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Comparative phytochemical analyses of *Stemona mairei* and related species

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Katharina Sandler BSc

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1. Introduction

Stemona Lour. species are known for their accumulation of alkaloids with a unique chemical structure, which have not been found in any other plant family so far. These so called *Stemona* alkaloids are characterized by either a pyrrolo- or a pyrido[1,2-α]-azepine backbone and are probably an apomorphy of the family Stemonaceae. In the past, phytochemical investigations lead to an identification of a huge number of derivates, up to now more than 150 derivatives are described (Chen et al., 2017a).

Besides the highly interesting structure, *Stemona* alkaloids are well known for various biological activities. Especially, the roots of *Stemona tuberosa* Lour*., S. sessilifolia* (Miq.) Miq. and *S. japonica* (Blume) Miq. have been in long usage in traditional Chinese and Japanese folk medicine. The roots have a broad range of applications like as anticough remedy, against ectoparasites on humans and cattle or as insecticides (Sakata et al., 1978). Due to the similar shape of the fleshy storage roots, roots of different *Stemona* species can be purchased under the same vernacular name as ‘‘Bai bu’’ in China, ‘‘Pong Mot Ngam’’ or ‘‘Non Tai Yak’’ in Thailand, ‘‘Bach bo’’ in Vietnam at local markets. Even similar looking roots of other plant families were offered under those names. This led to a far-reaching confusion in scientific literature (Brem et al., 2002; Greger, 2006). Furthermore, chemical instability of *Stemona* alkaloids has be taken into account during the extraction and isolation process, which can lead to a transformation or degradation of the compounds resulting in an inactivation of biological functions as well as in formation of artifacts (Greger, 2006). In the past, not all *Stemona* species have been well investigated, especially those ones, which had less importance for ethnomedical application in the eastern Asian countries.

The focus of the present study was on the Chinese endemic species *Stemona mairei* H. Léveillé and on *Stemona aphylla* Craib., which is endemic to Thailand. The unstudied *S. mairei* was collected in nine different locations in its natural habitat in southern China. A phytochemical screening for alkaloids and tochopherols was performed to get an insight in the accumulation patterns within this species. Beside *S. mairei*, five other species, collected in China, were also screened for alkaloids and tocopherols to receive an overview on the structural diversity in the genus *Stemona*. In addition, *S. aphylla* was investigated for its alkaloid and stilbenoid accumulation pattern. Furthermore, crude methanolic root extracts of five different *Stemona* species were tested on the free-living soil nematode *Panagrellus*

redivivus Linné to understand better the previously reported insecticidal properties of *Stemona* roots and their accumulated compounds.

1.1. Taxonomy, Evolution, Phylogeny and Morphology

The rather small monocotyledonous family Stemonaceae consists of 33 species in three genera: *Stemona* Lour., *Croomia* Lour. and *Stichoneuron* Hook. f. The main distribution area of the family is in southeast Asia with the most southern occurrence in northern Australia. One species of *Croomia* is even found in the United States, in Florida. The Stemonaceae are characterized as perennial herbs, subshrubs or vines with tuberous roots or a creeping rhizome. The leaves are petiolate with conspicuous veinlets between the primary veins. The tetramerous flowers are bisexual, tepals vary in color and the anthers have mostly an appendage. (Ji and Duyfejes, 2000)

Stemonaceae is an old group, which was already present in the Cretaceous. Based on rbcL sequence data, the crown node is dated back to 84 mya and its stem node to 108 mya (Janssen and Bremer, 2004). The family Stemonaceae was previously placed in the order Discoreales, based on the shared reticulate leaf venation, tuberous roots and prolongation of the anther connective (Caddick et al., 2002). Molecular analyses supported the inclusion of the family Stemonaceae into the monophyletic order Pandanales, including Pandanaceae, Velloziaceae, Cyclanthaceae, and Triuridaceae (APG, 1998; Caddick et al., 2002). Discovering the presence of pandamine, which is a rare type of alkaloid and only known from the family Pandanaceae, in the genus *Stemona,* underlines the close relationship of those two families from a chemotaxonomic view (Greger et al., 2009). Discussion about the placement of the taxon *Pentastemona* persists to date and ranges from classification within Stemonaceae to separate as its own family Pentastemonaceae (APG, 1998; Ji and Duyfejes, 2000; Caddick et al., 2002; Rudall and Bateman, 2006; Mennes et al., 2013).

Stemona is the largest genus within the Stemonaceae and compromises around 25 species. The plants are mainly perennial vines or subshrubs with fleshy tuberous storage roots. *Stemona* species prefer a seasonal climate and dry habitats (Kaltenegger et al., 2003). The tetramerous flowers have ovules which are basally attached to the placenta and possess conspicuous appendages at the anthers (Ji and Duyfejes, 2000). In a recent publication is shown that the flowers of different *Stemona* species emit a foul odor generated by a wide range of different degradation products, further lack of nectar. Flowers are visited by different

sapromyophilous flies, indicating that Stemona is a typical sapromyophilous taxon (Chen et al., 2017b). The seeds sit on a different long funiculus and elaiosomes are attached to it (Inthachub, 2008). Notably is, that the diaspores of *S. tuberosa* are dispersed not only by ants but as well as by hornets like *Vespa velutina* Lepeletier (Chen et al., 2016).

1.2. *Stemona mairei* H. Leveille

The perennial vine *S. mairei* is found on limestone rocks in different habitats like scrub, mountain slopes or on dry grassland with a wide range of altitude (800 to 3000 m). The storage roots are oblong to ovoid and vary in colors and length. The leaves are ovate to linear and have 3–5 primary veins. The inflorescence is a one to two flowered raceme having tetramerous flowers with 2–3 cm in size (Figure 1). The flower has white tepals, which are tinged pinkish, and stamens shorter than the perianth. Flower time is between April and July. (Ji and Duyfejes, 2000)

Figure 1. *Stemona mairei* is a perennial vine, which is endemic to China. (A) Storage roots are oblong and 5–10 cm long. (B) Stem with ovoid-linear leaves and flowers. (C) The tetramerous flower has white tepals and a size of about 3 cm. (Photos by Gao Chen)

1.3. *Stemona aphylla* Craib.

Stemona aphylla Craib. is a twiner with stems up to 4 m long. It is endemic to Thailand and grows in either dry evergreen forests or in deciduous forests on shale bedrock or on limestone. The broadly ovate leaves have 9–11 veins and 4–6.5 cm long petiole (Figure 2). The roots are tuberous, spindle shaped and up to 30 cm long, but only about 1 cm thick. The inflorescence is sessile or on short peduncles with 1–3 flowers, which are flowering from February to May. The narrowly triangular tepals vary in color from pink, yellowish pink to greenish purple. The four stamens are pinkish to reddish and the thecal appendage is absent. (Inthachub, 2008; Inthachub et al., 2010)

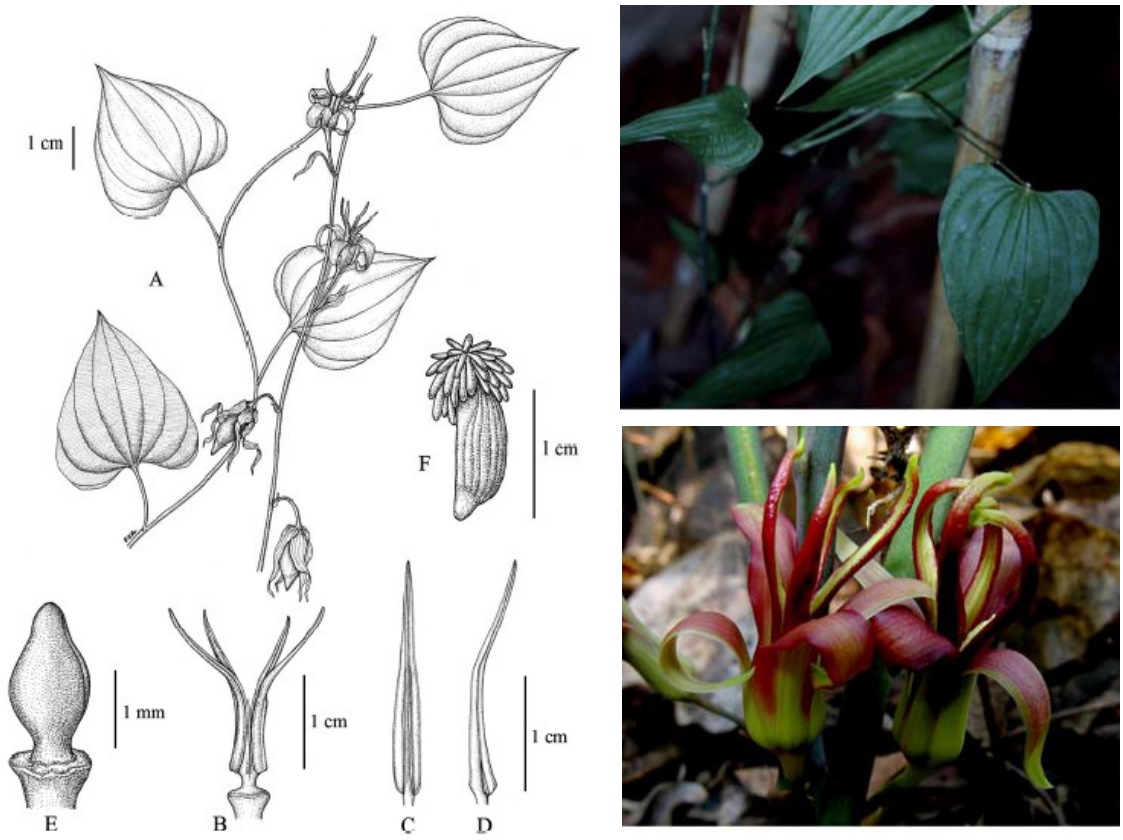

Figure 2. *Stemona aphylla* is endemic to Thailand. The left figure shows a drawing of *S. aphylla* by P. Inthachub. (A) Stem with leaves and flowers. (B) Androecium. (C–D) Stamen. (E) Ovary. (F) Seed. Right above figure shows leaves of *S. aphylla* with the typical parallel primary nerves of monocots. The right bottom figure shows the flowers with red colored stamen and greenish-reddish tepals. (Photos by P. Inthachub)

1.4. Secondary metabolites

Plants are sessile organisms, which cannot avoid any biotic and abiotic stress. Additionally, they are photoautotroph and heterotrophic organisms relying on their produced organic compounds via photosynthesis as a source. These conditions lead to the evolution of different strategies of the plant to cope with its hostile environment. Besides a high capacity of regeneration or other mechanical and morphological protection strategies, plants have evolved an enormous variety of secondary metabolites in the last 500 million years (Wink, 2010a) to interact with its biotic and abiotic environment. These secondary metabolites serve not only as protection from herbivores, pathogens, or from competing plants, but they also play a role in attracting different animals for pollination and dispersing seeds. Some secondary metabolites exhibit both function as signal and as defense compounds (Wink, 2010a; Wink, 2010b).

Secondary metabolites emerge from primary metabolism, which is essential for growth, development, and reproduction. In contrast, secondary metabolites are indispensable for the survival and fitness of the plant in its environment. Secondary metabolism has a high genetic plasticity and diversity (Hartmann, 2007), which enables the plant to continuously modify it and adapt so to the changing biotic and abiotic environment. Biosynthesis and the accumulation of secondary metabolites is regulated in space and time, in an organ-, tissue- and development-specific way. Usually vulnerable organs and organs used for reproduction show higher amounts of secondary metabolites (Wink, 2010a). Some secondary metabolites are not always present in the plant, they are produced in reaction to herbivores or pathogens as an induced defense mechanism. On the contrary, the constitutive defense mechanism is characterized by compounds which are always present in the plant. Furthermore, some of the secondary metabolites have restricted distribution within the plant kingdom, and these can be used as chemotaxonomic markers to underline relationships of taxonomic groups (Wink, 2010b).

1.4.1. *Stemona* alkaloids

Stemona alkaloids represent a typical chemical character of the family Stemonaceae, which have not been found in any plant family so far. The unique class of secondary metabolites are structurally characterized by a pyrrolo- or pyrido[1,2-α]-azepine core, which is usually linked with two carbon chains mostly forming a lactone ring (Figure 3). Greger (2006) grouped these alkaloids into three skeletal types according to different carbon chains attached to the C-9 of the backbone: stichoneurine-, protostemonine- and croomine-type alkaloids, but other classification systems have also been suggested (Pilli et al., 2010). Protostemonine-type alkaloids have not been detected in the genus *Croomia* and *Stichoneuron* so far. However, all three types are present in the genus *Stemona*. Based on the accumulation trends towards either stichoneurine or protostemonine-type alkaloids, *Stemona* species fall into two groups, whereas croomia-type alkaloids are detected in both groups (Schinnerl et al., 2007). Generally, the highest alkaloid content is found together with other bioactive compounds in the underground parts of *Stemona* species (Greger, 2006).

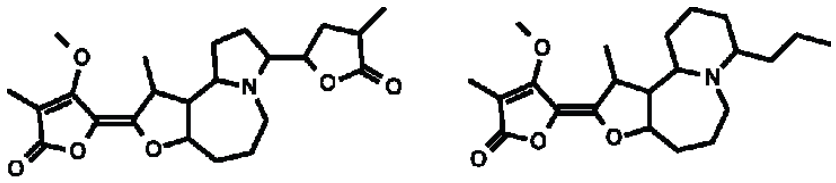

Figure 3. *Stemona* alkaloids are characterized either by a pyrrolo- or pyrido-azepine backbone (Chen et al., 2018, subm.).

The biosynthesis of *Stemona* alkaloids has not been elucidated so far. Their formation might originate from ornithine via homospermidine and this pathway was hypothesized by Seger et al. (2004). In this context, the detection of pandamine, a secondary amine, in *Stichoneuron cacliola* Inthachub, is of special interest. First, it underlines the close relationship of Stemonaceae with Pandanaceae, from which pandamine was known so far (Greger et al., 2009). Second, pandamine is a structural precursor of croomine and was suggested as biogenetic precursor for *Stemona* alkaloids (Greger et al., 2009). The pyridoazepines are proposed to derive from pyrroloazepines through cleavage of the pyrrolidine ring and incorporation of C-18 from the butyl side chain forming the characteristic piperidin ring of the pyridoazepines (Kaltenegger et al., 2003).

Phytochemical investigation of the little studied species *S. mairei* resulted in isolation of maistemonine, oxymaistemonine (Lin et al., 1991), tuberostemonine and several of its derivates (Cai and Luo, 2007). The underground parts of *S. kerrii* can be clearly distinguished from other *Stemona* species by the accumulation of pyridoazepine type alkaloids stemokerrine, oxystemokerrine and oxyprotostemonine (**6**) (Schinnerl et al., 2007; Kongkiatpaiboon et al., 2011). So far, no research was done on *S. shandongensis* D.K. Zang. and no phytochemical data are available. The well-studied species *S. japonica* and *S. sessilifolia* are characterized by pyrroloazepine derivatives: protostemonine (**1**), stemonine and their derivates (Greger, 2006). Furthermore, parvistemonine, also a protosemonine derivate, was isolated from *S. parviflora* (Greger, 2006). A recent study of Huang et al. (2016) indicates accumulation trends towards protostemonine (**1**) and stemofoline (**4**) in *S. parviflora*. *Stemona collinsiae* Craib. is clearly characterized by the presence of didehydrostemofoline as the major alkaloid accompanied by smaller amounts of stemofoline (**4**) (Kongkiatpaiboon et al., 2011). The chemical composition of *S. aphylla* shows some variability, either accumulating pyrroloazepine type alkaloid protostemonine (**1**) or different pyridoazepines as major component (Kongkiatpaiboon et al., 2011). It is remarkable that *S. aphylla*, *S. rupestris* Inthachub and *S. cochinchinensis* Gagnep. exhibit a general reduction in the alkaloid content (Kongkiatpaiboon et al., 2011).

Various *Stemona* species are known for different bioactivities of leaf or roots extracts and for toxicity against insects. The root extract of *S. collinsiae* exhibits high toxic properties against *Spodoptera littoralis* Boisduval (Brem et al., 2002) and *Plutella xylostella* Linné (Jiwajinda et al., 2001). These activities may be contributed to the main accumulated alkaloid didehydrostemofoline and the less toxic stemofoline (**4**). Furthermore, in the study of Huang et al., (2016) is shown that the root extract of *S. parviflora* and the pure alkaloids stemofoline (**4**) and protostemonine (**1**) have nematicidal activity. Due to their various bioactivities (Brem et al., 2002) *Stemona* roots may have a broad range of practical application in agriculture and pharmacy, but the metabolites play a more important role for the plant. Tang et al. (2008) suggests that plants use stemofoline (**4**) and its derivates for defense against herbivores and pathogens, for example by preventing the plant from herbivore biting in leaves and stems.

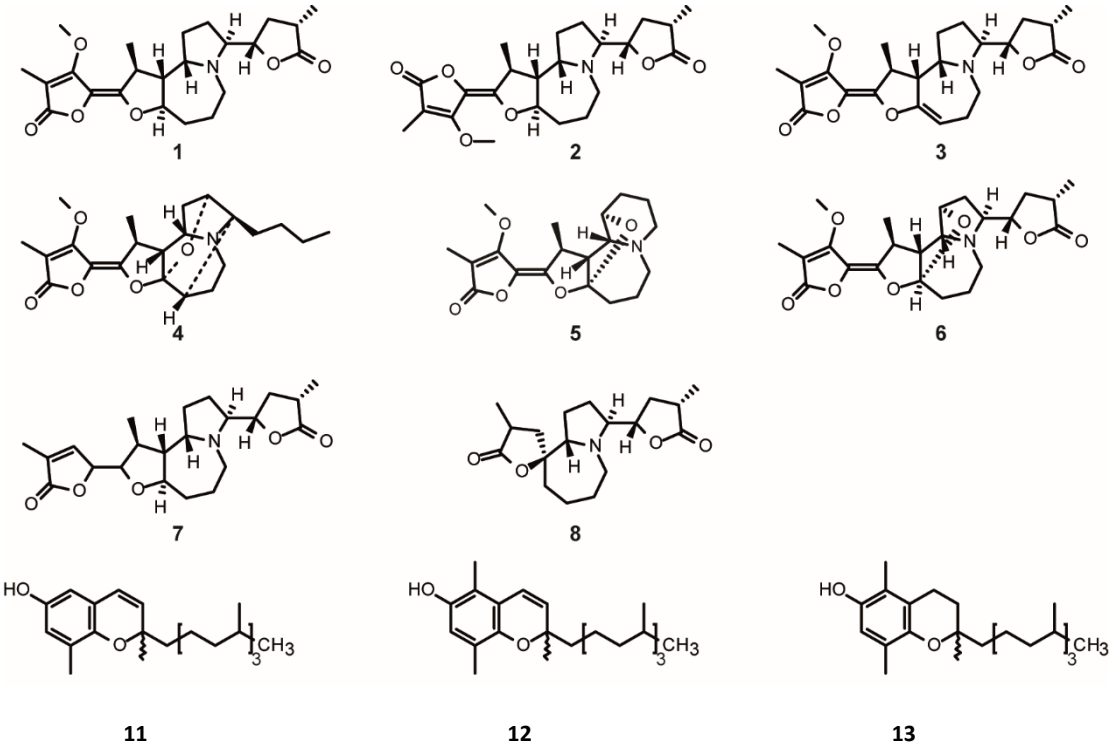

Figure 4. Chemical structures of detected alkaloids (**1**–**8**) and tocopherols (**11**–**13**). Protostemonine (**1**), isoprotostemonine (**2**), dehydroprotostemonine (**3**), stemofoline (**4**), stemocurtisine (**5**), oxyprotostemonine (**6**), stemocochinin (**7**), croomine (**8**), 3,4-dehydro-δ-tocopherol (**11**), 3,4-dehydro-β-tocopherol (**12**) and β-tocopherol (**13**). (Chen et al., 2018, subm.)

1.4.2. Tocopherols

Beside the accumulation of *Stemona* alkaloids, the occurrence of anti-oxidative tocopherols and dehydrotocopherols has been reported for a few *Stemona* species (Brem et al., 2004; Kil et al., 2015; Chen et al., 2017a). The lipid-soluble tocopherols are synthesized only by photosynthetic organism and belong together with tocotrienols to the vitamin E group (Munné-Bosch and Alegre, 2002).

Structurally, tocopherols are characterized by a polar chromanol core linked to a hydrophobic phytol side chain, which is fully saturated. Tocotrienols differ from tocopherols by having a 3-fold unsaturated side chain. Further classification of the two vitamin E subgroups can be done according to the number and position of the methylation of the chromanol core, forming four types: α-, β-, γ- and δ-tocopherol (Munné-Bosch and Alegre, 2002). The structurally related dehydrotocopherols are characterized by a double bond between C3 and

C4. The biosynthesis of tocopherols takes place in the plastid compartment and requires two substrates. The chromanol headgroup is formed out of homogentisate (HGA), which is derived via tyrosin from the shikimate pathway. The second substrate is phytyl diphosphate (PDP), which is synthesized by the methylerythritol phosphate (MEP) pathway. (Munné-Bosch and Alegre, 2002; Hussain et al., 2013)

In the past, various studies have mainly focused on the antioxidant activity of tocopherols in humans, plants and animals. Lately, other functions beyond antioxidative activity were suggested in plants. Tocopherols may affect different physiological processes in the plant like cell signaling, germination, export of photo-assimilates or response to abiotic stress (Munné-Bosch and Alegre, 2002; Falk and Munné-Bosch, 2010; Hussain et al., 2013). The biological role of tocopherols in *Stemona* species is poorly understood. Brem et al. (2004), who described first dehydrotocopherols as characteristic compounds for *Stemona* species, proved their antioxidant activity and that the double bond has no influence on the antioxidative capacity. Recently, dehydrotocopherol derivates were isolated from *S. tuberosa*, which showed some cell proliferation effects (Kil et al., 2015). Due to the co-occurrence of tocopherols and alkaloids in *Stemona* roots, the function in *Stemona* plants may be protecting other compounds, in this case alkaloids from oxidation (Chen G. et al., 2017a).

Stemona species accumulate different dehydrotocopherols in their storage roots with a species-specific pattern (Brem et al., 2004; Chen et al., 2017a). *Stemona tuberosa* group shows a clear preponderance towards dehydro-δ-tocopherol (**11**), whereas *S. curti*sii Hook. S. is characterized by dehydro-γ-tocopherol followed by smaller amounts of dehydro-α-tocopherol. *Stemona kerrii* diverges by the accumulation of tocopherol (chromanol) derivatives (Brem et al., 2004).

1.4.3. Stilbenoids

In addition to alkaloids and dehydrotocopherols, other biological active compounds are accumulated in the storage roots of *Stemona* species (Pacher et al., 2002). The occurrence of these polyphenolic compounds, namely stilbenoids, is restricted to the genus *Stemona* within the family Stemonaceae (Greger, 2012). Generally, stilbenoids are of limited but heterogeneous distribution in the plant kingdom (Rivière et al., 2012).

Stilbenoids, structurally characterized by a 1,2-diphenylethylene core, are synthesized via the phenylpropanoid pathway from the amino acids phenylalanine and tyrosine (Greger,

2012; Dubrovina and Kiselev, 2017). In the first step of the biosynthesis, stilbene synthase catalyzes the formation of the stilbenoid backbone from three malonyl- CoA and one CoA-ester of different cinnamic acid derivatives. Then, other enzymes glycosylate, methylate and prenylate the backbone leading to a wide range of stilbenoids (Greger, 2012; Dubrovina and Kiselev, 2017). In the genus *Stemona*, a typical chemical feature of stilbenoids is the C-methylation of mainly the aromatic ring B, which is rarely observed in the plant kingdom (Pacher et al., 2002; Greger, 2012). Stilbenoids are grouped according to their biosynthetic origin into dihydrostilbenes, stilbenes, dihydrophenanthrenes and phenylbenzofurans, respectively (Greger, 2012). Due to absence or presence of phenylbenzofurans, *Stemona* species can be separated into two groups (Greger, 2012) that are also supported by morphological characters and molecular data (Vongsak et al., 2008; Kongkiatpaiboon et al., 2010).

Pacher et al. (2002) described as first the antifungal properties of *S. collinsae* extract, which could be attributed to all four groups of stilbenoids. In the review of Greger (2012) is summarized that MeOH extracts of 14 *Stemona* species show antifungal activity with the fungus *Cladosporium herbarum* Pers. and that the stilbenoid content increases after fungal infection, thus suggesting a role in plant defense. Furthermore, some stilbenoids isolated of *S. sessilifolia* and *S. tuberosa* exhibited antibacterial activities against various taxa (Yang et al., 2006; Lin et al., 2008). Several analyses deal with their valuable pharmaceutical effects, their biosynthesis, metabolic engineering for their production and antifungal properties in plants (Dubrovina and Kiselev, 2017). Recent studies show that stilbene biosynthesis is induced in plants by different abiotic stress factors like ozone, drought or heat stress (Dubrovina and Kiselev, 2017). It can be expected that stilbenoids have other functions in *Stemona* species apart from plant defense mechanism against pathogens. As *Stemona* species occur often in dry habitats, the stilbenoids may influence the dormancy of these plants (Greger, 2012).

2. Material and Methods

2.1. Plant material

The roots of *S. mairei* and five other *Stemona* species were collected in various localities (Table 1) in SW-China either in 2015 or 2016 and identified by Gao Chen (Kunming Institute of Botany, China). Voucher specimen were deposited at the Herbarium of Kunming Institute of Botany, the Chinese Acadamy of Sciences (KUN). The roots were dried at 40 °C. The plant material of *S. aphylla* was collected in eleven localities in the province Nakhon Ratachasima in East-Thailand by Gao Chen in 2016.

2.2. General Experimental Procederes

HPLC-UV analyses were performed on Agilent 1100 series using a reversed phase column (Hypersil BDS-C18, 250 x 4.6 mm, 5 μm particle size) and as mobile phase MeOH (B) and a 10 mM ammonium acetate (A) aquaeous solution was used. The eluent gradient started with 55% to 90% B in A for 19 min, 90% B was accelerated to 100% in A for 1 min and 100% B was kept for 12 min. The flow rate was 1 mL/min and 10 μL the injection volume. UV-diode array detection was at 230/254/280/310 nm.

For monitoring the isolation process and getting an impression of the variety of compounds in the extracts, Thin layer chromatography (TLC) analyses were performed with silica gel 60 F_{254} plates, thickness 0.20 mm (Merck). The TLC plates were developed with following eluents PE/Aceton (2:1 or 9:1) and $CHCl_3$/MeOH (8:2). For post-chromatographic detection, UV lamps at 254 nm/366 nm were used to visualize compounds with a chromophore and staining reagents to observe colorless compounds on the TLC plate. Dragendorff reagent is used to detect alkaloids and in general N-containing compounds, whereas anisaldehyde reagent reacts unspecific.

For NMR spectrometry the isolated compounds were dissolved in either chloroform or methanol and transferred to 5 mL NMR tubes. The NMR-Spectra were measured on a Bruker DRX-600 at 600.13 MHz (^{1}H) or 150.61 MHz (^{13}C) using the Topspin 3.2 software. Measurement temperature was 298 K ± 0.05 K. Residual $CHCl_3$ was used as internal standard for ^{1}H (δ_H 7.26) and $CDCl_3$ for ^{13}C (δ_C 77.01) measurements, respectively. 1D spectra were recorded and Fourier transformed to spectra with a range of 7,200 Hz (^{1}H) and 32,000 Hz (^{13}C), respectively. To determine the 2D spectra 128 experiments with 2,048 data points each were

recorded and Fourier transformed to ranges of 6,000 Hz and 32,000 Hz for ^{1}H and ^{13}C, respectively.

Mass spectra were recorded on a high-resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive and negative ionization mode (mass accuracy ± 5 ppm). TOF MS measurements have been performed within the selected mass range of m/z 1002500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 3050 nA. Nitrogen temperature was maintained at 180 °C using a flow rate of 4.0 L min^{-1} and the N_2 nebulizer gas pressure at 0.3 bar.

2.3. Stemona mairei

2.3.1. Screening for Alkaloids and Tocopherols in roots of *S. mairei* and Calibration Parameter of Protostemonine

Extraction. 100 mg ±1 mg of air dried, ground *Stemona* roots were extracted with 1 mL MeOH in Eppendorf tubes under sonication for 20 min, respectively. After centrifugation at 12,500 rpm for 20 min the supernatant was concentrated to determine the weight of the crude extract, yielding minimum 19 mg and maximum 41 mg (Table 1). Furthermore, deep-frozen roots (-80 °C) of seven accessions of *S. mairei* and one leaf sample (Table 2) were squeezed with a hydraulic press to collect the root/leaf sap with a pipette. 1.5 mL sap were centrifuged in Eppendorf tubes at 14 000 rpm for 20 min. The supernatant was poured in a new Eppendorf tube and 0.5 mL MeOH was added, respectively.

For HPLC Analyses, 10 mg mL^{-1} of each MeOH extract of the dried roots and standards of protostemonine (**1**) with the concentrations of 125, 250, 500, 750 and 1000 μg mL^{-1} were prepared and subjected to HPLC. Standards were measured three times. The MeOH extract of the deep-frozen roots were measured without any specific concentration.

Identification. HPLC chromatograms were compared in retention times (rt) and in their corresponding UV spectra. Compounds were identified by analytical data of known reference compounds or with earlier reported data.

Calibration Curve. To determine the concentration of protostemonine (**1**) in the different samples, the corresponding peak area of the five external standards of protostemonine (**1**) in the HPLC chromatogram were assessed and were plotted against the amounts of the

standards. The concentration of protostemonine (**1**) were calculated for each sample (Table 3).

Table 1. Screening for Alkaloids and Tocopherols in several *Stemona* species. Origin and weight of used plant material (roots).

Taxon	Orgin	Year	Herbarium Specimen	Dried roots [mg]	Crude extract [mg]
S. mairei	Derong	2016	CHEN20160702	100.9	34.1
	Taoyuan	2016	CHEN20160707	100.3	28.1
	Qina	2016	CHEN20160630	100.7	19.3
	Huangping	2016	CHEN20160704	100.7	27.9
	Panzihua	2016	CHEN20160523	101.1	23.7
	Jiangbiancun	2016	CHEN20160703	100.5	20.8
	Fulongqiao	2016	CHEN20160701	101.1	33.5
	Panzihua	2015	CHEN20140327	100.5	36.2
	Dequin	2015	CHEN20110707	100.6	32.0
S. kerrii	Xiaoganba	2016	CHEN20160722	101.0	40.9
	Xishuangbanna	2015	CHEN20150208	100.2	43.0
S. japonica	Tianmushan	2015	CHEN20120709	99.9	40.0
S. parviflora	Baisha	2015	CHEN20100903	100.6	37.2
S. sessilifolia	Qixiashan	2015	CHEN20140511	100.8	25.2
S. shandongensis	Taishan	2015	CHEN20140506	100.5	31.8

Table 2. Comparative HPLC analyses of *S. mairei* roots, deep frozen at -80 °C. Origin and weight of frozen roots and leaf samples.

Taxon	Plant organ	Origin	Dried plant material [g]
S. mairei	roots		
		De Rong	27.67
		Taoyuan	18.63
		Qina	11.55
		Huangping	17.91
		Jiangbiancun	6.49
		Fulongqiao	15.17
		Xilong	5.10
S. mairei	leaves		0.13

Table 3. Calculated quantities of protostemonine (**1**) in various *Stemona* species.

Taxon	Orgin	Year	Protostemonine % [w/w]
S. mairei	Derong	2016	0.18
	Taoyuan	2016	0.26
	Qina	2016	0.32
	Huangping	2016	0.48
	Panzihua	2016	0.34
	Jiangbiancun	2016	0.91
	Fulongqiao	2016	0.18
	Panzihua	2015	0.04
S. japonica	Tianmushan	2015	0.15
S. parviflora	Baisha	2015	0.11
S. sessilifolia	Qixiashan	2015	0.16
S. shandongensis	Taishan	2015	0.07

2.3.2. Isolation

Extraction. Dried roots of *S. mairei* (501 g) were ground and extracted three times with MeOH at room temperature for 4 days. The crude extract was filtered and concentrated, yielding 88.62 g crude MeOH extract (Figure 6). After evaporation of the solvent, the crude MeOH extract was partitioned between PE with 5% EtOAc and dH_20, gaining 3.66 g PE phase. The H_20 phase was partitioned with $CHCl_3$ (3.36 g) and once more with EtOAc to afford 2.38 g EtOAc phase.

Separation of PE phase. One part of the PE phase (0.8 g) was subjected to a column chromatography using Sephadex LH20 as stationary phase and eluted isocratically with MeOH/Aceton 70:30, collecting 18 fractions. After comparative TLC analyses with anisaldehyde reagent, fractions 6 to 10 were pooled together to gain 450 mg. Fraction 12 to 14 reacted with anisaldehyde in greyish, intense red and red-brownish colored dots. The latter were combined, yielding 67.3 mg. In the next step, combined fractions 12–14 were further separated by preparative TLC (Merck silica gel 60, F_{254}; PE/EtOAc, 95:5) to afford 5 mg of fraction A. Staining the developed TLC plate of fraction A with anisaldehyde resulted in three colored spots: one intense red, one dark and bright blue-greyish spot. The pooled fractions 6–10 (350 mg) were chromatographed by MPLC over silica gel 60 using an eluent gradient of PE/EtOAc with increasing polarity. The eluent gradient started with 80:20 (PE/EtOAc) until 100% EtOAc was reached. 26 fractions were collected. Comparative HPLC-UV

analyses lead to the identification of stemofoline (**4**) in fraction 25 and both substances 3,4-Dehydro-δ-tocopherol (**11**) and 3,4-Dehydro-β-tocopherol (**12**) in fraction 3 and 4. After analyses with HPLC and TLC using anisaldehyde reagent, fraction 16 (KS-Mai 11) and fraction 19 (KS-MAI 12) were prepared for NMR analyses.

Another part of the PE phase (1 g) was roughly separated by CC (Merck silica gel 60, 0.04–0.063 particle size) with solvent mixtures of hexane/PE/isopropanol or PE/EtOAc and EtOAc/MeOH with increasing polarity. 23 fractions were collected with a volume of 30–40 mL. Fraction 15 (8.8 mg) reacted with anisaldehyde in a single blueish colored spot and showed no chromophore after UV light detection and no clear peak with HPLC analyses. The latter fraction was prepared for NMR analyses (KS MAI 13). Fraction 4 amounted in 100 mg and the compounds of it reacted with anisaldehyde in intense red, blue and grey-blue colored spots (Figure 5). 15 mg therefrom were purified by preparative TLC (silica gel 60 F_{254} glass plates, thickness 0.25 mm, Merck) using two eluents. First, the TLC plate was eluted twice with PE/Aceton (9:1) and afterwards twice with $CHCl_3$/EtOAc/MeOH/EtO_2 (58:38:2:2), gaining 3 fractions. Following, Fraction 2 (4.7 mg) was further purified with a MPLC using an eluent gradient of PE/EtOAC (99:1) with an increasing polarity until 50:50. TLC derivatization with anisaldehyde produced one red-brownish spot in Fraction 2 of previous MPLC and the same one in fraction 3 of the last preparative TLC, while UV light analyses resulted in two to three different dots. The latter two fractions were combined, yielding 3.3 mg. Finally, a preparative TLC of combined fraction 2 and 3 were performed using an analytical plate and PE/Acetone (2:1) as eluent. Fraction 2 yielded 1.7 mg and was prepared for NMR analysis (KS MAI14).

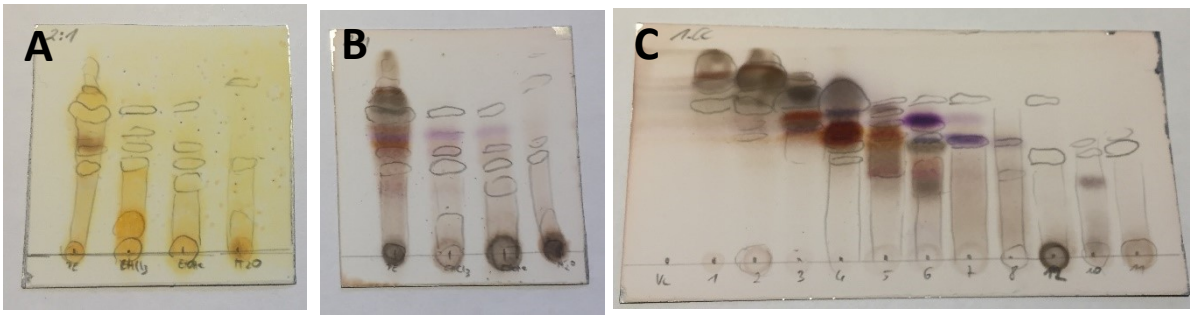

Figure 5. TLC plate (Silica gel, PE/Acetone (2:1)) of Liquid-liquid-separation phases (PE, $CHCl_3$, EtOAc and H_20 phase) was sprayed with Dragendorff (A) and with anisaldehyde (B). TLC plate of fractions 1–12 of CC (silica gel) of PE/EtOAc phase (C) were sprayed with anisaldehyde.

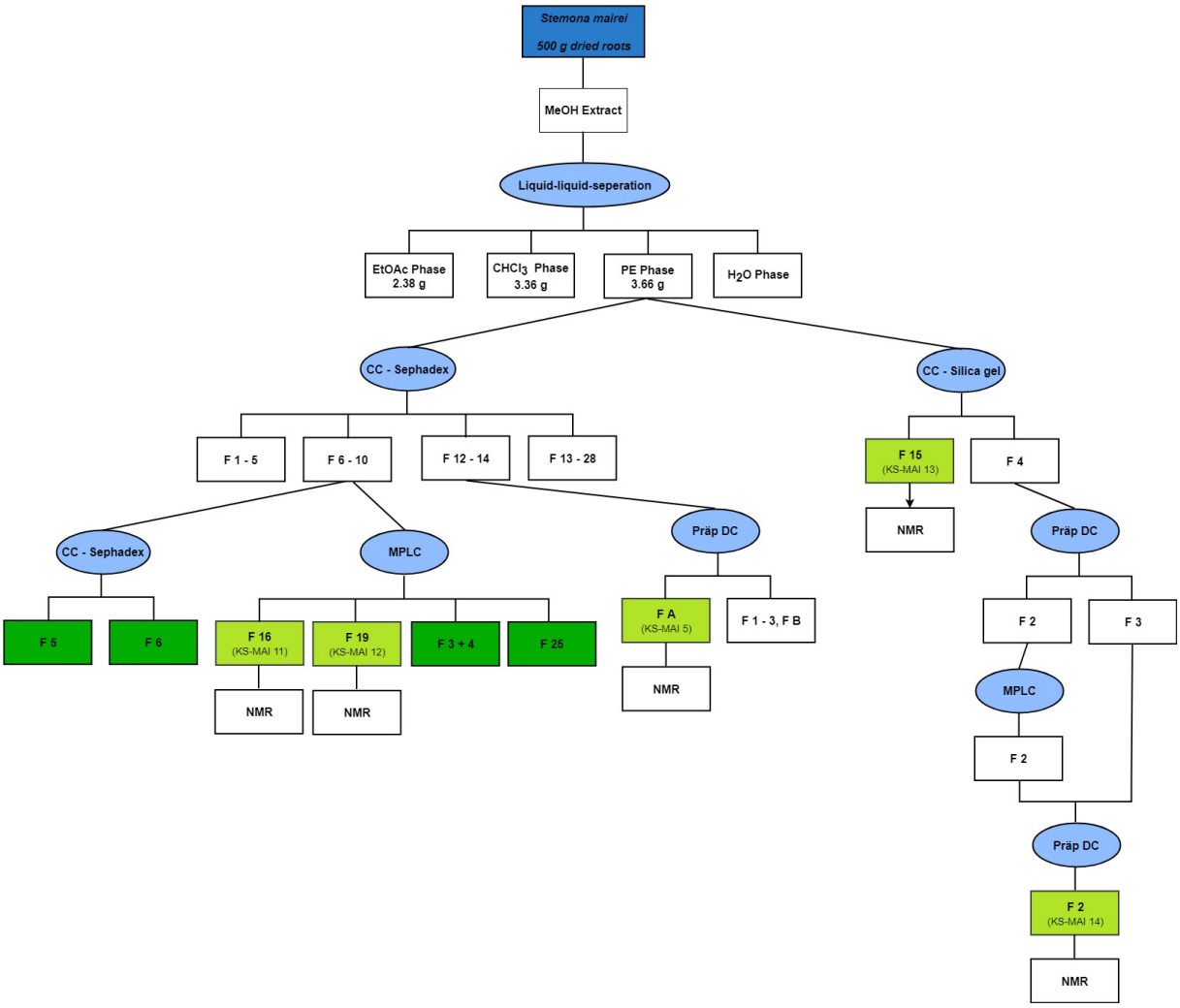

Figure 6. Schematic representation of the extraction and isolation process of the crude MeOH root extract of *Stemona mairei*. Dried roots of *S. mairei* (500 g) were extracted with MeOH and further separated with different chromatographic methods, indicated by the light blue colored ellipses. The rectangles represent different fractions of the separation process. The light green colored fractions were analyzed with NMR, whereas the dark green ones indicate fractions with identified compounds. In the PE phase, comparative HPLC-UV analyses lead to the identification of stemofoline (**4**) in fraction 25 and both substances 3,4-Dehydro-δ-tocopherol (**11**) and 3,4-Dehydro-β-tocopherol (**12**) in fraction 3, 4, 5 and 6 of two different separation steps of the PE phase. The five analyzed NMR samples were impure and the attempt to isolate new compounds was not successful.

2.3.3. Isolation of Protstemonine

Stemona mairei roots of the accession collected in De Rong (200g) were extracted three times with acetone to yield 3.5 g crude extract (Figure 7). The obtained crude extract was subsequently partitioned between $CHCl_3$ and H_20. Furthermore, the concentrated $CHCl_3$ phase was partitioned between PE and H_20 again. A part of the water phase (100 mg) was separated by open CC (Sephadex LH-20) and was eluted isocratically with MeOH, collecting 13 fractions. Comparative HPLC profiling resulted in identifying protostemonine (**1**) in fraction 5. In the next step, fraction 5 (8.3 mg) was purified by MPLC using an eluent gradient with an increasing polarity. The eluent gradient started with PE/EtOAc (90:10) until 100 % EtOAc was reached. Fraction 10 (1.6 mg) reacted with Dragendorff reagent in one orange spot and was prepared for NMR analyses.

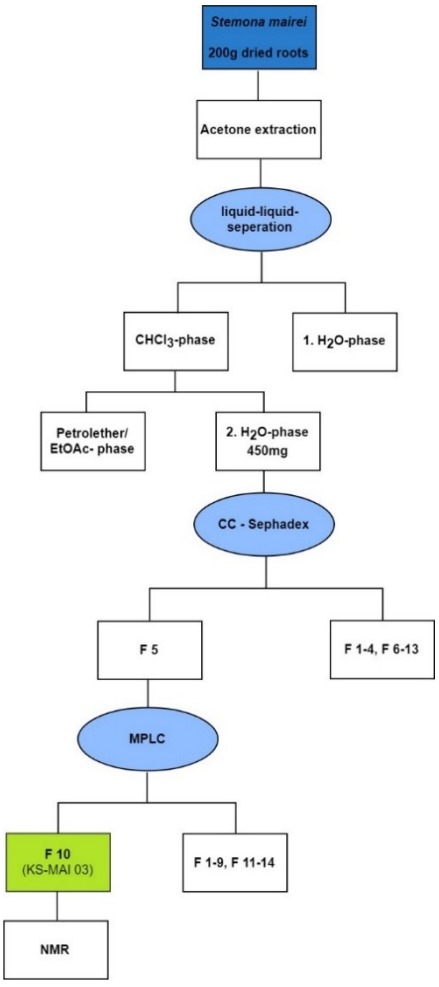

Figure 7. Schematic representation of the extraction and isolation process of Protostemonine in *Stemona mairei.*

Protostemonine (**1**) - Amorphous powder; HR-ESI-MS (pos. mode) [M+H+] m/z 418.2224 (calc. for C23H32NO6, m/z 418.2279), (neg. mode) [M-H-] m/z 416.2106 (calc. for C23H30NO6, m/z 416.2078); 1H NMR (CDCl3, 600 MHz, δH) 4.25 (1H, H-18), 4.15 (1H, H-20), 4.08 (3H, H-23), 4.07 (1H, H-8), 3.69 (1H, H-9a), 3.52 (1H, H-5a), 3.25 (1H, H-3), 2.95 (1H, H-5b), 2.90 (1H, H-10), 2.47 (1H, H-9), 2.33 (1H, H-19a), 2.28 (1H, H-7a), 2.04 (3H, H-16), 1.89 (1H, H-1a), 1.86 (1H, H-2a), 1.67 (1H, H-6b), 1.59 (1H, H-1b), 1.53 (1H, H-19b), 1.49 (1H, H-6a), 1.48 (1H, H-7b), 1.46 (1H, H-2b), 1.42 (3H, H-17), 1.29 (3H, H-22); 13C NMR (CDCl3, 150 MHz, δC) 178.6 (s, C-21), 169.9 (s, C-15), 163.0 (s, C-13), 149.0 (s, C-11), 125.2 (s, C-12), 97.6 (s, C-14), 82.8 (d, C-18), 82.4 (d, C-8), 64.0 (d, C-3), 59.8 (d, C-9a), 59.1 (q, C-23), 56.2 (d, C-9), 47.2 (t, C-5), 39.3 (d, C-10), 34.6 (d, C-20), 33.6 (t, C-19), 34.2 (t, C-7), 27.1 (t, C-2), 26.3 (t, C-1), 20.4 (t, C-6), 20.6 (q, C-17), 14.9 (q, C-22), 8.9 (q, C-16).

2.3.4. Nematode Bioassay with *Panagrellus redivivus*

Extraction. For the nematode assay, the dried roots (200–680 mg) of the *Stemona* species *S. mairei, S. kerrii., S. curtisii and S. tuberosa* were grounded with a coffee mill. The obtained powder was extracted twice with acetone under sonication for 20 min, then concentrated to yield between 6–15 mg crude extract (Table 4). An MeOH extract of *S. collinsiae* (Sri Racha, Thailand, 2004) was used for comparison.

Nematode Cultivation. The nematode *Panagrellus redivius* were bought at an online webstore (http://www.garnelen-krebs-co.at/, May 2017) and cultivated on a medium made of ground oat (*Avena sativa*, ca. 20 g) mixed with a smashed banana (*Musa sp*., ca. 30 g). A small plastic box (5 x 5 x 5 cm) was covered with the homogenous medium and afterwards inoculated with the nematodes, which were bred under dark conditions at room temperature (23 °C) for five days.

Nematode Bioassay. The nematicidal activity was tested of crude methanolic root extracts with concentration of 2.5 mg/mL and 1 mg/mL, respectively. To obtain the 0.5 mL final test solution, 1.25 mg and 0.5 mg of plant material was dissolved in 10 µL dimethylsulfoxid (DMSO) and diluted with deionized water (dH_2O) containing 0.3% v/v Tween-20. The test solution was added in 5.5 cm petri dishes. After the nematodes were collected with a spatula of the medium surface, a suspension was prepared with dH_2O. 60 µL of the obtained nematode suspension were added to the test solution in the petri dishes. Nematodes were incubated under dark conditions at room temperature (23 °C) for 24 hrs. All tests were performed in triplicates. As control a solution of 10 µL dimethylsulfoxid mixed with 490 µL dH_2O containing 0.3% v/v Tween-20 was used.

Evaluation. After 24 hours, the petri dishes containing the nematodes and test solution were photographed using the camera Mamiya Leaf Credo 80. Nematodes, both alive and dead ones, were assessed by counting them on each photograph. Dead nematodes appeared as straight lines and alive ones moved fast and were pictured as bent and curled lines, often s- or u-shaped.

Calculations. For each sample, the Lethality (L) was assessed by using the formula $L = DN/SN \times 100\%$. DN is the number of dead nematodes and SN is the sum of all counted, dead and alive, nematodes per petri dish. The mean value of three replicates was calculated and used for comparison.

Table 4. Nematode assay. Origin and weight of used plant material (roots).

Taxon	Origin	Year	Dried roots [mg]	Crude extract [mg]
S. mairei	Derong	2016	400.9	14.4
	Taoyuan	2016	201.3	7.8
	Qina	2016	201.3	6.9
	Huangping	2016	200.0	8.6
	Panzihua	2016	509.5	9.4
	Jiangbiancun	2016	200.9	8.5
	Fulongqiao	2016	200.4	9.1
S. kerrii	Xiaosanbashan	2016	401.3	8.6
S. curtisii	Trang	2016	675.7	15.0
S. tuberosa	Chaheyuxi	2016	506.4	8.1
S. collinsiae	Sri Racha	2004	-	22.9

2.4. Phytochemical Screening of *Stemona aphylla* roots

Extraction. Dried roots (260–270 mg) of eleven *S. aphylla* accessions were ground and extracted with 3 mL MeOH in Eppendorf tubes under sonication for 15 min, respectively. After centrifugation at 12.500 rpm for 15 min, the supernatant was concentrated to determine the weight of the crude extract (Table 5). The extraction step was done twice. Following, the dried crude MeOH extract was extracted twice with 0.75 mL $CHCl_3$, while vortexing for 1 min. The $CHCl_3$ extract was evaporated to dryness to yield between 3.3 and 11.5 mg.

Comparative HPLC Profiling. For HPLC measurements, the chloroform extract was dissolved in MeOH to obtain a concentration of 10 mg mL^{-1}. The HPLC profiles were compared in retention times and in their corresponding UV spectra. Analytical data of the identified compounds were compared with data of known reference compounds

Table 5. Phytochemical screening of *S. aphylla*. Origin and weight of plant material (roots).

Taxon	Origin	Dried roots [mg]	$CHCl_3$ Extract [mg]
S. aphylla	#1	262.2	3.4
	#2	262.5	4.4
	#3	266.7	6.5
	#4	265.9	7.8
	#5	266.3	3.4
	#6	266.5	7.7
	#7	268.8	5.7
	#8	263.8	3.3
	#9	265.6	3.6
	#10	262.5	3.5
	#11	268.5	11.5

1-11 are eleven accessions of *S. aphylla* collected in Sa Kaeo, Thailand.

2.5. UV Spectra

The UV Spectra of different compounds in the extracts of *Stemona* species were recorded with the UV detector of the HPLC and compared to UV spectra of known reference compounds. In Figure 8 and Figure 9, UV Spectra of different un- and identified compound groups are displayed. Noticeable by comparing the UV spectra of different chemical classes is that the UV absorption spectra is unique, and the UV spectra curve differs in how many absorption maxima (λ_{max}) it has and in which region the absorption maxima are.

Stemona alkaloids have a characteristic, uniform UV absorption spectra, they exhibit one major absorption in the region of 296–312 nm. This peak is associated with the chromophore structure of the *Stemona* alkaloids, which is identical in the various *Stemona* alkaloids. This similarity of the UV spectra makes it so difficult to differentiate the *Stemona* alkaloids, only in the combination with their retention time it is possible, but the retention time varies with the eluent. In this study the eluent MeOH (B) and 10 mM ammonium acetate (A) was used for HPLC analyses. The UV spectra of protostemonine (**1**) shows beside its λ_{max} a small depression on the ascending part of the curve at 260–280 nm and this small depression can be found in protostemonine (**1**) derivatives as well. The two identified *Stemona* alkaloids oxyprotostemonine (**6**) and stemocurtisinol (**9**) exhibit the typical UV absorption spectra of *Stemona* alkaloids, both substances have the λ_{max} at 298 nm.

The identified two tocopherols have a completely different UV spectra with more than one peak. One major peak occurs in the region of 220–230 nm with a second small peak at the top, which are followed by two smaller peaks in the region of ±270 and ±335 nm (Figure 8). 3,4-dehydro-δ-tocopherole (**9**) has its absorption maxima at 230, 264, 334 nm and 3,4-dehydro-β-tocopherole (**10**) at 220, 228, 270, 278 and 336 nm.

Corresponding to the aromatic system of stilbenoids the UV spectra of stilbenoids exhibits two absorption maxima. The UV spectra of Stemofuran N (**14**) possess an absorption maximum at 210 and the other one, which is broader, at 304 nm. Unidentified compound S01 bears resemblance to the UV spectrum of Stemofuran N (**14**), the UV spectra is featured with two absorption maxima at 210 and 296 nm. Unidentified compounds U01 and U02 show multiple peaks. Compound U01 with rt 2.641 min possess λ_{max} at 204, 234, 296 and 324 nm, whereas of compound U02 is characterized by three λ_{max}: 204, 234 and 298 nm.

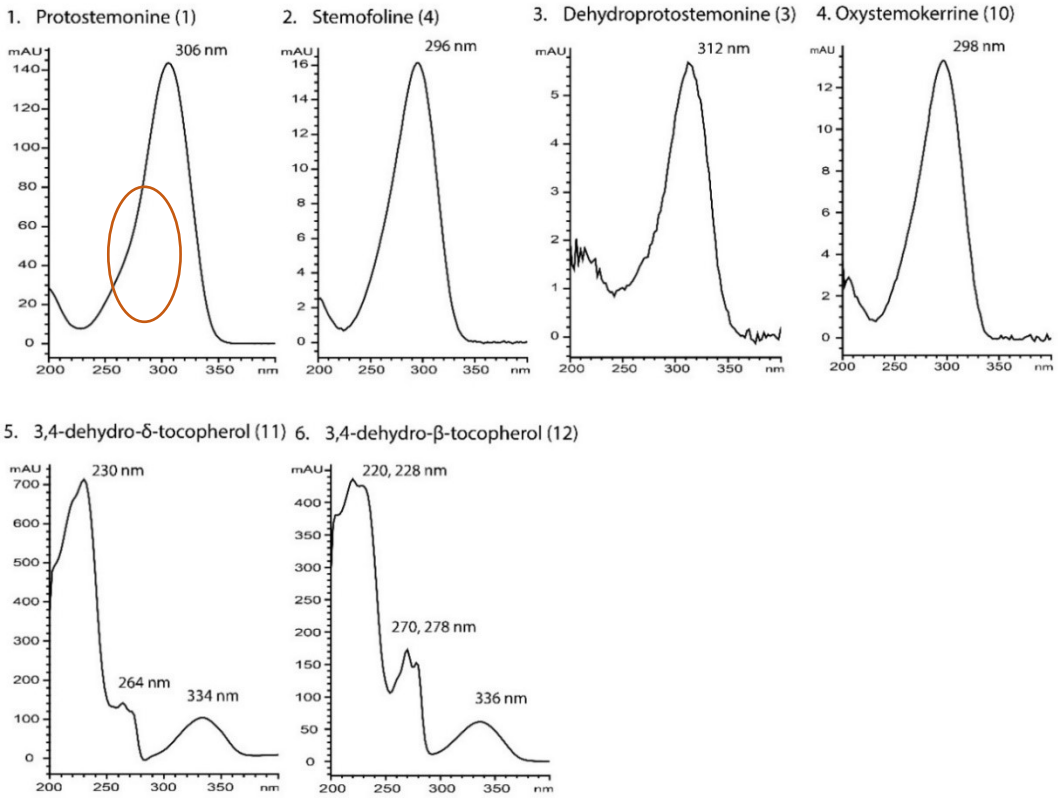

Figure 8. UV-Spectra of identified *Stemona* alkaloids (A) and tocopherols (B) in *Stemona* species with their absorption maxima (λ_{max}). The brown circle indicates a small depression in the UV spectra characteristic for protostemonine (**1**).

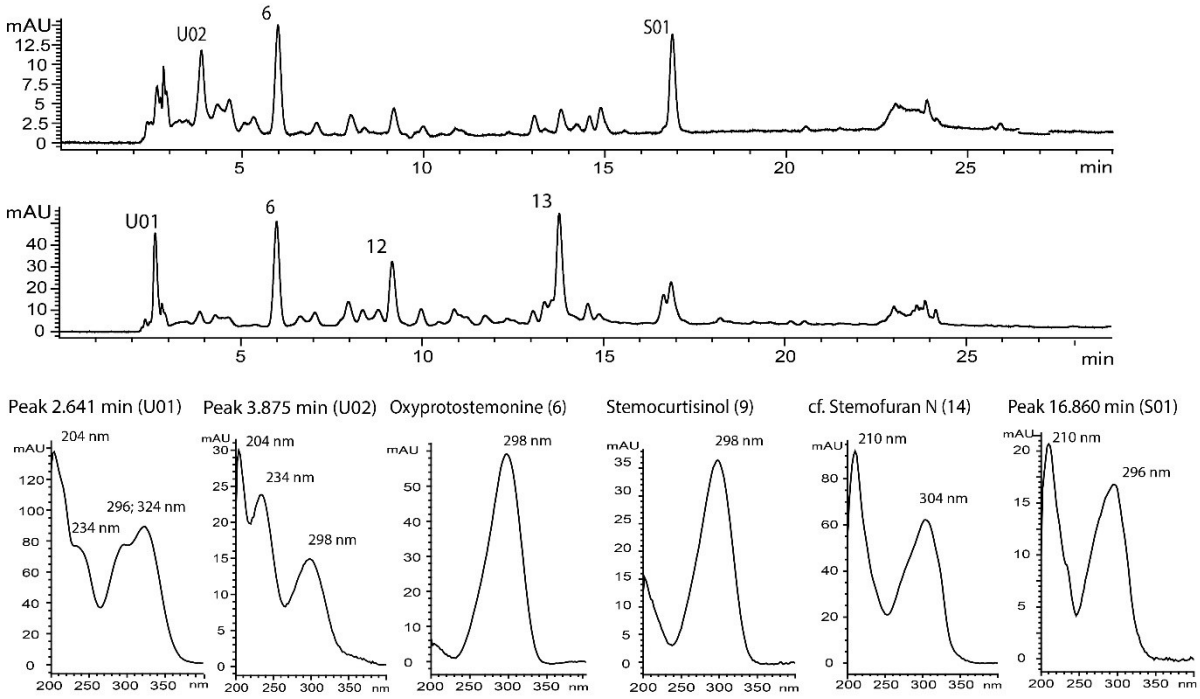

Figure 9. (A) HPLC Profiles of *S. aphylla* root extracts: UV detection was at 306nm. (B) UV Spectra of un- and identified chemical compounds: Compounds **6** and **9** are *Stemona* alkaloids. Compound **6** was identified as Oxyprotostemonine and compound **9** as Stemocurtisinol. Compound **14** was identified as Stemofuran N (**14**) and compound S01 could only be classified as stilbenoid, but not further identified by comparative HPLC spectra analysis.

3. Results

3.1. Phytochemical Screening of *Stemona mairei* and related species

Crude methanolic extracts of air dried roots of *S. mairei* and five other *Stemona* species were investigated for their alkaloid and tocopherol accumulation patterns by comparing their HPLC profiles. The focus was on *S. mairei*, from which nine accessions were used to examine species specific accumulation trends. The results of the phytochemical screening are displayed in Figure 10, Figure 11, Figure 12 and Table 6.

3.1.1. Alkaloids

All nine examined accessions of S*. mairei* showed little variation in their HPLC profiles. The HPLC profiles exhibit peaks in two regions (Figure 10). The first peaks are between retention time 2–4 min and can be classified as phenolic compounds due to their UV spectra. The other compounds with rt 4–11 min were identified as *Stemona* alkaloids. *Stemona mairei* accessions were characterized by protostemonine (**1**) as the major component. Beside the prominent peak of protostemonine (**1**), all nine accessions exhibited a peak at rt 8 min in their HPLC profile proving the occurrence of isoprotostemonine (**2**) and another one at rt 7 min a yet unidentified compound. The latter one might be a potostemonine derivate, because it possessed a small depression in the ascending part of the UV spectra curve like it is typical for protostemonine (**1**). Only in five out of nine samples stemofoline (**4**) was accumulated in minor amounts (Figure 10).

The screening of the sap of deep-frozen *S. mairei* roots at -80°C (Figure 11) showed similar results like the MeOH extracts of the corresponding air-dried roots (Figure 10). All seven investigated accessions are characterized by the occurrence of protostemonine (**1**) as the major alkaloid, followed by smaller amounts of isoprotostemonine (**2**). In four out of seven accessions stemofoline (**4**) is accumulated in the roots. In the HPLC profile between retention time 2 and 4 min, a few compounds can be also classified as phenolic compounds due to their UV spectra. In contrast to the root samples, the HPLC profile of the leaf extract is totally different. Outstanding is, that neither alkaloids, nor stilbenoids were detected in bigger amounts. A few phenolic compounds with the retention time between 2 and 4 min were accumulated.

The comparative HPLC profiling of the other five investigated *Stemona* species resulted in similar looking HPLC profiles with one exception (Figure 12). The four *Stemona* species *S. parviflora*, *S. sessilifolia*, *S. shandongensis* and *S. japonica* were characterized by the pyrroloazepine-type alkaloid protostemonine (**1**) as the major accumulated alkaloid, which was accompanied by small amounts of isoprotostemonine (**2**). The exception is *S. kerrii*, which diverged from the other five species including *S. mairei* by accumulating the pyridoazepine-type stemocurtisine (**5**) as the dominant alkaloid. Addionally, oxyprotostemonine (**6**), protostemonine (**1**) and dehydroprotostemonine (**3**) were identified in *S. kerrii*.

As observed in HPLC profiles of *S. mairei*, all five investigated *Stemona* species exhibit an accumulation of a few compounds between retention time 2–4 min, which possess a UV-spectra similar to phenolic compounds.

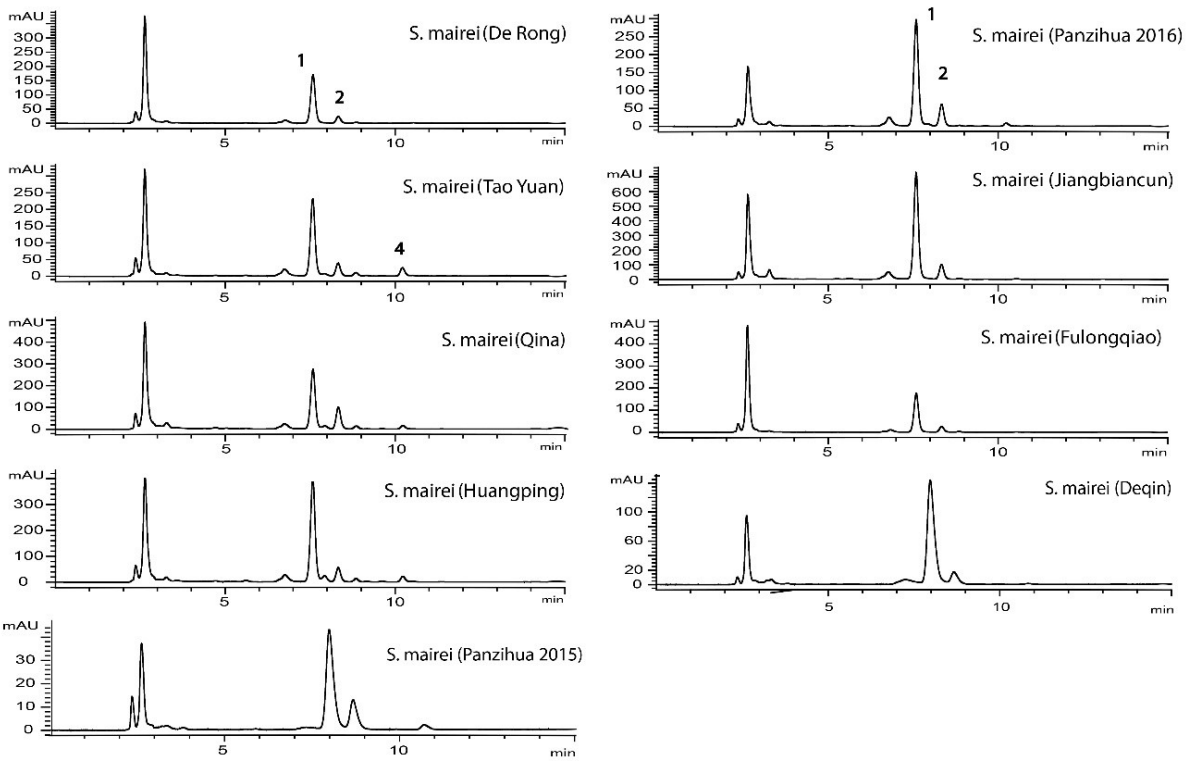

Figure 10. In crude methanolic root extracts of nine accessions of *Stemona mairei*, three alkaloids were identified: Protostemonine (**1**), isoprotostemonine (**2**) and stemofoline (**4**). Peaks between rt 2–4 min were identified as phenolic compounds. UV detection at 306nm.

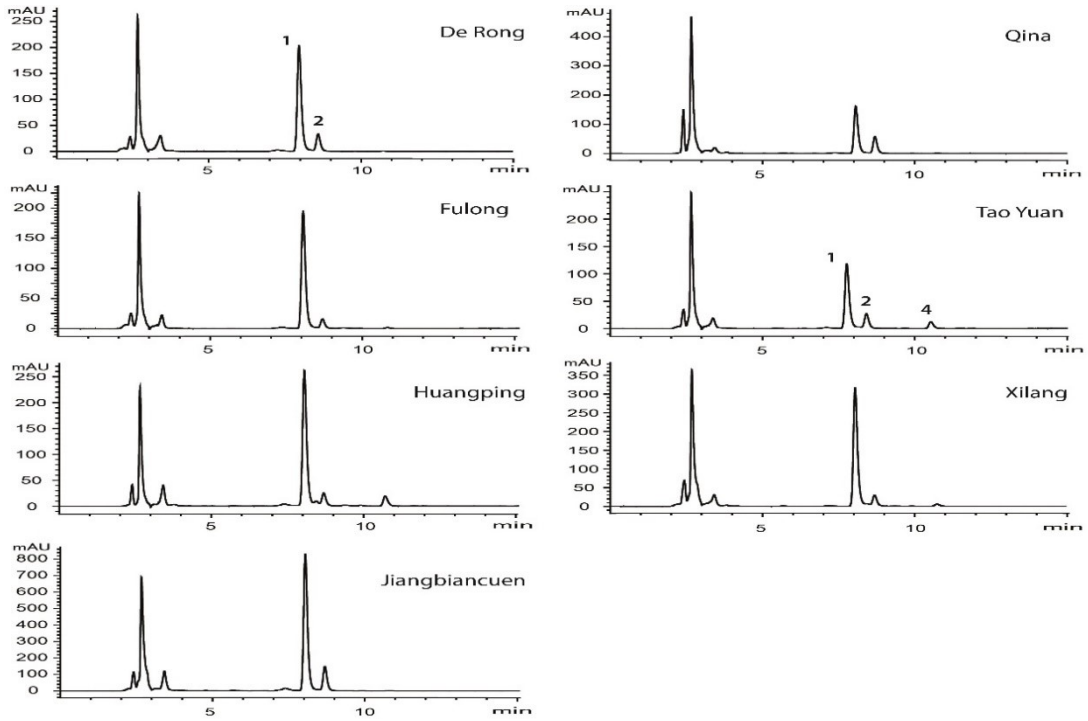

Figure 11. HPLC Profiles of the sap of deep-frozen *S. mairei* roots at -80°C. Protostemonine (**1**) was identified in major amounts, whereas isoprotostemonine (**2**) and stemofoline (**4**) only in minor. UV detection at 306 nm.

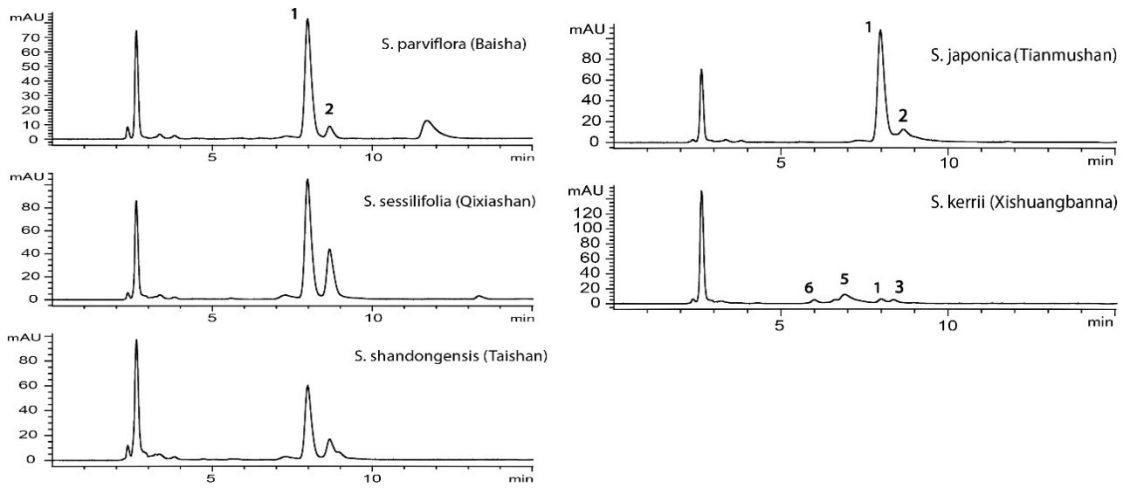

Figure 12. HPLC chromatograms of crude methanolic root extracts of five studied *Stemona* species. Protostemonine (**1**), isoprotostemonine (**2**), dehydroprotostemonine (**3**), stemocurtisine (**5**), oxyprotostemonine (**6**). UV detection at 306nm.

3.1.2. Tocopherols

Beside alkaloids, tocopherols were identified with a rt 26–29 min by comparative HPLC analysis (Table 6). *Stemona mairei* accessions were characterized by 3,4-dehydro-δ-tocopherol (**11**) as the main tocopherol, which was accompanied by small quantities of 3,4-dehydro-β-tocopherol (**12**). The results of the phytochemical screening of the other five studied *Stemona* species showed that not only *S. mairei* (Table 6), but also *S. japonica* accumulated 3,4-dehydro-δ-tocopherol (**11**) in major amounts, which was also accompanied by smaller amounts of 3,4-dehydro-β-tocopherol (**12**). On the contrary, *S. kerrii*, *S. shandongensis* and *S. sessilifolia* were characterized by accumulation of β-tocopherol (**13**) as the major tocopherol derivative.

Table 6. Results of Phytochemical Screening for Alkaloids and Tocopherols in eight different *Stemona* species.

Taxon	Orgin	Year	Alkaloids								Tocopherols		
			1	2	3	4	5	6	7	8	11	12	13
S. mairei	Derong	2016	●	○							▪	□	
	Taoyuan	2016	●	○		○					▪	□	
	Qina	2016	●	○		○					▪	□	
	Huangping	2016	●	○		○					▪	□	
	Panzihua	2016	●	○		○					▪	□	
	Jiangbiancun	2016	●	○							▪	□	
	Fulongqiao	2016	●	○							▪	□	
	Panzihua	2015	●	○		○					▪	□	
	Dequin	2015	●	○							▪	□	
S. kerrii	Xiaoganba	2016	○		○		●	○					▪
	Xishuangbanna	2015	○		○		●	○					▪
S. japonica	Tianmushan		●	○							▪	□	
S. parviflora	Baisha		●	○									
S. sessilifolia	Qixiashan		●	○									▪
S. shandongensis	Taishan		●	○	○				○	○			▪

Numbering of assigned compounds: Protostemonine (**1**), isoprotostemonine (**2**), dehydroprotostemonine (**3**), stemofoline (**4**), stemocurtisine (**5**), oxyprotostemonine (**6**), stemocochinin (**7**), croomine (**8**), 3,4-dehydro-δ-tocopherole (**11**), 3,4-dehydro-β-tocopherole (**12**) and β-tocopherole (**13**). Major compounds are indicated by a **●/▪** and minor ones with an ○/□.

3.1.3. Quantification

Five external standards of protostemonine (**1**) were prepared with concentrations between 125 to 1000 μg mL^{-1} to quantify the amount of protostemonine (**1**) in the samples. In Table 3, the contents of protostemonine (**1**) in various *Stemona* species are listed. The parameters of the calibration curve were y = 7.4372x - 328.97 and r^2 = 0.9989. Protostemonine (**1**), the major alkaloid in *S. mairei* ranged in eight accessions from 0.04 to 0.91% (w/w). The concentration of protostemonine (**1**) in *S. shandongensis* was 0.07%, in *S. japonica* 0.15%, in *S. parviflora* 0.11% and in *S. sessilifolia* 0.16%.

3.1.4. Isolation

The PE-phase was separated in different steps by CC (sephadex or silica gel), by MPLC and preparative TLC. Comparative HPLC and UV analyses of MPLC fractions with analytical data of known reference compounds resulted in identifying protostemonine (**1**), stemofoline (**4**), 3,4-Dyhdro-δ-tocopherol (**10**) and 3,4-Dyhdro-β-tocopherol (**11**). NMR analyses of sample KS-MAI11 and KS-MAI12 were not successfully interpretable, but the analyses suggest the presence of a mixture of different fatty acids in both samples. Further identification of these fatty acid mixtures is with HPLC and NMR not possible, suggestable would be further analysis with Gas Chromatography (GC). The fractions with the desired target compounds were difficult to separate and isolate, so any further isolation attempt of the PE phase lead to more impure fractions (KS-MAI13 and KS-MAI14) and were not successful in isolating new compounds.

3.1.5. Nematode Bioassay

To verify previous observed insecticidal properties of *Stemona* roots, crude methanolic root extracts of five different *Stemona* species were tested on the free-living soil nematode *Panagrellus redivivus*. Nematodes were exposed to two different concentration (2.5 and 1.0 mg mL^{-1}) of the root extracts for 24 hrs, respectively. The lethality (L) for the seven-different tested *S. mairei* extracts with 2.5 mg mL^{-1} ranged from 50 to 58.8% and the ones with 1.0 mg mL^{-1} from 23.6 to 48.4% (Table 7). The root extracts of *S. kerrii, S. curtisii* and *S. tuberosa* showed similar lethalities in both concentrations, an exception was the root extract of *S. collinsiae*. The nematicidal activity showed a higher activity against *P. redivivus* with 77.1% in the 2.5 mg mL^{-1} conc. root extract and 33.5% in conc. 1.0 mg mL^{-1} (Table 7).

Table 7. Nematicidal activities of *Stemona* crude methanolic root extracts.

Taxon	Origin	Lethalities (%)	
		2.5 mg mL $^{-1}$	1 mg mL^{-1}
S. mairei	Derong	58.8	31.8
	Taoyuan	55.5	23.6
	Qina	53.1	25.1
	Huangping	51.9	38.1
	Panzihua 2016	55.2	40.5
	Jiangbiancun	54.2	35.9
	Fulongqiao	50.0	48.4
S. kerrii	Xiaosanbashan	52.4	35,0
S. curtisii	Trang	47.6	31.8
S. tuberosa	Chaheyuxi	54.9	37.2
S. collinsiae	Sri Racha	77.1	35.5

Lethality was calculated for each extract. The value is the mean of three replicates.

3.2. Phytochemical Screening of *S. aphylla* accessions

The crude MeOH extracts of eleven accessions of *S. aphylla*, collected in Thailand, were concentrated, extracted again with $CHCl_3$ and analyzed by HPLC. HPLC and UV profiles were compared to see differences in intraspecific accumulation trends of different chemical compound classes.

3.2.1. Alkaloids and Stilbenoids

Comparative analysis revealed variable HPLC profiles with the occurrence of different phenolics, alkaloids and stilbenoids in variable amounts and of different accumulation patterns (Figure 9). The HPLC profiles of *S. aphylla* accessions (Figure 8) were measured with the concentration of 10 mg mL^{-1} as well as the HPLC profiles of *S. mairei* in Figure 9. Comparing the HPLC profiles of these two species (Figure 8, Figure 9) exhibit low mAU (milli absorption unit) values in the HPLC profiles of *S. aphllya* indicating a low alkaloid content in *S. aphylla* roots. Following two alkaloids and one stilbenoid could be identified in this species by analytical data of known reference compounds: oxyprotostemonine (**6**), stemocurtisinol (**9**) and cf. stemofuran N (**14**). Notable by comparing the HPLC profiles of *S. aphylla* is, that all eleven accessions accumulate oxyprotostemonine (**6**) in major amounts (Table 8), whereas additionally either cf. stemofuran N (**14**) (in accession #1, #3, #5, #6, #9) or the yet unidentified stilbenoid S01 with the rt 16.862 is present in major amounts in accession #1 and #2. The

unidentified substance U01 with retention time 2.64 min and UV spectra indicative of a phenolic structure occurs in accession #3 and #6 in major amounts. In accession #1, another unidentified substance (U02) with retention time 3.9 min is accumulated in larger amounts. To sum up, there is no uniform accumulation trend within *S. aphylla*. One accumulation trend is towards oxyprotostemonine (**6**) as major constitute which is accompanied by only minor amounts of other yet unidentified substances, which is found in five accessions (#4, #7, #8, #10, #11). Besides the major accumulated substances, many other phenolics and stilbenoids were detected in smaller amounts in HPLC profiles, but they could not be identified so far.

Table 8. Minor and major amounts of un- and identified compounds in eleven accessions of *S. aphylla.*

HPLC Peaks		**Accessions of *S. aphylla***										
Substance	rt (min)	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
Unident. (U01)	2.649	o	o	**x**	o	o	**x**	o	o	o	o	o
Unident. (U02)	3.875	o	**x**	o	o	o	o	o	o	o	o	
Oxyprotostemonine (**6**)	5.890	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
Stemocurtisinol (**9**)	9.174	o	o	o	o	**x**	**x**	o	o	o	o	
cf. Stemofuran N (**14**)	13.791	**x**	o	**x**	o	**x**	**x**	o	o	**x**	o	o
Unident. (S01)	16.862	**x**	**x**	o	o	**x**	o	o	o	o	o	o

Major accumulated compounds are assigned with an **x** and minor amounts with an o. Generally, *S. aphylla* shows a reduction in alkaloid content (Kongkiatpaiboon et al., 2011), this should be kept in mind for the interpretation of these results. In comparison to the HPLC profiles of *S. mairei* (Figure 10), the HPLC profiles of *S. aphylla* accessions (Figure 8) shows only low mAU (milli absorption unit) values indicating the low alkaloid content of *S. aphylla* roots. In Figure 8 and Figure 10, the samples were measured with the same concentration of 10 mg mL^{-1}. #1 –#11 are collections of different localities in Thailand.

4. Discussion

Stemona alkaloids with their unique chemical structure and their various biological activities are still subject of recent phytochemical studies (Kongkiatpaiboon et al., 2011; Huang et al., 2016; Chen et al., 2017a), but due to the ethnomedical application of *S. tuberosa, S. japonica* and *S. sessilifolia* in southeast Asia the focus was mainly on these three *Stemona* species. The current study concentrated on five *Stemona* species collected in China and on one species collected in Thailand.

Stemona alkaloid accumulation in *Stemona mairei* and related species

Analyzing the intraspecific alkaloid and tocopherol accumulation patterns of nine *S. mairei* individuals, collected in different localities, resulted in uniform HPLC profiles with only little variations (Figure 1). In all nine examined accessions, the pyrroloazepine alkaloid protostemonine (**1**) was identified as the predominant alkaloid and appears as chemical marker for this less studied species. Apart from the prominent peak of protostemonine (**1**), HPLC profiles of the accessions are characterized by small amounts of isoprotostemonine (**2**) and a yet unidentified compound at rt 7 min. Among the nine samples, only four exhibited minor amounts of stemofoline (**4**). Interestingly, data of the present study does not support previous findings of tuberostemonine and its derivates (Cai and Luo, 2007). Maistemonine was not detected in *S. mairei* (Lin et al. 1991). These contradictory results may be caused by using misidentified plant material in their studies. Considering the chemical instability of *Stemona* alkaloids, transformation or degradation of the secondary metabolites can be expected during the extraction and isolation process, leading to artefacts accompanied by changes of their biological functions (Greger, 2006). Artefacts may be caused by extensive acid-base treatment, exposition of the plant material and extracts to air and light causing oxidation processes or photoisomerization (Greger, 2006; Kongkiatpaiboon et al., 2011). Therefore, the sap of seven deep frozen S. *mairei* roots at -80 °C were analyzed by HPLC to test if sample preparation, drying plant material at 40 °C and immediate extraction after grinding, has an impact on the secondary metabolites. Comparing the HPLC profiles of the sap (Figure 9) to the corresponding MeOH extracts of the air-dried roots (Figure 10) resulted in no obvious differences, concluding that transformation and degradation of the secondary metabolites plays a subordinary role in the used extraction method. In comparison to the HPLC profile of

the sap of deep-frozen roots, the one of the deep-frozen leaves stuck out by the absence of alkaloids and stilbenoids indicating that the roots are the main storage organs for alkaloids and stilbenoids. This finding is in congruence with previous literature of alkaloids, stilbenoids and as well of tocopherols in the genus *Stemona* (Pacher et al., 2002; Brem et al., 2004; Greger, 2006; Chen et al., 2017a).

Furthermore, five other *Stemona* species were investigated for their species-specific accumulation pattern of alkaloids. *Stemona shandongensis* was studied phytochemically for the first time. This species is characterized by the pyrrolazepine alkaloid protostemonine (**1**) as the main alkaloid co-occurring with minor amounts of isoprotostemonine (**2**). Additionally, small amounts of dehydrostemofoline (**3**), stemocochinin (**7**) and croomin (**8**) were identified in *S. shandongensis* (Figure 2). In *Stemona parviflora*, protostemonine (**1**) was detected as the dominant alkaloid together with small amounts of isoprotostemonine (**2**). These findings are in congruence with the study of Huang et al. (2011), which reports the occurrence of protostemonine (**1**) and its isomer isoprotostemonine (**2**) besides 23 other identified alkaloids in this species. The present study supports the results of the study of Li et al. (2007) showing that *S. parviflora* is characterized by major amounts of protostemonine (**1**). Whereas, the other two reported alkaloids croomine and maistemonine were not detected in this study. *Stemona sessilifolia* and *S. japonica* are two of the intensely studied *Stemona* species in the past. In both species, protostemonine (**1**) was detected together with isoprotostemonine (**2**). The identification of these two alkaloids in *S. sessilifolia* are in accordance with previous studies (Schinnerl et al., 2007; Yang et al., 2009), whereas the other reported alkaloids like stemonine or maistemonine were not identified. In the study of Tang et al. (2008), 15 alkaloids have been reported *for S. japonica* amongst them are protostemonine (**1**) and isoprotostemonine (**2**). The alkaloid accumulations pattern of *S. kerrii* diverged from the other examined species by stemocurtisine, which has a pyrido[1,2-α]-azepine nucleus, as predominant component. On the contrary, pyrrolo[1,2-α]-azepine type alkaloids like protostemonine (**1**), oxyprotostemonine (**6**) and dehydroprotostemonine (**3**) occurred only as minor compounds. The main alkaloid component of *S. kerrii* was not identified neither in the study of Kaltenberger et al. (2003) nor in Kongkiatpaiboon et al. (2011). But the minor alkaloids identified in this study have already been detected by Kaltenegger et al. (2003). Even though, stemokerrine is identified in both studies (Kaltenegger et al., 2003; Kongkiatpaiboon et al.,

2011) as major alkaloid, it was not detected in *S. kerrii* in the present study. These intraspecific accumulation patterns may be explained by the big distribution area of *S. kerrii* or general environmental factors.

Secondary metabolites of *Stemona aphylla* accessions

The Thailand endemic species *S. aphyll*a exhibits besides different accumulation trends a general reduction in alkaloid content like *S. rupestris* Inthachub and *S. cochinchinensis* Gagnep. (Kongkiatpaiboon et al., 2011). The chemical composition of *S. aphylla* varies either accumulating pyrroloazepine type alkaloid protostemonine (**1**) or different pyridoazepines as major component (Kongkiatpaiboon et al., 2011). On the bases of the present investigations, comparative analysis confirmed variable HPLC profiles with the occurrence of different alkaloids and stilbenoids in varying amounts and of different accumulation patterns (Figure **9**). In all eleven investigated accessions the main alkaloid component was oxyprotostemonine (**6**) accompanied by stemocurtisinol (**9**) in varying amounts. Beside the two alkaloids of the pyridoazepine type, no pyrroloazepine type alkaloids like protostemonine (**1**) were detected. This finding is in contradiction with the study of Kongkiatpaiboon et al. (2011) that reported protostemonine (**1**) accumulating individuals. In this study, the eleven MeOH extracts of *S. aphylla* were analyzed with the same concentration 10 mg mL^{-1} as the nine accessions of *S. mairei* MeOH extracts. Comparing the HPLC profiles of these two species resulted in low mAU (milli absorption unit) values for *S. aphylla* (Figure 9), ranging from 12–50 mAU, whereas *S. mairei* possessed values from 300 mAU up to 450 mAU (Figure 10). This trend indicates a reduction of alkaloid formation in *S. aphylla* and supports the results of Kongkiatpaiboon et al. (2011). Apart from the alkaloids, stilbenoids were present in all accessions of *S. aphylla*. The HPLC profile revealed the occurrence of the stilbenoid stemofuran N (14) and a yet unidentified stilbenoid (S01). In a previous phytochemical investigation of *S. aphylla,* Stemofuran N (**14**) has already been detected together with other stemofuran derivatives (Sastraruji et al., 2011).

Chemotaxonomic aspects

From previous literature, it became apparent that *Stemona* species fall into two main groups concerning the alkaloid accumulation (Schinnerl et al., 2007, Kongkiatpaiboon et al., 2011). The metabolic differences are in the accumulation trend either towards protostemonine- or

stichoneurine-type derivatives (Schinnerl et al., 2007), when following Greger's (2006) classification system. Most *Stemona* species are characterized by the occurrence of protostemonine derivatives, which were investigated in this study. The *Stemona tuberosa* complex is clearly segregated by accumulating tuberostemonine and its derivatives, having a stichoneurine-skeleton (Greger, 2006; Chen et al., 2017a). Moreover, in *Stemona phyllantha* the stichoneurine-type alkaloid parvistemonine has been detected and can be grouped together with *S. tuberosa* (Schinnerl et al., 2007, Kongkiatpaiboon et al., 2011). In the present study, the accumulation trend towards protostemonine type alkaloids can be confirmed for *S. mairei*, *S. sessilifolia* and *S. japonica.* Furthermore, *S. shandongensis,* which was studied for the first time, was also characterized by protostemonine derivatives. Overall, the results of this study support the taxonomic splitting of the genus *Stemona* into two subgroups. Furthermore, the occurrence of the pyrroloazepine type alkaloid protostemonine (**1**) as major alkaloid in *S. mairei, S. sessilifolia, S. japonica, S. parviflora* and *S. shando*ngensis suggests a close relationship of these five species. The exception of the five Chinese collected species was *S. kerrii,* which was well diversified from the other *Stemona* species by the occurrence of pyridoazepine-type alkaloid stemocurtisine (**5**) as major alkaloid component. This in accordance with previous reports (Schinnerl et al., 2011) and underlines that *S. kerrii* is a rather homogenous species.

Occurrence of Tocopherols

Stemona species accumulate different antioxidative tocopherol and dehydrotocopherol derivatives mainly in their tuberous roots (Brem et al., 2004; Chen et al., 2017a). The structurally closely related dehydrotocopherols diverge from chromanols by a double bond between C-3 and C-4 position. Beside the well-known *Stemona* alkaloids, they appear as typical chemical character for the genus *Stemona* (Brem et al., 2004). The results of the current study support these previous findings of the occurrence of chromanol and dehydrotocopherol (chromenol) derivatives in *Stemona* species. *Stemona mairei* diverged from the other species by accumulating only dehydrotocopherol derivatives. 3,4-dehydro-δ-tocopherol were detected in major amounts together with minor amounts of 3,4-dehydro-β-tocopherol. *Stemona japonica* possessed the same accumulation pattern as *S. mairei*. As previously reported, *S. kerrii* exhibited also a preponderance towards chromanol derivatives in the present study (Brem et al., 2004). In contrast to the study of Brem et al. (2004), neither the

occurrence of δ-tocopherol nor of γ-tocopherol could be confirmed for *S. kerri*, but the accumulation of β-tocopherol was affirmed. However, *S. parviflora* diverged from the others by the absence of chromanol and chromenol derivatives. The biological function of these tocopherol and dehydrotocopherol derivatives in the genus *Stemona* have not been elucidated so far. In recent studies, other functions beyond antioxidative activities were suggested in plants (Munné-Bosch and Alegre, 2002, Falk and Munné-Bosch, 2010; Hussain et al., 2013). Considering the instability of the *Stemona* alkaloids and the co-occurrence of these alkaloids together with tocopherols derivatives, the latter may have the function of preventing alkaloids from oxidation, which was suggested by Chen et al. (2017a) for tuberostemonine. Further research is required to prove this hypothesis. Moreover, it would be interesting, if there is another abiotic or biotic factor causing oxidative stress in belowground organs. UV light can be excluded for producing oxidative stress through radical oxygen species (ROS) in the roots. In previous reports the antioxidant activity of tocopherol derivatives was discussed to protect the photosystem and membranes of the plants (Falk and Munné-Bosch, 2010) from ROS. However, further studies are required to get an insight in the biological function of tocopherol derivatives in the roots of *Stemona* species.

Nematicidal Activity

Comparing the lethality values of five tested *Stemona* species showed that *S. collinsiae* stuck out with the highest nematicidal activity with 77.1% in conc. of 2.5 mg mL^{-1}. Previous studies have already reported the anti-insecticidal properties of this species against *Plutella xylostella* (Jiwajinda., 2001) and *Spodoptera littoralis* (Brem et al., 2002). The chemical constituent, to which the most insecticidal activity can be contributed, is didehydrostemofoline (**3**), the major accumulated alkaloid in the roots of *S. collinsiae* (Kongkiatpaiboon et al., 2011). It is accompanied by stemofoline (**4**), which is also effective against insects (Brem et al., 2002; Kaltenegger et al, 2003). In contrast to *S. collinsiae*, all the crude methanolic root extracts of *S. mairei* showed less nematicidal activity with lethality rates between 50 and 58.8% in the higher concentrated samples. Huang et al. (2016) tested beside the crude extracts of *S. parviflora*, pure alkaloids like protostemonine (**1**) and stemofoline (**4**) against *P. redivius*. This study shows nematicidal bioactivity of both alkaloids and as well of the crude EtOH extract of *S. parviflora* (Huang et al., 2016). Nematicidal activity was also shown for the root extracts of *S. curtisii, S. tuberosa* and *S. kerrii*. Whereas, *S. curtisii* had the lowest activity in the

2.5 mg mL^{-1} concentrated root extract with the value 47.6%. It is known according to Kongkiatpaiboon et al. (2011) that this species accumulates either stemofoline (**4**) or stemocurtisine as main alkaloid. The lethality values of *S. kerrii* and *S. tuberosa* are in the same range like *S. mairei*, though the main chemical constituent are different. S*temona kerrii* is characterized by stemocurtisine and traces of protostemonine (**1**) and *S. tuberosa* by tuberostomine- and croomine- type alkaloids (Greger, 2005).

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6. Abstract

The Chinese endemic species *Stemona mairei* and five other *Stemona* species, collected in China, were examined for alkaloids and tocopherols by comparative HPLC analyses to get an insight in the accumulation pattern within species. Additionally, a Thai endemic species, namely *S. aphylla,* was analyzed for its alkaloids and stilbenoids. Crude methanolic root extracts of nine accessions of *S. mairei* exhibited uniform alkaloid and tocopherol accumulation patterns with only little variations. Generally, this unstudied species is characterized by the accumulation of the pyrroloazepine-type alkaloid protostemonine (**1**) as major component. In the four species *S. japonica, S. sessilifolia, S. parviflora* and *S. shandongensis* protostemonine (**1**) was identified as the main alkaloid component. In contrast to the afore mentioned species, *S. kerrii* diverged by accumulating pyridoazepine type alkaloids, whereas stemocurtisine (**5**) was identified as the major alkaloid. *Stemona aphylla* exhibited variable HPLC profiles with accumulation of alkaloids and stilbenoids in various amounts and generally in reduced amounts. In all eleven examined accessions the pyridoazepine-type alkaloid oxyprotostemonine (**6**) was identified. Furthermore, crude methanolic root extracts of five *Stemona* species were tested for its bioactivity and showed in general toxic properties against the nematode *Panagrellus redivivus* Linné, whereas *S. collinsiae* showed the highest nematicidal activity. From a chemotaxonomic point of view, the results of this study suggest a close relationship of *S. japonica, S. sessilifolia, S. parviflora* and *S. shandongensis* with *S. mairei*. Moreover, the obtained results are shortly discussed beside the chemotaxonomic view in a biological context.

7. Zusammenfassung

Die in China endemische Kletterpflanze *Stemona mairei* und fünf andere *Stemona* Arten, welche in China gesammelt wurden, wurden phytochemisch auf Alkaloide und Tocopherole untersucht, um einen Einblick auf artspezifische Akkumulierungstrend zu bekommen. Zusätzlich wurde eine in Thailand endemische Art, nämlich *S. aphylla*, auf Stilbenoide und Alkaloide analysiert. Die MeOH Wurzelextrakte der neun untersuchten Aufsammlungen von *Stemona mairei* weisen einheitliche Alkaloid und Tocopherol Verbreitungsmuster auf. Diese wenig untersuchte Art zeichnet sich allgemein durch Akkumulation von dem Pyrroloazepin-Alkaloid Protostemonine (**1**) aus. Die vier Arten *S. japonica, S. sessilifolia, S. parviflora* und *S. shandongensis* zeigen die Tendenz Protostemonine (**1**) als Hauptalkaloid-Komponente anzureichern. Im Gegensatz zu den genannten Arten, unterscheidet sich die Ausnahme *S. kerrii* durch die Akkumulierung von Pyridoazepinen, wobei hauptsächlich Stemocurtisine (**5**) identifiziert wurde. *Stemona aphylla* reichert unterschiedliche Mengen von verschiedenen Alkaloiden und Stilbenoiden an, wobei allgemein ein geringer Gehalt an Inhaltsstoffen festgestellt wurde. In den elf untersuchten Aufsammlungen wurde das Pyrridoazepin-Alkaloid Oxyprotostemonine (**6**) identifizert. Außerdem wurden MeOH Wurzelextrakte von fünf *Stemona* Arten auf ihre toxischen Effekte gegen den Nematoden *Panagrellus redivivus* getestet. Die Resultate zeigen, dass alle Extrakte toxische Effekte gegen P. redivivus aufweisen, wobei der Extrakt von *S. collinsiae* die höchste Aktivität hat. Aus chemotaxonomischer Sicht deuten die Ergebnisse dieser Studie auf eine nahe verwandtschaftliche Beziehung von *S. japonica, S. sessilifolia, S. parviflora* und *S. shandongensis* mit *Stemona mairei* hin. Außerdem werden die aktuellen Ergbenisse neben chemotaxonomischer Sicht in einem biologischen Kontext diskutiert.

8. Appendix

Phytochemical Characterization of the Chinese Endemic species *Stemona mairei* and five other *Stemona* species

Gao Chen, Lothar Brecker, Katharina Maria Sandler, Xiang-hai Cai, Sumet Kongkiatpaiboon, Karin Valant-Vetschera, Johann Schinnerl

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Phytochemical Characterization of the Chinese Endemic species *Stemona mairei* and five other *Stemona* species

Gao Chen [a], Lothar Brecker [b], Katharina Maria Sandler [c], Xiang-hai Cai [d], Sumet Kongkiatpaiboon [e], Karin Valant-Vetschera [c], Johann Schinnerl [c, *]

[a] *Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming 650204, Yunnan, P.R.China.*

[b] *Department of Organic Chemistry, University of Vienna, Währinger Strasse 38, A-1090, Vienna, Austria.*

[c] *Chemodiversity Research Group, Division of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria.*

[d] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, People's Republic of China*

[e] *Drug Discovery and Development Center, Thammasat University, Rangsit Campus, PathumThani 12121, Thailand.*

* Corresponding author: Tel.: +43 1 4277 54072; eFax: +43-1-4277-854072

E-mail address: johann.schinnerl@univie.ac.at (Johann Schinnerl)

Highlights

- Six *Stemona* species from 15 locations were studied phytochemically
- Nine samples of *Stemona mairei* from different locations were analyzed
- Eight *Stemona* alkaloids and three tocopherols were identified
- *Stemona mairei* accumulate protostemonines and Tocopherols
- The main alkaloid and Tocopherols were assessed quantitatively
- *Stemona mairei* extracts exhibited nematicidal activities

Abstract

Comparative HPLC-PDA analyses of methanolic root extracts of the almost unstudied Chinese endemic species *Stemona mairei*, collected from nine locations throughout its natural habitat in northern Yunnan, led to the identification of the pyrroloazepine-type alkaloid protostemonine (**1**) as the main alkaloid and 3,4-dehydro-δ-tocopherol (**9**) as the main tocopherol derivative. Analysis of the five further Chinese *Stemona* species *S. sessilifolia*, *S. japonica*, *S. parviflora* and *S. shandongensis*, collected in adjacent locations, resulted in identification of eight *Stemona* alkaloids, with seven of them assumed to be structurally derived from **1**. Additionally, three tocopherols could be identified co-chromatographically. From *S. shandongensis*, the alkaloids stemocochinin (**7**) and croomine (**8**) were isolated and their structures confirmed by NMR and MS. Additionally, the quantitative content of protostemonine (**1**) and the tocopherols **9** and **10** were assessed. Concerning the tocopherols, *S. mairei* differs by the occurrence of chromenol derivatives from the other species which accumulate both chromenol and chromanol derivatives. Furthermore, root extracts of *S. mairei* exhibited nematicidal activities against *Panagrellus redivivus*. The achieved results are briefly discussed in a biological and chemotaxonomic context, respectively.

Keywords

Stemona mairei; *Stemona shandongensis*; Protostemonine; Stemocochinin; Stemonaceae; Tocopherols

1. Introduction

The genus *Stemona* comprises of 33 species and is distributed in SE Asia. Due to their ethnomedicinal importance in this area, roots from several species have been subject of phytochemical studies. In particular, roots of the species *S. tuberosa* Lour., *S. sessilifolia* (Miq.) Miq. and *S. japonica* (Blume) Miq. were analyzed for the accumulation and bioactivity of specific alkaloids considered as unique for this genus (e.g. Greger, 2006; But et al., 2012; Wang and Chen, 2014). These so called *Stemona* alkaloids are characterized by a pyrrolo[*1*,*2*-*a*]azepine nucleus usually linked with two carbon chains mostly forming terminal lactone rings. According to Greger (2006), three different skeletal types may be distinguished: Tuberostemonine (stichoneurine)-, croomine-, and protostemonine type alkaloids. All of these three types may be found in species of *Stemona*.

In comparison to the already mentioned species, far less attention was paid to other *Stemona* species which are not extensively used in traditional medicine. Only a few reports addressing the diversity of accumulated alkaloids within this genus were published within the last years from samples mainly collected in Thailand (Kongkiatpaiboon et al., 2011; Schinnerl et al., 2007). Tuberostemonine and maistemonine were reported together with several derivatives from the endemic Chinese taxon *S. mairei* (H.Lév.) K.Krause (Figure 1) (Cai and Luo, 2007; Lin et al., 1991b). The pyridoazepine derivatives stemokerrine and oxystemokerrine were isolated from *S. kerrii* Craib (Kaltenegger et al., 2003; Kongkiatpaiboon et al., 2011). *Stemona sessilifolia* and *S. japonica*, are known for accumulation of protostemonine (**1**) and maistemonine (Li et al., 2007), whereas *Stemona parviflora* C.H. Wright yielded parvistemonine (Kaltenegger et al., 2003). Recently, several alkaloids together with the pyrroloazepines protostemonine (**1**), stemofoline (**4**) and several other alkaloids were reported from this genus (Huang et al., 2016).

[Here Figure 1]

In course of phytochemical studies within the genus *Stemona,* we focused on the diversification of alkaloid accumulation in different accessions of *S. mairei* (Figure 2) since this species is not well studied yet.. All of the samples were collected in their natural habitat in Southwest China. Additionally, several other species were analyzed for their alkaloid complement, among them *S. shandongensis* (Miq.) Miq., which has not been studied phytochemically so far. Apart from alkaloids, we also investigated the occurrence of anti-oxidative tocopherols since a constant co-occurrence of tuberostemonine and 3,4-dehydro-δ-tocopherol was reported for *S. tuberosa* recently (Chen et al., 2017). In order to confirm the supposed bioactivity of root alkaloids against herbivores, we performed an assay against the sour paste nematode *Panagrellus redivivus* L. Respective results are discussed shortly in a biological and chemotaxonomic context.

[Here Figure 2]

2. Results

2.1 Alkaloid pattern in the examined species

Fifteen individuals out of six *Stemona* species were studied for their root alkaloid profile. In sum, eight *Stemona* alkaloids and three Tocopherol derivatives could be identified (Figure 3). Comparative HPLC profiling indicated the presence of various types of alkaloids in crude methanolic extracts obtained from air-dried roots. Of the alkaloids, protostemonine (**1**), a pyrroloazepine derivative, proved to be the predominant compound in most of the studied samples. However, *S. kerrii* diverged by the accumulation of stemocurtisine (**5**), a pyrido[*1,2-α*]azepine derivative, as the major alkaloid. The alkaloids **1**, **7** and **8** were isolated and identified from *S. shandongensis.* Their structure was elucidated on basis of NMR and MS, and the other alkaloid compounds were identified on basis of co-chromatography using authentic samples from previous studies (Kaltenegger et al., 2003; Kongkiatpaiboon et al., 2011). Results are presented in Table 1; HPLC chromatograms of *S. mairei* are presented in Figure 4, and those from the other species in Figure 5, respectively.

[Here Figure 3]

[Here Table 1]

2.1.1. Stemona mairei

This species was phytochemically studied the first time in a broader sampling. Protostemonine (**1**), its isomer isoprotostemonine (**2**) and a yet unidentified protostemonine derivative (rt of 7 min) were detected in all of the nine accessions. Additionally, stemofoline (**4**) was found in five out of nine samples. Tuberostemonine and its derivatives as well as maistemonine could not be detected in these samples neither by TLC nor by HPLC with combined UV-DAD and ELS detection, respectively. Hence, the obtained results contradict previous reports concerning these alkaloids (Cai and Luo, 2007). Explanations for that divergent results are difficult, probably misidentification of the roots happened previously.

[Here Figure 4]

2.1.2. Stemona kerrii

This species is characterized by the occurrence of stemocurtisine (**5**) as the main alkaloid (Figure 5). In the current study, **1**, **3** and **6** were identified as minor compounds. The predominance of pyrido[*1,2-α*]azepine derivates was confirmed, whilst pyrrolo[*1,2α*]azepine derivatives are only accumulated in much lower amounts. In contrast to previous studies on this species collected in Thailand (Kaltenegger et al, 2003, Konkiatpaiboon et al., 2011) neither stemokerrine nor its derivative could be detected in both samples. Due to the large distribution area of this species, infraspecific variation concerning formation of pyrido[*1,2-α*]azepine derivates may be expected.

2.1.3. Stemona japonica and S. parviflora

These two species are largely characterized by the accumulation of protostemonine (**1**), with *S. japonica* producing compound **2** in addition**.** (Figure 5). None of the other alkaloids reported recently (Yi et al., 2015) could be detected in our sample. Protostemonine (**1**) was the only alkaloid found in the sample of *S. parviflora,* which is in accordance with previous reports (Li

et al., 2007). The presence of other alkaloids as reported in the past (Li et al., 2007; Lin et al., 1991a; Lin et al., 1990; Huang et al., 2016) could not be confirmed now, neither by HPLC combined with UV/ELS detection nor by TLC sprayed with Dragendorff' reagent. This may be due to the little amount of available plant material.

2.1.4. Stemona shandongensis and S. sessilifolia

Stemona shandongensis was studied phytochemically now the first time. The HPLC profile revealed the occurrence of protostemonine (**1**) accompanied by isoprotostemonine (**2**), dehydroprotostemonine (**3**) and a still unknown derivative showing an identical UV spectrum to that of **1**. The comparison on TLC revealed the presence of Dragendorff' positive spots lacking a chromophore. This led to the isolation of **1** as the main and **7** and **8** as minor compounds. Structure elucidation on basis of NMR and MS techniques evidenced the presence of **1**. However, the unknown compound could not be isolated in sufficient amounts for further structural analysis.

In *S. sessilifolia*, only protostemonine (**1**) was detectable as root constituent. This is in accordance with previous studies (Kongkiatpaiboon et al., 2011; Schinnerl et al., 2007). Contrary to literature reports, alkaloids lacking a chromophore could neither be detected by TLC combined with spraying by Dragendorff' reagent nor by employing HPLC combined with UV/ELSD detection in this species.

[Here Figure 5]

*2.2. Quantification of protostemonine (**1**) and tocopherols (**9**) and (**10**) by HPLC-UV DAD*

The quantification of **1** was performed by using the external standard method over a conc. range of 125–1000 μg mL^{-1}. Five different conc. of the isolated protostemonine (**1**) were used for calculating the calibration parameters. For the tocopherols **9** and **10** the recently published calibration parameter were used (Chen et al., 2017). The calibration curve for these compounds ranged between 12.5 and 500 μg mL^{-1}. The results are presented in Table 2. The amounts of **1** ranged between 0.04 and 0.91% dry weight in *S. mairei*. Within the species *S. sessilifolia, S.*

japonica, S. shandongensis and *S. parviflora* ranged the conc. of **1** between 0.07% in *S. shandongensis* and 0.16% in *S. sessilifolia* (Table 2). Zhang et al. (2007) reported 0.22–0.36% in *S. japonica* and *S. sessilifolia*. By contrast, Li et al. (2007) stated much higher quantities of this alkaloid in samples of both species. If this discrepancy is due to influences from biotic or abiotic factors, remains open at present. The conc. of the 3,4-dehydrotocopherols **9** and **10** are much lower, ranging between 0.03 and 0.06% dry weight.

[Here Table 2]

2.3. Tocopherols

The anti-oxidative tocopherol derivatives 3,4-dehydro-δ-tocopherol (**9)** and 3,4-dehydro-β-tocopherol (**10)** showing the unsaturated chromenol moiety were detectable in all samples of *S. mairei* (Figure 4) and also in *S. japonica*. However, *S. kerrii*, *S. shandongensis* and *S. sessilifolia* exhibited the chromanol derivative β-tocopherol (**11**) as the only vitamin E derivative. In *S. parviflora* neither of these derivatives could be detected. Compounds **9**, **10** and **11** were identified by co-chromatography using reference compounds obtained during a former study (Brem et al., 2004).

2.4. Nematicidal activities

Crude methanolic extracts of *S. mairei* were tested in the conc. of 2.5 mg mL^{-1} and 1.0 mg mL^{-1} against the nematode *P. redivivus*. In short, the nematodes were exposed to the extracts mixed with DMSO and water in petri dishes and the numbers of survivors counted after 24 h. Lethalities were observed in the range between 58.8 and 50% in a conc. of 2.5 mg mL^{-1} and 23.6 and 48.4% at a conc. of 1.0 mg mL^{-1} (Table 3). For comparison, an extract of *S. collinsae* was used as reference because of the accumulation of the potential insecticide *1,2*-didehydrostemofoline in the roots of this species. Our results are not directly comparable to the data reported by Huang et al. (2016), as they had tested with the isolated alkaloids protostemonine and stemofoline. As *Stemona* alkaloids are the predominant secondary metabolites in root extracts, the obtained results are indicative of nematicidal activities

underlining the defense character of *Stemona* alkaloids against nematodes and insects (e.g. Brem et al., 2002, Tang et al., 2008).

[Here Table 3]

3. Discussion

3.1 Chemotaxonomic significance of protostemonine (1)

Protostemonine and its derivatives are a characteristic feature of many *Stemona* species (Kongkiatpaiboon et al., 2011; Schinnerl et al., 2007). Structurally, featuring a 5,7,5-ring core structure, and are thus different from the tuberostemonines, characterized by a 5,7,6,5-ring core structure (Greger, 2006). Protostemonines further include the pyridoazepines, possessing a 6,7,5-ring core, and they are considered to be related to protostemonine derivatives (Kaltenegger et al., 2003). For respective structures see Figure 1. Earlier, these structural differences were suggested to be of chemotaxonomic relevance within *Stemona* and the Stemonaceae (Greger, 2006). Infraspecific variation is crucial to chemical character analysis, and this was elaborated for the first time for *S. mairei* individuals from different locations. The observed phytochemical profiles are relatively uniform, showing little variation, but a prominent occurrence of **1** in all of the studied samples. These are characterized by the dominant peak of **1** in the HPLC-chromatograms as compared to small amounts of the co-occurring related compounds **2** and **4.** This pattern suggests that enzymatic transformation of **1** into **2** and **4** plays a subsidiary role only. Thus, the pyrroloazepine alkaloid protostemonine (**1)** appears to be as a chemical marker for this species. Furthermore, the occurrence of **1** as the main alkaloid suggests a close relationship to *S. sessilifolia*, *S. japonica*, *S. parviflora* and *S. shandongensis*, with exception of *S. kerrii*. The latter species is characterized by the accumulation of alkaloids with a pyridoazepine core structure, containing alkaloids bearing a pyrroloazepine core present in much lower amounts only. Considering the occurrences of **1** or its derivatives within hitherto examined Chinese *Stemona* species, the existence of two main groups becomes apparent. On the one hand, the protostemonine (**1**) accumulating group is represented by species examined

in this study, and on the other hand, a tuberostemonine accumulating group was reported recently (Chen et al., 2017). Corresponding chemical differentiations had already been observed for species distributed in Thailand (Schinnerl et al., 2007; Kongkiatpaiboon et al., 2011). Similarly, our results coincide with the suggested grouping of *Stemona* alkaloids based upon structural similarities and suggested biosynthetic pathways (Greger, 2006). Knowing more about their biosynthetic relationships on the enzyme level would further strengthen the application of *Stemona* alkaloids as chemical characters. These results mirror the widely preliminary DNA sequencing data, in which *S. mairei* clusters together with *S. sessilifolia*, *S. japonica* and *S. parviflora* (Chen et al., 2018, in prep.). Such connection between DNA sequencing data and distribution pattern of *Stemona* alkaloids was already shown by superimposing the studies of Kongkiatbaipoon et al. (2011) and Keeratinijakal (2005) for *Stemona* species collected in Thailand.

3.2. Occurrences of tocopherols

The accumulation of antioxidative tocopherol derivatives in root tissue of the investigated species suggests a protective role against oxidative stress. Since an excess of UV light cannot cause oxidative stress by production of radical oxygen species (ROS) in belowground organs, there should be another explanation for their presence. As the present alkaloids are prone to oxidation processes and at the same time important defense compounds, tocopherols might act as antioxidants to prevent their breakdown, an idea already suggested for tuberostemonine by Chen et al. (2017). Further studies are required for confirming this assumption.

4. Conclusion

Phytochemical analyses of nine samples of the almost unstudied Chinese endemic species *S. mairei* revealed the accumulation of protostemonine in its roots. Only little variation in the alkaloid pattern could be observed in this species, although collected throughout its whole natural distribution area. The other studied Chinese *Stemona* taxa are largely characterized by the occurrence of protostemonine (**1**) or its derivatives. From the chemotaxonomic point of

view, the prominent accumulation of this compound in *S. mairei* suggests a close relationship to *S. japonica*, *S. sessilifolia* and also *S. parviflora*. Only *S. kerrii* differs by the accumulation of the pyridoazepine derivative stemocurtisine as the major alkaloid. The similarities in the accumulation pattern of alkaloids show also some parallelism to the accumulation of antioxidative tocopherol derivatives. Preliminary DNA sequencing results mirror the distribution pattern of protostemonine and its derivatives in Stemonaceae. The obtained results suggest that these derivatives may be useful as chemical markers.

5. Experimental

5.1 Plant material

Plant material (Table 2) was collected in 2015 and 2016 and identified by Gao Chen from Kunming Institute of Botany. *Stemona mairei* was collected throughout its whole natural distribution area, the other species were only collected punctually. Herbarium specimens were deposited at the Herbarium of Kunming (KUN) at the Institute of Botany (http://www.kun.ac.cn/).

5.2 General experimental procedures

HPLC analyses were performed on Agilent 1100 series with simultaneously UV-diode array detection at 230/254/280/310 nm and evaporative light scattering detection (ELSD) (N_2: 3.6 bar; 40 °C). HPLC methods were employed using a Hypersil BDS-C18 (250 x 4.6 mm, 5 µm particle size) column. Methanol (MeOH) (B) and an aqueous solution containing 10 mM ammonium acetate (A) from 55–90% B in A within 19 min, 90–100% B in A within 1 min, 100% B was kept for 12 min; inj. vol. 10 µL at a flow rate of 1.0 mL/min.

Thin layer chromatography (TLC) analyses were done on silica gel 60 F_{254} plates, thickness 0.20 mm (Merck) developed with CH_2Cl_2/EtOAc/MeOH/NH_4OH (70:25:5.5:0.5) or (50:25:5.5:0.5) and sprayed with Dragendorff and anisaldehyde, respectively. For prep. TLC, silica gel 60 F_{254} glass plates, thickness 0.25 mm (Merck) were used. The stationary phases for CC was Sephadex LH-20 (GE Healthcare) eluted with methanol.

All of the preparative separation steps were monitored by analytical TLC and HPLC, respectively. Co-chromatographically identified compounds were assigned by comparison of retention times and UV spectra using authentic samples isolated in previous studies (Kongkiatpaiboon et al., 2011; Schinnerl et al., 2007).

5.3 NMR spectroscopy

For NMR spectroscopic measurements each compound was dissolved in $CDCl_3$ (~3.0 mg in 0.7 mL) and transferred into 5 mm high precision NMR sample tubes. All spectra were measured on a Bruker DRX-600 at 600.13 MHz (^1H) or 150.61 MHz (^{13}C) using the Topspin 3.2 software. Measurement temperature was 298 K ± 0.05 K. Residual $CHCl_3$ was used as internal standard for ^1H (δ_H 7.26) and $CDCl_3$ for ^{13}C (δ_C 77.01) measurements, respectively. 1D spectra were recorded and Fourier transformed to spectra with a range of 7,200 Hz (^1H) and 32,000 Hz (^{13}C), respectively. To determine the 2D spectra 128 experiments with 2,048 data points each were recorded and Fourier transformed to ranges of 6,000 Hz and 32,000 Hz for ^1H and ^{13}C, respectively.

5.4 Mass spectrometry

Mass spectra were recorded on a high resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive and negative ionization mode (mass accuracy ± 5 ppm). TOF MS measurements have been performed within the selected mass range of m/z 100–2500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 30–50 nA. Nitrogen temperature was maintained at 180 °C using a flow rate of 4.0 L min^{-1} and the N_2 nebulizer gas pressure at 0.3 bar.

*5.5 HPLC analyses and calibration curve of **1***

Sliced roots were ground using a coffee mill and 50 ± 5 mg of the obtained powder was extracted using 0.7 mL methanol p.A. in an Eppendorf cup under sonication for 20 min. After

centrifugation at 12,500 rpm for 20 min, 0.5 mL of the supernatant was set to a conc. of 10 mg ml^{-1} and subjected to HPLC.

For calibration, standards in the conc. of 1000, 750, 500, 250 and 125 µg mL^{-1} of protostemonine (**1**) were prepared in methanol and measured by HPLC. For the assessment of tocopherols were the recently published parameter used (Chen et al., 2017). In short, from compound **9** standards containing 12.5, 125, 250 and 500 µg mL^{-1} were prepared. Each standard was subjected to HPLC three times. The calibration curves were obtained by plotting the peak area versus the amounts of the standards. The calculated results are displayed in Table 2.

5.6 Extraction and Isolation

Stemona shandongensis (CHEN20140506)–140 g dried roots were extracted with MEOH to yield 9.0 g crude extract. The obtained crude extract was subsequently partitioned between chloroform and water to yield 2.4 g lipophilic phase. A part of this fraction (1.0 g) was separated by open CC (silica gel 60, 40–60 µm) eluted isocratic with 300 mL of DCM/EtOAc/MeOH (70:25:5) collected in 10 fractions of 20–30 mL each. The pooled fractions 4–7 (135 mg) were subjected to CC over Sephadex LH20/MeOH followed by MPLC eluted with mixtures of DCM and MeOH. The joint fractions eluted with 20 and 30% MEOH in DCM (12 mg) were finally purified by prep. TLC which led to 3.0 mg of **1**, 1.8 mg of **7** and 1.1 mg of **8**.

5.7. Identification and structure elucidation

All compounds were identified by comparison of their analytical data with those of known reference compounds or with data reported earlier. In particular retention time in HPLC measurements as well as UV, MS and NMR spectra were used to compare substances with reference materials. For identification of all other compounds a detailed structural analysis was made. The analytical data, especially from NMR measurements, were evaluated by finding spectral identities and similarities in the combined CSEARCH and SPECINFO database system (Robien, 2009; Schütz et al., 1997). All data were compared with the originally published data.

Protostemonine (**1**) - Amorphous powder; HR-ESI-MS (pos. mode) [M+H$^+$] *m/z* 418.2224 (calc. for $C_{23}H_{32}NO_6$, *m/z* 418.2279), (neg. mode) [M-H$^-$] *m/z* 416.2106 (calc. for $C_{23}H_{30}NO_6$, *m/z* 416.2078); ^{1}H NMR ($CDCl_3$, 600 MHz, δ_H) 4.25 (1H, H-18), 4.15 (1H, H-20), 4.08 (3H, H-23), 4.07 (1H, H-8), 3.69 (1H, H-9a), 3.52 (1H, H-5^a), 3.25 (1H, H-3), 2.95 (1H, H-5^b), 2.90 (1H, H-10), 2.47 (1H, H-9), 2.33 (1H, H-19^a), 2.28 (1H, H-7^a), 2.04 (3H, H-16), 1.89 (1H, H-1^a), 1.86 (1H, H-2^a), 1.67 (1H, H-6^b), 1.59 (1H, H-1^b), 1.53 (1H, H-19^b), 1.49 (1H, H-6^a), 1.48 (1H, H-7^b), 1.46 (1H, H-2^b), 1.42 (3H, H-17), 1.29 (3H, H-22); ^{13}C NMR ($CDCl_3$, 150 MHz, δ_C) 178.6 (s, C-21), 169.9 (s, C-15), 163.0 (s, C-13), 149.0 (s, C-11), 125.2 (s, C-12), 97.6 (s, C-14), 82.8 (d, C-18), 82.4 (d, C-8), 64.0 (d, C-3), 59.8 (d, C-9a), 59.1 (q, C-23), 56.2 (d, C-9), 47.2 (t, C-5), 39.3 (d, C-10), 34.6 (d, C-20), 33.6 (t, C-19), 34.2 (t, C-7), 27.1 (t, C-2), 26.3 (t, C-1), 20.4 (t, C-6), 20.6 (q, C-17), 14.9 (q, C-22), 8.9 (q, C-16). NMR spectroscopic data were in agreement with those reported for this compound (Irie et al., 1970; Ye et al., 1994).

Stemocochinin (**7**) - Amorphous powder; HR-ESI-MS (pos. mode) [M+H$^+$] *m/z* 390.2267 (calc. for $C_{22}H_{32}NO_5$, *m/z* 390.2275); ^{1}H NMR ($CDCl_3$, 600 MHz, δ_H) 7.06 (1H, H-13), 4.88 (1H, H-12), 4.03 (1H, H-18), 3.83 (1H, H-8), 3.80 (1H, H-11), 3.47 (1H, H-9a), 3.85 (1H, H-5^a), 3.29 (1H, H-5^b), 3.41 (1H, H-3), 2.69 (1H, H-20), 2.26 (1H, H-19^a), 2.35 (1H, H-10), 2.10 (1H, H-9), 2.54 (1H, H-7^a), 1.95 (3H, H-16), 2.11 (1H, H-2^a), 2.01 (1H, H-1^a), 1.95 (1H, H-1^b), 1.94 (1H, H-6^a), 1.44 (1H, H-19^b), 1.92 (1H, H-2^b), 1.74 (1H, H-6^b), 1.51 (1H, H-7^b), 1.29 (3H, H-22), 1.09 (3H, H-17); ^{13}C NMR ($CDCl_3$, 150 MHz, δ_C) 177.5 (s, C-21), 174.1 (s, C-15), 145.8 (s, C-13), 131.2 (s, C-14), 84.7 (d, C-18), 83.0 (s, C-11), 80.1 (d, C-8), 79.3 (d, C-12), 62.7 (d, C-3), 53.8 (d, C-9), 51.0 (d, C-9a), 48.8 (t, C-5), 40.0 (d, C-10), 35.7 (t, C-7), 34.7 (d, C-20), 34.0 (t, C-19), 26.1 (t, C-2), 25.9 (t, C-1), 17.9 (t, C-6), 15.3 (q, C-17), 14.6 (q, C-22), 10.8 (q, C-16). NMR spectroscopic data were in reasonable good agreement with those reported for this compound (Kaltenegger et al., 2003).

Croomine (**8**) Amorphous powder; HR-ESI-MS (pos. mode) [M+H^+] *m/z* 322.2006 (calc. for $C_{18}H_{28}NO_4$, *m/z* 322.2013), [M+Na^+] *m/z* 344.1824 (calc. for $C_{18}H_{27}NO_4Na$, *m/z* 344.1832); ^{1}H NMR ($CDCl_3$, 600 MHz, δ_H) 4.21 (m, 1H, H-14), 3.89 (m, br, 1H, H-5^b),3.51 (m, 1H, H-3), 3.50 (m, 1H, H-9a), 3.43 (m, 1H, br, H-5^a), 2.84 (m, 1H, H-11), 2.69 (m, 1H, H-16), 2.53 (m, br, 1H, H-15^a), 2.35 (m, br, 1H, H-8^a), 2.34 (m, 1H, H-10^a), 2.25 (m, 1H, H-8^b), 1.85 (m, br, 1H, H-6^a), 1.82 (m, br, 1H, H-7^a), 1.79 (m, 1H, H-7^b), 1.71 (m, br, 1H, H-1^b), 1.69 (m, br, 1H, H-2^b), 1.65 (m, 1H, H-1^a), 1.64 (m, br, 1H, H-6^b), 1.61 (m, 1H, H-10^b), 1.44 (m, br, 1H, H-15^b), 1.35 (m, 1H, H-2^a), 1.24 (d, J=7.2 Hz, 3H, H-13), 1.18 (d, J=6.9 Hz, 3H, H-18); ^{13}C NMR ($CDCl_3$, 150 MHz, δ_C) 4.21 (m, 1H, H-14), 3.89 (m, br, 1H, H-5^b),3.51 (m, 1H, H-3), 3.50 (m, 1H, H-9a), 3.43 (m, 1H, br, H-5^a), 2.84 (m, 1H, H-11), 2.69 (m, 1H, H-16), 2.53 (m, br, 1H, H-15^a), 2.35 (m, br, 1H, H-8^a), 2.34 (m, 1H, H-10^a), 2.25 (m, 1H, H-8^b), 1.85 (m, br, 1H, H-6^a), 1.82 (m, br, 1H, H-7^a), 1.79 (m, 1H, H-7^b), 1.71 (m, br, 1H, H-1^b), 1.69 (m, br, 1H, H-2^b), 1.65 (m, 1H, H-1^a), 1.64 (m, br, 1H, H-6^b), 1.61 (m, 1H, H-10^b), 1.44 (m, br, 1H, H-15^b), 1.35 (m, 1H, H-2^a), 1.24 (d, J=7.2 Hz, 3H, H-13), 1.18 (d, J=6.9 Hz, 3H, H-18). NMR spectroscopic data were in reasonable good agreement with those reported for this compound (Noro et al., 1979; Jiang et al., 2006).

5.8. Assay on Panagrellus redivivus

Cultivation: The nematodes (*Panagrellus redivius*) were bought at an online webstore (http://www.garnelen-krebs-co.at/, May 2017) and cultivated on a medium made of ground oat (*Avena sativa*, ca. 20 g) mixed with a smashed banana (*Musa* sp., organic, ca. 30 g). A small plastic box (5 x 5 x 5 cm) was covered with the homogenous medium and afterwards inoculated with the nematodes and reared in darkness at room temperature (23°C) for five days.

Crude methanolic root extract were tested in concentrations of 2.5 mg mL^{-1} and 1.0 mg mL^{-1}, respectively. 1.25 mg and 0.5 mg of crude plant extract, respectively, were dissolved in 10 µL dimethylsulfoxid (DMSO) and then diluted with deionized water (dH_2O) containing 0.3% (v/v) Tween-20 to obtain 0.5 mL final test solution. The solution was added in 55 mm

petri dishes. The nematodes were taken off the growth medium and suspended in deionized water. 60 µL of the obtained nematode suspension were added to the test solution in the petri dishes, and these were incubated under dark conditions at room temperature (23°C) for 24 hrs. A solution of 10 µL DMSO and 490 µL dH_2O containing 0.3% v/v Tween-20 was used as control. All experiments were performed in triplicate. After 24 hours, the petri dishes containing the nematodes and test solution photographed (camera Mamiya Leaf Credo 80). Both, dead and alive nematodes were assessed by counting on each photograph. Dead nematodes appeared as straight lines and the alive ones were pictured as bent and curled lines, often s- or u-shaped.

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Table 1. Screening results of alkaloids and tocopherols of the studied *Stemona* species.

Taxon	Origin	Herbarium specimen	Alkaloids								Tocopherols		
			1	2	3	4	5	6	7	8	9	10	11
S. mairei	Panzihua 2015	CHEN20140327	●	○		○					◈	◇	
	Deqin	CHEN20110707	●	○							◈	◇	
	Derong	CHEN20160702	●	○							◈	◇	
	Taoyuan	CHEN20160707	●	○		○					◈	◇	
	Qina	CHEN20160630	●	○		○					◈	◇	
	Huangping	CHEN20160704	●	○		○					◈	◇	
	Panzhihua 2016	CHEN20160523	●	○		○					◈	◇	
	Jiangbiancun	CHEN20160703	●	○							◈	◇	
	Fulongqiao	CHEN20160701	●	○							◈	◇	
S. japonica	Tianmushan	CHEN20120709	●	○							◈	◇	
S. sessilifolia	Qixiashan	CHEN20140511	●	○									◈
S.shandongensis	Taishan	CHEN20140506	●	○	○				○	○			◈
S. parviflora	Baisha	CHEN20100903	●	○									
S. kerrii	Xiaoganba	CHEN20160722	○		○		●	○					◈
S. kerrii	Xishuangbanna	CHEN20150208	○		○		●	○					◈

The numbering of the compounds is according to Figure 3. The stereochemistry of the Tocopherols was not determined. The symbols ●, ◈, ○, and ◇, respectively, were used to distinguish between major and minor amounts within each class of compounds.

Table 2. Contents of protostemonine (**1**) and tocopherols **9** and **10** in *S. mairei* and other species. The values are shown in % (w/w).

species	**Origin**	**1**	**Σ 9 and 10**
S. mairei	Panzihua 2015	0.04	0.02
	Derong	0.18	0.05
	Taoyuan	0.26	0.31
	Qina	0.32	0.04
	Huangping	0.48	0.04
	Panzhihua 2016	0.34	0.03
	Jiangbiancun	0.91	0.06
	Fulongqiao	0.18	0.03
S. japonica		0.15	
S. sessilifolia		0.16	
S. shandongensis		0.07	
S. parviflora		0.11	

The linear calibration parameters for protostemonine (**1**) are: $y = 7.4372x - 328.97$ (r^2= 0.9989) and $y = 15.958x + 189.79$ (r^2= 0.9942) for the tocopherols (Chen et al., 2017), respectively.

Table 3. Nematicidal activities of *S. mairei* extracts. Each value represents the mean of three replicates. For comparison, a crude extract of *S. collinsiae* was tested because of the accumulation of the potent insecticide *1*,*2*-didehydrostemofoline in the roots of that species (Tang et al., 2008).

	lethalities [%]	
origin	**2.5 mg mL^{-1}**	**1 mg mL^{-1}**
Derong	58.8	31.8
Taoyuan	55.5	23.6
Qina	53.1	25.1
Huangping	51.9	38.1
Panzhihua 2016	55.2	40.5
Jiangbiancun	54.2	35.9
Fulongqiao	50.0	48.4
S. collinsiae	77.1	35.5

Figure 1. Basic structures of pyrrolo- and pyridoazepines type alkaloids.

Figure 2. *Stemona mairei* in its natural environment. The size of the flowers is approx. 3 cm (A) and the storage roots (B) are approx. 5–10 cm in length.

Figure 3. Structural formulae of alkaloids (**1**–**8**) and tocopherols (**9**–**11**) detected in the studied *Stemona* species. Protostemonine (**1**), isoprotostemonine (**2**), dehydroprotostemonine (**3**), stemofoline (**4**), stemocurtisine (**5**), oxyprotostemonine (**6**), stemocochinin (**7**), croomine (**8**), 3,4-dehydro-δ-tocopherol (**9**), 3,4-dehydro-β-tocopherol (**10**) and β-tocopherol (**11**). The stereochemistry of the Tocopherols were not determined.

Figure 4. HPLC profiles of methanolic crude extracts of *S. mairei*. Numbering of the assigned compounds is according Figure 3. A= Derong, B= Taoyuan, C= Qina, D= Huangping, E= Panzihua 2016, F= Jiangbiancun, G= Fulongqiao, H= Panzihua 2015, I= Deqin.

Figure 5. HPLC Profiles of *S. kerrii* and the other studies *Stemona* species. The prominent peak at rt 2.5 min is caused by an unidentified phenolic compound. Numbering of the assigned compounds is according Figure 3.