Carbon based secondary metabolites in African savanna woody species in relation to anti-herbivore defense

Dawood Hattas

February 2014

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biological Sciences

UNIVERSITY OF CAPE TOWN

Supervisors:

JJ Midgley, PF Scogings and R Julkunen-Tiitto

Declaration

I Dawood Hattas, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorize the University to reproduce for the purpose of research either the whole or a portion of the content in any manner whatsoever.

This thesis includes two publications that were published in collaboration with research colleagues. Thus I am using the format for a thesis by publication. My collaborators have testified that I made substantial contributions to the conceptualization and design of the papers; that I independently ran experiments and wrote the manuscripts, with their support in the form of comments and suggestions (see Appendix).

Published papers

Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F., Rooke, T., 2011. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. Phytochemistry 72: 1796-1803 (Chapter 2).

Hattas, D., Julkunen-Tiitto, R., 2012. The quantification of condensed tannins in African savanna tree species. Phytochemistry Letters 5: 329-334 (Chapter 1).

Signature: …… Date: …….

This thesis is dedicated to my late parents Aboebaka and Gadija Al-Attas (Hattas); my loving wife Faiza Achmat and adorable daughters Ganaan, Amina and Thanaa. Without your unwavering love, support and patience this journey would not have been possible.

Acknowledgements

Algamdulillah (All praise is due to Allah).

I would like to thank the numerous people who I have interacted with regarding the work presented here. You may not be mentioned here, but I would like to acknowledge your contribution to this effort.

I would like to express my sincere gratitude to my supervisors and inspirational mentors. Peter Scogings persuaded me to embark on this academic journey. Your belief in me (even when I doubted myself), patience and encouragement was the fuel that drove me to this point, thank you. To Riitta Julkunen-Tiitto, Kiitos for the belief, warmth, consideration and care that you showed during my visits to your lab. Your guidance was invaluable. To Jeremy Midgley, thank you for asking the right questions and your eye for detail which guided me in my development as a research scientist. It has indeed been a privilege to have worked so closely with you over the years.

I would like to thank my colleagues from the academic staff ranks in the former Botany and now Biological Sciences department. Adam West is thanked for his wise words of encouragement and providing perspective, especially when I hit those far too many productivity lows. To William Bond, Mike Cramer, Tony Verboom, Ed February and Samson Chimphango thank you for invaluable advice and encouragement. To Tshifhiwa Mandiwana, Lesego Khomo, Timm Hoffman, Muthama Muasya, John Bolton, Terry Hedderson, Lindsey Gillson, Anusuya Chinsamy-Turan, Coleen Maloney, Roishin Kelly-Laubser, Deena Pillay, Justin O'Riain and Cecile Reed, thank you for your encouragement and support. I am privileged to be surrounded by such enthusiastic academic excellence, I can't but be inspired.

The Zululand/Sweden Kruger Browse Project team members and assistants; Tuulikki Rooke, Luthando Dziba, Joakim Hjältén, Alpheus Zobolo, Ntuthuko Mkize,

Basanda Nondlazi, Julius Tjelele, Gilbert Pule and all the Kruger Scientific Services game guards who kept us safe while we played in the trees, are gratefully acknowledged.

My Finnish colleagues (and family) Sinikka Sorsa, Saarita Keski-Saari, Eveliina Hiltunen, Anneli Salonen and Anu Lavola are acknowledged for their technical assistance, warmth and kindness.

Special mention to Gonzalo Aguilar for unselfishly carrying my load during my many bouts of absence, as well as his friendship and encouragement. To Sandy Smuts, thank you to your belief, encouragement and acknowledgement even for my smallest achievements over the years. Those acknowledgements really put the wind back in my sails. My other colleagues and friends in the Department of Biological Sciences, Desmond Barnes, Robert Jacobs, Nazli Davids, Zamikaya Jikumlambo, Francois Majola, Liesl Phigeland, Gugu Ginindza and Tamara Nozewo are likewise thanked for their encouragement and patience.

My late father Aboebaka Hattas, Thandeka Mamashela and Patricia Shabangu are thanked for help with the collection of leaf material. Edward Chirwa assisted with feeding and harvesting of plants. Scientific Services in Kruger Park provided critical logistical support for fieldwork. The Seed Room at Kirstenbosch Botanical Gardens generously allowed me to collect leaf material. Neil Fishwick from Fishwick's Nursery is thanked for providing saplings.

Funding was provided by the National Research Foundation, South Africa (GUIN 46939) and the Dorothy Cameron Bequest. Funding for the Zululand/Sweden Kruger Browse Project was provided by the National Research Foundation (South Africa), University of Zululand, Agricultural Research Council (South Africa), Swedish Research Council and the Swedish International Development Agency.

Many colleagues and friends across UCT and elsewhere provided continuous encouragement. Thank you to Praveen Maistry, Simon Power, Mark Rothman, Benny Lemaire, Nicholas Wilding, Charles Stirton, Dalielah Jappie, Terry Trinder-Smith, Cornelia Klak, Gabi de Bie, Faezah Davids, Ntuthuko Mkize, Particia Thomson, Abdu Mohamed, Neil Bredekamp, Ullrich Mutzeck, Madhu Chauhan and Derrick September. I am grateful to my siblings Wagied, Adiel, Riefaat, Soliegah and Nadeema Hattas for their love, support and patience. My friends Buteri Calana, Ashia Petersen, Faghrie Mitchell, Zuhayr Kafaar, Yaqoob Thomas, Fatima Shabodien, Shameem Jaumdally and the many others are thanked for continuous encouragement and patience.

To Faiza Achmat, I cannot thank you enough for your sacrifice and infinite patience over so many years. Thank you for being the present parent for our lovely daughters. I can never repay you for your selflessness. You are truly a blessing in my life.

To Ganaan, Amina and Thanaa, thank you for those broad smiles and hugs that greeted me after a long day, but often only when you see me in the morning after a late night. Your unconditional love and affection drives me.

To my late parents Aboebaka and Gadija Al-Attas (Hattas), this is as much your achievement as it is mine. Everything I achieve and have is due to your hard work and sacrifices. THANK YOU, May Allah grant you a high place in Jannah, Insha-Allah.

Abstract

Chemical defense in African savanna woody species have been inferred from foliar condensed tannin and total phenolic concentrations. However these measures are controversial since condensed tannin chemical heterogeneity compromises its simple quantification and total phenolics, which is a measure of general foliar phenolics, includes many different low molecular weight phenolics. Some of these low molecular weight phenolics have been shown to deter herbivory individually or in combination with others. The growth differentiation balance hypothesis predicts a trade-off between costs of chemical defenses or carbon based secondary metabolites relative to the demand for photosynthate by growth. However, this hypothesis was developed in northern boreal and temperate forests and the extent to which it applies to African savanna woody species remains unclear. In this study we: 1) Compared the use of *Quebracho* with *Sorghum* tannin as standards for condensed tannin quantification, relative to absolute condensed tannin concentration in selected species; 2) Determined low molecular weight phenolic profiles of 6 species with different life history, morphological and functional traits; and 3) Investigated whether the growth differentiation balance hypothesis explains allocation to carbon based secondary metabolites in *Combretum apiculatum* along a 6-level N gradient; and following 100% simulated herbivory along the N gradient.

Condensed tannins were extracted from 5 tree species and purified. Condensed tannin concentrations were determined using the acid-butanol assay and expressed as *Quebracho*, *Sorghum* and absolute tannin concentrations. Chemical composition of different tannin polymers were determined by thiolysis using HPLC and HPLC-MS. Photosynthesis, growth variables and phenotypic phytochemical responses to the different N and N x herbivory treatments were measured in *C. apiculatum*. Low

molecular weight phenolics were determined and identified using HPLC and HPLC-MS respectively.

Results from this study suggest: that the continued use of *Quebracho* tannin as condensed tannin standard in the acid-butanol assay is unjustified; that generalizations based on life history, morphological and functional traits may be misleading since foliar low molecular weight phenolics are not necessarily related to these traits; and that the growth differentiation balance hypothesis does not explain allocation to carbon based secondary metabolites in *C. apiculatum*.

List of Abbreviations

Abs	Absorbance
AE	*Acacia exuvialis*
AG	*Acacia grandicornuta*
C	Carbon
C:N	Carbon:nitrogen ratio
CA	*Combretum apiculatum*
CBSM	Carbon based secondary metabolites
CNBH	Carbon nutrient balance hypothesis
CT	Condensed tannin
DC	*Dichrostachys cinerea*
ED	*Euclea divinorum*
FG	Flavonol glycosides
GDBH	Growth differentiation balance hypothesis
GF	*Grewia flavescens*
HPLC	High performance liquid chromatography
LAR	Leaf area ratio
lmwp	low molecular weight phenolic
M	Molar
mDP	Mean degree of polymerization
Mr	Molecular weight
MS	Mass spectrometer
NADH	Nicotinamide adenine dinucleotide
NAR	Net assimilation rate

nMDS	Non-metric multi-dimensional scaling
QT	*Quebracho* tannin
QTE	*Quebracho* tannin equivalents
RGR	Relative growth rate
Rt	Retention time
RWR	Root weight ratio
SIMPER	Similarity percentage
SM	*Scutia myrtina*
ST	*Sorghum* tannin
STE	*Sorghum* tannin equivalents
TP	Total polyphenolics

Contents

1. General Introduction

This thesis concerns plant secondary metabolites in African savanna tree species that may influence diet selection in mammalian herbivores. Specifically I investigate the appropriate use of reference material for a ubiquitously used condensed tannins method, identify and quantify low molecular weight phenolics, and evaluate whether the growth differentiation balance hypothesis (Loomis 1932, Herms & Mattson 1992) explains allocation to carbon based secondary metabolites in tree species.

Plants are preyed upon by a myriad of different herbivores, from tiny insects to large mammals (White 2005). To survive such herbivore stress, they appear to have evolved an array of chemical compounds that serve as antifeedants (Agrawal 2006, Bryant et al. 1991, Leimu & Koricheva 2006, Moore et al. 2014, Stamp 2003). The bulk of these compounds are carbon based secondary metabolites (CBSMs) that do not have any apparent role in the cell's primary functions such as growth and reproduction (Salminen & Karonen 2011). CBSMs are thought to act as protective agents against UV-B radiation, desiccation, pathogens, and as anti-feeding agents (Harborne 1991, Moore et al. 2014, Rhoades 1979, White 2005) that protect plants against herbivory.

Vegetation in African savannas supports, amongst others, an enormous diversity of insects and the largest number of extant ungulate species in the world (du Toit 2003). Together insect and large mammalian herbivores have been shown to consume ca. 5% of tree leaves in a broad-leaved savanna (i.e. infertile savanna) in the Nylsvley Nature Reserve, Limpopo, South Africa (Scholes & Walker 1993). Comparatively, indigenous large mammalian herbivores alone have been shown to consume 4.5% of available browse in fine-leaved savanna (i.e. fertile savanna) in the Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa (Owen-Smith & Danckwerts 1997). However, occasional outbreaks of lepidopteran larvae may result in as much as 80% of *Burkea africana*

leaves being consumed. Furthermore, only ca. 20% of the available browse species in the Nylsvley broad-leaved savanna was selected by kudu (*Tragelaphus strepsiceros)*, impala (*Aepyceros melampus)* and domestic goats (*Capra aegagrus hircus*) (Scholes & Walker 1993). Therefore, apart from the severe defoliation associated with lepidopteran outbreak, very little of the available browse is consumed. It is therefore reasonable to assume that these avoided plants may have chemical defenses that deterred herbivory.

Chemical defenses or CBSMs together with other plant traits, e.g. spines, therefore act as mediating agents in plant-herbivore interactions (Harborne 1991, Haukioja 2006, Rosenthal & Janzen 1979, Owen-Smith 2002), and have been shown to vary with nutrient availability (Glynn et al. 2007, Herms & Mattson 1992, Kleiner et al. 1999, Moore et al. 2014, Stamp 2003) and herbivory (Haukioja 2006, Massey 2005, Rooke and Bergström 2007, Scogings et al. 2011). Generally CBSMs have been shown to be high in slow growing and long-lived or evergreen plants from nutrient poor environments, whereas plants in high nutrient environments have low CBSMs (Coley et al. 1985, Stamp 2003). While fast growing deciduous plants from high nutrient environments store C reserves in their roots (Stamp 2003), evergreen plants store C and nutrient reserves in their leaves (Bryant et al. 1992).Browsing would therefore be detrimental for evergreen plants, and hence their leaves usually have higher CBSMs (Bryant et al. 1992, Coley & Barone 1996, Coley et al. 1985, Stamp 2003). Induced response in CBSMs following defoliation is inconsistent, but has been shown to depend on the severity of defoliation, e.g. 100% defoliation decreased total phenolics, but 50% had no effect in *Combretum apiculatum* (Rooke & Bergström 2007). Other factors such as shade (Bryant et al. 1988), as well as type (e.g. leaf picking or stripping vs. twig biting) and timing (e.g. winter vs. summer browsing) of herbivory (Bergström 1990) may also affect CBSM expression.

1.1. Secondary metabolites in African savanna woody species

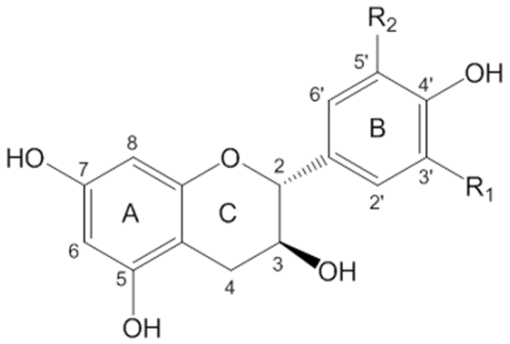
The avoided broad-leaved tree species in the Nylsvley study mentioned above generally had no thorns (Scholes & Walker 1993), but contained high levels of condensed tannins (Owen-Smith & Cooper 1987). Similarly, feeding preferences in steenbok (*Raphicerus campestris*), impala and kudu was also negatively correlated with CTs on fertile basalt plains in Kruger National Park (du Toit 2003). Recently, the degree of chemical defense in African savanna woody species has been inferred from measures of foliar condensed tannin (CT) and total phenolic (TP) concentrations (du Toit 2003, Hattas et al. 2005, Hattas et al. 2011, Hrabar et al. 2009, Rohner & Ward 1997, Rooke & Bergström 2007, Scogings & Macanda 2005, Scogings et al. 2011, 2012, 2013 & 2014). However, these measures are controversial since CT chemical heterogeneity compromises its simple quantification and total phenolics, which is a measure of general foliar phenolics and does not discriminate between tannin and non-tannin sources (Hagerman & Butler 1989), is too broad a group of compounds and may mask changes within the group. We know very little about what makes up this group of compounds.

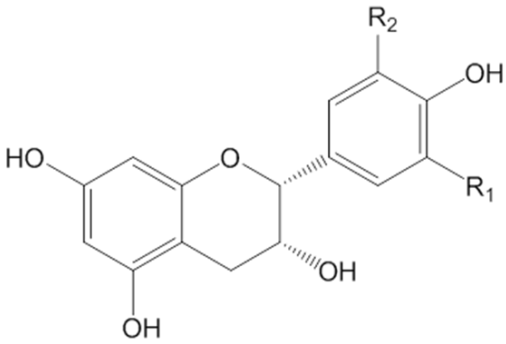
Low molecular weight phenolics form part of the total phenolic group of compounds and are involved in biological interactions (Harborne 1991, Siegler 1998). Flavonoids specifically have been shown to deter herbivores individually (Lawler et al. 2000, Moore et al. 2014, Wiggins et al. 2006), as subgroups (Marsh et al. 2007, Moore et al. 2005), or in combination with others (Stolter et al. 2005). These active agents against herbivory are often present in low concentrations (Moore et al. 2014). Thus qualifying and quantifying individual chemicals that make up this TP group, is therefore an important first step to aid our understanding of the role of CBSMs in anti-herbivore defense. Such knowledge is particularly important in African savannas where an array of

different mammalian herbivores may base their food selection on the presence/absence and quantity of these compounds.

1.2. Condensed tannin chemistry

CTs or proanthocyanidins are oligomers and polymers of flavan-3-ols (+)-catechin and (-)-epicatechin, produced in the flavonoid biosynthetic pathway (Dixon et al. 2005, Salminen & Karonen 2011, Xie & Dixon 2005). Depending on the hydroxylation in the B-ring, the monomer units may be catechin, epicatechin, gallocatechin, epigallocatechin, afzelechin or epiafzelechin (Fig. 1). These units may occur as either extender or terminal units in the CT molecule, and have been shown to determine reactivity and chromophore yield in the acid-butanol assay, e.g. cyanidins (catechin and epicatechin) and delphinidins (gallocatechin and epigallocatechin) have different extinction coefficients (Li et al. 2010). Therefore differences in CT cyanidin and/or delphinidin composition will affect chromophore yield. Chemical composition of CT molecules have been shown to be species specific (Gu et al. 2003, Scioneaux et al. 2011, Waghorn 2008), which suggest that reactivity would differ between plant species. The use of an appropriate standard should therefore be carefully considered and knowledge of the chemical composition of CT molecules is important to inform this decision.





R1=R2=H, (+)-afzelechin

R1=OH, R2=H, (+)-catechin

R1=R2=OH, (+)-gallocatechin

R1=R2=H, (-)-epiafzelechin

R1=OH, R2=H, (-)-epicatechin

R1=R2=OH, (-)-epigallocatechin

Figure 1. Structures of flavan-3-ol monomers (adapted from Xie & Dixon 2004).

1.3. Ecological significance of condensed tannins

CTs, due to their ecological significance, are the most commonly measured CBSMs (Salminen & Karonen 2011, Moore et al. 2014). Historically CTs have been viewed as harmful or toxic to mammalian herbivores, however, CTs have also been shown to be beneficial in ruminant diets. Foliage containing CTs have been shown to improve utilization of protein, accelerate live weight and wool growth rates, increase milk yield, increase fertility and improve animal wellbeing by preventing bloat and limiting worm infestations (Mueller-Harvey 2006). Nevertheless, some CTs have also been shown to negatively affect forage quality for herbivores (du Toit 2003, Mueller-Harvey 2006, Owen-Smith 2002, Scholes & Walker 1993).

CTs form insoluble tannin-protein complexes that impart an astringent taste in foliage by precipitating salivary proteins (Hagerman and Butler 1991, Hagerman et al. 1992, Scogings et al. 2004) and reduce dietary protein digestibility (Brunt et al., 2006; Dixon et al. 2005, Hagerman and Butler 1991, Hagerman et al. 1992, Mueller-Harvey

2006, Waghorn 2008). However, the effects of dietary [CT] on mammalian herbivore performance show inconsistent results. A diet containing 2.6% *Ceratinia siliqua* CTs reduced lamb growth by 63%, whereas 7.2% *Hedysarum coronarium* CTs had no effect on lamb weight gain (Mueller-Harvey 2006, Waghorn 2008). Mueller-Harvey (2006) reported that diet containing <5% CTs may be beneficial, and African indigenous ungulates selected browse containing <5%, but rejected browse containing >5% CTs (Cooper & Owen-Smith 1985). Thus a pattern seems to be emerging. However, to confirm that 5% is indeed the benefit/adverse effect threshold, we need to ensure that [CT] is reliably quantified by using an appropriate CT standard.

1.4. Condensed tannin standard

Despite empirical evidence that *Quebracho* tannin overestimates [CT] in the acid-butanol assay (Giner-Chavez et al. 1997, Salminen & Karonen 2011), it is still used as reference material (e.g. MacKown et al. 2008, Mattson et al. 2005, Shrader et al. 2012, Ward & Young 2002). However, *Sorghum* tannin, the proposed alternative, may also be inappropriate as standard. Nevertheless, knowledge of the amount of foliar CTs is essential for reliable assessments of nutritive value and to explain mammalian herbivore diet selection. To reliably quantify [CT] it is necessary to use CT from the plant under investigation, however, the extraction and purification process is laborious (Hagerman & Butler 1989, Rautio et al. 2007). Consequently researchers use generic standards in the acid-butanol assay. However, chemical composition of the generic CT standard may be different from the plant under investigation.

CT chemical composition, which may affect its reactivity in the acid-butanol assay for CTs, have been shown to differ between plants (Gu et al. 2003, Scioneaux et al. 2011, Waghorn 2008). Considering the wide variety of naturally occurring CTs, the use

of generic CT standards such as the commercially available *Quebracho*, and *Sorghum* tannin may produce erroneous results since its chemical composition and thus its reactivity, may not match that of the species under investigation. As a result there is no universal CT standard (Hagerman 1989, Salminen & Karonen 2011) and the aforementioned generic standards may not be ideal. These generic standards are still being used and an investigation into their appropriate use as CT standard in African savanna species is required.

1.5. Phenotypic expression of carbon based secondary compounds

Phenotypic expression of CBSMs along a resource gradient has been explained in terms of the growth differentiation balance hypothesis (GDBH, Loomis 1932, Lorio 1986, Herms & Mattson 1992). The GDBH provides a framework which predicts a trade-off between costs of differentiation processes in the plant relative to the demand for photosynthate by growth, where differentiation constitutes all processes not involved in growth, including formation of CBSMs (Fig. 2, Herms & Mattson 1992, Stamp 2003). See chapter 3 for a detailed explanation of the hypothesis.

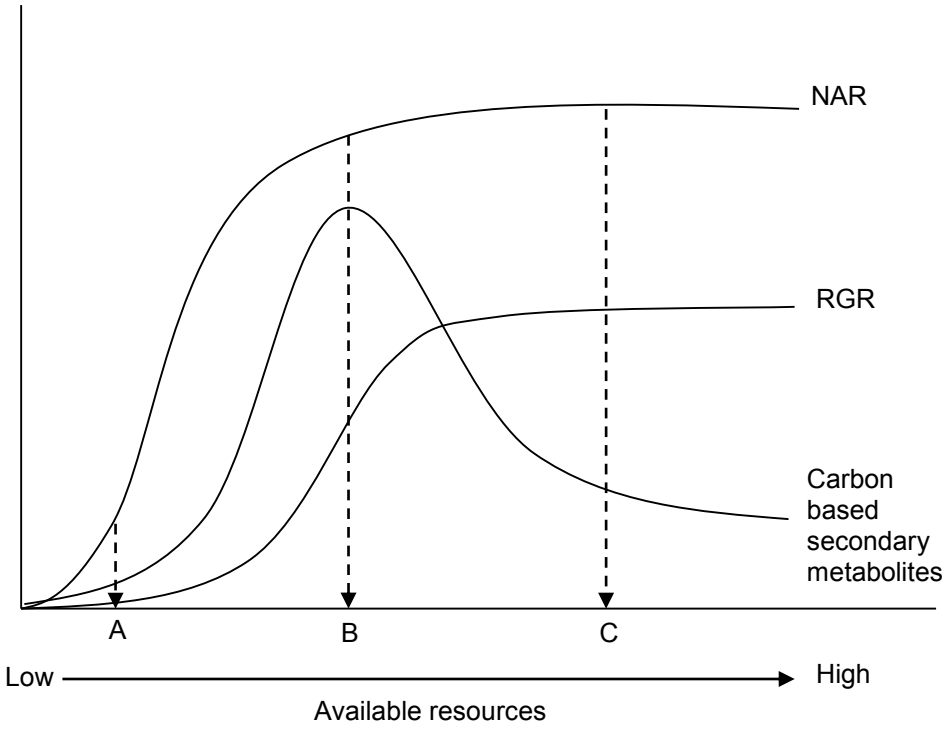


Figure 2. Relationship between net assimilation rate (NAR), relative growth rate (RGR) and carbon based secondary metabolite production. Letters A, B and C represent low, intermediate and high resource levels, respectively (adapted from Herms & Mattson 1992, Stamp 2003 and Glynn et al. 2007).

The cornerstone prediction of the GDBH is the curvilinear response pattern of CBSMs and is only detectable when using a resource gradient with minimum of five levels (Stamp 2004). The GDBH was developed from empirical evidence from northern boreal and temperate forests and has been supported in *Salix sericea* and *S. eriocephala* (Glynn et al. 2007). However, African savanna woody species may be chemically different from boreal and temperate woody species since it has presumably coevolved with a diversity of different browsing mammalian herbivores for almost 1 Myr BP (Cerling et al. 1999). Over time, this chemical “arms race” is expected to result in an

adaptation-counter adaptation reciprocity (Spencer 1988) which may ultimately result in divergent lineages in both woody species and its associated mammalian herbivores.

The GDBH predicts that herbivory would induce CBSMs in damaged plants (Herm & Mattson 1992). However, African savannas have diverse herbivores that impart differential damage with varying phytochemical consequences for plants. For example, defoliation by mopane caterpillar (*Imbrasia belina*) have been shown to decrease total polyphenol concentration in *Colophospermum mopane* (Hrabar et al. 2009). Bryant et al. (1991) showed that defoliation by the saturniid moth (*Cirina forda*) decreased total phenol and condensed tannin concentrations in fast growing deciduous species (*Grewia flavescens*, *Acacia tortilis* and *Dichrostachys cinerea*), but increased total phenol and condensed tannin concentrations in slow growing deciduous species (*Burkea africana*, *Ochna pulchra* and *Euclea natalensis*). Furthermore, severe pruning by African elephant (*Loxodonta africana*) had no effect on total polyphenol concentration in *C. mopane* (Hrabar et al. 2009), but increased foliage quality, as indicated by decreased tannin:protein ratio (Hrabar & du Toit 2014). Therefore, whether the GDBH explains allocation to CBSMs in African savanna woody species is questionable.

1.6. Research questions

To test whether *Quebracho* and *Sorghum* tannin are appropriate standards for CT quantification in the acid-butanol assay, I extracted and purified CTs from five woody species and address the following questions: (1) To what extent does *Quebracho* tannin overestimate [CT] in selected woody species? (2) Is *Sorghum* tannin an appropriate CT standard for these species? and (3) To what extent do CT chemical characteristics determine its reactivity in the acid-butanol assay? (Chapter 1)

Low molecular weight phenolics (lmwp's) were identified and quantified in six dominant woody species that differ in life history, morphological and functional traits, and grow in different habitats from an experimental site in Kruger National Park, South Africa. Specifically, I address the following questions: 1) Which chemical components contribute most to differences among species? 2) Are similarities in lmwp composition among species associated with phylogenetic relatedness? 3) Are spinescent plants less chemically defended than spineless plants? and 4) Is the evergreen *Euclea divinorum* chemically more defended relative to deciduous species? (Chapter 2)

To investigate whether the GDBH explains allocation to CBSMs in an African savanna woody species, I tested the hypothesis in the deciduous woody species *Combretum apiculatum*. Here I address the following questions: (1) Does the GDBH explain carbon allocation to CBSMs along a 6-level N gradient? and (2) Does the GDBH explain carbon allocation to CBSMs following 100% simulated herbivory along a 6-level N gradient? (Chapter 3)

This chapter was published in Phytochemistry Letters 5: 329-334, 2012

2. The quantification of condensed tannins in African savanna tree species

Dawood Hattas[a,b,c*], Riitta Julkunen-Tiitto[c]

[a*] Permanent address: Department of Biological Sciences, H.W. Pearson Building, University Avenue, University of Cape Town, Rondebosch, 7701, Cape Town, South Africa, Dawood.Hattas@uct.ac.za, Tel: +27216502443, Fax: +27216504041

[b]ARC-Range and Forage Institute, Department of Botany, University of the Western Cape, Private Bag X17, Bellville, 7535, Cape Town, South Africa.

[c]Natural Product Research Laboratory, Department of Biology, University of Eastern Finland, Joensuu, Finland, RJT@uef.fi (Riitta Julkunen-Tiitto)

2.1. Abstract

We compared *Quebracho* with *Sorghum* tannin as standards for condensed tannin (CT) quantification in selected African savanna tree species in relation to the acid-butanol assay for CTs. Without exception, the use of *Quebracho* tannin as standard overestimated CTs, ranging from 0.7 to as much as 8.3 times. *Sorghum* tannin underestimated CTs by 0.26-0.79 times, except in one species where there was no difference in the CT concentration. Condensed tannins in African savanna trees showed qualitative and quantitative differences in chemical composition which may explain the variable reactivity in the acid-butanol assay. We propose the use of condensed tannins

purified from the plant under investigation be used as standard since it will closely represent the CT structure and presumably chemical reactivity in the acid-butanol assay.

Key Words: *Acacia karroo*, *Acacia grandicornuta*, *Euclea divinorum*, proanthocyanidin, cyanidin, delphinidin

2.2. Introduction

African savannas are inhabited by an array of different mammalian herbivores and their choice of specific plants as food have been explained in terms of plant condensed tannin (CT) concentration amongst other variables. Thus quantitative assessments of CTs have been used to determine forage quality (du Toit, 2003; Fornara and du Toit, 2008; Hattas et al., 2005; Hattas et al., 2011; Hrabar et al., 2009; Scogings et al., 2011; Woolnough and du Toit, 2001) and to explain mammalian diet selection (e.g. Shrader 2012; Ward and Young, 2002; Woolnough and du Toit, 2001) in African savannas. In a recent review, Mueller-Harvey (2006) presented data on herbivore performance in relation to CT concentration, some of which reported a beneficial effect of CT concentrations of <5%. In contrast other studies showed that concentrations <3% had no or negative effects on mammalian herbivores performance. We suggest the key to these conflicting results may lie in the standard used to quantify CTs.

The acid-butanol assay (Porter et al., 1986), despite the problems associated with it (see Schofield et al., 2001), is one of the most commonly used CT quantification methods (Mueller-Harvey, 2006; Schofield et al., 2001). The assay is easily performed and highly reproducible, however, the natural chemical diversity of condensed tannins has resulted in there being no single universal CT reference standard (Hagerman et al., 1992; Li et al., 2010; Schofield et al., 2001). Researchers have thus been using different

means of expressing CT concentrations. Following a proposal by Hagerman et al. (1992), many studies have used the commercially available *Quebracho* tannin (QT) as reference material (e.g. Mattson et al., 2005; Ward and Young, 2002; MacKown et al., 2008; Shrader 2012). However, QT which is a 5-deoxy proanthocyanidin is resistant to oxidative cleavage and as a result produces less color per unit mass in the acid-butanol assay relative to other standards (Li et al., 2011; Mueller-Harvey, 2006; Rautio et al., 2007; Schofield et al., 2001). More recently the moderately sized *Sorghum* tannin (ST) has been deemed an appropriate CT standard (e.g. Hattas et al., 2011; Hattas et al., 2005; Hrabar et al., 2009; O'Reilly-Wapstra et al., 2005; Scogings et al., 2011; Woolnough and du Toit, 2001). Expressing tannin concentration as absolute is only possible by purifying CTs from the plant being studied (Hagerman and Butler, 1991), but the extraction and purification is laborious (Hagerman and Butler, 1989; Rautio et al., 2007). Furthermore, this is not always possible, especially in material from controlled environment experiments where plant matter is limited (e.g. small scale elevated atmospheric CO_2 experiments). However, a procyanidin dimer and trimer purified from fresh Granny Smith apples (*Malus domestica*) was recently proposed as universal standard (Li et al., 2011). This proposal considers the ubiquitous availability of Granny Smith apples and the ease of confirming the chemical characteristics of its procyanidins dimer and trimer.

Condensed tannins have been shown to be qualitatively different (Gu et al., 2003; Kennedy et al., 2000; Scioneaux et al., 2011) and these differences in chemical structure and Mr have been shown to be related to reactivity and consequently color yield in the acid-butanol assay (e.g. Li et al., 2010). For example, the molar extinction coefficients for cyanidins (catechin and epicatechin) and delphinidins (gallocatechin and epigallocatechin) have been shown to be different in the acid-butanol assay (24,500 and

21,200 M^{-1} cm^{-1} respectively, Li et al., 2010). It is therefore important that the standard used is chemically similar to the CT in the plant source under investigation. This is important in African savanna ecosystems where management decisions towards sustainable carrying capacities may be informed by potentially erroneous forage quality estimates, due to use of inappropriate reference materials.

In this paper we address the following questions: (1) To what extent does *Quebracho* tannin overestimate CT concentration in *Acacia karroo* Hayne (AK), *Acacia grandicornuta* Gerstner (AG, both Fabaceae), *Combretum apiculatum* Sond

. (CA, Combretaceae), *Euclea divinorum* Hiern (ED, Ebenaceae) and *Scutia myrtina* (Burm.f.) Kurz. (SM, Rhamnaceae); (2) Is *Sorghum* tannin an appropriate CT standard for these species; and (3) To what extent does CT chemical characteristics determine its reactivity in the acid-butanol assay? We hypothesize that: (1) Due to its inferior reactivity in the acid-butanol assay, *Quebracho* will overestimate CT concentration; (2) In the absence of an absolute standard (i.e. tannin extracted from the plant under investigation) the moderately sized *Sorghum* tannin is an appropriate CT standard for these species; and (3) That CT chemical characteristics will determine reactivity and thus color yield in the acid-butanol assay.

Study species were selected as they are important forage for a wide range of wild mammalian herbivores in African savannas (e.g. impala (*Aepyceros melampus*), giraffe (*Giraffa camelopardalis*) and kudu (*Tragelaphus strepsiceros*)), whereas ED is mostly unpalatable (Fornara and du Toit, 2008). This study presents, for the first time, qualitative and quantitative chemical data for purified CTs in these species. Furthermore, absolute CT concentrations have not previously in presented in these species.

2.3. Results and discussion

Quebracho overestimated CTs compared to ST. This is indicated by different regression line slopes between different tannin species (Fig. 1). Regression line slopes amongst different tannin species were significantly different (Fig. 1, ANCOVA, $F_{6,91}$ = 231.8, P<0.001). When comparing slopes of QT and ST with other tannin sources, QT was as expected significantly different from all other CTs (Fig. 1, P<0.05). The slope in ST did not differ from AK, CA, ED or SM (P>0.05), but differed significantly from AG tannin (P<0.001).

Condensed tannin concentration differed relative to standard used. When comparing CT concentration expressed as absolute, *Sorghum* tannin equivalents or *Quebracho* tannin equivalents, CT concentration was significantly different within all species (Fig. 2, AG, $F_{2,6}$=235.3, P<0.001; AK, $F_{2,6}$=5567.7, P<0.001; CA, $F_{2,6}$=2545.6, P<0.001; ED, $F_{2,6}$=3765.6, P<0.001; and SM, $F_{2,6}$=2357.9, P<0.001). The between standard comparison for each species revealed that QT significantly overestimated CT concentration in all cases, by 70, 575, 218, 422 and 825% in AG, AK, CA, ED and SM, respectively (Fig. 2, P<0.001 in all cases except in AG, P<0.01). Conversely, compared to absolute, ST significantly underestimated CT concentration in AG, AK, CA and ED by 79, 26, 73 and 47%, respectively (Fig. 2, P<0.001 in all cases), whereas CT concentration in SM was not different when expressed as absolute or as STE (P>0.05). These results show that due to its inferior reactivity in the acid-butanol assay, QT is grossly inappropriate as a condensed tannin standard for the species studied. The range of QT overestimation (0.7 to as much as 8.3 times) is in agreement with Giner-Chavez et al. (1997) who reported overestimation of 2.2 to 9.5 times when using QT as an external standard for *Desmodium ovalifolium*, *Manihot esculenta* and *Gliricidia*

sepium. Our results show that the use of *Sorghum* tannin as CT standard was appropriate only in SM.

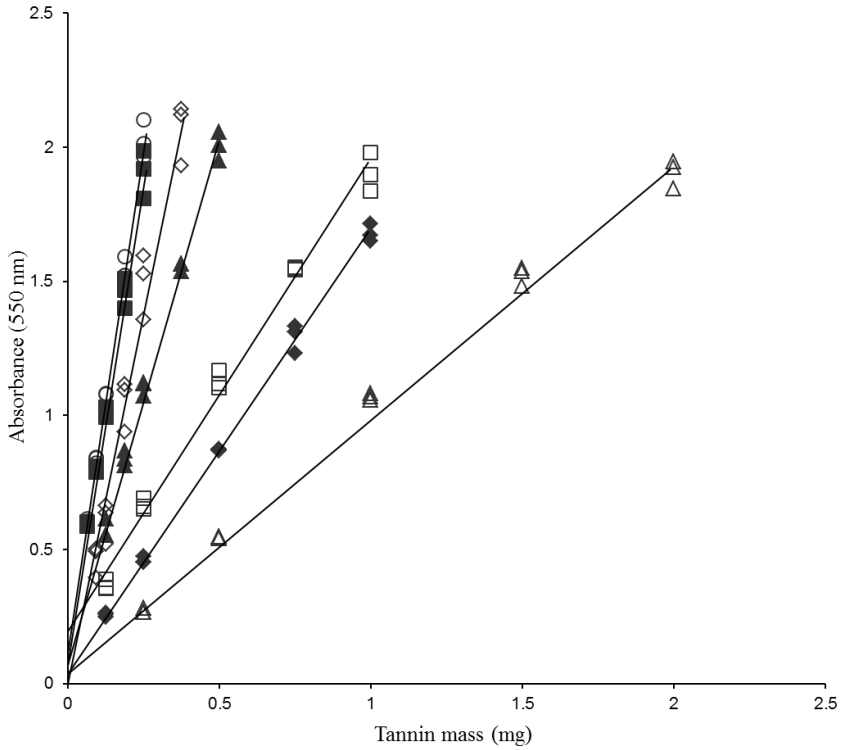


Figure 1. Regression lines for purified *Sorghum* (■, $y = 7.017\ x + 0.146$, $R^2 = 0.996$); *S. myrtina* (○, $y = 7.598\ x + 0.123$, $R^2 = 0.996$); *A. karroo*, (◇, $y = 5.854\ x + 0.072$, $R^2 = 0.972$); *E. divinorum* (▲, $y = 3.795\ x + 0.125$, $R^2 = 0.996$); *C. apiculatum* (□, $y = 1.774\ x + 0.208$, $R^2 = 0.991$); *A. grandicornuta* (◆, $y = 1.634\ x + 0.054$, $R^2 = 0.998$) and *Quebracho* (△, $y = 0.940\ x + 0.076$, $R^2 = 0.994$) tannin in the acid butanol assay. Standard curves were constructed following the acid butanol assay for proanthocyanidins. All data points are shown ($n = 3$).

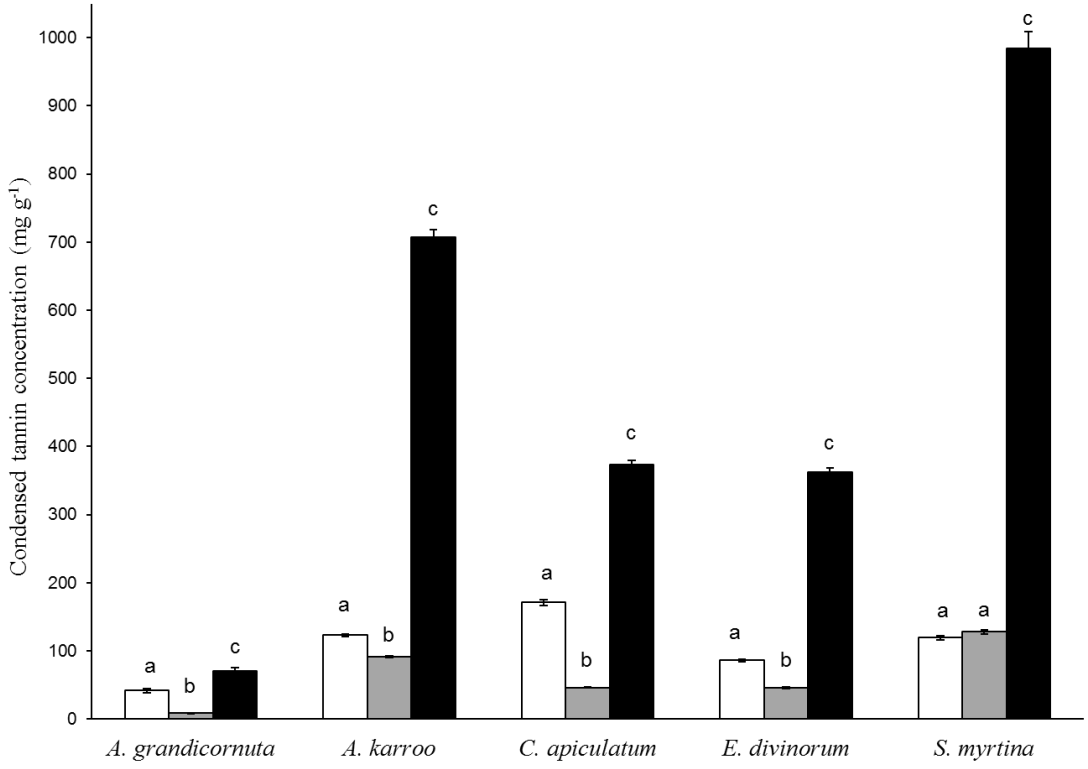


Figure 2. Condensed tannin concentrations in *A. karroo*, *A. grandicornuta*, *C. apiculatum*, *E. divinorum* and *S. myrtina* expressed as absolute (Abs, open bar), *Sorghum* tannin equivalents (STE, grey bar) and *Quebracho* tannin equivalents (QTE, black bar). Values are means ± s.e. Different letters indicate significant differences within species for different standards used following Tukey's multiple range test (P<0.05).

Molecular weight of different tannin species ranged widely. Similar Mr in ST and SM appear to explain the success of ST in predicting absolute CT concentration in SM (Table 3, Fig. 2). However, Spearman's correlation coefficient showed no relationship between Mr and slopes of all the tannin sources (P>0.05) indicating that Mr may not explain reactivity in the acid-butanol assay. The success of ST in predicting CT concentration in SM may be related to the chemical characteristics of the CT polymers. Condensed tannin polymers in ST and SM are cyanidin based (i.e. catechin and

epicatechin units), with ST being entirely procyanidin and SM having small numbers of pelargonidin (i.e. epiafzelechin units) and dephinidin units (i.e. gallocatechin and epigallocatechin units, Table 2). Indeed, CA and AG tannin which had almost identical cyanidin:delphinidin ratios (Table 3) and the same slope (P<0.05), but different Mr, showed a similar result.

Table 1. Compounds identified after thiolysis in *A. grandicornuta*, *A. karroo*, *C. apiculatum*, *E. divinorum*, *S. myrtina* and *Sorghum* tannin.

Peak no.	Rt (min)	Chemical compound	Source of confirmation
1	4.34	gallocatechin	UV, commercial standard
2	9.53	(+)-catechin	UV, commercial standard
3	11.99	(-)-epicatechin	UV, commercial standard
4	13.75	epigallocatechin 3-gallate	UV, commercial standard
5	23.53	gallocatechin benzylthioether	429 (M+1), 451 (M+23)
6	27.26	epigallocatechin benzylthioether	413 (M+1), 435 (M+23), 847 (2M+1)
7	28.51	catechin benzylthioether	413 (M+1), 435 (M+23), 847 (2M+1)
8	31.16	epigallocatechingallate benzylthioether	581 (M+1), 603 (M+23)
9	33.15	epicatechin benzylthioether	413 (M+1), 435 (M+23), 847 (2M+1)
10	36.63	epicatechin-3-*O*-gallate benzylthioether	565 (M+1), 587(M+23)
11	36.70	epiafzelechin benzylthioether	397 (M+1), 419 (M+23), 815 (2M+1)

Table 2. Extender and terminal units after thiolysis in *A. grandicornuta* (AG), *A. karroo* (AK), *C. apiculatum* (CA), *E. divinorum* (ED), *S. myrtina* (SM) and *Sorghum* tannin (ST).

Tannin	Extender units (%)							Terminal units (%)			
	GCat thiol	eGCat thiol	Cat thiol	eGCatG thiol	eCat thiol	eCatG thiol	eAfz thiol	GCat	Cat	eCat	eGCat
AG	0,7	0,9	1,9	5,8	10,5	80,3	nd	31,8	37,3	7,4	23,5
AK	6,9	60,4	0,3	28,2	3,3	0,9	nd	100,0	nd	nd	nd
CA	nd	0,4	1,8	1,2	11,4	85,3	nd	nd	70,0	16,1	13,9
ED	nd	14,1	nd	84,5	0,5	0,9	nd	0,3	nd	nd	99,7
SM	0,9	5,1	11,8	nd	81,8	nd	7,8	3,1	26,4	70,4	nd
ST	nd	nd	2,8	nd	97,2	nd	nd	nd	96,9	3,1	nd

Abbreviations: GCat, gallocatechin; eGCat, epigallocatechin; Cat , catechin; eGCatG, epigallocatechingallate; eCat, epicatechin; eCatG, epicatechin-3-*O*-gallate; eAfz, epiafzelechin; nd, not detected

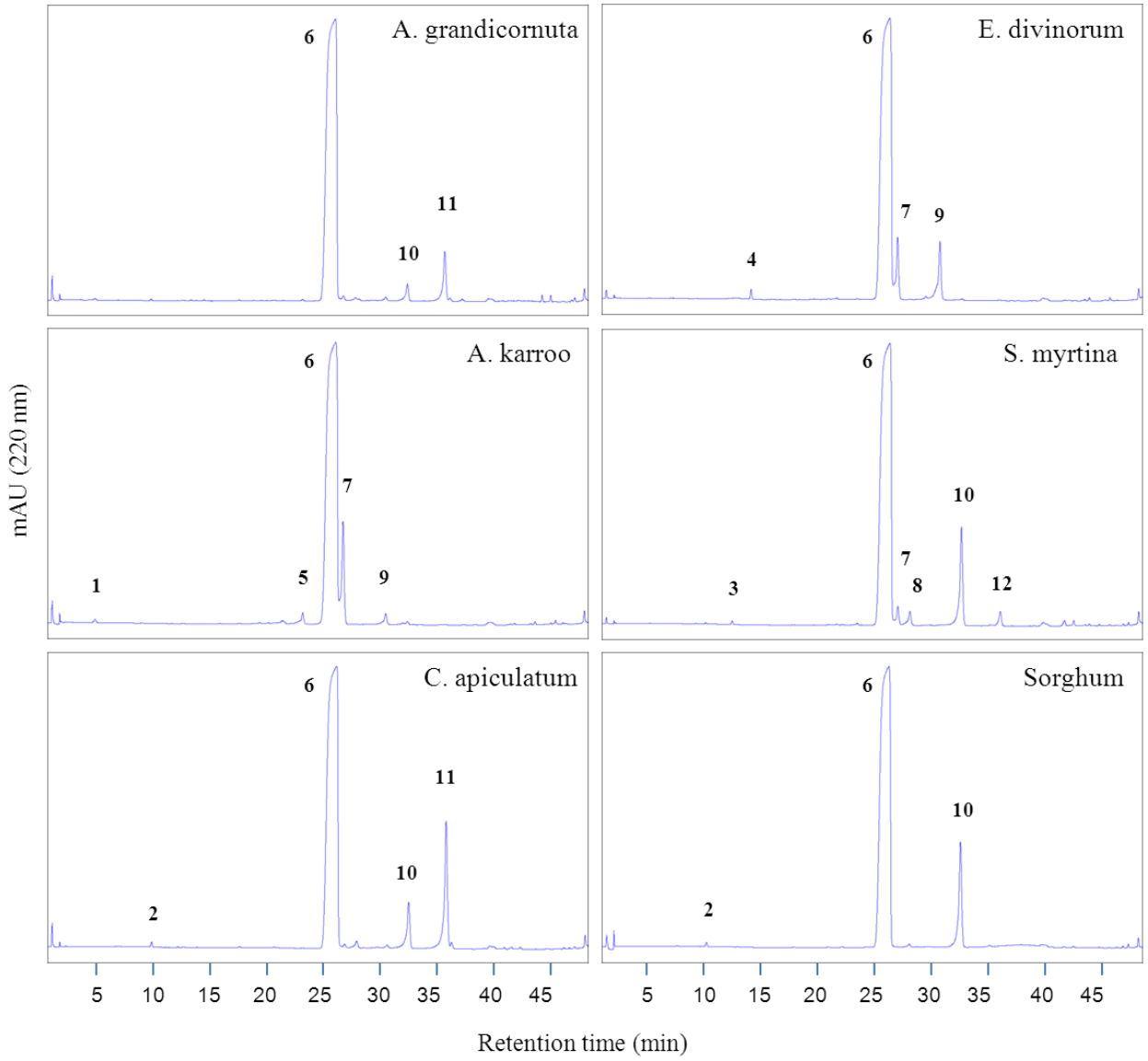


Figure 3. HPLC UV chromatogram showing thiolysis products in *A. karroo*, *A. grandicornuta*, *C. apiculatum* and *E. divinorum, S. myrtina*, and *Sorghum* tannin. Peak identity, R_t-value, UV and MS data are listed in Table 1.

Table 3. Chemical characteristics of *A. grandicornuta* (AG), *A. karroo* (AK), *C. apiculatum* (CA), *E. divinorum* (ED), *S. myrtina* (SM) and *Sorghum* tannin (ST).

Tannin	mDP	MW	PC:PD	C:D
AG	50.4	15,200	12:88	45:55
AK	45.8	13,854	4:96	100:0
CA	79.1	23,857	13:87	86:14
ED	10.7	3,242	1:99	0:100
SM	28.0	8,144	93:6	97:3
ST	28.7	8,310	100:0	100:0

Abbreviations: mDP, mean degree of polymerization; PC:PD, procyanidin:prodelphinidin; C:D, cyanidin:delphinidin ratio

HPLC and HPLC MS analysis of thiolysis products showed 11 degradation products (Fig. 3, Table 1 and 2). Thiolysis revealed qualitative and quantitative differences in the chemical composition of CT sources (Table 2). Extender units in the tannin of related *Acacia* species were qualitatively similar, but quantitatively different. Extender units in AG, AK, CA and ED tannin were predominantly prodelphinidins, whereas SM tannin were procyanidin based and ST was exclusively procyanidin (Table 2 and 3). This finding for ST is consistent with that reported by Gu et al. (2003) who showed that *Sorghum* only had epicatechin extender units. Terminal units in AK and ST were cyanidins, whereas that of ED tannin was entirely delphinidin. CA and SM tannin showed predominantly cyanidin terminal units, whereas AG tannin showed more delphinidin than cyanidin terminal units (Table 2 and 3).

To conclude, our results show the possible differences that may occur when different external standards are used. In particular, our results suggest that the continued use of QT as standard in the acid-butanol assay is unjustified. Using the recently proposed procyanidin dimer and trimer from apples as standard (Li et al., 2011) may allow inter laboratory comparison in a wide variety of plants, but it is not appropriate for all species due to its potentially different tannin character. We

therefore propose the use of absolute standards (Hagerman and Butler, 1991; Schofield et al., 2001) since this will closely represent the CT structure (Giner-Chavez et al., 1997) and presumably chemical reactivity of the studied species in the acid-butanol assay (Hagerman and Butler, 1989; Mueller-Harvey, 2006).

2.4. Experimental

2.4.1. General experimental procedures

Analytical reagent grade methanol, ascorbic acid, acetone, ethyl acetate, n-butanol, hydrochloric acid, ferric ammonium sulphate dodecahydrate and benzyl mercaptan were purchased from Merck (Darmstadt, Germany). Sephadex LH-20, (+)-catechin and (-)-epicatechin was purchased from Sigma-Aldrich (St Louis, MO, USA), (-)-gallocatechin and (-)-epigallocatechin 3-gallate from PhytoLab (Nürnberg, Germany), tetrahydrogenfuran from VWR International (Radnor, PA, USA), phosphoric acid and HPLC grade methanol form Lab-Scan Analytical Sciences (Gliwice, Poland). HPLC analyses were performed using an Agilent 1100 Series HPLC DAD system. Chemical identities were confirmed with HPLC MS (positive ion API-ES, Agilent Technologies, CA, USA) using a Hypersil Rp C-18, 2 mm ID, 10 cm long column (Thermo Fisher Scientific, MA, USA) at an ES fragmentor voltage of 80 V.

2.4.2. Plant material - sources and treatment

A. karroo and *S. myrtina* leaves were collected from mature trees from Kirstenbosch Botanical Gardens, Cape Town, South Africa (33°59'05.44"S, 18°25'50.81"E). *Sorghum bicolor* (L.) Moench seed (SA 423) was supplied by the Agricultural Research Council (ARC-Small Grain Institute, South Africa). Crude *Quebracho* tannin was obtained from Prof. Ann E Hagerman (Miami University,

USA). *A. grandicornuta*, *C. apiculatum* and *E. divinorum* were collected around Skukuza, Kruger National Park, South Africa (24°59'46.48"S, 31°35'30.81"E), a sub-tropical savanna habitat. Mature leaf material was air-dried at room temperature not exceeding 25 °C and away from direct sunlight. Sub-samples of AK, AG, CA, ED and SM were ground to pass through a 0.5 mm screen for use in CT quantification in leaf material. *Sorghum* seed were ground in a Waring blender and crude tannin was extracted from 200 g of each plant source.

2.4.3. Analytical procedures

2.4.3.1. Extraction and purification of condensed tannins

Crude QT was purified in accordance with Asquith and Butler (1985) as modified by Hagerman (2002). The extraction and purification method for *Sorghum* tannin (Hagerman and Butler, 1980) as modified by Hagerman (2002) was used to extract and purify CTs from *Sorghum* seed, and the leaves of the five savanna tree species. This extraction was performed in aqueous methanol, using Sephadex LH-20 to adsorb the tannin, a method that has previously been used to extract and purify hydrolysable tannins (see Nonaka, 1989). Therefore, to determine whether the purified CTs contained hydrolysable tannins, we quantified hydrolysable tannins in the different tannins after dissolving it in methanol using an HPLC as described by Julkunen-Tiitto and Sorsa (2001), standardizing against pentagalloylglucose (supplied by Ann E Hagerman).

2.4.3.2. Thiolysis

Condensed tannin polymers were degraded with thiolysis reagent benzyl mercaptan as described in Scioneaux et al. (2011) with some modifications. About 1 mg of each crude extract was dissolved in 1 ml of methanol. Twenty microliters of

acidic methanol (3.2 M HCl) was added to 200 µl of the tannin solution followed by 48 µl of 5% benzyl mercaptan in methanol (v/v). The sample was mixed and incubated at 40 °C for 30 min, after which samples were immediately transferred into a freezer at -18 °C for 5 min. Samples were then filtered through a 0.45 µm cellulose filter (Agilent Technologies, CA, USA) and thiolysis products were separated by HPLC as outlined in Julkunen-Tiitto and Sorsa (2001) using a 20 µl injection volume. The UV-response for catechin, epicatechin, gallocatechin as well as epigallocatechin and their benzylthioether adducts has been shown to be equivalent at 220 nm (Scioneaux et al., 2011). Thus the average degree of polymerization (mDP) was calculated using peak areas from the area ratio of the sum of all the flavan-3-ol units (benzylthioether adducts and terminal units) to the terminal flavan-3-ol units at 220 nm. The benzyl mercaptan reagent peak was identified from its Rt and UV spectrum. Identities of unreacted terminal units were confirmed from Rt and UV spectrums of pure catechin, epicatechin, gallocatechin and epigallocatechin 3-gallate. Epicatechin benzylthioether was identified from the major benzylthioether adduct following thiolysis of *Sorghum* tannin (Gu et al., 2003; Li et al., 2010; Scioneaux et al., 2011), whereas catechin benzylthioether, which eluted earlier was identified using Rt. The identities of both were confirmed by HPLC MS as described in Julkunen-Tiitto and Sorsa (2001). Gallocatechin benzylthioether and its epimer were identified by Rt and HPLC MS, with the epimer eluting later. Thiolysis degradation was performed in duplicate. Cyanidins, delphinidins and their thiol adducts were used to calculate the differential cyanidin:delphinidin as well as procyanidin:prodelphinidin ratios in the different tannins. Chemical composition as revealed by thiolysis was used to calculate Mr of the different tannin species.

2.4.3.3. Condensed tannin assay

Extractable condensed tannins in leaf material were quantified in triplicate for all species using the acid-butanol assay for proanthocyanidins (Porter et al., 1986) as modified by Hagerman (2002). This method was used to construct standard curves for the purified tannins of each tannin source (from three replicate absorbance values at each concentration). The concentration range for AK was 0.1-0.38 mg ml^{-1}, for AG and CA 0.125-1.0 mg ml^{-1}, for ED 0.125-0.5 mg ml^{-1}, for SM and ST 0.06-0.25 mg ml^{-1} and for QT 0.25-2.0 mg ml^{-1} of 70% acetone. Condensed tannin concentrations in all tree species were expressed as absolute (Abs), *Sorghum* tannin equivalents (STE) and *Quebracho* tannin equivalents (QTE). Standard curves were corrected for the presence of hydrolysable tannins where detected.

2.4.3.4. Statistical analysis

Slopes of different tannin standard curves were compared using a One-way analysis of covariance (ANCOVA) for independent samples. Standard curve data as well as condensed tannin concentrations were log transformed (log (1+x) in the case of standard curve data) to reduce the inequalities of variance. Differences within species, when using different standards, were analyzed using a one-way ANOVA. Significant differences were separated with Tukey's multiple range test. Spearman's product-moment correlation coefficient was determined to evaluate the relationship between Mr and reactivity of the CT sources in the acid-butanol assay, comparing Mr with slope for the different tannins. Statistical analysis were performed with Statistica v10 (2011, StatSoft, Inc. USA).

Acknowledgements

Funding was provided by the National Research Foundation, South Africa (GUIN 46939) and the Dorothy Cameron Bequest (Department of Botany, University of Cape Town, South Africa). We acknowledge Aboebaka Hattas, Thandeka Mamashela and Patricia Shabangu for help with the collection of leaf material. Comments from Jeremy Midgley and three anonymous reviewers improved the manuscript.

2.5. References

Asquith, T.N., Butler, L.G., 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannins. J Chem Ecol 11, 1535-1544.

du Toit, J.T., 2003. Large herbivores and savanna heterogeneity, in: du Toit, J., Rogers, K., Biggs, H. (Eds.). The Kruger experience. Ecology and management of savanna heterogeneity. Island Press, Washington, pp. 292-309.

Fornara, D.A., du Toit, J.T., 2008. Browsing-induced effects on leaf litter quality and decomposition in a southern African savanna. Ecosystems 11, 238-249

Giner-Chavez, B.I., Van Soest, P.J., Robertson, J.B., Lascano, C., Reed, J.D., Pell, A.N., 1997. A method for isolating condensed tannins from crude plant extracts with trivalent ytterbium. J Sci Food Agric 74, 359-368.

Gu, L., Kelm, M.A., Hammerstone, J.F., Beecher, G., Holden, J., Haytowitz, D., Prior, R.L., 2003. Screening of food containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. J Agric Food Chem 51, 7513-7521.

Hagerman, A.E., 2002. Tannin Handbook
URL [http://www.users.muohio.edu/hagermae/tannin.pdf]; downloaded March 2004.

Hagerman, A.E., Butler, L.G., 1980. Condensed tannin purification and characterization of tannin-associated proteins. J Agric Food Chem 28, 947-952.

Hagerman, A.E., Butler, L.G., 1989. Choosing appropriate methods and standards for assaying tannin. J Chem Ecol 15(1), 1795-1810.

Hagerman, A.E., Butler, L.G., 1991. Tannins and Lignins. In Herbivores: Their Interactions with Secondary Plant Metabolites (2nd edition) Vol 1. Rosenthal GA, Berenbaum MR (Eds.) Academic Press, New York, pp. 355-388.

Hagerman, A.E., Robbins, C.T., Weerasuriya, Y., Wilson, T.C., McArthur C., 1992. Tannin chemistry in relation to digestion. J Range Manage 45, 57-62.

Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F., Rooke, T., 2011. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. Phytochemistry 72, 1796-1803.

Hattas, D., Stock, W.D., Mabusela, W.T., Green, I.R., 2005. Phytochemical changes in leaves of subtropical grasses and fynbos shrubs at elevated atmospheric CO_2 concentrations. Glob Plan Change 47, 181-192.

Hrabar, H., Hattas, D., du Toit, J.T., 2009. Differential effects of defoliation by mopane caterpillars and pruning by African elephants on the regrowth of *Colophospermum mopane* foliage. J Trop Ecol 25, 301-309.

Julkunen-Tiitto, R., Sorsa. S., 2001. Testing the drying methods for willow flavonoids, tannins and salicylates. J Chem Ecol 27(4), 779-789.

Kennedy, J.A., Matthews, M.A., Waterhouse, A.L., 2000. Changes in grape seed polyphenols during fruit ripening. Phytochemistry 55, 77-85.

Li, C., Trombley, J.B., Schmidt, M.A., Hagerman, A.E., 2010. Preparation of an acid-butanol standard from fresh apples. J Chem Ecol 36, 453-460.

MacKown, C.T., Carver, B.F., Edwards, J.T., 2008. Occurrence of condensed tannins in wheat and feasibility for reducing pasture bloat. Crop Sci 48, 2470-2480.

Mattson, W.J., Julkunen-Tiitto, R., Herms, D.A., 2005. CO_2 enrichment and carbon partitioning to phenolics: do plant response accord better with the protein competition or growth differential balance models? Oikos 111, 337-347.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J Sci Food Agric 86, 2010-2037.

Nonaka, G., 1989. Isolation and structure elucidation of tannins. Pure Appl Chem 61(3), 357-360.

O'Reilly-Wapstra, J.M., Potts, B.M., McArthur, C., Davies, N.W., 2005. Effects of nutrient availability on the genetic-based resistance of *Eucalyptus globulus* to a mammalian herbivore and on plant defensive chemistry. Oecol 142, 597-605.

Porter, L.J., Hrstich, L.N., Chan, B.C., 1986. The conversion of procyanidins and prodelphinins to cyanidin and delphinin. Phytochemistry 25, 223-230.

Rautio, P., Bergvall U.A., Karonen M., Salminen J.P., 2007. Bitter problems in ecological feeding experiments: Commercial tannin preparations and common methods for tannin quantifications. Biochem Syst Ecol 35, 257-262.

Schofield, P., Mbugua, D.M., Pell, A.N., 2001. Analysis of condensed tannins: a review. Anim Feed Sci Tech 91, 21-40.

Scioneaux, A.N., Schmidt, M.A., Moore, M.A., Lindroth, R.L., Wooley, S.C., Hagerman, A.E., 2011. Qualitative variation in proanthocyanidin composition of *Populus* species and hybrids: genetics is the key. J Chem Ecol 37, 57-70.

Scogings, P.F., Hjältén, J., Skarpe, C., 2011. Secondary metabolites and nutrients of woody plants in relation to browsing intensity in African savannas. Oecol 167, 1063-1073.

Shrader, A.M., Bell, C., Bertolli, L., Ward, D., in press. Forest or the trees: At what scale do elephants make foraging decisions? Acta Oecol (2011), doi:10.1016/j.actao.2011.09.009

Ward, D., Young, T. P., 2002. Effects of large mammalian herbivores and ant symbionts on condensed tannins of *Acacia drepanolobium* in Kenya. J Chem Ecol 28(5), 291-237.

Woolnough, A.P., du Toit, J.T., 2001. Vertical zonation of browse quality in tree canopies exposed to a size-structured guild of African browsing ungulates. Oecol 129, 585-590.

This chapter was published in Phytochemistry 72: 1796-1803, 2011

3. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense

Hattas, Dawood[a*], Joakim Hjältén[b], Riitta Julkunen-Tiitto[c], Peter F Scogings[d]; and Tuulikki Rooke[e]

[a*]Department of Biological Sciences, H.W. Pearson Building, University Avenue, University of Cape Town, Rondebosch, 7701, Cape Town, South Africa, Dawood.Hattas@uct.ac.za, Tel: +27216502443, Fax: +27216504041

[b]Department of Wildlife, Fish, and Environmental Studies, Swedish University of Agricultural Sciences, Sweden, Joakim.Hjalten@slu.se

[c]Natural Product Research Laboratory, Department of Biology, University of Eastern Finland, Finland, RJT@uef.fi

[d]Department of Agriculture, University of Zululand, South Africa, pscoging@pan.uzulu.ac.za

[e]Swedish Environmental Protection Agency, Stockholm, Tuulikki.Rooke@naturvardsverket.se

3.1. Abstract

Low molecular weight phenolics are suggested to have a role in mediating diet selection in mammalian herbivores. However, very little is known about low molecular weight phenolic profiles of African savanna woody species. We determined low molecular weight phenolic profiles of 6 woody species with different life history, morphological and functional traits. We investigated interspecific phytochemical variation between species and found that: 1) Related *Acacia* species were chemically dissimilar; 2) Similarity percentage analysis revealed that *Acacia grandicornuta* was most dissimilar from other species and that the evergreen and unpalatable *Euclea divinorum* had a qualitatively similar chemical profile to the deciduous and palatable *Acacia exuvialis* and *Combretum apiculatum*; 3) *Combretum apiculatum* had the highest chemical diversity; 4) Relative to spineless plants, spinescent plants contained significantly less HPLC phenolics and condensed tannins; and 5) The major quantitative difference between the evergreen and unpalatable *E. divinorum* and other species was its high myricitrin concentration.

Keywords: *Low molecular weight phenolics, Tannins; Spines; Herbivory; Acacia; Euclea; Myricitrin*

3.2. Introduction

Extensive empirical evidence shows that plant secondary metabolites are effective defensive compounds against insect herbivores (e.g. Siegler 1998; Harborne 1999, 2001; Niemi et al., 2005; Andrew et al., 2007; Wennström et al., 2010 and references therein). Emerging evidence suggests that these compounds may also have a role as defense agents against mammalian herbivores (Lawler et al., 1998; Pass et al., 1998; Harborne 1999; Lawler et al., 2000; Harborne 2001;

Moore et al., 2005; Stolter et al., 2005; Torregrossa and Dearing 2009). However, the role of plant phenolics in mammalian herbivore feeding ecology in African savannas remains enigmatic. It is likely that an individual or a subclass of phenolic constituents may be the active antifeeding agents (Harborne 1999) against mammalian herbivory in these systems.

Evidence of the deterrent effects of individual (or groups of) phenolics on mammalian herbivore feeding is emerging. Lawler et al. (1998) showed that the formylated phloroglucinol compound, macrocarpal G was the active antifeedant in *Eucalyptus viminalis* that deterred ringtail possum feeding. This result was further reinforced by Lawler et al. (2000) and Wiggins et al. (2006) who found that a sideroxylonal (a formylated phloroglucinol compound) was responsible for an observed decrease in dry matter intake by the common ringtail possum feeding on eucalypts. Moreover, Moore et al. (2005) and Marsh et al. (2007) showed that the presence of formylated phloroglucinol compounds explained the observed feeding deterrence in koala feeding on eucalypts. These findings suggest that foliar phenolics have an important mediating role in mammalian herbivore diet selection. It therefore follows that the ubiquitous phenolic compounds in African woody species may have a similar mediating role, but we know little about individual constituents that make up polyphenolic compounds in these species.

Mammalian herbivory in African savannas may also be affected by plant life history, morphological and functional traits. It has been shown that slow-growing, long-lived leaves are chemically more defended than fast-growing, short-lived leaves (Coley et al., 1985; Coley et al., 1988; Coley and Barone 1996). Empirical evidence suggests that spines negatively affect bite size and feeding efficiency in mammalian herbivores (Cooper and Owen-Smith 1986; Milewski et al., 1991; Gowda 1996; Owen-Smith 2002; Wilson and Kerley 2003a, 2003b), which presumably negates the need for chemical defenses in spinescent plants (Ward, 2010). Furthermore,

phylogenetically related woody species have been shown to have different chemical profiles (Julkunen-Tiitto, 1986, 1989; Julkunen-Tiitto et al., 1996; Bacerra 1997; Lawler et al., 1998; Keinänen et al., 1999; Nyman and Julkunen-Tiitto 2005; Orians, 2005) which may affect palatability. What this implies is that phylogenetically related African woody species with similar life history, morphological and functional traits could have different chemical profiles which may affect trait-based generalizations about ecosystem functioning.

The aim of this study was to screen low molecular weight phenolic constituents in 6 African savanna woody species that differ in life history, morphological and functional traits, and grow in different habitats, *viz*. the deciduous and palatable *Acacia exuvialis* I. Verd., *Acacia grandicornuta* Gerstner, *Dichrostachys cinerea* subsp. a*fricana* (L.) Wight and Arn. (all Fabaceae), *Combretum apiculatum* Sond. (Combretaceae), *Grewia flavescens* Juss. (Tiliaceae), as well as the evergreen and unpalatable *Euclea divinorum* Hiern. (Ebenaceae). Specifically, we addressed the following questions: 1) What chemical components contribute most to differences among species? 2) Are similarities in low molecular weight phenolic composition among species associated with phylogenetic relatedness? 3) Are spinescent plants less chemically defended than spineless plants? and 4) Is the evergreen *E. divinorum* chemically more defended relative to deciduous species? We hypothesize that: 1) Phylogenetically related *Acacia* species have different chemical profiles; 2) Spinescent plants should be chemically less defended relative to spineless plants; and 3) The evergreen *E. divinorum* should be better defended than the deciduous species. The last hypothesis is premised upon *E. divinorum* inhabiting a predominantly nutrient poor environment - due to limited precipitation and thus nutrient paucity outside the growing season. Consequently the species is inherently slow growing and is predicted to be chemically more defended (Coley et al., 1985).

No previous data on phenolic profiles of *A. exuvialis* and *A. grandicornuta* are available, and limited chemical work has been done on *D. cinerea* (Bryant et al., 1991; Aworet-Samseny et al., 2011), *G. flavescens* (Bryant et al., 1991) and *E. divinorum* (Mebe et al., 1998). However, some ethnobotanical investigations on the medicinal properties of *C. apiculatum* have been conducted (Eloff et al., 2001; Fyhrquist 2007; Eloff et al., 2008).

3.3. Results and discussion

The nMDS plot and SIMPER analysis indicate that *A. grandicornuta* was most dissimilar to other species with regard to phenolic composition. By contrast, *E. divinorum* was most similar to *A. exuvialis,* and *C. apiculatum* than the other species, whereas *D. cinerea* and *G. flavescens* were most similar to each other (Figure 2 and Table 3). SIMPER analysis suggests that dissimilarity, in most cases, was mainly due to qualitative (presence/absence) rather than quantitative differences between species. This assertion is made because the substances that explained most of the differences in chemistry in the pair-wise comparisons between plant species were usually present in one of the species and absent in the other: e.g. Luteolin-diglucoside and Quercitrin in the *A. grandicornuta* - *A. exuvialis* and the *A. grandicornuta* - *C. apiculatum* comparison; Luteolin-diglucoside and Methylluteolin-glucuronide in the *A. grandicornuta* - *D*. *cinerea* and *A. grandicornuta* - *G. flavescens* comparison; Myricitrin and Luteolin-diglucoside in the *A. grandicornuta* - *E. divinorum,* and Quercitrin and Aglucose of salidroside in the *C. apiculatum* - *G. flavescens* comparison (Table 1 and 4). Quantitative differences in specific chemicals were also important determinants for the differences between species but qualitative differences were consistently most influential (Table 4). Luteolin-diglucoside and methylluteolin-glucuronide were consistently amongst the top 6 chemical species accounting for 4.5-7% and 3.9-6.1% of the average dissimilarity between *A.*

grandicornuta and other species, respectively. Quercitrin and flavokawain b contributed most to the dissimilarity between *C. apiculatum* and *D. cinerea* and together accounted for more than 10% of the dissimilarity (Table 4). These chemicals along with kaempferol 3-rhamnosode, dimer of flavokawain b, quercetin 3-glucoside and apigenin derivative 2 were good discriminators between *C. apiculatum* and *D. cinerea*.

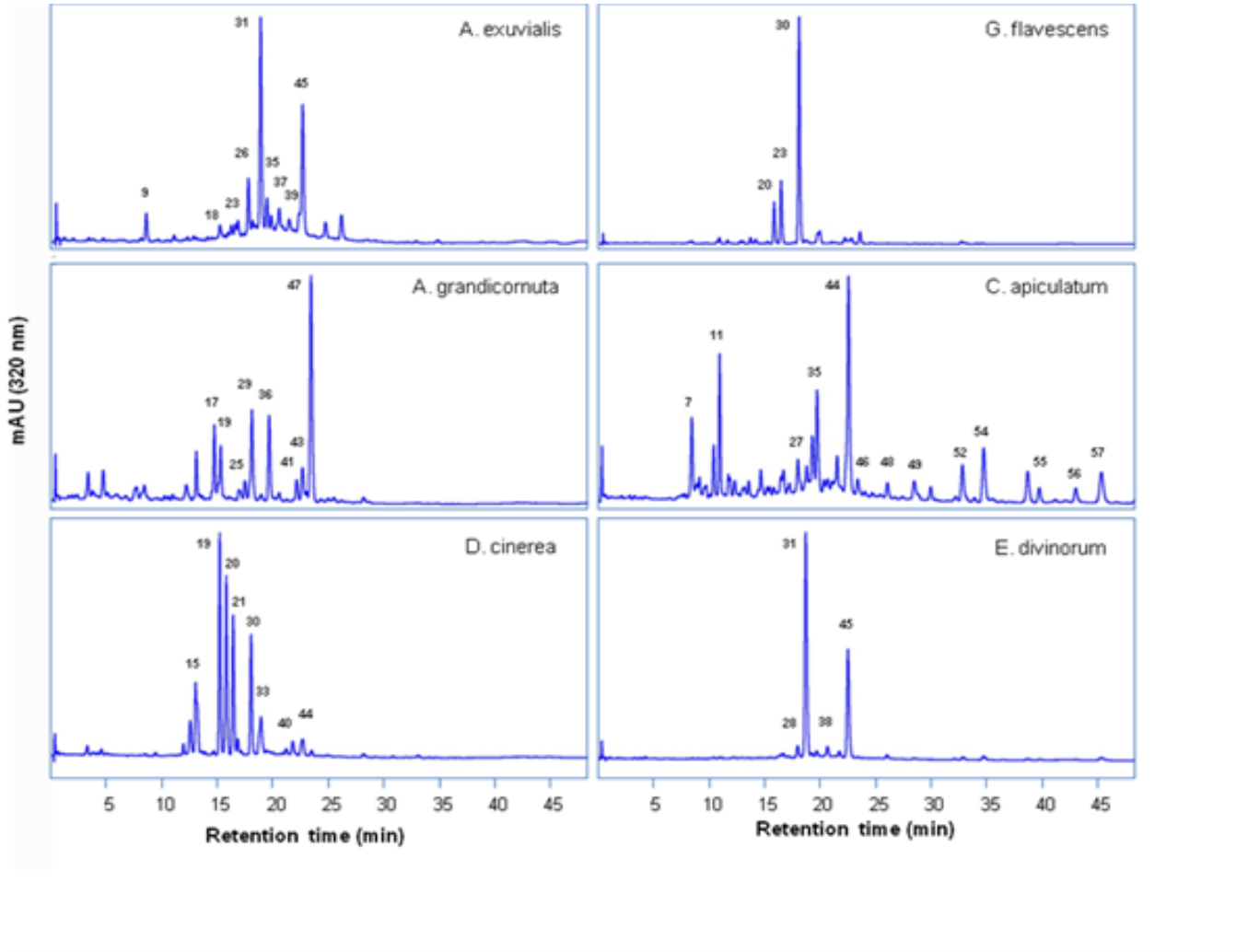


Figure 1. HPLC-UV chromatograms of *A. exuvialis*, *A. grandicornuta*, *D. cinerea*, *G. flavescens, C. apiculatum* and *E. divinorum* at 320 nm. Peak identity, R_t-value, UV and MS data are listed in Table 1.

Table 1. Detected compounds in the leaves of *A. exuvialis* (AE), *A. grandicornuta* (AG), *C. apiculatum* (CA), *D. cinerea* (DC), *E. divinorum* (ED) and *G. flavescens* (GF). (Rt= retention time, identification: UV= UV-spectrum, MS= quadrupole MS with molecular ion and possible fragments). All fragments are positive, except in *C. apiculatum* where some negative ions are also reported.

Peak	Chemical compound	Rt (min)	Molecular fragment/s	AE	AG	CA	DC	ED	GF
1	Gallotannin derivative 1	0.965	UV	x					
2	Ellagitannin derivative 1	1.026	UV	x	x	x	x		x
3	Gallic acid	1.707	UV, 171 (M+1)	x		x	x	x	x
4	aglucose of Salidroside	2.062	UV				x		
5	Cinnamic acid derivative 1	5.097	UV			x			
6	Ellagitannin derivative 2	7.370	UV			x			
7	(+)-Catechin + B3	7.589	UV¸ 291 (M+1, Catechin) and 579 (M+1, B3), 577 (M-1, B3)			x			
8	(+)-Catechin	7.864	UV, 291 (M+1, Catechin)					x	x
9	Chlorogenic acid	8.348	UV	x					
10	Gallotannin derivative 2	9.071	UV	x		x			
11	Cinnamic acid derivative 2	10.616	UV			x			x
12	Gallotannin derivative 3	10.751	UV	x					
13	Cinnamic acid derivative 3	10.816	UV						x
14	Cinnamic acid derivative 4	12.704	UV						x
15	Apigenin derivative 1	12.767	UV				x		
16	Apigenin derivative 2	12.992	UV				x		
17	Apigenin-diglucoside	13.808	UV, 595 (M+1), 617 (M+23)		x				x
18	Myricetin-diglucoside	14.635	UV	x					
19	Luteolin (5/7)-glucoside	14.802	UV , 449 (M+1)		x		x		x
20	Luteolin (5/7)-glucuronide	15.426	UV, 463 (M+1),485 (M+23)				x		x
21	Apigenin-diglucoside (isomer)	15.906	UV, 595 (M+1), 617 (M+23)				x		x

22	Apigenin-glucoarabinofuranoside	15.924	UV, 565 (M+1), 587 (M+23)		x				
23	Myricetin 3-galactoside	15.998	UV	x					
24	Myricetin 3-glucuronide	16.228	UV	x					
25	Apigenin-glucoarabinoside (pyranoside)	16.907	UV, 565 (M+1), 587 (M+23)		x				
26	Quercetin 3-glucoarabinoside	17.134	UV, 303 (M+1, Quercetin), 465 (M+1) and 597 (M+1), 619 (M+23)	x					
27	Ellagic acid glucoside	17.410	UV, 301 (M-1, Ellagic acid), 463 (M-1)			x			
28	Myricetin-glycoside	17.497	UV					x	
29	Luteolin-diglucoside	17.517	UV		x				
30	Isovitexin	17.652	UV, 433 (M+1)				x		x
31	Myricitrin	18.193	UV, 319 (M+1, Myricetin), 487 (M+23)	x				x	x
32	Quercetin 3-galactoside (Hyperin)	18.196	UV, 303 (M+1, Quercetin), 465 (M+1), 487 (M+23)	x					
33	Apigenin derivative 3 (rhoifolin)	18.512	UV, 579 (M+1), 601 (M+23)				x		
34	Apigenin derivative 4 (methyl-rhoifolin)	18.764	UV, 593 (M+1), 615 (M+23)				x		
35	Quercetin 3-glucoside	18.883	UV, 303 (M+1, Quercetin), 465 (M+1), 487 (M+23)	x		x			
36	Luteolin 7-glucuronide (isomer)	19.008	UV, 463 (M+1),485 (M+23)		x				
37	Quercetin 3-arabinofuranoside	19.196	UV	x					
38	Quercetin 3-arabinoside	20.063	UV, 303 (M+1, Quercetin), 435 (M+1), 457 (M+23)			x		x	
39	Quercetin 3-arabinopyranoside	20.666	UV	x					
40	Luteolin derivative 1	21.095	UV				x		
41	Apigenin 7-glucoside + glucuronide	21.482	UV, 433 (M+1), 455 (M+23) and 449 (M+1), 469 (M+23)		x				x
42	Luteolin derivative 2	21.592	UV				x		
43	Methylluteolin derivative 2	21.932	UV		x				
44	Apigenin-glucuronide	21.952	UV, 447 (M+1), 469 (M+23)				x		
45	Quercitrin	21.962	UV, 303 (M+1, Quercetin), 449 (M+1), 447 (M-1), 471 (M+23)	x		x		x	
46	Kaempferol 3-arabinoside	22.621	UV			x			

47	Methylluteolin-glucuronide	22.667	UV, 477 (M+1), 499 (M+23)	x			
48	Kaempferol 3-rhamnoside	25.350	UV		x	x	
49	Pinocembrin-glucoside	27.546	UV, 257 (M+1, Pinocembrin), 255 (M-1, Pinocembrin), 417 (M-1), 419 (M+1), 441 (M+23)		x		
50	Acetyl-Quercetin-rhamnoside	28.974	UV, 489 (M-1), 513 (M+23)		x		
51	Luteolin derivative 3	31.578	UV				x
52	dimer of Alpinetin	31.723	UV, 271 (M+1, Alpinetin), 269 (M-1, Alpinetin), 563 (M+23)		x		
53	dimer of Flavokawain b	31.732	UV, 285 (M+1, Flavokawain), 307 (M+23), 591(M+23)		x		
54	Flavokawain b	33.576	UV		x		
55	Pinocembrin	38.402	UV, 257 (M+1, Pinocembrin), 255 (M-1, Pinocembrin), 279 (M+23)		x		
56	dimethylpinocembrin + other	41.568	UV, 257 (M+1, Pinocembrin), 255 (M-1, Pinocembrin)		x		
57	dimer of methylflavokawain b	43.801	UV, 299 (M+1, methylflavokawain), 321 (M+23); 619 (M+23)		x		

Table 2. Tannins, total HPLC phenolics and flavonoid concentration in *A. exuvialis*, *A. grandicornuta*, *C. apiculatum*, *D. cinerea*, *E. divinorum* and *G. flavescens* (n=3). Values are mean ± SE, and values in brackets are percentage contribution to total HPLC phenolics; ND = not detected.

Species	Life strategy/ spinescence	Condensed tannins (mg g^{-1})	Total HPLC phenolics (mg g^{-1})	Hydrolysable tannins (mg g^{-1})	Myricetin conjugates (mg g^{-1})	Quercetin conjugates (mg g^{-1})	Kaempferol conjugates (mg g^{-1})	Apigenin conjugates (mg g^{-1})	Luteolin conjugates (mg g^{-1})
A. exuvialis	Deciduous-spinescent	11.3 ± 1.3	11.3 ± 1.2	0.42 ± 0.06 (4)	1.26 ± 0.3 (11)	9.1 ± 0.9 (80)	ND	ND	ND
A. grandicornuta	Deciduous-spinescent	5.9 ± 0.8	17.6 ± 3.0	0.15 ± 0.01 (0.8)	ND	ND	ND	5.1 ± 2.0 (29)	12.3 ± 1.1 (70)
C. apiculatum	Deciduous-spineless	86.7 ± 35.6	32.0 ± 0.87	1.36 ± 0.12 (4)	ND	12.0 ± 0.1 (38)	2.04± 0.20 (6)	ND	ND
D. cinerea	Deciduous-spinescent	14.3 ± 6.7	25.8 ± 3.6	0.28 ± 0.08 (1)	2.1 ± 0.6 (23)	ND	ND	8.9 ± 0.7 (34)	7.4 ± 1.2 (29)
E. divinorum	Evergreen-spineless	21.3 ± 1.4	24.2 ± 3.4	ND	16.4 ± 3.7 (70)	5.7 ± 1.5 (23)	0.32 ± 0.17 (1)	ND	ND
G. flavescens	Deciduous-spineless	31.5 ± 26.3	19.6 ± 3.8	0.17 ± 0.02 (1)	0.3 ± 0.2 (2)	ND	ND	14.2 ± 3.9 (72)	3.3 ± 0.7 (17)

Table 3. Average percentage dissimilarity between species from SIMPER analysis for *A. exuvialis* (AE), *A. grandicornuta* (AG), *C. apiculatum* (CA), *D. cinerea* (DC), *E. divinorum* (ED) and *G. flavescens* (GF).

Species	AG	CA	DC	ED	GF
AE	82.6	67.4	78.4	62.8	77.8
AG		87.2	84.3	85.7	76.3
CA			82.9	63.3	79.2
DC				80.3	59.9
ED					69.7

Table 4. SIMPER analysis showing average dissimilarity of the 10 compounds which contributed most to the dissimilarity between species. Species are abbreviated as follows; *A. grandicornuta* (AG) and *A. exuvialis* (AE), *C. apiculatum* (CA), *D. cinerea* (DC), *E. divinorum* (ED) and *G. flavescens* (GF). Only species comparisons that showed > 80% average dissimilarity is shown (see Table 3).

Chemical compound	Av. diss. (%)	Av. diss. (%)	Av. diss. (%)	Av. diss. (%)	Av. diss. (%)	Av. diss. (%)
	AG-AE	AG-CA	AG-DC	AG-ED	AG-GF	CA-DC
Myricitrin				9.22		
Luteolin-diglucoside	6.09	4.47	5.55	7.00	6.02	
Quercitrin	5.48	4.76		6.92		4.38
Methylluteolin glucuronide	5.26	3.87	4.80	6.05	5.20	
Luteolin 7-glucuronide	4.79	3.53	4.37	5.51	4.74	
Apigenin diglycoside	4.35	3.20	3.97	5.00	4.30	
Apigenin glucoarabinoside (pyranoside)	4.13			4.75	4.08	
Quercetin 3-arabinoside		3.88				3.56
Quercetin 2-gallactoside (Hyperin)	5.38					
methylluteolin der 1	3.99			4.58		
Apigenin glucoarabinofuranoside	3.89			4.47		
Isovitexin					6.84	
Apigenin diglucoside (isomer)			4.05		4.95	
Luteolin glucuronide					4.45	
(+)-Catechin					4.30	
Apigenin 7-glucoside + glucuronide	3.99			4.59	3.95	
Aglucose of salidroside			5.31			3.93
Luteolin (5/7)-glucoside			5.06			3.75
Luteolin (5/7)-glucuronide			4.89			3.62
Apigenin derivative 2			4.74			3.51
Myricetin derivative 1			4.30			3.19

Flavokawain b	4.64	4.27
Condensed tannins	3.91	
(+)-Catechin + B3	3.73	3.43
Pinocembrin-glucoside	3.32	3.05

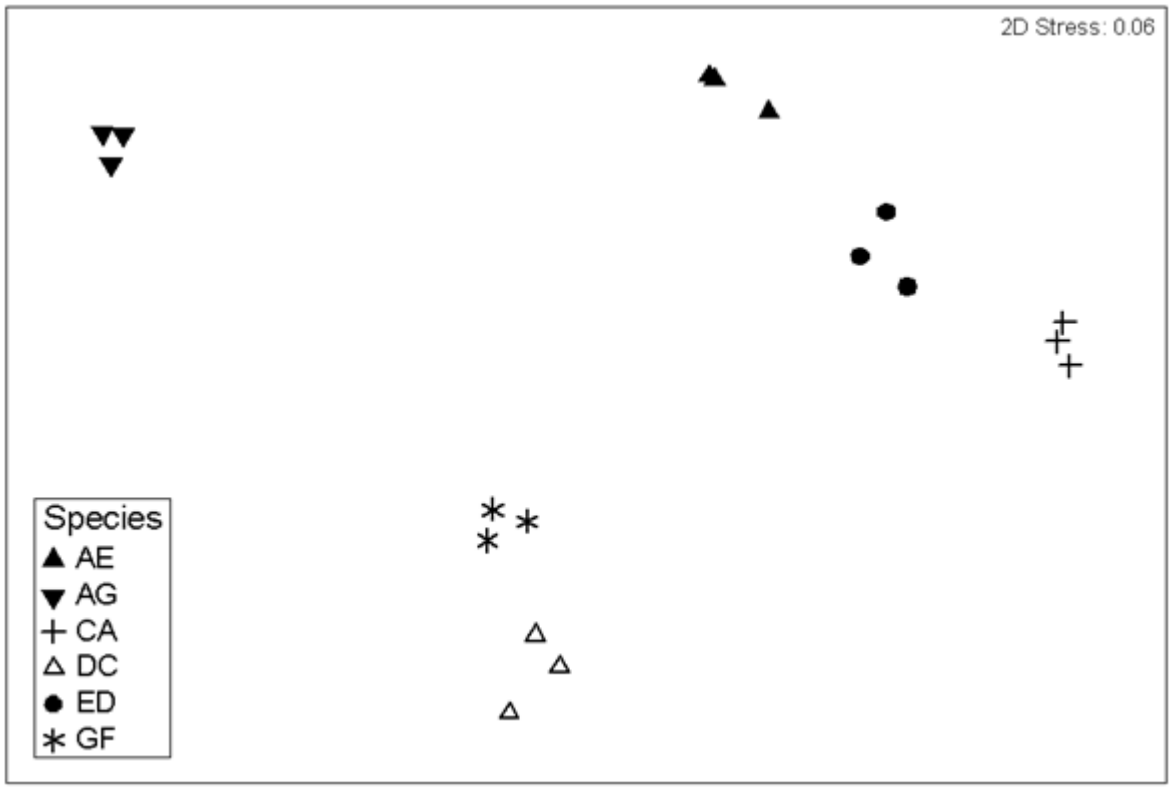


Figure 2. Non-metric multi-dimensional scaling ordination stress plot to visualize chemical (dis)similarities between *A. exuvialis* (AE), *A. grandicornuta* (AG), *C. apiculatum* (CA), *D. cinerea* (DC), *E. divinorum* (ED) and *G. flavescens* (GF).

Composition of HPLC phenolics was species specific with *C. apiculatum* showing the highest chemical diversity (Table 1 and Figure 1). HPLC phenolics in phylogenetically related *A. exuvialis and A. grandicornuta* were different from each other (Table 1, Figure 1 and 2). *Acacia exuvialis*, *C. apiculatum* and *E. divinorum* constituted exclusively flavonol glycosides, whereas flavone glycosides characterised *A. grandicornuta*. *Dichrostachys cinerea* contained both flavone glycosides and a single myricetin derivative, whereas *G. flavescens* contained flavone glycosides and myricitrin (Table 1 and 2, Figure 1). Consistent with previous findings (Julkunen-Tiitto, 1986, 1989; Julkunen-Tiitto et al., 1996; Bacerra 1997; Lawler et al., 1998; Keinänen et al., 1999; Nyman and Julkunen-Tiitto 2005; Orians, 2005), our results show that phylogenetically related species, in our case acacias, have different chemical profiles and end products (flavones and flavonols in *A. grandicornuta* and

A. exuvialis, respectively) that are synthesized following different branching points in the phenylpropanoid pathway (Siegler 1998; Winkel-Shirley 2001; Nyman and Julkunen-Tiitto 2005). *Acacia grandicornuta* grows in a nutrient rich clayey substrate and is thus predicted to have a growth strategy that favours allocation to growth over secondary defense compounds (Herms and Mattson 1992; Stamp 2003). Indeed, *A. grandicornuta* does not have flavonol glycosides and contains low condensed tannin and total HPLC phenolics concentrations.

Acacia grandicornuta and *D. cinerea* had flavones which have been shown to induce transcription of root nodulation genes that code for nodule formation and symbiotic N_2 fixation (Dakora 1995; Siegler 1998; Winkel-Shirley 2001). Both *A. grandicornuta* and *D. cinerea* are from the Fabaceae which are well known for nodulating. However, *A. exuvialis,* which also belongs to this family, does not contain flavones. Nodulation in *Acacia* species has been shown to be plastic, being phenotypically expressed when resources are limited (Cramer et al., 2007, 2010; Zharare and Scogings 2011). Thus the absence of flavones in *A. exuvialis* does not imply that the species is non-nodulating. Similarly the presence of flavones does not imply that a species is nodulating since *G. flavescens* has been shown to be non-nodulating (Aranibar et al., 2005). Indeed, flavones have also been shown to inhibit plant growth regulating hormones and NADH dehydrogenase, which consequently interrupted the electron transport chain of respiration and photosynthesis in potato tubers (Siegler 1998).

Total HPLC phenolics and condensed tannin concentration in spineless plants was significantly higher than that of spinescent plants ($t_{(16)}$=2.130, p=0.049 and $t_{(16)}$=2.300, p=0.035, respectively). Total HPLC phenolics concentration in spineless plants was 1.4 times more than spinescent plants (25.8 ± 2.52 and 18.2 ± 2.51 mg g^{-1}, mean ± SE respectively), whereas condensed tannin concentration in spineless plants was more than 4 times higher than that in spinescent plants (46.5 ± 16.3 and

10.5 ± 2.33 mg g^{-1}, mean ± SE respectively). These results show that chemical defenses are better expressed in the absence of spines, which is contrary to previous findings that found no trade-off between chemical and mechanical defenses (Koricheva et al., 2004; Rohner and Ward 1997). However, HPLC phenolics and condensed tannin in spinescent plants may have functions other than defense, e.g. polyphenolics and tannins have also been shown to protect plants against fungal and microbial attack (Scalbert 1991; Schultz et al., 1992; Siegler 1998); and condensed tannins have been reported to protect plants against photodamage (Close and McArthur 2001).

Compared to other species, the evergreen and unpalatable *E. divinorum* contained similar, but fewer, phenolic compounds. However, its myricitrin concentration was 55 times greater than that in *G. flavescens* and 129 times greater than that detected in *A. exuvialis* (Table 2). Furthermore, myricitrin in *E. divinorum* accounted for 68% of its total HPLC phenolic concentration and 1.6% of the foliar dry matter content of the species. Myricetin has been shown to have negative effects on insect herbivores (Mutikainen et al., 2000; Roitto et al., 2008). An artificial diet containing myricitrin (0.1%) and quercitrin (0.5%) significantly decreased consumption by winter moth larvae (Lavola et al., 1998), while quercitrin deterred feeding in monarch butterfly larvae (Vickerman and de Boer 2002). It is therefore likely that 0.6% quercitrin coupled with a high myricitrin concentration may render *E. divinorum* toxic to insects, since artificial diets containing more than 0.2% of many common flavonoid glycosides were toxic to tobacco budworm, bollworm and the pink bollworm (Harborne 1991; Siegler 1998). Further research is required to test whether myricitrin is indeed a deterring agent against mammalian herbivory in *E. divinorum*. However, it has to be noted that antifeeding (Harborne 1991; Bryant and Julkunen-Tiitto 1995; Harborne 1998, 2001; Isman 2002) and potentially toxic triterpenoids

(Harborne 1991, 2001) have been isolated from the root bark of *E. divinorum* (Mebe et al., 1998), but these compounds may be absent in its leaves (Grubb 1992).

Quercetin glycoside concentration in *C. apiculatum* and *A. exuvialis* were also higher than the purported 0.2% tolerance threshold (1.2 and 0.9%, respectively, Table 3) and may afford these species some protection against insect herbivores. In addition to its high quercetin glycoside concentration, *C. apiculatum* also contained relatively high condensed and hydrolysable tannins (gallo- and ellagitannins) (8.7 and 0.14%, respectively, Table 2). Condensed tannins in excess of 5% deterred feeding in kudu and impala as well as goats (Cooper and Owen-Smith 1985) and hydrolysable tannins have been shown to negatively affect moth pupal growth (Rossiter et al., 1988; Lill and Marquis 2001) and cause moth pupal mortality (Karowe 1989) and poisoning in mammalian herbivores (Harborne 2001; McSweeney et al., 2001; Makkar 2003). It is thus possible that these chemical defenses may reduce palatability of *C. apiculatum* for herbivores. It has to be noted though that we sampled mature leaves from adult trees late in the growing season, and it has been empirically shown that allocation to defense varies with ontogeny (Barton and Koricheva 2010). Thus flavonoid glycoside and condensed and hydrolysable tannin concentrations reported here may only be relevant to this particular life stage.

3.4. Concluding remarks

The limited number of species in this study precludes generalizations to be made, and our conclusions are restricted to this group of species. However, our results show that phylogenetically related *Acacia* species, and species with similar life history, morphological and functional traits had different foliar low molecular weight phenolic profiles. Furthermore, the evergreen-spineless and unpalatable *E. divinorum* and the deciduous-spinescent and palatable *A. exuvialis* had qualitatively similar

chemistry. These results suggest that low molecular weight phenolic profiles are not necessarily related to life history, morphological or functional traits. Therefore, trait-based generalizations that exclude foliar low molecular weight phenolic chemistry may be misleading.

Detailed phenolic composition reported here for the 6 woody African savanna species have not previously been published. This information provides a platform from which we can further pursue our understanding of plant secondary chemical defenses in savanna woody species in relation to herbivory.

3.5. Experimental

3.5.1. Study site

The study was carried out in the Nkuhlu area, along the Sabie River, about 20 km south east of Skukuza in Kruger National Park, South Africa (24°59'23.57"S, 31°46'28.55"E). The site is situated along a catena with nutrient poor granite derived sand on the crest and relatively nutrient rich sodic, clayey soil on the footslope. The two soil types are characterised by distinctive vegetation, with *Acacia grandicornuta* the dominant species on the footslope (Venter et al., 2003) and *Combretum apiculatum* dominating the crest (Siebert and Eckhardt 2008). The site, which runs along the length of the catena and includes both soil types, were enclosed with wire fencing in 2002 to exclude elephants (Siebert and Eckhardt 2008), but effectively exclude all mammalian herbivores bigger than a hare.

The mean annual rainfall for Skukuza, the closest weather station, is 587 mm with a 29% coefficient of variation (*n*= 52, 1956-2009, South African Weather Service), 89% of which falls in the growing season, between October and April (February and Higgins 2010). The average minimum and maximum temperature at Skukuza in the growing season is 19 and 31 °C, respectively (1956-2009, South African Weather Service).

3.5.2. Tree species

Six tree species from amongst the most abundant trees in the Nkuhlu area (Siebert and Eckhardt 2008) were selected for this study. All individuals were reproductive adult trees of 2-4 m high, of which 5 were deciduous *viz*. *A. grandicornuta*, *C. apiculatum*, *G. flavescens*, *A. exuvialis*, *D. cinerea* and one evergreen - *E. divinorum*. The two *Acacia* species are phylogenetically close, both falling within the *Vachellia* clade of the Mimosoideae legumes, with a divergent time of ca. 14 Ma (Bouchenak-Khelladi et al. 2010). The selected species comprise a range of morphological and functional traits; *A. grandicornuta*, *A. exuvialis* and *D. cinerea* have bi-pinnate compound leaves and spines, whereas the rest have simple leaves and no spines. They also include a range of apparent palatability (Van Wyk 1972; Coats Palgrave 2002) with the evergreen *E. divinorum* the only unpalatable species (Coats Palgrave 2002; Fornara and du Toit 2008). *Euclea divinorum* leaves outside the exclosure showed some signs of herbivory at certain times of the year, but no sign of mammalian herbivory (Zululand/Sweden Kruger Browse Project, unpublished data).

3.5.3. Sample collection

Ten leaves from 3 random adult trees per species were collected in March 2007. Samples were immediately frozen in liquid N, kept frozen until they were lyophilized in a New Brunswick freeze dryer (New Brunswick Scientific Co., Inc., New Jersey, USA), and milled to a fine powder using a Retsch ball mill (F. Kurt Retsch, GmbH and Co. KG, Haan, Germany).

3.5.4. Chemical analysis

Low molecular weight phenolics were extracted from 5 mg of plant material in 700 µl of cold methanol and homogenized using an Ultra-Turrax T25 homogeniser (Ika-Labortechnik, Staufen, Germany) for 20 s. The extracts was allowed to stand for 15 min and again homogenized for 20 s and centrifuged at 13000 g for 3 min. The supernatant was saved and re-extracted 3 more times using a 5 min waiting period - combining the supernatants. The residue was evaporated under N-gas and stored at -20 °C until analyzed. Prior to HPLC analysis, the residue was dissolved in 600 µl methanol-water (1:1) and identified and quantified using a Hewlett-Packard HPLC-DAD system (Hewlett-Packard, Avondale, Pa., USA) using parameters described by Julkunen-Tiitto et al. (1996). Chemical identities were confirmed by HPLC-MS (API-ES positive ions as well as negative ions in the case of *C. apiculatum*) using a Hypersil Rp C-18, 2 mm ID, 10 cm long column and a flow rate of 0.4 ml min^{-1}. The ES fragmentor voltage ranged from 80-120 (Julkunen-Tiitto and Sorsa, 2001). Chemical identification was based on retention time, chromatographic UV spectra and HPLC-MS data. Cinnamic acids, quercetins, kaempferols and catechin were standardized against chlorogenic acid, isoquercitrin, Kaempferol 3-glucoside and (+)-catechin, respectively. Alpinetin, pinocembrin and flavokawain b and their derivatives were standardized against eriodictyol 7-glucoside. Condensed tannins were determined using the acid-butanol assay (Porter et al., 1986) as modified by Hagerman (2002), and using *Sorghum* tannin as reference material (Hattas & Julkunen-Tiitto 2012).

3.5.5. Data analysis

We tested for chemical similarities between plant species using non-metric methods. These methods make no assumptions about the form of the data, which

makes them widely applicable, leading to greater confidence in interpretation. Prior to this analysis, data were fourth-root transformed to reduce the influence of dominant chemical compounds. This transformation further allows mid-range and rare chemical compounds to exert some influence on the calculation of the (dis)similarity between plant species (Clarke and Warwick 2001). Relative dissimilarities between plant species were visualized using a non-metric Multi-Dimensional Scaling (nMDS) ordination technique (Clarke 1993). nMDS essentially constructs a map which display the relative separation between plant species with regards to their chemical composition, i.e. the further apart two species, the more dissimilar they are. Similarity percentage (SIMPER) breakdown analysis (Clarke 1993) was used to determine the contribution of individual chemical constituents to the separation of plant species as reflected in the nMDS plot. SIMPER analyses are generally used to explore differences in community structure (Clarke 1993), but have also been used to assess chemical differences in soil and plants: Carney and Matson (2005) used SIMPER analysis to determine whether phospholipid fatty acids accounted for differences in soil microbial communities; Ens et al. (2010) used SIMPER analysis to assess differences in hydrophobic chemicals signatures in soil to determine differences in allelopathy of different soils; whereas Nahrung et al. (2009) used SIMPER analysis to assess differences in foliar chemistry by assessing which GC-MS peaks were most important in contributing to differences between allopatric pure and a commercial hybrid of *Corymbia* species. SIMPER analysis is not a statistical test, but an exploratory analysis based on ranks that indicate which chemical compounds are principally responsible for the observed clustering or differences between samples (species). To identify characteristic features of the chemistry of a specific plant, SIMPER calculates the average Bray-Curtis similarity between all pairs of intra-group samples. Good discriminator compounds have consistent quantitative presence, which result in a high average dissimilarity/SD ratio. This allows for identifying

chemical compounds that significantly (Torok et al. 2008) and consistently contributed to the dissimilarity between plant species (Clarke and Warwick 2001). Analysis was performed using PRIMER v6 (Clarke and Gorley 2006). To test whether spinescent plants are more defended than spineless plants, condensed tannins and total HPLC phenolic concentrations for the different life strategies were pooled. Condensed tannin concentration was log-transformed to correct for normality and a Student's *t*-test was performed to determine whether condensed tannin and total HPLC phenolic concentrations were different between spinescent and spineless plants (n=9 in both cases, for both variables).

Acknowledgements

We thank Jeremy Midgley, William Bond and two anonymous reviewers for helpful comments which improved this manuscript. Funding for the Zululand/Sweden Kruger Browse Project was provided by the National Research Foundation (South Africa), University of Zululand, Agricultural Research Council (South Africa), Swedish Research Council and Swedish International Development Agency.

3.6. References

Andrew, R., Wallis, I., Harwood, C., Henson, M., Foley, W., 2007. Heritable variation in the foliar secondary metabolite sideroxylonal in *Eucalyptus* confers cross-resistance to herbivores. Oecologia 153, 891–901.

Aranibar, J., Otter, L., Macko, S., Feral, C. W., Epstein, H., Dowty, P., Eckardt, F., Shugart, H., Swap, R., 2005. Nitrogen cycling in the soil-plant system along a precipitation gradient in the Kalahari sands. Global Change Biology 10, 359–373.

Aworet-Samseny, R., Souza, A., Kpahé, F., Konaté, K., Datté,J., 2011. *Dichrostachys cinerea* (L.) Wight *et* Arn (Mimosaceae) hydro-alcoholic extract action on the contractility of tracheal smooth muscle isolated from guinea-pig. BMC Complementary and Alternative Medicine, 11:23.

Bacerra, J., 1997. Insects on plants: macroevolutionary chemical trends in host use. Science 276, 253-255.

Barton, K., Koricheva, J., 2010. The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. The American Naturalist 175, 481-493.

Bouchenak-Khelladi, Y., Maurin, O., Hurter, J., Van der Bank, M., 2010. The evolutionary history and biogeography of Mimosoideae (Leguminosae): an emphasis on African acacias. Mol. Phylogenet. Evol. 57, 495-508.

Bryant, J., Heitkonig, I., Kuropat, P., Owen-Smith, N., 1991. Effects of severe defoliation on the long-term resistance to insect attack and on leaf chemistry in six woody species of the southern African savannah.The American Naturalist 137(1), 50-63.

Bryant, J., Julkunen-Tiitto, R., 1995.Ontogenic development of chemical defense by seedling resin birch: Energy cost of defense production. Journal of Chemical Ecology 21, 883-896.

Clarke, K. R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18, 117-143.

Carney, K., Matson, P., 2005. Plant communities, soil microorganisms, and soil carbon cycling: Does altering the world belowground matter to ecosystem functioning? Ecosystems 8, 928-940.

Clarke, K. R., Gorley, R. N., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

Clarke, K.R., Warwick. R.M., 2001. Changes in marine communities: an approach to statistical analysis and interpretation, 2nd edition. PRIMER-E: Plymouth.

Close, D., McArthur, C., 2002. Rethinking the role of many plant phenolics – protection from photodamage not herbivores? Oikos99, 166–172.

Coates Palgrave, M., 2002. Trees of Southern Africa. Struik Publishers, Cape Town.

Coley, P., 1988. Effects of plant growth rate and leaf lifetime on the amount and type of anti-herbivore defense. Oecologia 74, 531-536.

Coley, P., Barone, J., 1996. Herbivory and plant defenses in tropical forests. Annual Review Ecology and Systematics 27, 305–335.

Coley, P., Bryant, J., Chapin, F. S., 1985. Resource availability and plant antiherbivore defense. Science 230, 895-899.

Cooper, S., Owen-Smith, N., 1985. Condensed tannins deter feeding by browsing ruminants in a South African savanna Oecologia 67, 142-146.

Cooper, S., Owen-Smith, N., 1986.Effects of plant spinescence on large mammalian herbivores. Oecologia 86, 446-455.

Cramer, M., Chimpanhgo, S., Van Cauter, A., Waldram, M., Bond, W., 2007. Grass competition induces N_2 fixation in some species of African *Acacia*. Journal of Ecology 95, 1123–1133.

Cramer, M., Van Cauter, A., Bond, W., 2010. Growth of N_2-fixing African savanna *Acacia* species is constrained by below-ground competition with grass. Journal of Ecology 98, 156–167.

Dakora, F., 1995. Plant flavonoids: biological molecules for useful exploitation. Australian Journal of Plant Physiology 22, 87-99.

Eloff, J., Jager, A., van Staaden J., 2001.The stability and relationship between anti-inflammatory activity and antibacterial properties of southern African *Combretum* species. South African Journal of Science 97, 291-293.

Eloff, J., Katerere, D., McGaw, L., 2008. The biological activity and chemistry of the southern African *Combretaceae*. Journal of Ethnopharmacology 119, 686-699.

Ens, E.-J., French, K., Bremner, J., Korth, J., 2010. Novel technique shows different hydrophobic chemical signatures of exotic and indigenous plant soils with similar effects of extracts on indigenous species seedling growth. Plant Soil 326, 403-414.

February, E., Higgins, S., 2010. The distribution of tree and grass roots in savannas in relation to soil nitrogen and water. South African Journal of Botany 76, 517-523.

Fornara, D., Toit, J. D., 2008. Browsing-induced effects on leaf litter quality and decomposition in a southern African savanna. Ecosystems 11, 238-249.

Fyhrquist, P., 2007. Traditional medicinal uses and biological activities of some plant extracts of African *Combretum* Loefl., *Terminalia* L. and *Pteleopsis* Engl. species (*Combretaceae*). PhD Thesis, University of Helsinki.

Gowda, J., 1996. Spines of *Acacia tortilis*: What do they defend and how? Oikos 77, 279-284.

Grubb, P., 1992. Presidential address: A positive distrust in simplicity - lessons from plant defences and from competition among plants and among animals. Journal of Ecology 80, 585-610.

Hagerman, A. E., 2002. Tannin handbook (http://www.users.muohio.edu/hagermae/tannin.pdf, downloaded in March 2004. Miami University, Ohio, USA.

Harborne, J. B., 1991. The chemical basis of plant defense. In: Palo RT and Robbins CT. eds. Plant defenses against mammalian herbivory. CRC Press, New York, USA.

Harborne, J. B., 1998. Phytochemical Methods: A guide to modern techniques of plant analysis. Chapman & Hall.

Harborne J. B., 1999. Recent advanced in chemical ecology. Natural Products Report 16, 509-523.

Harborne J. B., 2001. Twenty-five years of chemical ecology. Natural Products Report 18, 361-379.

Herms, D., Mattson, W., 1992. The dilemma of plants: To grow or defend. The Quarterly Review of Biology 67, 283-335.

Isman, M., 2002. Antifeedants: Insect antifeedants. Pesticide Outlook, August, 152-157.

Julkunen-Tiitto, R., 1986. A chemotaxonomic survey of phenolics in leaves of northern salicaceae species. Phytochemistry 25, 663-667.

Julkunen-Tiitto, R., 1989. Phenolic constituents of Salix: A chemotaxonomic survey of further Finnish species. Phytochemistry 28, 2115-2125.

Julkunen-Tiitto, R., Rousi, M., Bryant, J., Sorsa, S., Keinänen, M., Sikanen, H., 1996. Chemical diversity of several *Betulaceae* species: comparison of phenolics and terpenoids in northern birch stems. Trees 11, 16-22.

Julkunen-Tiitto, R., Sorsa, S., 2001. Testing the drying methods for willow flavonoids, tannins and salicylates. *Journal of Chemical Ecology* 27(4), 779-789.

Karowe, D., 1989. Differential effect of tannic acid on two tree-feeding Lepidoptera: implications for theories of plant anti-herbivore chemistry. Oecologia 80, 507-512.

Keinänen, M., Julkunen-Tiitto, R., Rousi, M., Tahvanainen, J., 1999. Taxonomic implications of phenolic variation in leaves of birch (*Betula* L.) species. Biochemical Systematics and Ecology 27, 243-254.

Koricheva, J., Nykänen, H., Gianoli, E., 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? The American Naturalist 163, E64-E75.

Lavola, A., Julkunen-Tiitto, R., Roininen, H., Aphalo, P., 1998. Host-plant preference of an insect herbivore mediated by UV-B and CO_2 in relation to plant secondary metabolites. Biochemical Systematics and Ecology 26, 1-12.

Lawler, I. R., Foley, W. J., Eschler, B., Pass, D. M., Handasyde, K., 1998. Intraspecific variation in secondary metabolites determines food intake by folivorous marsupials. *Oecologia* 116, 160-169

Lawler, I., Foley, W., Eschler, B., 2000. Foliar concentration of a single toxin creates habitat patchiness for marsupial folivore. Ecology 81, 1327-1338.

Lill, J., Marquis, R., 2000. The effects of leaf quality on herbivore performance and attack from natural enemies. Oecologia 126, 418-428.

Makkar, H., 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Research 49, 241-256.

Marsh, K., Wallis, I., Foley, W., 2007. Behavioural contributions to the regulated intake of plant secondary metabolites in koalas. Oecologia 154, 283-290.

McSweeney, C., Palmer, B., McNeill, D., Krause, D., 2001. Microbial interactions with tannins: Nutritional consequences for ruminants. Animal Feed Science and Technology 91, 83-93.

Mebe, P., Cordell, G., Pezzuto, J., 1998. Pentacyclictriterpenes and naphthoquinones from *Euclea divinorum*. Phytochemistry 47, 311-313.

Milewski, A., Young, T., Madden, D., 1991. Thorns as induced defenses: Experimental evidence. Oecologia 86, 70-75.

Moore, B. D., Foley, W. J., Wallis, I.R., Cowling, A., Handasyde, K. A., 2005. A simple understanding of complex chemistry explains feeding preferences of the koala. *Proceedings of the Royal Society:* Biology Letters 1, 64-67

Mutikainen, P., Walls, M., Ovaska, J., Keinänen, M., Julkunen-Tiitto, R., Vapaavuori, E., 2000. Herbivore resistance in *Betula pendula*: effect of fertilization, defoliation, and plant genotype. Ecology 81, 49-65.

Nahrung, H., Waugh, R., Hayes, R., 2009. Corymbia species and hybrids: Chemical and physical foliar attributes and implications for herbivory. Journal of Chemical Ecology 35, 1043-1053.

Niemi, L., Wennström, A., Ericson, L., 2005. Insect feeding preferences and plant phenolic glucosides in the system *Gonioctenalinnaeana -Salix triandra*. Entomologia Experimentalis et Applicata 115, 61-66.

Nyman, T., Julkunen-Tiitto, R., 2005. Chemical variation within and among six northern willow species. Phytochemistry 66, 2836-2843.

Orians, C., 2005. Herbivores, vascular pathways, and systemic induction: facts and artefacts. Journal of Chemical Ecology 31, 2231-2242.

Owen-Smith, R., 2002. Adaptive Herbivore Ecology. Cambridge University Press.

Pass, D. M., Foley, W. J., Bowden, B., 1998. Vertebrate herbivory on *Eucalyptus* - identification of specific feeding deterrents for common ringtail possums

(*Pseudocheirus peregrinus*) by bioassay-guided fractionation of *Eucalyptus ovate* foliage. *Journal of Chemical Ecology* 24(9), 1513-1527.

Porter, L. J., Hrstich, L. N., Chan, B. C., 1986. The conversion of procyanidins and prodelphinins to cyanidin and delphinin. Phytochemistry 25, 223-230.

Rohner, C., Ward, D., 1997. Chemical and mechanical defense against herbivory in two sympatric species of desert *Acacia*. Journal of Vegetation Science 8, 717-726.

Roitto, M., Rautio, P., Markkola, A., Julkunen-Tiitto, R., Varama, M., Saravesi, K., Tuomi, J., 2008. Induced accumulation of phenolics and sawfly performance in Scots pine in response to previous defoliation. Tree Physiology 29, 207-216.

Rosenthal, J., Kotanen, P., 1994. Terrestrial plant tolerance to herbivory. Trends in Ecology and Evolution 9, 145-148.

Rossiter, M., Schultz, J., Baldwin, I., 1988. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. Ecology 69, 267-277.

Scalbert, A., 1991. Antimicrobial properties of tannins. Phytochemistry 30, 3875-3883.

Schultz, J., Hunter, M., Appel, H., 1992. Antimicrobial activity of polyphenols mediates plant-herbivore interactions. In: Hemingway, R., Laks, P. (Eds.), Plant polyphenolics: Synthesis, properties and significance. Plenum Press, New York.

Siebert, F., Eckhardt, H., 2008. The vegetation and floristics of the Nkuhlu exclosures, Kruger National Park. Koedoe 50, 126-144.

Siegler, D., 1998. Plant Secondary Metabolism. Kluwer Academic Press, Massachusetts, USA.

Stamp, N., 2003. Out of the quagmire of plant defense hypotheses. The Quarterly Review of Biology 78, 23-55.

Stolter, C., Ball, J. P., Julkunen-Tiitto, R., Lieberei, R., Ganzhorn, J. U., 2005. Winter browsing of moose on two different willow species: food selection in relation to plant chemistry and plant response. Canadian Journal of Zoology 83, 807-819.

Torok, V., Ophel-Keller, K., Loo, M., Hughes, R., 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. Applied and Environmental Microbiology 74, 783-791.

Torregrossa, A., Dearing, M., 2009. Nutritional toxicology of mammals: regulated intake of plant secondary compounds. Functional Ecology 23, 48-56.

Van Wyk, P., 1972. Trees of the Kruger National Park. Purnell and Sons (Pty.) Ltd, Cape Town.

Venter, F., Scholes, R., Eckhardt, H., 2003.The abiotic template and its associated vegetation pattern. In: du Toit, J., Rogers, K., Biggs, H. The Kruger Experience Ecology and Management of Savanna Heterogeneity. Island Press, USA.

Vickerman, D., de Boer, G., 2002. Maintenance of narrow diet breadth in the monarch butterfly caterpillar: response to various plant species and chemicals. Entomologia Experimentalis et Applicata 104, 255-269.

Ward, D., 2010. The effects of apical meristem damage on growth and defences of two *Acacia* species in the Negev Desert. Evolutionary Ecology Research 12, 589-602.

Wennström, A., Hjulström, L., Hjältén, J., Julkunen-Tiitto, R., 2010. Mother really knows best: host choice of adult phytophagous insect females reflects a within-host variation in suitability as larval food. Chemoecology 20, 35-42.

Wiggins, N., Marsh, K., Wallis, I., Foley, W.J., McArthur, C., 2006. Sideroxylonal in *Eucalyptus* foliage influences foraging behaviour of an arboreal folivore. Oecologia 147, 272-279

Wilson, S., Kerley, G., 2003a. Bite diameter selection by thicket browsers: the effect of body size and plant morphology on forage intake and quality. Forest Ecology and Management 181, 51-65.

Wilson, S., Kerley, G., 2003b. The effect of plant spinescence on the foraging efficiency of bushbuck and boergoats: browsers of similar body size. Journal of Arid Environments 55, 150-158.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology 126, 485-493.

Zharare, G., Scogings P.F., 2011\. Responses of potted *Acacia natalitia* and *Scutia myrtina* saplings to type of fertiliser and rate of application. African Journal of Agricultural Research.

4. Does the growth differentiation balance hypothesis explain allocation to carbon based secondary metabolites in *Combretum apiculatum*, an African savanna woody species?

Dawood Hattas, Department of Biological Sciences, H.W. Pearson Building, University Avenue, University of Cape Town, Rondebosch, 7701, Cape Town, South Africa, Dawood.Hattas@uct.ac.za, Tel: +27216502443, Fax: +27216504041

Peter F Scogings, Department of Agriculture, University of Zululand, South Africa, scogingsp@unizulu.ac.za

Riitta Julkunen-Tiitto, Natural Product Research Laboratory, Department of Biology, University of Eastern Finland, Finland, RJT@uef.fi

4.1. Abstract

The growth differentiation balance hypothesis (GDBH) provides a framework that predicts a trade-off between costs of carbon based secondary metabolites (CBSMs) relative to the demand for photosynthate by growth. However, this hypothesis was developed using empirical evidence from northern boreal and temperate forests and the extent to which it is applicable to African savanna woody species remains unclear. The objective of this study was to investigate whether the GDBH explains allocation to CBSMs in the deciduous African savanna woody species *C. apiculatum* along a 6-point N gradient. The cornerstone prediction of the GDBH, i.e. the parabolic response in CBSMs along the N gradient, was not observed, with CBSMs showing chemical-specific responses. Quercetin, myricetin and kaempferol glycoside concentrations, all produced via the same pathway, responded differently across the N gradient. Flavonol glycoside concentration decreased as N became more abundant along the gradient which provides partial support for the carbon nutrient balance hypothesis. Simulated herbivory had no effect on photosynthesis, decreased foliar N and consequently increased C:N ratio, but did not induce an increase in CBSMs, with condensed tannins and flavonol glycosides being unaffected. Defoliated plants at low N levels expressed a capacity to compensate for lost biomass, which suggests a tolerance response, but plants at higher N levels were evidently C limited and thus unable to compensate. Our results show that the GDBH does not explain allocation to CBSMs in *C. apiculatum* and suggest that mechanistic explanations of plant allocation to CBSMs should not be limited to specific or general pools of defense compounds, but should include low molecular weight phenolic conjugates as they may respond differently.

4.2. Introduction

Mechanisms addressing phenotypic plasticity of plant secondary metabolites in relation to resource availability and environmental stress have been the subject of debate for many decades (Stamp 2003). Empirical evidence (Koricheva 1998 & 2002, Stamp 2003, Lambers & Poorter 2004, Glynn et al. 2003 & 2007) suggests that the growth differentiation balance hypothesis (GDBH, Loomis 1932, Lorio 1986, Herms & Mattson 1992) effectively explains the allocation of resources to carbon based secondary metabolites (CBSMs). Originally proposed by Loomis (1932), Herms & Mattson (1992) used this hypothesis to describe how the physiological trade-off between growth and differentiation processes interacts with nutrient availability to influence phenotypic expression of CBSMs (Stamp 2003).

The GDBH provides a framework that predicts a trade-off between costs of differentiation processes relative to the demand for photosynthate by growth, i.e. growth is inversely related to differentiation processes in the plant (Fig. 1, Herms & Mattson 1992, Glynn et al. 2007). Differentiation includes all processes not involved in growth, including the synthesis of CBSMs (Herms & Mattson 1992, Stamp 2003). The hypothesis assumes that nutrient limitation has a more adverse effect on growth than photosynthesis, which is predicted to result in an accumulation of carbohydrates in excess of growth demand, particularly if foliar N is lower than the level required for growth. The excess carbohydrates, manifested by an increased foliar C:N ratio, can then be inexpensively converted to CBSMs. Furthermore, the hypothesis assumes that herbivory is a strong selective force for the production of CBSMs.

The GDBH predicts that plants at low N availability should be limited in growth and photosynthetic capacity. Under these conditions, photosynthetic rate would be low due to a limited amount of N and consequently Rubisco, chlorophyll and phospholipid contents (Bryant et al. 1992). The limited assimilated C would then be allocated to the little growth that does occur, resulting in growth being favoured over

differentiation processes. Plants are therefore predicted to have a low growth rate and low secondary metabolites (Fig. 1). At high N availability plants have sufficient resources for rapid growth. Under these conditions plants should not be limited by photosynthesis and a greater proportion of photosynthate should be allocated for growth rather than secondary metabolites. However, at intermediate N availability, growth is limited by N, whereas photosynthesis would be less affected, i.e. net assimilation rate is at its maximum, whereas growth rate is constrained by limited N. This should result in an accumulation of excess carbohydrates which can be allocated to secondary metabolites (Loomis 1932, Herms & Mattson 1992, Stamp 2003). The resulting negative quadratic response pattern of secondary metabolites (Fig. 1) is only detectable experimentally when using a resource gradient with at least five levels (Stamp 2004). A recent test of the GDBH, which included a five level N gradient, found that *Salix sericea* and *S. eriocephala* generally responded as predicted by the GDBH (Glynn et al. 2007). The GDBH was developed using empirical evidence from northern boreal and temperate forests and the extent to which it is applicable to African savanna woody species remains unclear.

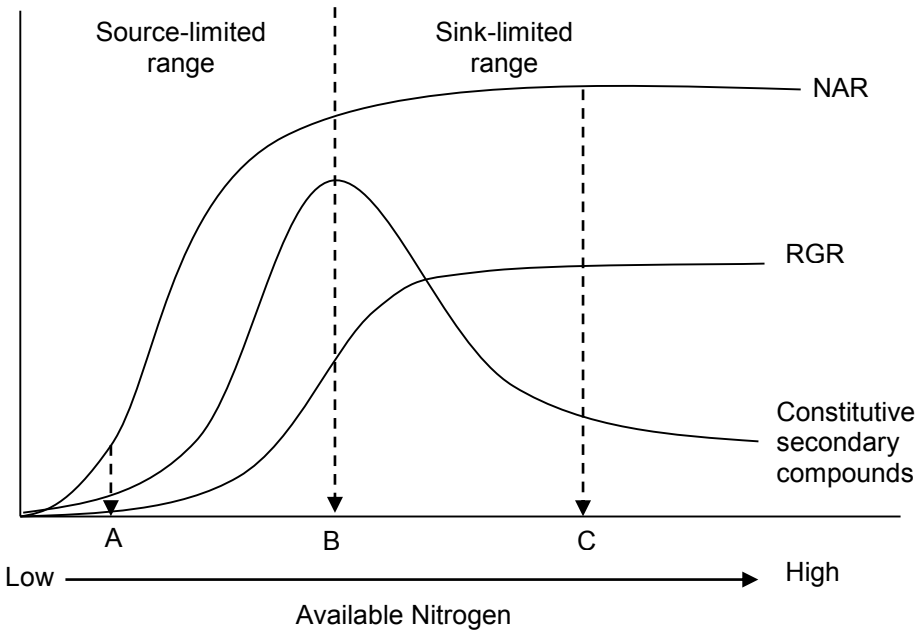


Figure 1. Relationship between net assimilation rate (NAR), relative growth rate (RGR) and constitutive secondary compound production along a N gradient. Letters A, B and C represent low, intermediate and high N levels, respectively (adapted from Herms & Mattson 1992, and Glynn et al. 2007).

It is well known that herbivory can induce increases in CBSMs (Haukioja 2006), although the mechanism by which this occurs remains contentious (Massey et al. 2005). Herbivory involves the removal of photosynthetic source tissue, i.e. leaves. This removal of source tissue results in a source-sink imbalance, to which plants have been shown to respond by increasing photosynthetic rate to acquire more assimilate to replace the removed material and re-establish the source-sink balance (McCormick et al. 2006). Herms & Mattson (1992) extended the GDBH, predicting that herbivory would cause an increase in CBSMs. However, the limited data on plant CBSM allocation following herbivory in African savanna woody species is inconsistent (Bryant et al. 1991, Rohner & Ward 1997, Scogings & Macanda 2005, Rooke and Bergström 2007, Scogings et al. 2011, Scogings et al. 2013). While Scogings et al. (2011) measured CBSMs along a browsing gradient, none of the

studies had a resource gradient, thus making it impossible to fully relate these results to predictions of the GDBH. These results suggest that more research is needed (Scogings 2005) to improve our understanding of allocation to CBSMs in savannas, and to establish whether these patterns can be explained in terms of the GDBH, as well as the role of herbivores in mediating phenotypic expression of CBSMs.

The GDBH was developed using empirical evidence from northern boreal and temperate forests, but has not been tested for African savanna woody species. The objective of this study was to investigate whether the GDBH explains allocation to CBSMs in the deciduous woody species *Combretum apiculatum* Sond. (Combretaceae). This species was selected because it is an important browse plant in African savannas that produces both condensed and hydrolysable tannins as well as flavonol glycosides (Hattas et al. 2011). Specifically, we address the following questions: (1) Does the GDBH explain carbon allocation to CBSMs along a 6-level N gradient? and (2) Does the GDBH explain carbon allocation to CBSMs following 100% simulated herbivory along a 6-level N gradient?

As predicted by the GDBH, we hypothesized that plants at the lower end of the N gradient would grow slowest and have the lowest foliar N and photosynthetic rate, since N is limiting. These plants would also have low CBSMs. Herbivory would constitute severe stress on these plants and with limited available N, these plants are expected to show low capacity for regrowth and little or no change in foliar N and consequently C:N ratio and photosynthesis. Allocation to CBSMs would be low since the limited assimilate would be used for the little growth (and maintenance respiration) that does occur. Plants in the middle of the N gradient would grow at an intermediate rate, have intermediate levels of foliar N, but high C:N ratio and CBSMs as a consequence of carbon accumulation, due to photosynthesis being less affected than growth. When subjected to herbivory, these plants are hypothesized to increase carbon allocation to above ground growth, i.e. leaf weight and area, as plants attempt

to restore source-sink imbalance, producing new growth with higher foliar N and consequently lower C:N ratios and elevated photosynthetic rates. CBSMs are expected to increase since the GDBH predicts that herbivory selects for defense. Plants at the high end of the N gradient would grow faster due to higher available N, have higher photosynthetic rates, but lower CBSMs than plants at the middle of the gradient. These plants are hypothesized to respond to herbivory by increasing carbon allocation to above ground growth, i.e. leaf weight and area, as plants attempt to restore source-sink imbalance, producing new growth with higher foliar N and consequently lower C:N ratios and higher photosynthetic rates. CBSMs are expected to increase since the GDBH predicts that herbivory selects for defense.

4.3. Materials and methods

4.3.1. Experimental setup

Combretum apiculatum saplings were bought from Fishwick's Wholesale Nursery, Nelspruit, South Africa. Saplings were transplanted into 5 litre pots filled with acid washed sand and allowed to acclimate to controlled chamber conditions, i.e. 25 ºC, PAR of 600-850, photoperiod of 16 h light and 8 h dark cycle and relative humidity of 54%, for 3 weeks while being fed twice a week with Long-Ashton solution (pH 5.5) containing 2 mM of $NaNO_3$ l^{-1} flushing with water before each feeding event and watering on days when not fed to prevent drought stress. After the acclimation period, ten plants per treatment were fed twice a week with Long-Ashton solution containing 0.25, 0.5, 1, 2, 3 and 4 mM of $NaNO_3$ l^{-1}, flushing with water before each feeding event and watering on days when not fed. Plants were arranged in accordance with a completely randomised single block design and rotated weekly to account for differences in environmental conditions within the chamber.

4.3.2. Photosynthesis and carbon allocation to biomass

Testing the GDBH requires a measure of net carbon assimilation rate (NAR) and relative growth rate (RGR), the interrelationship of which determines allocation to CBSMs (Herms & Mattson 1992, Stamp 2004, Glynn et al. 2007). NAR is the product of carbon gain through photosynthesis and carbon loss through respiration and leaching (Stamp 2004). Notwithstanding carbon losses, measuring photosynthetic rate nevertheless provides an impression of how variation in N level affects phenotypic carbon acquisition. Measures of above ground weight gain, leaf area ratio (LAR) which have been shown to be tightly correlated with RGR (Poorter & Remkes 1990, Lambers et al. 2008), and root weight ratio (RWR), provide information on the relative carbon investment to above- and below-ground resources, i.e. plant growth strategy in response to stress.

After six weeks herbivory was simulated by removing all the leaves including petiole and shoot apexes (100% simulated herbivory) from 5 plants per treatment. Simulated herbivory does not account for the different enzymatic response to damage from different elicitors (Karban & Baldwin 1997), but can be a useful tool to investigate effects of herbivory on growth and general physiological responses, e.g. changes in plant allocation to CBSMs at different nutrient levels (Hjältén 2004, Hrabar et al. 2009). Six weeks after the simulated herbivory event, photosynthetic rates were determined, using a LI-6400 Infra-red gas analyzer (LI-COR, Lincoln, Nebraska, USA), on 3 youngest fully expanded leaves of each tree, from which a mean value per sapling was calculated. These measurements were performed on consecutive days, measuring plants in the 0.25, 0.5 and 1 mM $NaNO_3$ l^{-1} treatments on the first day and the remaining treatments on the following day (~4.5 h uninterrupted/day). Plants were watered 1 h before measurements commenced to ensure fully hydrated plants to achieve maximum photosynthetic rates.

On the day immediately following the photosynthesis measurements, 5 youngest fully expanded leaves per sapling were collected, scanned for leaf area using the LI-3100 leaf area meter (LI-COR, Lincoln, Nebraska, USA), flash-frozen in liquid N and freeze-dried in a New Brunswick freeze dryer (New Brunswick Scientific Co., Inc., New Jersey, USA). Dried leaves were weighed and milled in a Retsch ball mill (F. Kurt Retsch, GmbH and Co. KG, Haan, Germany) to a fine powder. Dry weights were added to the weight of the rest of the leaves of the particular plant. On the same day as the leaf sampling, the entire plant was harvested and separated into leaves, shoots and roots. Plants were then dried at 60 ºC until constant weight and weighed to determine dry mass.

4.3.3. Chemical analysis

Foliar N and C were determined using a Delta Plus XP isotope ratio mass spectrometer (Thermo Electron, Germany) following combustion in a Flash EA 1112 elemental analyzer (Thermo Finnigan, Germany) and delivery via a Conflo III gas controlling unit (Thermo Finnigan, Italy). Nitrogen and carbon were standardized against atmospheric N and two in-house standards (relative to PDB-belemnite standard), respectively. Low molecular weight phenolics were extracted in methanol as outlined in Hattas et al. (2011) and identified and quantified using high-performance liquid chromatography (HPLC) as described by Julkunen-Tiitto and Sorsa (2001). Chemical identities were confirmed by HPLC-MS (Hattas et al. 2011). Condensed tannins (CTs) were determined using the acid-butanol assay (Porter et al. 1986) as modified by Hagerman (2002) and standardized against purified *Sorghum* tannin (Hattas & Julkunen-Tiitto 2012). Hydrolysable tannins, cinnamic acid derivatives; myricetin, quercetin and kaempferol glycosides were quantified separately, and flavonol glycosides (FG) constituted the sum of myricetin, quercetin and kaempferol glycosides. To account for the dilution effects due to growth and

storage (Koricheva 1999, Häring et al. 2007), leaf area was used to express foliar chemical concentrations on a per unit area basis.

4.3.4. Statistical analysis

Except for quercetin and kaempferol glycoside concentrations, data were normally distributed and thus did not require transformation. However, root weight ratio (RWR) being a proportion was arcsine transformed. Two-way ANOVA (Type III sums of squares) measured differences in plant biomass, photosynthesis and chemical variables between N fertilization concentration ([N]) and herbivory treatments. Means were separated with the Unequal N HSD post hoc test. The nonparametric Kruskal-Wallis ANOVA was performed to test for differences between [N] and herbivory treatments within N level for quercetin and kaempferol glycoside concentrations. Where differences were detected we employed multiple comparison tests to determine its source. Reported data are mean ± standard error. Analysis was performed using Statistica v10 (2011, StatSoft Inc., USA).

4.4. Results

Photosynthetic rate was not significantly affected by [N] ($F_{(5,156)}$=1.002, P=0.418) or herbivory treatments ($F_{(1,156)}$=0.526, P=0.469). Leaf weight showed significant interaction between [N] and herbivory treatment (Table 1, Fig. 2). Control plants showed a general increase in leaf weight with increase in [N], however, defoliated plants did not compensate for lost leaf weight at high [N], but did compensate at low [N]. Total plant weight showed a significant herbivory effect (Table 1), where total plant weight of control plants at the highest [N] was more than 3-fold that of simulated herbivory plants (P=0.007).

Table 1. F-ratios of 2-way ANOVAs for main and interactive effects of N fertilization level and simulated herbivory treatment on measured variables in *C. apiculatum*. LAR = Leaf area ratio, RWR = Root weight ratio and C:N = carbon:nitrogen ratio.

Variable	Main effect		Interaction
	N level	Herbivory	N level x herbivory
Leaf weight	4.554**	7.704**	2.866*
Total weight	1.843^{NS}	8.002**	2.032 ^{NS}
LAR	3.280*	6.838*	0.336^{NS}
RWR	5.848***	3.816 ^{NS}	1.142^{NS}
N	1.913^{NS}	33.68***	0.598^{NS}
C:N	6.474***	24.35***	1.460^{NS}
Cinnamic acid der conc.	3.434*	0.020^{NS}	0.473^{NS}
Flavonol glycosides	11.27***	0.106^{NS}	1.354^{NS}

Significant differences at ***P<0.001; **P<0.01; *P<0.05; NS – not significant

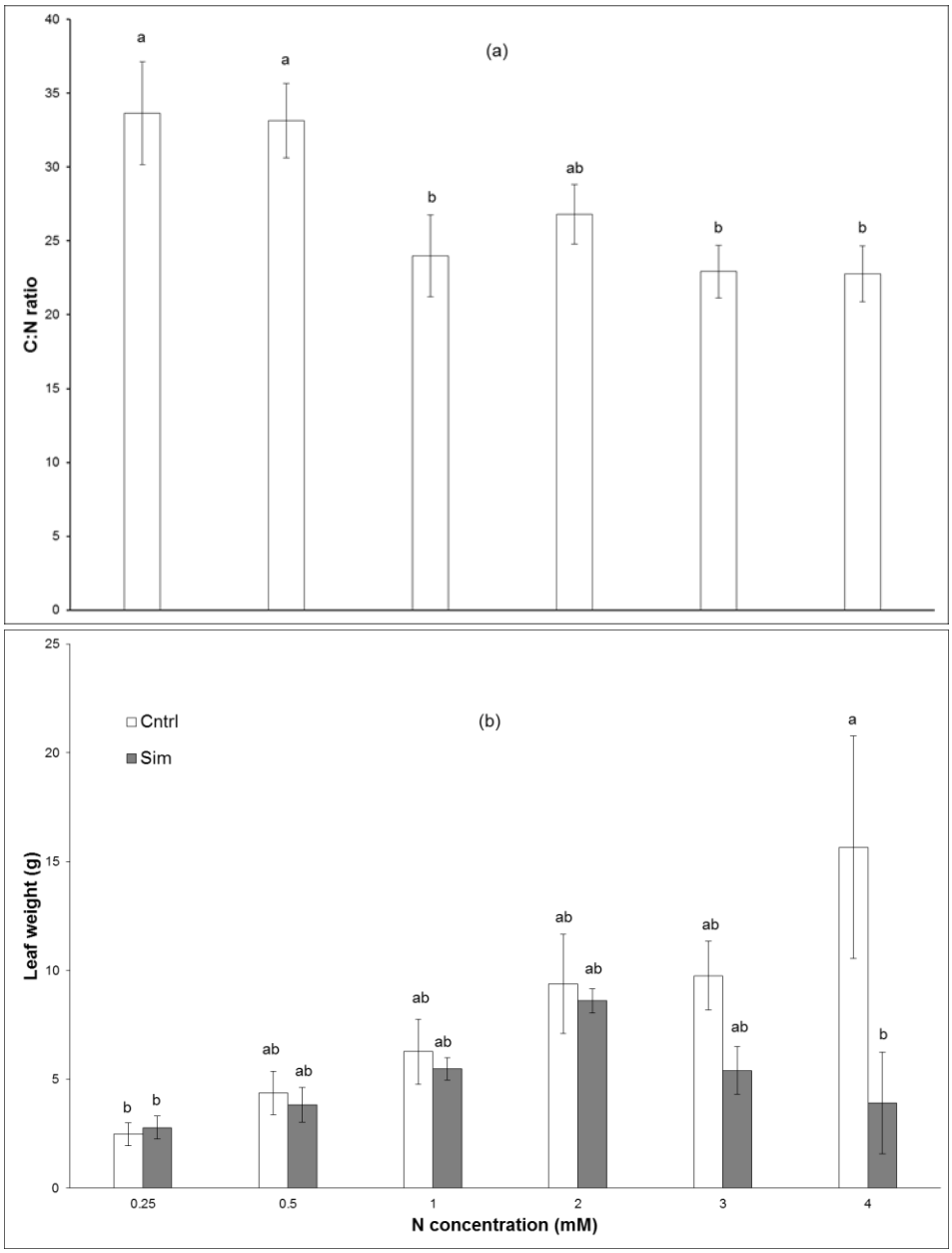


Figure 2. Effects of N concentration on foliar C:N ratio (a); and the effects of N concentration and 100% simulated herbivory on leaf weight (b) in *C. apiculatum*. Values are means ± SE and different letters (a and b) indicate significant differences among means (P<0.05).

Leaf area ratio increased, whereas RWR decreased with increasing [N] (Table 1, Fig. 3). LAR in defoliated plants was significantly higher (31%, P=0.01) relative to control plants. Foliar N concentration showed a significant defoliation effect

(Table 1), where N levels in control plants were 69% higher than plants subjected to simulated herbivory (P=0.0001). C:N ratio showed significant N and herbivory effects (Table 1, Fig. 4), where C:N ratio showed a significant decrease with increasing [N] in both treatments. C:N ratio in defoliated plants was 37% higher than control plants (P=0.0001).

Cinnamic acid and quercetin glycoside concentrations showed significant N effects (P=0.01, Table 1; and Kruskal-Wallis ANOVA, χ^2= 36.105, df = 5, P<0.0001; respectively), where concentrations at lower [N] (0.25 and 0.5 mM N) were generally high relative to that at higher N levels in both cases (Fig. 4). Similarly, a Kruskal-Wallis ANOVA revealed a significant N effect for kaempferol glycoside concentration (χ^2= 14.500, df = 5, P=0.01), showing a positive quadratic response to increasing [N] (Fig. 4). Flavonol glycoside concentration ([FG]) showed a significant N effect (Table 1), where concentrations at low [N] were generally higher than that at high [N] (Fig. 4). Condensed and hydrolysable tannin, as well as myricetin glycoside concentrations did not show a significant N or herbivory effect ([Condensed tannin]: P=0.253 and P=0.186; [Hydrolysable tannin]: P=0.120 and P=0.277; and [myricetin glycoside]: P=0.086 and P=0.527 for N and herbivory effects respectively).

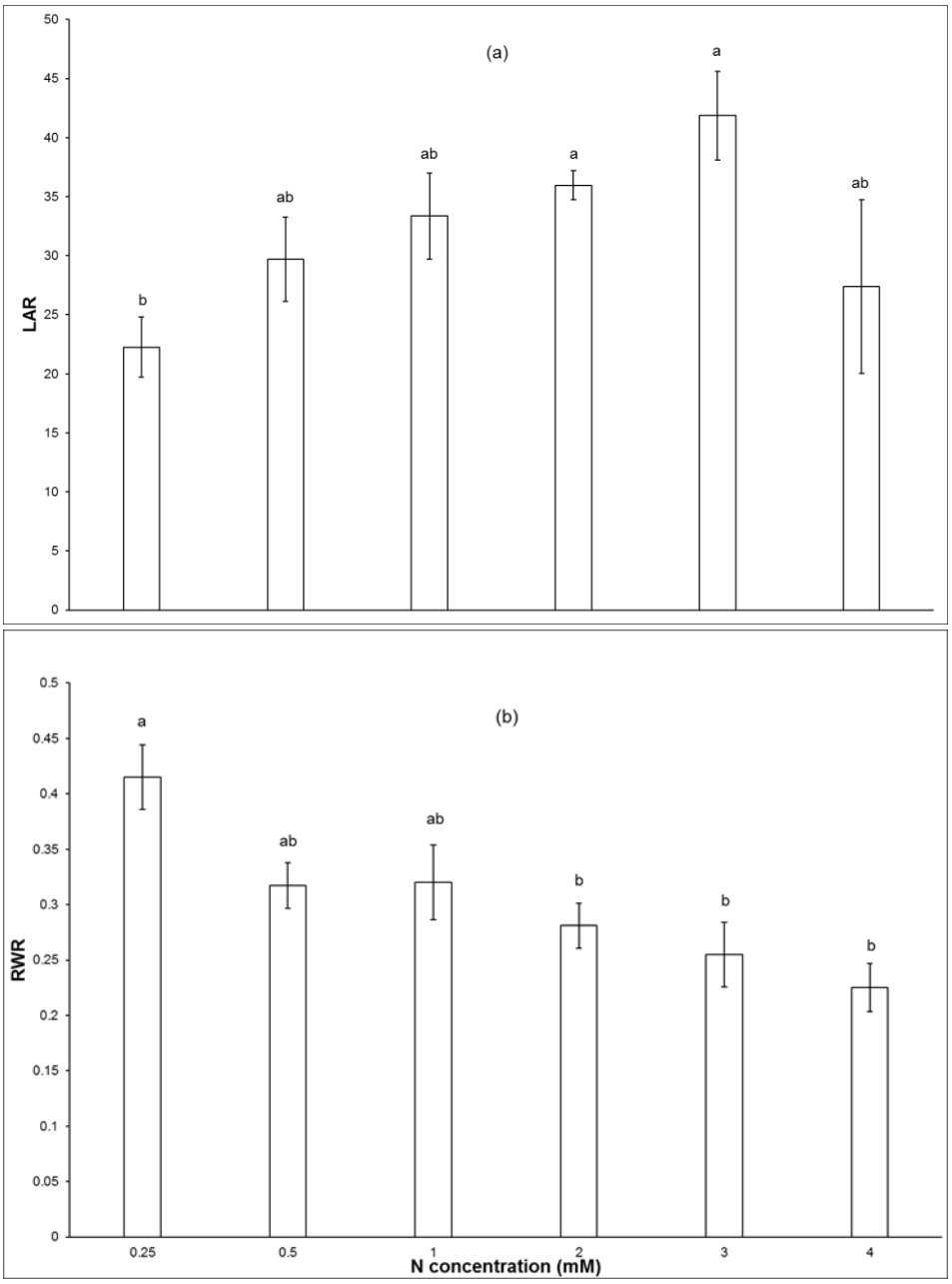


Figure 3. Effects of N concentration on leaf area ratio (LAR, a) and root weight ratio (RWR, b) in *C. apiculatum*. Values are means ± SE and different letters (a and b) indicate significant differences among means (P<0.05).

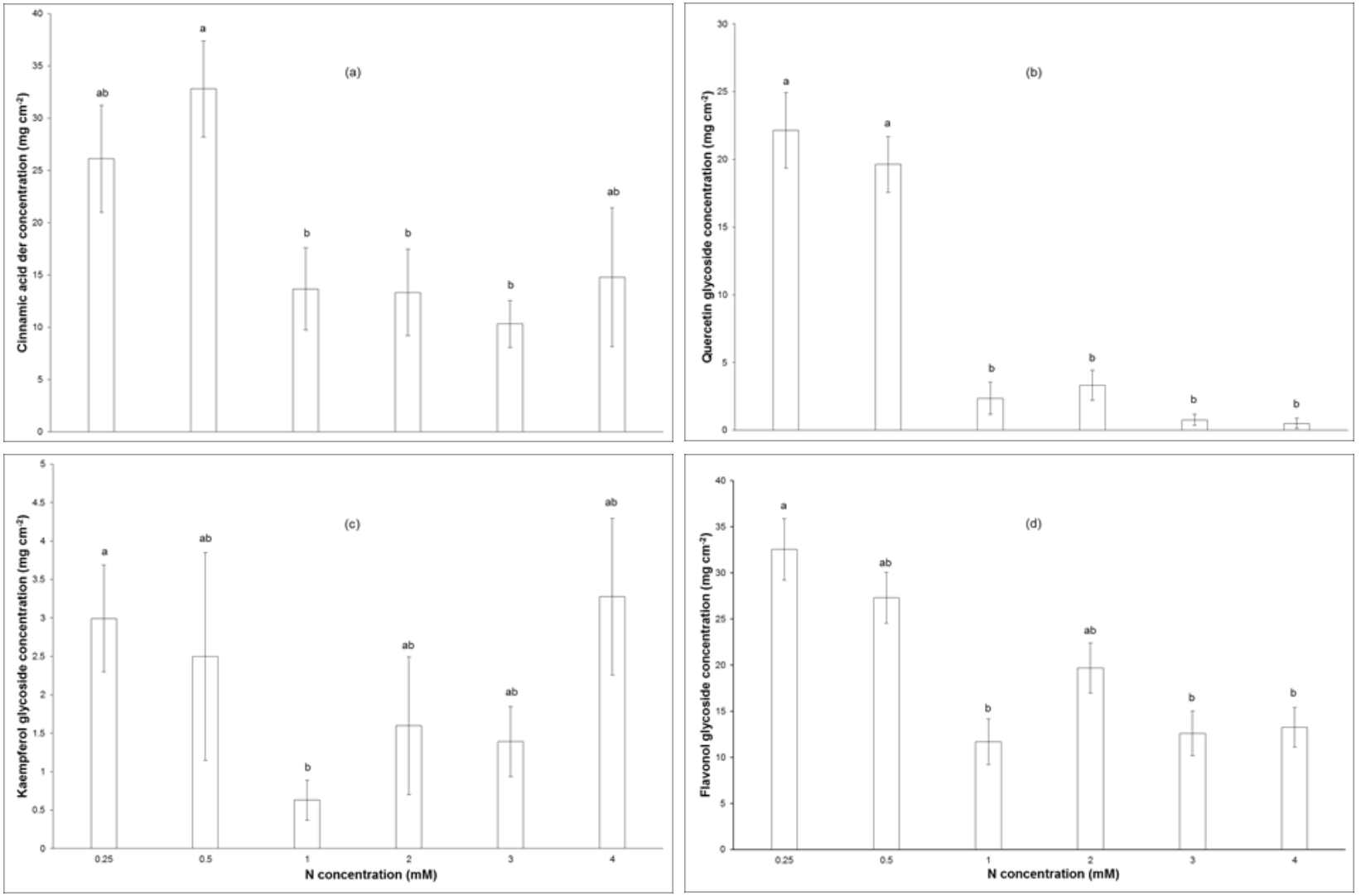


Figure 4. Effects of N level on cinnamic acid derivative (a), quercetin (b), kaempferol (c) and flavonol glycoside concentration (d) in *C. apiculatum*. Values are means ± SE and different letters (a and b) indicate significant differences between means (P<0.05).

4.5. Discussion

As predicted by the GDBH, we hypothesized that photosynthesis, leaf weight and LAR would increase, and that CBSMs would show a parabolic response with increasing [N]. The parabolic response in CBSMs is the cornerstone prediction of the GDBH (Stamp 2003, Glynn et al. 2007), however, this response pattern was not observed. Instead, CBSMs showed chemical-specific responses across the N gradient, which were associated with unchanged photosynthetic rates, an increase in leaf production and LAR, and a decrease in RWR. The stable end product CTs, which are putatively correlated with total allocation to secondary metabolites (Stamp 2004), was unaffected by treatment. Conversely, [FG] showed a general decrease, but its components showed varied responses with increasing [N]. Quercetin glycoside response pattern seemed to resemble that of cinnamic acid, its precursor (also precursor for kaempferol and myricetin glycosides), but kaempferol showed a positive quadratic response trend (Fig. 4), whereas myricetin glycoside concentration was unaffected. This differential response is not surprising since individual secondary chemicals are not necessarily correlated with total allocation to CBSMs (Stamp 2004) or flavonol glycosides (see Hattas et al. 2011).

Simulated herbivory had no effect on photosynthesis, but decreased foliar N and consequently increased C:N ratio. However, this apparent C surplus did not induce an increase in CBSMs in defoliated plants. Instead, our results show that defoliated plants at low N levels expressed a capacity to compensate for lost biomass (Fig. 2), which suggests a tolerance response. However, plants at high N levels tended to undercompensate. This disparity may be due to changing C/N dynamics imposed by defoliation. Plants are N limited at low N availability, but C limited relative to N at high N availability (because N is now abundant). Consider that defoliation removes both C and N, then already C limited plants at high N availability would experience exaggerated C shortages (Wise & Abrahamson 2005). Responding

to above ground biomass loss, the deciduous savanna tree *Acacia karroo* has been shown to draw on root C reserves (Schutz et al. 2009 & 2011), probably to activate dormant meristems and promote regrowth (Strauss & Agrawal 1999, Tiffin 2000). Plants at high N availability had lower RWR and therefore root mass and storage capacity, thus drawing from a smaller storage pool relative to plants at lower N levels. This withdrawal of C from an already limited storage supply would presumably exacerbate C limitation to the extent that plants may experience chronic C starvation which could affect its ability to compensate for biomass losses. Rooke & Bergström (2007) also showed that *C. apiculatum* saplings subjected to 100% defoliation could not compensate (in terms of regrowth) for lost biomass. Furthermore, Gao et al. (2008) showed that the perennial grass *Leymus chinensis* was unable to compensate for lost biomass at high nutrient treatment following 75% clipping, but could compensate at 50% clipping. This suggests that severe herbivory in nutrient rich environments may negatively affect plant fitness and may even be detrimental to its survival.

Simulated herbivory applied here ignores chemical elicitors present in herbivore salivary enzymes which have been shown to differentially affect growth response in *C. apiculatum* following simulated herbivory with or without goat saliva (Rooke 2003). The differential response, due to the absence of chemical elicitors here, may also affect expression of CBSMs. Furthermore, phytochemical expression in juvenile plants has been shown to be plastic relative to other ontogenetic phases (Koricheva & Barton 2012). Indeed, irrespective of treatment, [CT] here was ca. 50% that found by Hattas et al. (2011) and Scogings et al. (2011) for adult trees, while myricetin glycosides were present in juvenile trees here, but not detected in adult *C. apiculatum* trees (see Hattas et al. 2011). Results presented here may therefore not apply to other ontogenetic phases of *C. apiculatum*.

Nevertheless, our results show that CTs and FGs were unaffected by simulated herbivory. Higher C (indicated by foliar C:N ratio) in defoliated plants was used for compensatory regrowth at low [N], whereas defoliated plants at higher [N] were evidently C limited and thus unable to compensate. This inability of plants to compensate for losses at high nutrient concentration has been found elsewhere (see Wise & Abrahamson 2005, 2007) and is consistent with the predictions of the limiting resource model. This model incorporate considerations of whether the focal resource that differs between environments (i.e. [N] in our case) is limiting plant fitness and whether biomass removal affects mainly the focal resource or an alternate resource (Wise & Abrahamson 2005, 2007). The evidence presented here suggests that defoliation does indeed affect an alternate resource, i.e. plant C concentration, which indicates that N availability alone may not predict CBSMs, but that knowledge of plant C status as affected by herbivory is also important.

In summary, our findings did not support predictions of the GDBH, but the trend showed by [FG] provides partial support for the carbon nutrient balance hypothesis (CNBH) which explains how plant carbon:nutrient ratio influences the phenotypic expression of plant genetic potential for defense (Bryant et al. 1983). As predicted by the CNBH, plants showed a physiological response to low [N], by decreasing growth rate (relative to other [N] levels) and increasing carbon allocation to [FG]. Furthermore, as predicted, [FG] decreased as [N] became more abundant along the gradient. This is consistent with the CNBH which predicts that herbivore losses at high nutrient availability would be easily replaced by rapid regrowth, and that the production of CBSMs would be constrained (Bryant et al. 1983, Massey et al. 2005).

The observed decrease in [FG], in association with unchanged photosynthetic rate and increase in LAR with increasing N gradient, resembles the response pattern predicted by the GDBH between intermediate and high N level (see Fig. 1).

Therefore, our lowest N level may be equivalent to what the GDBH depicts as intermediate nutrient level. Nevertheless, our results show that mechanistic explanations of plant allocation to CBSMs should not be limited to specific or general pools of defense compounds (e.g. condensed and hydrolysable tannins or FGs), but should include low molecular weight phenolic conjugates because they may respond differently. Here we show that quercetin, myricetin and kaempferol glycoside concentrations, all produced via the same pathway, responded differently across the N gradient. The mechanisms controlling transcriptional regulation that promote gene expression for specific compounds need to be investigated. Our study is limited to phenolics derived from cinnamic and shikimic acid precursors and did not investigate N-rich alkaloids which have previously been reported in Combretaceae (see Ogan 1972 and Fiot et al. 2006). However, the presence of alkaloids in *Combretum* has not been confirmed (Eloff et al. 2008).

We studied one species in the sapling phase, which precludes generalization about the inadequate explanatory power of the GDBH in African savanna species. However, our results show that theories developed in northern boreal forests may not be directly applicable to Africa savanna species. Additional research is needed to test the GDBH in other African savanna woody species, as well as within ontogenic phases of the same species. This will add to our knowledge and understanding of phenotypic expression of CBSMs in African savannas.

5. Synthesis

I showed that CT chemical composition affects reactivity in the acid-butanol assay, and that reactivity is unrelated to MW. I further showed that *Quebracho* tannin consistently overestimated [CT], and by more than 8 times in certain species, thus its use as a CT standard cannot be justified. Conversely, *Sorghum* tannin proved to be an appropriate standard for *S. myrtina*. My findings show that using purified CTs from a phylogenetically related plant species may not be appropriate as CT standard since its chemical composition may differ. This was evident in the two *Acacia* species that showed qualitatively similar extender units, however, quantitative differences in extender units and differences in terminal units produced different CT regression slopes. This suggests that using purified CTs from a related plant species may not be an appropriate CT standard since its chemical composition may differ.

CT heterogeneity makes it impossible to find a generic CT standard that would be appropriate for all species. The proposed use of procyanidin based Granny Smith apple CTs (Li et al. 2010) would be inappropriate in four of the five tree species studied in chapter 2 since these four are predominantly prodelphinidin based. Similarly, *Sorghum* tannin, being 100% procyanidin (see Chapter 1, Table 3), was also inappropriate, underestimating [CT] in the four species. Comparing *Sorghum* with Granny Smith tannin as standard showed that *Sorghum* underestimated [CT] by 29% relative to Granny Smith tannin when quantifying *Ficus* [CT] (Li et al. 2010), but differences in using one or the other standard would depend on the chemical composition and reactivity of the CT in the species under investigation. Therefore, it is difficult to predict which standard would be more appropriate without information on chemical composition and reactivity of the species under investigation.

The acid-butanol assay is easy to replicate and requires minimal infrastructure to perform. However, the drawback of this assay is that it requires an

appropriate standard (Hagerman & Butler 1989). As mentioned earlier, purifying *Sorghum* and apple tannin is laborious, and challenging where the infrastructure does not exist. I therefore propose that in the absence of purified *Sorghum* and apple tannin, that the regression line for *Sorghum* tannin as reported in chapter 2 (Fig. 1) be used. To use this regression line, analysts would have to ensure that all reagents are chemically pure, solutions prepared exactly as outlined in Hagerman (2002) and that a 1 cm cuvette be used. Furthermore, the regression lines for the other study species could also be used to determine absolute tannin concentration where these species are studied. It would have to be acknowledged though that environmental factors may influence CT chemical composition and therefore reactivity, but using an absolute regression line, despite not being from the actual plant under investigation, would be more preferable than a generic standard.

In chapter 3 I showed that lmwp profile is not related to life history, morphological or functional traits. Myricitrin concentration was the only chemical amongst the ones determined that could explain why *E. divinorum* was mostly unpalatable. To test whether myricitrin is indeed the agent that deters feeding, it is proposed that a feeding trial be performed where the dietary [myricitrin] is varied in an artificial diet. An investigation into how [myricitrin] changes over time within a growth-year may also be useful since the little herbivory that does occur in *E. divinorum* has been shown to be concentrated during the dry-season when other food is not available (Scogings et al. 2014). I further showed that phylogenetically related *Acacia* species had different lmwp profiles. These differences could be explored further by sampling of *Acacia* to uncover the phylogeny of phenolic synthesis.

I showed that phylogenetically unrelated species had similar lmwp profiles. This suggests that the same phenylpropanoid pathways may enjoy genetic preference (Scioneaux et al. 2011) in these unrelated species. Assuming that the

genes controlling lmwp compounds production are similarly transcribed when subjected to the same environmental stresses, then we would be able to predict CBSM allocation amongst chemically similar functional types (chemotypes). This would provide another layer of functional traits that could strengthen our trait based predictive ability of plant response to different environmental stresses.

My findings revealed a trade-off between investments in CBSMs relative to spines, but the number of species here precludes generalization to be made. Additional work on a larger group of phylogenetically independent species is required to enable such generalization. Furthermore, I sampled mature trees from a herbivore exclosure to attain a baseline measure of lmwp's in the selected trees. Future work should focus on the effects of herbivory on the expression of these compounds. Though I measured the effects of simulated herbivory in *C. apiculatum*, these plants were saplings and CBSMs have been shown to differ with ontogeny (Barton & Koricheva 2010, Koricheva & Barton 2012), as has been confirmed here where myricitrin glycosides were present in juvenile but not in adult trees of *C. apiculatum*.

The GDBH did not explain allocation to CBSMs in *C. apiculatum*, with its characteristic quadratic response at intermediate nutrient concentration being absent. Instead CTs were unaffected and flavonol glycosides showed a decrease with increasing N gradient, whereas lmwp conjugates produced in the same pathway showed chemical-specific responses. Defoliation had no effect on CBSMs. However, experimental shortcomings have to be acknowledged: 1) The absence of salivary elicitors (see Rooke 2003) may have affected the response to defoliation; 2) Replication was small, which resulted in a lack of statistical power. This lack of power may have affected the ability to detect statistical differences and consequently committing Type II errors; 3) Nutrient addition here did not follow a steady state approach. Steady state nutrition requires the concentration of nutrients to be increased exponentially over time to match the exponential growth of the plant

(Ingestad & Lund 1986, Stamp 2004). Nevertheless, growth in general showed a linear increase over the N gradient, which suggest that nutrients were not limiting.

The usefulness the GDBH as a predictive model for CBSM allocation in African savanna woody species is being questioned. In response to stress, plants have been shown to mitigate its effects by up-regulating resource acquisition which mitigates any response to an extent that no trade-off between the synthesis of CBSMs and growth is detected (Mitra & Baldwin 2008, Moore et al. 2014). It is therefore not surprising that predictive models of plant defense are often not supported, as found here. Furthermore, when investigating [CT] allocation along a browsing intensity gradient, Scogings et al. (2011) found that *A. grandicornuta* was the only one out of four species that showed a quadratic response as predicted by the GDBH, whereas *C. apiculatum*, *G. flavescens* and *D. cinerea* responded differently. Nevertheless, due to the paucity of available data, it may be premature to dispose of the GDBH as a mechanistic model of defense allocation in African savanna woody species.

Results here show that chemical profiles change with ontogenic growth phase, it is therefore also possible that CT chemical composition may show similar ontogenic changes. Scioneaux et al. (2011) proposed that CT chemical composition may be controlled by dominance of specific phenylpropanoid pathway and proanthocyanidin-polymerization genes. Thus qualitative and quantitative variation in CTs, as well as lmwp's, may be controlled by distinct genetic mechanisms (Moore et al. 2014). Investigations into genetic mechanisms that control CT and lmwp chemical composition, and how these change with ontogeny are required.

An important source of variation that was not specifically investigated here is qualitative and quantitative intraspecific variation of CTs and lmwp's, but also intraspecific variation between different plant parts, e.g. leaves and shoots. We know

that different herbivores consume different plant parts, e.g. insects mostly consume only parts of the leaf, whereas different ungulates differentially consume leaves and twigs, and even remove entire branches (i.e. elephants). Furthermore, it has been shown that herbivores also feed on different zones of the same tree (Woolnough & du Toit 2001). Intraspecific variation between different parts and zones of the tree therefore suggests that different herbivores may consume biomass of potentially different quality.

The lmwp profile has been shown to change with increasing nutrient availability in *C. apiculatum* (Hattas et al. unpublished data) which suggests that future studies, particularly related to atmospheric change, should include qualitative in combination with quantitative measures of lmwp's. This will provide insight into chemical traits that are under genetic control as opposed to those that are plastic. Such knowledge may be important since anti-herbivore agents may be subject to phenotypic changes as a result of a changing environment.

Bibliography

Agrawal, A.A., 2007. Macroevolution of plant defense strategies. Trends Ecol. Evol. 22, 103-109.

Bergström, R., 1992. Browse characteristics and impact of browsing on trees and shrubs in African savannas. J. Veg. Sci. 3, 315-324.

Brunt, C., Read, J., Sanson, G.D., 2006. Changes in resource concentration and defence during leaf development in a tough-leaved (*Nothofagus moorei*) and soft-leaved (*Toona ciliate*) species. Oecologia 148, 583-592.

Bryant, J.P., Heitkonig, I., Kuropat, P., Owen-Smith, N., 1991. Effects of severe defoliation on the long-term resistance to insect attack and on leaf chemistry in six woody species of the southern African savanna. Am. Nat. 137, 50-63.

Bryant, J.P., Reichardt, P.B., Clausen, T.P., 1992. Chemically mediated interactions between woody plants and browsing mammals. J. Range Manage. 45, 18-24.

Bryant, J.P., Tuomi, J., Niemala, P., 1988. Environmental constraint of constitutive and long-term inducible defenses in woody plants, in: Spencer, K. (Ed.), Chemical Mediation of Coevolution. Academic Press Inc., pp. 367-389.

Cerling, T.E., Harris, J.M., Leakey, M.G., 1999. Browsing and grazing in elephants: the isotope record of modern and fossil proboscideans. Oecologia 120, 364-374.

Coley, P., Barone, J., 1996. Herbivory and plant defenses in tropical forests. Annual Review Ecology and Systematics 27, 305–335.Coley, P.D., Bryant, J.P., FS, Chapin III, 1985. Resource availability and plant antiherbivore defense. Science 230, 895-899.

Cooper, S.M., Owen-Smith, N., 1985. Condensed tannins deter feeding by browsing ruminants in a South African savanna. Oecologia 67, 142-146.

Dixon, R.A., Xie, D., Sharma, S.B., 2005. Proanthocyanidins: A final frontier in flavonoid research? New Phytol. 165, 9-28.

du Toit, J.T., 2003. Large herbivores and savanna heterogeneity, in: du Toit, J.T., Rogers, K.H., Biggs, H.C. (eds.), The Kruger Experience: Ecology and Management of Savanna Heterogeneity. Island Press, pp. 292-309.

Eloff, J., Katerere, D., McGaw, L., 2008. The biological activity and chemistry of the southern African Combretaceae. J. Ethnopharmacol. 119, 686-699.

Fiot, J., Sanon, S., Azas, N., Mahiou, V., Jansen, O., Angenot, L., Balansard, G., Ollivier, E., 2006. Phytochemical and pharmacological study of roots and leaves of *Guiera senegalensis* JF Gmel (Combretaceae). J. Ethnopharmacol. 106, 173-178.

Gao, Y., Wang, D., Ba, L., Bai, Y., Liu, B., 2008. Interactions between herbivory and resource availability on grazing tolerance of *Leymus chinensis*. Environ. Exp. Bot. 63, 113-122.

Giner-Chavez, B.I., Van Soest, P.J., Robertson, J.B., Lascano, C., Reed, J.D., Pell, A.N., 1997. A method for isolating condensed tannins from crude plant extracts with trivalent ytterbium. J. Sci. Food Agric. 74, 359-368.

Glynn, C., Herms, D.A., Egawa, M., Hansen, R., Mattson, W.J., 2003. Effects of nutrient availability on biomass allocation as well as constitutive and rapid induced herbivore resistance in poplar. Oikos 101, 385-397.

Glynn, C., Herms, D.A., Orians, C.M., Hansen, R.C., Larsson, S., 2007. Testing the growth differentiation balance hypothesis: Dynamic responses of willows to nutrient availability. New Phytol. 176, 623-634.

Gu, L., Kelm, M.A., Hammerstone, J.F., Beecher, G., Holden, J., Haytowitz, D., Prior, R.L., 2003. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. J. Agric. Food Chem. 51, 7513-7521.

Hagerman, A.E., 2002. Tannin handbook. URL [http://www.users.muohio.edu/hagermae/tannin.pdf]; downloaded March 2004.

Hagerman, A. E., Butler, L.G., 1991. Tannins and lignins, - In: Rosenthal, G.A., Berenbaum, M.R. (eds.), Herbivores: Their interactions with secondary plant metabolites (2nd Edition). Academic Press: New York, pp. 355-388.

Hagerman, A.E., Butler, L.G., 1989. Choosing appropriate methods and standards for assaying tannin. J. Chem. Ecol. 15, 1795-1810.

Hagerman, A.E., Robbins, C.T., Weerasuriya, Y., Wilson, T.C., McArthur, C., 1992. Tannin chemistry in relation to digestion. J. Range Manage. 45, 57-62.

Harborne, J.B., 2001. Twenty-five years of chemical ecology. Nat. Prod. Rep. 18, 361-379.

Harborne, J.B., 1991. The Chemical Basis of Plant Defense. CRC Press, Inc.

Häring, D., Suter, D., Amrhein, N., Lüscher, A., 2007. Biomass allocation is an important determinant of the tannin concentration in growing plants. Ann. Bot. 99, 111-120.

Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F., Rooke, T., 2011. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. Phytochemistry 72, 1796-1803.

Hattas, D., Julkunen-Tiitto, R., 2012. The quantification of condensed tannins in African savanna tree species. Phytochemistry Lett. 5: 329-334

Hattas, D., Stock, W., Mabusela, W., Green, I., 2005. Phytochemical changes in leaves of subtropical grasses and fynbos shrubs at elevated atmospheric CO_2 concentrations. Global Planet. Change 47, 181-192.

Haukioja, E., 2006. Tree defenses against insects. Springer Science and Business, Media Inc.

Herms, D.A., Mattson, W.J., 1992. The dilemma of plants: To grow or defend. Q. Rev. Biol. 67, 283-335.

Hjältén, J. 2004. Simulating Herbivory: Problems and possibilities. – In: Weisser W.W. and Siemann E. (eds.). Insects and Ecosystem Function. Springer-Verlag, Berlin, pp 243-256.

Hrabar, H., Hattas, D., Toit, J., 2009. Differential effects of defoliation by mopane caterpillars and pruning by African elephants on the regrowth of *Colophospermum mopane* foliage. J. Trop. Ecol. 25, 301-309.

Ingestad, T., Lund, A., 1986. Theory and techniques for steady state mineral nutrition and growth of plants. Scand. J Forest Res. 1, 439-453.

Julkunen-Tiitto, R., Sorsa, S., 2001. Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. J. Chem. Ecol. 27, 779-789.

Karban, R., Baldwin, I., 1997. Induced responses to herbivory University of Chicago Press. Chicago, Illinois, USA.

Kleiner, W.K., Raffa, K.F., Dickson, R.E., 1999. Partitioning of ^{14}C-labeled photosynthate to allelochemicals and primary metabolites in source and sink of aspen: evidence for secondary metabolite turnover. Oecologia 119, 408-418.

Koricheva, J., 2002. Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. Ecology 83, 176-190.

Koricheva, J., 1999. Interpreting phenotypic variation in plant allelochemistry: problems with the use of concentrations. Oecologia 119, 467-473.

Koricheva, J., Barton, K. E. 2012. Temporal changes in plant secondary metabolite production: Patterns, causes and consequences. – In: Iason G.R., Dicke M., Hartley S.E. (eds.). The ecology of plant secondary metabolites: From genes to global processes. Cambridge University Press, New York, USA, pp. 34-55.

Koricheva, J., Larsson, S., Haukioja, E., Keinänen, M., 1998. Regulation of woody plant secondary metabolism by resource availability: Hypothesis testing by means of meta-analysis. Oikos 83, 212-226.

Lambers, H., Chapin III, F.S., Pons, T.L., 2008. Plant Physiological Ecology. Springer.

Lawler, I.R., Foley, W.J., Eschler, B.M., 2000. Foliar concentration of a single toxin creates habitat patchiness for marsupial folivore. Ecology 81, 1327-1338.

Leimu, R., Koricheva, J., 2006. A meta-analysis of tradeoffs between plant tolerance and resistance to herbivores: Combining the evidence from ecological and agricultural studies. Oikos 112, 1-9.

Li, C., Trombley, J.D., Schmidt, M.A., Hagerman, A.E., 2010. Preparation of an acid butanol standard from fresh apples. J. Chem. Ecol. 36, 453-460.

Loomis, W., 1932. Growth-differentiation balance vs. carbohydrate-nitrogen ratio. 29, 240-245.

Lorio, P.L., 1986. Growth-differentiation balance: A basis for understanding southern pine beetle-tree interactions. For. Ecol. Manage. 14, 259-273.

MacKown, C.T., Carver, B.F., Edwards, J.T., 2008. Occurrence of condensed tannins in wheat and feasibility for reducing pasture bloat. Crop Sci. 48, 2470-2480.

Marsh, K.J., Wallis, I.R., Foley, W.J., 2007. Behavioural contributions to the regulated intake of plant secondary metabolites in koalas. Oecologia 154, 283-290.

Massad, T.J., Dyer, L.A., Vega C., G., 2012. Costs of defense and a test of the carbon-nutrient balance and growth-differentiation balance hypotheses for two co-occurring classes of plant defense. PLoS ONE 7, 1-8.

Massey, F.P., Press, M.C., Hartley, S.E., 2005. Long- and short-term induction of defences in seedlings of *Shorea leprosula* (Dipterocarpaceae): Support for the carbon: nutrient balance hypothesis. J. Trop. Ecol. 21, 195-201.

Mattson, W. J., Julkunen-Tiitto, R., Herms, D. A., 2005. CO_2 enrichment and carbon partitioning to phenolics: Do plant responses accord better with the protein competition or the growth differentiation balance models? Oikos 111, 337-347.

Mitra, S., Baldwin, I.T., 2014. RuBPCase activase (RCA) mediates growth-defense trade-offs: silencing RCA redirects jasmonic acid (JA) flux from JA-isoleucine to methyl jasmonate (MeJA) to attenuate induced defense responses in *Nicotiana attenuata*. New Phytol. 201, 1385-1395. doi: 10.1111/nph.12591 [doi].

Moore, B.D., Andrew, R.L., Külheim, C., Foley, W.J., 2014. Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New Phytol. 201, 733-750.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010-2037.

Müller, V., Lankes, C., Zimmermann, B.F., Noga, G., Hunsche, M., 2013. Centelloside accumulation in leaves of *Centella asiatica* is determined by resource partitioning between primary and secondary metabolism while influenced by supply levels of either nitrogen, phosphorus or potassium. J. Plant Physiol. 170, 1165-1175

Ogan, A., 1972. The alkaloids in the leaves of *Combretum micranthum*. Planta Med. 21, 210-217.

Owen-Smith, N., 2002. Adaptive herbivore ecology. Cambridge University Press.

Owen-Smith, N., Cooper, S.M., 1987. Palatability of woody plants to browsing ruminants in a South African savanna. Ecology, 319-331.

Owen-Smith, N., DANCKWERTS, J., 1997. Herbivory, in: Cowling, R.M., Richardson, D.M., Pierce, S.M. (Eds.), Vegetation of Southern Africa, pp. 397-420.

Poorter, H., Remkes, C., 1990. Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia 83, 553-559.

Porter, L.J., Hrstich, L.N., Chan, B.G., 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25, 223-230.

Rautio, P., Bergvall, U., Karonen, M., Salminen, J., 2007. Bitter problems in ecological feeding experiments: Commercial tannin preparations and common methods for tannin quantifications. Biochem. Syst. Ecol. 35, 257-262.

Rhoades, D.F., 1979. Evolution of plant chemical defense against herbivores. – In: Rosenthal, G., Janzen, D. (eds.). Herbivores: Their interaction with secondary plant metabolites. Academic Press, New York, USA, pp. 3-54

Rohner, C., Ward, D., 1997. Chemical and mechanical defense against herbivory in two sympatric species of desert *Acacia*. J. Veg. Sci. 8, 717-726.

Rooke, T., 2003. Growth responses of a woody species to clipping and goat saliva. Afr. J. Ecol. 41, 324-328.

Rooke, T., Bergstrom, R., 2007. Growth, chemical response and herbivory after simulated leaf browsing in *Combretum apiculatum*. Plant Ecol. 189, 201-212.

Rosenthal, G., Janzen, D., 1979. Herbivores: Their Interaction with secondary plant metabolites. Academic Press. New York.

Salminen, J., Karonen, M., 2011. Chemical ecology of tannins and other phenolics: We need a change in approach. Funct. Ecol. 25, 325-338.

Scholes, R.J., Walker, B.H., 1993. An African savanna: Synthesis of the Nylsvley study. Cambridge University Press.

Schutz, A.E., Bond, W.J., Cramer, M.D., 2011. Defoliation depletes the carbohydrate reserves of resprouting *Acacia* saplings in an African savanna. Plant Ecol. 212, 2047-2055.

Schutz, A.E., Bond, W.J., Cramer, M.D., 2009. Juggling carbon: Allocation patterns of a dominant tree in a fire-prone savanna. Oecologia 160, 235-246.

Scioneaux, A.N., Schmidt, M.A., Moore, M.A., Lindroth, R.L., Wooley, S.C., Hagerman, A.E., 2011. Qualitative variation in proanthocyanidin composition of *Populus* species and hybrids: Genetics is the key. J. Chem. Ecol. 37, 57-70.

Scogings, P. F., 2005. Rapid chemical responses of *Acacia karroo* to early dormant season defoliation in a semi-arid subtropical savanna. J. Arid Environ. 62, 225-233.

Scogings, P.F., Dziba, L.E., Gordon, I.J., 2004. Leaf chemistry of woody plants in relation to season, canopy retention and goat browsing in a semiarid subtropical savanna. Austral Ecol. 29, 278-286.

Scogings, P. F., Hjältén, J., Skarpe, C., 2013. Does large herbivore removal affect secondary metabolites, nutrients and shoot length in woody species in semi-arid savannas? J. Arid Environ. 88, 4-8.

Scogings, P.F., Hjältén, J., Skarpe, C., 2011. Secondary metabolites and nutrients of woody plants in relation to browsing intensity in African savannas. Oecologia 167, 1063-1073.

Scogings, P.F., Hjältén, J., Skarpe, C., Hattas, D., Zobolo, A., Dziba, L., Rooke, T., 2014. Nutrient and secondary metabolite concentrations in a savanna are independently affected by large herbivores and shoot growth rate. Plant Ecol. 215, 73-82.

Scogings, P. F., Macanda, M., 2005. *Acacia karroo* responses to early dormant season defoliation and debarking by goats in a semi-arid subtropical savanna. Plant Ecol. 179, 193-206.

Scogings, P.F., Mamashela, T.C., Zobolo, A.M., 2012. Deciduous sapling responses to season and large herbivores in a semi-arid African savanna. Austral Ecol. 38: 548-556.

Shrader, A.M., Bell, C., Bertolli, L., Ward, D., 2012. Forest or the trees: At what scale do elephants make foraging decisions? Acta Oecol. 42, 3-10.

Siegler, D.S., 1998. Plant Secondary Metabolism. Kluwer Academic Press, Massachusetts, USA.

Stamp, N., 2004. Can the growth-differentiation balance hypothesis be tested rigorously? Oikos 107, 439-448.

Stamp, N., 2003. Out of the quagmire of plant defense hypotheses. Q. Rev. Biol. 78, 23-55.

Stolter, C., Ball, J.P., Julkunen-Tiitto, R., Lieberei, R., Ganzhorn, J.U., 2005. Winter browsing of moose on two different willow species: Food selection in relation to plant chemistry and plant response. Can. J. Zool. 83, 807-819.

Strauss, S.Y., Agrawal, A.A., 1999. The ecology and evolution of plant tolerance to herbivory. Trends Ecol. Evol. 14, 179-185.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production - Progress and challenges. Anim. Feed Sci. Technol. 147, 116-139.

Ward, D., Young, T.P., 2002. Effects of large mammalian herbivores and ant symbionts on condensed tannins of *Acacia drepanolobium* in Kenya. J. Chem. Ecol. 28, 921-937.

White, T.C., 2005. Why does the world stay green? Nutrition and survival of plant-eaters. CSIRO Publishing.

Wiggins, N.L., Marsh, K.J., Wallis, I.R., Foley, W.J., McArthur, C., 2006. Sideroxylonal in *Eucalyptus* foliage influences foraging behaviour of an arboreal folivore. Oecologia 147, 272-279.

Wise, M.J., Abrahamson, W.G., 2007. Effects of resource availability on tolerance of herbivory: A review and assessment of three opposing models. Am. Nat. 169, 443-454.

Wise, M.J., Abrahamson, W.G., 2005. Beyond the compensatory continuum: Environmental resource levels and plant tolerance of herbivory. Oikos 109, 417-428.

Woolnough, A.P., du Toit, J.T., 2001. Vertical zonation of browse quality in tree canopies exposed to a size-structured guild of African browsing ungulates. Oecol 129, 585-590.

Xie, D., Dixon, R.A., 2005. Proanthocyanidin biosynthesis - still more questions than answers? Phytochemistry 66, 2127-2144.

Appendix: Authorship statements

FACULTY OF SCIENCE AND AGRICULTURE

Department of Agriculture

UNIVERSITY OF ZULULAND

Website: http://www.unizulu.ac.za

Private Bag X1001
3886 KwaDlangezwa

Tel: 035 902 6063
Fax: 035 902 6056

RESTRUCTURED FOR RELEVANCE

4 February 2014

To whom it may concern:

I hereby testify that the first author of the following published articles was the lead researcher in the work done towards completing the articles.

Mr Hattas conceptualised the research, designed and conducted the data collection and analysis, compiled the figures and tables and wrote the articles. The co-authors contributed by facilitating the work through providing specialist expertise, funds, facilities, supervision and editorial advice.

The papers are as follows:
Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F., Rooke, T., 2011. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. Phytochemistry 72: 1796-1803.
Hattas, D., Julkunen-Tiitto, R., 2012. The quantification of condensed tannins in African savanna tree species. Phytochemistry Letters 5: 329-334.

Yours sincerely,

P. F. Scogings, PhD

UNIVERSITY OF
EASTERN FINLAND

To the UNIVERSITY OF CAPE TOWN

From Prof. Riitta Julkunen-Tiitto
University of Eastern Finland
Department of Biology
Natural Product Research Laboratory
Yliopistokatu 7
FI-80100 Joensuu, Finland

I hereby testify that Mr. Dawood Hattas made a substantial contribution to the conceptualization and designed of the project. He independently ran the experiments, performed the chemical and physiological, as well as statistical analysis. He independently wrote the manuscript with comments and suggestion from supervisors and submitted and dealt with referees' comments in collaboration with co-authors/myself.

I contributed to the conceptualization (and/or design) of this research project and provided comments and suggestions on the manuscript.

In Joensuu 5th of February,

Riitta Julkunen-Tiitto

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Forestry/Department of Wildlife, Fish, and Environmental Studies
Joakim Hjältén

Certificate

To Whom It May Concern,

I hereby testify that Dawood Hattas made a substantial contribution to the conceptualization and design of the research project that ultimately resulted in the publication: Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F., Rooke, T., 2011. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. Phytochemistry 72: 1796-1803. He independently ran the experiments, performed the chemical and physiological, as well as statistical analysis. He independently wrote the manuscript supported with comments and suggestion from the co-authors. He was the corresponding author of the manuscript and as such submitted and dealt with referees' comments in collaboration with co-authors/myself.

My contribution to the project/paper was conceptual and I also provided comments and suggestions on the manuscript.

Umeå, 5 February 2014

Joakim Hjältén
Professor
Department of Wildlife, Fish, and Environmental Studies
Swedish University of Agricultural Sciences
Umeå, Sweden

Swedish University of Agricultural Science
Faculty of Forestry
Department of Wildlife, Fish,
and Environmental studies
SE-901 83 UMEÅ, SWEDEN

SE-90183 Umeå, Sweden
Besök/visits: Skogsmarksgränd
www.slu.se/xxx

tel: +46-70-3178183
mob: +46-70-3178183
joakim.hjalten@slu.se

To Whom It May Concern,

I hereby testify that Dawood Hattas made a substantial contribution to the conceptualization and design of the research project that ultimately resulted in the publication: *Hattas, D., Hjältén, J., Julkunen-Tiitto, R., Scogings, P.F., Rooke, T., 2011. Differential phenolic profiles in six African savanna woody species in relation to antiherbivore defense. Phytochemistry 72: 1796-1803.* He independently ran the experiments, performed the chemical and physiological, as well as statistical analysis. He independently wrote the manuscript supported with comments and suggestion from the co-authors. He was the corresponding author of the manuscript and as such submitted and dealt with referees' comments in collaboration with co-authors.

I was co-leader for the main project (the Zululand/Sweden Kruger Browse Project) within which this research was undertaken. My contribution to this specific project/paper was conceptual.

Östersund, 6 February 2014

TUULIKKI ROOKE
Senior Research officer

SWEDISH ENVIRONMENTAL PROTECTION AGENCY
Research and Assessment Department, Biodiversity Unit
SE-106 48 Stockholm, Sweden