1, followed by senecionine (*N*-oxide:free base ratio of 3.8:1) (Table 3.1). The two unidentified alkaloids represented only a minor proportion of the total alkaloids in *S. inaequidens*. One of the unidentified PA's with MM 338 (NI1) had a *N*-oxide:free base ratio of 53.6:1 and the other unidentified PA with MM 368 (NI2) a *N*-oxide:free base ratio of 0.1:1. In *Senecio latifolius* the major constituent was another unidentified PA with MM 388 (NI4), followed by retrorsine and by the unidentified PA with MM 370 (NI3). Senecionine and the unidentified PA with MM 338 (NI1) were minor constituents. In *S. consanguineus* only retrorsine was identified at very low concentrations (Table 3.1). *Senecio retrorsus* had the same PA composition as *S. latifolius* (Table 3.3).

The total alkaloid (free base plus *N*-oxide) content in the dried, milled *S. inaequidens* plant material was 0.18%; in *S. latifolius* 1.12% and in *S. consanguineus* merely 0.01% (Table 3.1). The average total PA concentration in plant parts of *S. inaequidens* collected at Frankfort was 0.81% (Table 3.2), whereas the average total PA concentration in plant parts of *S. retrorsus* was 1.62% (Table 3.3).

Table 3.1 PA concentrations (µg/g retrorsine or retrorsine equivalents for S, NI1, NI2, NI3 and NI4) of dried, milled *S. inaequidens*, *S. consanguineus* and *S. latifolius* plant material.

	S. inaequidens					***S. latifolius***						***S. consanguineus***	
	R	S	NI1	NI2	**TOT**	R	S	NI1	NI3	NI4	**TOT**	R	**TOT**
A	1358.4	359.2	32.8	39.9	**1790.4**	3628	199	7	2120	5321	**11275**	88.9	**88.9**
B	265.1	74.5	0.6	35.9	**376.1**	243	6	0	103	438	**790**	77.3	**77.3**

R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI2 = not identified (MM 368); NI3 = not identified (MM 370); NI4 = not identified (MM 388). A = reduced (total alkaloids); B = not reduced (free basic alkaloid).

The ratio of total *N*-oxides:free bases in *Senecio inaequidens* was 3.7:1. Comparatively, *S. latifolius* had a ratio of total *N*-oxides:free bases of 13.2:1 and that of *S. consanguineus* was 0.15:1 (Table 3.1).

The pyrrolizidine alkaloid concentrations of the different parts (leaves, flowers/seeds and stems) of the *S. inaequidens* plant material obtained from Frankfort, Ermelo and Queenstown are reflected in Table 3.2. Flowers/seeds of *S. inaequidens* from the three localities analyzed had higher concentrations than the leaves and stems. In Table 3.3, the concentrations of PA's of different parts of the *S. retrorsus* collected at Molteno are indicated for comparison.

Zinc reduced samples (A) representing the total PA content, generally had higher concentrations than non-reduced fractions (B) representing the free basic alkaloids. This indicates that the majority of PA's are present in the *N*-oxide form.

Table 3.2 Concentrations of PA's (µg/g retrorsine or retrorsine equivalents for S, NI1, NI2) in different parts of *S. inaequidens* collected at Frankfort, Ermelo and Queenstown.

		Frankfort					Ermelo					Queenstown				
		R	S	NI1	NI2	**TOT**	R	S	NI1	NI2	**TOT**	R	S	NI1	NI2	**TOT**
Lv	A	4795	1196	60.4	48.8	**6100**	448.9	60.9	0	18.3	**528.1**	45.1	4.5	0	4.6	**53.4**
	B	316.4	124.9	1.1	110.9	**553.3**	28.4	0	0	0	**28.4**	1	6.4	0	1.9	**1.3**
F/S	A	13451	1804	32.3	0	**15287**	12440	947.7	81.9	142.9	**13613**	72.1	0	0	0.3	**101.8**
	B	1580	235.9	0	161.4	**1977**	707.9	26.3	0	142.5	**876.7**	3.5	0	0	1.5	**5**
St	A	2557	351.5	50	4.9	**2963**	703.9	165.1	52.6	8.2	**929.8**	27.5	3.9	0	0	**31.4**
	B	125.9	33	0.7	42.1	**201.7**	59.57	4.5	0	4.6	**68.7**	0	0	0	0	**0**

Lv = leaves; F/S = flowers and seeds; St = stems. R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI2 = not identified (MM 368). A = reduced; B = not reduced.

Table 3.3 Concentrations of PA's (µg/g retrorsine or retrorsine equivalents for S, NI1, NI3 and NI4) in *S. retrorsus* obtained from Molteno.

S. retrorsus		R	S	NI1	NI3	NI4	**TOT**
Lv	A	932	20	4	1619	4094	**6669**
	B	86	0	0	131	268	**485**
F/S	A	13092	229	56	3677	13814	**30868**
	B	639	0	0	211	1087	**1934**
St	A	4728	120	17	1927	4404	**11196**
	B	146	0	0	97	311	**554**

Lv = leaves; F/S = flowers and seeds; St = stems. R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI3 = not identified (MM 370); NI4= not identified (MM 388). A = reduced; B = not reduced.

3.4 DISCUSSION

Chemical analyses by LC-MS/MS and GC-MS revealed the presence of four pyrrolizidine alkaloids in *S. inaequidens*, namely retrorsine, the major constituent, which accounted for 75.8% of total PA's, followed by senecionine (20%) and two unidentified compounds, which represented a minor proportion (<5%). The content of PA's in *Senecio* species varies enormously, being dependent on season, meteorological conditions, growth stage and geographic distribution of the plant (Kellerman *et al.,* 2005). Macel, Vrieling and Klinkhamer (2004), reported that variation in the PA patterns of *Senecio* species might also have a genetic basis.

Röder *et al*. (1981) identified two alkaloids, senecionine and retrorsine in *S. inaequidens,* while Bicchi *et al.* (1985) isolated five different alkaloids from the plant, namely retrorsine (27.6%), senecionine (21.3%), senecivernine (16.4%), integerrimine (4.7%) and a retrorsine analogue (1.2%). In addition, two unidentified compounds were assumed to be PA's. The difference in the number of PA's identified in the present study compared to the results of Bicchi *et al.* (1985), can be attributed to the difficulties in identifying unknown PA's with spectral electron impact (EI) libraries (as used in the present study) due to the similar fragments derived from the necine base and the low abundance of the molecular ions (Rösemann, 2007).

The dried, milled *Senecio inaequidens* plant material (subsequently used in dosing trials) yielded a crude extract containing 0.18% total alkaloids. The average total concentration of alkaloids in plant parts of *S. inaequidens* collected during the field outbreak was 0.81%. Comparatively, *S. latifolius* and *S. retrorsus,* two important poisonous species in South Africa, contained 1.12% and 1.62% total alkaloids, respectively. On the other hand, the non-toxic *S. consanguineus* yielded only 0.01% total alkaloids. In a previous study, Bicchi *et al.* (1985) reported total alkaloids in the range of 0.3-0.4% in *S. inaequidens* dry plant material. *Senecio* species with PA concentrations ranging from 0.03 to 0.25% green material, and up to 1.2% dry weight, have been reported to cause outbreaks in livestock (Craigmill, 1981; Karam, Soares, Haraguchi, Riet-Correa, Méndez and Jerenkow, 2004).

The concentrations of PA's as well as the specific alkaloids present in a plant are crucial factors when estimating the toxicity of the plant. *Senecio inaequidens* plant material collected at Frankfort, during the outbreak and responsible for the intoxication of cattle, had higher PA concentrations (total alkaloids = free base + *N*-oxide) than the material from Ermelo and Queenstown (Table 3.2). Retrorsine, the most abundant PA identified in *S. inaequidens*, and senecionine are hepatotoxic with LD_{50} values for male rats of 38 mg/kg and 85 mg/kg, respectively (Cheeke, 1998).

The ratios of total *N*-oxides:free bases were 3.7:1 in *S. inaequidens* and 13.2:1 in *S. latifolius*. In *Senecio inaequidens*, ratios of *N*-oxide:free bases of 4.12:1 and 3.8:1 were determined for retrorsine and senecionine, respectively. These results concur with published data, which states that PA's are maintained in plants mainly as *N*-oxides (Mattocks, 1986; Hartmann, 1999). Zinc reduced (A) samples, which reflect the total PA's (free base + *N*-oxide) are expected to have similar or higher concentrations to B samples (only basic alkaloids). In the present study some B samples were higher than their related A samples. This may be explained by PA loss during the extraction and reduction processes.

According to Hartmann and Dierich (1998) the differences between the quantitative and qualitative patterns of *Senecio* PA's involve interacting factors, which include amongst others *de novo* synthesis of PA precursors, efficiency of translocation between plant organs and efficiency of tissue storage.

Pyrrolizidine alkaloid concentrations in the flowers/seeds of *S. inaequidens* were higher than those in the leaves and stems, from all localities analyzed. This is in agreement with previous observations which report that inflorescences have higher PA concentrations than leaves and stems (Kellerman *et al*., 2005). Craigmill (1981) ranked plant parts in decreasing concentration of PA's as follows: flowers and seeds > leaves > stems > roots.

The average PA concentration in plant parts of *S. inaequidens* (Table 3.2) was 8116.3 µg/g, which is much higher than the concentration of 1790.4 µg/g, determined in the dried, milled plant material (Table 3.1) subsequently used in the dosing trials. The *S. inaequidens* plant material represented in Table 3.1 was collected seven weeks after the collection of plant material in Table 3.2, when the quantity of flowers/seeds was much less. In the current outbreak, the attending veterinarian specifically commented on the abundance of a shrub with yellow flowers that was heavily grazed, which transpired to be *S. inaequidens*. *Senecio* species are reported to be more toxic in the pre-flowering stage. This is a very interesting observation as animals are more likely to graze on immature plants (Watt and Breyer-Brandwijk, 1962).

Chapter 4

CONFIRMATORY DOSING TRIALS

INTRODUCTION

Senecio inaequidens was incriminated as the cause of intoxication in cows near Frankfort, Free State Province, South Africa. As far as could be ascertained, no incidence of poisoning involving this *Senecio* species has previously been reported in South Africa.

In a pilot study, rats were dosed with a crude extract prepared from *S. inaequidens* to confirm toxicity. In an attempt to reproduce the intoxication a sheep was also dosed*.

4.1 PILOT STUDY

4.1.1 MATERIALS AND METHODS

Four male Sprague Dawley rats (No 1 - 4), aged 8-9 weeks and weighing 115 –140.5 g were purchased from the University of North-West, Potchefstroom, South Africa. The initial administered dose to the rats in this trial was intended to be equivalent to 10 g dried plant material per kg body weight (b.w.). The rats were dosed by oral gavage with a *S. inaequidens* crude extract obtained from 50 g dried, milled plant material, which yielded 0.28 g crude extract. The extract was prepared as described in Section 3.2.3. The chemical composition and PA concentrations in the dried, milled *S. inaequidens* are presented in Table 3.1.

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* Ethical approval was only granted by the Animal Use and Care Committee, University of Pretoria to dose four rats and one sheep.

One gram dried plant material contained 1.35 mg retrorsine (basic alkaloid plus *N*-oxide). The doses administered to the rats ranged from 0.049 - 0.245 mg crude extract/g b.w., which was equivalent to 0.012 - 0.06 mg retrorsine/g b.w. (Table 4.1)

The rats were observed at least three times a day. Based on their habitus and clinical signs the rats were terminated with an overdose of pentobarbitone sodium administered intraperitoneally. When deeply anaesthetized blood samples were collected for clinical pathology by intracardiac puncture. The following parameters in the serum were analyzed: total serum protein (TSP), albumin (ALB), globulin (GLOB), albumin/globulin ratio (A/G), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), total bilirubin (Bil T), bile acids (Bile A), urea and creatinine. To determine enzyme activities and serum protein, urea and creatinine concentrations, an automated chemical analyzer (Technicon RA-XT system, Miles Inc. Diagnostics Division, Tarrytown, New York) was used following the manufacturer's methods and reagents. Total bilirubin was determined with the NexCT^{TM} Total Bilirubin Reagent Kit using the NExCT^{TM} clinical chemistry system. Bile acids were detected with the formazan method, an enzymatic colourimetric method developed by Next/Vetex Alfa Wassermann Analyser, The Netherlands.

From all four experimental rats and one control rat, liver, lung and kidney samples were collected in 10% buffered formalin and routinely processed for light microscopical examination. In addition, small blocks measuring 0.5–1 mm were cut from the middle of the liver (parietal surface) and fixed in 2.5% gluteraldehyde (pH 7.2 to pH 7.4) for 24 hours. Selected blocks were post-fixed in 2% osmium tetroxide for one hour, dehydrated in a graded ethanol series (50–100%), passed through propylene oxide as the intermediate solvent and embedded in EMBed 812. Thick (1–2 micron) sections were cut for tissue orientation and stained with toluidine blue. Thin sections from selected blocks were stained at room temperature for 20 min. in a saturated aqueous solution of uranyl acetate, rinsed and then post-stained for 3 min. in Reynold's lead citrate.

Fresh tissue (liver, kidney and lung) samples were collected and stored frozen (-25°C) to determine PA concentrations and possibly PA metabolites. The PA's were extracted and analyzed using the same methods as previously described (see Section 3.2.3).

4.1.2 RESULTS

4.1.2.1 Clinical signs

Rat 1 did not exhibit any noticeable clinical signs. The other rats dosed with the *S. inaequidens* crude extract initially became depressed with a decreased habitus. The rats also demonstrated pilo-erection and developed an unsteady gait and icterus, noticeable at the ears (Fig 4.1). The dosing regimen, clinical signs and macroscopic lesions observed are summarized in Table 4.1.

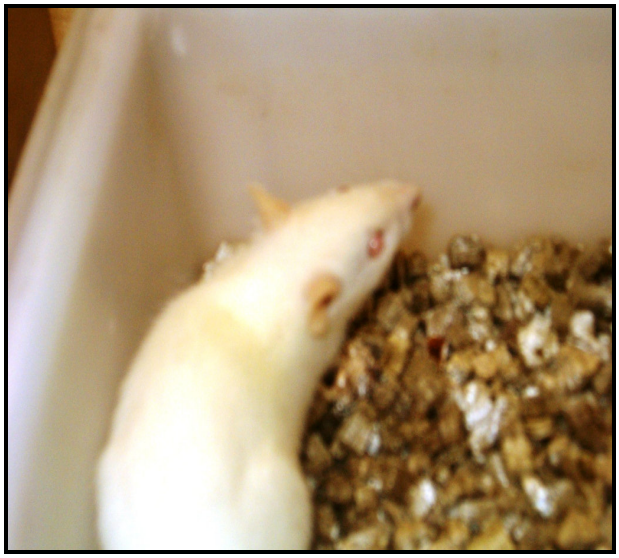


Fig. 4.1 Rat 2. Icteric ears, Day 2 after dosing.

Table 4.1 Dosing regimen, clinical signs and macroscopic lesions of the rats dosed with *S. inaequidens* crude extract.

Rat			Dosing regimen		Clinical signs*	Macroscopic lesions
No	**Age (weeks)**	**Body mass (g)**	**Day**	**Dose (mg/g)**		
1	8	115.5	0;1	0.049	N/a. Euth (D6)	N/a
2	8	118	0	0.142	Depression (D1)**; pilo-erection, jaundice of ears, unsteady gait (D2); weight loss (D3); Euth (D4)	Liver necrosis.
3	9	138.6	0	0.196	Depression, swaying gait (6h); pilo-erection, jaundice of ears (D1); Euth (D1)	Congestion of the liver. Liver necrosis.
4	9	141.3	0	0.245	Depression, pilo-erection (7h); slow movements (12 h); unsteady gait, jaundice of the ears (D2); Euth (D2)	Jaundice of the skin. Liver necrosis

N/a = Nothing abnormal noticed;
* = Clinical signs in order of appearance;
** = Time post dosing;
Euth = Euthanased

4.1.2.2 Clinical pathology

Clinical chemistry revealed a marked increase in ALP (except Rat 4), AST and GGT activities. Total bilirubin concentrations were also increased. Decreased TSP, albumin and globulin concentrations were noticed in all four rats with especially the globulin fraction in Rats 2, 3 and 4 severely reduced. The A/G ratio increased in all animals (Table 4.2).

Table 4.2 Clinical chemistry parameters of the rats dosed with a crude extract prepared from *S. inaequidens.*

Analytes	Reference Values [a]	Rat 1	Rat 2	Rat 3	Rat 4
TSP (g/l)	58.5 (±2.3)	55.2	39.5	40.2	33.2
ALB (g/l)	30.8 (±1.1)	30.5	26.2	29.8	26.0
GLOB (g/l)	31-33[b]	24.7	13.3	10.4	7.2
A/G	0.95-0.96[c]	1.23	1.97	2.87	3.61
ALP (U/l)	290 (±63)	862	1035	690	35
AST (U/l)	78.1 (±13.0)	320	1194	19170	12600
GGT (U/l)	5-6[b]	10	14	23	17
Bil T (µmol/l)	1.4 (±0.6)	9.1	49.7	92.3	97.2
Urea (mmol/l)	9.46 (±0.84)	7.7	8.2	24.9	15.1
Creat (µmol/l)	47.6 (±7.4)	50	42	30[d]	25[d]
Bile A (µmol/l)	20 – 60[e]	58.6	111.3	79.9	88.6

[a] Mean (s.d.) values of male Sprague-Dawley rats (Lillie, Temple and Florence, 1996).
[b]Normal range values (I.S.I.S., 1999).
[c]Calculated from the reference values.
[d]Serum icteric, creatinine results may be influenced by colour.
[e]Mean control ranges in CD Rats (Derelanko and Hollinger, 2002).

4.1.2.3 Pathology

Macroscopic lesions

With the exception of Rat 1, who received the lowest dose of the extract, all the rats showed marked hepatic lesions characterized by congestion, accentuated lobulation, multifocal to coalescing pale areas (liver necrosis) and jaundice that ranged in extent from mild to moderate (Table 4.1 and Fig. 4.2 - 4.3).

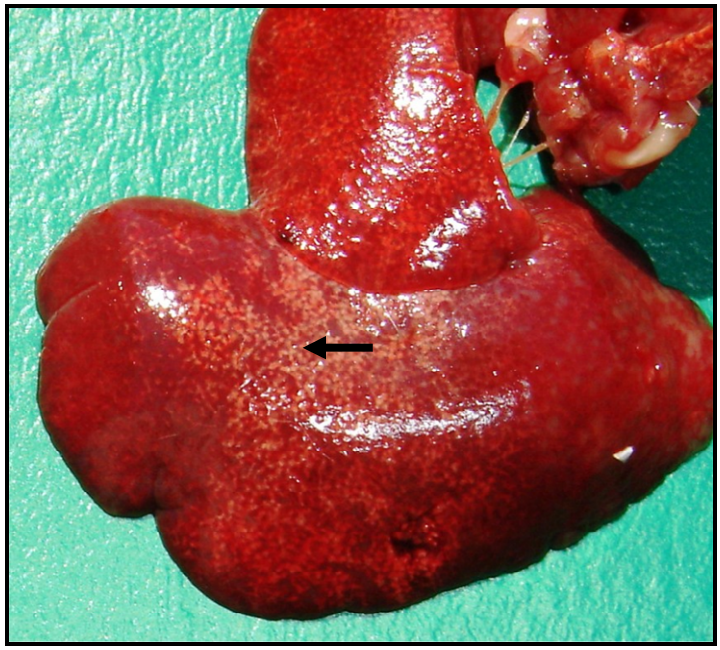


Fig. 4.2 Rat 2: Multifocal to coalescing pale, necrotic areas (arrow) and congestion.

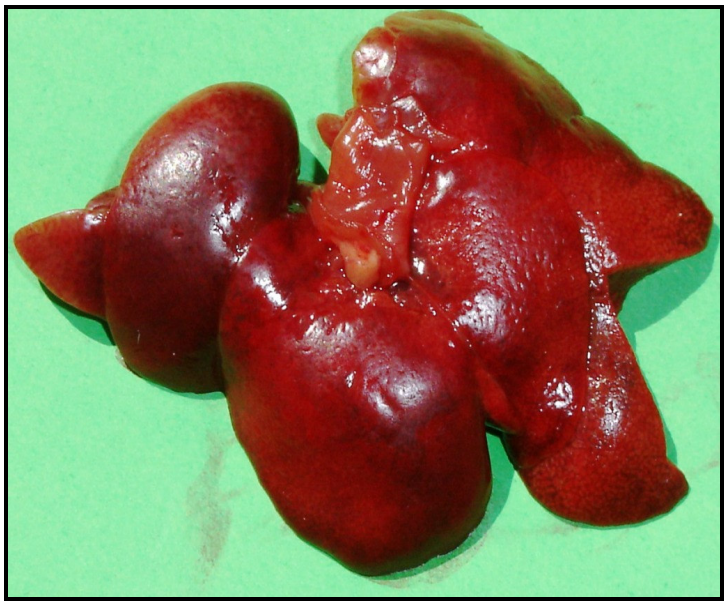


Fig. 4.3 Rat 4: Extensive hepatic necrosis characterised by light brown discolouration.

Histopathology

Rat 1: Throughout the liver the hepatocytes were swollen with a loss of cellular detail and the presence of large intracellular empty spaces (Fig. 4.4). No lesions were identified in the other organs examined.

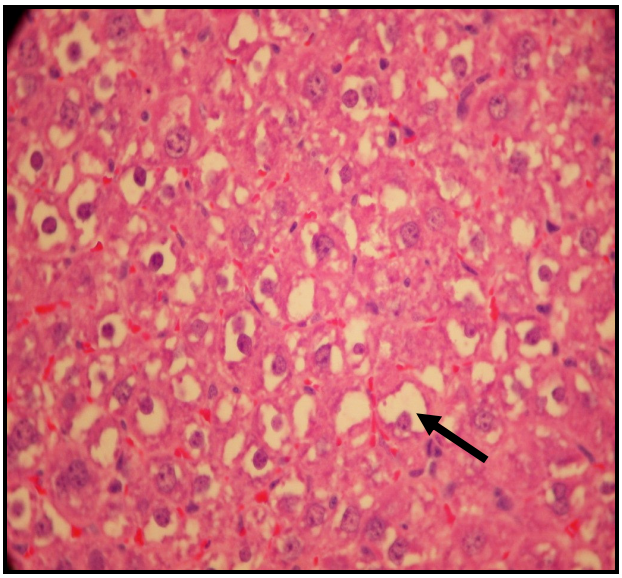
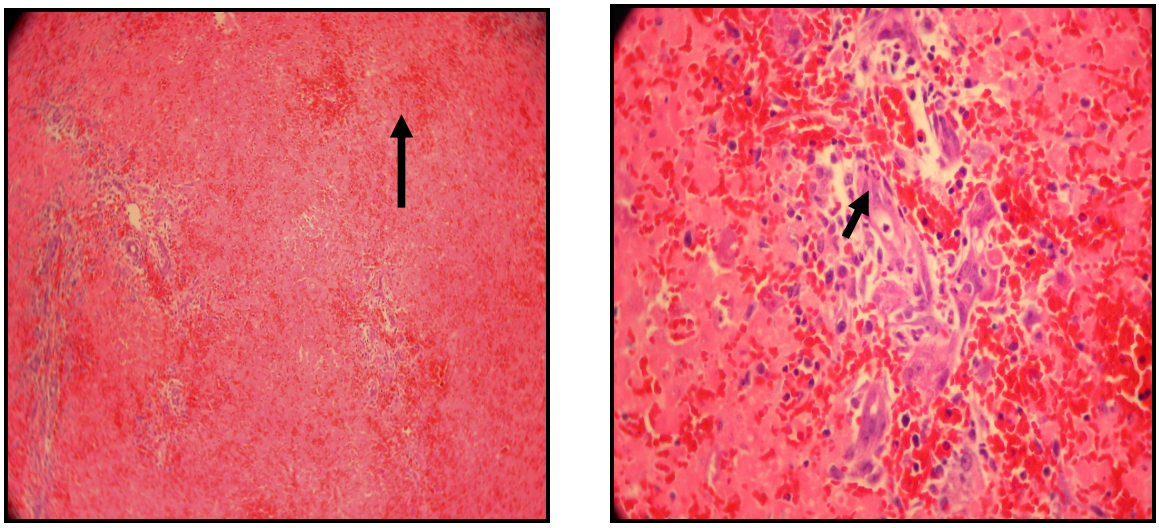


Fig. 4.4 Rat 1: Swollen hepatocytes with loss of cellular detail. Note the large empty intracytoplasmic spaces (arrow) (HE, 200x).

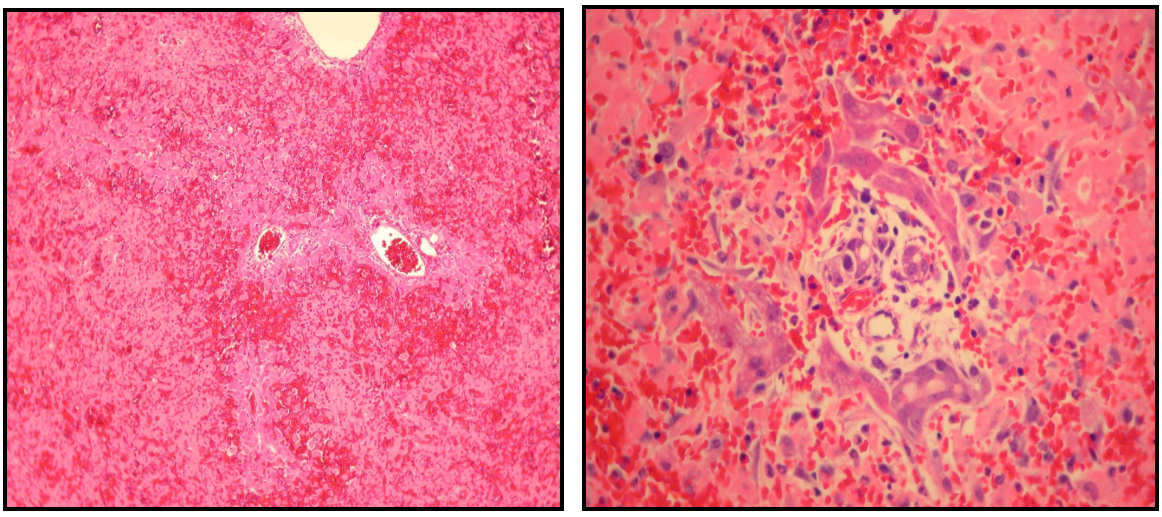
Rat 2: Hepatic lesions were characterized by extensive coagulative to lytic necrosis of the centrilobular and midzonal areas, sparing the portal hepatocytes, accompanied by extensive haemorrhage, blood pooling and congestion (Fig. 4.5 a). Hepatocytes in the portal areas that were not necrotic were swollen with a fine granular cytoplasm. Mild bile ductular proliferation (Fig. 4.5 b), portal fibrosis and oedema accompanied by a mild purulent infiltration were also present.



(a) (b)

Fig. 4.5 a and b Rat 2: Severe hepatic necrosis with extensive haemorrhage and blood pooling (arrow) [(a) HE 100x]. Note bile ductular proliferation (arrow) [(b) HE 200x].

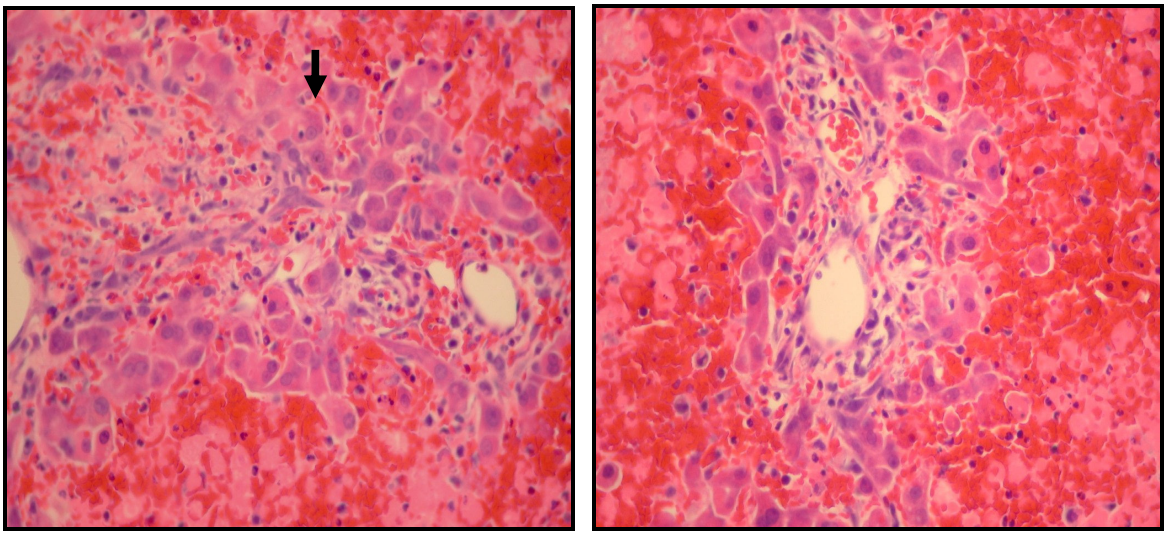
Rat 3: The liver lesions were similar to those reported in Rat 2, but were more severe (Fig. 4.6). Mild nephrosis characterized by swelling of the epithelial cells, mainly in the proximal convoluted tubules, was also present.



(a) (b)

Fig. 4.6 a and b Rat 3: Severe hepatic necrosis [(a) HE 100x (b) HE 250x].

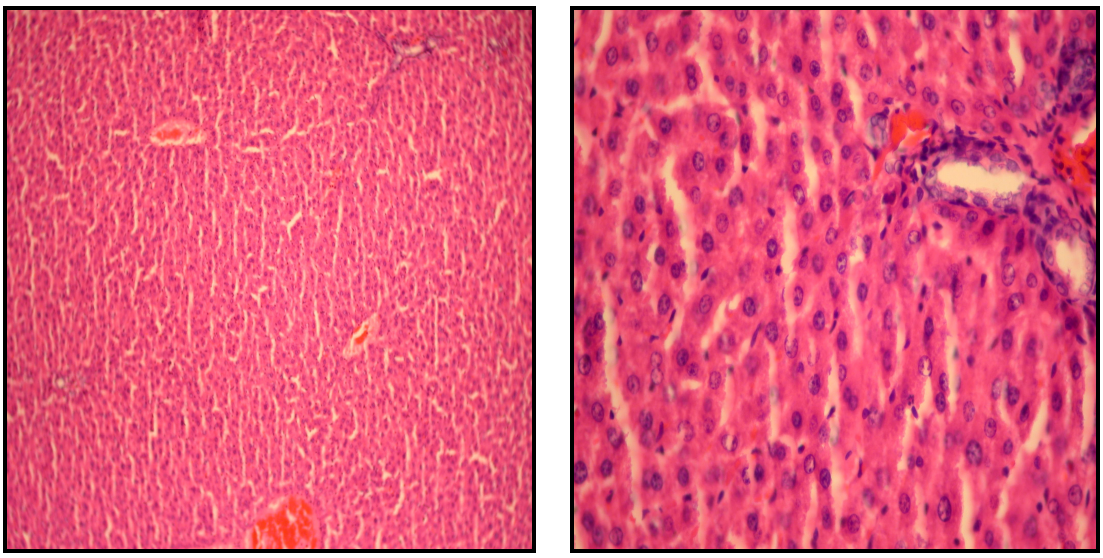
Rat 4: Extensive hepatic pannecrosis with only one to two cell layers of viable hepatocytes bordering the portal triads (Fig. 4.7a and b). The cytoplasm of viable hepatocytes demonstrated an increased basophilia. A mild inflammatory response (scattered neutrophils, Kupffer cell proliferation and fibroblasts) was associated with the necrosis. Lymph vessels in the portal areas were dilated, indicating portal oedema.



(a) (b)

Fig. 4.7 a and b Rat 4: A few viable hepatocytes are present in the periportal region (arrow). Note extensive hepatic necrosis, haemorrhage, blood pooling and congestion [(a) and (b) HE 300x].

The normal architecture of the rat liver (control) is presented in Fig. 4.8.



(a) (b)

Fig. 4.8 a and b Control Rat: Normal architecture of rat liver [(a) HE 100x (b) HE 200x].

Transmission Electron Microscopy (TEM)

Rat 1

In some hepatocytes the cytoplasm showed greatly increased numbers of smoothly-contoured, single membrane-bound bodies with an electron-dense appearance (lysosomes) compared to the normal cells in the control animal. Furthermore, some hepatocytes were characterized by the presence of large areas of cytoplasm containing medium electron-dense material devoid of organelles (vacuoles).

Rats 2, 3 and 4

Nuclear changes ranged from chromatin margination to karyopyknosis and karyorrhexis. A few pyknotic nuclei were visible and were recognized by the shrunken nucleus with diffuse condensation of the chromatin. Chromatin margination representing the early stages of karyolysis was evident as condensation of the chromatin in irregular clumps along the inner membrane of the nuclear envelope, with disappearance of the chromatin from other areas of the nucleus (Fig. 4.9 and 4.10).

The cytoplasm of necrotic hepatocytes contained severely morphologically distorted organelles, some of which could not be identified (Fig. 4.9). The swollen/distorted mitochondria were encircled by free ribosomes dispersed in the cytoplasm. Also present in necrotic hepatocytes were autolysosomes, also known as autophagic vacuoles.

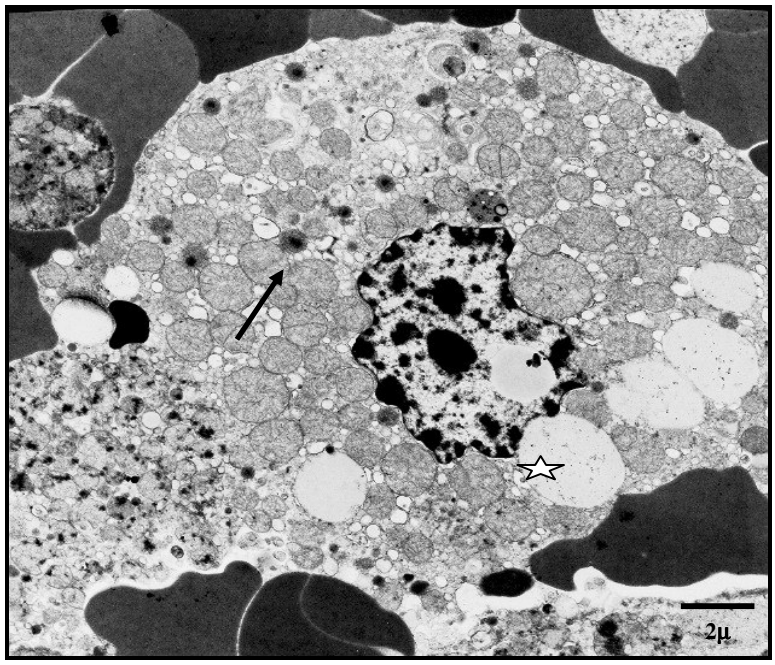


Fig. 4.9: Electron micrograph of the liver of Rat 3. The mitochondria are swollen (arrow) and intracytoplasmic vacuoles are evident (star). Also note chromatin condensation (karyopyknosis).

In less severely affected hepatocytes, mitochondria were generally mildly swollen and often contained intramitochondrial inclusions (Fig. 4.10). These were irregularly shaped, medium to electron-dense and had woolly, filamentous borders, which gave them a flocculant and woolly appearance. Also present was dilatation of the endoplasmic reticulum with vesiculation of the rough endoplasmic reticulum and degranulation of ribosomes.

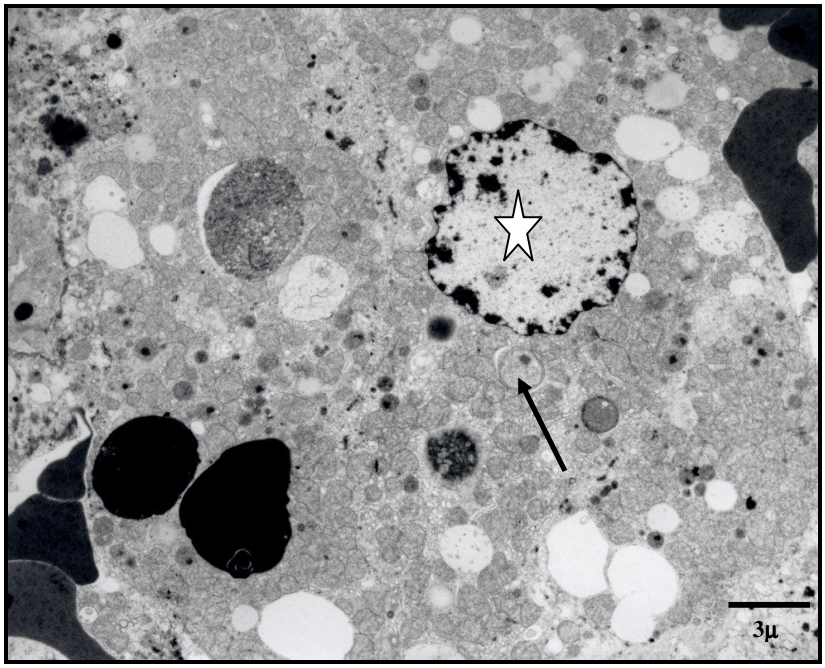


Fig. 4.10: Electron micrograph of the liver of Rat 4. Note the electron-dense inclusions in swollen mitochondria (arrow) and chromatin margination (star).

In a few sections dark cells were noted directly adjacent to customary lighter staining cells (Fig. 4.11). This is known as the so-called “dark cell-light cell phenomenon”.

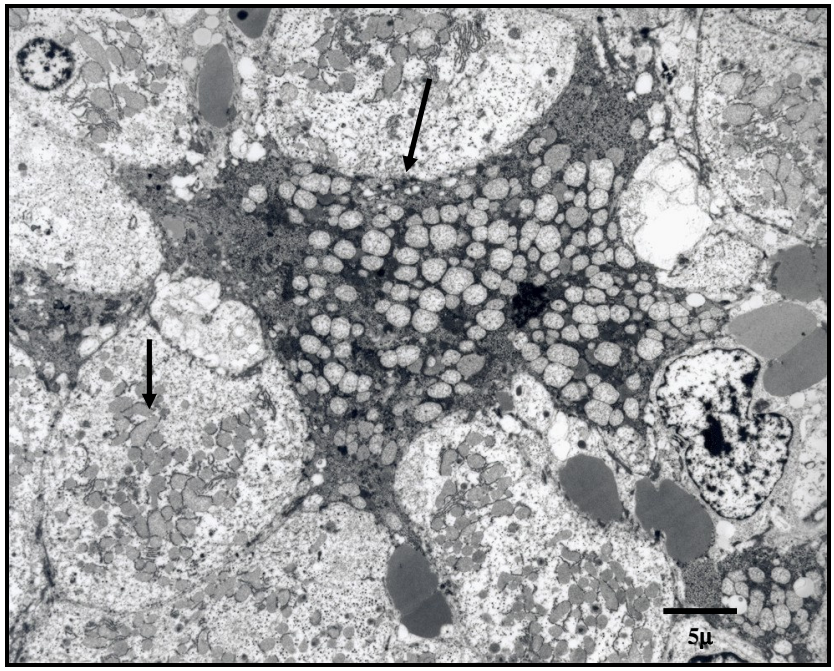


Fig. 4.11: Electron micrograph of rat liver showing the “dark cell-light cell phenomenon”; dark cell (top arrow) adjacent to lighter staining cells. Note the distorted cytoplasmic morphology in a light cell (bottom arrow).

4.1.2.4 Extraction of pyrrolizidine alkaloids in tissues

Pyrrolizidine alkaloids were detected in the livers of all experimental rats. The concentration of PA's detected in rat livers was inversely proportional to the amount of the extract dosed to the rats. Thus, Rat 1 had higher PA concentrations in the liver compared to those of Rat 4 (Table 4.3). Pyrrolizidine alkaloids were also detected in the kidneys of Rat 2 and Rat 4 and in the lungs of Rat 4. In these cases, the concentrations of PA's were higher than in the liver.

The alkaloids detected in the tissues were the same as those in the extracts of *Senecio inaequidens* (Table 3.1).

Table 4.3 Concentration of PA's (µg/g retrorsine or retrorsine equivalents for S, NI1, NI2) in the liver, kidney and lung of the experimental rats.

		RAT 1					**RAT 2**			**RAT 3**			**RAT 4**		
		R	S	NI1	NI2	**TOT**	R	S	**TOT**	R	S	**TOT**	R	S	**TOT**
Liver	A	175.1	35	3.1	1.6	**214.8**	15.63	3.6	**19.23**	14.8	2.5	**17.3**	0.1	0	**0.1**
	B	83.1	14.3	0	0	**97.4**	-			21.6	2.6	**24.2**	5.93	1.1	**7.03**
Kidney	A	0	0	0	0	**0**	48.8	5.7	**54.5**	0	0	**0**	31.2	0	**31.2**
	B	*	*	*	*	*	37.9	5.2	**43.1**	0	0	**0**	0.2	0	**0.2**
Lung	A	0	0	0	0	**0**	0	0	**0**	0	0	**0**	0.5	0	**0.5**
	B	*	*	*	*	*	*	*	*	0	0	**0**	32.7	0	**32.7**

R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI2 = not identified (MM 368). A = reduced; B = not reduced. * = not expected to exceed A which is zero. (-)= glass test tube containing the sample broke

4.1.3 DISCUSSION

Using retrorsine as a reference (LD_{50} = 38 mg/kg for male rat) (Cheeke, 1998), Rat 1 was dosed with the equivalent of 23.4 mg/kg of retrorsine, which is 0.6 times the LD_{50}. Rat 2 received approximately 3 times the initial dose, which was close to, but still below, the LD_{50}. Rat 3 and Rat 4 received 1.2 times and 1.5 times the LD_{50}, respectively. Rats gavaged with doses similar to and exceeding the LD_{50} of retrorsine, manifested overt clinical signs of PA poisoning, while Rat 1 only developed

histopathological and ultrastructural changes. Although retrorsine is the major PA constituent of *Senecio inaequidens,* the toxicity of the plant to the rats is not ascribed only to retrorsine. Other PA's such as senecionine and the two unidentified PA's detected in the plant are highly likely to be involved in the toxicity. As no comparative toxicity trials with *S. inaequidens* in rats are available, dosing of *S. inaequidens* extracts similar to and above the retrorsine LD_{50} may induce acute hepatotoxicity in rats.

With the exception of Rat 1, who did not show any noticeable clinical signs, the clinical signs exhibited by the other rats were those of acute liver failure and have been described in other species (Stegelmeier *et al*., 1999). The first signs were observed between 6 and 24 hours after a single large dose and manifested as decreased habitus, depression and pilo-erection. The animals deteriorated rapidly, exhibited slow movements with an unsteady gait, which was probably due to the debilitating condition. Jaundice was often present in poisoned animals and is ascribed to increased bilirubin in the blood due to decreased bile secretion (Cheeke, 1998). In this study icterus was particularly noticeable in the ears. Ascites, a consequence of the decreased liver synthesis of plasma proteins, resulting in decreased oncotic pressure (Mattocks, 1986; Cheeke, 1998), was not seen in any of the experimental animals. Acutely affected animals usually die within 1-4 days after a large PA dose (Mattocks, 1986). In this study all animals were euthanased for humane reasons when overt clinical signs manifested. Rat 1 was only euthanased on Day 6 as no clinical sign was observed.

Clinicopathological changes detected in the experimental rats were comparable to those previously described in other animal species, although small differences were noticed (Johnson *et al*., 1985; Lessard *et al*., 1986; Craig *et al*., 1991). Pearson (1990) reported that GGT and ALP tend to be consistently elevated as the lesions are mainly in the portal region. Liver enzyme activities increase during periods of hepatocyte destruction. In this study, one rat (Rat 4) did not develop an increased ALP activity, in

fact ALP activity decreased with no plausible explanation. The use of this enzyme to aid in the diagnosis of PA toxicosis is limited due to its lack of specificity for the liver. Determination of ALP activity is probably useful when performed in conjunction with other liver specific enzyme analyses (Craig *et al*., 1991). Although AST is not specific for hepatic damage, in the absence of lesions in other organs or tissues, it is a good indicator for liver damage and a rise in activity generally precedes that of GGT (Johnson *et al*., 1985). Total serum protein concentration tends to be normal and only terminally does the albumin level decrease due to deficient hepatic synthesis (Pearson, 1990). In the current study all rats presented with decreased TSP, albumin and globulin concentrations, suggesting that impairment of liver protein synthesis occurs early in the course of the disease in rats. In equines, bile acids and bilirubin tend to increase later in the course of the disease (Pearson, 1990). In the present study however, rats that showed clinical signs displayed a dramatic increase in bilirubin concentration.

Macroscopic and histopathological lesions observed in the experimental rats were consistent with those described by several authors in species other than rats (Mattocks, 1986; Stegelmeier *et al.,* 1999; Kellerman *et al*., 2005). Usually the liver is congested, with accentuated lobulation and rounded edges. Liver necrosis is extensive, with haemorrhage and minimal inflammation. In the present study, moderate jaundice was also evident. Microscopic lesions were characterized by centrilobular to midzonal hepatic necrosis and proliferation of bile ducts. The reason why the centrilobular region is particularly affected has been attributed to the high cytochrome P-450 activity in the region (Kellerman *et al*., 2005). Pyrrolizidine alkaloids are bioactivated to pyrrolic derivatives via the cytochrome P-450 system. Furthermore, there is a poor supply of oxygen and nutrients in the blood reaching this zone. According to Mattocks (1986), the zone of necrosis is dependent on the animal species, nutritional status and chemical pretreatment. Rats dosed with retrorsine may have centrilobular necrosis, while in the hamster for example, it is periportal. Severe poisonings may affect the whole lobule. Brain lesions and nephrosis have also been

reported with PA toxicosis (Seaman, 1987; Barros *et al*., 1992; Kellerman *et al*., 2005). In the current study, only Rat 2 showed a mild nephrosis.

In Rat 1, ultrastructural alterations included increased lysosomes in some hepatocytes and the presence of large to medium electron-dense areas in others, with few identifiable organelles. The medium electron-dense material was most probably due to intracellular oedema, representing the large empty spaces noted during light microscopical examination.

In the rats that presented with clinical signs, hepatocyte nuclear changes were characterized by chromatin margination. This represents the early stages of karyolysis and was also described by Molineux *et al*. (1988) and Barros *et al*. (1992). It is regarded as an early change associated with irreversible cell injury and is a much more common and consistent EM finding than karyopyknosis and karyorrhexis (Ghadially, 1997).

Necrotic hepatocytes contained numerous intracytoplasmic autolysosomes enclosing organelles in various stages of breakdown and inclusions such as glycogen and lipids. The increased number of autolysosomes indicates intracellular injury and their formation is the mechanism by which the cell disposes of old or damaged organelles, which are abundant in necrotic cells (Ghadially, 1997).

The presence of intramitochondrial inclusions in the moderately affected hepatocytes is an important observation and is usually not reported in relation to PA poisoning. According to Ghadially (1997), the presence of woolly densities within mitochondria is the most reliable early manifestation of irreversible cell injury. Numerous mitochondria must contain woolly densities, as seen in this case, before cell death can be confirmed. Woolly densities have been described after *in vivo* ischaemia, for example after myocardial infarcts, heavy metal and other types of poisoning and immune cytolysis (Ghadially, 1997). They are believed to be precipitated by denatured mitochondrial matrix proteins, as well as proteins and lipids released from

disintegrating cristae in irreversibly damaged cells, where mitochondrial function becomes disorganized.

The “dark cell-light cell phenomenon” may or may not have significance with respect to PA poisoning. The phenomenon may be seen in normal and pathological conditions, especially in the liver. It has been described with hepatotoxic damage to the liver, but also as a non-specific or agonal change and in some cases it may develop as an artifact of liver fixation. Dark cells may represent dehydrated or necrotic cells (Ghadially, 1997).

Changes observed in the endoplasmic reticulum in this experiment included dilatation with vesiculation and degranulation. These changes have been previously described by Ilha *et al*. (2001) as dilatation of the rough endoplasmic reticulum and in certain areas moderate hyperplasia of the smooth endoplasmic reticulum. The distinction between dilatation and vesiculation of the rough endoplasmic reticulum is often not possible. The term vesiculation implies that the rough endoplasmic reticulum has broken up into numerous discrete vesicles. The dilatation of the endoplasmic reticulum is probably due to ingress of water and solutes into the damaged cells. Ingress of water and solutes into a cell results in dilatation of various membrane–bound compartments, including the endoplasmic reticulum and mitochondria as was seen in the liver of the experimental animals in this study. Swelling of the rough endoplasmic reticulum results in degranulation of ribosomes (Ghadially, 1997).

Pyrrolizidine alkaloids were extracted from the liver, kidney and lung of experimental rats. It is reported that PA's are metabolized very rapidly. Those that are not biotransformed to toxic metabolites are converted to harmless compounds or excreted unchanged (Mattocks, 1986). In the present study the PA’s extracted from the liver of Rat 1 were the same as those detected in *S. inaequidens* plant material, i.e. retrorsine, senecionine and the two unidentified compounds. In the tissues of Rats 2, 3 and 4 only retrorsine and senecionine were distinguished.

Quantification of individual PA's in the organ samples was problematic, probably due to PA losses during preparation and extraction. However, as this is not a kinetic study, the information gathered is still useful, as detection of PA's in tissues of affected animals may assist in confirming a diagnosis of intoxication.

4.2 SHEEP DOSING TRIAL

4.2.1 MATERIALS AND METHODS

A male Dorper sheep, aged 10 months and weighing 40 kg, was purchased from a supplier in Upington, Northern Cape Province, South Africa. During the trial the sheep received lucerne hay and a pelleted concentrate, and water was available *ad lib*. Following an adaptation period of approximately four weeks the animal was dosed with a crude extract. The crude extract (total yield = 21.2 g) was prepared from 4.66 kg dried, milled *Senecio inaequidens* plant material following the method described in Section 3.2.3. Chemical composition and PA concentrations of the *S. inaequidens* crude extract are presented in Table 4.5, i.e. 1 g dried, milled plant material contained 1.51 mg retrorsine. The sheep was dosed with the crude extract on four consecutive days, by stomach tube. Incremental doses, starting from 49.5 mg/kg body weight on Day 0 and Day 1, 99.0 mg/kg on Day 2 and 198 mg/kg on Day 3 were administered. The extract was mixed with 10 ml cellofas and approximately 50 ml of water.

Clinical examination (respiration, pulse, temperature, colour of mucous membranes and ruminal motility) was performed daily. The sheep was observed twice a day for clinical signs.

Blood samples and urine were collected before dosing, five and two times, respectively, and every day during the dosing period. Blood was collected from the *Vena jugularis* to determine clinical chemistry parameters, haematology and PA concentrations. Urine was obtained for PA determination after placing the sheep in a metabolic crate. The following clinical pathology parameters in the serum were determined: total serum protein (TSP), albumin (ALB), globulin (GLOB),

albumin/globulin ratio (A/G), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), glutamate dehydrogenase (GLDH), total bilirubin (Bil T), bile acids (Bile A), urea and creatinine, using the same methodology as described in the rat pilot study. Haematology was performed using the automated analyzer Cell-dyne 3700 (Abbot Laboratories, South Africa). The following parameters were determined: haemoglobin concentration (Hb), red cell count (RCC), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white cell count (WCC), mature neutrophils (N mat) immature neutrophils (N imm), lymphocytes, monocytes, eosinophils, basophils and thrombocyte count (Thr C). The clinical chemistry parameters were determined by the Clinical Pathology Laboratory, Faculty of Veterinary Science, University of Pretoria, which also supplied the reference ranges.

The sheep was euthanased on Day 4 by administering an overdose of sodium pentobarbitone intravenously. A necropsy was conducted and samples were collected and processed for microscopical examination. For EM, liver samples were collected from the parietal surface as well as from the middle of the left and right lobes. The samples were prepared and processed as described previously for the rat pilot study.

4.2.2 RESULTS

4.2.2.1 Clinical signs

On Day 1 of the dosing trial, the sheep's ruminal motility decreased. No other clinical signs were observed until Day 4 when the sheep refused to eat lucerne hay and only ingested 150 g of pellets. The sheep was subsequently euthanased.

4.2.2.2 Clinical pathology

Haematological values obtained before dosing (Days -18; -17; -14 and Day 0) and during the dosing trial, from Day 1 to Day 4, fluctuated within normal reference ranges.

Clinical chemistry analyses (Table 4.4) revealed that albumin and albumin/globulin ratios were below the normal reference range before and during the dosing trial. Total serum protein was below normal on Day -18, Day -3 and Day 4, and within the normal reference range on the remaining experimental days. AST and GLDH activities were above the reference ranges during the entire experimental period. AST activity increased slightly on Day 1 of the dosing trial and GLDH activity was elevated on Day 3 and Day 4. The remaining analytes were within the reference ranges or did not differ much from the values determined during the pre-dosing period.

Table 4.4 Clinical chemistry parameters of the sheep before and during the dosing trial.

Analytes	Ref Values	D -18	D -17	D -14	D -3	D 0	D 1	D 2	D 3	D 4
TSP (g/l)	60-75	57.8	59.7	60.4	58.9	63.7	67.3	61.9	60.9	59.8
ALB (g/l)	28-34	22.5	23.2	23.5	23.6	23.8	27.3	23.9	23.8	24.4
GLOB (g/l)	32-43	35.3	36.5	36.9	35.3	39.9	40.0	38.0	37.1	35.4
A/G	0.7-1.0	0.64	0.64	0.64	0.67	0.60	0.68	0.63	0.64	0.7
ALP (U/l)	33-426	390	372	414	335	375	347	330	318	301
AST (U/l)	10-125	173	165	167	179	208	228	208	202	209
GGT (U/l)	4-57	68	57	51	50	55	53	52	55	56
GLDH (U/l)	0-2	27	33	33	34	37	27	22	53	48
Bil T (µmol/l)	0-6.8	6.7	6.1	6.2	5.6	5.0	5.8	5.0	5.0	6.5
Urea (mmol/l)	2.65-6.64	13.0	11.7	13.3	11.2	12.2	13.9	10.9	8.6	10.4
Creat (µmol/l)	44-150	64	70	44	64	65	58	50	57	63
Bile A (µmol/l)	0-50*	54.1	42.7	40.7	40.2	29.4	26.5	40.4	52.7	45.8

* Reference range from Biochemistry Data Sheet 5, 2002. Bile acids.

4.2.2.3 Pathology

Macroscopic lesions

On necropsy a congested carcass and a pale, swollen liver with rounded edges were observed.

Histopathology

Histopathological examination revealed swollen hepatocytes with fine vacuolization of the cytoplasm. Mild oedema was depicted as dilatation of lymph vessels in the portal area. Necrosis of single cells with mild neutrophil infiltration was also observed. The spleen was congested with white pulp hyperplasia. A mild interstitial infiltration by mononuclear cells and mild neutrophilic leukostasis were noticed in the lungs. Severe accumulation of mononuclear cells in the mucosa and submucosa of the small intestines and a dispersed distribution of coccidian parasites were present.

Transmission electron microscopy

Hepatic lesions in the left lobe included chromatin margination as described in the rats (*vide supra)* and the presence of electron-translucent variably sized, spherical lipid droplets in the cytoplasm of hepatocytes. Mitochondria were mainly pyknotic with an increase in intramitochondrial inclusions that were irregularly shaped, medium to electron-dense with woolly, filamentous borders as described in the rat hepatocytes (*vide supra*). Also present in the cytoplasm were aggregates of smooth membrane-bound ramifying tubules, which were interpreted as smooth endoplasmic reticulum proliferation.

Compared to the left side, lesions in the hepatocytes of the right lobe were much more pronounced (Fig. 4.12 a and b). The morphology of the organelles was distorted and large vacuoles, often with an uneven outline, were scattered throughout the

cytoplasm. Some of the vacuoles contained a faint electron-translucent material, intermingled with multilaminated structures and small electron-dense granules, interpreted as either glycogen or free ribosomes. Chromatin margination of hepatic nuclei was common (Fig. 4.12 a) and identification of organelles in the hepatocytes was problematic. Some of the pyknotic or swollen mitochondria identified in the affected hepatocytes (Fig. 4.12 b) contained electron-dense inclusions similar to those described in the experimental rats (*vide supra*) and the endoplasmic reticulum was dilated. The latter lesion and the presence of large, empty intracytoplasmic spaces were indicative of cellular oedema, confirmed by light microscopy of this case.

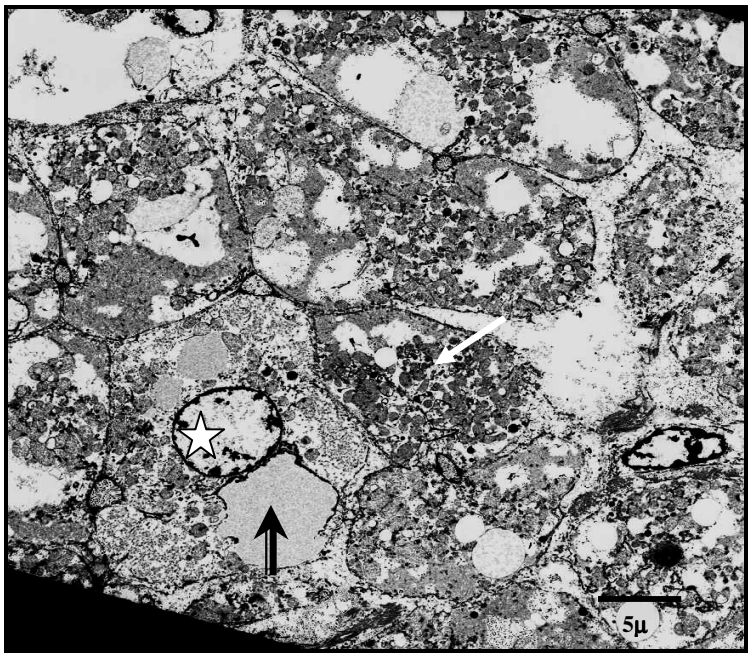


Fig. 4.12 a Electron micrograph of the right lobe of the sheep liver. The morphology of the organelles is distorted (white arrow). Note the large intracytoplasmic vacuole (black arrow) and chromatin margination (star).

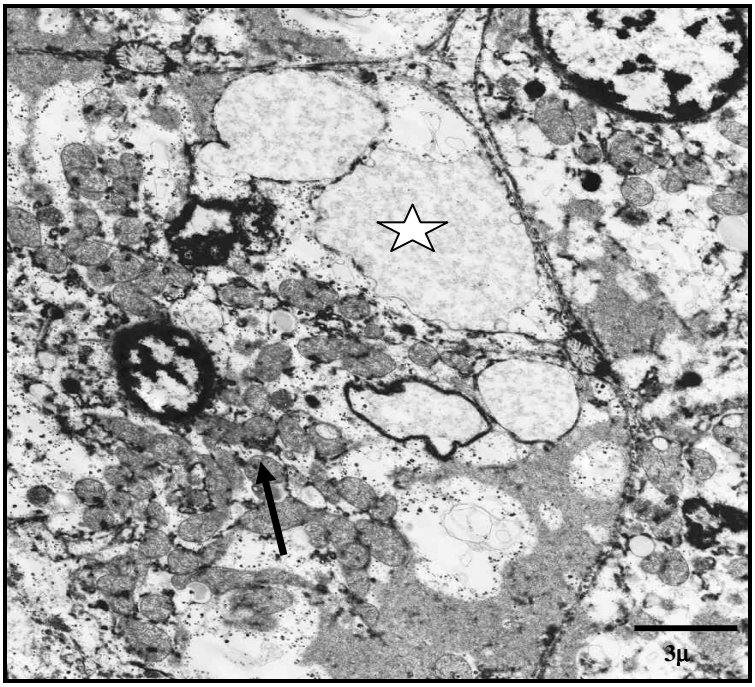


Fig. 4.12 b Electron micrograph of the right lobe of the sheep liver. Note distorted hepatocyte with large intracytoplasmic vacuoles (star) and pyknotic mitochondria (arrow).

4.2.2.4 Extraction of PA's in body fluids and tissues of the sheep

The composition and concentration of PA's in the dosing extract and in some organs and body fluids of the experimental sheep were determined. Save for *N*-oxides, the analytical methods utilized could not detect the pyrrolic and other metabolites. The PA composition of the dosing extract was the same as that determined for *S. inaequidens* plant material and presented in Table 3.1. In the tissues and body fluids only retrorsine and senecionine were recovered. The highest concentration of retrorsine was detected in the urine on Day 4 (one day after the last dose), followed by the liver and the kidney. The total and specific PA concentrations in the organs and fluids are presented in Table 4.5.

Pyrrolizidine alkaloids were not detected in urine and serum samples collected before dosing and in the serum samples after dosing. The rumen content collected at necropsy also did not contain any detectable PA's.

Table 4.5 Concentration of PA's (µg/g retrorsine or retrorsine equivalents for S, NI1 and NI2) in the dosing extract, tissues and body fluids of the sheep.

		R	S	NI1	NI2	**TOT**
Dosing extract	A	1518.6	1194	188.8	40.9	**2942.3**
Urine (Day 3)	A	22.3	0	0	0	**22.3**
	B	0	0	0	0	**0**
Urine (Day 4)	A	82	0	0	0	**82**
	B	12.4	0	0	0	**12.4**
Bile	A	0	0	0	0	**0**
	B	6	0	0	o	**6**
Liver	A	42.4	10.7	0	0	**53.1**
	B	43.1	10.8	0	0	**54.9**
Kidney	A	28.9	10.9	0	0	**39.8**
	B	29.4	0	0	0	**29.4**
Lung	A	11.5	0	0	0	**11.5**
	B	16.5	0	0	0	**16.5**

R = retrorsine; S = senecionine; NI1 = not identified (MM 338); NI2 = not identified (MM 368). A = reduced; B = not reduced.

4.2.3 DISCUSSION

For the sheep dosing trial the initial dose of plant material was estimated at 10 g/kg b.w. Taking into consideration the concentration of retrorsine in the *S. inaequidens* extract used for dosing [1 g dried plant material contained 1.51 mg retrorsine (Table 4.5)], the sheep was dosed with 15 mg retrorsine/kg b.w. At the end of the dosing trial the sheep had received a total dose of 4.8 g retrorsine, which equated to about 3.2 kg of dried plant material, representing 8% of the sheep's body weight. According to Johnson and Molineux (1984), for toxicosis to develop, there is a threshold level of PA that must be exceeded. The toxic dose of dried *Senecio* plant material as a percentage of body weight is estimated to be more than 100% for sheep and goats (Craigmill, 1981). This may explain in part why the sheep did not manifest overt clinical signs, even though the appetite decreased and the ruminal movements were reduced on Day 4 of the trial.

Clinical chemistry analysis did not reveal any major aberrations and only GLDH increased slightly on Days 3 and 4. This increased GLDH enzyme activity could indicate liver cell damage. GGT, which is a good marker for biliary damage, and AST, a marker for hepatocyte damage, were within reference ranges. Thus, no significant clinicopathological changes, which could be related to PA poisoning, were observed in the sheep.

No major macroscopic lesions were seen in the experimental animal. Apart from the swollen liver with rounded edges, no other lesions associated with seneciosis were seen in the experimental sheep. On the other hand, histopathological lesions in the liver were more consistent with those reported in affected animals, i.e. swollen and vacuolized hepatocytes, oedema and single cell necrosis.

The most significant changes in this case were noticed with TEM analysis. The nuclear and cytoplasmic changes were consistent with those described by Ilha *et al*. (2001) in *Senecio* poisoned sheep. The lesions observed were also similar to those described in acutely affected rats in the pilot study. It could be concluded that, *Senecio inaequidens* causes ultrastructural lesions similar to those reported by other researchers (Ilha *et al*., 2001).

Senecionine and retrorsine were recovered from the tissues, but only retrorsine was isolated from bile and urine. The highest concentration of PA's, mainly retrorsine *N*-oxide, was recovered from the urine on Day 4, one day after the dosing ceased. Although it was only determined in one animal, this could indicate that urinary excretion of *N*-oxides plays an important role in the elimination of PA's in sheep. The quantities of PA's extracted from the bile were very small, and none was detected in the serum after dosing. This could mean that biliary excretion of unchanged PA's in the sheep is limited compared to urinary excretion. According to White (1977), biliary excretion is important in the elimination of retrorsine metabolites, but not of the parent compounds. Unchanged PA's were also detected in the liver, kidneys and lungs.

No PA's were recovered from the ruminal fluid of the sheep. Rösemann (2007) isolated PA's of *S. inaequidens* from the rumen content of cattle poisoned during the field outbreak. Failure to detect PA's in the sheep's rumen content in the present experiment raises various questions, amongst them the much debated theory of ruminal metabolism and detoxification of PA's by sheep.

Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

Four pyrrolizidine alkaloids were isolated and identified by LC-MS/MS and GC-MS from *Senecio inaequidens* DC., namely retrorsine, senecionine and two unidentified compounds. The unidentified compounds were assumed to be PA's given the presence of fractions 94, 120 and 138, which were also visible in the spectra of the known PA's retrorsine and senecionine. Pyrrolizidine alkaloids of *S. inaequidens* were mainly in the *N*-oxide form (ratio of *N*-oxides to free bases was 3.71:1). This implies that to exert their toxic effect, the *N*-oxides should first be reduced to the corresponding basic alkaloids, a process suggested to be performed by enzymes produced by the intestinal flora (Mattocks, 1986).

Retrorsine and senecionine were also detected in *S. latifolius* and *S. retrorsus,* the two *Senecio* species most often implicated in livestock poisonings in South Africa (Kellerman *et al*., 2005). These PA's are known to be hepatotoxic with LD_{50} values of 38 mg/kg and 85 mg/kg for male rats, respectively. Retrorsine was the most abundant PA in *S. inaequidens* and accounts for 75.8% of the total PA's of the plant.

The toxicity of *Senecio* species to animals depends on the PA composition of the plant species, the total PA content of the plant, the animal's susceptibility and the relative toxicity of the pyrrole metabolites formed in the liver after the animal has ingested the plant (Johnson and Molineux, 1984). The PA content of *S. inaequidens,* as in other *Senecio* species, varies enormously and depends on the growth stage, season and location of the plant. This was demonstrated by analyzing *S. inaequidens* plant

material obtained from three different localities in South Africa, namely Frankfort, Ermelo and Queenstown, and during three different seasons. Considering the PA concentration and specific alkaloid composition of *S. inaequidens,* it must be regarded as potentially toxic and dangerous to livestock in areas where it grows.

Rats dosed with *S. inaequidens* crude extract equal to or exceeding the LD_{50} of retrorsine exhibited clinical signs of acute pyrrolizidine alkaloid poisoning, while the rat gavaged with the crude extract at a dose lower than the LD_{50} of retrorsine did not show any clinical signs. The sheep dosed with *S. inaequidens* crude extract, equivalent in dried plant material to 8% of body weight, did not present clinical signs that could be related to PA intoxication.

Clinical chemistry and gross pathological changes consistent with acute PA poisoning were observed only in the clinically affected rats. Macroscopic and histological lesions of acutely affected rats were similar to those described in the cows that died in the Frankfort outbreak. Thus, *S. inaequidens* was shown to be capable of inducing acute hepatoxicity in rats when administered at levels equivalent to the LD_{50} of retrorsine.

All experimental animals (rats and a sheep) developed histopathological and ultrastructural lesions comparable to PA poisoning. The *S. inaequidens* crude extract administered at sub-lethal doses to a rat and the sheep did not produce overt clinical signs, but induced hepatic changes noticeable at cellular and ultrastructural level. The margination of chromatin in the nucleus of the hepatocytes and the presence of woolly densities within numerous mitochondria observed in the experimental rats and the sheep are considered early signs of irreversible cell injury.

Retrorsine and senecionine were recovered from the liver of the experimental rats as well as from the liver of the sheep. Retrorsine was also detected in the kidneys of the sheep and two of the rats (Rats 2 and 4). In addition, retrorsine was also detected in the lungs, bile and urine of the sheep. Although limited dosing trials were conducted, it

appears that detection of PA's in tissues of poisoned animals may be useful in confirming a diagnosis of PA intoxication.

In conclusion, the *S. inaequidens* collected at Frankfort, where an outbreak of hepatotoxicity in cattle occurred, contained known hepatotoxic PA's. In addition, the crude extract prepared from plant material of this species was highly toxic when administered to rats. Although the intoxication could not be reproduced in a sheep, this was probably not the ideal species to use in an attempt to reproduce the toxicity in ruminants, in the light of the reported resistance to PA toxicity of sheep. It can be deduced that *S. inaequidens* DC. was most probably responsible for the cattle mortalities experienced in Frankfort.

Chapter 6

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