

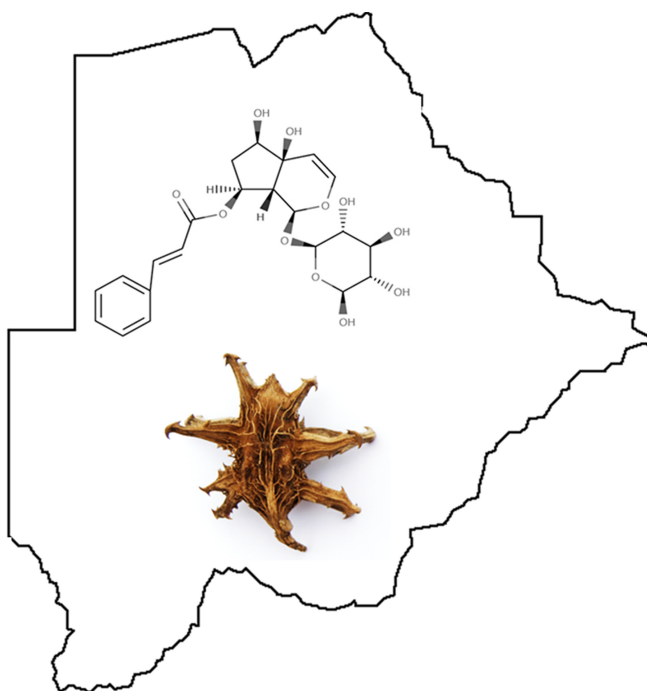


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FACULTY OF LANDSCAPE ARCHITECTURE, HORTICULTURE
AND CROP PRODUCTION SCIENCE

Genetic, Morphological and Chemical Variation in the Genus *Harpagophytum*

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Cover: Inside the map of Botswana is a seed capsule of *Harpagophytum procumbens* and the chemical structure of the main ingredient of harpagoside, harpagide.

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Abstract

Harpagophytum procumbens is a valuable medicinal plant but mistakes with plant identification at harvesting, and wilful adulteration, using the presumably less efficient *H. zeyheri*, are suggested. A multidisciplinary study with plant material from Botswana was conducted: (1) Morphometric measurements on seed capsules showed continuous variation between and, in some cases, within locations indicating occurrence of interspecific hybridization. (2) Mitotic and meiotic chromosome counts, that need to be ascertained, suggest $2n=20$, $20/22$ and 24 in the studied materials. Meiosis in a putative hybrid appeared regular but occurrence of dyads and triads at the tetrad stage indicated disturbances. (3) DNA markers for seedlings from field-collected seed capsules suggest that outcrossing is prevalent. While the two species showed significant differentiation, some morphologically deviating plants had intermediate DNA profiles possibly representing introgression products. (4) Analysis of selected biochemical compounds in peel and pulp of the tubers showed that content of the main bioactive compound harpagoside was significantly higher in *H. procumbens* compared to putative hybrids and *H. zeyheri*. In both peel and pulp, contents of isoverbascoside, verbascoside, acetylacteoside and pagoside were higher in *H. procumbens* and putative hybrids compared to *H. zeyheri*. (5) Tuber extracts were investigated for antioxidant capacity, content of total phenols, important phenylpropanoids and selected iridoids, and capacity to suppress respiratory burst in terms of reactive oxygen species produced by human neutrophils challenged with phorbol myristate acetate, opsonized *Staphylococcus aureus* and *Fusobacterium nucleatum*. A variety of *H. procumbens* ssp. *transvaalense* showed the highest degree of antioxidant capacity, whereas an anti-inflammatory effect could not be corroborated for any of the tested extracts.

Keywords: antioxidant, anti-inflammatory, devil's claw, DNA marker, harpagoside, HPLC, ISSR, medicinal plant, RAPD, ROS

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Dedication

Dedicated to all those who have been with me through thick and thin to turn the PhD dream into a success story.

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text.

- I Muzila, M., Werlemark, G., Ortiz, R., Sehic, J., Fatih, M., Setshogo, M., Mpoloka, W. and Nybom, H. (2014). Assessment of diversity in *Harpagophytum* with RAPD and ISSR markers provides evidence of introgression. *Hereditas* 151: 91–101.
- II Muzila M., Ekholm A., Nybom H., Widén C. and Rumpunen K. *Harpagophytum* germplasm varies in tuber peel and pulp content of important phenylpropanoids and iridoids. Manuscript submitted.
- III Muzila, M., Rumpunen, K., Wright, H., Roberts, H., Grant, M., Nybom, H., Sehic, J., Ekholm, A. and Widén, C. Alteration of neutrophil reactive oxygen species production by extracts of devil's claw (*Harpagophytum*). Manuscript submitted.

The contribution of Mbaki Muzila to the papers included in this thesis was as follows:

- I Planned the study with co-authors. Collected seed capsules in Botswana. Germinated seedlings in Sweden, performed all experimental work and data analysis, produced the first draft and then wrote the manuscript together with co-authors.
- II Collected tubers and seed capsules in Botswana. Performed all experimental work, performed statistical analyses together with co-authors, produced the first draft and then wrote the manuscript together with co-authors.
- III Collected tubers in Botswana. Prepared *Harpagophytum* extracts. Performed all experimental work together with co-authors on extracts and neutrophils for the first set of five patients. Produced the first draft and then wrote the manuscript together with coauthors.
- M+C Performed all sampling and experimental work for the morphological (M) and cytological (C) research which is also described in this thesis. Performed the statistical analyses and wrote the text together with supervisors.

1 Introduction

1.1 Overview of the genus *Harpagophytum*

Harpagophytum is a genus with two closely related species, both of which are native to Southern Africa (Ihlenfeldt 1988). The species are *H. procumbens* (Burch.) DC. ex Meisn. and *H. zeyheri* Decne. The genus belongs to Pedaliaceae, a family of 13 genera with approximately 60 species. Most family members are in Africa, and one genus (*Uncarina* (Baill.) Stapf) is endemic to Madagascar. Some genera are, however, found in India, Sri Lanka (*Sesamum* L.) and Australia (*Josephinia* Vent.). Both *Sesamum indicum* L. and *H. procumbens* have economic importance for oil and medicine, respectively (Ihlenfeldt 1988).

The *Harpagophytum* plant is a xerophytic, multi-stemmed and perennial herb, which survives unfavourable weather through its succulent underground tubers. The primary tuber forms a tap root, which grows vertically to 1 m below ground. The secondary tubers radiate in different directions reaching a distance of up to 1 m from the taproot. Rain triggers re-sprouting of the trailing stems, which usually reach 60 cm, rarely 1 m.

Leaves occur in opposite arrangement along the entire semi-woody branches of the herb, and are petiolate, with wavy scalloped margins. *Harpagophytum procumbens* (Figure 1) generally has a more narrow leaf lamina and smaller lobes than *H. zeyheri* (Figure 2). However leaf size and shape is affected by environmental conditions, and leaves are therefore not very useful for classification.

Flowers are perigynous, hermaphroditic, perfect, zygomorphic, solitary and non-sessile. Colours of the perianth tube include purplish to pink, white, yellowish or greenish with purplish tinges. *Harpagophytum* plants are outcrossing and bee pollinated (MCAN 2011).



Figure 1. *Harpagophytum procumbens*; deeply lobed bluish green leaves. Photo Ilze Vermaak.



Figure 2. *Harpagophytum zeyheri*; bright green leaves, lobes broader than in *H. procumbens*. Photo Mbaki Muzila.



Figure 3. *Harpagophytum procumbens* in flower and with fruit (seed capsule). Photo Ilze Vermaak.

Seed capsules (Figure 3) are woody, longitudinally dehiscent, almost flattened, generally elliptic except in *H. zeyheri* ssp. *schiffii* Ihlenf. & H.E.K. Hartmann, and armed along the edges. There are two obtuse protuberances on each face, and rows with curved arms along the edges. These curved arms manifest in various forms and are often used for classification of the different taxa of *Harpagophytum*. Each one of these curved arms bears re-curved spines.

Inside the capsule, seeds are stacked in rows within each loculus. The elliptic seeds are enclosed in two seed coats (Jordaan 2011). The outer seed coat is brownish black, imperfectly rectangular with sharp edges, rough textured and fibrous. The second coat is light brownish, highly tenacious and almost elastic when soaked in water.

Plant extracts derived from *H. procumbens* have been reported to cure a number of ailments, which include fever, skin lesions, rheumatism, intestinal disorders, headache, arteriosclerosis, kidney problems, liver bladder diseases and osteoarthritis (Ihlenfeldt 1988, Chantre et al. 2000). By contrast, *H. zeyheri* was reported as a medicinal plant only by Baghdikian et al. (1997), while Chantre et al. (2000) dismissed ethnobotanical claims about *H. zeyheri* except for its use as food for insect larvae.

Accurate identification of *Harpagophytum* species before use as a medicine or propagation for medicinal purposes is thus crucial. One

laboratory-based procedure, that claims to accurately distinguish *H. procumbens* from *H. zeyheri*, depends on evaluating the ratio of harpagoside to 8-coumaryl harpagide (Baghdikian et al. 1997). This procedure has been applied at the species level, but its performance at the subspecies level is yet to be determined.

Specimens, that are difficult to classify into one of the two species, are sometimes encountered in areas where the distributions overlap (Muzila et al. 2011, Mncwangi et al. 2013, Muzila et al. 2014), leading to the hypothesis that gene transfer may occur between *H. procumbens* and *H. zeyheri*.

1.1.1 Socio-economic importance of *Harpagophytum*

Harpagophytum plant materials are harvested mainly from Botswana, Namibia and South Africa. Between the years 1992 and 2001 Botswana exported in total 117 metric tons of *Harpagophytum* tubers, Namibia 3400 tons and South Africa 176 tons (Raimondo and Donaldson 2002).

Much of the plant material is sent to Germany where there are numerous patent holders of *Harpagophytum* products. In 2003 about 57 *Harpagophytum*-based medicinal products were marketed by 46 companies in Germany (Kathe et al. 2003). These products are then exported to others in Europe (e.g. Italy and Sweden), Africa, USA and worldwide (Kathe et al. 2003). In 2004, the German Commission (a panel of medicinal doctors and pharmacists), however, removed *Harpagophytum* products from the list of medicinal drugs covered by medical aid (Stewart and Cole 2005).

At the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) meeting in April 2000 in Kenya, Germany made a proposal to list *Harpagophytum* in Appendix II of CITES (Strohbach and Cole 2005, Setshogo 2013). Botswana, Namibia and South Africa rejected this proposal since it would disadvantage about 10,000 poor families in Africa who depend exclusively on *Harpagophytum* sales for income generation (Strohbach and Cole 2007). These families belong to the poorest sectors of the economy and live mainly in rural areas, where job opportunities are insufficient. Factors leading to the exploitation of *Harpagophytum* harvesters include lack of education and legal representation. There are normally no contracts between the buyer and the seller (vendor), and the markets are informal, with very low levels of value addition (Strohbach and Cole 2007).

Harvesting of *Harpagophytum* tubers is very labour-intensive (Figures 4 and 5) and time-consuming despite the low payments. In 2003 and 2004, local vendors in Botswana were paid US\$ 1.5/kg. In Namibia vendors were paid US\$ 2.7/kg in 2002 (Stewart and Cole 2005), while in the same year vendors in South Africa were paid US\$ 0.15-3.0/kg (Raimondo et al. 2003). Price bargaining occurs mainly during the purchasing from vendors, and because of the desperate need for cash, the vendors eventually succumb to the exploitation. Cole and Du Plessis (2001) estimated the benefits obtained by vendors from *Harpagophytum* sales to reach only 1 to 2% of the value of the international *Harpagophytum* trade, which is estimated to be 30 million Euros per year (Stewart and Cole 2005).

1.1.2 Harvesting of *Harpagophytum* plants

Generally there are two types of harvesters: herbalists (e.g. traditional medicinal doctors) and vendors. The herbalists normally require small quantities (≈ 100 g per month) of just a secondary tuber for a portion of the required medicinal formula. However, they will usually not cover up the plant afterwards since ancient myth says that filling up the pit is synonymous to burying the dead, and will invite bad spirits to kill and bury the people whom the medicine was intended to heal.

The vendors dig up *Harpagophytum* plants for direct sales and will normally take as much of the secondary roots as possible. However, since their livelihoods are entirely dependent on *Harpagophytum*, sustainable harvesting methods have been developed. Damage to the plant is minimized by avoiding contact with the primary root upon digging out the secondary roots that are found at 20 to 120 cm underground, depending on the age of the plant (Strohbach and Cole 2007) (Figures 4 and 5). Plant survival appears to be high when harvesting is performed in this manner (Stewart 2009).

The *Harpagophytum* plant life cycle reportedly has seven age stages (Strohbach and Cole 2007) which can guide vendors about the readiness of the plant for harvesting; seed, seedling, juvenile, young reproductive, mature reproductive, old reproductive and senile stages. Yield reaches a maximum in 5–10 year old plants, producing tubers of up to 1 kg. *Harpagophytum* plants can be harvested every two years, but the optimum for long-term sustainability is every four years.



Figure 4. Harpagophytum procumbens plant with secondary tubers.
Photo Ilze Vermaak.



Figure 5. *Harpagophytum* harvesting procedure. Photo by Mashi Crafts by permission.

1.2 Systematics

1.2.1 Taxonomy

The type specimen of the genus *Harpagophytum* was described as *Uncaria procumbens* in 1822 by Burchell. Sometime between 1824 and 1839, De Candolle nullified the name in his *Prodromus*, based on illegitimacy since the genus *Uncaria* already existed in Rubiaceae. After being edited by Meissner, the genus *Harpagophytum* was validly published in 1840. *Harpagophytum procumbens* (Burch.) D.C. ex Meissn., as usually presented in taxonomic text, is thus a summary of events that led to the publication and acceptance of *Harpagophytum*. Other proposed binomial names include *H. burchellii* coined by Decaisne in 1846, but rejected based on the principle of priority. An attempt to re-publish the same name in 1865, but in a different journal, was also rejected due to the principle of priority. *Harpagophytum belckii*, proposed by Asher. & Schinz, was also rejected, due to the lack of a formal description (*nomen nudum*).

The species and subspecies so far described in the genus are mainly differentiated on reproductive characters, especially the seed capsule. The following characters have been much used as diagnostic features: 1) the number of arms; 2) fruit shape excluding wings or arms; 3) ratio of the longest arm to the capsule width; 4) the number of seed rows; and 5) presence or absence of wings. In addition, the different taxa have, on the whole, only slightly overlapping distributional areas according to Ihlenfeldt and Hartmann (1970).

Harpagophytum procumbens ssp. *procumbens* DC. ex Meisn.

Diagnostic features include a fruit capsule with 8 long curved arms on each half of the capsule, and each arm with scattered re-curved spines along the sides and at the tip (Figure 6). Length of the longest arm is about five times the width of the fruit capsule proper. Sometimes there are only three arms in a row, but the lowest arm is then much broader and imperfectly divided. The fruit capsule has 19–70 seeds (Ernst et al. 1988), arranged in four rows in each seed locule. This nominal subspecies grows in almost all of Namibia except the coastal area, in southwestern Botswana and in northern and central parts of South Africa.



Figure 6. Seed capsule of *H. procumbens* ssp. *procumbens*. 3 arms per row, with the lower right arm much wider and bifurcating into 2 subarms.

Harpagophytum procumbens ssp. *transvaalense* Ihlenf. & Hartm.

The fruit capsule usually has 3 arms in each row, and the longest arm does not exceed twice the width of the fruit capsule proper (Figure 7). This subspecies has a restricted distribution covering the eastern tip of Botswana, the northeastern tip of South Africa and the southernmost tip of Zimbabwe.

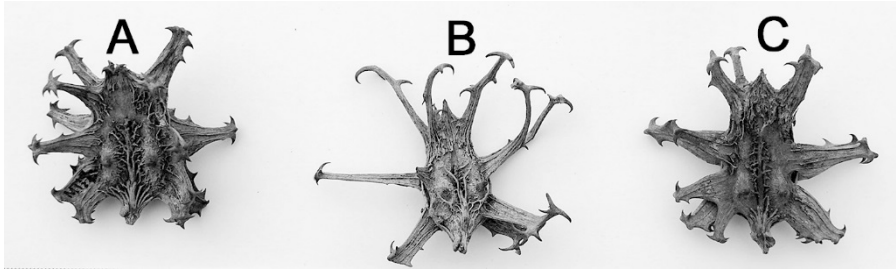


Figure 7. Seed capsule variation in *H. procumbens* ssp. *transvaalense*. A) 3 arms per row, one of the lower arms much wider (new variety); B) 3 arms per row, symmetrical; C) 3 arms per row, asymmetrical.

Harpagophytum zeyheri ssp. *zeyheri* Ihlenf. & Hartm.

The fruit capsule has 8 short and slightly curved arms on each half of the capsule, with recurved spines at the tips (Figure 8, see also Figure 10). The longest arm does not exceed the width of the fruit capsule proper. Sometimes the arms are much shorter and form four rigid wings with re-curved spines. The wings sometimes bifurcate into short arms in the upper portion of the fruit. There are usually two seed rows in each locule, with a total of 11–26 seeds in a capsule (Ernst et al. 1988). This subspecies has its main distribution in northeastern South Africa but extends into southeastern Botswana.

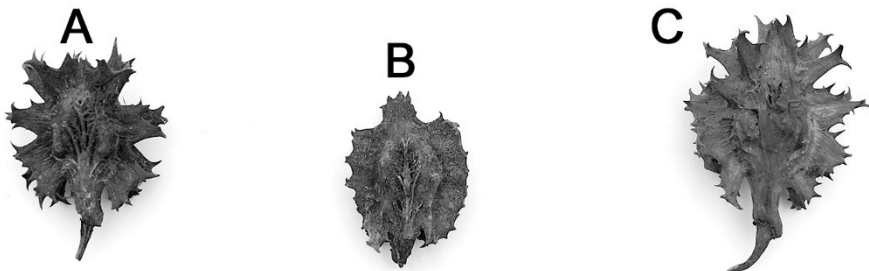


Figure 8. Seed capsule variation in *H. zeyheri* ssp. *zeyheri*.

Harpagophytum zeyheri ssp. *sublobatum* (Engl.) Ihlenf. & Hartm.

The fruit capsule has broad arms, with the length of the longest arm being nearly half the width of the fruit capsule proper, but also with spines along the length of the arms and at their tips (Figure 9). This subspecies has a wide distribution and occurs in six countries; southern Angola, northern Namibia, northern and eastern Botswana, western and southern Zambia, western and southern Zimbabwe and southwestern Mozambique.

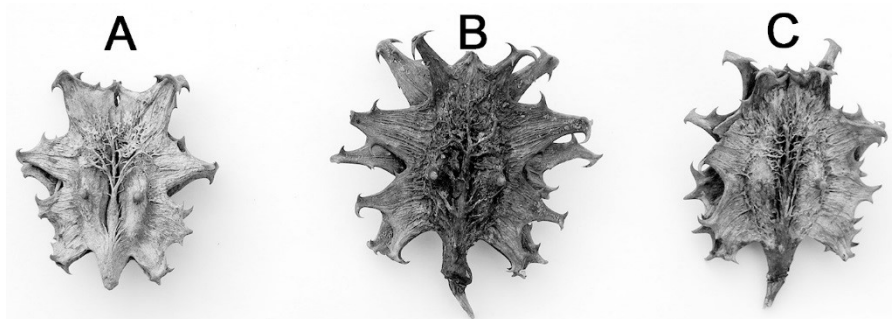


Figure 9. Seed capsule variation in *H. zeyheri* ssp. *sublobatum*: A) 3 arms/winglike appendages per row; B) 3 arms per row; C) 2 arms on the upper half of the capsule and 1 big wing-like appendage on the lower half.

Harpagophytum zeyheri ssp. *schijffii* Ihlenf. & Hartm.

This subspecies is little known and often omitted in monographs, but it has a unique morphology. Unlike other *Harpagophytum* taxa, it generally lacks arms and instead carries two or three winglike appendages on either side, giving it an overall triangular to lanceolate shape resembling a launching spaceship (Figure 10). This subspecies is reportedly endemic to Kruger National Park in northeastern South Africa near the border to Mozambique.



Figure 10. Fruit capsule of *H. zeyheri* ssp. *schijffii* mounted on a herbarium sheet (Type specimen). Courtesy of Pretoria National Herbarium.

1.2.2 Introgression

The occurrence of two seed rows in each loculus of the seed capsule is a diagnostic character for *H. zeyheri*, while the occurrence of four rows is diagnostic for *H. procumbens* (Ihlenfeldt 1988). Seed capsules of *Harpagophytum* are, however, quite variable in their morphology, and intermediate morphotypes have been regarded as evidence of hybridization within the genus (Muzila et al. 2011). Similarly, Ihlenfeldt and Hartmann (1970) suggested the existence of introgressive populations in northern Namibia and northern Transvaal. Henceforth Hachfeld (2004) concluded that “further taxonomic research on genetic differences between the different species of *Harpagophytum*, could help to determine and distinguish between introgressive populations”.

Animal movement and the human exodus of Nguni tribes, particularly Batswana as they migrated from South Africa into Botswana (and within Botswana), have probably played a prominent role in dispersal of *Harpagophytum* seed capsules (Ihlenfeldt and Hartmann 1970), and the resulting admixture of different taxa may have set the scene for establishment of introgression zones.

Hybridization between different taxa can lead to formation of hybrid swarms, caused by gene exchange. Interspecific and intergeneric introgression can also lead to speciation, by the stabilization of a particular hybrid to become a recognized species. The exchange of genetic material can be very complex, with introgression detected in the morphology of the species but not in the nuclear DNA. In other situations, genetic exchange can be detected in the nuclear DNA and not in the cytoplasmic DNA, and *vice-versa*. A combination of methods to detect introgression (e.g. cytology, morphometry and nuclear DNA markers) can be valuable for the determination of the direction of gene transfer and the age of the hybrid swarm. In the case of *Harpagophytum*, analysis of chemical contents in the secondary roots can also be important, both as an indicator of introgression, and for learning more about the potential medicinal efficacy of different species and possible hybrids.

1.3 Distribution

Botswana, Namibia and South Africa are considered as the range states for *Harpagophytum*. The geographical distribution of the five

acknowledged taxa is shown in Figure 11. Plant inventories carried out in Botswana have, however, shown that several of these taxa are more widely distributed in this country (e.g. M. Muzila, unpublished collection records) and possibly also in the surrounding countries.

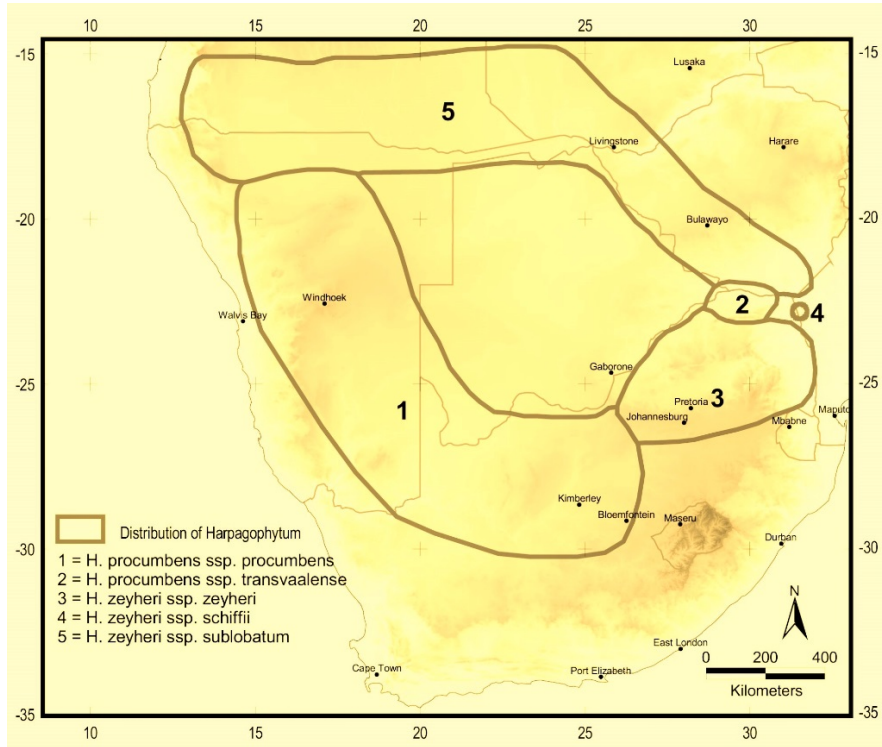


Figure 11. Distribution of *Harpagophytum* in Southern Africa (Ihlenfeldt and Hartmann 1970). Reproduced with permission.

1.3.1 Habitats

Based on electronic maps of Botswana (Surveys and Mapping 2014), habitat attributes of *Harpagophytum* can be summarised as follows: 1) annual rainfall 300–500 mm; 2) minimum temperature 3.0–3.5 °C; 3) maximum temperature 32–36 °C; 4) soil type variable but sands dominating; 5) altitude 700–1400 m above sea level, but with some notable differences between taxa; *H. procumbens* ssp. *procumbens* is found mostly at 1000–1400 m, ssp. *transvaalense* at 700–1400 m, *H. zeyheri* ssp. *zeyheri* at 900–1000 m, *H. zeyheri* ssp. *sublobatum* at 700–1400 m, and *H. zeyheri* ssp. *schiffii* at 1000–1200 m.

Harpagophytum procumbens ssp. *procumbens* grows mainly in the Kalahari dune veld in Southwestern Botswana. Trees/shrubs that characterise this vegetation type include *Acacia haematoxylon*, *A. mellifera*, *Boscia albitrunca* and *Rhigozum trichotomum*. The grasses found are, e.g., *Stipagrostis amabilis*, *S. uniplumis*, *S. ciliata*, *Eragrostis trichophora*, *Brachiaria glomerata*, *Centropodia glauca* and *Schmidtia kalahariensis*. Prominent species in the herbaceous cover include *Harpagophytum procumbens*, *Jatropha erythropoda* and *Dipcadi gracillimum*. The substrates are calcrete outcrops (Thomas and Twyman 2004, Mosweu et al. 2013) in dune valleys and depressions, and are characterised by whitish calcaric and compact sandy soils. Soil colour ranges from greyish to reddish. The greyish type is rich in lime and alkali. Reddish types are acidic and poor in nutrients. The dominant fraction is sand (Surveys and Mapping 2014). The area receives summer rains that average 150 to 230 mm per year, and the rainfall pattern is classified as unreliable (Skarpe 1986, Tyson 1986). Rainy months are January–April, with March normally being the wettest. Winter temperatures range from 2 to 4 °C, while summer temperatures can reach up to 45 °C (Van Rooyen et al. 1990, Mosweu et al. 2013).

Harpagophytum procumbens ssp. *transvaalense* is mainly found in eastern Botswana, particularly at the Oodi introgression zone. Here, the vegetation type is *Acacia* shrubland with the dominant tree species being *Acacia erubescence* and *A. tortilis*. *Ehretia rigida*, *Grewia flava*, *G. retinervis*, *Boscia albitrunca*, *B. foetida* and *Dichrostachys cineria* also occur but sparsely. Grasses species include *Eragrostis* sp., *Digitaria* sp., *Aristida* sp., *Commelina* sp., and *Evolvulus* sp. Herbaceous cover is dominated by other members of the Pedaliaceae which include *Dicerocaryum eriocarpum*, *D. senecioides*, *Ceratotheca* sp., *Sesamum* sp. and the monotypic *Holubia saccata*. The two genera *Dicerocaryum* and *Harpagophytum* grow in close proximity. Since *Harpagophytum* plants are generally difficult to find, occurrence of *Dicerocaryum* sp., which are easy to see, can be indicative of *Harpagophytum* presence.

The soils are ferric lxisols (SMAS 1989, Surveys and Mapping 2014) including deep brown to red sandy loam to sandy clay-loam (Figure 2) soils that are moderately to well-drained. The clay content increases with depth, and they are acidic and less fertile (Surveys and Mapping 2014). Around the Oodi, Leshibitse, Malotwana and Tlokweng areas, where this species occurs, mean minimum

temperatures range from -4 °C in July to 20 °C in January, while mean maximum temperatures range from 22 °C in July to 33 °C in January (SMAS 1989).

Harpagophytum zeyheri ssp. *sublobatum* often grows in Mopane (*Colophospermum mopane*) woodland in Northern and Eastern Botswana, with trees also of *Acacia arenaria*, *A. ataxacantha*, *A. erioloba*, *A. hebeclada*, *A. fleckii*, *A. luederitzii*, *A. mellifera*, *A. tortilis*, *Albizzia versicolor*, *Bauhinia petersiana*, *Boscia albitrunca*, *Catophractes alexandrii*, *Combretum albopunctatum*, *C. collinum*, *C. hereroense*, *Commiphora africana*, *Dichrostachys cineria*, *Grewia bicolor*, *G. flava*, *G. flavescens*, *Lonchocarpus nelsii*, *Rhus tenuinervis*, *Terminalia* sp., *Ochna pulchra*, *Ximenia americana* and *X. caffra* (Ringrose et al. 2003). Herbaceous cover includes *Asparagus* sp., while the dominant grasses are *Aristida meridionalis*, *Eragrostis pallens* and *Anthepora pubescens*. The soils are calcisols, which are characterised by high levels of calcium carbonate in coarse to silty sandy loams. Soil colour ranges from grey to pale brown. Drainage is moderate to high. In general, this subspecies occurs in wetter areas receiving an annual precipitation of 380 mm in northeastern Botswana, and 640 mm in northern Botswana. The plant thrives in temperatures ranging from 25 °C to 34 °C.

Harpagophytum zeyheri ssp. *zeyheri* grows in arid sweet bushveld and semi-sweet mixed bushveld, e.g. at localities like Bela-Bela, Kumakwane, Leshibitse, Oodi and Tlokweng where *H. procumbens* ssp. *transvaalense* also grows.

1.3.2 Vulnerability and conservation status

Harpagophytum tubers are harvested almost exclusively from wild-growing plants in Botswana, Namibia and South Africa. There is a huge risk of overexploitation and plant density has been reported to decrease in some harvested areas (Hachfeld 2003). Plant replacement in the wild is erratic since *Harpagophytum* plants are very sensitive to competition for water and nutrients, and ample rain is required for fruit maturation (Stewart 2009). In addition, seeds have a prolonged dormancy associated with a dense seed coat which physically retards radicle emergence (Jordaan 2011).

Using red data listing terminology, *H. procumbens* is currently classified as Lower Risk, near threatened (LR/nt) in Botswana, which includes taxa that are not critically endangered, endangered or vulnerable, but not far from qualifying for a threatened category in

the near future (Golding 2002). The red data status of *H. zeyheri* is unknown. In Namibia the red data status of *Harpagophytum* is unclear, but both *H. procumbens* ssp. *procumbens* and *H. zeyheri* ssp. *sublobatum* have been proposed for listing under protected plants of Namibia (Craven and Kolberg 2016). In South Africa the red data status of *H. procumbens* ssp. *procumbens* and *H. procumbens* ssp. *transvaalense* is unknown (i.e., not evaluated), while all the *H. zeyheri* subspecies have been listed (SANBI 2015) as of Least Concern (LC).

Since previous efforts to get *H. procumbens* listed under the CITES were rejected by the range states Botswana, Namibia and South Africa, it is now up to these countries to ensure sustainable usage of the plant, or enforce conservation measures through state protected areas.

The situation in Botswana is presently as follows: populations of *H. procumbens* ssp. *procumbens* in the southwest occur in wildlife management areas and national parks and are therefore safe, whereas populations in the south occur on communal land and are more vulnerable to overexploitation. All populations of *H. procumbens* ssp. *transvaalense*, including those in putative introgression zones, grow on communal land and are therefore vulnerable.

Northeastern populations of *H. zeyheri* ssp. *sublobatum* also occur on communal land. In the more northern areas, most populations instead grow in wildlife management areas, national parks, game reserves and forest reserves, and are therefore protected to some extent. However the drawback with forest reserves is that they are not fenced. *Harpagophytum zeyheri* ssp. *zeyheri* is found mainly within the putative introgression zone stretching from southeastern Botswana (Gaborone and Tlokweng) through Kgatlang district (Oodi, Artesia, Dibete) and into the Central district (Mahalapye all the way to Bobirwa area), where populations grow mainly on communal land and therefore are vulnerable.

Some efforts have also been made to introduce farmer-based cultivation trials for production of *H. procumbens* in, e.g., Botswana with the hope to improve sustainability of wild populations while still generating income for local farmers (Motlhanka and Makhabu 2011).

1.4 Chemical contents and medicinal properties

1.4.1 Phytochemistry

Tubers of *Harpagophytum* (Figure 12) have been used for medicinal purposes in Africa for centuries, and in Europe since at least the 1960s, when ‘*Radix Harpagophyti*’ was introduced for manufacturing remedies against arthritis and other ailments (Feistel and Gaedcke 2000). *Harpagophytum procumbens*, the more thoroughly investigated of the two species in the genus *Harpagophytum*, is characterized by the presence of iridoid glycosides, acetylated phenolic glycosides and terpenoids in the tubers that are being used for manufacturing drugs with medicinal properties (Burger et al. 1987, Kundu et al. 2005, Stewart and Cole 2005, Mncwangi et al. 2014).

The major iridoid and phenylethanoid glycosides found in these tubers include acetylacteoside, coumaroylharpagide chebuloside II, diacetylacteoside, decaffeoylverbascoside, harpagide, harpagoside, isoverbascoside (*syn.* isoacteoside), verbascoside and 8-coumaroylharpagide (Boje et al. 2003, Clarkson et al. 2003, Munkombwe 2003, Mncwangi et al. 2014), with harpagoside (Figure 13) generally assumed to be the main active ingredient (Georgiev et al. 2013).

Beneficial effects have also been reported from verbascoside (Gyurkovska et al. 2011). Exact mode of action is mostly unknown but it has been suggested that the efficacy of *H. procumbens* is dependent on synergistic or antagonistic interactions of the ratios of the four compounds 8-coumaroylharpagide, harpagide, harpagoside, and verbascoside (Abdelouahab and Heard 2008). In addition, other constituents such as sugars, polyphenols and their derivatives may also have medicinal effects (Abdelouahab and Heard 2008, Georgiev et al. 2010).

Some efforts have recently been made to grow *Harpagophytum in vitro*, but the tissue-cultured explants (stems, leaves and callus) usually contain mainly isoverbascoside and verbascoside instead of the more desirable harpagoside (Georgiev et al. 2010, Grąbkowska et al. 2014). Tubers harvested from *in vitro*-derived plants grown in a greenhouse for 6 months did, however contain harpagoside in levels comparable to seed-derived plants but harpagide contents were much lower (Grąbkowska et al. 2014).



Figure 12. Secondary tuber of *Harpagophytum*. Photo Ilze Vermaak.

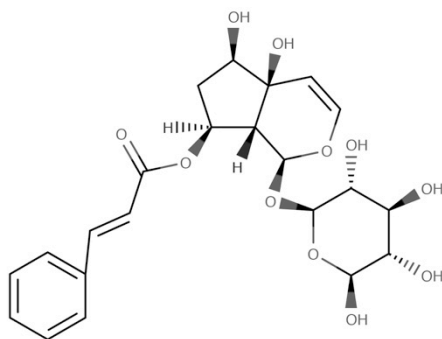


Figure 13. Molecular structure of the harpagoside molecule, assumed to be the main active ingredient of *Harpagophytum* tubers. Picture drawn by the software MarvinSketch.

1.4.2 Medicinal effects

Harpagophytum plants have been used for centuries by the Khoisan people of southern Africa for treating a wide range of ailments including fever, gastro-intestinal problems, diabetes, hypertension and blood diseases (Stewart and Cole 2005). In the last 60 years, various scientific investigations have also indicated an effect of *Harpagophytum* tuber extracts for treating rheumatoid arthritis, osteoarthritis, tendonitis, kidney inflammation and heart disease. For example, Warnock et al. (2007) conducted a clinical study with more

than 200 patients suffering from rheumatic disorders. After 8 weeks of ingesting *Harpagophytum* tablets, a significant improvement was documented based on patient assessments of global pain, stiffness and function.

Adverse effects reported have been few, but Cuspidi et al. (2015) described one case where a patient developed hypertension after self-medication with two daily tablets of *Harpagophytum procumbens* for two weeks.

Clinical studies often lack a control group making it difficult to properly evaluate the effects of the tested drugs. However, use of a control group was a requirement for inclusion in a recently published large review on the efficiency of various plant-based medicinal drugs for treatment of lower back pain (Gagnier et al. 2016). *Harpagophytum procumbens* appeared to reduce pain more than placebo but the evidence was reported to be of moderate quality only.

Another potential problem is the quality of the preparations used in various trials. Chemical contents and anti-inflammatory effects documented by an *ex-vivo* porcine skin model, proved to be very variable when six commercial *H. procumbens*-derived products were analysed (Ouitas and Heard 2010).

1.4.3 Medicinal action

Although *Harpagophytum* is being used for a wide array of ailments, laboratory research has focused mainly on anti-inflammatory activity. Compounds like harpagoside, 8-coumaroylharpagide and verbascoside in the tubers of *Harpagophytum* are known to inhibit the arachidonic, cyclooxygenase 2 (COX-2), and lipo-oxygenase (LOX) pathways (McGregor et al. 2005, Abdelouahab and Heard 2008). By contrast, *Harpagophytum* is apparently ineffective against the COX-1 subtype (Abdelouahab and Heard 2008, Grant et al. 2007). *Ex-vivo* application of *H. procumbens* to porcine skin suppressed the COX-2 products, prostaglandin E₂ (PGE-2) and lipoxygenase (5-LOX), suggesting an ability to cure inflammation in deeper subcutaneous tissues as in arthritis (Ouitas and Heard 2009).

Extracts from tubers of *H. procumbens* have been shown to suppress lipopolysaccharide-stimulated (LPS) expression of COX-2, and inducible nitric oxide synthase (iNOS) in cell lines (Jang et al. 2003). *Harpagophytum procumbens* also inhibits the release of TNF α , interleukin (IL)-6, IL-1 β and prostaglandin E₂ (PGE₂), which

prevents gene expression of TNF α and IL-6 mRNA in human monocytes, and COX-2 in RAW 264.7 cells (Inaba et al. 2010). Similarly, effects were noted on TNF α -induced mRNA synthesis and protein production of the atherogenic adipokines including IL-6, PAI-1 and MCP-1, suggesting that harpagoside may prevent obesity-induced atherosclerosis (Kim and Park 2015). Extracts from *H. procumbens* also block the AP-1 pathway via inhibition of the transcription of AP1 genes that are stimulated by the LPS (Fiebich et al. 2012).

In-vitro and *ex-vivo* human blood sample tests suggest that the anti-inflammatory effects of *Harpagophytum* are associated with an ability to interact with eicosanoid biosynthesis (Loew et al. 2001). Eicosanoids (hormones) are signaling molecules originating from the oxidation of 20 carbon fatty acids, whose imbalance between omega-6 and omega-3 fatty acids can trigger the onset of cardiovascular diseases, blood pressure or arthritis.

Antimutagenic properties of harpagoside have been indicated in *in-vitro* tests of *H. procumbens* against the mutagenic and carcinogenic 1-nitropyrene (Luigi et al. 2015). In the same experiment, other *H. procumbens* compounds affected human lymphocytes before and after exposure to 1-nitropyrene (i.e., pre and post-treatment) suggesting that these compounds also are important for medicinal efficacy.

For evaluation of the potential to counteract osteoporosis, *in-vitro* analyses were conducted using mice osteoblastic cells together with evaluation of *in-vivo* bone status of mice (Chung et al. 2016). The results indicated that harpagide can stimulate osteoblast differentiation and maturation, as well as inhibit osteoclast formation. Unfortunately the source material was reported as *H. procumbens* ssp. *sublobatum*, which is an invalid name, so it is not possible to tie the observations to a specific taxon.

An anti-Alzheimer effect has also been indicated, with verbascoside apparently being the crucial component for inhibition of cholinesterases (Bae et al. 2014). Even an anti-obesity effect has been indicated; due to suppression of the hunger hormone ghrelin, mice lost their appetite when treated with an *H. procumbens* extract (Torres-Fuentes et al. 2014).

2 Objectives

2.1 Problem statement

- Numerous morphotypes, with almost continuous variation between the two species *H. procumbens* and *H. zeyheri*, have been found in wild populations in Botswana, in an area where several taxa overlap. However, the ranges of intraspecific variation are not well documented and outliers therefore difficult to define properly.
- Different opinions have been voiced concerning the medicinal efficacy of *Harpagophytum*-derived products, and allegations of adulteration by adding *H. zeyheri* to the marketed plant material have surfaced. The acceptance of both species as medicinal equivalents has also been proposed.
- Chemical contents of *Harpagophytum* tubers have been analysed using equipment like HPLC-MS (high pressure liquid chromatography-mass spectrometry) and NMR (nuclear magnetic resonance), but information on the medicinal activity of the different compounds is still lacking. Nevertheless, it can—perhaps—be stated that *H. zeyheri* should not be regarded as an acceptable substitute for *H. procumbens* since the two species appear to be chemically distinct.

2.2 Hypotheses

- Variation seen in seed capsule morphology of *Harpagophytum*, can also be reflected in genetic variation estimated by DNA markers, in chemical contents and in medicinal effects.
- Samples with an intermediate morphology may derive from introgression between the two species.

2.3 Main objectives

The main goal of this research was to investigate how morphological, genetic, and chemical variation is inter-connected in a set of *Harpagophytum* samples growing in localities within a putative introgression zone.

The study was divided into five components as follows: 1) morphometric assessment; 2) chromosomal analyses; 3) DNA profiling; 4) phytochemical profiling; and 5) antioxidant and anti-inflammatory experiments. Components 1 and 2 will just be shortly described, whereas components 3 to 5 are reported in three separate manuscripts.

Paper I: Assessment of diversity in *Harpagophytum* with RAPD and ISSR markers provides evidence of introgression.

Specific objectives

- 1) Assess seedling similarity and determine breeding system.
- 2) Estimate differentiation among different taxonomic units of *Harpagophytum*.
- 3) Investigate possibilities of introgression (gene flow) between different taxa of *Harpagophytum*.

Paper II: *Harpagophytum* germplasm varies in tuber peel and pulp content of important phenylpropanoids and iridoids.

Specific objectives

- 1) Study content of selected biochemical compounds in peel and pulp of *Harpagophytum* tubers of different taxa.
- 2) Identify possible inter-taxon differentiation in content (profile and amount) of selected biochemical compounds
- 3) Investigate associations between content of biochemical compounds and sample origin.

Paper III: Alteration of neutrophil reactive oxygen species production by extracts of devil's claw (*Harpagophytum*).

Specific objectives

- 1) Use neutrophils as a model to explore mechanisms and possible therapeutic modulation of inflammation by *Harpagophytum* extracts.
- 2) Screen genetically diverse *Harpagophytum* plant material representing the pharmacological capability of the genus.

- 3) Determine the potential of root tuber ethanol extracts of various *Harpagophytum* taxa to suppress the production of ROS in human neutrophils.

3 Materials and Methods

3.1 Plant material

3.1.1 Seed capsules for morphometric analysis

Seed capsules were collected from a total of 21 plants found at six locations. They were Sekoma in southern Botswana where presumably only *H. procumbens* ssp. *procumbens* occurs, and Bela-Bela, Oodi, Kumakwane, Leshibitse and Mmamashia in southeastern Botswana where both *H. procumbens* and *H. zeyheri* may occur as well as introgression products (Figure 14). The seed capsules were deposited at UCBG herbarium in Gaborone, Botswana. For each plant, a maximum of five capsules were taken.

3.1.2 Seedlings for DNA analysis

For paper I, seed capsules were collected from six locations, Sekoma in southern Botswana, and Bela-Bela, Oodi, Kumakwane, Leshibitse and Tlokweng in southeastern Botswana (Figure 14). Seeds from the collected capsules were germinated on petri dishes at 30 °C at Balsgard, SLU, Sweden. For paper III, seed capsules were collected from Matlapaneng in northern Botswana, Mmathethe in southern Botswana, and Bela-Bela, Kumakwane, Malotwana, Oodi and Tlokweng in southeastern Botswana (Figure 14), and seedlings were germinated as above.

3.1.3 Tubers for phytochemical and Reactive Oxygen Species (ROS) analysis

For paper II, secondary root tubers and about five seed capsules per *Harpagophytum* accession were collected at seven different locations, Matlapaneng in northern Botswana, Mmathethe in

southern Botswana, and Bela-Bela, Kumakwane, Malotwana, Oodi and Tlokweng in southeastern Botswana (Figure 14). After harvest, the tubers were stored at room temperature in paper bags until they were freeze dried and milled.

A total of 37 samples were collected including 5 samples of *H. procumbens* ssp. *transvaalense*, 14 samples of *H. zeyheri* and 18 samples of putative *Harpagophytum* hybrids. The tubers were used in the biochemical analyses, while the seed capsules were used in morphological verification of the species.

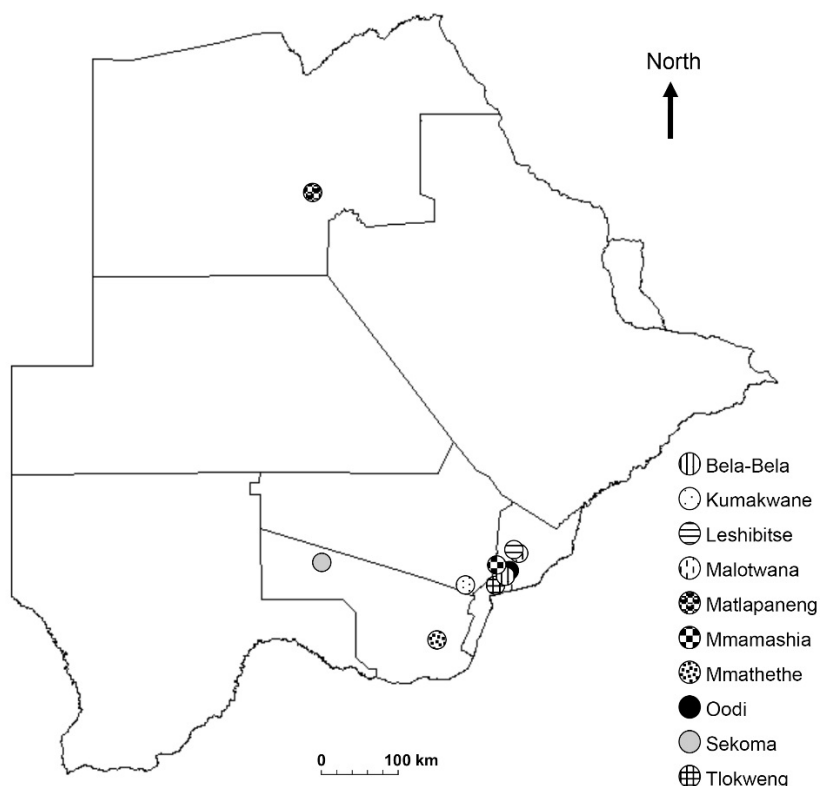


Figure 14. Map of Botswana with all locations where *Harpagophytum* was sampled for analyses included in this thesis.

3.1.4 Flower buds for cytological analysis

Flower buds at different developmental stages were collected from Mmathethe, Oodi, and Tlokweng (Figure 14). Younger buds (5–10 mm) were used in cytological analyses. The flower buds were bisected into equal halves using a razor blade, and fixed in Farmer's fluid (3 parts ethanol 95%:1 part acetic acid) (Berlyn et al. 1976). After 24 hours the flower buds were transferred to 70% ethanol and stored at -4°C until squashing.

3.2 Methods and analyses

3.2.1 Morphometry

Five seed capsules from each sampled plant were used for morphometric analyses. The characters analysed were as follows: 1) length of the longest arm; 2) widest part of the longest arm (arm width); 3) width of seed capsule; 4) length of seed capsule; 5) height of column that encloses the seed rows; 6) seed capsule circumference. In addition, number of seed rows was determined for one capsule of each sampled plant.

3.2.2 Cytology

Flower buds were soaked in iron aceto-carmin stain for 1–2 weeks, and then acidified with 1 M HCl for a minimum of 1 hour. The HCl produces a transparent cytoplasm and softens the tissues, while the iron enhances the staining. Flower buds were dissected under a stereo dissection microscope to remove the calyx and other debris. The Darlington and La Cour (1976) squash method was modified by mixing aceto-carmin with Euparal in a 1:1 ratio, after which the anthers were squashed in the mixture.

3.2.3 DNA analysis

For Paper I, DNA was extracted from 96 plantlets and analysed with polymerase chain reaction (PCR) using standard RAPD and ISSR primers. A total of 30 RAPD primers were tested for reproducibility and polymorphism, and 10 of these were retained for further analyses. The PCR products were separated by electrophoresis in 1.8% agarose gel, stained and photographed under ultraviolet illumination. Only clearly visible DNA fragments with lengths

between 150 and 2176 bp were scored. Similarly, 25 ISSR primers were screened for reproducibility and polymorphism, and 6 of these were retained for analysis. Detection and scoring of PCR products were performed as described for RAPD.

For Paper III, DNA was extracted from 17 plantlets representing one accession each. These were analyzed together with reference samples of 7 accessions from the previous DNA study (Paper I) representing three taxa, and using the same primers and methods as described above.

3.2.4 Phytochemical compounds

Sample preparation and extraction

Tubers were cut into two parts and the peel separated from the pulp. Both peel and pulp were freeze-dried, and then milled to a fine powder in a laboratory mill. Twenty-five mg of milled sample was weighed into a micro tube and extracted with 50% acidified ethanol water solution (3.75 mL H₃PO₄ in 1000 mL ethanol). The mixture was vortexed and extracted in an ultrasonic water bath at 24 °C, and then centrifuged at 13 000 rpm. Then 300 µL of the supernatant was transferred directly into an HPLC vial, and diluted with 900 µL of the 50% acidified ethanol water solution. Three replicates were made per sample.

HPLC-DA and HPLC-ESI-MS analysis of phenylpropanoids and iridoids

The chemical compounds were determined on a Shimadzu HPLC system equipped with a diode-array detector. A Phenomenex Synergi Hydro-RP 80A column and a guard C18 precolumn were used at 24 °C. Detection was carried out at 280 nm with a total run time of 35 minutes. Evaluation of chromatograms was carried out with Shimadzu Class-VP software. Confirmation of compounds and peak identity were done with a Sciex API 150EX single Quadrupole mass spectrometer equipped with a Turbo Ion Spray interface. The peak area under curve units (AUC) was used for biochemical profiling of accessions. For direct quantification acetylacteoside and pagoside were quantified against harpagoside, and isoverbascoside was quantified against verbascoside.

3.2.5 ROS determination

Extracts for ROS analyses

The 100% stock solution (250 µg/mL) extracted using an acidified alcohol method, was diluted (v/v) with phosphate-buffered saline (PBS) to produce final concentrations of 50%, 10% and 5%.

Determination of total phenolics

Total phenolic content was determined according to the Folin-Ciocalteu method (Singleton et al. 1999). For comparison, total phenolic content was analyzed also in commercially obtained, external standards of 8-O-p-coumaroyl-harpagide, harpagoside and verbascoside.

Determination of Ferric Reducing Ability of Plasma

The ferric reducing ability of plasma (FRAP) of the extracts was measured according to Benzie and Strain (1996). The absorbance was measured at 595 nm after 4 min on a plate reader. Fe^{2+} was used as a standard and L-ascorbic acid was used as a control.

HPLC analysis of ascorbate

Samples were placed in ultrasonic bath and then centrifuged. The supernatant was filtered directly into an HPLC-vial. The analysis was made on a Shimadzu HPLC-system consisting of a communication bus module and a pump using an ACE (5 µm) for separation. For detection, a variable SPD-10A UV-VIS detector set to 254 nm was used. The peak was identified by retention time of an ascorbate standard, and quantification was carried by peak area.

Collection of venous blood and preparation of neutrophils

Venous blood was drawn from 10 healthy volunteers. Neutrophils were isolated, washed and then re-suspended in a phosphate buffered saline (GPBS) containing 1 mM glucose and cations. Only samples with more than 98% viability (Trypan blue) were retained for further analysis.

Cell viability assay

Cell viability was measured using the CellTiter-Glo™ Reagent (Promega).

Detection of ROS by chemiluminescence

Chemiluminescence assays were performed using luminol to detect total oxygen radical (HOCl and H₂O₂) generation (intra- and extra-cellular) as described in Matthews et al. (2007). Neutrophils were stimulated with either PMA (Phorbol 12-myristate 13-acetate, 25 nM), *Fusobacterium nucleatum* (MOI 1:100), opsonised *S. aureus* (MOI 1:300) or with PBS (control). For each subject, all samples were analyzed in triplicate and light emission in relative light units (RLUs) was recorded throughout the experiment.

3.3 Statistical analyses

3.3.1 Morphometry

Mean values for the seed capsule characters were recorded for each plant. Data for all characters except seed row number were evaluated using box and whisker plot analysis showing mean value for each character and location, as well as quartiles and range. In addition, similarities between all of the 21 sampled plants were investigated in a principal component analysis (PCA), and a biplot showing the contribution of the different characters on PCA generation. All analyses were executed in Minitab.

3.3.2 DNA analysis

For Paper I, RAPD and ISSR bands were scored for 96 plants, and polymorphic bands were then used in the subsequent analyses. For each primer, polymorphism information content (PIC) was determined as the average PIC for each polymorphic locus detected by that primer. A matrix with pairwise genetic similarities (band sharing) between all 96 plants was calculated using Jaccard's similarity coefficient.

Partitioning of genetic variation among and within different groups of samples (taxa and putative hybrids) was investigated with analysis of molecular variance (AMOVA), performed with GenAlEx using a matrix with Euclidean distances.

Dissimilarity between groups of samples was quantified with a cluster analysis, using the standard distance matrix in PAUP ver. 4.0 (Swofford 1998), and the unweighted pair group method with arithmetic averages (UPGMA) mode. Branch support was tested with 1,000 jackknife replicates (Davis 1993). The strict consensus

criterion was adopted to obtain a tree with components common to all parsimonious trees.

A principal component analysis (PCA) and a multi-dimensional scaling (MDS) analysis were also conducted. Both methods used a matrix of standardized covariates as input variable. When plotted into a coordinate system, degree of similarity between taxa is indicated but the MDS goes beyond the PCA by preserving reproducibility of distances between points.

A Bayesian analysis of genomic structure and gene flow was performed using the program Structure 2.3.1 (Pritchard et al. 2000) which quantifies the number of genomes that can be derived from a population, i.e., the entire set of *Harpagophytum* samples, and how these are represented within each sample.

For paper III, a PCA was conducted as described above.

3.3.3 Phytochemistry and ROS analysis

Associations between chemical compounds content among different taxa were assessed with PCA. General Linear Model (GLM) analyses, followed by *post hoc* tests, were used to determine significant differences among sample means for the taxonomic units.

Associations between the content of chemical compounds in the six samples used for the ROS analysis were investigated with PCA. Descriptive statistics for mean values and standard deviations were calculated on data for the enhanced chemiluminescence assays, and mean values, standardized to RLUs for each test subject respectively, were compared using a Wilcoxon signed-rank test.

4 Results and Discussion

4.1 Morphology and cytology

Two preliminary studies (not described in any of the attached papers in this thesis) on seed capsule variation and cytology (chromosome counts, chromosomal pairing behaviour and tetrad stage) in *Harpagophytum* will be shortly presented here.

4.1.1 Seed capsule variation

The measured characters indicated large and almost continuous variation in seed capsule morphology of the 21 sampled plants. A comparison of the average values calculated for each plant, shows that arm length ranged from 1 to 79 mm, arm width from 2 to 15 mm, seed column height from 2 to 10 mm, seed capsule length from 21 to 72 mm, seed capsule width from 15 to 37 mm, and seed capsule circumference from 51 to 167 mm. Most plants had seed capsules with two seed rows in each locule, which is indicative of *H. zeyheri*. By contrast, all three plants from the westernmost location Sekoma had four seed rows, indicative of *H. procumbens*. In addition, one plant was found that had three seed rows in each locule, while three plants had three seed rows in one locule and two in the other. These may result from interspecific hybridization.

The box and whisker plots (Figure 15) show how the character means vary between the six different sampling locations, as well as the amount of variation among plants within each location.

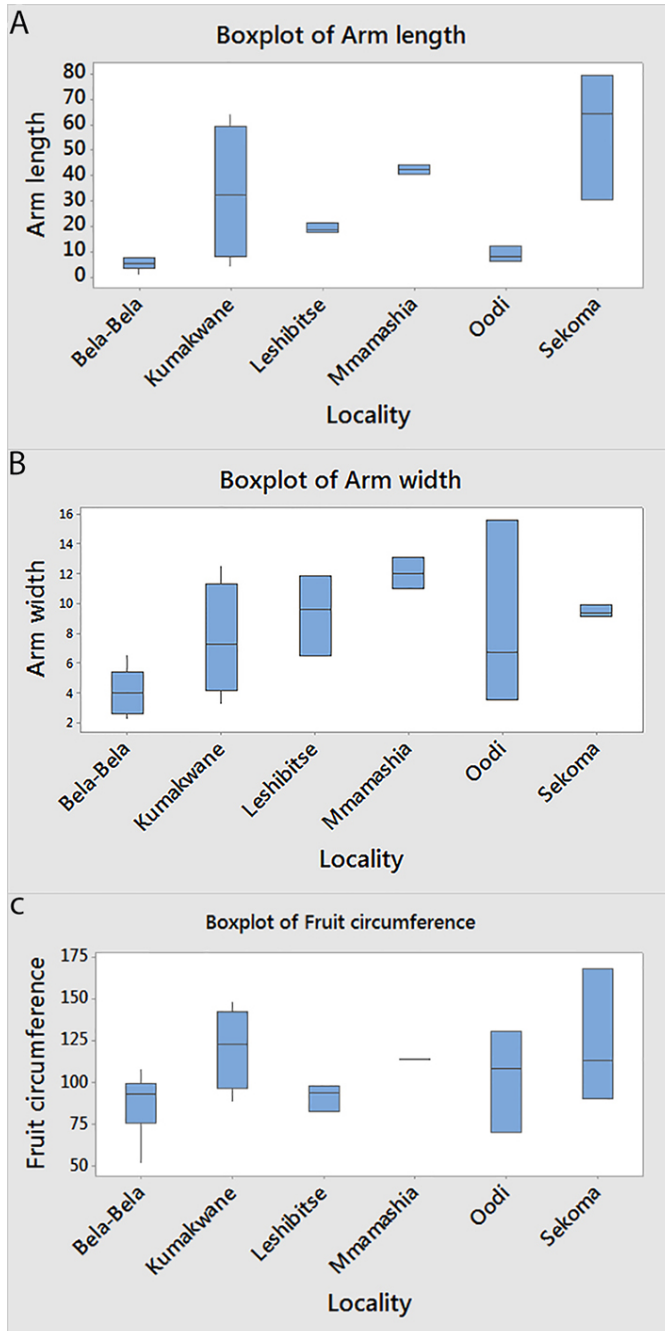


Figure 15. A) Box and whisker plots showing mean arm length, B) mean arm width and C) mean fruit circumference for the six locations.

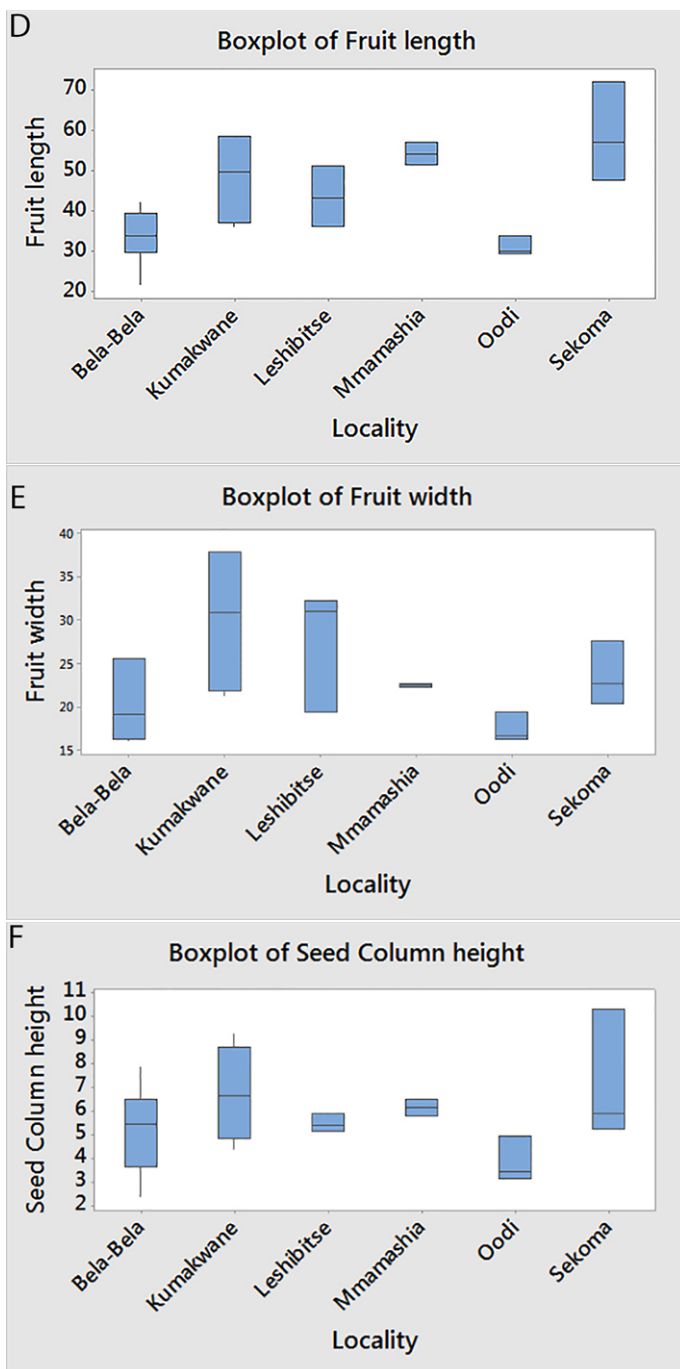


Figure 15. D) Mean fruit length, E) mean fruit width, F) mean seed column height for the six locations.

Character values were overall homogeneous in Bela-Bela, Oodi, Leshibitse and Mmamashia except for some variation in capsule circumference in Oodi. Moreover, in these populations, seed capsules were rather short and had short arms, although slightly longer at Mmamashia. More variable characters were encountered in Kumakwane and Sekoma, and here both arm length and fruit circumference reached higher values than in the other four locations.

Although most plants sampled in Bela-Bela, Oodi, Leshibitse and Mmamashia could be classified as *H. zeyheri* based on seed capsule shape, four of these plants did not have the expected two seed rows in each locule of the seed capsule. Instead one of the plants at Leshibitse had 3 + 3 seed rows and a second plant had 3 + 2. Similarly, one plant each at Oodi and Mmamashia had 3 + 2 seed rows. Both plants from Mmamashia also had slightly longer arms and more width of seed capsule. Probably some of these plants with deviating seed row number and/or slightly longer arms could be hybridogenous.

Both seed capsule morphology and seed row number indicate that the three plants sampled in Sekoma should be classified as *H. procumbens*. This was expected since Sekoma occurs in the area where *H. procumbens* grows allopatrically (Ihlenfelt and Hartmann 1970). By contrast, the four plants sampled in Kumakwane exhibit a more complex picture, with seed capsule morphology leaning towards *H. procumbens* but with only two seed rows in three of the plants and three seed rows in the fourth. Possibly these also represent introgression products.

No clear grouping of locations was observed when investigating genetic distances in a principal component analysis (PCA) (Figure 16). Still, there was a tendency for plants from the same location to occur together. In the upper right corner, plants from Sekoma (presumably pure *H. procumbens*) were found together with two plants from Mmamashia and one each from Oodi and Kumakwane. In the lower right corner, another two Kumakwane plants grouped on the basis of wider fruits with taller seed columns. In the lower left corner most Bela-Bela plants, characterized by short arms, grouped together, presumably representing pure *H. zeyheri*. The Leshibitse accessions grouped in the middle, and may represent introgression products, together with some plants from Kumakwane, Mmamashia and Oodi.

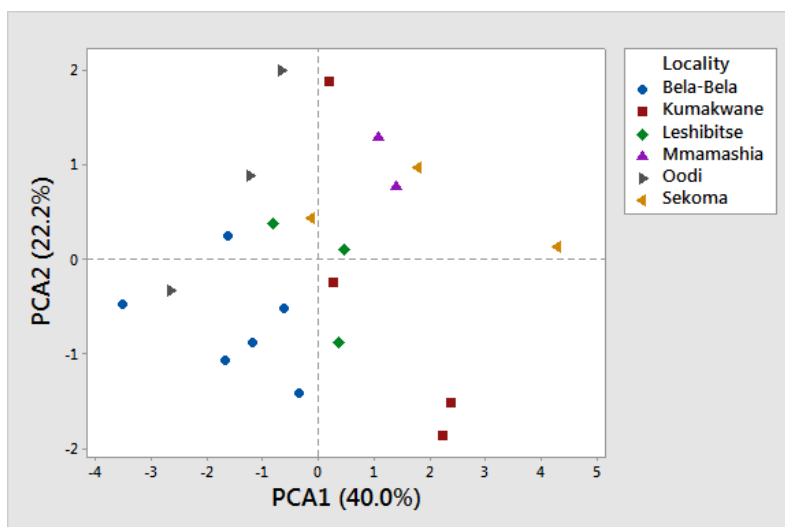


Figure 16. PCA of *Harpagophytum* collected at six locations, and analysed for six seed capsule characters.

Finally, the biplot (Fig 17) shows that all characters are relatively closely correlated, and may thus be under the control of a rather restricted set of genes. This kind of situation is not ideal for plant taxonomy, and more efforts should be made to enlarge the basis for classification of *Harpagophytum* into species and subspecies.

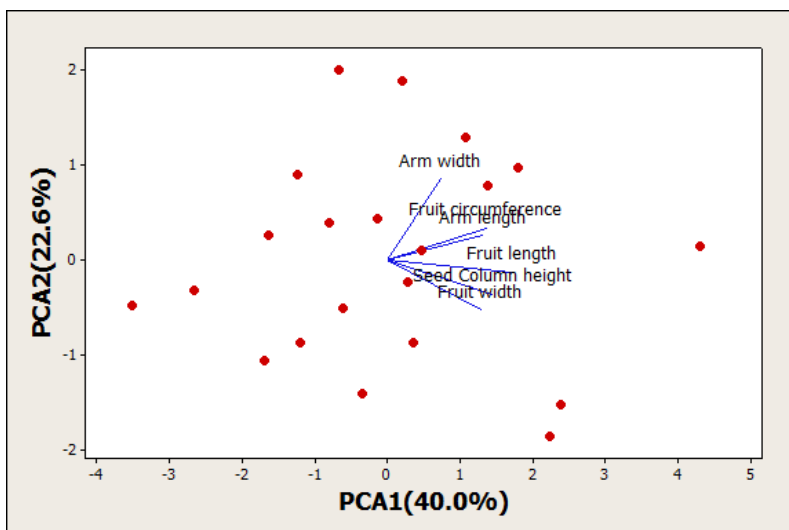


Figure 17. Biplot showing the six seed capsule characters used for analysis of 21 *Harpagophytum* samples.

4.1.2 Cytology

Somatic chromosome counts were performed on tapetal cells of the anthers, while meiotic counts were done on pollen mother cells. However focusing the chromosomes onto a common plane for photography was quite difficult. Digestion with enzymes for easier flattening of cells failed to improve the situation. Pollen mother cells and tapetal cells of *Harpagophytum* appear to be highly tenacious. To promote cell flattening, a possible approach is acid hydrolysis and Feulgen staining as an alternative to aceto-carmin staining. More work is also needed to define optimal flower bud developmental stages for analysis of mitotic divisions in tapetal cells and meiotic divisions in pollen mother cells.

The chromosomes are small in size, about 2 μm in meiotic cells (Figure 19). Chromosome numbers are presented in Table 1. Somatic chromosome counts of $2n = 22$ and 24 were recorded in the putative hybrid accessions Oodi1-13a and Oodi7-10 (Figure 18), respectively. *Harpagophytum zeyheri* ssp. *zeyheri* (accessions TLK1-12 and TLK2) had $2n = 24$ chromosomes.

Meiotic chromosomes were counted on the *H. procumbens* ssp. *transvaalense* accession MTT8b, which had $2n = 20$ chromosomes (Figure 19). For the putative hybrid accession Oodi1-13a, 10 or 11 bivalents could be counted at diakinesis and metaphase I, and 10 or 11 chromosomes in the polar groups of anaphase I (Table 2).

Table 1. Chromosome counts in Harpagophytum taxa.

Accession	Taxon	Cell type	Chromosome counts
Oodi7-10	Putative hybrid	Somatic	$2n = 24$
Oodil-13a	Putative hybrid	Somatic	$2n = 22$
Oodil-13a	Putative hybrid	Meiotic	$2n = 20/22$
MTT8b	<i>H. procumbens</i> ssp. <i>transvaalense</i>	Meiotic	$2n = 20$
TLK1-12	<i>H. zeyheri</i> ssp. <i>zeyheri</i>	Somatic	$2n = 24$
TLK2	<i>H. zeyheri</i> ssp. <i>zeyheri</i>	Somatic	$2n = 24$

Table 2. Examples of the meiotic chromosome constitutions recorded in the putative *Harpagophytum* hybrid Oodi1-13a.

Meiotic stage	Chromosome behaviour	Chromosome counts
Diakinesis	cf. 11 II	$2n = 22$
Metaphase I	11 II	$2n = 22$
Metaphase I	10 II/11 II	$2n = 20/22$
Metaphase I	11 II	$2n = 22$
Anaphase I	cf. 11 + 11	$2n = 22$
Anaphase I	cf. 10 + 10	$2n = 20$
Anaphase I	cf. 10 + 10	$2n = 20$
Anaphase I	cf. 11 + 11	$2n = 22$

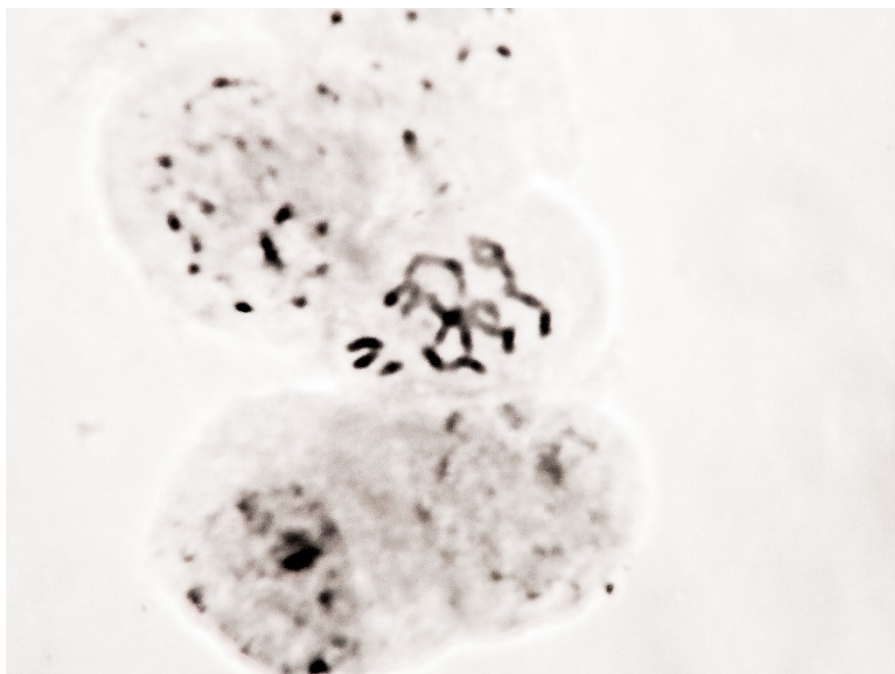


Figure 18. Somatic chromosomes ($2n = 24$) of the putative hybrid Oodi7-10A.

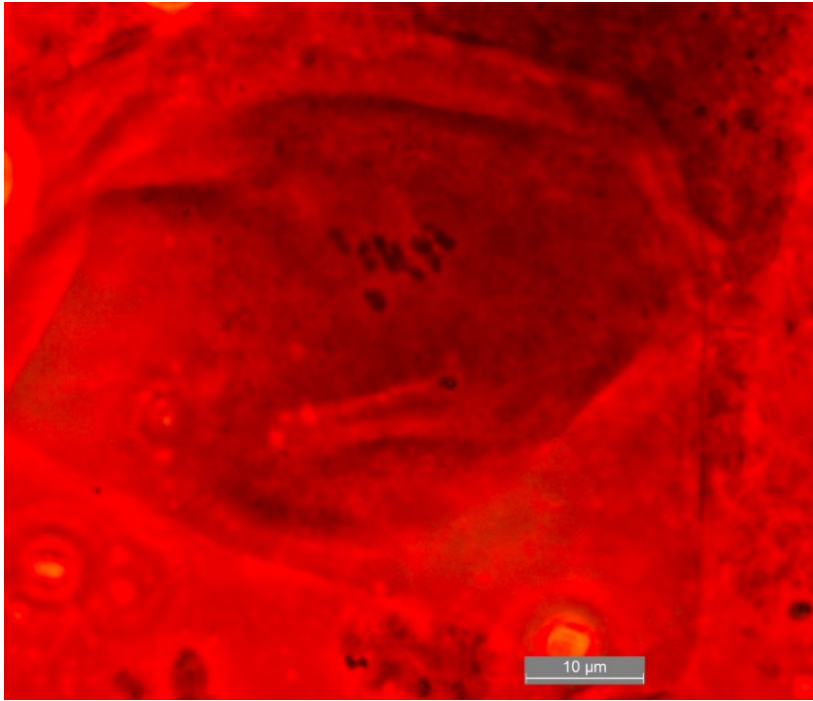


Figure 19. Meiotic chromosome count of 10 bivalents ($2n = 20$) in accession MTT8b (*H. procumbens* ssp. *transvaalense*).

Bivalent pairing was the most frequent type of pairing behaviour, as exemplified by Oodi1-13a (Table 2). This accession, identified as a putative hybrid, showed an association of one bivalent with a nucleolus (Figure 20).



Figure 20. Pollen mother cell with a bivalent-nucleolus association in the putative hybrid Oodi1-13a.

Analysis of pollen mother cells in the putative hybrid accession Oodi1-13a, showed occurrence of triads and dyads in addition to tetrads (Figure 21). The apparent discrepancy with regular bivalent pairing (Table 2), and an irregular/aberrant tetrad stage in the same plant warrants further studies.

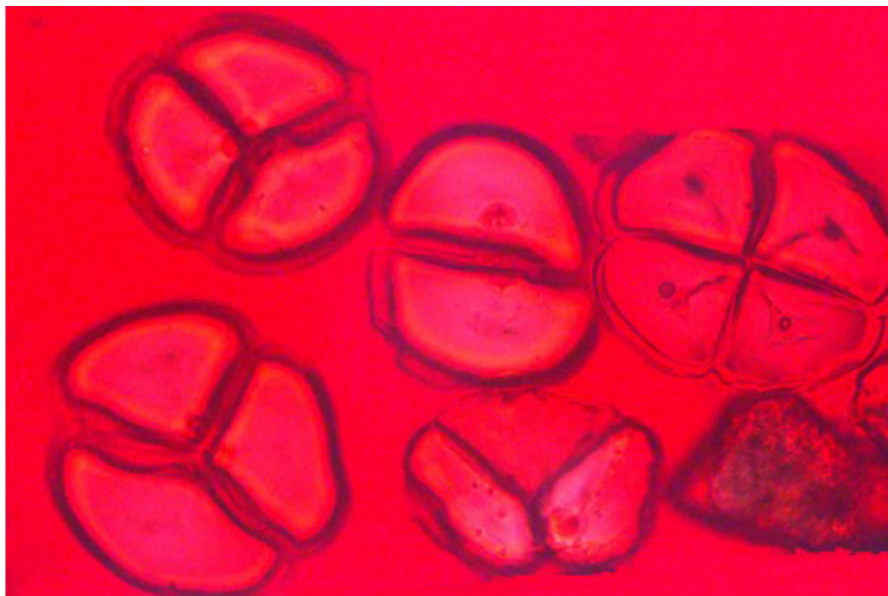


Figure 21. Co-occurrence of triads and tetrads in the putative hybrid Oodi1-13a.

The chromosome counts reported here should be regarded as preliminary results to be used as reference points for future studies. To the best of our knowledge, chromosome counts have never before been published for *Harpagophytum*. There is likewise very little cytological information for other species in the Pedaliaceae family except for those belonging to the economically important genus *Sesamum* with $x = 8$ and $x = 13$, the latter probably deriving from ancient polyploidy (Zhang et al. 2013). Most species of *Sesamum* are diploid (e.g. *Sesamum indicum* $2n = 26$) or tetraploid (e.g. *S. angolense* $2n = 32$), or sometimes even octaploid (*S. radiatum* $2n = 64$). In addition, $2n = 16$ in *Pedaliium murex* (Srinivasan 1942) and $2n = 32$ in *Ceratotheca sesamoides* (Adéoti et al. 2011). Obviously, the here reported chromosome counts of $2n = 20$ and $2n = 24$ for the

two *Harpagophytum* species would represent two new basic numbers for the family, and these counts must therefore be properly verified.

The bivalent-nucleolus configuration observed in accession Oodil-13a reflects the association of the nucleolar chromosomes that harbour the ribosomal genes (rDNA), with the sites of ribosome synthesis and assembly, the nucleoli (Kim et al. 2004). The chromosomal rDNA-rich region contains the 16S, 5.8S and 28S genes, which are often studied in the reconstruction of species phylogenies.

The occurrence of dyads and triads at the tetrad stage in the putative hybrid accession Oodil-13a is a feature associated with hybrids (e.g. Silkova et al. 2016), and could support the concept of hybridisation within *Harpagophytum*.

4.2 Genetic variation

4.2.1 Evaluation of primers

All bands were scored as present or absent in the 96 samples, producing a total of 138 polymorphic and 31 monomorphic bands for the ISSR and RAPD primers taken together. The highest PIC values were obtained with RAPD primers OPT-B11 (PIC = 0.420) and OPT-J06 (PIC = 0.423) while average PIC values were relatively similar for RAPD and ISSR primers (0.202 and 0.220, respectively) in spite of the fact that ISSR primers contain repetitive elements that are expected to guide the amplification to variable sites in the genome. While ISSR markers are highly reproducible (Khan and Shah 2016), we obtained adequate reproducibility also with RAPD, and both marker types are likely to remain a much appreciated tool in laboratories with restricted access to expensive equipment and chemicals.

4.2.2 Seedling similarity and breeding system

Amount of variation within a plant population or an offspring group depends to a very large extent on breeding system. Generally, outcrossing species have significantly larger within-population diversity than self-fertilizing species (Nybom 2004). Moreover, in an outcrossing species, seedlings from different fruits are usually more heterogeneous than seedlings from the same fruit since the former are more likely to derive from different pollination events. Jaccard's

similarity coefficients among seedlings from the same *Harpagophytum* seed capsule ranged from 0.84 to 0.96 with a mean of 0.89, while those from different seed capsules on the same plant ranged from 0.73 to 0.92, with a mean of 0.83. By comparison, seedlings from different mother plants of the same taxon growing at the same locality, showed Jaccard's similarity coefficients that ranged from 0.78 to 0.82, with a mean of 0.80.

The higher Jaccard's index similarities found in single-capsule offspring compared to among offspring from different capsules on the same plant, suggest that both species are cross-pollinated thereby facilitating interspecific hybridization and backcrossing.

4.2.3 Differentiation among taxa

Analysis of molecular variance (AMOVA) showed 39% variability between the two main species, *H. procumbens* and *H. zeyheri*, while the two recognized subspecies of *H. procumbens*; i.e., ssp. *procumbens* (PP) and ssp. *transvaalense* (PT), showed 15% intraspecific variability. Putative hybrids (PH), showed 21% differentiation when compared with PP, and 19% when compared with either PT or with *H. zeyheri* ssp. *zeyheri* (ZZ). A deviating morphotype, '*procumbens* new variety' (PN), showed only 9% differentiation when compared with PT, 22% when compared with PP or with PH, and 41% when compared with ZZ, suggesting that it belongs to *H. procumbens*, and is most closely affiliated with ssp. *transvaalense*. The unequal sample sizes can, however, cause some artifacts when different results are compared.

The UPGMA tree showed two major clusters representing the two recognized species of the genus. Putative hybrids were allocated to both clusters A and B, and to various subclusters.

The PCA and MDS analyses produced very similar results. Differentiation of the two *Harpagophytum* species was supported by two well-separated clusters along PCA1 (Figure 22). Furthermore, PCA1 also dispersed the putative hybrids from the edge of the *H. procumbens* cluster to the edge of the *H. zeyheri* cluster. The different taxa of *H. procumbens* were instead separated by PCA2, although with some overlap.

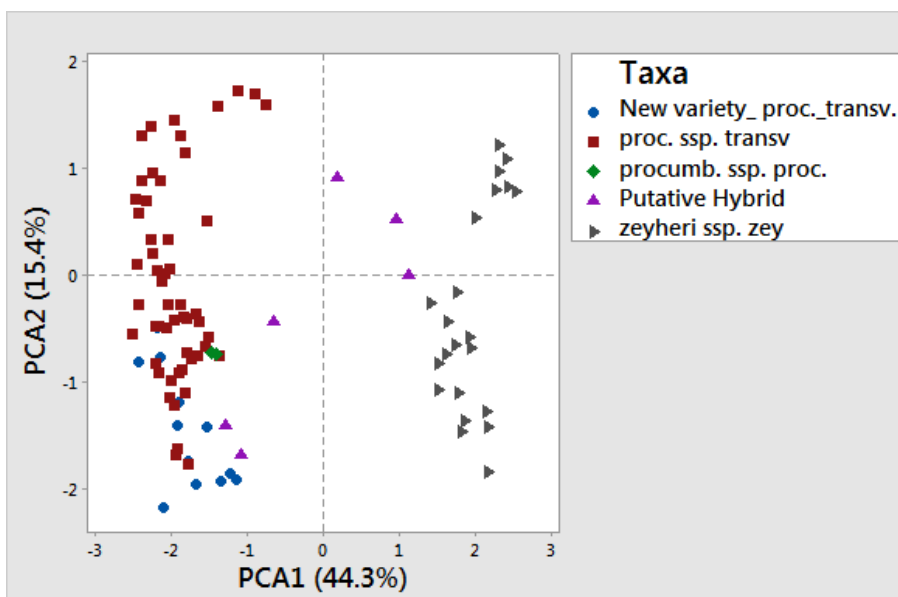


Figure 22. PCA showing phenetic relationships of *Harpagophytum* taxa based on DNA data.

The Bayesian analysis reached an equilibrium for the model at $K = 2$ roughly corresponding to the two species *H. procumbens* and *H. zeyheri* but with indication of some gene flow, especially in the samples denoted as putative hybrids. For $K = 3$, a similar result was obtained but with two subgenomes within *H. procumbens* ssp. *transvaalense*.

Our study supports the commonly accepted classification of *Harpagophytum* into two species, with substantial interspecific differentiation according to the AMOVA, occurrence of two well-separated clusters in the UPGMA cluster analysis and the PCA, and two well delimited genomes in the Bayesian structure analysis.

4.2.4 Genome structure and introgression

The five putative hybrid plants were equally well differentiated from the two possible parent taxa according to the AMOVA. Similarly, the PCA showed these five plants bridging the gap between the clusters representing the parental taxa. By contrast, these plants were scattered within the parental clusters in the dendrogram, which is typical of the topology of hybrids in hierarchical trees (Archibald et al. 2004). The two putative hybrid plants sampled at the same

location, O5PH and O9PH, had widely different capsules, and possibly one of these plants was a polyploid.

Plants with three seed rows in each locule but otherwise conforming to *H. procumbens* ssp. *transvaalense* were sampled at four locations (Tlokweneng, Oodi, Leshibitse and Kumakwane) and denoted as ‘*procumbens* new variety’ (PN). All the statistical analyses showed that PN is more similar to *H. procumbens* than to *H. zeyheri*, and there was no marker-based evidence of interspecific introgression despite the number of seed rows suggesting such an affinity. Possibly PN plants derive from backcrosses between hybrids and *H. procumbens* ssp. *transvaalense*.

This DNA study indicates the presence of introgression at Oodi, Leshibitse, Kumakwane and Tlokweneng, probably associated with the formation of patchy populations resulting from the mainly epizoochorous dispersal (attachment of fruit capsules to hooves, jaws and mouths of cattle and antelopes) of *Harpagophytum* (Ihlenfeldt 1967). The locations where these plants were collected are along an introgressive belt (Leshibitse, Oodi), which can be associated to routes followed by herders of livestock or near water points (Kumakwane, Oodi, Tlokweneng). The *procumbens* new variety was also found in villages of Mmathethe (about 110 km from the introgression zones) and in Matlapaneng, Maun (about 660 km from the introgression zones), again indicating that the distribution patterns and introgression zones of *Harpagophytum* in Botswana may be closely related to livestock movements.

4.3 Biochemical compounds

Extracts made from tuber peels and tuber pulp of 37 *Harpagophytum* accessions were analysed with HPLC. Based on the HPLC-chromatograms, five compounds; verbascoside, isoverbascoside, acetylacteoside, pagoside and harpagoside, were selected for further analyses based on their stability and reliability across taxa. The identities of these compounds were verified with HPLC-ESI-MS.

4.3.1 Chemical compounds in tuber peels and pulp

The content of harpagoside was significantly highest in HPT (*H. procumbens* ssp. *transvaalense*) samples (9.8 mg/g dry weight or dw), intermediate in HH (putative hybrids) (5.0 mg/g dw) and lowest in HZ (*H. zeyheri*) (1.5 mg/g dw). All other compounds were

generally highest in HH samples (Table 3). The range across samples for peel compounds was for verbascoside 0.2–8.0 mg/g dw, isoverbascoside 0.1–14.5, acetylacteoside 0.0–10.6, pagoside 0.0–4.4, and harpagoside 0.0–16.0. The range across samples for pulp compounds was for verbascoside 0.3–21.0 mg/g dw, isoverbascoside 0.2–13.2, acetylacteoside 0.0–14.8, pagoside 0–2.3, and harpagoside 0.0–23.9.

This study, as per our literature search, seems to be the first of its kind to report on biochemical composition of both peel and pulp of tubers of *Harpagophytum*. In general there was little difference between contents in peel and pulp for different compounds except for harpagoside. The harpagoside content in HPT was nearly double that of HH for both peel and pulp, and more than six times the quantity of HZ in both peel and pulp (Table 3).

The harpagoside (1.62%) content we obtained was within the range reported in other studies (e.g. 1.06%; Karioti et al. 2011).

4.3.2 Variation among samples in chemical composition

Two PCAs were calculated based on AUC chemical profiles, with the first two extracted principal components explaining 81% and 84% of the total variance for tuber peels and pulp, respectively. Due to the similarities between the two PCAs, only the pulp PCA is presented here (Figure 23).

Both PCAs indicated a grouping of samples into three clusters: 1) the HPT cluster; 2) the HZ cluster; and 3) the HH cluster. Some HZ samples were, however, found within the HPT cluster. Even though the HH samples tended to cluster in between of the HPT and HZ clusters, some of the HH samples also occurred within the HPT and HZ clusters.

In the bi-plot, harpagoside content mapped in the opposite direction of the acetylacteoside, isoverbascoside, pagoside and verbascoside (Figure 23A). There was also a general tendency of samples to group with respect to sampling location, even though some taxa occurred in areas other than their place of origin (Figure 23B).

Table 3. Average content of biochemical compounds analysed in peel and pulp of tubers for different *Harpagophytum* taxa (HH = putative *Harpagophytum* hybrids, N = 18; HPT = *H. procumbens* ssp. *transvaalense*, N = 5; HZ = *H. zeyheri*, N = 14). Significant difference among taxa for a specific compound is indicated by different letters (P<0.05); dw = dry weight.

Compound	Taxon	Peel (mg/g dw)	Pulp (mg/g dw)
Verbascoside	HH	2.50 ^a	4.56 ^a
	HPT	2.28 ^{ab}	2.15 ^b
	HZ	1.47 ^b	1.12 ^b
Isoverbascoside	HH	4.04 ^a	5.40 ^a
	HPT	1.70 ^b	2.45 ^b
	HZ	1.61 ^b	1.68 ^b
Acetylacteoside	HH	3.61 ^a	6.80 ^a
	HPT	4.24 ^a	6.18 ^a
	HZ	1.91 ^b	1.85 ^b
Pagoside	HH	0.69 ^a	0.64 ^a
	HPT	0.32 ^{ab}	0.55 ^a
	HZ	0.03 ^b	0.03 ^b
Harpagoside	HH	5.02 ^b	8.36 ^b
	HPT	9.83 ^a	16.22 ^a
	HZ	1.47 ^c	1.23 ^c

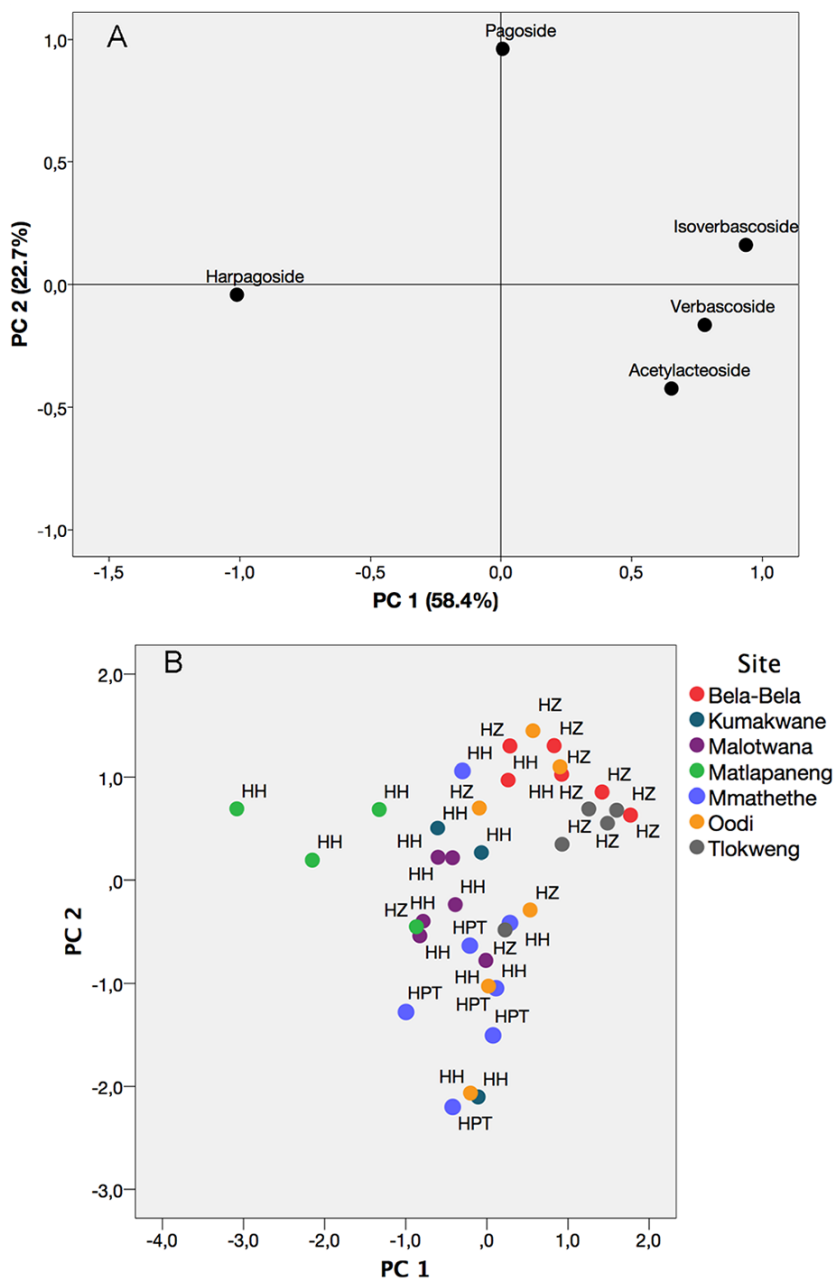


Figure 23. A) PCA for tuber pulp compounds based on their share of total AUC, and B) plot of regression scores of samples. (HH = putative *Harpagophytum* hybrid, HPT = *H. procumbens* ssp. *transvaalense*, HZ = *H. zeyheri*). Total variance explained by two principal components: 81.0%.

4.3.3 Variation among samples in chemical content by weight

The first two principal components extracted based on tuber peel and pulp chemical content by weight explained 77.1% and 74.6% of the variance, respectively. For both PCAs (not shown) there was a less clear grouping according to taxon compared to the PCAs calculated on chemical composition. The HZ samples did, however, form a cluster together with some HH samples. The HZ samples were characterized by a low content of all the biochemical compounds. One HPT sample was characterized by a very high content of pagoside.

In both PCAs, samples from Bela-Bela, Oodi and Tlokweng, which are located within a maximum distance of 20.2 km, formed a dense cluster, except for two samples. However, samples from Mmathethe and Malotwana also overlapped despite the 150 km distance between these localities.

4.4 Antioxidants and anti-inflammatory capacity

The antioxidant and anti-inflammatory capacity of different taxa of *Harpagophytum* was investigated using extracts from six samples (two *H. procumbens*, two *H. zeyheri* and two putative hybrids), for which taxonomic classification had been verified with DNA markers (results not shown here). Contents of total phenolics, individual phenylpropanoids and iridoids, and the ferric reducing ability of plasma of the six extracts was analysed. None of the extracts had significant lethal or suppressive effects nor any effect on cell viability of human neutrophils. Level of anti-inflammatory capacity was evaluated using chemiluminescence assays for detection of the extent to which ROS generation is suppressed in neutrophils treated with the different extracts as well as with commercially obtained standards of harpagide, harpagoside and verbascoside.

4.4.1 Effect of extracts on neutrophil ROS production

Unstimulated cells (PBS) treatment

The PBS treatment, herein termed unstimulated cells since it is a buffer with osmolarity and ion concentrations that mimic the human body (isotonic), indicated the following results. Accession 3 (new variety of *H. procumbens* ssp. *transvaalense*) showed a significant ($P<0.05$) anti-inflammatory effect when compared to accessions 16 (putative hybrid) and 24 (*H. zeyheri* ssp. *zeyheri*) (Figure 24). Accession 14 (*H. zeyheri* ssp. *sublobatum*) showed a significant anti-inflammatory effect when compared with accessions 16, 17 (*H. procumbens* ssp. *transvaalense*) and 24. Still, none of the accessions differed significantly from the buffer PBS control or the vehicle control (ethanol).

Staphylococcus aureus (SA) treatment

None of the accessions showed significant differences when compared with the buffer PBS or the vehicle control (ethanol) (Figure 25).

Fusobacterium nucleatum (FN)-toll like receptor ligand treatment

Accession 11 showed a significant pro-inflammatory effect when compared with the vehicle control (ethanol), and with accession 17 (Figure 26). None of the accessions showed significant differences when compared with the buffer PBS control.

PMA treatment

Accessions 14 and 17 showed the highest anti-inflammatory properties and differed significantly from the buffer PBS control (Figure 27). The level of anti-inflammatory activity also differed among the *Harpagophytum* accessions; accessions 3 and 11 showed a significantly lower anti-inflammatory activity compared to accessions 14 and 17.

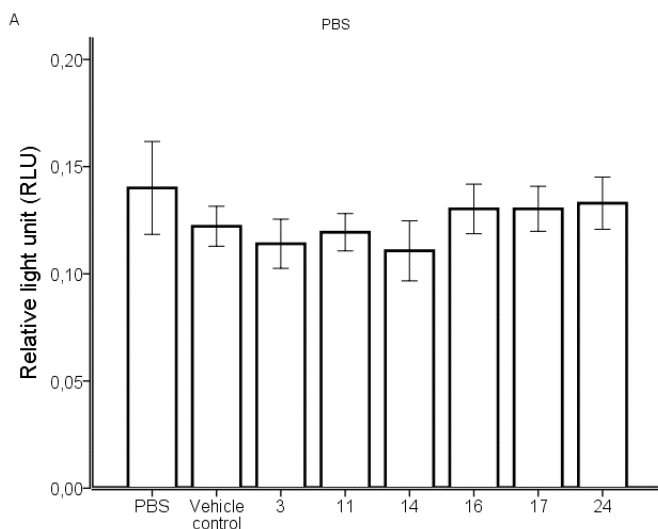


Figure 24. Luminol-detected total ROS production by neutrophils stimulated with PBS (no stimulus) in the presence of PBS, ethanol, and accessions 3, 11, 14, 16, 17 and 24 (n = 10). Error bars: 95% CI.

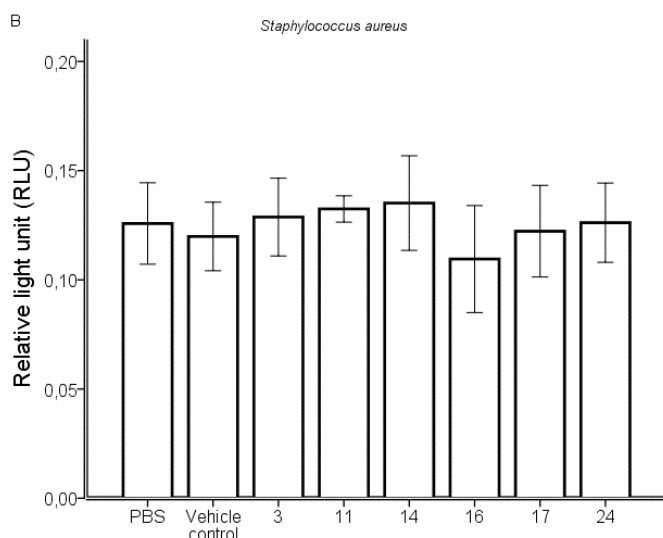


Figure 25. Luminol-detected total ROS production by neutrophils stimulated with *Staphylococcus aureus* (SA) in the presence of PBS, ethanol, and accessions 3, 11, 14, 16, 17 and 24 (n = 10). Error bars: 95% CI.

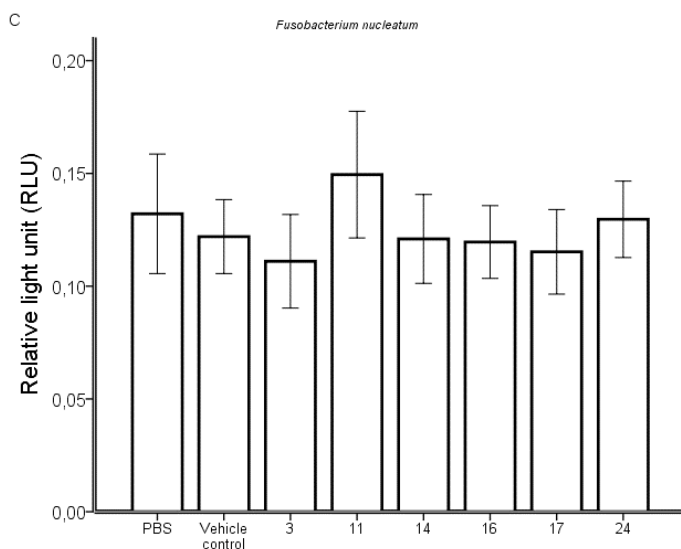


Figure 26. Luminol-detected total ROS production by neutrophils stimulated with *Fusobacterium nucleatum* in the presence of PBS, ethanol, and accessions 3, 11, 14, 16, 17 and 24 (n = 10). Error bars: 95% CI.

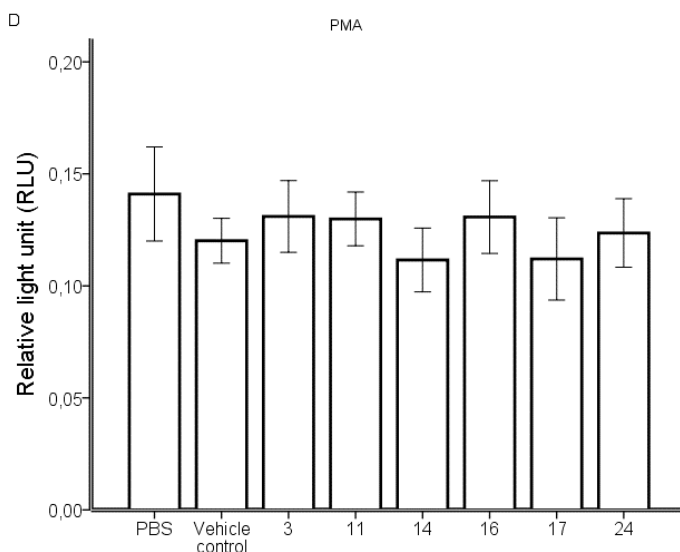


Figure 27. Luminol-detected total ROS production by neutrophils stimulated with PMA (25nM) in the presence of PBS, ethanol, and accessions 3, 11, 14, 16, 17 and 24 (n = 10). Error bars: 95% CI.

Effects of verbascoside and harpagoside standards on ROS inhibition

The verbascoside and harpagoside standards had no significant effects on either FN- or SA-treated neutrophils. Significant differences were only noted for PBS- and PMA-treated neutrophils. For PBS stimulation, the verbascoside (P=0.043) and harpagoside (P=0.043) standards inhibited ROS production better than PBS control. For PMA stimulation, the verbascoside standard inhibited ROS generation significantly better (P=0.043) than the harpagoside standard. The lack of a strong effect of either compound, when analysed by itself, suggests that putative anti-inflammatory effects of *Harpagophytum* extracts may be dependent on a synergistic interaction among several compounds.

4.4.2 Chemical contents and antioxidant capacity

Content of total phenols was highest in the extracts derived from the putative new variety of *H. procumbens* (accession 3, 28.9 mg GAE/g dw) and from *H. zeyheri* ssp. *sublobatum* (accession 14, 25.1 mg GAE/g dw), respectively (Table 4). The external standards of harpagide, harpagoside and verbascoside were also subjected to this analysis; low phenolic content was identified for the iridoids and high phenolic content for the verbascoside standard (Table 4).

Table 4. Content of total phenols (TP) and antioxidant capacity (FRAP) of *Harpagophytum* extracts; mean values with standard deviation. (GAE = gallic acid equivalents).

Accession	Total phenols (mg GAE/g dw)	FRAP ($\mu\text{mol Fe}^{2+}$ /g dw)
3 (new variety of ssp. <i>transvaalense</i>)	28.9 \pm 0.4	596.7 \pm 25.2
11 (putative hybrid)	13.5 \pm 0.6	204.8 \pm 0.1
14 (<i>H. zeyheri</i> ssp. <i>sublobatum</i>)	25.1 \pm 1.3	444.3 \pm 13.0
16 (putative hybrid)	5.1 \pm 0.3	80.8 \pm 13.2
17 (<i>H. procumbens</i> ssp. <i>transvaalense</i>)	2.1 \pm 0.4	33.8 \pm 0.2
24 (<i>H. zeyheri</i> ssp. <i>zeyheri</i>)	3.5 \pm 0.5	54.5 \pm 5.6
Harpagide standard	16.7 \pm 1.1	149.2 \pm 0.0
Harpagoside standard	0.0 \pm 0.0	612.3 \pm 3.2
Verbascoside standard	359.9 \pm 9.8	5854.2 \pm 52.7

Table 5. Contents of compounds in each *Harpagophytum* tuber extract; mean values with standard deviation, as mg per g dry weight of the plant material.

Accession	Verbascoside	Isoverbascoside	Harpagoside	8-O-p-coumaroyl-harpagide
3	3.04±0.11	21.75±1.16	4.09±0.34	0.04±0.00
11	1.28±0.11	6.02±0.50	9.08±0.97	4.04±0.41
14	4.22±0.33	15.62±1.32	7.07±0.60	3.29±0.35
16	0.64±0.09	2.47±0.27	0.96±0.15	0.07±0.01
17	0.33±0.05	0.62±0.08	1.71±0.24	0.09±0.01
24	0.39±0.08	0.64±0.14	0.16±0.01	0.01±0.00

In keeping with the above results, the FRAP assay showed that accession 3 had the highest antioxidant capacity (596.7 $\mu\text{mol Fe}^{2+}/\text{g dw}$), followed by accession 14 (444.3 $\mu\text{mol Fe}^{2+}/\text{g dw}$) (Table 5). Since there was no ascorbate in the samples, antioxidant capacity must derive from other compounds. The standards harpagide, harpagoside and verbascoside were also analysed and verbascoside was the most potent (5854.2 $\mu\text{mol Fe}^{2+}/\text{g dw}$) with approximately 10 \times the capacity of harpagoside and 40 \times the capacity of harpagide (Table 5).

The content of verbascoside was highest in accession 14 (4.2 mg/g dw) followed by accession 3 (3.0 mg/g dw). The content of isoverbascoside was highest in accession 3 (21.8 mg/g dw). Significant amounts of harpagoside and 8-O-p-coumaroyl harpagide were identified in accessions 11 and 14. Extracts from accessions 16, 17 and 24 contained low levels of verbascoside, isoverbascoside and iridoid compounds. Thus, the phytochemical analysis revealed substantial differences in chemical composition between the analyzed taxa but whether these differences are taxon-specific must be ascertained in future studies using a much larger material with several samples of each taxon.

Bivariate Pearson rank correlation analysis was used to verify antioxidant potentials of verbascoside and iso-verbascoside in standards.

Table 6. Bivariate Pearson rank correlation analysis amongst compounds and conducted tests. Numbers in brackets indicate P-values.

	Total phenols	FRAP	PBS
FRAP	0.991 (0.000)	-	
PBS	-0.888 (0.018)	-0.847 (0.033)	-
Verbascoside (standard)	0.934 (0.006)	0.903 (0.014)	-0.931 (0.007)
Isoverbascoside (standard)	0.985 (0.000)	0.999 (0.000)	-0.846 (0.034)

The positive correlations between contents of total phenols, and the verbascoside and iso-verbascoside standards (Table 6), and the negative correlations obtained when comparing total phenols and FRAP (Table 4) with the PBS control (which imitates conditions of the human body), indicate that verbascoside is an important antioxidant phenol in *Harpagophytum*. Accessions 3 and 14, which had the highest verbascoside levels, also had high antioxidant activity in the unstimulated cell PBS control treatment. High levels of total phenols, isoverbascoside and verbascoside characterise antioxidant properties of many plant species (Kostyuk et al. 2011). Verbascoside is known to scavenge reactive oxygen species, repair skin tissues and facilitate the iron chelation process (Vertuani et al. 2011).

4.5 Implications of biochemical and ROS studies on medicinal relevance

Contrary to results reported by El Babili et al. (2012) in an *in vitro* study aimed at establishing a tool for quantification of harpagoside in *H. procumbens*, our data did not support a positive association between anti-inflammatory effects and quantity of harpagoside in the investigated extracts. The discrepancy may be due to our low sample size, or to the fact that *Harpagophytum* compounds could be functioning in a synergistic fashion (Ebrahim and Uebel 2011). Although accession 3 had only a medium-high harpagoside content,

it had, together with accession 14, the highest values in the FRAP assay. Antioxidant activity in extracts of *Harpagophytum* tubers appears to be closely dependent on contents of verbascoside and isoverbascoside. Part of the efficacy of *Harpagophytum* extracts may be related to the antioxidant activity, as suggested by the beneficial effects of verbascoside (Gyurkovska et al. 2011).

The connection between harpagoside content and medicinal activity of *Harpagophytum* is still unclear. Baghdikian et al. (1997) found quantities of harpagoside in *H. zeyheri* to be similar to those of *H. procumbens*, and therefore argued that both species should be regarded as medicinally active. According to Mncwangi et al. (2014) the two species are instead significantly dissimilar, which is more in line with our results. Thus, the harpagoside content in *H. procumbens* was 13 times higher than that of *H. zeyheri* in the pulp, and six times higher in the peel. More data from a both geographic and taxonomically wider array of samples are, however, needed before drawing any final conclusions.

5 Conclusions and perspectives

A large group of rural poor in Botswana, Namibia and South Africa, depend on harvesting *Harpagophytum* tubers for survival. It is, however, very difficult to classify the species properly based on just the seed capsules, and therefore vendors may occasionally misidentify the species when harvesting tubers in areas where both species, and possibly also hybrids, grow. Since the species differ significantly in chemical contents, it is conceivable that such mistakes eventually contribute to chemical inconsistencies in the commercial products derived from *Harpagophytum*.

Domestication of *Harpagophytum* has already been initiated in Botswana (Motlhanka and Makhabu 2011) in order to allow for production of tubers for medicinal purposes without jeopardizing the survival of the species. The studies presented in this thesis may contribute to domestication strategies, multiplication and genetic manipulation for plant breeding purposes.

Depending on the intentions of the breeder, for instance, directional selection of plants with high harpagoside content may be desirable. So far, no genetic lineage has been found to have high quantities of all compounds. Our results indicate that improved plant material of *Harpagophytum* can be produced through conventional breeding methods, combining beneficial traits from several genotypes. Moreover, based on the PCA data, we suspect that the biochemicals in *Harpagophytum* may be controlled by a restricted group of genes. We therefore postulate that gene manipulation procedures could also be useful for plant improvement.

Selection of valuable germplasm could also involve ROS studies for identification of genotypes with superior antioxidant and anti-inflammatory qualities. Our results indicate that *Harpagophytum* has a genetically influenced effect on suppressing or stimulating reactive

oxygen species. One *H. zeyheri* ssp. *sublobatum* accession displayed antioxidant and anti-inflammatory properties, while a *H. zeyheri* ssp. *zeyheri* accession was the only one that failed all the medicinal efficacy tests. Although the two accessions belong to the same species, they behaved very differently, perhaps due to genetically determined subspecies differences or to environmental factors.

While testing, verification and improvement of germplasm, may require advanced research facilities, multiplication of the finally selected germplasm could be accomplished in a rural setup, through the establishment of community-funded greenhouse facilities and gardens. Carefully organized and monitored, the availability of high-quality *Harpagophytum* plants for commercial harvesting has the potential to benefit the rural communities.

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