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South African Journal of Botany 77 (2011) 387–396

SOUTH AFRICAN JOURNAL OF BOTANY

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Seasonal variation in antimicrobial and phytochemical properties of frequently used medicinal bulbous plants from South Africa

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Received 22 July 2010; received in revised form 5 October 2010; accepted 5 October 2010

Abstract

The growing popularity of traditional medicine and the unrestricted collection of medicinal plants from the wild have put many of the slow growing bulbous plant species at the risk of over-exploitation and extinction in South Africa. This study was aimed at comparing the phytochemical composition and biological (antibacterial and anticandidal) activities of bulb and leaf extracts of *Tulbaghia violacea*, *Hypoxis hemerocallidea*, *Drimia robusta* and *Merwilla plumbea* between spring, summer, autumn and winter seasons, with the view of promoting the use of leaves, as a conservation strategy. Antibacterial and anticandidal activities of petroleum ether (PE), dichloromethane (DCM), 80% ethanol and water extracts of bulbs and leaves were tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* bacteria and the fungus *Candida albicans* using the microdilution bioassay. Spectrophotometric methods were used to evaluate saponin and phenolic compositions for the four seasons. Leaf and bulb extracts exhibited comparable anticandidal activity (MIC < 1 mg/ml) in all the plant species in all seasons. Only ethanol and water extracts of *H. hemerocallidea* corms (autumn and winter) showed correspondingly good fungicidal activity amongst the bulbs tested. Antibacterial activity was fairly comparable between bulbs and leaves with at least one extract of each plant species showing some good MIC values in most of the seasons. The best antimicrobial activities were recorded in winter and autumn seasons, with MIC values as low as 0.2 mg/ml in the DCM bulb extracts of *T. violacea* (winter) against *K. pneumoniae* and *S. aureus*. The amounts of total phenolic compounds in all plant samples were generally higher in spring compared to the other seasons. Condensed tannin, gallotannin and flavonoid levels, depending on the sample, were either higher in spring or winter except for *H. hemerocallidea* (corm) which had higher gallotannin levels in autumn. Total saponin levels were higher in winter in all plant samples. Although variation was observed in the phytochemical concentrations between the bulbs and leaves of each plant species, their antimicrobial activities were fairly comparable. Leaves may be used as substitutes for bulbs in the treatment of bacterial and fungal ailments.

Keywords: Antimicrobial activity; Medicinal bulbs; Phenolic compounds; Saponins; Seasonal variations

1. Introduction

Medicinal plants are used by billions of people in most developing countries because of the frequently inadequate provision of modern medicine, their low cost, effectiveness, as well as cultural beliefs and preferences (Shanley and Luz, 2003; Sheldon et al., 1997). In 2000 an estimated 60% of the South African population were using indigenous, traditional plant medicines for primary health care purposes (Van Wyk and Gericke, 2000).

In South Africa, bulbs are one group of plants that are highly valued and extensively used in the traditional medicine system. Bulbs are used in the treatment of various ailments (Hutchings, 1989; Katerere and Eloff, 2008; Taylor and Van Staden, 2001; Zschocke et al., 2000) and considerable research resources have been directed towards screening for biologically active compounds. Further investigations on their ethnopharmacological and phytochemical properties can serve to validate their use in traditional medicine and provide new pharmaceutical leads.

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doi:10.1016/j.sajb.2010.10.004

The scale and intensity of human interactions with plants have led to progressive and wide-scale habitat loss, degradation and fragmentation, with subsequent loss of plant species. As a result of urbanization and increased demand for medicinal plants, however, harvesting has often become the domain of untrained, and often unconcerned, commercial gatherers with no other income sources (Williams et al., 2000). Bulbs are ranked as one of the most popular plant groups sold at most of the traditional medicinal markets in South Africa (Mander, 1997). Their use in herbal medicine involves destructive uprooting of the whole plant. Harvesting of whole plants, roots and bulbs account for approximately 50% of the more than 500 species and 48% of the volume of plants sold in the traditional medicine market in Johannesburg (Williams, 2003). Mander (1998) reported that of the most important bulbs traded in the markets of KwaZulu-Natal, approximately 28% had the whole plant harvested. This non-sustainable harvesting not only threatens the survival of valuable medicinal plant species, but also the livelihoods of people that depend on them. It is for this reason that medicinal bulbous plants, whether listed as threatened species or not, by virtue of their economic and cultural significance, are at the risk of over-exploitation and extinction, and needs renewed attention with respect to their conservation.

The harvesting of leaves is considered less destructive, although intensive pruning can affect reproductive performance (Gaoue and Ticktin, 2007). A conservation strategy that would take into consideration sustainable harvesting and perhaps simultaneously provide similar medicinal benefits would be the substitution of bulbs with leaves of the same plant (Lewu et al., 2006; Zschocke et al., 2000). However, secondary metabolite production, which happens to be responsible for the bioactivity in plants, is determined by a number of factors, chief amongst them being climatic (Koptur, 1985; Nacif de Abreu and Mazzafera, 2005).

This study was aimed at addressing the conservation of *Tulbaghia violacea*, *Hypoxis hemerocallidea*, *Drimia robusta* and *Merwilla plumbea* through the concept of plant part substitution. Antibacterial and anticandidal activities together with phenolic and saponin composition in bulbs (currently the more widely used part) and leaves of these four species were evaluated during summer, autumn, winter and spring.

2. Materials and methods

2.1. South African seasons

South Africa's eastern seaboard has a weather pattern with four distinct seasons. Winter is the coldest and driest season and covers the period May to July. Most vegetation shed leaves during this dry winter period. Following winter is the hot, windy and dry spring season which is characterised by flowering in most of the plant species and occurs from mid-August to late-October. Summer succeeds spring and is the longest of the seasons stretching from November to February. Summer is the rainy season and is characterised by high temperatures. All the vegetation is green in summer with a predominantly rich geophyte component. Autumn marks the transition from summer into winter and usually falls between March and April. Plant leaves begin senescing during this season.

2.2. Plant material

The plant materials used in this study were collected in summer (December), autumn (March), winter (June) and spring (September) from the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa and separated into bulbs/corms and leaves. Voucher specimens (Table 1) were deposited in the Bews Herbarium (NU) at the University of KwaZulu-Natal, Pietermaritzburg. The samples were then dried in an oven at a constant temperature of 50 °C for five days and ground into fine powders.

2.3. Preparation of plant extracts

The ground samples were sequentially extracted with 20 ml/g of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water respectively in a sonication bath containing ice for 1 h. The crude extracts were then filtered under vacuum through Whatman No. 1 filter paper and the organic extracts were concentrated *in vacuo* at 35 °C using a rotary evaporator. The concentrated extracts were subsequently dried at room temperature under a stream of cold air. Water extracts were freeze dried and kept in airtight containers.

2.4. Antibacterial activity

Minimum inhibitory concentrations (MIC) of extracts for antibacterial activity were determined using the microdilution bioassay as described by Eloff (1998). Overnight cultures (incubated at 37 °C in a water bath with an orbital shaker) of two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) bacterial strains were diluted with sterile Mueller-Hinton (MH) broth to give a final inoculum of approximately

Table 1
Bulbous medicinal plants used in this study.

Family	Species	Voucher number	Medicinal uses
Alliaceae	*Tulbaghia violacea* Harv.	NCUBE 04 NU	Bulbs and leaves used for the treatment of gastrointestinal ailments, asthma, tuberculosis, colds and fever (Hutchings et al., 1996; Van Wyk et al., 2009).
Hypoxidaceae	*Hypoxis hemerocallidea* Fisch.& C.A. Mey	NCUBE 01 NU	Plant decoctions have purging effects (Watt and Breyer-Brandwijk, 1962).
Hyacinthaceae	*Drimia robusta* Bak	NCUBE 03 NU	Bulbs used to treat urinary infections, diseases of the uterus (Hutchings, 1996; Van Wyk et al., 2009).
Hyacinthaceae	*Merwilla plumbea* (Lindl.) Speta	NCUBE 02 NU	Decoctions are used for wound healing (Van Wyk et al., 2009).

10^6 CFU/ml (colony forming units). The dried crude organic plant extracts were resuspended in 70% ethanol to a concentration of 50 mg/ml while water extracts were dissolved in distilled water to the same concentration. One hundred microlitres of each extract was serially diluted two-fold with sterile distilled water in a 96-well microtitre plate for each of the four bacterial strains. A similar two-fold serial dilution of neomycin (Sigma-Aldrich, Germany) (0.1 mg/ml) was used as a positive control against each bacterium. One hundred microlitres of each bacterial culture was added to each well. Water and 70% ethanol were included as negative and solvent controls respectively. The plates were covered with parafilm and incubated at 37 °C for 24 h. Bacterial growth was indicated by adding 50 μl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and a further incubation at 37 °C for 2 h. Since the colourless tetrazolium salt is biologically reduced to a red product due to the presence of active organisms, the MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. Bacterial growth in the wells was indicated by a reddish-pink colour. The assay was repeated twice with two replicates per assay.

2.5. Anticandidal activity

A microdilution method as described by Eloff (1998) and modified for fungi (Masoko et al., 2007) was used to determine the antifungal activity of the extracts against *Candida albicans* (ATCC 10231). An overnight yeast culture was prepared in Yeast Malt (YM) broth. Four hundred microlitres of the overnight culture was added to 4 ml of sterile saline and absorbance was read at 530 nm. The absorbance was adjusted with sterile saline to match that of a 0.5 M McFarland standard solution. From this standardised candidal stock, a 1:1000 dilution with sterile YM broth was prepared giving a final inoculum of approximately 10^6 CFU/ml. Dried organic extracts were resuspended in 70% ethanol to a concentration of 50 mg/ml and water extracts were dissolved in water to the same concentration. One hundred microlitres of each extract was serially diluted two-fold with sterile water in a 96-well microtitre plate. A similar two-fold dilution of Amphotericin B (Sigma-Aldrich, Germany) (2.5 mg/ml) was used as the positive control while water and 70% ethanol were used as negative and solvent controls respectively. Dilute fungal culture (100 μl) was added to each well. The plates were covered with parafilm and incubated at 37 °C for 24 h after which 50 μl (0.2 mg/ml) INT was added and incubated for a further 24 h at 37 °C. The wells remained clear where there was inhibition of fungal growth. MIC values were recorded as the lowest concentrations that inhibited fungal growth after 48 h. To determine the fungicidal activity, 50 μl of sterile YM broth was added to all the clear wells and further incubated at 37 °C for 24 h after which the minimum fungicidal concentrations (MFC) were recorded as the last clear wells. The assay was repeated twice with two replicates per assay.

2.6. Phenolic content determination

2.6.1. Preparation of extracts

Phenolic compounds were extracted from plant materials as described by Makkar (1999). Dried plant samples (2 g) were extracted with 10 ml of 50% aqueous methanol by sonication on ice for 20 min. The extracts were then filtered under vacuum through Whatman No. 1 filter paper.

2.6.2. Determination of total phenolic compounds

The amounts of total phenolic compounds in plant samples were determined using the Folin Ciocalteu (Folin C) assay for total phenolics as described by Makkar (1999) with slight modification (Ndhlala et al., 2007). Fifty microlitres of each extract from the plant samples were transferred into test tubes into which 950 μl of distilled water was added followed by 1 N Folin C phenol reagent (500 μl) and 2% sodium carbonate (2.5 ml). A blank that contained aqueous methanol instead of plant extracts was also prepared. The test mixtures were incubated for 40 min at room temperature and the absorbance was read at 725 nm using a UV–vis spectrophotometer (Varian Cary 50, Australia). Three replicates of each extract were tested. Total phenolic concentrations were expressed as gallic acid equivalents (GAE).

2.6.3. The butanol–HCl assay for condensed tannins (proanthocyanidins)

Three millilitres of butanol–HCl reagent (95:5, v/v) was added to 500 μl of each extract, followed by 100 μl ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The test combination was vortexed and placed in a boiling water bath for 60 min. The absorbance was then read at 550 nm using a UV–vis spectrophotometer against a blank prepared by mixing the extract (500 μl) with butanol–HCl reagent (3 ml) and ferric reagent (100 μl), but without heating. Each extract had three replicates. Condensed tannin (%) was calculated as leucocyanidin equivalents using the formula developed by Porter et al. (1986):

$$\text{Condensed tannin (\%)} = (A_{550\ \text{nm}} \times 78.26 \times \text{Dilution factor}) \div (\%\ \text{dry matter})$$

This formula assumes that the effective $E^{1\%,\ 1\ \text{cm},\ 550\ \text{nm}}$ of leucocyanidin is 460. The dilution factor is 0.5 ml/(volume of extract taken).

2.6.4. Vanillin assay for flavonoids

Plant extracts (50 μl), were made up to 1 ml with methanol in test tubes before adding 2.5 ml methanolic–HCl (95:5, v/v) and 2.5 ml vanillin reagent (1 g/100 ml acetic acid). Similar preparations of a blank that contained methanol instead of plant extracts were made. After 20 min at room temperature, absorbance was read at 500 nm using a UV–vis spectrophotometer. The flavonoid levels were expressed as catechin equivalents (CTE) (Hagerman, 2002).

2.6.5. Rhodanine assay for gallotannins

Plant extracts (50 μl) in test tubes were made up to 1 ml with distilled water. One hundred microlitres of 0.4 N sulphuric acid

and 600 µl of rhodanine were added to the diluted extracts. After 5 min, 200 µl of 0.5 N potassium hydroxide was added followed by 4 ml distilled water after a further 2.5 min. The mixtures were left for an additional 15 min at room temperature, after which the absorbance at 520 nm was read using a UV–vis spectrophotometer against a blank that contained methanol instead of plant extract. Each extract was evaluated in triplicates and gallotannin concentrations were expressed as gallic acid equivalents (GAE) (Makkar, 1999).

2.7. Saponin content

2.7.1. Qualitative determination of saponins

Ten millilitres of distilled water was added to 0.1 g of ground samples in test tubes. The test tubes were corked and vigorously shaken for 2 min. The appearance of stable and persistent foam on the liquid surface for 15 min indicated the presence of saponins (Tadhani and Subhash, 2006). Presence of saponins was confirmed by the formation of an emulsion upon addition of ten drops of olive oil to the 2 ml aqueous extract.

2.7.2. Saponin extraction

Saponins were extracted from the plant material as described by Makkar et al. (2007). The dried and ground plant samples were defatted with hexane in a Soxhlet apparatus for 3 h. After air-drying, saponins were extracted twice from the defatted samples (10 g) in 100 ml of 50% aqueous methanol by incubating at room temperature overnight with continuous stirring. The extracts were then centrifuged at 3000 rpm for 10 min and the supernatant collected. The procedure was repeated with the original residue to obtain a second supernatant. The first and second supernatants were combined and filtered under vacuum through Whatman No. 1 filter paper. Methanol from the filtrate was evaporated from the solution under vacuum at 40 °C to yield the saponin sample in the aqueous phase. The aqueous phase was then centrifuged at 3000 rpm for 10 min to remove water insoluble materials, and transferred to a separating funnel and extracted three times with an equal volume of chloroform to remove pigments. The concentrated saponins in the aqueous solution were then extracted twice with an equal volume of *n*-butanol. The *n*-butanol was evaporated under vacuum at 45 °C. The dried fractions containing saponins were dissolved in 10 ml of distilled water and freeze dried.

2.7.3. Quantitative determination of total saponins

Total saponin content was determined using a spectrophotometric method as described by Hiai et al. (1976) with modifications. The crude saponin extracts were dissolved in 50% aqueous methanol to a concentration of 10 mg/ml. From this, aliquots of 250 µl (in triplicate) of each sample were transferred to test tubes into which an equal volume of vanillin reagent (8 g/100 ml ethanol) was added followed by 2.5 ml of 72% (v/v) sulphuric acid. The mixture was mixed with a vortex and placed in a water bath adjusted at 60 °C for 10 min. The tubes were cooled on an ice-cold water bath for 3 to 4 min and absorbance was measured at 544 nm using a UV–vis spectrophotometer against a blank that contained 50% aqueous methanol instead of sample extract. The saponin concentrations were expressed as diosgenin equivalents (DE) calculated from a standard curve.

2.7.4. Quantitative determination of total steroidal saponins

The amounts of total steroidal saponins in plant samples were determined using the method described by Baccou et al. (1977). Crude saponin extracts were dissolved in 50% aqueous methanol (0.1 mg/ml) from which 300 µl aliquots (corresponding to a sapogenin content of between 1 and 40 µg) were transferred into test tubes and placed in a boiling water bath at 100 °C to remove the methanol by evaporation. After cooling, 2 ml of ethyl acetate was added followed by 1 ml of anisaldehyde–ethyl acetate reagent (0.5:95.5, v/v) and 1 ml sulphuric acid–ethyl acetate reagent (50:50, v/v). The test combination was mixed with a vortex and incubated in a water bath at 60 °C for 20 min. After cooling for 10 min in a water bath at room temperature, absorbance was measured at 430 nm using a UV–vis spectrophotometer against a blank that contained ethyl acetate instead of plant extract. Each extract was evaluated in triplicate and steroidal saponin concentrations were expressed as diosgenin equivalents (DE) calculated from a standard curve.

3. Results and discussion

The minimum inhibitory concentrations of different sample extracts evaluated for antibacterial activity are presented in Table 2. Winter leaf samples of *D. robusta* were unavailable because the plant had shed leaves when samples were collected. In this study, only MIC values less than 1 mg/ml were considered as good activity (Aligiannis et al., 2001). With the exception of *H. hemerocallidea* in summer and *M. plumbea* in winter, at least one extract of the leaves of the screened plant species exhibited good antibacterial activity against at least one of the test bacteria in all seasons. Bulb extracts of *D. robusta* did not show good activity in any of the four seasons. This is surprising due to its widespread utilisation in traditional medicine for the treatment of urinary infections and diseases of the uterus (Hutchings et al., 1996) which may be caused by the test organisms *K. pneumoniae* and *E. coli*. The other three bulb extracts, except *H. hemerocallidea* (spring) and *M. plumbea* (summer), were active against at least one test bacterial strain in each season. The best antibacterial activities were recorded in the winter season from the DCM bulb extracts of *T. violacea* against *K. pneumoniae* and *S. aureus* and PE corm extracts of *H. hemerocallidea* against *B. subtilis*, with all having an MIC value of 0.2 mg/ml. This good activity in winter particularly from the bulb extracts, suggest that the plants could be minimising metabolic cost by retrieving metabolites from senescing leaf tissues and transporting compounds to the underground parts (Mckey, 1979).

Relatively low antibacterial activity was observed in most of the water extracts. MIC values were greater than 1 mg/ml in all except the corm and leaf extracts of *H. hemerocallidea*, for

Table 2
Antibacterial activity (MIC mg/ml) in bulbs/corms (B)/(C) and leaves (L) of *T. violacea*, *H hemerocallidea, D. robusta* and *M. plumbea* sample extracts in spring, summer, autumn and winter seasons.

Plant name	Part	Extract	Spring				Summer				Autumn				Winter			
			Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa	Bs	Ec	Kp	Sa
Tulbaghia violacea	B	PE	**0.78**	3.13	1.56	**0.78**	3.13	3.13	1.56	3.13	3.13	3.13	3.13	3.13	**0.78**	3.13	1.56	**0.39**
		DCM	**0.78**	3.13	**0.78**	**0.78**	**0.78**	3.13	1.56	3.13	**0.78**	3.13	3.13	3.13	**0.39**	1.56	**0.20**	**0.20**
		EtOH	6.25	3.13	1.56	12.5	6.25	3.13	1.56	12.5	3.13	1.56	1.56	6.25	6.25	3.13	3.13	3.13
		Water	>12.5	>12.5	3.13	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	12.5	>12.5	12.5	12.5	6.25	12.5
	L	PE	1.56	3.13	1.56	3.13	**0.78**	1.56	1.56	1.56	**0.78**	1.56	3.13	**0.78**	1.56	1.56	6.25	3.13
		DCM	**0.78**	3.13	1.56	**0.78**	**0.39**	1.56	1.56	3.13	**0.78**	1.56	1.56	3.13	**0.78**	1.56	3.13	3.13
		EtOH	6.25	3.13	12.5	12.5	6.25	1.56	1.56	6.25	6.25	3.13	1.56	6.25	3.13	3.13	3.13	12.5
		Water	>12.5	12.5	12.5	12.5	>12.5	12.5	6.25	12.5	>12.5	6.25	12.5	12.5	12.5	>12.5	6.25	12.5
Hypoxis hemerocallidea	C	PE	1.56	6.25	3.13	6.25	3.13	3.13	3.13	6.25	3.13	3.13	3.13	3.13	**0.20**	1.56	**0.78**	1.56
		DCM	1.56	3.13	3.13	3.13	3.13	3.13	3.13	3.13	1.56	3.13	3.13	3.13	1.56	3.13	1.56	1.56
		EtOH	3.13	3.13	1.56	3.13	**0.78**	1.56	1.56	3.13	3.13	3.13	1.56	3.13	3.13	3.13	1.56	3.13
		Water	>12.5	12.5	3.13	12.5	**0.78**	**0.78**	1.56	**0.39**	1.56	**0.78**	1.56	**0.78**	**0.78**	1.56	**0.39**	**0.78**
	L	PE	**0.78**	3.13	3.13	3.13	3.13	3.13	3.13	6.25	3.13	3.13	3.13	3.13	3.13	3.13	3.13	6.25
		DCM	**0.39**	6.25	3.13	1.56	3.13	3.13	1.56	3.13	3.13	3.13	3.13	3.13	1.56	1.56	0.78	1.56
		EtOH	1.56	3.13	1.56	**0.78**	1.56	1.56	1.56	1.56	3.13	1.56	1.56	**0.78**	1.56	1.56	1.56	1.56
		Water	>12.5	>12.5	3.13	>12.5	3.13	3.13	3.13	1.56	1.56	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**
Drimia robusta	B	PE	3.13	3.13	3.13	6.25	3.13	3.13	3.13	6.25	3.13	3.13	3.13	3.13	3.13	3.13	3.13	6.25
		DCM	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	6.25	3.13	1.56	3.13	3.13	3.13
		EtOH	>12.5	3.13	1.56	1.56	6.25	3.13	3.13	12.5	6.25	3.13	3.13	12.5	6.25	6.25	6.25	12.5
		Water	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	12.5	>12.5	>12.5	>12.5	>12.5	12.5	6.25	12.5	12.5
	L	PE	1.56	3.13	1.56	1.56	**0.78**	3.13	3.13	3.13	1.56	1.56	3.13	3.13	–	–	–	–
		DCM	**0.78**	3.13	3.13	3.13	1.56	1.56	3.13	3.13	**0.78**	1.56	1.56	**0.78**	–	–	–	–
		EtOH	3.13	3.13	1.56	3.13	3.13	1.56	3.13	3.13	6.25	1.56	1.56	1.56	–	–	–	–
		Water	>12.5	6.25	6.25	3.13	3.13	3.13	3.13	3.13	6.25	3.13	6.25	3.13	–	–	–	–
Merwilla plumbea	B	PE	6.25	6.25	6.25	12.5	3.13	6.25	3.13	12.5	3.13	3.13	3.13	3.13	6.25	6.25	6.25	12.5
		DCM	**0.78**	3.13	3.13	12.5	3.13	3.13	3.13	3.13	**0.78**	1.56	3.13	3.13	**0.78**	3.13	**0.78**	**0.78**
		EtOH	3.13	3.13	3.13	1.56	12.5	6.25	6.25	12.5	12.5	6.25	6.25	3.13	6.25	6.25	6.25	12.5
		Water	>12.5	>12.5	>12.5	12.5	3.13	6.25	12.5	3.13	3.13	6.25	3.13	**0.78**	12.5	12.5	>12.5	>12.5
	L	PE	6.25	6.25	3.13	6.25	1.56	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13
		DCM	1.56	3.13	3.13	**0.78**	1.56	3.13	1.56	1.56	3.13	1.56	3.13	**0.78**	1.56	1.56	1.56	1.56
		EtOH	3.13	3.13	3.13	3.13	3.13	1.56	3.13	3.13	6.25	1.56	3.13	1.56	3.13	3.13	3.13	3.13
		Water	6.25	12.5	>12.5	12.5	3.13	3.13	3.13	**0.78**	3.13	6.25	3.13	6.25	1.56	1.56	3.13	3.13
Neomycin (μg/ml)			1.95	0.98	0.49	1.95	1.95	0.98	0.49	1.95	1.95	0.98	0.49	1.95	1.95	0.98	0.49	1.95

Bs = *Bacillus subtilis*, Ec = *Escherichia coli*, Kp = *Klebsiella pneumoniae*, and Sa = *Staphylococcus aureus.*
Values boldly written are considered very active (<1 mg/ml).

Table 3
Anticandidal activity (MIC and MFC mg/ml) in bulbs/corms (B)/(C) and leaves (L) of *T. violacea*, *H. hemerocallidea, D. robusta* and *M. plumbea* sample extracts in spring, summer, autumn and winter seasons.

Plant Name	Part	Extract	Spring		Summer		Autumn		Winter	
			MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Tulbaghia violacea	B	PE	6.25	6.25	3.13	6.25	**0.78**	1.56	1.56	3.13
		DCM	3.13	6.25	1.56	3.13	1.56	3.13	0.78	1.56
		EtOH	6.25	12.5	3.13	6.25	3.13	6.25	**0.78**	3.13
		Water	>12.5	>12.5	>12.5	>12.5	>12.5	>12.5	12.5	>12.5
	L	PE	**0.78**	1.56	**0.39**	6.25	**0.78**	**0.78**	3.13	3.13
		DCM	1.56	1.56	**0.78**	**0.78**	**0.78**	**0.78**	1.56	1.56
		EtOH	3.13	6.25	3.13	6.25	3.13	3.13	3.13	3.13
		Water	12.5	12.5	12.5	12.5	12.5	12.5	>12.5	12.5
Hypoxis hemerocallidea	C	PE	6.25	6.25	6.25	6.25	3.13	6.25	3.13	6.25
		DCM	6.25	6.25	3.13	3.13	**0.78**	1.56	3.13	3.13
		EtOH	3.13	3.13	3.13	3.13	**0.78**	**0.78**	3.13	3.13
		Water	3.13	3.13	3.13	3.13	**0.39**	**0.39**	**0.39**	**0.39**
	L	PE	6.25	6.25	**0.78**	6.25	3.13	3.13	3.13	6.25
		DCM	**0.78**	3.13	1.56	1.56	1.56	3.13	3.13	3.13
		EtOH	**0.78**	3.13	3.13	3.13	3.13	3.13	1.56	3.13
		Water	**0.78**	**0.78**	3.13	3.13	**0.39**	**0.78**	**0.78**	3.13
Drimia robusta	B	PE	6.25	6.25	**0.78**	6.25	**0.78**	3.13	3.13	6.25
		DCM	6.25	6.25	6.25	6.25	3.13	3.13	3.13	6.25
		EtOH	**0.39**	3.13	6.25	6.25	6.25	6.25	3.13	3.13
		Water	>12.5	>12.5	>12.5	>12.5	12.5	12.5	12.5	12.5
	L	PE	**0.39**	3.13	**0.78**	6.25	**0.78**	**0.78**	–	–
		DCM	**0.78**	**0.78**	1.56	1.56	**0.39**	**0.78**	–	–
		EtOH	6.25	6.25	**0.78**	3.13	1.56	3.13	–	–
		Water	3.13	3.13	3.13	6.25	3.13	3.13		
Merwilla plumbea	B	PE	6.25	6.25	6.25	6.25	3.13	3.13	3.13	6.25
		DCM	6.25	6.25	3.13	6.25	3.13	3.13	1.56	6.25
		EtOH	6.25	12.5	3.13	12.5	3.13	6.25	1.56	6.25
		Water	>12.5	>12.5	>12.5	>12.5	3.13	6.25	12.5	12.5
	L	PE	3.13	6.25	1.56	6.25	1.56	1.56	3.13	3.13
		DCM	**0.39**	**0.78**	3.13	3.13	**0.78**	**0.78**	3.13	3.13
		EtOH	3.13	6.25	3.13	3.13	**0.78**	**0.78**	3.13	6.25
		Water	6.25	6.25	6.25	12.5	3.13	3.13	6.25	6.25
Amphotericin B (μg/ml)			9.77	78.1	9.77	78.1	9.77	78.1	9.77	78.1

Values boldly written are considered very active (<1 mg/ml).

which MIC values ranged from 0.39 to 0.78 mg/ml in summer, autumn and winter and *M. plumbea* bulb in autumn. In contrast, the corm extracts of *H. hemerocallidea* did not show good activity in spring but the activity shifted from the leaf to the corm in summer. The shift in the activity between plant organs and different seasons suggests a possible corresponding shift in and/or accumulation of some compounds responsible for the activity (Koptur, 1985) since most of these phytochemicals are produced in response to external stimuli (Derita et al., 2009; Kubo et al., 1976) such as light intensity, moisture stress and temperature amongst others. It is possible therefore, that, according to the season of the year, the content and presence of these bioactive compounds could vary in parallel with the presence or absence of the stimuli, resulting in changing antibacterial properties. In addition to the intrinsic morphological, physiological and biochemical differences between bulbs and leaves, the observed differences in the activity between leaves and bulbs of the same plant could also be explained by dynamics in the production of the active compounds in response to stimulation factors.

The low activity shown by most extracts against the Gram-negative bacteria was not surprising since these bacteria are more tolerant than Gram-positive ones (Chariandy et al., 1999; Paz et al., 1995; Rabe and Van Staden, 1997) owing to them having an outer membrane which presents a barrier to various antimicrobial molecules (Sleigh and Timbury, 1998).

The leaf extracts of all four plant species showed good anticandidal activity (MIC) (Table 2) against *C. albicans* in all seasons except for *T. violacea* (winter) and *M. plumbea* (summer and winter). Very few bulb extracts showed good fungistatic (MIC) activities, with *M. plumbea* yielding no active extracts in any of the seasons. However, in determining the minimum fungicidal concentrations (MFC), some of the extracts with good MIC (<1 mg/ml) values did not have a correspondingly good MFC suggesting such extracts have only an inhibitory effect on the fungus. Most of the fungicidal activities were shown by the extracts of plant material gathered in autumn. This could be as a result of the accumulation of the fungicidal compounds during this season in response to favourable stimuli. The compounds, on the other

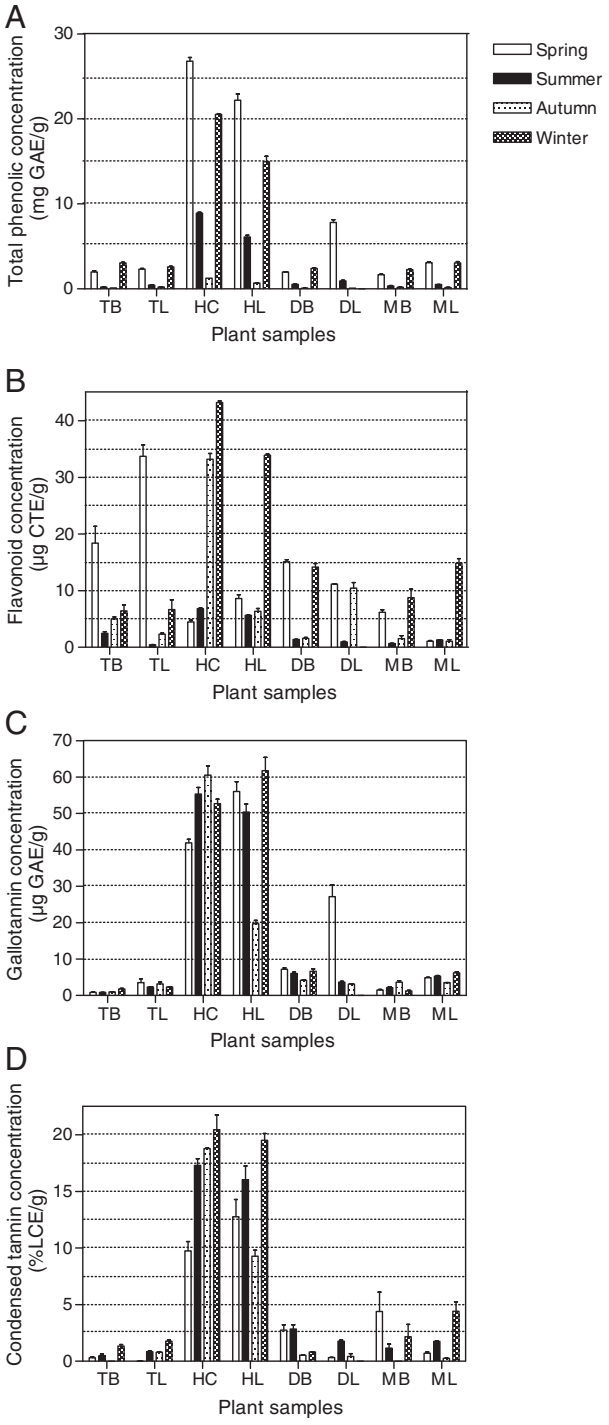

Fig. 1. Total phenolic compounds as gallic acid equivalents (GAE) (A), flavonoid concentrations as catechin equivalents (CTE) (B), gallotannin concentrations as gallic acid equivalents (GAE) (C) and percentage condensed tannin concentrations as leucocyanidin equivalents (LCE) (D) per dry matter in *T. violacea* bulbs (TB), *T. violacea* leaves (TL), *H. hemerocallidea* corms (HC), *H. hemerocallidea* leaves (HL), *D. robusta* bulbs (DB), *D. robusta* leaves (DL) and *M. plumbea* bulbs (MB) and *M. plumbea* leaves (ML) in summer, autumn, winter and spring.

hand could have accumulated during the preceding spring and summer seasons and reached high concentration levels in autumn. Although good MIC activity in the leaf extracts was maintained across seasons, there tended to be a shift in some cases in the activity from one solvent extract to the other. For example, with *H. hemerocallidea* leaf extracts, an MIC value of 0.78 mg/ml was recorded from the DCM, ethanol and water extracts in spring, and in summer, this activity was lost from these three extracts and shifted to the PE extract. This sudden shift suggests that the activity cannot solely be attributed to a single compound but rather different compounds or combinations with the same and/or synergistic effects on the fungus. Particularly interesting is that only ethanol and water corm extracts of *H. hemerocallidea* showed good MFC activity in autumn and winter amongst all the bulb extracts. Lack of fungicidal activity (MFC > 1 mg/ml) from bulb extracts could be due to lack or low concentration of the active compound(s) in these extracts (Nacif de Abreu and Mazzafera, 2005; Rabe and Van Staden, 1997). Another possible explanation for this could be that the compounds present might not be fungicidal.

It is interesting to note that in most traditional herbal preparations, water is used as the major extractant (Hoffmann, 1989), yet most of the water extracts tested in this study revealed poor antibacterial and antifungal activity. The trend is, however, consistent with most of the findings in other studies (McGaw et al., 2001; Rabe and Van Staden, 1997). This suggests that most of the active constituents in these plant species are non-polar since water extracts mainly contain polar compounds. However, our results indicate that at least some of the biologically active compounds in *H. hemerocallidea* are

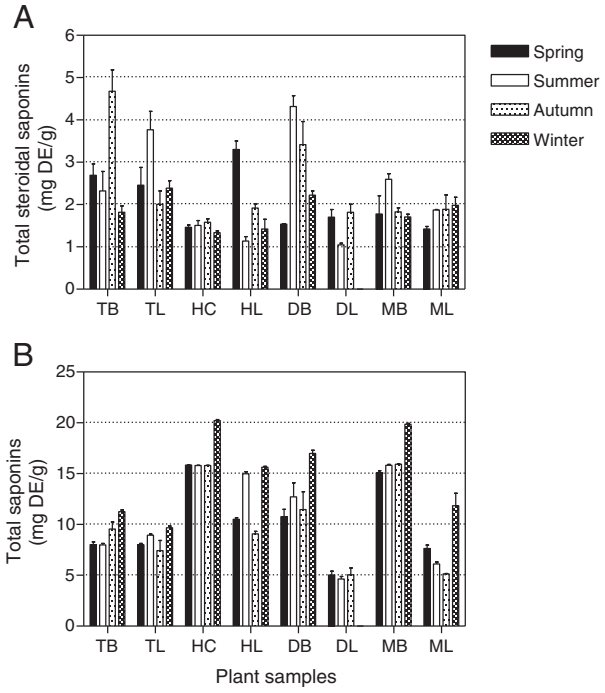

Fig. 2. Total steroidal saponin concentrations (A) and total saponin concentrations (B) as diosgenin (DE) equivalents per dry matter in *T. violacea* bulbs (TB), *T. violacea* leaves (TL), *H. hemerocallidea* corms (HC), *H. hemerocallidea* leaves (HL), *D. robusta* bulbs (DB), *D. robusta* leaves (DL) and *M. plumbea* bulbs (MB) and *M. plumbea* leaves (ML) in summer, autumn, winter and spring seasons.

polar and exhibit greater activity in autumn and winter (Tables 2 and 3).

The total phenolic concentrations of the screened plant species are presented in Fig. 1A. The highest concentrations of total phenolic compounds in all the tested plant species were detected in spring in both leaves and bulbs/corms. Considering that light plays an essential role in phenol biosynthesis (Treutter, 2001), Amaral et al. (2004) attributed the increase in the production of phenolic compounds to high values of solar radiation. These findings supports the observation by Derita et al. (2009) who reported variations in the percentages of polygodial in *Polygonum acuminatum* extracts collected in the four seasons of the year and their corresponding variation in antifungal activity.

Phenolic compounds are produced in plants to serve a diverse range of purposes such as defence against pathogens and different forms of environmental stress, amongst them heat stress, moisture stress, UV radiation (Dey and Harborne, 1989; Treutter, 2001). It is for this reason that the observed differences between different seasons could be explained on the basis of the differences in the climatic, biotic and environmental conditions experienced in different seasons. In relation to the results of the present study, high phenolic concentrations could notably be due particularly to the highly fluctuating temperatures and dry conditions experienced in spring. Another explanation to this phenomenon could be that in spring, vegetative material starts to develop. Immature plant tissues have been reported to produce more phenolic compounds especially tannins compared to old and mature tissues (Heldt, 2005). Phenolic compounds have some important pharmacological value and have been reported to have antioxidative, anticarcinogenic, antibacterial and anti-inflammatory effects (Bruneton, 1995; Kuda et al., 2005; Sharma et al., 1994).

A similar trend to that of total phenolic compounds was observed for flavonoids, gallotannins and condensed tannins (Figs. 1B, C and D) but with high concentrations observed in winter in most of the plant species. The exceptions were *T. violacea* and *D. robusta* in which there were higher concentrations of flavonoids and gallotannins in spring than in other seasons.

The content of some phenolic compounds and their seasonal alterations determined in the present study, point to the fact that there is a connection between their different functions in different plants and the stimuli triggering their synthesis. The results show that each phenolic group is characterised by a pattern of seasonal fluctuations in concentrations. It can also be noted that the amount of total phenolic compounds, flavonoids, gallotannins and condensed tannins in bulbs was significantly different from the leaves in each of the plant species, although the pattern of seasonal fluctuations in most cases were similar.

Whereas the biosynthesis and accumulation of phenolic compounds depend on exogenous factors, it is the endogenous factors, developmental stage and tissue differentiation (Mirdehghan and Rahemi, 2007; Treutter, 2001) that determine the site of synthesis in plants. Differences between leaves and bulbs observed in this study could therefore be explained on the same basis. The high levels of flavonoid, gallotannin and condensed tannin content in winter are probably in response to cold and water stress experienced during this period. During leaf senescence in winter, metabolic cost is minimised by concentrating and retrieving metabolites from senescing tissues, transporting them to other organs (Mckey, 1979; Waterman and Mole, 1994). This may account for the observed trend in the results of this study.

Flavonoids have been reported to posses some antibacterial properties (Tapas et al., 2008; Tapiero et al., 2002) and could have made some contribution to the good activity shown by some extracts.

The qualitative froth test for the presence of saponins was positive for all samples. Like phenolic compounds, saponin concentrations in this study were found to vary with season and plant organ and were consistently higher in winter than in other seasons (Fig. 2). Generally, saponins are found in tissues that are most vulnerable to fungal or bacterial attack or insect predation (Wina et al., 2005). Therefore, it is believed that one of their roles in plants is to act as a chemical barrier against potential pathogens, which would account for their antimicrobial activity (Osbourn, 1996, 2003). The concentrations of saponins in different parts of the plant would therefore be expected to correlate with levels and sites of pathogenic stimulation. This also serves to explain the variations between bulbs and leaves as well as between seasons.

Spring and summer seasons appeared to have favoured production and accumulation of steroidal saponins in most plant species compared to other seasons (Fig. 2A). Although seasonal fluctuations existed in different plant species, in contrast with phenolic compounds, the magnitude of the relative variation was less in the total saponin concentrations. An overall assessment of the published literature reveals a surprisingly poor understanding of the role of environmental factors (light, temperature, moisture, and nutrition) on saponin levels. Nonetheless, the marked seasonal variations in the steroidal saponins of some plant species suggest that one or more of these factors might contribute to the observed differences. Due to their toxicity to various organisms, saponins can be utilised for their insecticidal, antibiotic, fungicidal, and pharmacological properties.

4. Conclusions

The results of the present work show that leaves have comparable, and in some cases, better antimicrobial activity than bulbs. The leaves of these plant species could feasibly be harvested for medicinal use against fungal and bacterial infections to improve the sustainability of these medicinal plant resources. Harvesting of leaves inflict much less damage on the plant than harvesting bulbs/corms. However, the time of harvest should also depend on the plant part to be used since it has been demonstrated that the level of bioactivity and biologically active compounds can vary in different plant parts at different seasons. Leaves seemed to demonstrate a more stable antimicrobial activity across the four seasons compared

to bulbs. Regarding, the marked differences in antifungal activities noticed between the leaves and bulbs, the use of leaves in the treatment of fungal infections would be more rational than the use of bulbs. Phenolic compounds and saponin levels appeared to be higher in winter and autumn, a phenomenon which implies that the periods coincide with the favourable stimuli for the production of these metabolites. Research aimed at identifying specific compounds responsible for the observed bioactivity and environmental conditions that enhance production of these and other compounds could have positive impact on the commerce of these medicinal plants and their future role in public health.

Acknowledgements

Mrs Alison Young (Horticulturalist) of the University of KwaZulu-Natal Botanical Garden and Dr Gary Stafford of the Research Centre for Plant Growth and Development, UKZN are thanked for their assistance with plant collection and identification. The National Research Foundation (NRF), Pretoria and the University of KwaZulu-Natal are gratefully acknowledged for financial assistance.

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Edited by AK Jäger