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„ Aspects of organ-specific alkaloid distribution in *Catharanthus roseus* from Thailand“

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Massah Massaquoi

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1. Abstract

The ornamental plant species *Catharanthus roseus* G. Don (Magdagascar perinkle; Apocynaceae) collected in Thailand was studied for its alkaloid composition. Phytochemical investigations, applying mainly high performance liquid chromatography (HPLC) with UV-diode array detection, and thin layer chromatography (TLC) with UV detection at 254 respective 366 nm and subsequent derivatization by Dragendorff's reagent, led to the isolation and identification of the diastereomers alstonine and serpentine from the methanolic root extract. Analytical comparison by HPLC and TLC of the crude extracts obtained from pinkish and white flowering individuals did not show any differences, therefore individuals of both morphs were pooled. Comparative analyses of the methanolic extracts from leaves, stems and roots revealed similarities in the accumulated compounds of the roots and stem extracts whilst the leaf extract showed clear differences. These data indicate a clear differentiation in the alkaloid composition of the studied organs. Investigations of the exudates obtained by dipping leaves in acetone for a short time did not show the presence of monoterpene indole alkaloids on the leaf surface as reported by Roepke et al. (2010). Additionally, acid-base extraction of the methanolic crude extract from stems showed the sensitivity of serpentine against a higher pH value. This experiment led to a reduction of the amount of this alkaloid whereas the amount of alstonine increased. This result suggests that application of acid-base extraction for gaining a crude alkaloidal fraction may not always be the best choice because of the possibility of formation of artifacts. The results from anti-feedant assays using neonate larvae of the insect pest cotton leafworm *Spodoptera littoralis* Boisduval (Lepidoptera, Noctuidae) tested in concentrations between 250 and 2000 ppm of the crude extracts revealed a clear growth reduction of the larvae by the leaf extracts. The calculated values of the highest tested conc. ranged between 74% after four and 97% after seven days. In contrast, the growth reduction of the larvae by the root and stem extracts at 2000 ppm ranged between 31% for the root extract and almost 12% for the stem extract after four days. After seven days these values increased up to 52% for the root extract and 41% for the stem extract. Increased lethality could not be observed during this experiment. These data indicate the presence of compounds with strong anti-feedant properties in the leaf extract and also-with some restrictions- in the stem and root extracts. Overall, the obtained results are in accordance with reported literature.

2. Introduction

Plants are an important source of chemical compounds, the so-called secondary or specialized metabolites. These phytochemicals are derived from the primary metabolism by enzymatic modifications. Basically, metabolites derived from the primary metabolism are necessary for growth and development of plants whereas specialized metabolites are important for interactions of plants with the environment. According to Ehrlich & Raven (1964), the structural diversity is one of the results of the so-called "co-evolutionary arms race". This process describes a reciprocal process involving the evolution of structural modifications of phytochemicals as reaction to herbivory, and subsequent and the changes in herbivorous organisms in order to adapt with these changes, leading again to a change in the plant's chemistry. Thus, this "co-evolutionary arms race" results in an uncountable number of specialized metabolites which are divided in classes of compounds, mostly according to their biosynthesis. Interestingly, these compounds do not occur arbitrarily in the plant kingdom, they rather show a distinct pattern of distribution which is reflected by the term chemotaxonomy (Singh et al., 2019).

Historically, research in this field was focused on medicinally important plant species due to their use as remedies for many diseases. The use of plant-derived remedies is still of importance for many people, especially in underdeveloped countries whereas in well-developed countries the discovery of novel chemical structures is an essential factor. To date, research on biological functions of such compounds become more significant because of their interaction role of organisms.

The aim of this study was to identify constituents in the leaves, stems, and roots of *Catharanthus roseus* (L.) G. Don (Figure 1) collected in Thailand, their organ-specific accumulation, and to reveal anti-feedant or insecticidal properties of the extracts. Furthermore, experiments testing the hypothesis of occurrence of *Vinca* alkaloids on the surface of leaves as proposed by Roepke et al. (2010), and also testing the stability of a plant extract against acid-base extraction were performed.

Figure 1. Habitus of *Catharanthus roseus* (Photo: S. Vajrodaya).

2.1. Apocynaceae Juss.

The plant family Apocynaceae, also known as dogbane family, is listed together with Rubiaceae, Gentianaceae, Loganiaceae and Gelsemiaceae in the order Gentianales (www.mobot.org, retrieved 20200219). This taxon is one of the largest angiosperm families and comprises around 4,500 species classified in five subfamilies and 370 genera (Chase et al., 2016; Endress et al., 2014; Fishbein et al., 2018). Species of this plant family are mostly distributed in tropical and subtropical regions, but some members also occur in mediterranean and temperate regions (Figure 2). According to AGP IV there are five subfamilies are Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae and Asclepiadoideae (Figure 3). Members of this family usually have simple leaves, milky sap, five-numbered calyx, five large petals joined at the base and five stamens (Retna & Ethalsa, 2013). This family comprises species with a wide range of growth forms, such as trees, lianas, vines, cacti-similar succulents as well as herbs. Members of this family show a combination of distinct flower characters and the presence of latex. Based on these hallmarks Apocynaceous species are easily distinguishable from other species listed in the order Gentianales.

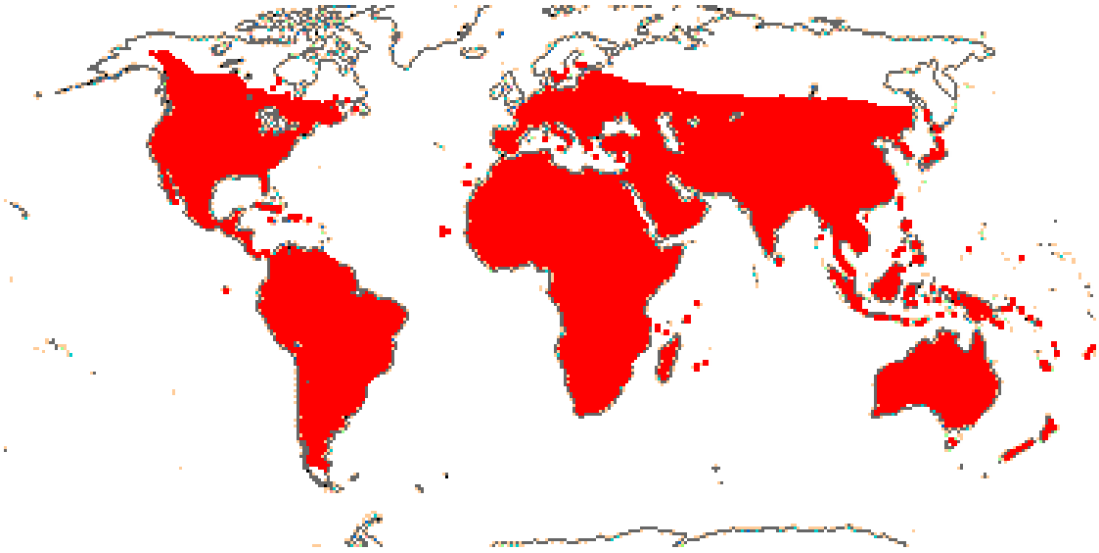

Figure 2. Red marked areas show the distribution of Apocynaceae (http://www.mobot.org/MOBOT/research/APweb/ retrieved 20200302).

Morphology: The leaves are simple and mostly opposite. Inflorescences are mostly cymose and arranged either terminal or axillar. The flowers are pentamerous and rarely tetramerous and actinomorphic. Sepals often possess hairy structures (colleters) and the corollas are sympetalous with overlapping lobes. The ovaries are mostly superior with two separate carpels fused into one

style. The fruits are drupes, berries, capsules, or follicles. The seeds are winged and wrapped in an aril.

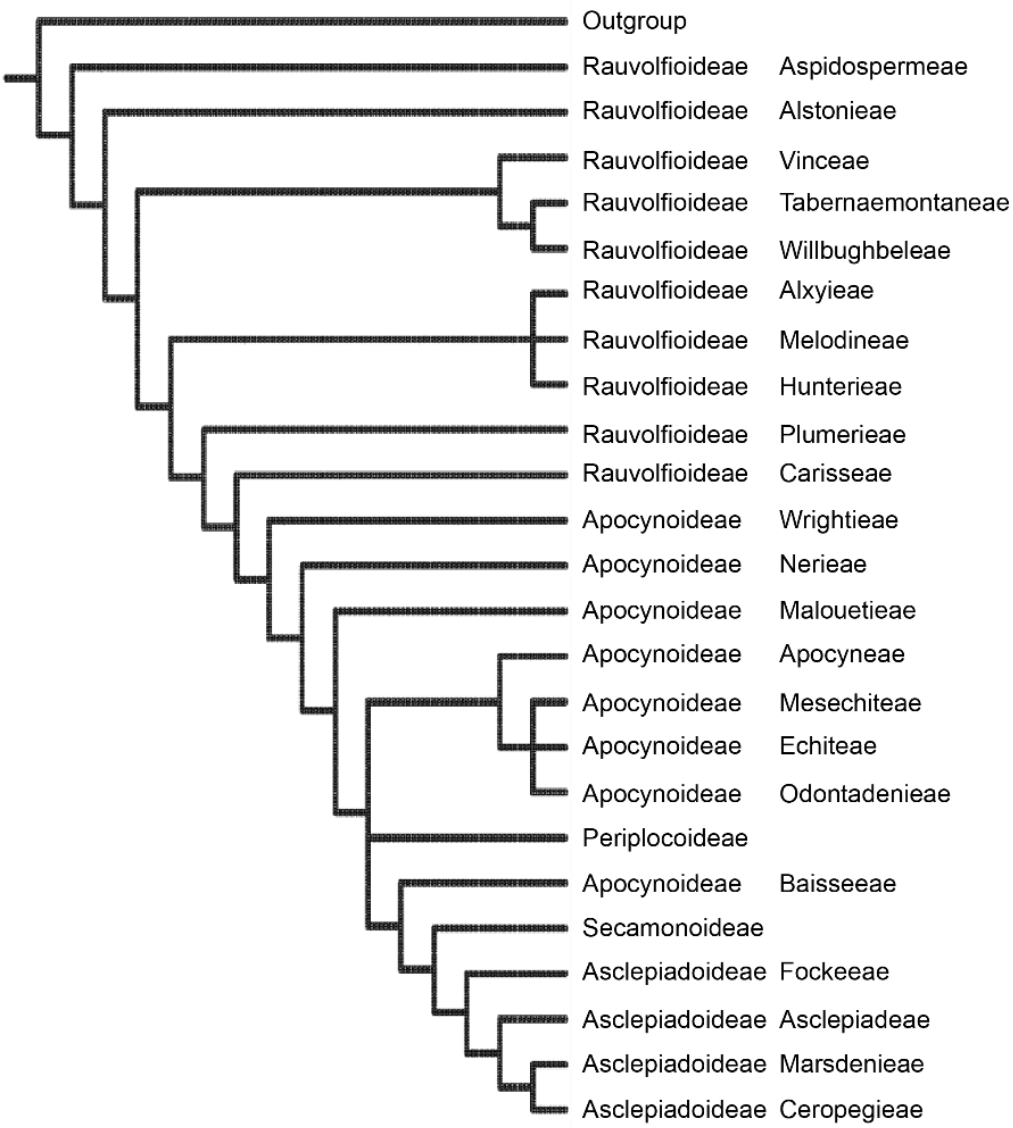

Figure 3. Cladogram showing the relationships between the five subfamilies and twenty-two tribes of Apocynaceae based on phylogenetic studies using data from Rapini et al., 2007; Simoes et al., 2007; Livshultz, 2010. Modified from Nazar et al. (2013).

Phytochemistry: On the subfamily classification level, this plant family shows some differentiation in the accumulation of secondary metabolites. Thus, indole-derived alkaloids are mainly present in members belonging to the subfamily Rauvolfioideae like in the genera *Catharanthus*, *Tabernaemontana*, *Alstonia*, and *Vinca* and many more taxa (Dey et al., 2017). Within these taxa, the *Tabernaemontana* species are a rich source of structurally rather complex monoterpene indole alkaloids (Wu et al., 2019; Yu et al., 2019). In contrast, members of the subfamily Apocynoideae are mostly accumulating cardiac glycosides, triterpenoid-derived glycosides, which cause the toxicity of e.g. *Nerium oleander* L., an ornamental shrub. Further known compounds are flavonoids and other phenolics (Bhadane et al., 2018). Within the subfamily Apocynoideae, the tribe Wrightieae is chemically quite different. *Wrightia* species accumulate certain indole-derived compounds, named as benzoxacinoids (Traxler et al., 2020, submitted). These compounds are structurally related to the dye indigo, but they are not biosynthesized from these pathways leading to monoterpene indole alkaloids. Structural formulae of some of these compounds are given in Figure 4. Tabernabovine A as an example of monoterpene indole alkaloids isolated from *Tabernaemontana bovina* (Yu et al., 2019), the toxic cardiac glycoside oleandrin from *Nerium oleander* (https://pubchem.ncbi.nlm.nih.gov/compound/oleandrin,retrieved 20200303) and the benzoxacinoid derivative blepharin from *Wrightia religiosa*). Glc refers to glucose.

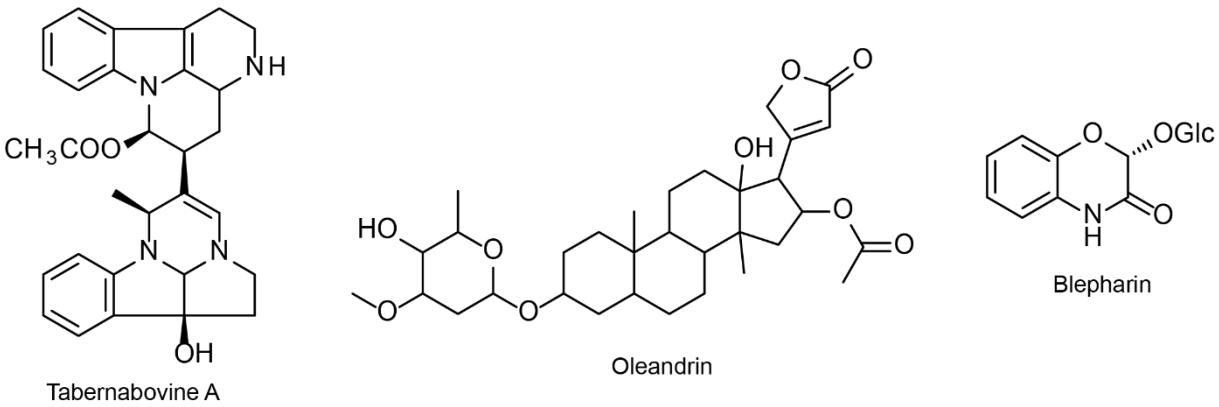

Figure 4. Chemical compounds known from Apocynaceae species. Tabernabovine A as an example of monoterpene indole alkaloids isolated from *Tabernaemontana bovina* (Yu et al., 2019), the toxic cardiac glycoside oleandrin from *Nerium oleander* (*https://pubchem.ncbi.nlm.nih.gov/compound/oleandrin*, retrieved 20200303) and the benzoxacinoid derivative blepharin from *Wrightia religiosa* (Teijsm. & Binn.) Benth. (Traxler et al.,2020 submitted). Glc refers to glucose.

2.2. Genus *Catharanthus* G. Don

The genus *Catharanthus* (Apoycynaceae) comprises nine species, namely *C. coriaceus* Markgr. *C. lanceus* (Bojer ex A.DC.) Pichon, *C. longifolius* (Pichon) Pichon, *C. ovalis* Markgr. *C. pusillus* G. Don, *C. roseus* (L.) G. Don., *C. scitulus* (Pichon) Pichon, *C. trichophyllus* (Baker) Pichon, *C. makayensis* L. Allorge, Phillipson & Razakamal. (Arora et al., 2010), seven of them are endemic to Madagascar and one species, *C. pusillus*, occurs naturally in India and Sri Lanka (Allorge et al., 2015). This genus is classified in the monophyletic tribe Vinceae (Endress et al., 2014). Historically, Carl von Linnaeus classified *Catharanthus roseus* as *Vinca rosea* due to similarities in morphology. However, genetic analyses revealed a relationship to *Catharanthus*. Most recent phylogenetic studies, combined with morphological and phytogeographic analyses of species classified in the tribe Vinceae, indicated the relationship of *Catharanthus* with the genera *Kamettia* and *Petchia*. According to the most recent classification by Simões et al. (2016), there are eight species listed in this genus. Due to human activities the distribution area is nowadays found in subtropical regions of the world and especially in Southern United States. Currently this genus is listed in the subfamily Vinceae (www.mobot.org, retrieved 20200219).

The herein studied perennial herb *Catharanthus roseus* (Figure 1 and Figure 5) also known as Madagascar periwinkle, is an important ornamental herbaceous plant in originating from Madagascar and now widespread in the tropical and subtropical regions worldwide. This species is nowadays a famous source of the antitumor drugs vinblastine and vincristine. Previously it was listed in the genera *Ammocallis*, *Lochnera, Pervinca* and later in *Vinca*. Latter genus is eponymous for a certain group of monoterpene indole alkaloids sometimes referred to as *Vinca*-alkaloids (see below). This plant species possesses a sprawling woody base and grows is approximately up to 80 centimeters. The leaves of the plant are petiolate, oblong in shape, thick and leathery in texture, with an opposite or alternate arrangement. The flowers are single with overlapping petals. The colours of the flowers are mostly pink, white or a mixture of both with a dark violet dot in the center (Hogan, 2003; Arora et al., 2009; Das & Sharangi, 2017). The stems are cylindrical in shape, green or dark red in color, and they contain a white latex (Tolambiya & Mathur, 2016). The leaves are oval to oblong, 2.5–9.0 cm long and 1–3.5 cm broad glossy green hairless with a pale midrib and a short petiole about 1–1.8 cm long, and they are arranged in opposite pairs (Gajalakshmi et al., 2013). The leaf tip is round to acute with a tiny point extending from the midrib. Flowers are borne in leaf axills, they are either singly or paired, on very short stalks (pedicels). The sepals are narrow, 5, 2–6 mm long, usually with hairs (pubescent). The corolla has a green long narrow tube

and lobes that spread perpendicular to the tube. The anthers are attached to the inside of the corolla tube in the upper portion and enclosed within it. The fruit is a follicle, which is 2.0–4.7 cm long, with numerous small black seeds (Tolambiya & Mathur, 2016). There are two common cultivars of *Catharanthus roseus*, named on the basis of their flower color, one producing pink flower "rosea" and the other, white flowers "alba". This species shows self-compatibility, in contrast to most of the other species in this plant family (Das & Sharangi, 2017; Tolambiya & Mathur, 2016). The Madagascar periwinkle does not usually exhibit intra floral self-pollination, that is the transfer of a pollen from an anther to the stigma within the same flower. The reason for this is the physical separation between the stigma and the anthers, which is known as herkogamy, meaning the stigma is attached below the anther (Kulkarni et al., 2001; Sreevalli et al., 2000).

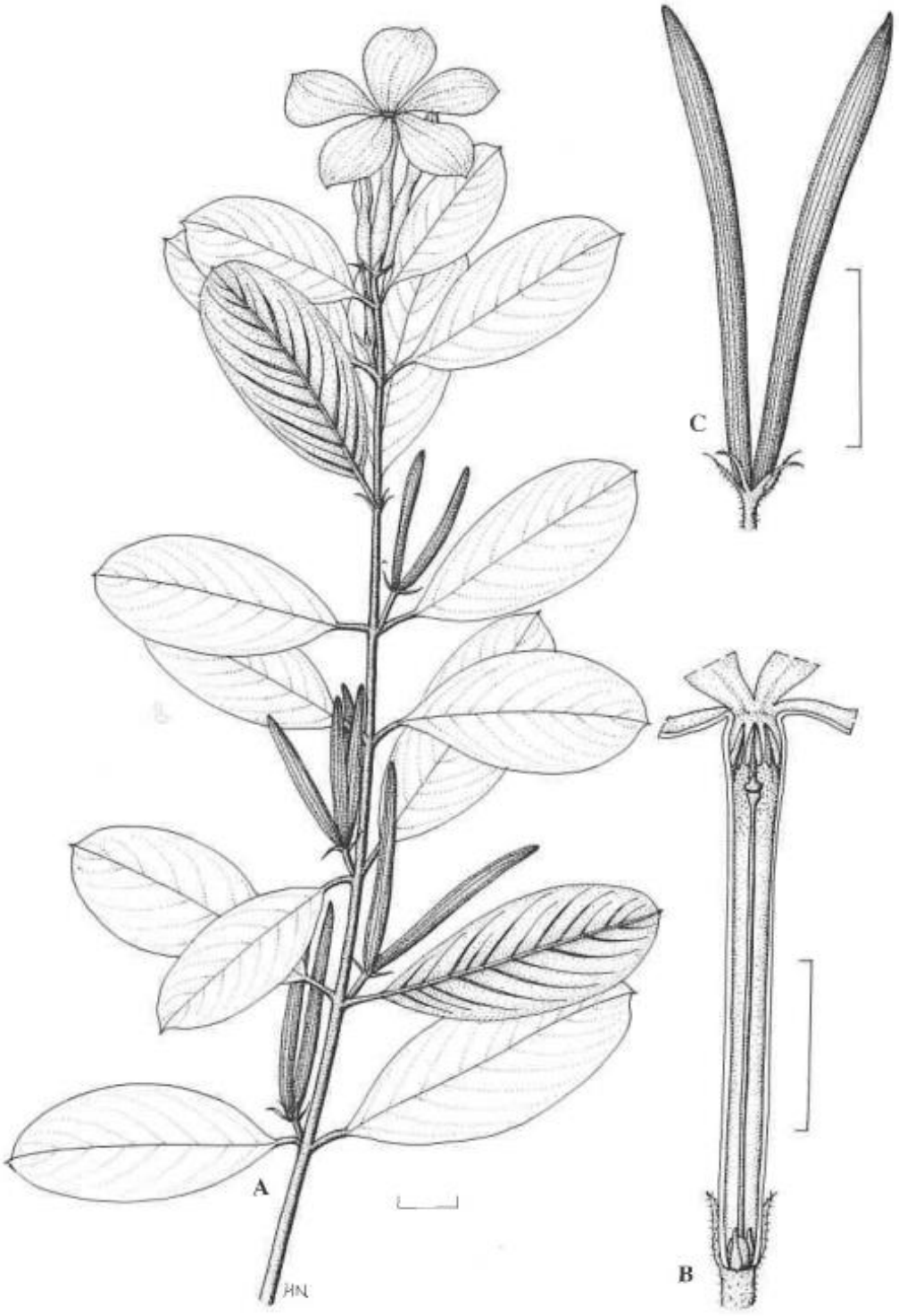

Figure 5. *Catharanthus roseus* in more detail. Modified from Middleton, 1999.

3. Phytochemistry

3.1. Secondary metabolites in general

Specialized plant metabolites are chemicals that are not essential for growth and development, but are involved in biotic interactions and interactions with the environment (Wink, 2010). According to Pyne et al. (2019) more than 200,000 secondary metabolites are known from plants.

Interactions between plants and animals is based upon chemical communication, such as attracting pollinators by emitting floral scents and seed dispersers by the color of fruits. A further important function of such compounds is their role as defense chemicals against herbivorous animals like insects, molluscs, mammals, and microbial pathogens such as bacteria, fungi or viruses (Wink, 2010). They are also important for plants to deal with environmental stresses (Labarrere et al., 2019). Apart from their ecological role, they are also important for humans for different purposes such as medicines, fragrances, flavors, nutrients, repellents, and dyes (Pyne et al., 2019).

3.1.1. Spatial segregation of compounds

To avoid self-poisoning, biosynthesis of reactive compounds is spatially segregated both, in cells and in plant tissue. Many species possess specialized cell types, the so-called laticifers, for effective transportation and storage of the milky sap. Laticifers are specialized cells with tube-like networks in the plant, which are involved in the biosynthesis of defense-related compounds and storage space of latex. The latex is released after the laticifers are damaged or ruptured. Latex consists of secondary metabolites (terpenoids, alkaloids, lignans, tannins etc.), and proteins which protect the plant against herbivores. Apart the possible presence of toxic compounds in the sap, it often also affects the feeding insects by trapping them in the coagulated sap after its exposure to air (Dussourd, 1995) According to Ramos et al. (2019) poly-isoprene serves as building block in the increasing coagulation of the latex. Nevertheless, many insect species are developed strategies to avoid such incidents (Dussourd and Eisner, 1987; Dussourd and Denno., 1991). Laticifers also play a key role in the biosynthesis, transport, and compartmentalization of secondary metabolites. For example, the laticifers of *Catharanthus roseus* are involved in the biosynthesis of monoterpenoid indole alkaloids. The proteins produced by the latex play a role in stress inducing signal cascades, e.g. the peroxidases in species of the spurge family (Euphorbiaceae) *Euphorbia characias* L. and allene oxide synthase from the rubber tree *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. (Hagel et al., 2008).

3.2. Alkaloids

Alkaloids are a structurally diverse class of compounds known from many plant taxa. Members of this class of secondary metabolites are mostly heterocylic chemical compounds, containing one or more nitrogen atoms, which are derived mostly from amino acids.

The alkaloid morphine was the first derivative isolated by Friedrich Sertürner in 1805 (Matsuura et al., 2017). Since then, more than 12,000 different structures have been described (Schläger & Dräger, 2016). Due to the structural diversity of these compounds a clear definition of alkaloids is difficult. A differentiation based on the nitrogen source allows a simple splitting of this class of compounds. This lead then to the three main groups: Proto-alkaloids, true alkaloids and pseudoalkaloids. True alkaloids and proto-alkaloids are derived from amino acids, whereas pseudoalkaloids are not derived from amino acids. According to Matsuura et al. (2017) alkaloids are predominant in higher plants, at least 25% of higher plants contain constitutively alkaloids. The structurally complexity of most of the known alkaloids enable them to act in various manners, which further lead to impressive bioactivities. This explains the broad usage of plants, or parts of plants for medicinal or other purposes especially by indigenous people. Apart from the commonly known alkaloid-accumulating taxa *Nicotiana* and *Atropa* (both Solanaceae), *Papaver* (Papaveraceae) and coffee (*Coffea arabica*; Rubiaceae) as well as further members of the Rubiaceae, especially the genus *Palicourea* (Berger et al., 2012; 2017), and also from the dogbane family (Apocynaceae) are well-known alkaloid plants. In Apocynaceae alkaloids are known mostly from following genera: *Rauvolfia* L., *Catharanthus* G. Don, *Tabernaemontana* L., *Voacanga* U., and *Alstonia* R. Br. (Matsuura et al., 2017). These genera produce monoterpene indole alkaloids (MIAs) also known as tryptamine-iridoid alkaloids. Biosynthetically, these alkaloids are derived from tryptamine as amino acid part and the iridoid glucoside secologanin. To date, more than 3,000 derivatives are known (Aniszewski, 2015; O'Connor & Maresh, 2006). Some of the alkaloids are depicted in Figure 6. Overview of important strictosidine-derived alkaloids. Modified from Stöckigt et al. (2011) are known from different plant sources. Ajmaline, reserpine and raubasine are known from *Rauvolfia serpentine* (Apocynaceae), quinine is known from the stem bark of *Cinchona species* (Rubiaceae), strychinine and *C*-toxiferine are known from *Strychnos* species (Loganiaceae), catharanthine, vinblastine and vincristine are known from *Catharanthus roseus*. Due to the medical importance of anti-tumor drugs vinblastine and vincristine, the biosynthesis of strictosidine and further derivatives well studied (Guirimand et al., 2010; O`Connor & Maresh,

2006). The following chapters focus on several aspects relating to structure, biosynthesis and function of these alkaloids.

3.2.1. *Vinca* alkaloids

Vinca alkaloids belong to MIAs and are known from *Catharanthus roseus* (*Vinca rosea*) since decades. These alkaloids were first discovered in the 1950's by Robert Noble and Charles Beer (Moudi et al., 2013) and they represent a typical chemical character of the genus. The most famous representatives of these alkaloids are the heterodimeric anticancer drugs vincristine and vinblastine (Johnson et al., 1963). These two compounds are only known from *Catharanthus roseus*. To date, more than 130 different MIAs are known from this genus (van der Heijden et al., 2004), some of these compounds are also known from members other taxa of the families Apocynaceae, Loganiaceae and Rubiaceae (Duge de Bernonville et al., 2015). All these families belong to the order Gentianales. Currently monomeric and dimeric structures of these alkaloids are known. In the study of Johnson et al. (1963) more than 30 *Vinca* alkaloids were identified from *Catharanthus roseus* (Table 1). Some of their structural formulae are given in Figure 6. The biosynthesis of such structurally complex compounds is highly complex (see the following chapters) and involves various cell organelles as well as cell types. Due to the medically importance of especially vincristine and vinblastine as antitumor drugs current research is focused on biosynthesis of these alkaloids in plants. This led hitherto to a vast number of studies describing enzymes, signaling cascades etc. A lot of effort is also undertaken in increasing the yield of the above-mentioned alkaloids in tissue cultures.

Table 1. Well-known *Vinca* alkaloids isolated from *Catharanthus roseus* according to van der Heijden et al. (2004).

compound	**organ**	
Ajmalicine	Pl, CC	monomeric
Alstonine	R, CC	monomeric
Serpentine	L, R, Sl, CC	monomeric
Lochnerine	CC	monomeric
Catharanthine	Pl, L, F, Sl, CC	monomeric
Vindoline	Pl, L, F, S,Sh	monomeric
Vincristine	PL, L	dimeric
Vinblastine	Pl, L, F, Sl, CC	dimeric
Yohimbine	PL, R, Sl, CC	dimeric
Strictosidine	Pl, R, L, F, CC	monomeric

Pl= whole plant extract, CC= cell culture, L= leaf, F= Flower, S= shoot, Sl=seedling, R= root

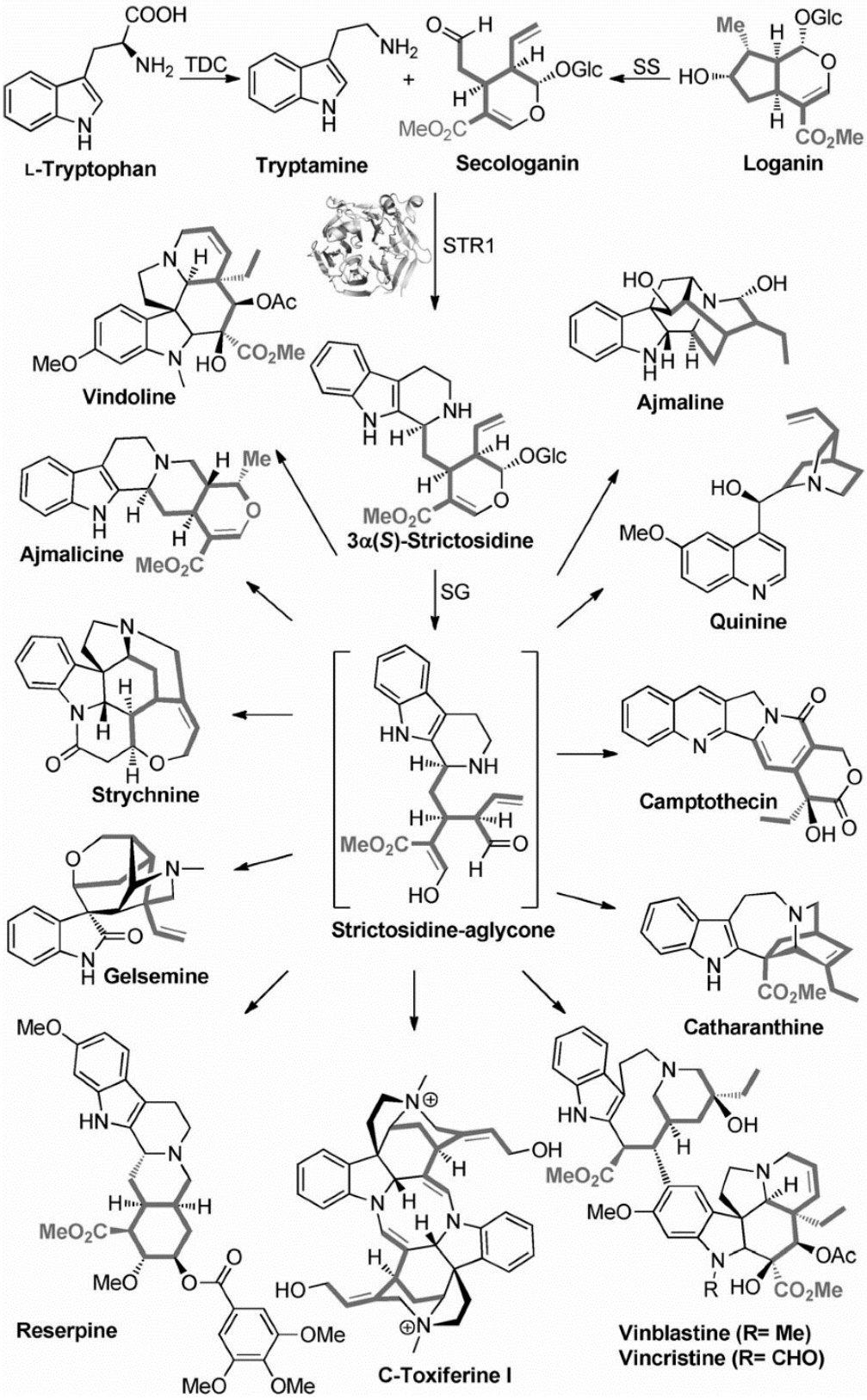

Figure 6. Overview of important strictosidine-derived alkaloids. Modified from Stöckigt et al. (2011).

3.2.2. Biosynthesis of the iridoid secologanin

Secologanin is a secoiridoid monoterpene and an important plant metabolite with anti-herbivorous properties. It is produced in a wide range of plant taxa. The biosynthesis of secologanin involves several steps as shown in the Figure 7. The methyl erythritol phosphate pathway (MEP) converts glyceraldehyde 3-phosphate (GAP) and pyruvate to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and it is localized in plastids. These steps are catalyzed by seven enzymes: 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2C- methyl-D-erythritol synthase, 4- diphoscytidyl-2C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol-2,4-cyclodiphoshate synthase, 1-hydroxy-2-methyl-2-butenyl-4-diphosphate synthase and 1-hydroxy-2-methyl-2-butenyl-4-diphosphate reductase. Isopentenyl diphosphate and dimethylallyl diphosphate are isoprenoid precursors. The interconversion of the isomers of IPP and DMAPP are catalyzed by IPP isomerase based on the needs of various tissues and organs for the terpenoid biosynthesis. These primary metabolites enter the iridoid biosynthesis first by the transfer of IPP on DMAPP catalyzed by the enzyme geranyl diphosphate synthase, leading to the formation of the monoterpene geraniol by geraniol synthase (Duge de Bernonville et al., 2015). Geraniol is subsequently converted by various steps into loganic acid in the internal phleom associated cells (IPAP). Loganic acid is transported to the leaf epidermis where it is converted to loganin and secologanin by loganic acid methyltransferase and secologanin synthase (De Luca et al., 2014; El-Sayed & Verpoorte, 2007).

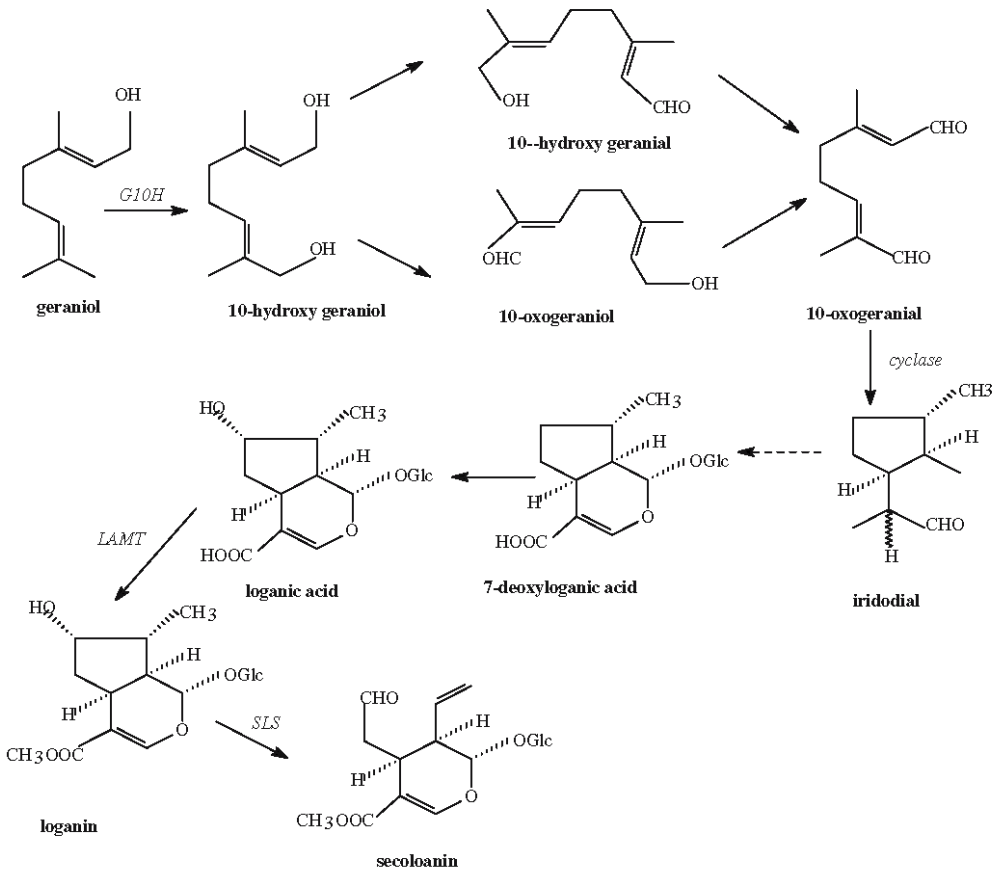

Figure 7. Biosynthesis of the iridoid secologanin starting from geraniol (modified from El-Sayed & Verpoorte, 2007).

3.2.3. Biosynthesis of *Vinca* alkaloids

The biosynthesis of *Vinca* alkaloids involves the condensation of tryptamine with secologanin, and this reaction is catalyzed by the enzyme strictosidine synthase (Duge de Bernonville et al., 2015). Three cells types, the internal phloem associated parenchyma cells IPAP; epidermis and lacticifers/idioblasts) and five subcellular compartments are involved in the biosynthesis (Duge de Bernonville et al., 2017). The first step is the formation of tryptamine from tryptophan by decarboxylation catalyzed by the enzyme tryptophan decarboxylase (TDC) (Guirimand et al., 2010). This tryptamine unit is subsequently linked with the iridoid secologanin. This reaction is catalyzed by the enzyme strictosidine-synthase. Strictosidine is the main precursor of most of the known monoterpene indole alkaloids. Figure 6, modified from Stöckigt et al. (2011), gives a simplified overview of strictosidine-derived alkaloids which are of importance for people. The enzymatic steps leading to various *Vinca* alkaloids are accomplished in various cell organelles (Table 2).

Table 2. Location of important biosynthetic enzymes within a cell according to El-Sayed & Verpoorte, 2007.

Enzyme	Abbreviation	Cofactors	Product	Localization
Anthranilate synthase	AS	Mg^{2+}	Anthranilate	Plastid
Tryptophan decarboxylase	TDC	PP, PQQ	Tryptamine	Cytosol
Isopentenyl diphosphate isomerase	IPP isomerase	Mg^{2+}, Mn^{2+}	3,3-Dimethylallyl diphosphate	Plastid
Geraniol 10-hydroxylase	G10H	Haeme	10-Hydroxygeraniol	Provacuolar membrane
NAPDH:cytochrome P450 reductase	CPR	NADPH, FAD, FMN	–	Provacuolar membrane
SAM:loganic acid methyltransferease	LAMT	SAM	Loganin	–
Secologanin synthase	SLS	NADPH	Secologanin	–
Strictosidine synthase	STR	–	Strictosidine	Vacuole
Strictosidine β-glucosidase	SGD	–	[Cathenamine]	Endoplasmic reticulum
Cathenamine reductase	CR	NADPH	Ajmalicine	–
Geissoschizine dehydrogenase	–	$NADP^{+}$	4,21-Dehydrogeis-soschizine	–
Tetrahydroalstonine synthase	THAS	NADPH	Tetrahydroalstonine	–
Tabersonine 16-hydroxylase	T16H	Haeme, NADPH	16-Hydroxytabersonine	Endoplasmic reticulum
11-Hydroxytabersonine *O*-methyltransferase	OMT	–	16-Methoxytabersonine	–
SAM:methoxy 2,16-dihydro-16-hydroxytabersonine *N*-methyltransferase	NMT	SAM	Desacetoxyvindoline	Thylakoid membranes
Desacetoxyvindoline 17-hydroxylase	D4H	2-	Oxoglutarate, Fe^{2+}, ascorbate	Desacetylvindoline
Cytoplasm				
Acetyl CoA:deacetylvindoline 17-*O*-acetyltransferase	DAT	Acetyl CoA	Vindoline	Cytoplasm
Acetyl CoA:minovincinine-*O*-acetyltransferase	MAT	Acetyl CoA	Echitovenine	Cytoplasm
Anhydrovinblastine synthase	AVLB synthase	–	Anhydrovinblastine	Vacuole

The biosynthesis of *Vinca* alkaloids starts with the formation of secologanin and tryptamine in the cytoplasm because both necessary enzymes, secologanin synthase (SLS) and tryptophan decarboxylase (TDC) are located in the cytoplasm. Both, secologanin and tryptamine are subsequently transported into the vacuole where the strictosidine-synthase is located. Strictosidine is then stored in the vacuole and for downstream processing transportation to the nucleus is necessary because the, for this process necessary enzyme, the strictosidine glucosidase, is located in the cell nucleus. A simplified overview of these steps is given in Figure 8.

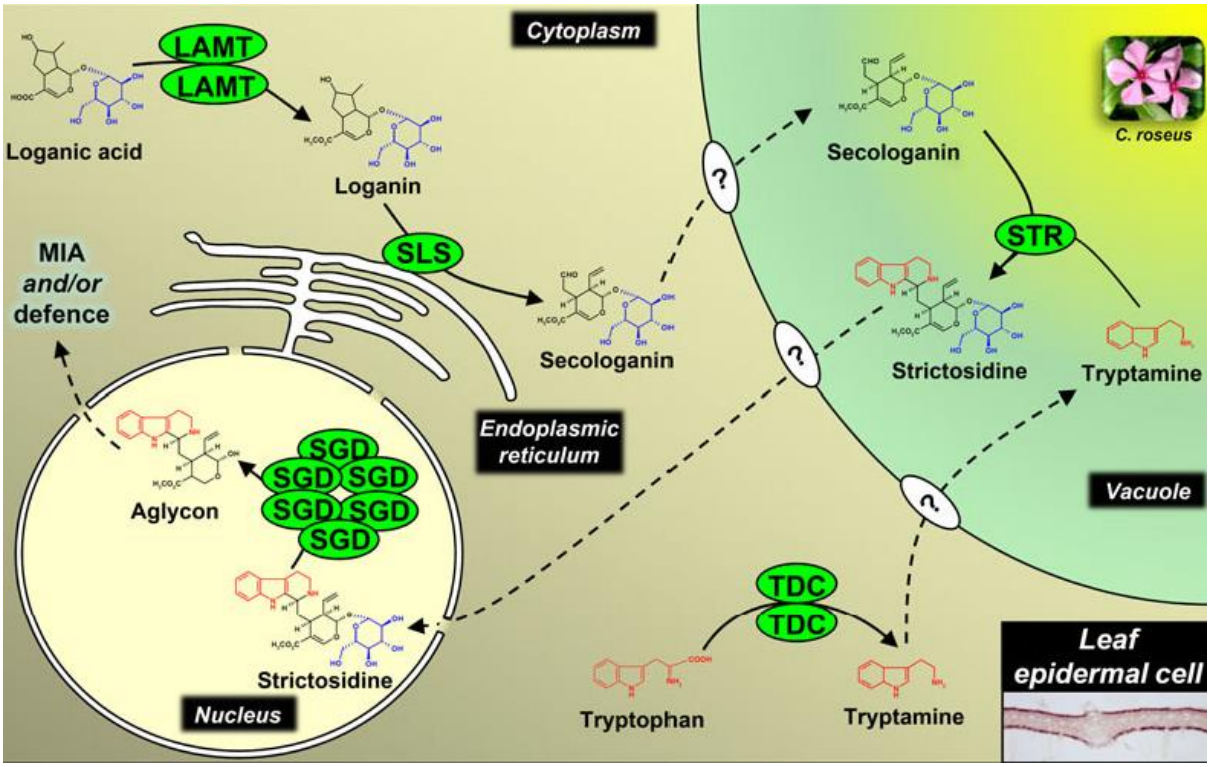

Figure 8. Simplified overview of the subcelluar organisation of the strictosidine biosynthesis. Modified from Guirimand et al. (2010).

3.3. Monoterpene indole alkaloids in plant defense

As mentioned above, alkaloids are mostly present constitutively in plant tissues. The main precursor of monoterpene indole alkaloids strictosidine (Figure 6), is present as a glucoside which is a rare condition in MIAs. Guirimand et al. (2010) showed that the sugar moiety stabilizes the molecule in intact plant tissue. Another prerequisite for keeping this system is the spatial segregation of the enzyme strictosidine glucosidase from strictosidine. This enzyme is located in the cell nucleus. In healthy plant tissues, the downstream rearrangement of strictosidine is accomplished by regulated transportation of this compound out from the vacuole to the nucleus. There enzymatic cleavages of the sugar moiety and modification of the aglycone can be accomplished by various enzymes or enzyme complexes under controlled conditions.

This is in contrast to the process going on in damaged tissues. A destroyed tonoplast leads to an uncontrolled outflux of strictosidine from the vacuole which further reacts with strictosidine glucosidase. Deglucosylation leads to a highly reactive aglycone which further easily reacts arbitrarily with peptides, proteins or other biosynthetic products in the plant cells and also after uptake by herbivorous insects in their gut (Guirimand et al., 2010). *Catharanthus roseus* exhibited two

types of reactions to defend itself when attacked by herbivores. The two types of defense mechanism are as follows:

a. Local response: This is induced faster and depends on the amount of strictosidine stored. The effective defense compound is deglucosylated strictosidine, transformed to a highly reactive dialdehyde. This reaction product may subsequently lead to proteine cross-linking and further to precipitation (Guirimand et al., 2010) (Figure 9).

b. Systemic and long-term response: This involves storage of toxic MIAs in developing organs, eventually also in unattacked plants (systemic response) (Dugé de Bernonville et al., 2017).

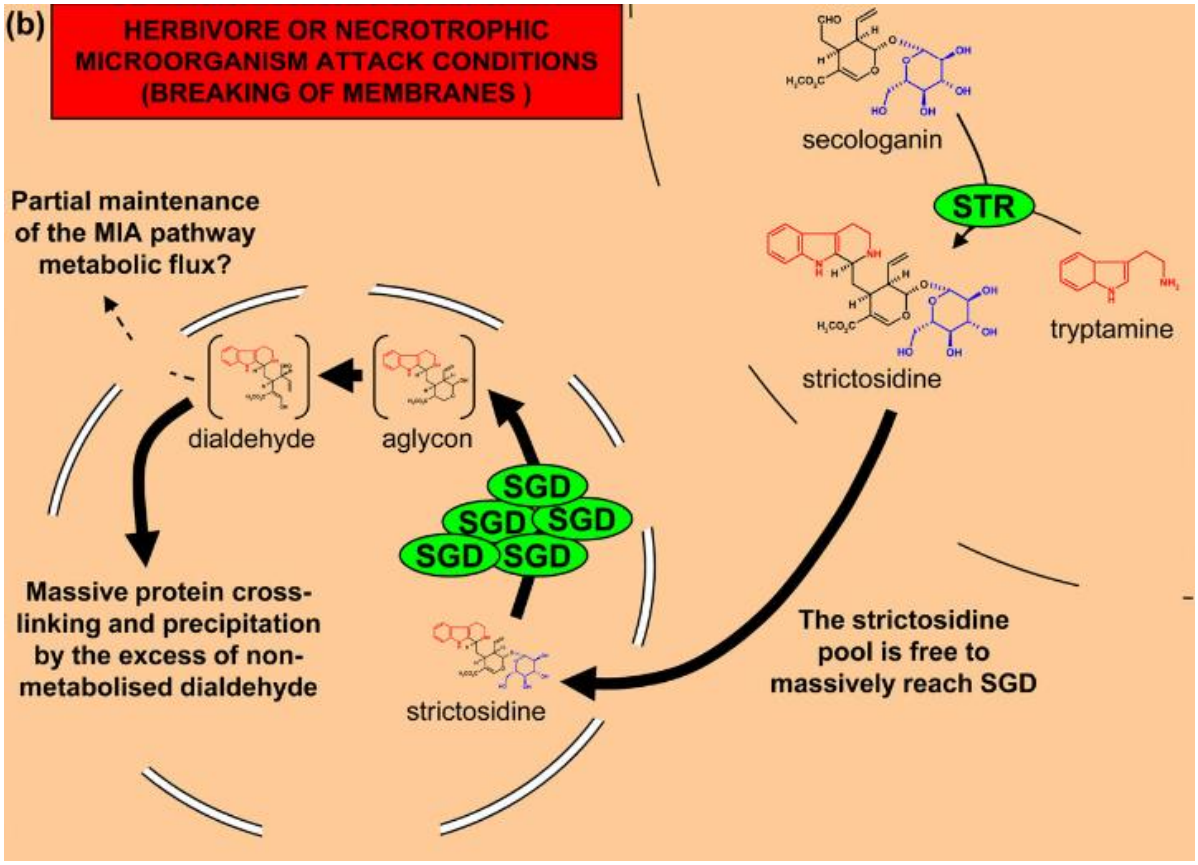

Figure 9. Local response by forming the highly reactive strictosidine aglycone (modified from Guirimand et al., 2010).

3.4. Importance of monoterpene indole alkaloids for humans

Apocynaceaeous plant species are a rich source of bioactive monoterpene indole alkaloids which are the reason why people used plant material from this family since immemorial times. Within this plant family, MIAs are significantly accumulated in species of the genera *Rauvolfia*, *Catharanthus*, *Alstonia*, and *Tabernaemontana* and some other genera as well. Their structural diversity enables them to fulfil many different, but mainly medicinally related purposes, but the mode of actions are mostly still unrevealed. Well-known examples of such alkaloids and their effects are reserpine (antihypertensive), ajmaline (antiarrhythmic), ajmalicine (antihypertensive), vincristine, vinblastine (anticancer), alstonine (antipsychotic) and cryptolepine with antimalarial activities (Dey et al., 2017). The *Vinca* alkaloids vinblastine and vincristine are the most common anticancer agents, they suppress growth of tumor cells which will lead to apoptosis (Almagro et al., 2015). Ajmalicine and serpentine are known from *C. roseus* and *Rauwolfia serpentina* and possess hypotensive effect and they are used in the treatment of Alzheimer disease (Almagro et al., 2015 Kumar et al 2016). Yohimbine from *Rauwolfia serpentina* (L.) Benth. ex Kurz is used to treat erectile dysfunction (Kumar et al 2016). Catharoseumine from *Catharanthus roseus* possesses antimicrobial activities and is used in the treatment of malaria (Almagro et al., 2015). Further examples apart from the alkaloids from members of the dogbane family are the antimalaria drug quinine from *Cinchona officinalis* L. (Rubiaceae) and the neurotoxine strychnine from *Strychnos nuxvomica* L. (Loganiaceae). Camptothecin obtained from *Camptotheca acuminata* Decne. (Nyssaceae) as well as *Ophiorrhiza pumila* Champ. ex Benth. (Rubiaceae) showed antitumor properties (De Luca et al., 2014). Some of these multiple bioactivities are summarized in Figure 10 and for some structural formulae see Figure 6. Overview of important strictosidine-derived alkaloids. Modified from Stöckigt et al. (2011).

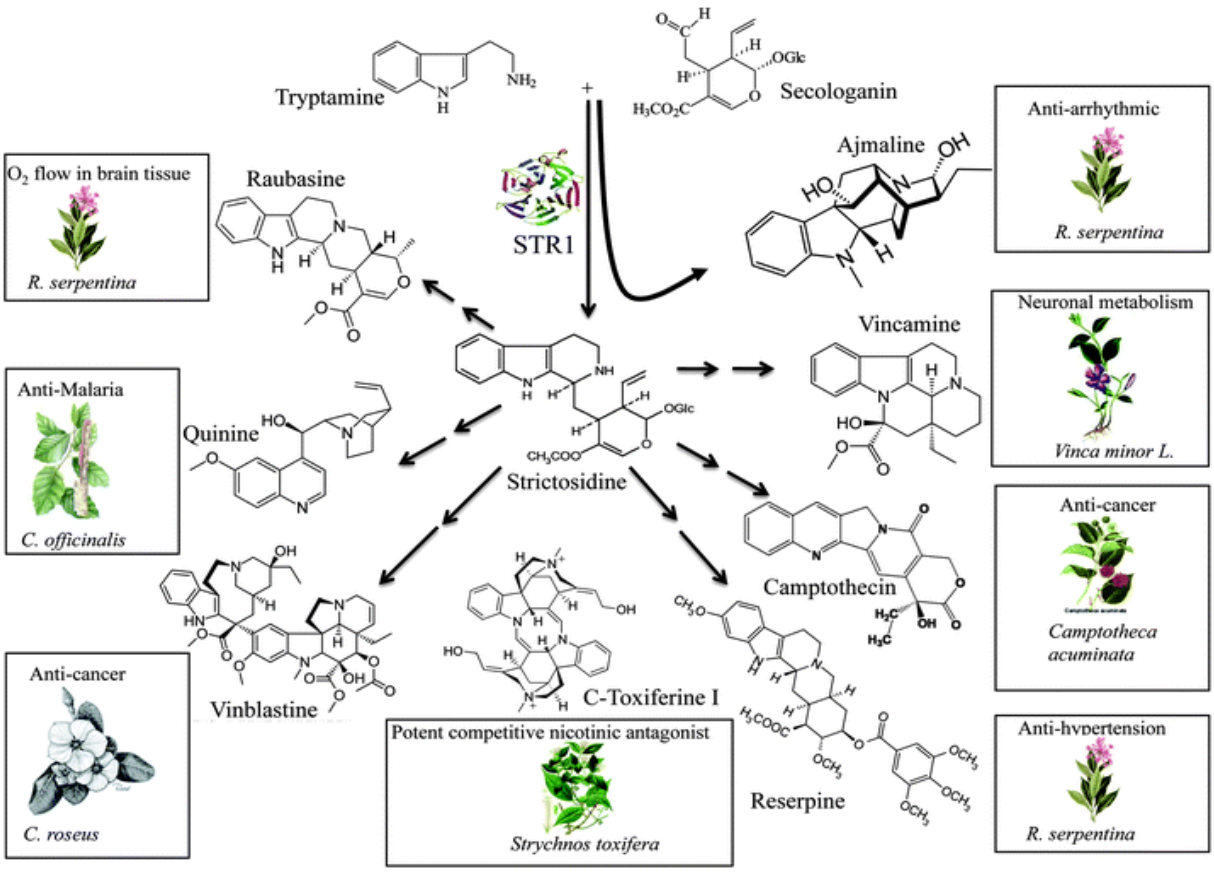

Figure 10. Summarized medicinally related bioactivities from selected monoterpene indole alkaloids (modified from Panjikar et al., 2012).

4. Material and methods

4.1. Plant material

Whole plants of pink and white flowering *Catharanthus roseus* were collected from the province of Chumphon in Thailand. For analyses, air-dried plant material was used. Chromatographic comparison by TLC and HPLC analyses revealed no differences between both morphs, therefore both were subsequently treated as one sample.

4.2. Analytical methods

4.2.1. Thin layer chromatography (TLC)

Silica gel 60 $_{F254}$ TLC plates with 0.2 mm or 0.25 mm layer thickness (Machery- Nagel/ Sigma Aldrich) were used to study the characteristics of the extract and to find ways for separation

and to control fractions after separation. Compounds with chromophoric moieties could be detected using UV light at 254 nm due to the extinction of florescence of the indicator and with UV light at 366 nm when substances show auto-fluorescence. The mobile phase was a solvent mixture of $CHCl_3$/MeOH/H_2O mixed in various volumes to obtain optimal separation. After detection under UV light, analyzed TLC plates were sprayed either with anisaldehyde reagent in order to detect substances without chromophoric group or with Dragendorff`s reagent to detect alkaloids and in general nitrogen-containing compounds in the sample.

Reagents for detection

Anisaldehyde reagent

A mixture of 85 mL methanol and 10 mL acetic acid was cooled under ice with caution, followed by adding of 8 mL conc. sulfuric acid and 0.5 mL anisaldehyde.

Dragendorff`s reagent

In beaker A, 0.85 g $Bi(OH)_2\,NO_3$ was dissolved in 10 mL concentrated acetic acid and 40 mL distilled H_2O. In beaker B, 8 g potassium iodide was dissolved in 20 mL distilled H_2O. For the usable reagent equal volumes of A and B were mixed using a magnetic stirrer. This reagent reacts with many alkaloids to orange-colored complex structures which are easy observable on TLC plates.

4.2.2. High Performance liquid chromatography (HPLC)

High Performance liquid chromatography was used for phytochemical profiling, analyzing fractions and identification of compounds by using UV spectra and retention times. The HPLC analyses were performed on Agilent 1100 series with UV- diode array detection at 230 nm. A Hypersil BDS-C18 (5 µm particle size, 4.6 x 250 mm) column was used for separation. The HPLC instrument consist of a reservoir of mobile phase, a pump, an injector, a separation column and a detector. Mobile phase: Eluent A (consisting of 15 mM phosphoric acid and 1.5 mM tetrabutylammoniumhydroxide) and MeOH (Eluent B), Flow rate: 1.0 mL/min, the injection volume was 10 uL. The wavelength of detection was set at 230 nm. The samples of the crude extracts were standardized to a concentration of 2 mg/mL to enable comparison of chromatograms.

4.3. Preparative methods

4.3.1. Liquid-liquid extraction

Liquid-liquid extraction is a basic technique to separate substances according to their polarity. Two immiscible fluids are mixed in a separation funnel and the dissolved compounds are subsequently extracted according to their solubility in the below-mentioned organic solvents. During this work, the plant extract was suspended in distilled water and the inmiscible organic solvents $CHCl_3$, ethyl acetate (EtOAc) and *n*-butanol, respectively, were added in equal volumes. Vigorous shaking of the funnel combined with patiently awaiting the separation of the layers together with five repetitions contributed to a good separation of the present compounds. Each step was repeated three to five times to ensure a good separation.

4.3.2. Column chromatography

Open column chromatography (CC) using silica gel 60 (0.04–0.063 mm) as stationary phase was used for the separation of compounds according to their polarity. A slurry out of silica gel and the starting eluent was used for the packing the column. As mobile phases were mixtures consisting of petrol ether (PE)-ethyl acetate (EtOAc) respective $CHCl_3$-MeOH at different polarity used. Their composition is given in Table 3. The sample to be separated was subjected on the top of the stationary phase and eluted with 100 mL of each solvent mixture and fractions of a volume of 50 mL each were collected. The collected fractions were combined according to the results obtained from TLC and HPLC analyses.

For size exclusion chromatography the dextrane gel Sephadex LH-20 was used as stationary phase. This stationary phase separates compounds according to their size. Smaller molecules fall into pores whilst bigger molecules cannot enter these pores, therefore smaller compounds remain longer in the column. For this separation step was MeOH used as eluent in isocratic mode.

Table 3. Composition of the eluents of column chromatography. Solvent mixtures given in A were used for the separation of the chloroform extract and in B were used for separation of the *n*-butanol extract.

A:.			**B**:	
PE	**EtOAc**	**MeOH**	**$CHCl_3$**	**MeOH**
100			100	
90	10		85	15
75	25		75	25
60	40		60	40
45	55		45	55
30	70		20	80
15	85			100
	100			
	80	20		
	50	50		
	20	80		
		100		

4.3.3. Preparative thin layer chromatography (TLC)

Merck silica gel 60 $_{F254}$ coated on glass plate (20 x 20 cm) with 0.5 mm thickness was used for preparative separation/purification. The sample was subjected as thin layer two centimeters above the bottom of the plate and developed in previous tested solvent mixtures. The separated compounds were detected by UV light and scratched off. The obtained silica gel powder was extracted with methanol, filtered using a glass frit and the purity checked by TLC and HPLC analyses.

4.3.4. Acid-base extraction of the stem extract

This experiment was performed in order to get insight whether the alkaloids present in the plant material are stable or unstable during acid-base extraction. Acid-base extraction is a widely used technique to obtain an alkaloidal-rich fraction. For this purpose, the aqueous crude extract is acidified first which leads to the formation of alkaloid ions. Extraction with chloroform removes lipophilic non-ionic constituents from this extract. Basifying of the remaining water phase by adding a base like ammonia solution transfers the alkaloid ions into their molecules which are subsequently extracted by chloroform.

4.4. Anti-feedant activities against *Spodoptera littoralis*

For getting insight into the anti-feedant properties of phytochemicals of *C. roseus* a *non*-choice feeding experiment was carried out using the insect pest *S. littoralis* Boisduval. For this purpose, food pellets spiked with different concentrations of crude extracts obtained from leaves, stems and roots of the studied plant species were prepared and freshly hatched larvae were reared for seven days. The evaluation took place four and seven days after preparation of the food pellet.

4.4.1. Rearing the larvae

Larvae of the nocturnal moth *S. littoralis* commonly also known as Egyptian cotton leaf worm, are cultivated on artificial diet consisting mainly of beans and yeast and stabilized with agar-agar (Table 4).This diet was supplemented was this diet with vitamins and *para*-hydroxy benzoic acid ethyl ester for avoiding the growth of mold (Table 4).

Table 4. Composition of the artificial diet.

Product	**amount**
White beans	150 g
Baker's yeast	30 g
Ascorbic acid	3 g
Nipagin [1]	3 g
Tap water	460 mL
Vitamin solution [2]	1.5 mL
Agar-Agar [3]	7 g

[1] Nipagin is the trivial name of *para*-hydroxy benzoic acid ethyl ester; [2] this solution consists of 2 mg myo-Inosit, 100 mg nicotinic acid, 100 mg calcium panthothenate, 50 mg riboflavin, 25 mg thiamine hydrochloride, 25 mg pyridoxin hydrochloride, 25 mg folic acid, 2 mg biotin dissolved in 100 mg destilled water; [3] the agar-agar was suspended in 280 mL tap water and boiled for 2–3 minutes. After cooling down to ca. 50 °C was this solution added to the ground mixture of beans, yeast and other compounds and further mixed for one minute.

5. Performed experiments

5.1. Extraction of the plant material

Ground rinsed roots, stems and leaves were extracted separately with methanol for 3 x 3 days at room temperature. The pooled extracts were filtered and concentrated under reduced pressure with rotary evaporator to obtain a viscous brownish residue.

5.2. Examination of exudates

Due to a previous report about the presence of monoterpenoid alkaloids in the leaves of *C. roseus* (Roepke et al., 2010.) a corresponding experiment was performed. Comparable areas of air-dried leaves were briefly rinsed with acetone and the obtained exudates analyzed by HPLC. For this purpose, the concentration of the injected samples was standardized to 2 mg/mL. These samples were also analyzed by TLC using Dragendorff's reagent for detection.

5.3. Examination of the organ-specific distribution of compounds

Comparable amounts (20 mg) of leaves, stems and roots of whitish- and pinkish-flowering individuals were ground using glass beads and a tissue-lyser (Qiagen). The powdered plant material was subsequently extracted with 500 µL methanol and subjected to sonication for 20 minutes. After centrifugation at 14,000 rpm for 20 minutes 350 µL of the supernatant of each sample separately was transferred into a HPLC vessel and analyzed. These extracts were also analyzed by TLC using Dragendorff's reagent for detection.

5.4. Isolation of plant constituents

Ground roots (19.2 g) were extracted as mentioned above. The obtained extract (2.2 g) was partitioned between $CHCl_3$ and H_2O to yield 0.33 g of $CHCl_3$ phase. The obtained lipophilic phase was separated by column chromatography (CC) over silica gel 60 (Merck, 0.2-0.5 mm) by using a solvent system of petrolether (PE), ethyl acetate (EtOAc) and methanol (MeOH) with increasing polarity (see Table 3). The fractions obtained were analyzed by HPLC and TLC. Fractions 14 and 15 were pooled and further separated over Sephadex LH-20 eluted with methanol (S1). Based on the HPLC and TLC analysis fractions S1-7 (4.8 mg) to S1-11 (6.9 mg) were pooled and purified by preparative TLC developed in $CHCl_3$/MeOH (96:4). This step yielded 0.9 mg of still impure **CR1** and **CR2** (3.3 mg).

The *n*-butanol phase (0.40 g) was first chromatographed over Sephadex LH-20 eluted with MeOH (S2). Based on the HPLC analysis fractions S2-5 to S2-10 were combined and further separated by column chromatography on silica gel 60 (Merck, 0.4-0.6 mm) by using a solvent system of $CHCL_3$ and MeOH (Table 3). The fractions obtained were analyzed by HPLC and TLC. Fraction S2-11 was further separated by column chromatography over Sephadex LH-20 to yield 3.6 mg of a fraction called **CR**. Nuclear magnetic resonance spectroscopy revealed the presence of two isomeric compounds in this sample, namely serpentine (**CR3**) and Alstonine (**CR4**).

5.5. Acid-base extraction of the stem extract

A small amount of the dried crude methanolic stem extract (2.5 mg) was suspended in 5 mL water and several drops of 2M HCl were added to reach a pH of 2. This aqueous solution was extracted three times with chloroform in a separation funnel. The remaining water phase was basified with 2 M ammonia solution (pH 9) and extracted again with chloroform. The obtained lipophilic phase was further analyzed by TLC and HPLC.

5.6. Anti-feedant activities of the crude extracts

For the performed anti-feedant assay stock solutions of the crude extracts in following concentrations were prepared: Leaves (8.7 mg/mL), stem (13 mg/mL) and roots (8.9 mg/mL). From the freeze-dried food powder, which consisted of ground beans, yeast and *para*-hydroxy benzoic acid ethyl ester, 367±1 mg were weighed, and the calculated volumes of the stock solutions added in order to reach the testing concentrations of 2000, 1000, 500 and 250 ppm. Afterwards methanol was added to the spiked food powder and the extracts evenly distributed. Then these samples were afterwards placed under the hood for 16 hours to ensure the evaporation of methanol. The next step was adding of 725 µL of solution of chloramphenicol dissolved in an aqueous solution, and of various vitamins (375 µg chloramphenicol dissolved in 22.5 µL vitamin solution and 702.5 µL destilled water; to each of the prepared samples (for composition of the vitamin solution see Table 4). These samples were stabilized with 1.1 mL of a warm (ca 50 °C) solution of 5 g agar-agar boiled in 140 mL water. The so prepared solid food pellets were afterwards transferred into petri dishes and ten freshly hatched larvae counted on each food pellet and kept in darkness at 26°C and 90% humidity. The surviving larvae were counted after four and seven days, and their weight accurately determined using the analytical balance. As basis for the assessment ten controls prepared and the averages of the weight and number of survivors set as 100%. This test was performed

in triplicate. The LD50 value and the growth inhibition rate were calculated based on the assessed data.

6. Results and discussion

The obtained results from the above-mentioned experiments are explained and discussed in detail, focusing on the points summarized below.

1) No alkaloids could be detected in the leaf exudates
2) Comparative analyses of different organs show organ-specific accumulation patterns
3) The alkaloids alstonine (**1**) and serpentine (**2**) were isolated from the stem extract
4) Serpentine (**2**) may be degraded by application of acid-base extraction

6.1. Exudates

Rinsing the leaves with pure acetone to obtain exudate did not result in weighable quantities, therefore HPLC samples were measured in a conc. range of 0.1 to 1.5 mg/mL. HPLC and TLC analyses combined with the use of Dragendorff' reagent did not reveal the presence of alkaloids in detectable amounts, although *Vinca* alkaloids in general react positively with this reagent. Prior microscopy of the rinsed leaves did not show the presence of secretory structures like glands etc. on the surface, which would be a prerequisite for secretion of compounds. The HPLC profile show two major and several smaller peaks (Figure 11). Comparison of the UV spectra of all peaks with a UV spectra database consisting of several hundred spectra did not reveal any similarities. Moreover, comparison of the UV spectra with spectra of peaks visible in the chromatograms of leaves, stem and root did not indicate the presence of *Vinca* alkaloids. These results do not confirm earlier studies claiming the presence of these alkaloids (Roepke et al. 2010). In this work, chloroform was used to gain exudates from the leaves of *Catharanthus roseus*. Abouzeid et al. (2019) proved on leaves of *Catharanthus roseus* and *Vinca minor* that chloroform destroys the integrity of cells which would lead then to a false-positive result as may have happened in the work of Roepke et al. (2010). In contrast to chloroform, acetone preserves the cell integrity when applied for a short period of time, therefore is this a proper solvent for obtaining exudates. Our result thus confirm with Abouzeid et al. (2019).

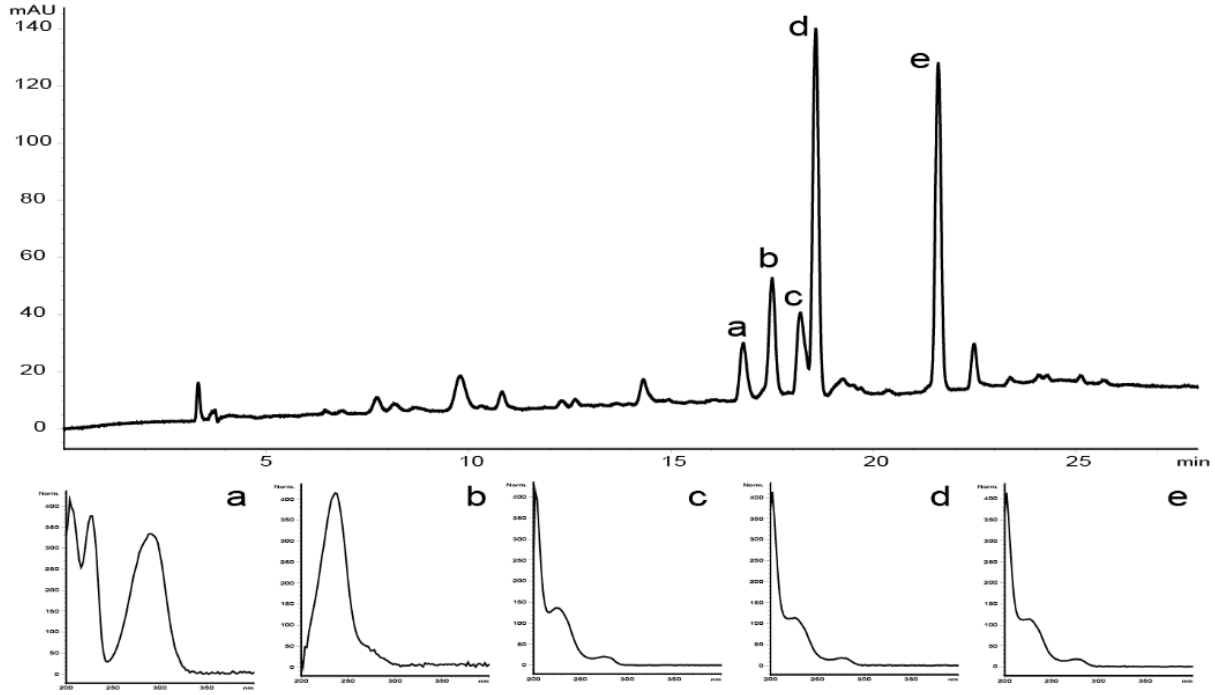

Figure 11. HPLC chromatogram and UV spectra of the most prominent peaks of the exudate.

6.2. Organ-specific distribution of alkaloids

Methanolic crude extracts obtained from air-dried leaves, previously with acetone rinsed, and of stems and roots of the studied plant species were analyzed comparatively in order to get insight into accumulation patterns of alkaloids. The chromatographic separation on TLC plates revealed clear differences between the extracts of the leaves and the other two extracts whilst the extracts of the stem and roots are quite similar (Figure 12). The leaf extract is lacking characteristic spots of compounds with chromophoric groups. Spraying with anisaldehyde reagent revealed mainly two reddish/violet lipophilic spots in the extracts of leaves and stems and much weaker in the root extracts indicating the presence of terpenoidic compounds without chromophoric groups. Application of anisaldehyde reagent also indicates the absence of iridoids, which are often present together with monoterpene indole alkaloids (Kornpointner et al., 2020). These compounds often show blueish spots after reacting with anisaldehyde reagent. The extracts obtained from the stems and roots are mainly characterized by a blue spot under 254 nm at an rf value of 0.16. The compounds responsible for this spot showed Dragendorff' positive reaction were later identified as alstonine (**1**) and serpentine (**2**) (see section 5.3. Isolation of secondary metabolites). Furthermore, a Dragendorff' positive spot appeared at an rf value of 0.9 in the stem extract. This spot is also present in the root extract. Latter extract showed also the presence of Dragendorff' positive spots

at the starting point. These spots indicate the presence of further alkaloids, which possess a more hydrophilic character than the identified compounds.

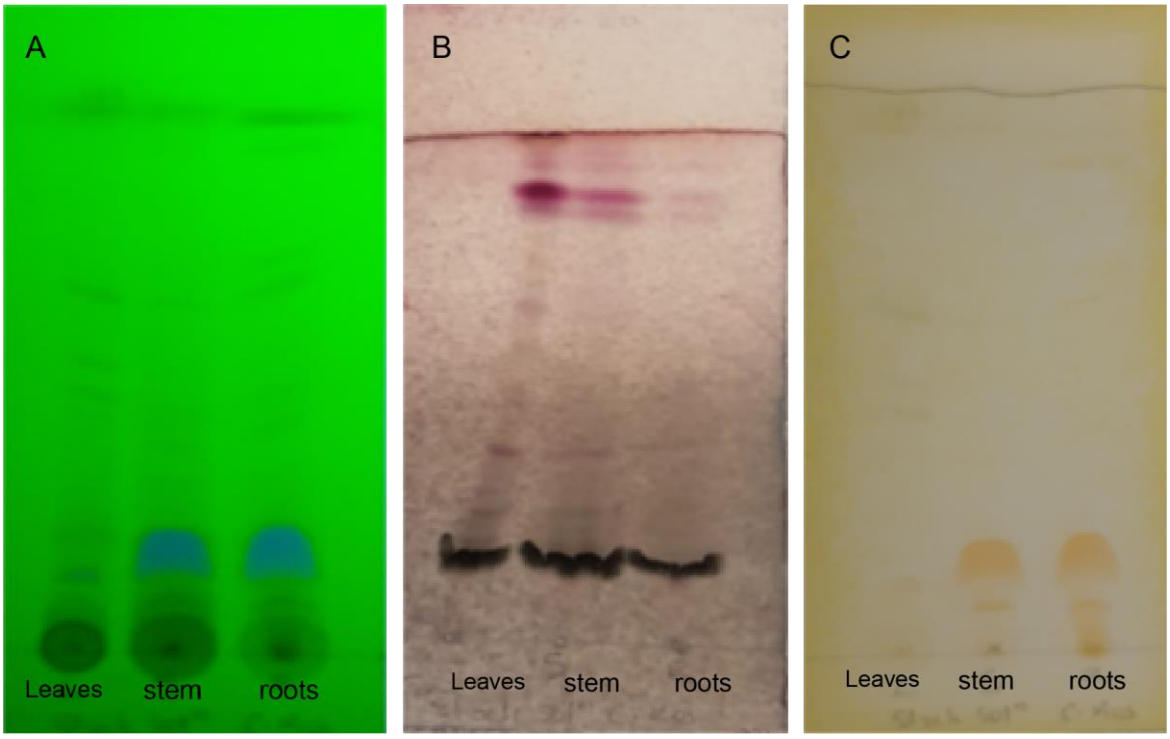

Figure 12. Comparison of crude extracts on TLC plates developed in $CHCl_3$/MeOH/H_2O (75:20:5). A: TLC plate under UV light at 254 nm; B: TLC plate sprayed with anisaldehyde reagent; C: TLC plate sprayed with Dragendorff' reagent.

The obtained HPLC chromatograms revealed interesting similarities and differences as depicted in Figure 13. The chromatogram of the leaf extract indicates the presence of the isolated alkaloids alstonine (**1**) and serpentine (**2**) at the retention times of 15.5 and 16 minutes together with more hydrophilic compounds in the retention time range of 9 to 11 minutes. These compounds show clearly different UV spectra. Comparison of UV spectra of the peaks at 10 and around 10.5 minutes indicate their nature as cinnamic acid derivatives. These compounds are present in probably all higher plant species and therefore of less characteristic value. The UV spectrum of the peak at ca. 12.5 minute indicates the presence of a quercetin glycoside. The presence of flavonoid derivatives in different organs of this plant species have been reviewed by Mustafa & Verpoorte (2007). In this review it has been shown that flavonol glycosides based upon kampferol, quercetin, syringetin etc. are present in stems and leaves. Even in hairy root cultures kampferol and Quercetin glycosides were detected (Chung et al., 2009).

The three peaks between 8.7 and 9.6 minutes and the smaller and less well separated peaks between 11 and 15 minutes showed UV spectra which may point to *Vinca* alkaloids. The peak at 13.1 minutes showed the UV spectrum of strictosidine. This compound has already been isolated from *Palicourea* species (Berger et al., 2017) (Rubiaceae) and was also found in *Catharanthus* species (Dugé de Bernonville et al., 2017). Summarizing, the chromatogram of the leaf extract shows many more UV active compounds than that of the stem and root extract. The chromatograms of the stem and the root extracts showed clearly the presence of alstonine (**1**) and serpentine (**2**) together with an unidentified and less well separated pair of peaks at 9.2 and 9.3 minutes. The chromatogram of the stem showed a small peak of a cinnamic acid derivative at 10.5 minutes identical with the peak in the leaf chromatogram.

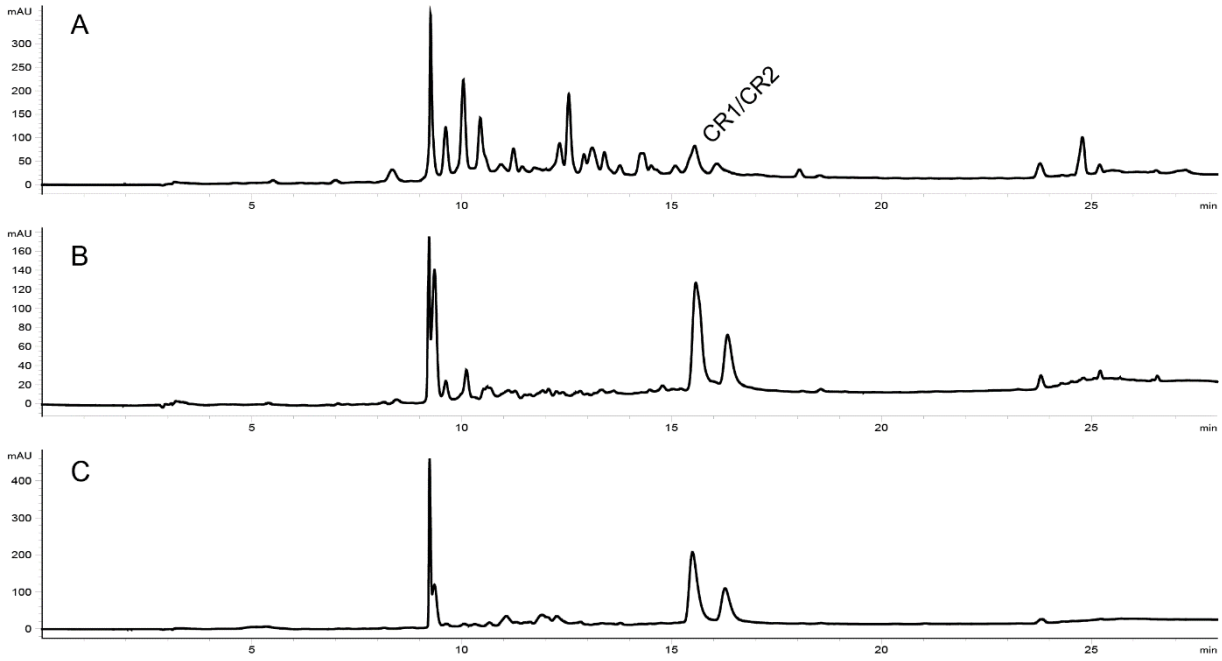

Figure 13. Comparative HPLC analyses of extracts (A) leaves, (B) stem, and (C) roots. CR1 and CR2 refer to the isolated mixture of alstonine (**1**) and serpentine (**2**). The extracts were measured at a conc. of 20 mg/mL.

6.3. Isolation of secondary metabolites

Chromatographic separation of the methanolic crude extracts obtained from the roots afforded the two compounds named as **CR** and **CR1**. However, **CR** was not pure enough for structure elucidation by NMR. Compound **CR1** showed one spot with tailing on the TLC plate and two

peaks. This mixture was inseparable by preparative chromatography and was later identified by NMR and MS as alstonine (**1**) and its diastereomer serpentine (**2**) (Figure 14). Both alkaloids were described firstly from the roots of *Catharanthus roseus* (published under *Lochnera rosea* (L.) Rchb. ex Endl. by Pillay et al. (1961). Both molecules possess a positive charged nitrogen which may explain the rather low rf value (see section 5.2).

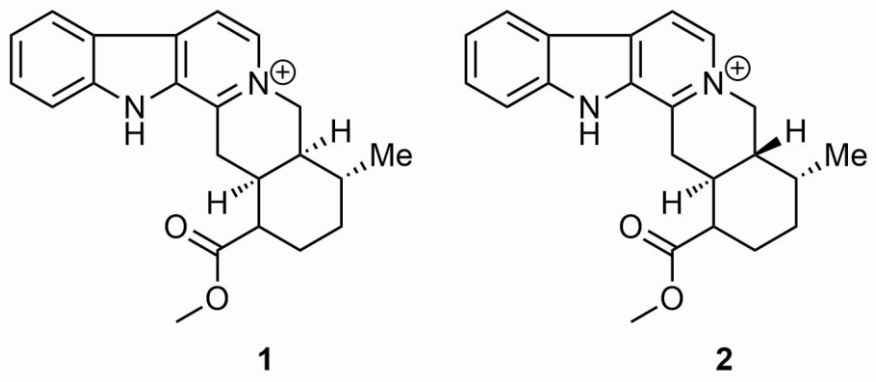

Figure 14. Structural formulae of the isolated diastereoisomers alstonine (**1**) and serpentine (**2**).

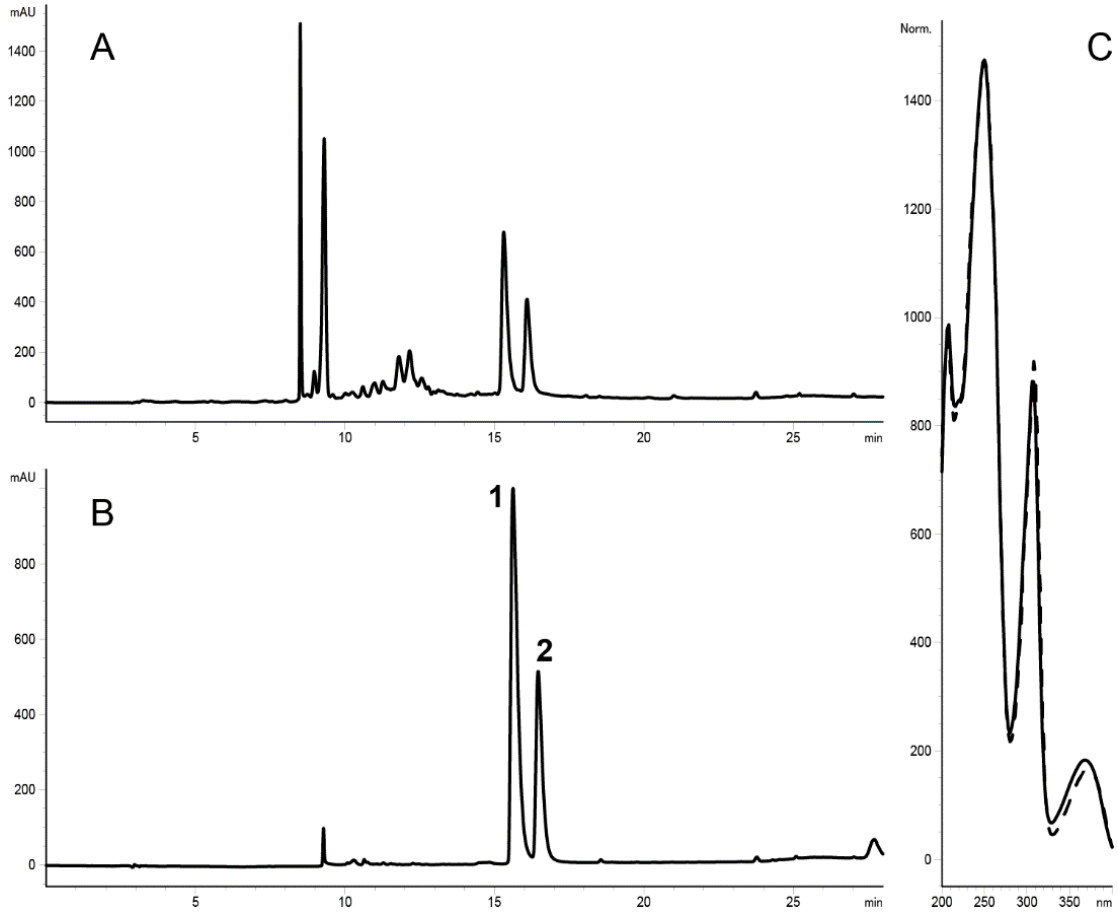

Figure 15. HPLC chromatogram of the crude extract from roots (A) and the isolated mixture of alstonine (**1**) and serpentine (**2**) (B). UV spectra of both compounds depicted right (C). The UV maxima of both compounds are at 208, 250, 308 and 368 nm. The dotted line indicates the UV spectrum of **2**.

Both alkaloids have already been reported for *Catharanthus roseus* several times in all organs, even in flowers, and they have also been found in other plant species (Table 5). Species of the genus *Rauvolfia* (Apocynaceae) are a rich source of these alkaloids. Since *Catharanthus roseus* is member of subfamily Rauvolfioideae (Nazar et al., 2013) occurrences of similar secondary metabolites is not completely unexpected. The report on the occurrence of serpentine (**2**) in leaves of the mangrove species *Rhizophora mucronata* (Gurudeenban et al., 2015) is remarkable. Alkaloids mentioned in this study were identified by comparison of retention times with respective peaks present in an extract obtained from *Catharanthus roseus* but without prior isolation and structure elucidation by NMR. Hence, the results present in this study are doubtful.

Table 5. Overview of distribution of the identified compounds alstonine (**1**) and serpentine (**2**).

species	compound	organs	reference
Catharanthus roseus	alstonine (**1**)	root, stem, leaves, flowers	Pillay et al., 1961
C. ovali		tissue culture	Stöckigt & Soll, 1980
Alstonia scholaris		leaves	Pratap et al., 2013
A. boonei *Picralima nitida* *Rauvolfia caffra* *R. vomitoria*		bark, fruit	Elisabetsky et al., 2006; Linck et al 2015; 2011
R. tetraphylla *R. serpentina* *R. verticillata* *R. vomitona* *R. hookeri* *R. micrantha* *R. canescens*	serpentine (**2**)	root, leaves	Kumar et al., 2016; Sagi et al., 2015
R. tetraphylla,		root, stem, leaves, flowers	Vema, 2017
Vinca minor		leaves	Liu et al., 2016
Rhizophora mucronata		leaves	Gurudeenban et al., 2015

6.4. Acid-base extraction of the stem extract

This performed experiment showed clearly the sensitivity of serpentine (**2**) to changes of the pH (Figure 16). Adding hydrochloric acid with subsequent extraction using chloroform did not cause any changes in HPLC analysis, neither in retention times nor in the UV-spectra (not shown) of the identified alkaloids. Adding 2M ammonium hydroxide solution reduced the amount of serpentine (**2**) whilst the estimated amount of alstonine (**1**) appears to remain equal (Figure 16). The finally remaining water phase only contained traces of alstonine (**1**). These results suggest that alstonine (**1**) is less sensitive to changes of the pH than serpentine (**2**), although both compounds are just diastereomers. Based on these results, it seems that some alkaloids are forming artifacts after they got in contact with strong acids and bases. Hence, application of this widely used extraction technique (Auterhof et al., 1998) should be tested beforehand on small amount of extracts otherwise the formation of artifacts may lead to wrong results.

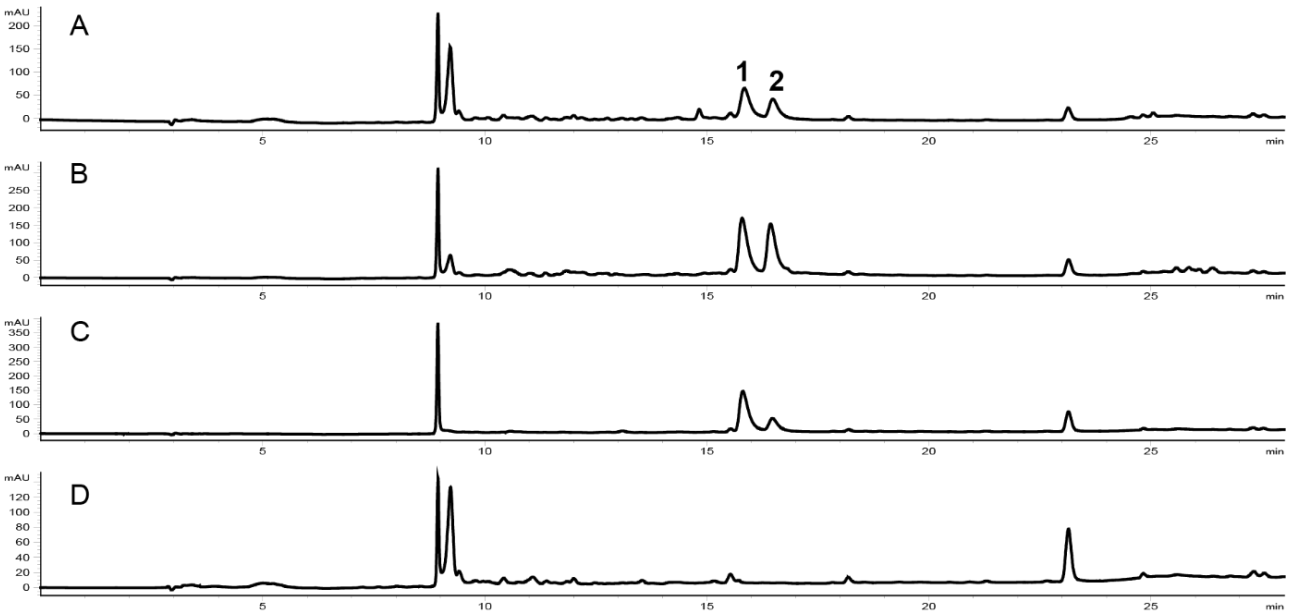

Figure 16. HPLC chromatograms after performing the acid-base extraction from the stem extract of *Catharanthus roseus*. A: crude methanolic extract; B: Chloroform-phase after addition of 2M HCl to the aqueous crude extract; C: Chloroform- phase after adding 2M ammoniak solution to the sample; D: remaining water phase; **1**= alstonine and **2**= serpentine.

Formation of artifacts during extraction of plant material should always be kept in mind when plant material is extracted or isolation of plant-derived compounds is taking place. This is especially of importance, when bioactivity tests should be performed during the study. According to Maltese et al. (2009), formation of artifacts may cause following effects:

- Formation of new compounds
- Loss of bioactivity
- Increasing bioactivities by formation of new structures
- Degradation of native compounds
- Loss of confirmability

Such effects may lead to wrong positive results, which may further cause additional problems. Concerning alkaloids, such formation of artifacts are known from the isolation of berberine by using chloroform. This halogenated solvent lead to the formation of berberine-chloroform (Figure 17). Formation of berberine-chloroform according to Maltese et al. (2009) and secologanin artifacts isolated from *Palicourea luxurians*. R1 and R2 show the position of methyl- and/or butyl-

groups derived from extraction solvent (modified from Kornpointner et al., 2020), which is a chlorinated berberine derivative not native in plant material. Berberine possesses a positively charged nitrogen like the identified alkaloids alstonine (**1**) and serpentine (**2**) (Figure 14) and this position may cause instabilities during extraction. Another example is the 3,4,5,6-tetradehydroreserpine which is formed on TLC plates after developing in chloroform containing solvents developed under UV light (Maltese et al., 2009). These effects are not only restricted to alkaloids as such, but they are also found in other classes of compounds. For examples, the iridoid secologanin forms methyl acetals or butyl acetals by extraction with methanol and/or butanol. This has been proven for *Lonicera japonica* Thunb. (Tomassini et al., 1995) and also for the neotropical plant species *Palicourea luxurians* (Rubiaceae) (Kornpointner et al., 2020). Secologanin possesses an aldehyde function which easily can react with alcohols like methanol or butanol, leading further to specific reaction products (Figure 17).

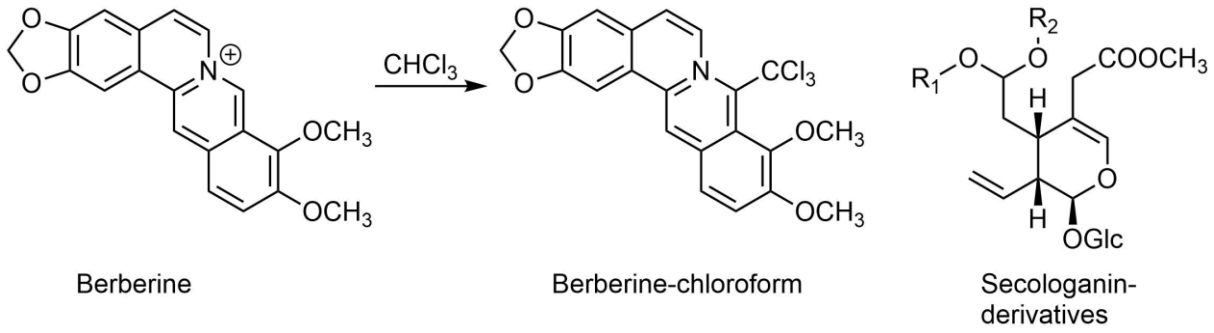

Figure 17. Formation of berberine-chloroform according Maltese et al. (2009) and secologanin artifacts isolated from *Palicourea luxurians*. $R_1$and R_2 show the position of methyl- and/or butyl-groups derived from extraction solvents (modified from Kornpointner et al., 2020).

6.5. Feeding assay

The performed feeding assay revealed clearly the anti-feedant properties of the leaf extracts in all of the tested concentrations, whereas the other tested extracts barely influenced the development of the caterpillars. Extraordinary lethality was not observed in this experiment (data not shown). This suggest, that the present compounds are either less toxic or-if toxic compounds are present- their concentration was too low in the tested plant material. The obtained data also show, that the leaf extract affected the development of the larvae throughout all tested concentrations much more than the extracts of the other plant organs (Table 6).

Table 6. Growth inhibition after four days of the tested plant extracts on *S. littoralis* larvae. Values are given in %. Nicotine was used as positive control.

Conc. [ppm]	roots	stems	leaves	nicotine
2000	31.6	11.6	74.2	n.t
1000	4.9	n.e.	79.2	55.2
500	0.1	15.4	87.0	14.5
250	5.5	n.e.	81.4	32.7
125	n.t.	n.t	n.t	11.0

n.e.= no effect; n.t= not tested

The picture of the growth inhibition became clearer after evaluation after seven days (Table 7). Values are given in %. Nicotine was used as appositive control). The leaf extract affected growth of the caterpillars heavily in the conc. 2000 to 500 ppm (Figure 18). Antifeedant assay of the leaf extract after four days (A) and seven days (B). Right: control; down right: 250 ppm, down left:500 ppm, up right:100 ppm and up left:1000 ppm). It is hypothesized that the strong effects may be caused by rather lipophilic compounds which are accumulated in the insect tissues shortly after ingestion. Apparently, these compounds are neither detoxified nor efficiently excreted, indicating a lack of respective mechanism in the *Spodoptera* larvae. The extract of the stems performed also well whilst the root extract only showed a higher effect at 2000 ppm.

Table 7. Growth inhibition after seven days of the tested plant extracts on *S. littoralis* larvae. Values are given in %. Nicotine was used as positive control.

Conc. [ppm]	roots	stems	leaves	nicotine
2000	52.4	40.7	97.4	n.t.
1000	18.8	32.4.	97.6	43.1
500	12.8	25.9	83.3	n.e.
250	7.7	20.9.	8.0	19.2
125	n.t.	n.t	n.t	n.e

n.e.= no effect; n.t= not tested

A **B**

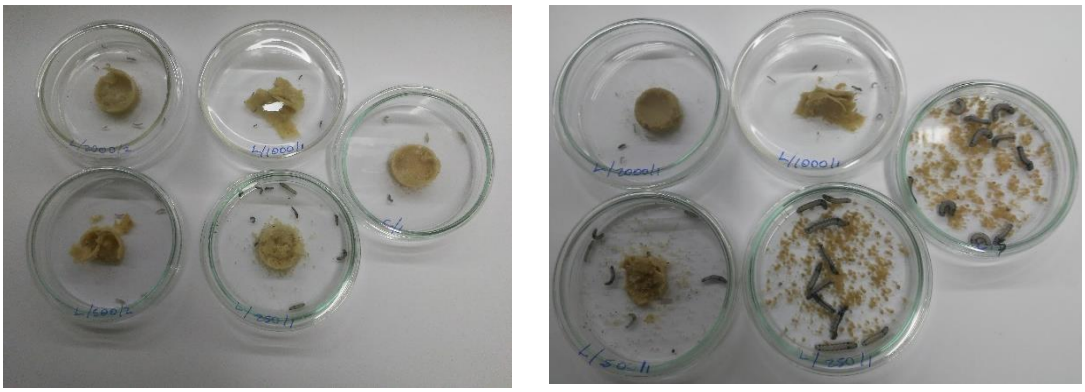

Figure 18. Antifeedant assay of the leaf extract after four (A) and seven days (B). Right: control; down right: 250 pmm, down left: 500 ppm; up right: 1000 ppm and up left 2000 ppm.

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8. Zusammenfassung

Die vorliegende Arbeit präsentiert die Ergebnisse von phytochemischen Untersuchungen an rosa und weiß blühenden Individuen vom Madagaskar Immergrün, *Catharanthus roseus* G. Don (Apocynaceae), aus Thailand. Zur vergleichenden Analyse wurden chromatographische Methoden wie HPLC mit UV-Diodenarray Detektion sowie Dünnschichtchromatographie (DC) herangezogen. Nach Besprühen der DC-Platten mit Dragendorff's Reagenz konnten keine Unterschiede zwischen den unterschiedlichen Blütenmorphen beobachtet werden. Daher wurden die entsprechenden Extrakte vereinigt und gemeinsam analysiert. Chromatographische Vergleiche zwischen den einzelnen Organen Wurzeln, Spross und Blättern, zeigten auffällige Ähnlichkeiten zwischen dem Spross- und dem Wurzelextrakt, während der Blattextrakt deutlich verschieden war. Diese Unterschiede sind auf Anreicherung weiterer, nicht-alkaloider Stoffe, in den Blättern zurückzuführen. Die präparative Auftrennung des Wurzelextraktes führte zur Isolierung der beiden bekannten Alkaloide Alstonin und Serpentin. Diese beiden Alkaloide sind auch im Sprossextrakt nachweisbar, fehlen jedoch im Blattextrakt. Zusätzlich durchgeführte Säure-Basen Extraktion führte zu einem Abbau von Serpentin, während Alstonin angereichert wurde. Daher sollte diese Methode mit Vorsicht angewandt werden, um Bildung von Artefakten zu vermeiden. In Blattexsudaten konnten keine Monoterpenindolalkaloide nachgewiesen werden. Damit widerlegen diese Ergebnisse, ebenso wie auch neuere Untersuchungen, frühere Behauptungen einer externen Alkaloidakkumulation. Das Vorkommen von möglichen fraß-hemmenden Substanzen wurde mit frisch geschlüpften Raupen der Ägyptischen Baumwolleule *Spodoptera littoralis* Boisduval in einem Bioassay getestet. Es zeigte sich eine klare Entwicklungshemmung der Raupen bei der Aufnahme von Blattextrakt. Nach vier Tagen betrug die Hemmung 74% und nach sieben Tagen 97% bei der getesteten Konzentration von 2000 ppm (parts per million). Diese starke Wirkung nach sieben Tagen war auch bei niedrigeren Konzentrationen des Blattextraktes nachweisbar. Die Wirkung der beiden anderen Extrakte war mit 52% für den Wurzelextrakt und 41% für den Sprossextrakt nach sieben Tagen weitaus schwächer. Diese Ergebnisse deuten auf die Präsenz von Substanzen mit stark fraßhemmenden Eigenschaften in den Blättern hin. Die erhaltenen Ergebnisse dieser Untersuchungen stimmen mit der Literatur überein und zeigen unter anderem auch, dass sich verschiedene Blütenfarbe nicht unbedingt in einer Diversifizierung der Inhaltsstoffmuster widerspiegeln muss.