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„Ohne Spekulation gibt es keine neue Beobachtung“
Charles Darwin

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Abstract

The genus *Notopleura* is member of the tribe Palicoureeae (Rubiaceae) within the speciose *Psychotria* alliance. This group is well known for the accumulation of bioactive monoterpene indole alkaloids. Comparable little is known about the secondary plant metabolites of *Notopleura* species. The aim of this study was to analyse the chemical composition of selected *Notopleura* species also with respect to organ-specific profiles, with some species studied for the first time. The extracts were purified by various chromatographic techniques and analysed by comparison of the HPLC-UV data. Four already known substances were isolated from *N. uliginosa* and structurally identified by ^{13}C -NMR spectroscopy. These include the naphthoquinone psychorubrin (**8**), the megastigmanes 3-oxo- α -ionol (**9**) and blumenol A (**10**), and the monoterpene hydroxylactone loliolide (**11**). No alkaloids could be detected in the studied samples. As far as organ-specific occurrence in *Notopleura* species is concerned, megastigmanes dominate in leaves, while there is a shift towards dominance of quinones in roots and stems. Interestingly, none of these compounds were detected in the inflorescences. This indicates expression of different pathways in the respective plant organs. The observed antioxidative activity of crude plant extracts could not be correlated experimentally with the four isolated compounds. As for chemotaxonomic relevance, the observed accumulation tendencies are in agreement with the current taxonomic position of this genus.

Key words: *Notopleura* – Rubiaceae; Naphthoquinones; Megastimanes; Monoterpene hydroxylactones.

1 Introduction

Members of the Rubiaceae are very diverse in their secondary metabolites, with many of them being highly bioactive. Therefore many species of that family have been of interest already for a long time. Beside scientific and medicinal aspects, many species are of socio-, or religio-cultural value. Some secondary metabolites can be used, due to their restricted distribution to specific plant groups, as a chemotaxonomic marker, while others are distributed irregularly. The object of the current study are members of the *Notopleura*, which was included earlier in the genus *Psychotria*, but later separated on basis of morphological and molecular evidence. Comparative phytochemical analyses, which could support its placement, were rarely done until now.

Isolated reports, however, exist and indicate the presence of different structures, mainly derived from the flavonoid-, quinone- and terpenoid pathway. In 2016 a new dihydroflavonol ((2R,3R)-7,4'-O-dimethyl-aromadendrin-5-O- β -D-apiofuranosyl-(1/6)- β -D-glucopyranoside) (**13**) was identified from a leaf extract of *Notopleura polyphlebia* (Berger et al., 2016). *Notopleura polyphlebia* leaves yielded, 2-aza-anthraquinone (**12**), the hydroquinone glycosides harounoside and roseoside, and an unnamed megastigmane glucoside (Berger, 2012; Kolar, 2012). Previously, the pyranonaphthoquinone psychorubrin (**8**) and 2-aza-anthraquinone (**12**) were isolated from *Notopleura camponutans* (Jacobs et al., 2008; Solis et al., 1995). The aim of this research was to identify secondary metabolites in selected species of *Notopleura* and their organ-specific occurrence. In addition, comparison of the HPLC profiles was done to reveal possible patterns in the distribution of compounds that could be used as chemical characters. All of the crude extracts were further tested for antioxidative activity.

2 Systematics

2.1 Rubiaceae Juss.

The Rubiaceae Juss., also known as the coffee family, contains about 13.500 species in 620 genera which makes it one of the largest known angiosperm families. It consists of three subfamilies, the Cinchonoideae, Ixoroideae and Rubioideae and the tribes Coptosapelteae and Luculieae (Bremer, 2009). Although the distribution is cosmopolitan, the tropics and subtropics have the highest species diversity (De Block et al., 2006). About one-third of the genera and almost one half of the species occur in the neotropics and have adapted to many different habitats (Delprete, 2004). Most Rubiaceae species in the tropics are small trees or shrubs, which constitute a major part of the understorey of low- and mid- altitude rainforests, less often are high trees, lianas, vines, and herbs, mostly terrestrial, rarely epiphytic or aquatic. In the temperate zone especially species of the tribe Rubieae are distributed, annuals and perennials herbs are dominating the region (Robbrecht, 1988).

2.2 Palicoureeae

The tribe Palicoureeae Robbr. & Manen belongs to the subfamily Rubioideae Verdc. which comprises 26 tribes, and shows a high occurrence of anthraquinones (Martins and Nunez, 2015). Palicoureeae is part of the *Psychotria* alliance and includes together with the sister tribe Psychotrieae 91% of all species in that alliance and 24% of all species in the Rubiaceae family. Palicoureeae contains at least 1500 species in the subtropical and tropical genera *Carapichea*, *Chassalia*, *Eumachia* (incl. *Chazaliella*, *Margaritopsis* and *Readea*), *Geophila*, *Hymenocoleus*, *Notopleura*, *Palicourea*, *Puffia*, and *Rudgea* (Razafimandimbison et al., 2014).

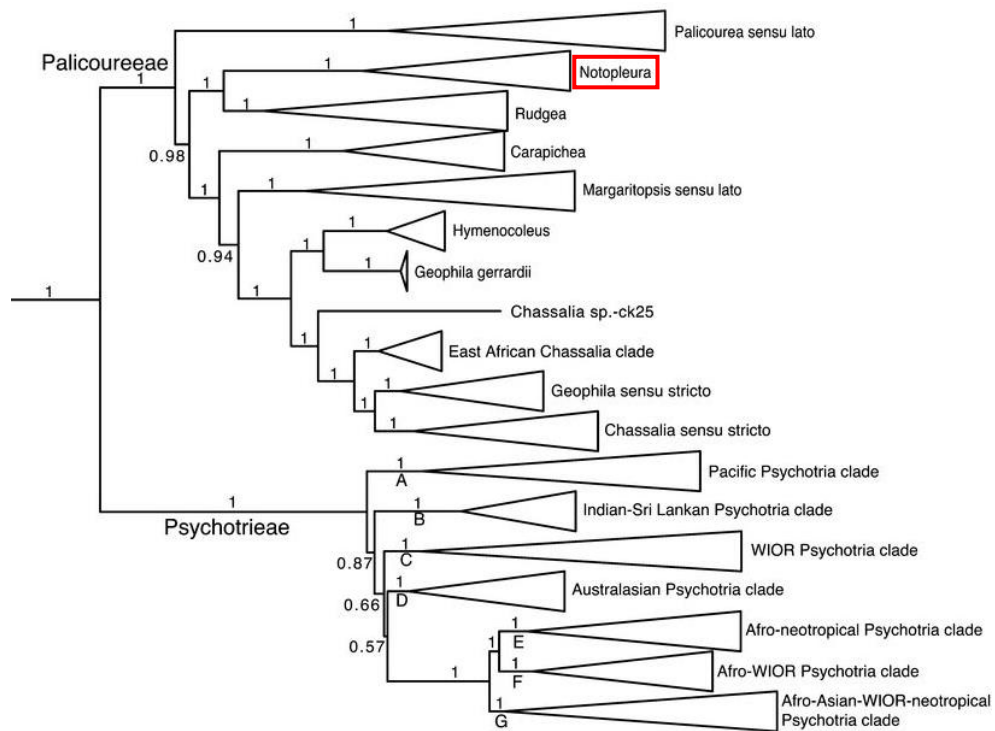


Fig. 1. Extract of the phylogenetic tree of the sister tribes Palicoureeae and Psychotrieae with the labelled genus *Notopleura*. The tree is based on molecular data from plastids and the nucleus. Adapted from Razafimandimbison et al., 2014.

2.3 *Notopleura*

The genus *Notopleura* (Benth. & Hook. f.) Bremek. is one of the largest genera of the tribe Palicoureeae. It comprises about 100 species and its occurrence is restricted to the neotropics. Species of this genus grow in wet tropical forests from Mexico and the Antilles south to Bolivia and Brazil (Taylor, 2001). Twenty-four *Notopleura* species are known to occur in Costa Rica, living as herbs or shrubs, five of them have an epiphytic lifestyle the rest is terrestrial (Hammel et al., 2014). Species of the subgenus *Notopleura* are terrestrial and are always found at wet microsites. Species of subgenus *Viscagoga* (Baill.) C. M. Taylor are epiphytic and are found at wet forest canopy (Taylor, 2001).

Before *Notopleura* was treated as a separate genus, first time by Bremekamp in 1934, it was included in the genus *Psychotria* sect. *Notopleura* Benth. & Hook. f. (1874). Steyermark (1972) reintegrated the genus as a section in *Psychotria* subg. *Heteropsychotria*, but due to molecular (Nepokroeff et al., 1999) and morphological (Taylor, 2001) evidence *Notopleura* is treated as a separate genus currently.

2.3.1 Botany of *Notopleura*

Plants of the genus *Notopleura* (Fig. 2), mostly herbs and subshrubs, are between 1- 3 m tall, succulent and often unbranched or clambering. They possess sheath like interpetiolar stipules with a medial, often glandular appendage, which is unique to the genus *Notopleura*. The inflorescence consists of quite small white flowers and is pseudoaxillary in subg. *Notopleura* whereas in subg. *Viscagoga* the inflorescences may be terminal or pseudoaxillary. In all plant tissue raphides are densely distributed. The fruits are spongy and succulent, the colour is species dependant from white to red or black. The seed dispersion is assumed to occur by birds (Taylor, 2001).

Nine of the selected *Notopleura* species are terrestrial including *N. anomothyrsa* (K. Schum. & Donn. Sm.) C. M. Taylor, *N. capacifolia* (Dwyer) C. M. Taylor, *N. costaricensis* C. M. Taylor, *N. nepokroeffiae* C. M. Taylor, *N. panamensis* (Dwyer) C. M. Taylor, *N. polyphlebia* (Donn. Sm.) C. M. Taylor, *N. siggersiana* (Standl.) C. M. Taylor, *N. tolimensis* (Wernham) C. M. Taylor and *N. uliginosa* (Sw.) Bremek. Only *N. parasitica* (Sw.) Hammel and *N. pithecobia* (Standl.) C. M. Taylor are epiphytic species belonging to subg. *Viscagoga*. More detailed description and keys of *Notopleura* species are given by Taylor (2001).

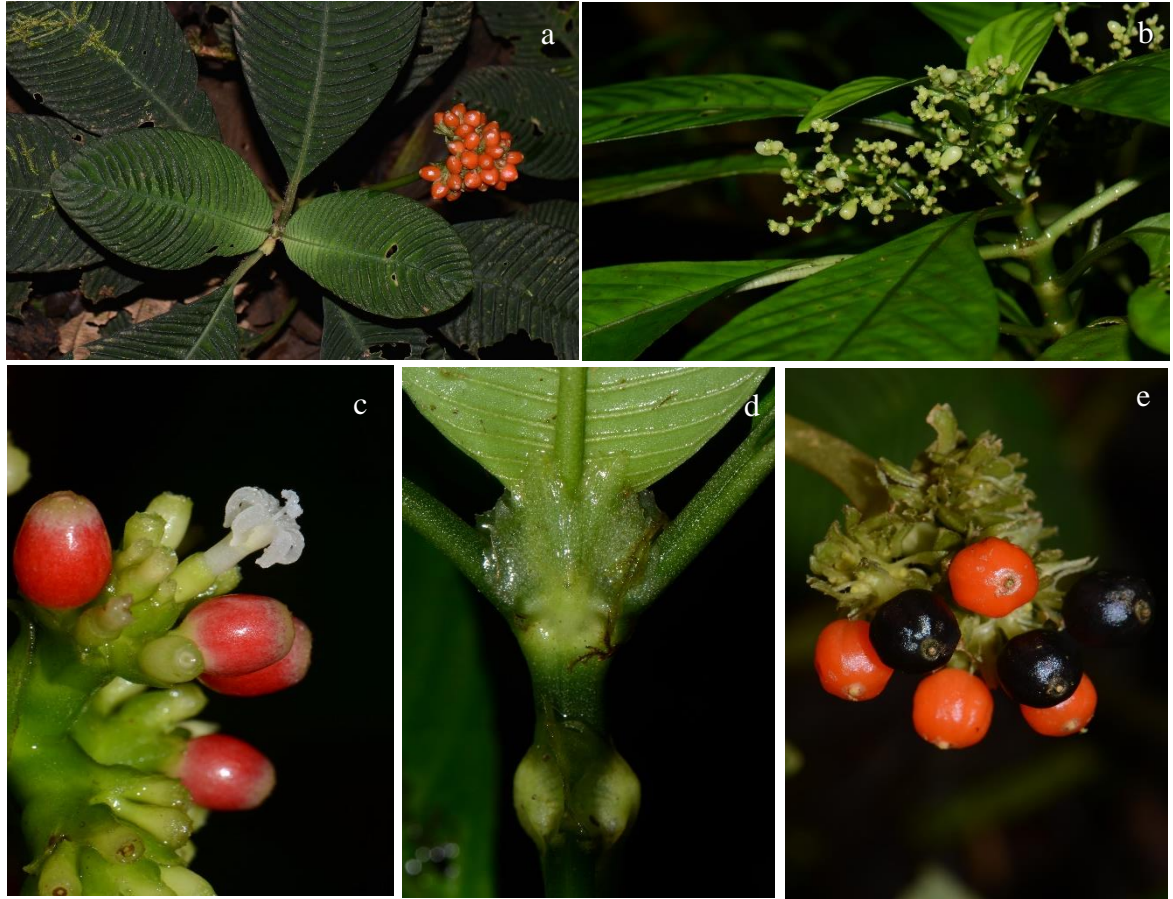


Fig. 2. Different Plant organs of *Notopleura* species. **a:** Decussated leaves and immature red fruits of *N. polyphlebia*. **b:** Inflorescence with maturing white fruits of *N. anomothyrsa*. **c:** Small flower and fruits of *N. uliginosa*. **d:** Sheath like interpetiolar stipules of *N. costaricensis*. **e:** Black ripe and red immature fruits of *N. polyphlebia*. (Photographs courtesy of Andreas Berger).

3 Secondary metabolites

Plants produce a variety of compounds during their lifecycle, which can be divided into primary and secondary metabolites. Kossel (1891) was the first in differentiating between primary and secondary metabolites, though they were thought to be “waste products” of the cell (quoted after: Bourgaud et al., 2001). Due to improvements of biochemical techniques in the last fifty years the importance of secondary metabolites for plants was revealed. Primary metabolites are synthesized for essential functions, such as growth and development, and are therefore present in all plants. Secondary metabolites are variously distributed in the plant kingdom and have different specific functions. They often occur in dedicated cells or specialized organs for example in oil cells or glandular hairs. Secondary metabolites contribute to the fitness by interacting with the ecosystem and in further consequence by adaptation of the plant to their environment (Bourgaud et al., 2001). Many of them have protective functions against pathogens and show antifungal, antibiotic, antiviral or antioxidant activities. Based upon their biosynthesis, three major groups of compounds may be distinguished: alkaloids, terpenes and phenolics. However, some rather complex molecules are often the products of more than one type of biogenetic precursor (Harborne, 1999). Secondary metabolites are widely distributed in the plant kingdom, as for example, flavonoids and carotenoids, which often function as floral pigments in flowering plants. Others like alkaloids are much more specific to defined plant lineages, which implies that the restricted distribution could be used as a chemotaxonomic marker (Bourgaud et al., 2001).

3.1 Antioxidants

In the environment, plants are exposed to different types of stress, which can induce the formation of reactive oxygen species (ROS) and which further can increase dramatically. Such triggers for environmental stress can be for instance drought (Smirnoff, 1993), UV radiation (Landry et al., 1995), salinity (Mittova et al., 2003) or wounding (Grantz et al., 1995). One group of secondary metabolites has a particular

role in the defence against oxidative stress, the phenolics. These are aromatic compounds bearing one or more hydroxyl groups, ranging from relatively simple structures to complex oligomers such as tannins, or polymers such as lignin. Different classes of phenols are distinguished: the hydroxycinnamic acids (HCAs), flavonoids, anthocyanins and tannins. Most phenolic compounds are synthesized from shikimic acid and further by the phenylpropanoid pathway, the first product being the HCAs that can serve as precursor or esters for phenolic based metabolites (Hahlbrock and Scheel, 1989). Flavonoids result from a combination of the phenylpropanoid pathway with the polyketide pathway, to form the flavan skeleton (Falcone Ferreyra et al., 2012). The efficient antioxidant activity of phenolic compounds is the result of the electron donating activity of the phenolic hydroxyl groups. The oxygen radicals have a higher one-electron reduction potential than phenolic radicals with the effects that ROS oxidize the phenolics in preference to other ROS (Bors et al., 1990; Buettner, 1993; Jovanovic et al., 1994). Furthermore, phenolics are less reactive when oxidised than oxygen radicals so they are able to interrupt the harmful oxidative chain reaction of ROS (Bors et al., 1994). Quinones are also known to act as electron transporters within plant cells and thus are crucial in different biochemical processes (Lenaz et al., 2004). Their free radical scavenging character depends on the oxidation state of the quinone. Under normal conditions, a plant cell maintains the redox balance in a reducing environment. By oxidation of hydroquinones, unstable intermediate semi-quinones and further quinones can be generated. They have the ability to operate as anti- or pro-oxidants, dependent on the ambient medium. (Gutteridge and Halliwell, 2006).

3.2 Secondary metabolites in Rubiaceae

Due to a large diversity of secondary compounds, including iridoids (Lopes et al., 2004), indole alkaloids (Berger et al., 2015; Lopes et al., 2004; Van De Santos et al., 2001), terpenoids (Moreno et al., 2014), anthraquinones (Feng et al., 2011) and flavonoids (Berger et al., 2016), many rubiaceae species are of medicinal and pharmacological interest. The probably most prominent example of a Rubiaceae of great economic importance with a high concentration of bioactive secondary metabolites is *Coffea arabica* L. (Złotek et al., 2016). Others are used in traditional medicine (Gong et al., 2017; Shan et al., 2016) or as a hallucinogen in shamanic rituals (Dobkin de Rios, 1972). The occurrence of structurally complex indole alkaloids is quite restricted to families belonging to the Gentianales order, in Rubiaceae, indole alkaloids are indicated as the main chemical markers (Martins and Nunez, 2015)

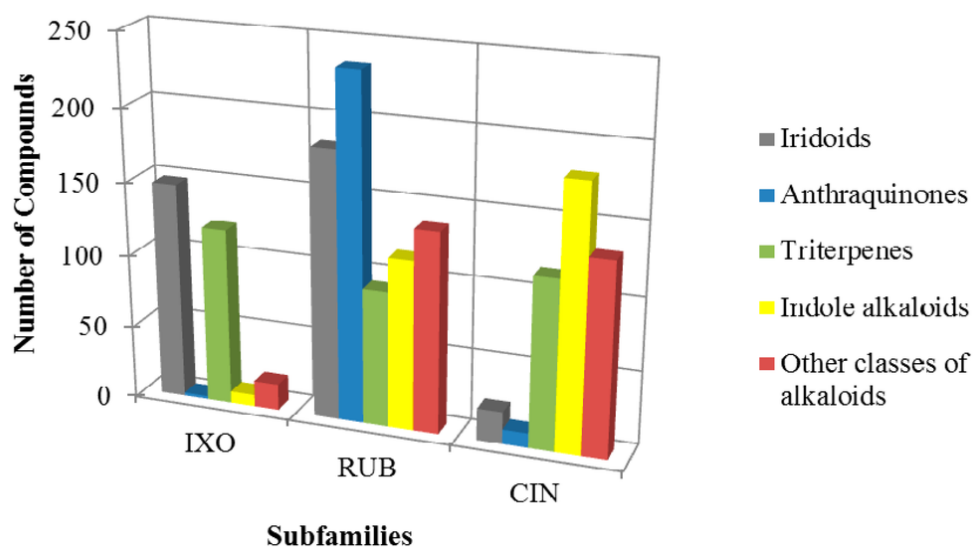


Fig. 3. Chemical diversity and major secondary metabolite distribution among Rubiaceae subfamilies. IXO: Ixoroideae, CIN: Cinchonoideae, RUB: Rubioideae. Adapted from Martins and Nunez, 2015.

3.3 Secondary metabolites in the *Psychotria* alliance

The *Psychotria* alliance can be separated in groups not only by morphology but also by the presence of different secondary metabolites. Thus, members of subgenus *Psychotria* were shown to accumulate primarily tannins, whereas members of subgenus *Heteropsychotria* are characterized by predominant monoterpene indole alkaloid occurrence (Lopes et al., 2004). In general, the tribe Palicoureeae exhibits alkaloids as the main compound group, except for the genus *Notopleura* where no alkaloids could be found so far, and quinones and megastigmanes are typical constituents. Similarly, no alkaloids have been detected so far in the genera *Puffia*, *Geophila* and *Hymenocoleus* (Berger, 2012). The following chapters give a short introduction into the major compound classes that have been detected in the studied *Notopleura* species.

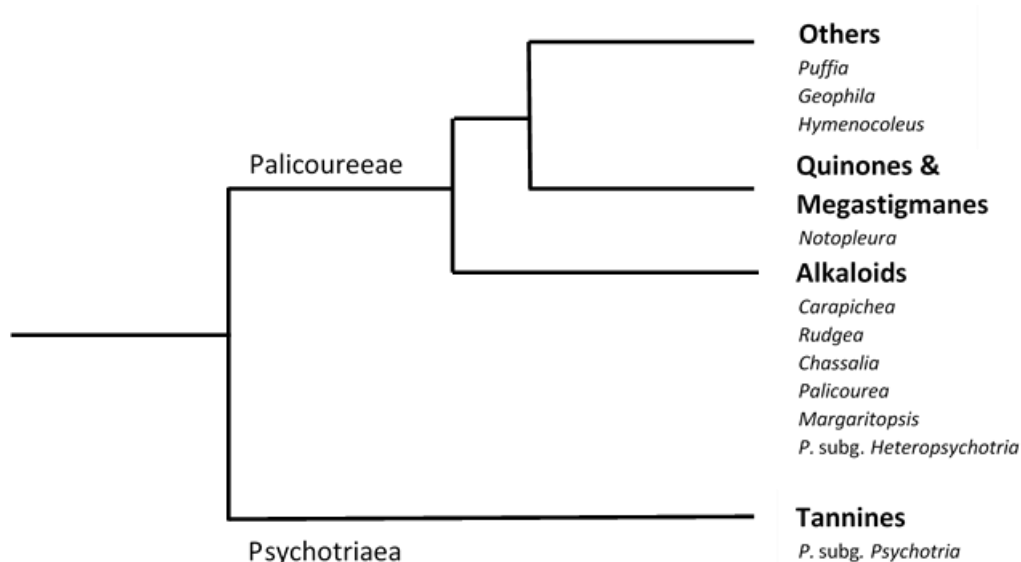


Fig. 4. Simplified classification of tribes and genera of the *Psychotria* alliance, due to the differences in their major secondary metabolite composition. (Adapted from Berger, 2012).

3.4 Benzoquinones, Naphthoquinones and Anthraquinones

Quinones, naphthoquinones and anthraquinones are found in different groups of plants. Some can be found in all green plant tissues such as vitamin K₁ and play crucial roles in primary plant metabolism. Others are also found in bacteria and fungi. They

occur free or as glycosides, and highly oxidized forms are coloured and thus serve as pigmentation molecules (Velisek et al., 2008).

The simplest forms of quinones are *para*-(**1**) and *ortho*-benzoquinones. Benzoquinones are found commonly in nature, for instance the primary metabolite ubiquinone, which plays a crucial role in the electron transport chain in mitochondria. Naphthoquinones are formed when a second aromatic ring is fused to the benzoquinone ring, which then results for instance in a 1,4-naphthoquinone (**2**). Many of them act as phytotoxins like for instance juglone (Stoessl, 1981), which was shown to have allelopathic effects in the field by inhibiting seed germination of other plants (Powell and Spencer, 1988). With about 700 described compounds, anthraquinones constitute one of the largest groups of natural pigments in nature. Most of them are produced by lichens and fungi and approximately 200 were described in flowering plants (Duval et al., 2016). They often occur as glycosides within the plant, and they may be hydrolysed to aglycones in defense reactions (Seigler, 1998).

A deep red or blue colour is characteristic for anthraquinones in basic solution, which causes the colour of several tropical wood (Seigler, 1998). Therefore, many anthraquinone containing plants were used for coloring purposes (Velisek et al., 2008). They have been found in all parts of the plants: roots, rhizomes, fruits, flowers and leaves (Dave and Ledwani, 2012). Different biosynthetic pathways have been described. Thus, many quinones derive from the acetate-malonate pathway, whereas others originate from the shikimate pathway (Harborne, 1999). In addition, anthraquinones may be generated via oxidative modification of other secondary metabolites as for instance from polyphenolics (Seigler, 1998).

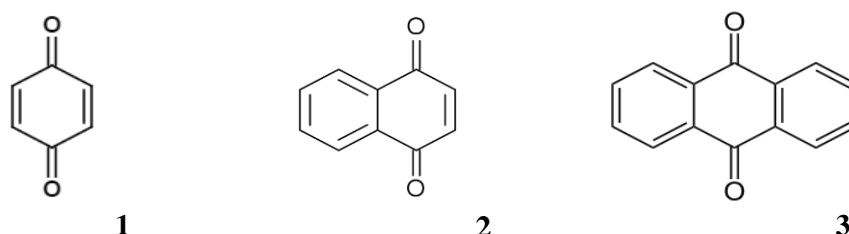


Fig. 5. Simplest structural formulas of different quinones. *para*-benzoquinone (**1**), 1,4-naphthoquinone (**2**) and 9,10-anthraquinone (**3**)

3.5 Terpenoids

This is by far the largest group of secondary metabolites found in all classes of organisms, with about 40.000 structures described from plants (Tholl, 2015). They are structurally and biosynthetically derived from a universal five-carbon building block called isoprene. The biosynthesis starts with isopentenyl pyrophosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP). In plants, two independent pathways, i.e. the cytosolic mevalonic acid (MVA) and the plastidial methylerythritol phosphate (MEP), build the C₅-isoprene units from which all terpenoids derive (Dudareva et al., 2005).

Classification of the terpenoids is done according to the number of isoprene units present in the molecules. Thus, monoterpenes (C₁₀) consist of two isoprene units, three units construct the sesquiterpenes (C₁₅) and four isoprene units characterize the diterpenes (C₂₀). Triterpenes (C₃₀) consist of 6, and tetraterpenes (C₄₀) of 8 isoprene units, respectively. Molecules with more than eight isoprene units are termed as polyterpenes (C_{5n}). Steroids are triterpenes composed of six cyclised isoprene units, folded to a multi-ring structure. Norisoprenoids are terpenoids where one or more carbon atoms are absent. The differential subcellular localization of MVA and MEP pathways leads generally to different end products. Monoterpenes and diterpenes are primarily synthesized via the MVA pathway, whereas sesquiterpenes and triterpenes largely derive from the MEP pathway. In addition, structural modification by cyclase enzymes are frequent, accounting for the structural diversity in plants. For an illustration of structures and biosynthetic sequences see Fig. 6.

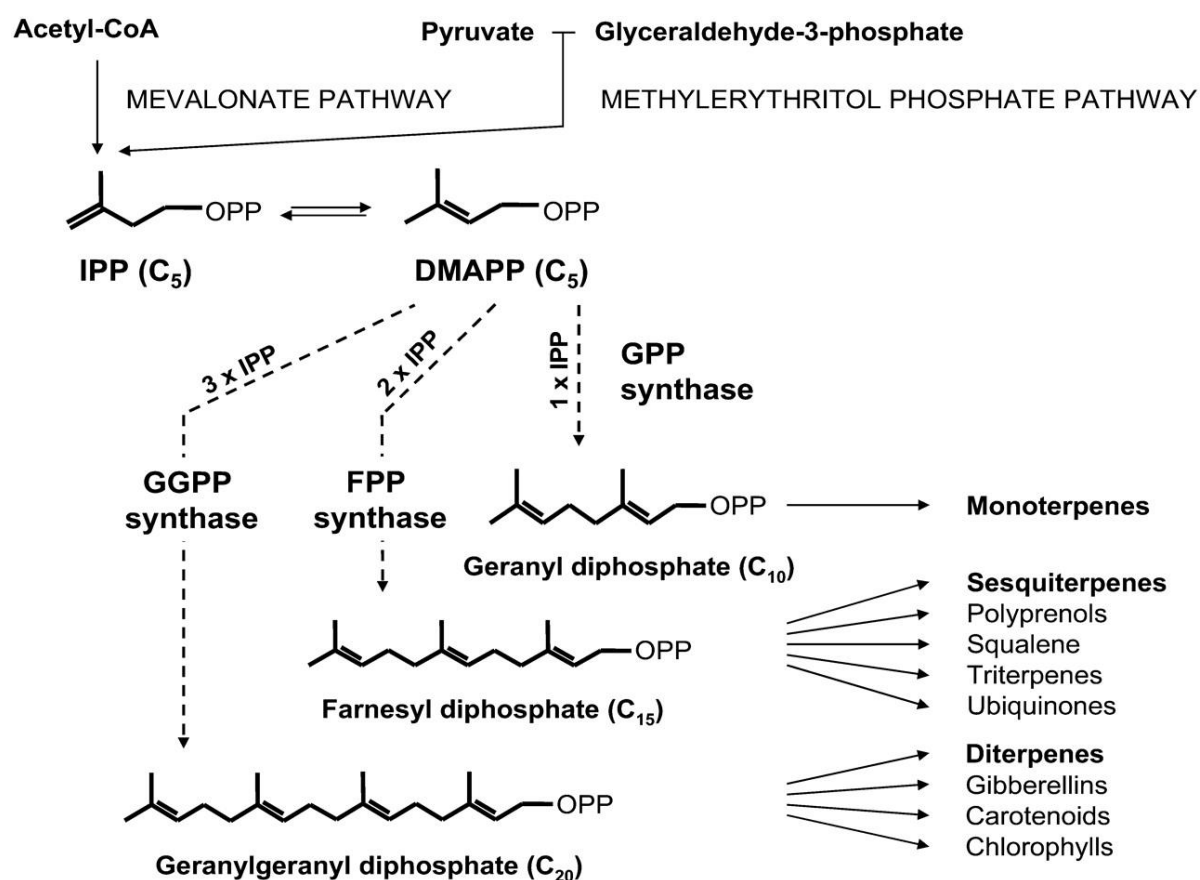


Fig. 6. Overview of the biosynthesis of different terpenoid classes. IPP: Isopentenyl pyrophosphate; DMAPP: Dimethylallyl pyrophosphate; GGPP: Geranylgeranyl pyrophosphate; FPP: Farnesyl pyrophosphate. Copyright by the American Society of Plant Biologists (Schmidt et al., 2010).

3.5.1 C13 Norisoprenoids (Megastigmanes)

C13 Norisoprenoids are structurally similar to sesquiterpenes and also known as norterpenoids, with most of them bearing an aromatic scent (Ayinampudi Sridhar Rao, 2017). They are thought to derive from carotenoid degradation, such as from β -carotene (4) or lutein (Fig. 7). Carotenoids are naturally unstable products because of the conjugated double-bond structure, which makes them highly reactive. In nature, the most widespread norisoprenoids are C13 compounds also known as megastigmanes and comprise structures such as ionones and damascones (Fig. 8). They are

differentiated due to their oxygen position which is located at C9 in the ionone group (**5**) and at C7 in the dasmascone group (**6**) (Mendes-Pinto, 2009; Winterhalter, 2001).

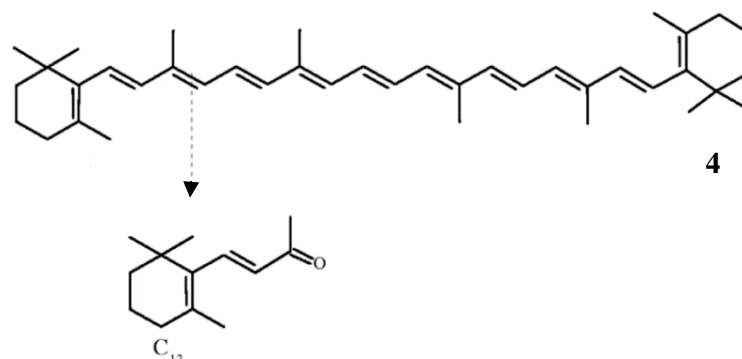


Fig. 7. Formation of the C₁₃ norisoprenoid compound from (**4**) β-carotene. (Adapted from Winterhalter, 2001)

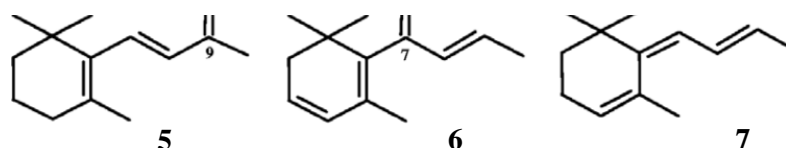


Fig. 8. Megastigmane carbon skeletons with different oxygen positions define the groups. β-ionone (**5**), β-damascenone (**6**) and (E,E)-megastigma-4,6,8-triene (**7**) (Adapted from Winterhalter, 2001).

Many C₁₃ norisoprenoids are of great importance as aroma compounds in plants. For instance the β-damascenone (**6**), first isolated from the essential oil of *Rosa damascena* Herrm., is a major contributor to the aroma of roses (Ohloff and Demole, 1987). Furthermore, C₁₃ norisoprenoids have been found and described in wine (Winterhalter et al., 1990), honey (Jerkovic and Kus, 2014), starfruits (Herderich et al., 1992) and tea (Yamaguchi and Shibamoto, 1981).

4 Material and Methods

4.1 Plant material

Most of the plant material was collected in the year 2010 or 2013 in the tropical rainforest of Costa Rica by A. Berger and J. Schinnerl. A list of the collected plants with details about collector, collecting number, origin and herbarium number is given in Table 5 (see Appendix). For the methanolic extraction, air dried plant material was used. Plants were separated into their organs leaf, stem and root for separate analysis.

For this study eleven *Notopleura* species have been selected (Tab. 1). The main focus was on *N. uliginosa*, due to the high diversity and concentration of secondary compounds.

Tab. 1. Studied *Notopleura* species, with details about dry weight and examined plant organs.

<i>N. anomothyrsa</i>	Stem	33.3 mg	<i>N. pithecobia</i>	Leaves	10.08 g
	Leaves	38.1 mg	<i>N. polyphlebia</i>	Stem	38 mg
	Inflorescence	33.1 mg		Leaves	38.3 mg
<i>N. capacifolia</i>	Stem	39.9 mg		Roots	33.9 mg
	Leaves	40.5 mg	<i>N. siggersiana</i>	Stem	38.4 mg
	Inflorescence	44.9 mg		Leaves	38.1 mg
<i>N. costaricensis</i>	Stem	40.9 mg	<i>N. tolimensis</i>	Stem	48.7 mg
	Leaves	49.1 mg		Leaves	44.1 mg
<i>N. nepokroeffiae</i>	Stem	36 mg		Inflorescence	49.2 mg
	Leaves	35.5 mg	<i>N. uliginosa</i>	Stem	38.2 mg
<i>N. panamensis</i>	Stem	40.5 mg		Leaves	51.6 g
	Leaves	46 mg		Roots	37.1 mg
<i>N. parasitica</i>	Stem	40.3 mg			
	Leaves	8.72 g			

4.2 Analytical methods

4.2.1 Thin Layer Chromatography (TLC)

This technique is used to separate single chemical compounds of a substance mixture. The method is based on the principle of interaction of a certain compound between a mobile phase and a stationary phase. In general, the stationary phase consists of a thin layer of silica gel, aluminium oxide or cellulose, coated on a glass plate, plastic or aluminium foil, which operates as an adsorbent material. The sample solvent is applied with a capillary as a small spot about 1.5 cm from the bottom edge. For the mobile phase different pure solvents or mixtures can be used. The TLC plate is placed into a chamber containing the mobile phase which is soaked upwards through capillary force. Due to varying strong interaction of the single molecules with the stationary phase (polar in case of silica gel), different ascend velocities are achieved and the compound becomes separated.

In our case we applied pre-coated silica gel on foil (silica gel 60, F254, Sigma-Aldrich). Different mixture ratios of methanol (MeOH) and chloroform (CHCl₃) were used for mobile phases. Molecules with a chromophore could be detected using UV₂₅₄ light due to the extinction of fluorescence of the indicator or with UV₃₆₆ light when substances show autofluorescence. For detection of substances without a chromophore, plates were sprayed with anisaldehyde reagent and developed with a heat gun. The Anisaldehyde reagent was prepared after Wagner and Bladt (1996).

4.2.2 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) can be used for identification, quantification and separation of mixed components. The technique is based on the same principles as the TLC. The sample is eluted with the mobile phase pumped through a column as the compact stationary phase. Due to varying interactions with the adsorbent material the compounds of the sample have different flow rates leading to a specific retention time for each substance. In addition, the HPLC can be

linked with an UV/VIS detector. UV sensitive compounds can be detected by their specific retention times and their characteristic UV-spectra.

The HPLC analyses were performed on Agilent 1100 series with UV- diode array detection. A Hypersil BDS-C18 (5µm particle size, 4.6 x 250 mm) column was used. A method of Berger et al., (2015) was used as followed. The eluent was a mix of MeOH (B) and an aqueous solution containing 10 mM ammonium acetate (A) from 40% to 90% B in A within 12 min, 90% to 100% B in A within 0.1 min, 100% B was kept for 5.9 min; inj. vol. 10 µL at a flow rate of 1.0 mL/min. The wavelength of detection was set at 230 nm.

4.2.3 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy is a technique applied for structure elucidation of molecules by using a strong magnetic field. In this method, atoms possessing a magnetic moment like ^1H or ^{13}C are excited by electric pulses and the released energies during the relaxation processes of the atoms is detected. The frequencies of the released energy provides detailed information about structure, dynamics and the individual functional groups of a molecule. These analyses and structure elucidation were performed at the Institute of Organic Chemistry, University of Vienna.

4.2.4 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH assay is a method to measure the antioxidant activity of a substance. DPPH is a stable free radical with an absorption at 517 nm, showing a violet colour in solution and becomes colourless when neutralized. This characteristic is ideal for using it for visual monitoring of reactions. When dosed with an antioxidant or other radicals the absorption at 517 nm is measured again. The measured change in the optical absorption can be counted for the number of initial radicals (Alger, 1997). The assay can be used as a spraying reagent or modified for microwell plates. As spraying reagent, DPPH is sprayed on the developed and dried TLC plates. For the spraying reagent, a

0.4 mM solution in Methanol of DPPH was used. For the microwell plates the assay described by Formagio et al., (2014) was modified. A two-fold serial dilution of the sample stock solutions (2.5 mg/mL) of the methanolic plant extracts was prepared with methanolic DPPH (0.2 mM). The mixture was shaken, measured at 517 nm and left to stand at room temperature. After 30 min, the absorbance was measured again against a blank containing all reagents except the test samples. Quercetin, ascorbic acid and quercetin-3-methylether were used as positive controls. The assays were carried out in duplicates.

4.2.5 β -carotene bleaching assay

The β -carotene bleaching assay is a method to measure the antioxidant activity of a substance. In this research the method of Formagio et al. (2014) was performed as followed.

“The β -carotene solution was prepared by dissolving 2 mg β -carotene in 10 mL chloroform. 1 mL of this β -carotene-chloroform solution was mixed with 20 mg linoleic acid and 0.2 g Tween 20. Subsequently, the chloroform was removed by a rotary evaporator at 45 °C. Distilled water (50 mL) was slowly added with vigorous agitation to form an emulsion. Emulsion aliquots (5 mL) were transferred with 0.2 mL of the extracts different concentrations (10–200 μ g/mL, sample stock 1.0 mg/mL).”

4.3 Preparative methods

4.3.1 Liquid-liquid extraction

Liquid-liquid extraction is a basic technique to separate substances due to their polarity. Two immiscible fluids are mixed in a separation funnel and subsequently extracted according to their solubility behaviour. In this case the plant extracts were suspended in distilled water and as nonpolar solvents CHCl_3 and EtOAc were used.

4.3.2 Column Chromatography (CC)

A simple column chromatography was performed after liquid-liquid extraction for further separation of the substance mixture. A glass column is filled with the stationary phase and saturated with the mobile phase. The sample is dissolved in a small volume of the mobile phase solvent and then applied on the top of the stationary phase. The sample is rinsed through the column with the mobile phase by gravity.

Approximately 50 g of silica gel (silica gel 60, 0.2-0.5 mm, Roth) were filled into a column of about 1.5 cm inner diameter. As mobile phase solvent mixtures of increasing polarity from PE, EtOAc to MeOH were used (Table). 100 mL of each solvent mixture were used. The eluent was collected in 20 x 50 mL flasks.

4.3.3 Gel permeation chromatography (GPC)

The gel permeation chromatography is a Column chromatography and is based on size exclusion of the analytes. In this case the stationary phase consists of porous beads packed in a column. Smaller molecules enter the pores of the beads and spend more time in the column leading to increased retention time. Larger molecules elude the pores due to its size and are eluted more quickly. In this work Sephadex LH-20 in Methanol was used.

4.3.4 Medium pressure liquid Chromatography (MPLC)

Medium pressure liquid chromatography is a technique which allows a better separation performance of pre-purified compounds. Due to the more densely packed column and a smaller particle size, pressure is needed to rinse the eluent through the column. Different eluent mixtures of petroleum ether and ethyl acetate were used. Silica gel (40-60 μm particle size) was used for the separation.

4.3.5 Preparative thin layer Chromatography (prTLC)

Preparative thin layer chromatography is similar to TLC but differ in the amount of sample added to the plate. The band of the substance on the silica gel can then be

scraped off from the plate and dissolved in MeOH in a frit. It was used for separating small amounts of compounds.

5 Extraction and Isolation

Of all the selected *Notopleura* species a methanolic extract of different plant organs, leaves, stem, roots or inflorescence, was prepared for HPLC-UV and TLC analysis.

5.1 *Notopleura uliginosa*

Leaves: 51.6 g of dry leaves were used to prepare a methanolic extract (3 x 3 d, 400 mL each) at room temperature, which was filtered and dried to give a crude extract of 4.39 g. Further the extract was partitioned by a separation funnel with different solvents each 3x 100 mL of CHCl₃, EtOAc and H₂O. The different phases had been evaporated to get a concentrated extract. Then 1.18 g of the CHCl₃ phase extract was separated with a separation funnel (3 x 50 mL) with H₂O' and a mixture of petrol ether and diethyl ether (PE:Et₂O 95:5). The PE:Et₂O phase (930.7 mg) was separated by CC (silica gel 60, 0.2–0.5 mm) with solvent mixtures of increasing polarity (Tab. 2). The fraction IV₂, eluted with PE/EtOAc (60:40) was further separated by GPC to give psychorubrin (0.8 mg) (**8**) 203.3 mg of the H₂O' phase was chromatographed by Sephadex LH-20 with MeOH. The fractions 3 and 4 were combined (54.3 mg) and separated by liquid-liquid extraction. 3 x 30 mL of H₂O'' and PE' (with 1% IPA) were used as solvents. CC with Sephadex LH-20 in MeOH was performed for 47.9 mg of the H₂O'' phase. Fraction 5 with 6 (9.8 mg) was pooled and fraction 7 with 8 (13.2 mg) was combined, respectively. The fraction 9 (12 mg), 5+6 and 7+8 were used for a Sephadex LH-20 column separation eluted with MeOH. The eluted fractions were combined according to HPLC-analyses and gave blumenol A (3 mg) (**10**), 3-oxo- α -ionol (1.2 mg) (**9**) and loliolide (7.9 mg) (**11**). For a better replicability further information is given in Fig. 9.

Tab. 2. Solvent mixtures and received fractions of the
CC of PE:Et₂O phase

PE %	EtOAc %	Fractions
90	10	I ₁ , I ₂
80	20	II ₁ , II ₂
70	30	III ₁ , III ₂
60	40	IV ₁ , IV ₂
50	50	V ₁ , V ₂

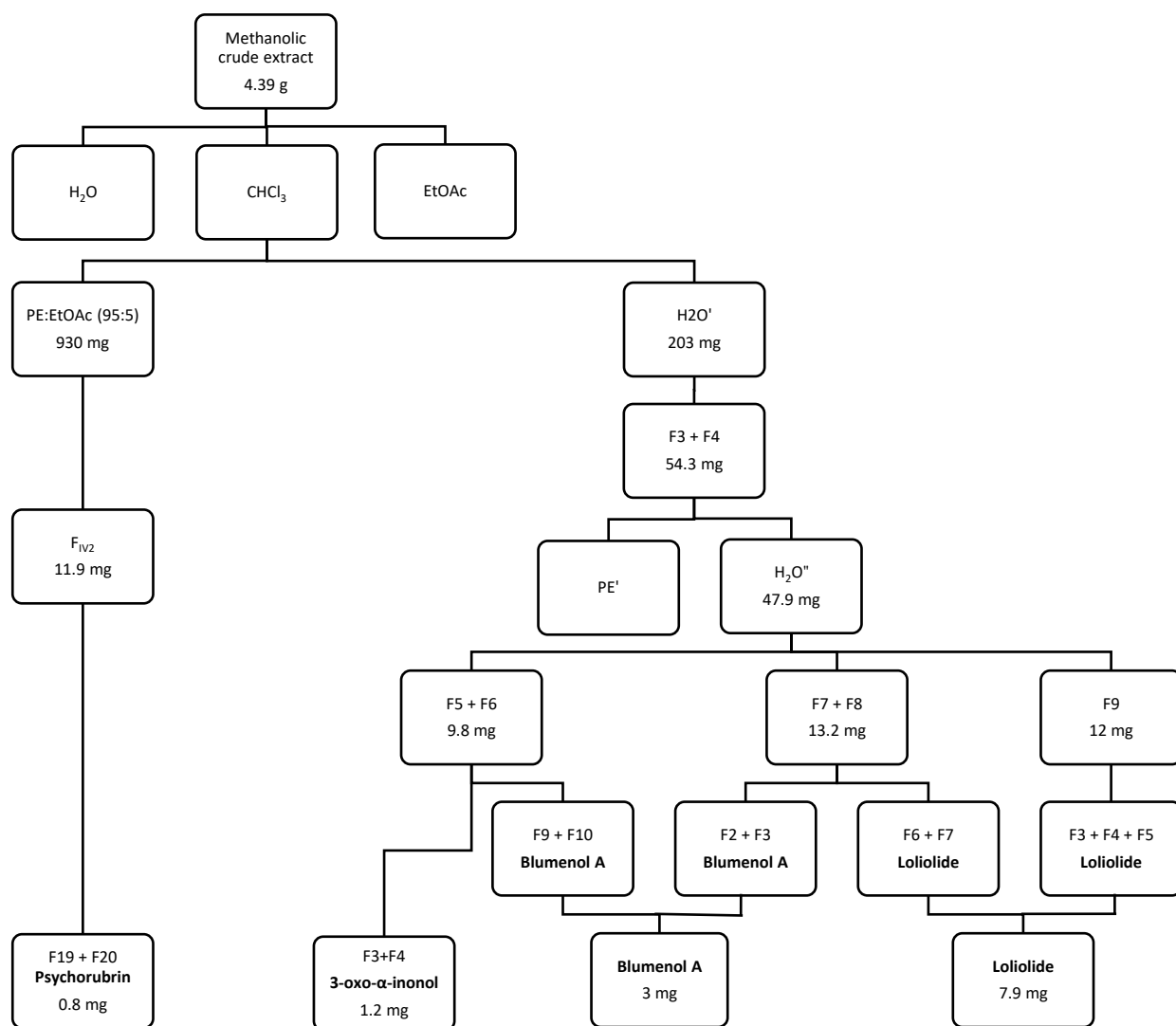


Fig. 9. Scheme of extraction from the methanolic crude extract to the pure substances of *N. uliginosa*. F: Fraction; H₂O: Water phase; CHCl₃: Chloroform phase; EtOAc: Ethyl acetate phase; PE: Petrol ether.

6 Results and Discussion

Comparative HPLC analysis revealed the presence of secondary metabolites from different biosynthetic pathways in selected *Notopleura* species, mainly quinones and terpenoids. For analysis of possible chemical diversification, leaf material was chosen. In addition, organ-specific accumulation was further addressed, and results are summarized and commented accordingly (Tab. 3). The study is complemented by testing antioxidant activities of crude extracts and isolated compounds. Investigation of leaves of *N. uliginosa* led to extraction of the pyranonaphthoquinone psychorubrin (**8**), the megastigmanes 3-oxo- α -ionol (**9**) and blumenol A (**10**), as well as the monoterpenoid hydroxylactone loliolide (**11**). These compounds were structurally identified on basis of their NMR spectroscopic data and by comparison of spectral identities and similarities in the combined CSEARCH and SPECINFO database system (Robien, 2009; V.Schütz et al., 1997). Additionally, 2-aza-anthraquinone (**12**) and the dihydroflavonol (**13**) were detectable in some species. Its presence was confirmed by comparative HPLC and UV spectra analysis. Under the conditions used in the HPLC method, psychorubrin (**8**) eluted at 8.9 min, 3-oxo- α -ionol (**9**) at 9.4 min, blumenol A (**10**) at 6.2 min, loliolide (**11**) at 6.8 min, 2-aza-anthraquinone (**12**) at 11.1 min and the dihydroflavonol (**13**) at 8.4 min.

Psychorubrin (**8**) was first isolated from *Psychotria rubra*, a plant used in Chinese folk medicine, and exhibits cytotoxicity against KB cells (Hayashi et al., 1987). Furthermore strong antiplasmodial (Endale et al., 2012), antibiotic and antileishmanial (Fabri et al., 2012) properties of the substance have been reported. The fact, that psychorubrin (**8**) was not reported outside the genera *Psychotria* (Hayashi et al., 1987), *Notopleura* (Jacobs et al., 2008; Solis et al., 1995), *Mitracarpus* (Fabri et al., 2012) and *Pentas* (Endale et al., 2012), which all belong to the Rubiaceae family, indicates that it could be used as chemotaxonomic marker on family level.

In 1992 Pabst et al. identified 3-oxo- α -ionol (**9**) as the aglycone of a glucoside isolated from *Rubus idaeus* (Rosaceae) for the first time. It is known to be an isoprenoid

flavour precursor with a spicy odour in the genus *Equisetum* (Fons et al., 2013). This compound is also found in invasive plant species such as in *Vulpia myuros* and in *Brachiaria brizantha* (both Poaceae), and it is reported to have allelopathic properties (Kato-Noguchi et al., 2014, 2010). Blumenol A (**10**) is well known from many different plant species. It was first described under the synonym vomifoliol, isolated from *Rauvolfia vomitoria* (Apocynaceae) (Pousset and Poisson, 1969) and three years later under bluminol A (**10**) isolated from *Nageia wallichiana* (Podocarpaceae) (Galbraith and Horn, 1972). It is thought to be biosynthesized from (+) abscisic acid by oxidative removal of the two terminal carbon atoms (Bhakuni et al., 1974), but its biosynthesis is still not fully resolved. Due to the fact that various C₁₃ norisoprenoids are found in dried tobacco leaves but not in fresh material (Winterhalter, 2001), it is not clear if the compounds **9** and **10** are biosynthesised *in vivo* or if some precursor compounds are converted during the drying process thus creating artefacts.

The monoterpenoid hydroxylactone loliolide (**11**) was first identified in the English Ryegrass *Lolium perenne* in 1964 from Hodges and Porte. This ubiquitous compound occurs in both marine and terrestrial animals and in plants (Grabarczyk et al., 2015). It is known from several other plant species, many of them are used for various applications in folk medicine (Geng and Liu, 2008; Sarker et al., 2000; Zajdel et al., 2012). It has been shown that loliolide exhibits inhibitory activity against cellular senescence (Yang et al., 2015). The yield of loliolide (**11**), reported from different plants in literature, was of small quantities (5.8×10^{-5} to 8×10^{-4} % of dry weight) (Grabarczyk et al., 2015), as compared to *N. uliginosa* where the yield was 10 times higher (1.5×10^{-2} %).

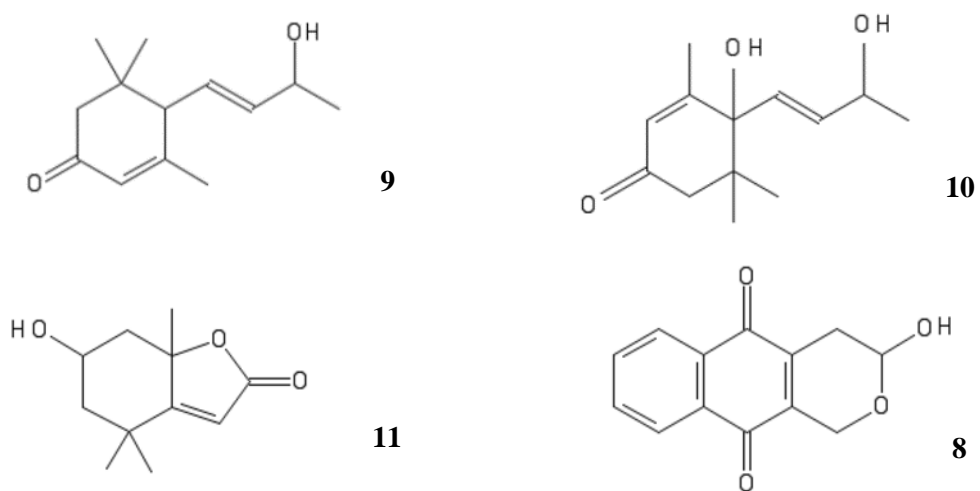


Fig. 10. Structural formula of the megastigmanes 3-oxo- α -ionol (**9**) and blumenol A (**10**), which differ in one additional hydroxyl group, the monoterpenoid hydroxylactone loliolide (**11**) and the naphthoquinone psychorubrin (**8**).

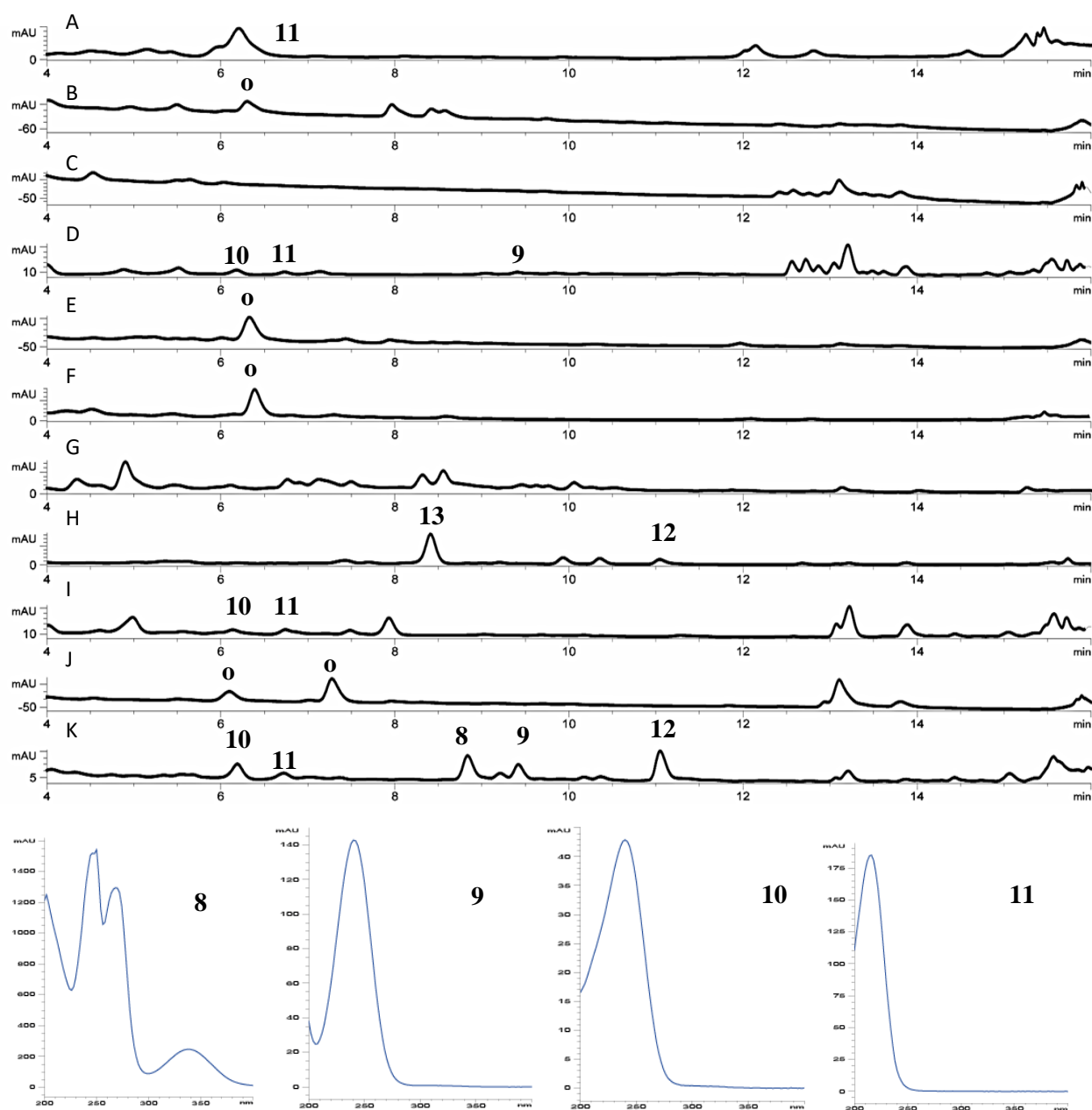


Fig. 11. HPLC profiles of methanolic leaf extracts and UV spectra of the assigned peaks of different *Notopleura* species. Psychorubrin (**8**), 3-oxo- α -ionol (**9**), blumenol A (**10**) and lolilide (**11**), 2-aza-anthraquinone (**12**), dihydroflavonol (**13**) and qercetin derivatives (**o**). A: *N. anomothyrsa*, B: *N. capacifolia*, C: *N. costaricensis*, D: *N. nepokroeffiae*, E: *N. panamensis*, F: *N. parasitica*, G: *N. pithecobia*, H: *N. polyphlebia*, I: *N. siggersiana*, J: *N. tolimensis*, K: *N. uliginosa*.

Tab. 3. Secondary metabolite distribution in surveyed *Notopleura* species.

Species	Megastigmanes			Quinones		Dihydroflavonol	Quercetin derivatives
	10	9	11	8	12	13	o
Leaves							
<i>N. anomothyrsa</i>			x				
<i>N. capacifolia</i>							x
<i>N. nepokroeffiae</i>	x	x	x				
<i>N. panamensis</i>							x
<i>N. parasitica</i>							x
<i>N. polyphlebia</i>	x		x		x	x	
<i>N. siggersiana</i>	x		x				
<i>N. tolimensis</i>							x
<i>N. uliginosa</i>	x	x	x	x	x		
Stems							
<i>N. capacifolia</i>					x		
<i>N. nepokroeffiae</i>	x		x				
<i>N. panamensis</i>					x		
<i>N. polyphlebia</i>				x	x		
<i>N. uliginosa</i>	x			x	x		
Roots							
<i>N. polyphlebia</i>				x	x		
<i>N. uliginosa</i>				x	x		

While in the leaves more terpenoids are present, in the stems and roots the quinones are dominating. None of the isolated substances were detectable in: a) the inflorescences of the selected *Notopleura* species (Tab. 1.), b) in leaves of *N. capacifolia*, *N. costaricensis*, *N. panamensis*, *N. parasitica*, *N. pithecobia* and *N. tolimensis*, and c) in stems of *N. anomothyrsa*, *N. costaricensis*, *N. parasitica*, *N. siggersiana* and *N. tolimensis*. **8**: Psychorubrin, **9**: 3-oxo- α -ionol, **10**: blumenol A, **11**: Loliolide, **12**: 2-aza-anthraquinone, **13**: dihydroflavonol and **o**: quercetin derivatives.

Comparative HPLC analysis revealed differences in the composition of leaf extracts as shown in Fig. 11. Thus, the isolated compounds **8**, **9**, **10** and **11** are unevenly distributed among the studied species (Tab. 3). Loliolide (**11**) occurs most common in the studied samples, followed by blumenol A (**10**). The remaining compounds are only of scattered distribution, and presence of psychorubrin (**8**) could only be confirmed for *N. uliginosa* and *N. polyphlebia*. Quite some variation was noted in the accumulation of terpenoids and quinones in the different plant organs (Tab. 3). It is evident that leaves

are the main accumulation sites of the terpenoids while the occurrence of the quinone seems to be predominant in stems and roots (Tab. 3).

Antioxidant activities of crude extracts and isolated compounds was monitored by two different assays as described in 5.2.4 and 5.2.5., respectively. Only the DPPH assay results were taken into account, because the β -carotene bleaching assay was not performed successfully due to unknown reasons. Though one investigated compound (**11**) is known to act as intracellular antioxidant there seems to be no clear correlation between activity of plant extracts and that of isolated compounds (c.f. Tab. 3 and Fig. 12). The crude extract of *N. panamensis* shows a relatively high DPPH inhibition rate, but none of the secondary metabolites isolated in this research (Tab. 3) was detected in the leaves of this species. The same applies for the studied species *N. capacifolia*, *N. parasitica*, *N. tolimensis* and *N. pithecolobium*. These results imply that other yet unidentified compounds are responsible for antioxidant activity of crude extracts. In this context, it has to be mentioned that the crude plant extracts of *N. panamensis*, *N. parasitica*, *N. capacifolia* and *N. tolimensis* contained relatively high amounts of quercetin derivatives as suggested from HPLC data. For this reason it is supposed that rather the polyphenolic compounds would be the antioxidative active substances. A more detailed analysis and quantification of these compounds is necessary for a clarification of their role as antioxidants.

7 Conclusion

1. The present study revealed the presence of blumenol A (**10**), 3-oxo- α -ionol (**9**), loliolide (**11**) and psychorubrin (**8**) in *Notopleura* species belonging to the *Psychotria* alliance. The naphthoquinone psychorubrin (**8**) has been found so far only in Rubiaceae and could thus be used as chemotaxonomic marker for this family. By contrast, the detected megastigmanes are not restricted to this family but of rather widespread occurrence. However, it appears that within the *Psychotria* alliance, their prime occurrence in *Notopleura* might be of relevance. None of the typical alkaloids could be detected in all of the studied *Notopleura* species. In combination with the predominant occurrence of megastigmanes and quinones, the genus is phytochemically unique within this alliance.
2. Megastigmanes and quinones have different distribution patterns in *Notopleura* plant organs. Whereas megastigmanes occur mainly in the leaves, quinones are primarily located in the stems and roots. Different biosynthetic routes are involved in the genesis of these compounds, also with respect to their intercellular localization. In particular, megastigmanes are derived from plastidial pathways probably expressed in photosynthetic tissues only.
3. Megastigmanes could theoretically result as artefacts from drying plant material. To avoid artefacts both, fresh and dry plant material of the same individual should be used for extraction. The quantification of the secondary metabolites before and after drying would be suggestive.
4. None of the isolated compounds seem to be the main reason for the observed antioxidant activity of the crude extracts of *Notopleura* species. However, it is supposed that yet unidentified flavonol derivatives and other polyphenols may be responsible for the antioxidant activity. Therefore, enhanced analysis of polyphenolic fractions should be carried out to identify the antioxidant compounds in *Notopleura* species.

8 References

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9 Zusammenfassung

Die Gattung *Notopleura* gehört innerhalb der *Psychotria*-Verwandtschaftsgruppe dem Tribus Palicoureeae (Rubiaceen) an. Diese Gruppe ist bekannt für die Akkumulation von bioaktiven Monoterpen-Indolalkaloiden. Da vergleichsweise wenig über die sekundären Metabolite der *Notopleura* Arten bekannt ist, war das Ziel dieser Arbeit ausgewählte *Notopleura* Arten hinsichtlich ihrer chemischen Zusammensetzung sowie der organspezifischen Akkumulation mittels vergleichender HPLC zu analysieren. Die Extrakte wurden mittels unterschiedlicher chromatografischer Verfahren gereinigt und anschließend durch vergleichende HPLC analysiert. Aus Blattextrakten von *N. uliginosa* wurde das Naphthochinon Psychorubrin (**8**), die Megastigmane 3-Oxo- α -ionol (**9**) und Blumenol A (**10**), sowie das Monoterpen-Hydroxylacton Loliolid (**11**) isoliert und die Struktur mit ^{13}C -NMR-Spektroskopie aufgeklärt. In der Verbreitung dieser Metabolite zeigen sich auffallende Unterschiede zwischen Blättern, Stängeln und Wurzeln der untersuchten Arten. Megastigmane dominieren in den Blättern, wohingegen Chinone vermehrt in Wurzeln und Stängeln vorkommen, was offensichtlich ein Ausdruck unterschiedlicher Expression der Biosynthesewege ist. Keiner dieser Stoffe wurde allerdings in den Infloreszenzen nachgewiesen. In keiner der untersuchten Arten konnten Alkaloide nachgewiesen werden. Die antioxidative Aktivität der Pflanzenrohextrakte konnte in dieser Arbeit nicht mit den vier isolierten Substanzen in Verbindung gebracht werden. Die beobachteten Akkumulationstendenzen unterstreichen die aktuelle taxonomische Stellung der Gattung und könnten somit chemosystematisch relevant sein.

10 Appendix

10.1 DPPH assay results

Graphs resulting from the DPPH tests (Fig. 12) and the percentages of inhibition of different *Notopleura* species in Table 4 for a better overview.

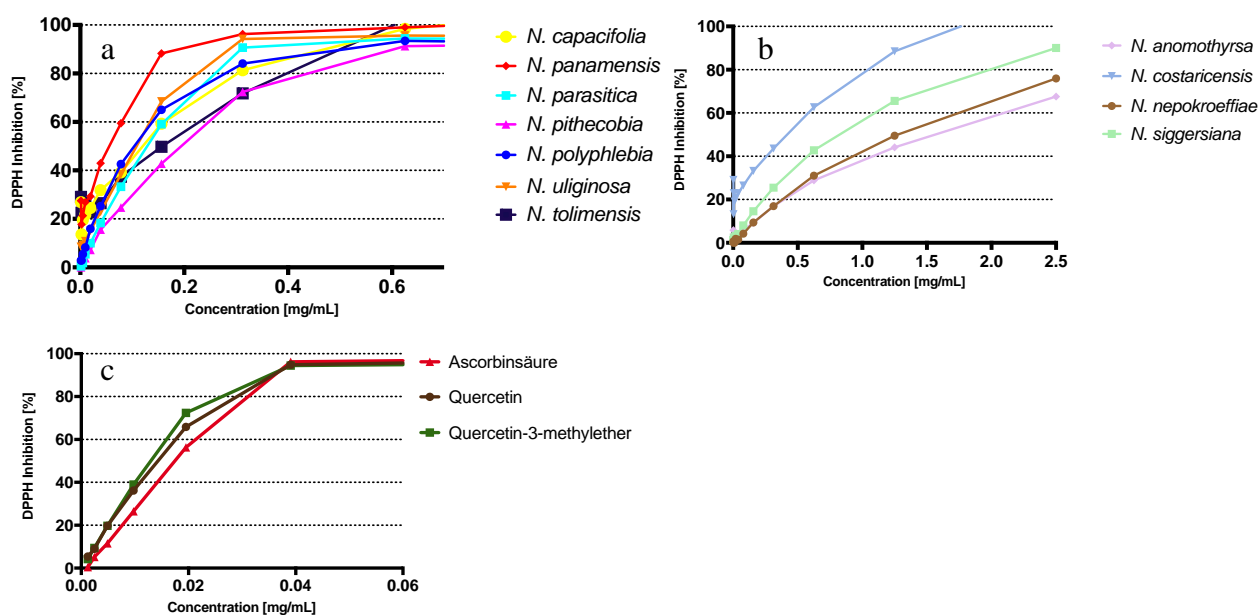


Fig. 12. DPPH Inhibition percentage to the concentration of **a** and **b**: the leave extracts of *Notopleura* species in mg/mL **c**: the standards quercetin-3-methylether, quercetin and ascorbic acid in mg/mL. The percentage of inhibition of DPPH (I%) was calculated using the following equation: $I\% = [(A_0 - A)/A_0] \times 100$. A_0 is the absorbance of the blank solution and A is the absorbance of the methanolic crude extract of the *Notopleura* species.

Tab. 4. Inhibition in % of *Notopleura* species at a concentration of 0.16 mg/mL of the crude Extract.

Species	I%	Species	I%
<i>N. anomothyrsa</i>	9.19	<i>N. parasitica</i>	59.07
<i>N. capacifolia</i>	59.26	<i>N. pithecobia</i>	42.77
<i>N. costaricensis</i>	33.23	<i>N. polyphlebia</i>	65.03
<i>N. nepokroeffiae</i>	9.39	<i>N. tolimensis</i>	49.73
<i>N. panamensis</i>	88.28	<i>N. siggersiana</i>	14.56
		<i>N. uliginosa</i>	68.41

10.2 List of taxa

Table 5 gives a list of all taxa surveyed in the present study with additional information on taxonomic authors, collectors and collection number of the plant material, as well as the origin and herbarium number of the specimens.

Tab. 5. List of all surveyed *Notopleura* species and their collection data.

Species	Collector	Origin	Date	Herbarium nr.
<i>Notopleura uliginosa</i> (Sw.) Beremek.	Berger, A. 1543 A	Costa Rica, Puntarenas	2013	WU 0082490
<i>Notopleura tolimensis</i> (Wernham) C. M. Taylor	Berger, A. & Schinnerl, J. 1463	Costa Rica, Cartago	2013	WU 0082488
<i>Notopleura anomothyrsa</i> (K. Schum. & Donn. Sm.) C. M. Taylor	Berger, A. AB 07021001	Costa Rica, Limon	2010	WU 0067350
<i>Notopleura siggersiana</i> (Standl.) C. M. Taylor	Berger, A. & Schinnerl, J. 1484	Costa Rica, Heredia	2013	WU 0082489
<i>Notopleura capacifolia</i> (Dwyer) C. M. Taylor	Berger, A. 05031003	Costa Rica, Puntarenas	2010	WU 0067352
<i>Notopleura costaricensis</i> C. M. Taylor	Berger, A. & Schinnerl, J. 1506	Costa Rica, Cartago	2013	WU 0082496
<i>Notopleura parasitica</i> (Sw.) Hammel	Berger, A. & Schinnerl, J. 1538	Costa Rica, Puntarenas	2013	WU 0082486
<i>Notopleura panamensis</i> (Dwyer) C. M. Taylor	Berger, A. & Schinnerl, J. 1539	Costa Rica, Puntarenas	2013	WU 0082495
<i>Notopleura nepokroeffiae</i> C. M. Taylor	Berger, A. & Schinnerl, J. 1507	Costa Rica, Cartago	2013	WU 0082503
<i>Notopleura polyphebia</i> (Donn. Sm.) C. M. Taylor	Berger, A. AB 19021001	Costa Rica, Puntarenas	2010	WU 0067354
<i>Notopleura piihecobia</i> (Standl.) C. M. Taylor	Berger, A. 1589	Costa Rica, Cartago	2015	WU 0082484