1. General Introduction

1.1 Natural products

Natural products are organic compounds produced by living organisms. Currently, there is an increasing interest in the study of natural products since they represent potentially useful leads for new medicines (Newman & Cragg, 2007). They are usually classified into two broad categories; primary and secondary metabolites. Primary metabolites comprise of compounds (e.g. nucleic acids, amino acids, sugars) which occur in all cells and play a central role in the growth and development of the organism (e.g. plants). Secondary metabolites, on the other hand, are compounds that are not directly involved in growth and development (Harborne, 1984), but are required for the plant to survive in its environment. Nevertheless, they can act as defensive, anti-feedants, attractants and pheromones substances which often account for the plant's colors, flavors and toxicities; of which alkaloids play an essential role.

1.1.1 Alkaloids

Alkaloids are one of the most important nitrogenous organic compounds which are produced by large varieties of organisms including plants, and to a lesser extent, microorganism and animals (Samuelsson, 1999). They mostly invoke bitter taste, while many of them are poisonous thus protecting the plants from herbivores (Rhoades, 1979). Alkaloids are believed to exhibit a broad range of physiological activities comprising antimicrobial, antiviral, antiparasitic, antioxidant, as well as cytotoxic and neurotoxic actions in animals and humans. In view of these, their presence in foodstuffs and medicines has attracted increasing interest around the globe. The free-base alkaloids are soluble in organic solvents but not in water; however, the protonation of the nitrogen in the free-base form usually results in water-soluble alkaloids. Besides, their ability to form salts with acids and to complex with metal ions facilitate their isolation and purification in plant material, which in turn form the basis of their selective isolation by liquid/liquid partitioning processes (Zanolari et al., 2003).

Alkaloids are divided, according to their structures and biological origins, into three main groups: (1) true alkaloids, (2) protoalkaloids and (3) pseudoalkaloids (Aniszewski, 2007). However, pyrrolizidine alkaloids (PAs), which are of particular interest due to their role in humans and livestock toxicities, fall under the true alkaloids.

1.2 Pyrrolizidine Alkaloids (PAs)

1.2.1 Occurrence

One important and well-known class of naturally occurring toxin-alkaloid in foodstuffs/herbal products is the PAs. PAs are a large group of plant-derived secondary metabolites that are produced all over the world by about 6000 or 3 % of the world's flowering plants (Smith & Culvenor, 1981) as protection against herbivores. They are mainly produced by plant species within the following families: Boraginaceae (*Heliotropium*, *Trichodesma*, *Symphytum*, and many other genera), Asteraceae (*Senecio*, *Eupatorium*, and other genera of the tribes Senecioneae and Eupatoriae) and Fabaceae (genus *Crotalaria*). But less frequently in Apocyanacae and Scrophulariaceae (genus *Castilleja*), Orchidaceae, Convolvulaceae and Poaceae (Smith & Culvenor, 1981; Stegelmeier et al., 1999). The PA-content of plant material depends on a large number of factors, notably plant species, age and organs, as well as the surrounding climate. Also, some insects, particularly certain moths and butterflies, accumulate the toxic PAs from plants, which they can then use as a defense against predators.

1.2.2 Chemistry of PAs

PAs are composed of two-fused five-ring structures (necine base) which share a common nitrogen at position 4, and their associated acid moieties, necic acids (Röder, 2000; see Figure 1-1). The necine bases may either be saturated or possess a double bond at the 1,2-position (Röder, 1995); whereas the latter is potentially hepatotoxic and/or carcinogenic (Mattocks, 1986), the former is relatively non-toxic. The necine bases are normally esterified with necic acids at position(s) C-7 and/or C-9 to form either monoester, open

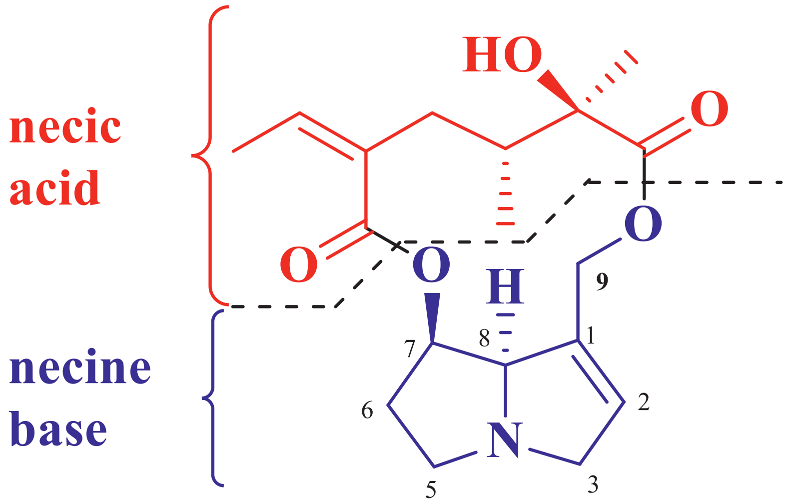
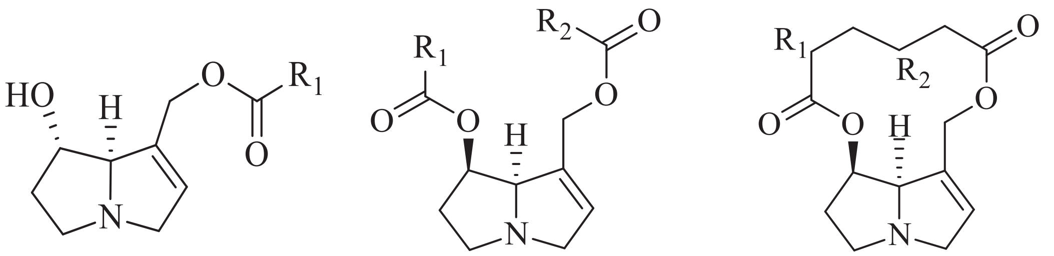


Figure 1-1. General PA structure showing necine base and necic acid

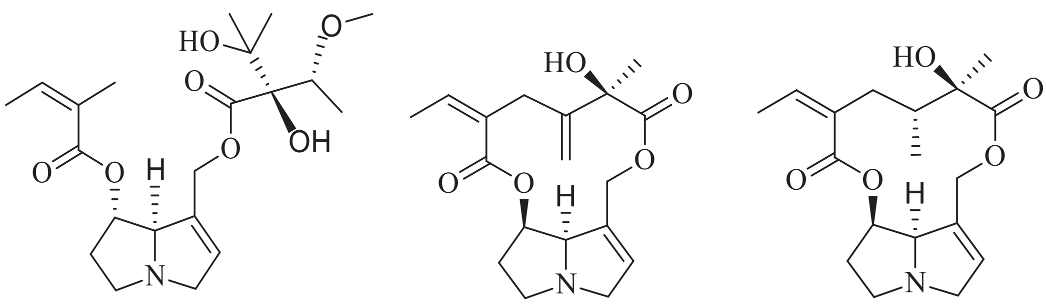
chain diester or, more frequent and most diverse class, macrocyclic diester PAs (Röder, 2000; see Figure 1-2). Generally, macrocyclic PAs are more toxic than diester PAs, which are also more toxic than monoester PAs. Most of the naturally occurring PAs are derived from the several variations of the necine bases: retronecine, heliotridine, platynecine and otonecine (see Figure 1-3). The otonecine is *N*-methylated necine base



Monoester PA Open chain diester PA Macrocyclic diester PA

Figure 1-2. PA structures showing the three main variations of the necine bases

(EFSA, 2007), while the platynecine-type PAs are considered non-toxic (Hartmann & Witte, 1995). Retronecine and heliotridine, on the other hand, are diasteroisomers at the C-7 position (Mattocks, 1986) and are considered to be toxic, similar to otonecine. Unlike the free PAs, their corresponding PA *N*-oxides (PANOs) are charged molecules which are, to a certain degree, soluble in water, but also in polar organic solvents. In addition, they are salt-like molecules and are generally considered non-toxic. Moreover, the reduction of PANOs and *N*-oxidation of PAs can occur naturally in the presence of chemicals/biological reducing agents (Hartmann & Toppel, 1987) and enzymes, respectively.



Lasiocarpine (Heliotridine) Seneciphylline (Retronecine) Senecionine (Retronecine)

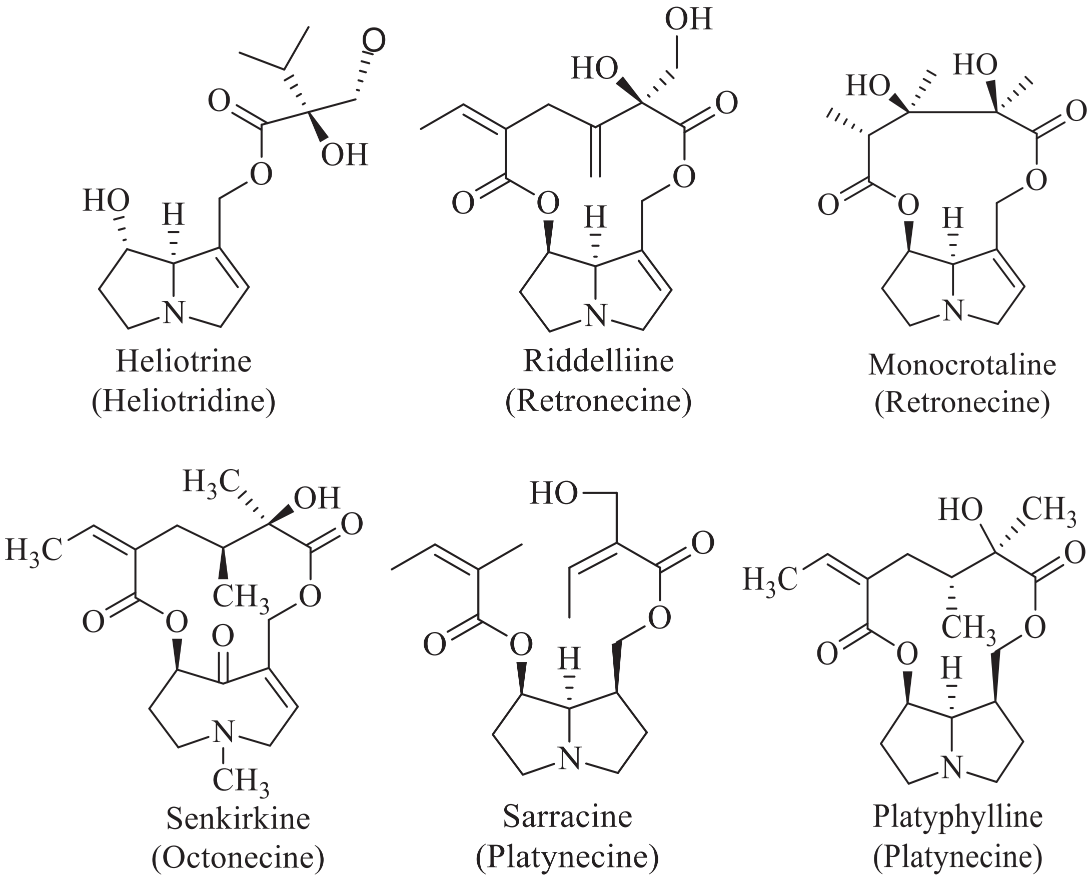


Figure 1-3. Structures of the four main necine base-type PAs

However, in humans and mammalian gut, they may be reduced to tertiary alkaloids and passively taken up into the blood and then the liver, which may potentially result in hepatotoxicity. The basic structure of hepatotoxic PAs is shown in Figure 1-4. The minimum structural requirements for toxicity are:

(1) a double bond in the 1,2 position of the pyrrolizidine ring;

(2) a hydroxyl substituent at the C-9 position and/or at the C-7 position(s);

(3) at least one of the hydroxyls must be esterified;

(4) the necic acid moiety has a branched chain (Mattocks, 1986).

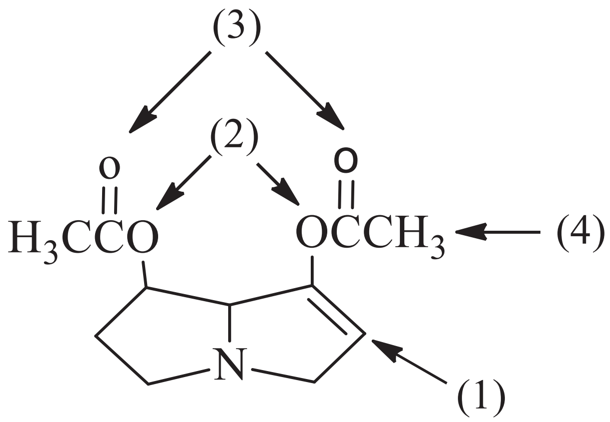


Figure 1-4. Requirements for hepatotoxicity of PAs
Modified according to Prakash et al. (1999)

1.2.3 Biosynthesis of PAs

PAs are synthesized in plants during amino acid metabolism in which the necine base is biosynthesized by the combination of either *L*-ornithine or *L*-arginine or both to form two molecules of putrescine, and proceeds *via* homospermidine. In contrast, necic acids are synthesized from amino acids such as *L*-valine, *L*-leucine, *L*-isoleucine and *L*-threonine by a route demonstrated by Röder (1995). The site of this process depends on the plant family. For example the site of PA biosynthesis within the Asteraceae (e.g. *Senecio* and *Eupatorium* spp.) lies in the roots (Hartmann, 1994), whereas in Orchidaceae, root tips have been identified as the site of PA biosynthesis (Frölich, 1996). However, within the Fabaceae, they have been reported to be synthesized exclusively in the shoots (Hartmann & Witte, 1995), but occurs within the shoots and roots of Boraginaceae. Generally, PAs are synthesized mainly as polar PANOs in the roots of most of the PA producing plants. Since these compounds are much more water-soluble than the free base, they are transported *via* the phloem of the plant to the younger leaves and flowering parts where they accumulate (Hartmann, 1999) and act as deterrent to herbivores. The PANOs are usually unstable, hence they are easily converted into the free base in the presence of weak reducing agents (Hartmann & Toppel, 1987).

1.2.4 Contamination of foodstuffs and herbal products with PAs

The exposure of humans to PAs and the potential poisoning thereafter is of great interest as it can take many forms (i.e. ranging from acute to chronic illness). Humans are usually exposed to PAs through plant products (e.g. herbal products or contaminated grain crops) or animal-derived products (e.g. honey, milk, eggs and meat) (ANZFA, 2001; WHO, 2011). PA contaminations of these foodstuffs are through several channels; including co-harvesting PA weeds with edible grains or medicinal plants, livestock grazing on PA plants, *via* chickens feeding on contaminated feeds or honey bees foraging on PA-producing plants. In general, plants that contain PAs are usually avoided by grazing animals due to their bitter taste, but there are reports that sheep and goats have some tolerance to these plants (Molyneux & James, 1990). Nevertheless, the risk of ingestion by grazing livestock on PA-containing plants is considered to be low under normal circumstances. However, in regions where there are severe and persistent drought or when PA-plants are accidentally harvested and mixed with forage, they are likely to be consumed and may be hepatotoxic to these animals (Shimshoni et al., 2015).

1.2.4.1 Grain

When wheat or other cereal grains are harvested, they are sometimes contaminated with PAs which are in turn incorporated into the flour during milling. Outbreaks resulting from PA-contaminated cereal grains have occurred in Africa, Central and South Asia, Caribbean and India (Molyneux et al., 2011). The first recorded case of human disease was reported in 1920 in South Africa where multiple cases of cirrhosis occurred following consumption of bread made from flour contaminated essentially with PA plant, *Senecio burchellii* (Willmot & Robertson, 1920). Since then, many more reports of human PA poisoning have occurred, including one particularly devastating outbreak, in which an estimated 35,000 people were exposed. This occurred in northwestern Afghanistan in 1976 following a two-year drought and was due to the contamination of bread with seeds from *Heliotropium* spp. (Kakar et al., 2010). Likewise, the contamination of local grains with seeds of *Crotalaria* spp. was found to be responsible for the death of 28 patients from 67 people affected in four villages in India (Tandon et al., 1976). Another similar but more recent outbreak occurred in Tajikistan in 1993 which was as a result of the contamination of bread with *Heliotropium lasiocarpum,* and led to about 3,906 reported cases of hepatotoxicity with a case fatality rate of 1.3% (Kakar et al., 2010).

1.2.4.2 Honey and Pollen

Human exposure to PAs from contaminated honey and pollen products can cause chronic diseases, such as liver cirrhosis, pulmonary hypertension and cancer (Edgar et al., 2011). PA content in honey from various plant species and countries has been measured and reported by several investigators at concentrations ranging from 1 to 13019 µg/kg of honey (Beales et al., 2004; Betteridge et al., 2005; Kempf et al., 2010; Dübecke et al., 2011). Even though PA contamination of honey has commonly been associated with nectar, pollens which are also actively collected by bees to make honey have also been shown to be the primary source of PA contamination (Edgar et al., 2011). Pollens from PA-containing plant species have been detected in honey emanating from the genera *Echium, Senecio*, *Eupatorium*, *Heliotropium*, *Borago*, *Myosotis*, as well as *Chromolaena*, *Petasites*, *Ageratum*, *Cynoglossum*, *Tussilago* and *Symphytum* (Beales et al., 2004; Betteridge et al., 2005; Edgar et al., 2002; Röder, 1995; Dübecke et al., 2011). In view of this, Boppre et al. (2005) suggested that in instances where honey is contaminated with PAs, the plant species responsible is usually the main nectar/pollen source in the locality of the apiary.

1.2.4.3 Milk

The ability of PAs and/or PANOs, consumed in feed or during grazing, to transfer into milk is well-known (EFSA, 2007). However, the more water-soluble PANO, which is the dominant form of PAs in plants, have been found to be more readily transferred into milk (Molyneux & James, 1990) even though the transfer tend to be quite low. Nevertheless, PAs had been found in human milk during PA-poisoning epidemics while cases of hepatic veno-occlusive disease (HVOD) have occurred in infants (ANZFA, 2001). Some other reports have also detected PA concentration in milk between 9.4 and 16.7 µg/100 mL (Dickinson et al., 1976) and between 0.05 and 0.17 µg/L (Mulder et al., 2015). Notwithstanding this, Molyneux and James (1990) have suggested that PA-contamination of commercial cow milk is unlikely to pose a significant health risk due to extensive mixing of these products from many sources, which results in the dilution of the PAs.

1.2.4.4 Eggs and Meat

There have been reports of poultry being contaminated with PAs (Peterson & Culvenor, 1983; ANFSA, 2001). For instance, PAs have been isolated from eggs at concentrations (1.15 to 9.7 µg/egg), while PA levels in Australian eggs were found ranging from 0.10 to 168 µg/kg (Edgar & Smith, 1999; ANZFA, 2001; Mulder et al., 2015).

PAs in meat of animals fed with PA-containing plant/feed before slaughter have been reported (ANZFA, 2001; EFSA, 2007), and the possibility of toxicity being caused through this medium was considered to be low, if non-existence. Report indicates that PA levels from 10 to 73 mg/kg have been found in the liver and kidney of domesticated animals (ANZFA, 2001), while a recent study detected no PAs in about 273 analyzed meat products (Mulder et al., 2015).

1.2.4.5 Herbal Products

The exposure of human to toxic PAs may be as a result of intentional or unintentional consumption of plant species of the genera *Echium*, *Borage*, *Anchusa*, *Symphytum* (comfrey), *Alkanna*, *Cynoglossum*, *Heliotropium, Jacobaea,* including *Tussilago*, *Erechthites*, *Lithospermum*, *Brachyglottis*, *Cineraria*, *Petasites, Senecio* and *Crotalaria* *via* the consumption of herbal medicinal products (Röder, 1995; Röder, 2000; Mulder et al., 2015). Consequently, their consumptions have resulted in adverse clinical signs in infants (Sperl et al., 1995) and adults (Ridker et al., 1985), while the use of comfrey, in particular, has been restricted in most countries due to its potential hepatotoxicity (EFSA, 2007). Total PA contents from 0 to 3430 µg/kg dry matter have been detected in the herbal tea and tea samples, including fennel tea, chamomile tea, peppermint tea, nettle tea and melissa tea, which could potentially be hepatotoxic when consumed for a longer-term (BfR, 2013; Mädge et al., 2015).

1.2.5 Toxicity and Metabolism

1.2.5.1 General toxicity

PAs and PANOs are pro-toxins which are activated *in vivo* by the CYP monooxygenases, hence may result in hepatotoxicity (Johnson et al., 1985), pneumotoxicity (Stalker &