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The rational usage of *Drimia robusta* Bak. in traditional medicine

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Drimia robusta was screened for biological activity using an antibacterial, the cyclooxygenase, and the angiotensin-converting enzyme assays. Ethanolic bulb extracts exhibited anti-inflammatory activity. Antibacterial activity was present in the ethyl acetate bulb extract. A phytochemical screening of *D. robusta* for alkaloids, saponins and cardiac glycosides was carried out. *D. robusta* does not contain alkaloids. Bulb and leaf extracts exhibited haemolytic activity, which is characteristic of saponins. The bulb contains 2-deoxy sugars, common components in the sugar moieties of cardiac glycosides. Chromatographic screening showed that the bulbs do not contain cardenolides. Bufadienolides were detected, one of which was identified as proscillaridin A.

Keywords: cardiac glycosides, *Drimia robusta,* proscillaridin A, saponins, traditional medicine.

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Introduction

Traditional medicine has maintained a virile people in Africa, where there is a greater variety of herbal traditions than in any other continent (Chevallier 1996). Plants used in traditional medicine are likely to yield pharmacologically active compounds. It is imperative, however, that these plants, as well as traditional methods of treatment, undergo scientific evaluation in order to facilitate the incorporation of traditional medicine into the health care system of South Africa.

Drimia robusta Bak. (Hyacinthaceae) is a southern African medicinal plant closely related to the genus Urginea - in fact, the genera are sometimes regarded as synonymous. *Urginea maritima*, commonly known as squill, has been used for centuries in Europe because of its powerful digitalis-like effect (Steyn & van Heerden 1998); in German phytomedicine it is used for mild cardiac insufficiency and impaired kidney function (Leung & Foster 1996). The bulbs of *U. altissima* (African Squill) have been sold as a squill substitute in Europe (Hutchings *et al.* 1996). The bulbs of *D. robusta* are used as expectorants and emetics, and as protective charm mixes known as intelezi (Hutchings 1996). Hot water infusions of pounded bulbs and leaves are used as enemas to treat feverish colds, and cut bulb scales are rubbed onto the chest for stabbing pains (Hutchings 1992). The leaves are diuretic and are used to clean the bladder and to treat diseases of the uterus (van Wyk *et al.* 1997). Decoctions and infusions of the bulbs have been used as arrow poisons.

D. robusta is reported to be toxic to stock (Watt & Breyer-Brandwijk 1962). Tests with rabbits produced symptoms of dysponoea and muscular paresis. *Urginea* species produce various cardiac glycosides of the bufadienolide-type. Upon enzymatic hydrolysis, these glycosides yield the medicinally important bufadienolide proscillaridin A (van Wyk *et al.* 1997).

Based on the medicinal usage and the physiological effects of *D. robusta*, as well as the close relationship of *Drimia* with *Urginea*, it is likely that *D. robusta* contains cardiac glycosides. However, *D. robusta* has not been studied chemically and the pharmacology of this species is unknown. The objective of this study was to investigate some of the pharmacological effects of *D. robusta*, and in so doing, substantiate its use in traditional medicine. *D. robusta* was screened biologically for antibacterial activity, for the presence of prostaglandin-synthesis inhibitors (anti- inflammatory activity), and for the inhibition of the angiotensin-converting enzyme (anti-hypertentive and diuretic activity), and phytochemically for the presence of alkaloids, saponins and cardiac glycosides.

Materials and Methods

Plant material

Mature plants of *D. robusta* Bak. were obtained from Silverglen Nursery in Durban. A voucher specimen has been deposited in the Herbarium of the Botany Department at the University of Natal, Pietermaritzburg under the number LUYT1UN. The plants were divided into leaf, bulb and root material and dried in an oven at 50°C for approximately 72 hours. The dried plant material was ground to a fine powder, and stored in airtight containers at room temperature in the dark until extraction.

Extraction of plant material

Powdered leaf and bulb material (500 mg) was extracted with 5 ml water, ethanol or ethyl acetate for 30 minutes in an ultrasonic bath. The extracts were filtered and dried. The residues were resuspended in water, ethanol or ethyl acetate to give 100 mg residue ml^{-1} solvent (antibacterial assay), 20 mg residue ml^{-1} ethanol or ethyl acetate and 2.5 mg residue ml^{-1} water (cyclooxygenase assay).

Antibacterial activity

The disc-diffusion assay (Rasoanaivo & Ratsimamandga-Urverg 1993) was used to determine the inhibition of bacterial growth by leaf and bulb extracts of *D. robusta*. Cultures of the following bacteria were used: *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermis*. The cultures were maintained on nutrient agar at 4°C. Base plates were prepared by pouring 10 ml Mueller-Hinton (MH) agar into sterile petri dishes (9 cm) and allowing them to set. Molten MH agar (5 ml) held at 48°C was inoculated with a broth culture of the test organism to give a dilution of approximately 10^5 cells ml^{-1} media, and poured over the base plates to form a homogenous top layer. Ten µl of plant extract suspension were applied to sterile filter paper discs (Whatman No.3, 6 mm diameter) so that each disc contained 1 mg of extract. Antibiotic control discs containing 2 µg neomycin were prepared in the same way. The discs were air-dried and placed onto the seeded top layer of the agar plates. Each extract was tested in quadruplicate (four discs containing the same plant extract per plate) with a neomycin disc in the centre as a positive control. The plates were evaluated after incubation at 37°C for 20 hours. Antibacterial activity was expressed as the

ratio of the inhibition zone (mm) produced by the plant extract to the inhibition zone caused by the neomycin control (Rabe & van Staden 1997).

Cyclooxygenase assay

The inhibition of prostaglandin synthesis by bulb and leaf extracts of *D. robusta* was investigated using the cyclooxygenase assay. The bioassay was performed according to Jäger *et al.* 1996. Ten μl of stock enzyme solution (sheep seminal vesicle microsomes stored at -70°C) were suspended in 90 μl Tris buffer. Twenty-five μl of this solution were suspended in 975 μl Tris buffer to give 0.25% of the stock. Two ml of cofactor solution (0.3 mg ml^{-1} L-adrenalin and 0.3 mg ml^{-1} reduced glutathione in 0.1 M Tris buffer, pH 8.2) and 400 μl of enzyme solution were mixed and incubated on ice for 15 minutes. Sixty μl of enzyme/cofactor solution were added to 20 μl of solvent/test solution (20 μl aqueous extracts; 2.5 μl ethanol extracts + 17.5 μl water; 2.5 μl ethyl acetate extracts + 17.5 μl water) or standard solution (2.5 μl ethanolic indomethacin solution + 17.5 μl water). Twenty μl ^{14}C-arachidonic acid (16 Ci/mole, 30 mM) were added and the assay mixture was incubated at 37°C for eight minutes. The reaction was terminated by adding 10 μl 2 N HCL. The enzyme activity of background samples was inactivated prior to adding ^{14}C-arachidonic acid, and kept in an ice bath. The ^{14}C-labelled prostaglandins synthesized during the assay were separated from unmetabolized arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane:dioxane:acetic acid 350:150:1) was packed in pasteur pipettes to a height of 3 cm. One ml of eluent 1 was added to each of the assay mixtures, which were then applied to the columns. The arachidonic acid was eluted from the columns with a further 4 ml of eluent 1 and discarded. The labelled prostaglandins were then eluted with 3 ml of eluent 2 (ethyl acetate:methanol 425:75) into scintillation vials. Four ml scintillation cocktail (Beckman Ready Solve) were added to the eluent. After 30 minutes the radioactivity of the samples was counted using a Beckman LS3801 scintillation counter. The percentage inhibition of the test solutions was obtained by comparing the amount of radioactivity present relative to the radioactivity in the solvent blank (McGaw *et al.* 1997). All experiments were performed in duplicate.

Angiotensin-converting enzyme assay (ACE)

Water and ethanol extracts (0.33 mg ml^{-1}) of *D. robusta* bulbs were tested in the ACE- assay (Duncan 1998).

Alkaloids

pH partitioning for alkaloids was performed according to Brimer *et al.* (1989). Two g of dried, powdered bulb material were extracted with 20 ml 96% ethanol for 30 minutes with constant stirring. The extract was filtered. Fifteen ml of water were added to the filtrate, and the ethanol was removed by gently heating the filtrate on a hot plate. The remaining extract was filtered, taken to pH 4.5 with 5 ml 2N NaOH, and partitioned against 10 ml dichloromethane. The organic phase was filtered and partitioned against 2 ml 0.1 N HCl. The HCl phase was divided into two parts. Dragendorff's reagent (Wagner *et al.* 1984) was added to one part, and Mayer's reagent (Wagner *et al.* 1984) to the other. Observations were made for the development of a red-orange precipitate on the addition of Dragendorff's reagent, and a white precipitate on the addition of Mayer's reagent.

Saponins

The haemolysis test (Anonymous) was used to screen for the presence of saponins in leaf and bulb extracts of *D. robusta*. Discs were removed from four areas of a blood agar plate to create cup-like wells in the agar. It was necessary to seal off the agar at the bottom of each cup in order to prevent the liquid samples from spreading beneath the surface of the blood agar. This was done by heating the tip of a pasteur pipette in a Bunsen burner flame, and pressing it gently against the sides of the wells causing some agar to melt onto the petri dish. Aqueous bulb or leaf extracts (10 mg ml^{-1}) were added to three of the four cups, and the fourth cup was filled with distilled water as a negative control. After two hours the blood agar plates were observed for clear zones of haemolysis surrounding the cups containing the extracts.

Cardiac glycosides

One g of dried powdered bulb material was boiled under reflux with 30 ml 53% ethanol containing 3% lead acetate (Jäger & van Staden 1995). The cooled extract was filtered, acidified with acetic acid, and partitioned three times against 15 ml dichloromethane. The combined dichloromethane extracts were filtered over anhydrous sodium sulphate and taken to dryness under vacuum. The residue was resuspended in 1 ml dichloromethane: methanol (1:1).

The Keller-Killiani test (Evans 1989) was used to screen for 2-deoxy sugars. The quantities for the extraction of cardiac glycosides from dried bulb material were increased three-fold. The residue was resuspended in 3 ml dichloromethane:methanol (1:1) and taken to dryness under nitrogen. This residue was resuspended in 2 ml glacial acetic acid, containing 2.2 mM $FeCl_3$. This solution was layered on top of 2 ml concentrated H_2SO_4. The mixture was observed for a reddish-brown ring at the interface. Digitoxin and digoxin were used as positive controls, and water as a negative control.

To test for the presence of an unsaturated lactone ring 50 μl of the extract was applied to a Merck Silica 60 F_{254} TLC plate (Jäger & van Staden 1995). Ten μg each of digitoxin and digoxin were used as controls. The TLC plate was developed in ethyl acetate:methanol:water (81:11:8). The plate was sprayed with Kedde's reagent (alkaline 3,5-dinitrobenzoic acid) (Wagner *et al.* 1984) which indicates the presence of the γ-lactone ring of cardenolides, but does not respond with bufadienolides. The plate was evaluated in visible light for pink/violet spots.

For further detection of bufadienolides, a second TLC plate was sprayed with chloramine T-trichloroacetic acid reagent (Wagner *et al.* 1984) and heated for 10 minutes at 100°C. The plate was viewed under ultraviolet light at 365 nm for yellow- orange zones. A third TLC plate was sprayed with antimony(III)chloride reagent ($SbCl_3$) (Wagner *et al.* 1984) and heated at 100°C for six minutes. The plate was observed under ultraviolet light at 365 nm for yellow to yellow-brown spots.

Extraction of bufadienolides

The procedure for extraction of bufadienolides was performed, with slight modifications, according to Krenn *et al.* (1988). Seventy g of dried, powdered bulb material were heated under reflux for two hours in 1400 ml 50% ethanol and 700 ml 3% lead acetate. The extract was filtered, reduced to 1800 ml, and acidified using acetic acid. Nine-hundred ml of the extract was partitioned three times in succession against 900 ml chloroform:isopropanol (3:2). The chloroform:isopropanol extract was filtered over anhydrous sodium sulphate and taken to dryness under vacuum.

Isolation of proscillaridin A

Twenty mg of the chloroform:isopropanol bulb extract were strip-loaded onto five glass Merck Silica 60 F_{254} TLC plates (0.25 mm). Proscillaridin A (Sigma) was used as reference. The plates were developed in ethyl acetate:methanol:water (81:11:8), and evaluated under ultraviolet light at wavelengths of 365 and 254 nm prior to chemical treatment. A strip approximately 2.5 cm wide was cut from each plate using a diamond knife, and subsequently treated with $SbCl_3$ reagent (Wagner *et al.* 1984) The strips were heated at 100°C for six minutes and re-evaluated alongside the untreated plates under ultraviolet light at wavelength 365 nm. The bands on the untreated plates that corresponded with the proscillaridin A standard on the treated strips were scraped off. The combined scraped bands were extracted with dichloromethane:methanol (1:1).

Table 1 Inhibition of cyclooxygenase by water, ethyl acetate and ethanol extracts of *D. robusta*

Plant part analyzed	Amount extract used (μg)	Inhibition (%)		
		Water	Ethyl acetate	Ethanol
Bulb	50	77	69	98
Leaf	50	37	21	31
Indomethacin[c]	0.5	n.a	n.a	94

[c]The concentration of the indomethacin standard was 20 mM.

The sample was filtered and taken to dryness. In order to remove excess traces of silica from the sample, it was dissolved in methanol and applied to a small Sephadex LH_{20} column (i.d. = 1.8 cm; length = 12 cm) and eluted with 25 ml methanol. The clean sample was then dried under nitrogen, whereafter it underwent structure elucidation with the use of ^1H-NMR spectroscopy. The ^1H-NMR spectrum of the sample dissolved in CD_3OD was recorded at 200 Mhz using a Kratos MS 80RF double-focussing magnetic sector instrument at 70 eV. TMS was used as an internal standard.

Results

A total of six extracts of *D. robusta* were tested against seven bacterial strains. Four of the bacterial strains were Gram-positive and three were Gram-negative. Neither the ethanol and water extracts, nor the ethyl acetate leaf extract, exhibited antibacterial activity. Antibacterial activity was present in the ethyl acetate bulb extracts, which inhibited five of the seven bacterial strains tested. The two strains which were not inhibited were *E. coli* (a Gram-negative bacterium) and *S. epidermis*. The inhibition ratios of the remaining Gram-negative bacteria, *K. pneumoniae* and *P. aeruginosa* were 0.07 and 0.08 respectively. The inhibition ratios of *B. subtilis* and *M. luteus* were 0.12 and 0.13 respectively, and the highest activity was a ratio 0.63 against *S. aureus*. These results indicate that the extracts of *D. robusta* are only slightly active against bacteria. Traditionally, plant extracts are prepared with water. Since the antibacterial activity was present only in the ethyl acetate extracts, it is not likely that the traditional healer extracts the compounds which were responsible for activity in these extracts. The negative results obtained against *E. coli* support the fact that Gram-negative bacteria are more resistant, probably because of their thick murein layer which prevents the entry of inhibitors (Martin 1995). However, even the slight activity of the ethyl acetate bulb extract against the other Gram-negative bacteria, as well as the relatively higher activity against *S. aureus* provides evidence of biological activity. The plant extracts, therefore, warrant further investigation.

The results of the screening for the inhibition of cyclooxygenase by extracts of *D. robusta* are given in Table 1. Indomethacin (0.5 μg) or 50 μg plant extract residue were added to the assay. The indomethacin standard inhibited cyclooxygenase to a level of 94% in ethanol. The water and ethyl acetate extracts, and the ethanolic leaf extracts, showed no significant inhibition of cyclooxygenase relative to the indomethacin standard. The ethanolic bulb extract showed higher inhibitory activity than indomethacin (98%).

If the release of prostaglandins is modulated, the result of the inflammation process may be affected, and the inhibition of prostaglandin action may also lead to the relief of headache pain and fever (McGaw *et al.* 1997). The inhibition of cyclooxygenase by the ethanolic bulb extract may, therefore, explain the use of *D. robusta* in traditional medicine for the treatment of feverish colds.

The water and ethanol extracts of the bulbs did not inhibit the angiotensin-converting enzyme (ACE) (Duncan 1998). The ACE is associated with high blood pressure. The negative result obtained from the bulb extracts does not necessarily negate the traditional usage of *D. robusta* as a diuretic. It is possible that the active compound(s) in the plant promote diuresis through processes not associated with ACE action; it could, for example, be facilitated by an effect on the heart by cardiac glycosides. *Digitalis* was originally used in European folk medicine to treat dropsy, working as a diuretic.

A red-orange precipitate did not develop on the addition of Dragendorff's reagent to the bulb extract. Similarly, Mayer's reagent did not produce a white precipitate. This suggests that alkaloids are not present. *U. altissima*, also known as *D. altissima*, is reported to contain alkaloids (Raffauf 1996). The finding that *D. robusta* does not contain alkaloids supports Raffauf (1996), who reported that alkaloids are not present in *D. alta*. *D. robusta* was previously known as *D. alta*.

Most saponins have haemolytic properties that can generally be attributed to their interaction with the sterols of the erythrocyte membrane (Bruneton 1995). This interaction causes the permeability of the membrane to increase which results in the loss of haemoglobin. Clear zones of haemolysis surrounded the cups containing the leaf and the bulb extracts, confirming the presence of saponins in both the bulb and the leaves of *D. robusta*. There were no signs of haemolysis surrounding the cups containing distilled water. Saponins are known to have anti-inflammatory properties (Lewis 1989). Although the inhibition of prostaglandin synthesis by saponins is not well investigated, it is possible that the positive test for the inhibition of cyclooxygenase, as well as the traditional usage of *D. robusta* for the treatment of feverish colds, may be attributed to the presence of saponins in *D. robusta*.

The Keller-Killiani reagent reacts with the deoxy-sugar moeity of cardiac glycosides. This test confirmed the presence of 2-deoxy sugars in the *D. robusta* bulb extract. A brown ring appeared at the interface of the bulb extract solution (upper phase) and the concentrated sulphuric acid. The upper phase turned blue, and became dark brown on standing.

Kedde's reagent indicated the presence of cardenolides in the standards - digoxin and digitoxin - only, and not in the *D. robusta* bulb extract. The bulb, therefore, does not contain cardenolides.

Chloramine T-trichloroacetic acid produced yellow-orange fluorescent bands in the bulb extract under ultraviolet light at a wavelength of 365 nm. This is the characteristic colour reaction of bufadienolides with this treatment. Several bands in the bulb extract appeared yellow-orange under ultraviolet light at a wavelength of 365 nm after spraying with $SbCl_3$. One band in particular, at R_f 0.53, produced an intense yellow fluorescent zone. The R_f value of digoxin was 0.55. According to Wagner *et al.* (1984), digoxin and proscillaridin have similar R_f values. Isolation of the compound causing the yellow fluorescence was carried out. Liquid-liquid partitioning of the bufadienolide-containing extract with chloroform:isopropanol 3:2 yielded 2.05 g. After thin-layer chromatography of 100 mg residue, the scraped bands yielded 3 mg. Following solvent extraction of the scraped bands, the sample and the proscillaridin A standard were chromatographed using TLC. The R_f values of the sample and the standard were identical (0.53). After treating the chromatogram with $SbCl_3$ reagent, the sample produced the same violet colour in visible light as that of the standard, and under ultraviolet light of a wavelength of 365 nm, both the sample and the standard fluoresced the characteristic intense yellow fluorescence of proscillaridin A. The following ^1H-NMR data were recorded for the sample: δ

0.80 (s, 3H, CH_3-18), δ 0.96 (s, 3H, CH_3-19), δ 1.14 (d, 3 H, CH_3-6), δ 5.26 (s, 1H, H-4), δ 7.14 (d, 1H, H 21), δ 7.70 (dd, 1H, H-22). Analysis of this data and comparison to NMR data obtained by Kopp *et al.* (1996), as well the data obtained from TLC, confirm the presence of proscillaridin A in *D. robusta*.

Cardiac glycosides assist in the transfer of fluid from tissue and the circulatory system, thereby promoting diuresis and lowering blood pressure. The negative result in the inhibition of the angiotensin-converting enzyme may, therefore, indicate that the diuretic action for which *D. robusta* is used in traditional medicine is due to cardiac glycosides. Additionally, bufadienolides such as proscillaridin A are used in modern medicine as heart tonics, and for the preparation of expectorants and emetics (Iwu, 1993). This further substantiates the use of *D. robusta* in traditional medicine.

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