The effect of cold storage during winter on the levels of COX-1 inhibitory activity of *Eucomis autumnalis autumnalis* extracts

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Levels of anti-inflammatory activity exhibited by *Eucomis autumnalis autumnalis* were affected by the environmental conditions during the period of winter dormancy. Bulbs stored at low temperatures (10°C) showed a limited period of time during which the anti-inflammatory activity of the bulb extracts was higher than that of the control. This effect was evident for a longer period in the leaves. Extracts prepared from *E. autumnalis* (subspecies *autumnalis*) bulbs, which were removed from the soil and stored at 8–10°C during the winter months, exhibited significantly higher COX-1 inhibitory activity than those extracts prepared from control bulbs maintained in the soil in a greenhouse (15–24°C) during winter, and from bulbs stored dry in paper bags (±18°C). A second harvest, performed on plants that had been returned to the soil after winter storage, was conducted half-way through the growing season. Higher COX-1 inhibitory activity was observed in the leaf extracts from the plants subjected to cold storage, than in the extracts from plants stored dry or in the greenhouse. No significant difference was observed at this stage between the activity of the bulb and root extracts from the different treatments. All extracts were prepared at a concentration of 250μg ml⁻¹ for comparison.

Introduction

The potency of medicinal plants can be affected by a number of factors including biochemical variation within individual species, and external factors such as climate, soil conditions and season (Prance 1995, Tunón et al. 1995). Changes to the environment can cause modifications in plant growth and development, which in turn can often result in changes in the natural product content (Mâthé 1988). Plants thus do not consistently produce the same chemicals, in the same quantities (Prance 1995). This has long been recognised by traditional healers in southern Africa. Factors such as locality, season, slope aspect, soil type and moisture, and the time of day, influence the choice and harvest of medicinal plants by experienced gatherers and healers (Van Wyk et al. 1997).

*Eucomis* L’Herit is a genus of deciduous geophytes, indigenous to southern Africa (Baker 1897, Pienaar 1984, Compton 1990). The genus belongs to the family Hyacinthaceae, and is classed in the order Asparagales (Dahlgren et al. 1985, Compton 1990). The genus comprises about thirteen species (Hutchings et al. 1996), with the species *E. autumnalis* further divided into three subspecies, *autumnalis; amaryllidifolia* and *clavata* (Reyneke 1980). The species are mostly summer-growing and become dormant in winter (Du Plessis and Duncan 1989). The bulbs are of great value in African traditional medicine, and are heavily harvested for trade in South Africa’s ‘muthi’ markets (Roberts 1990, Van Wyk et al. 1997).

The high rate of harvest of *Eucomis* species has seriously depleted natural populations in southern Africa (Cunningham 1988). *Eucomis* species propagate from seeds and from bulb offsets, but the rate of natural propagation is insufficient to sustain, or increase, population size in the wild. Species such as *E. bicolor* are classed as threatened in South Africa. The wide interest in this genus as an ornamental plant led to the cultivation of several species in Europe, and more recently locally in South Africa. In the southern African context the plant’s value is enhanced by the medicinal importance of the genus. *Eucomis* species are hardy geophytes, bearing attractive inflorescences. The plants show great horticultural potential, and would conceivably form a profitable small scale crop for both the horticultural market and the medicinal plant trade.

The commercial production of *Eucomis* species in Europe is purely for their ornamental value. Bulb production is, however, low (approximately 700 to 1 000 bulbs per acre). Bulb harvesting of commercial bulbs and planting stock takes place in November in the Northern Hemisphere. After lifting, bulbs are stored between 13–20°C in a well ventilated room during winter. Bulbs are maintained during storage and transport in frost-free conditions (De Hertogh and Le Nard 1993).

Of the genera in the family Hyacinthaceae used in Zulu medicine, most are considered highly toxic. Genera including *Bowles, Scilla* and *Urginea* contain cardiotoxic glyco-
sides (Hutchings et al. 1996). Scilla natalensis Planch., which is used as an anti-inflammatory, additionally contains homoisoflavonoids which are thought to be responsible for this pharmacological activity (Van Wyk et al. 1997). While Eucomis species are suspected of causing human and livestock poisoning (Watt and Breyer-Brandwijk 1962), no cardiac glycosides have been found in the plants (Hutchings et al. 1996). The genus Eucomis does contain saponins (Watt and Breyer-Brandwijk 1962) benzopyrones, steroidal triterpenoids and homoisoflavonoids (Van Wyk et al. 1997). Traditional remedies prepared from Eucomis extracts are used to treat a variety of ailments which exhibit symptoms of pain and inflammation. Previous studies (Jäger et al. 1996, Taylor and Van Staden 2001a) have indicated the presence of cyclooxygenase-1 (COX-1) inhibitors in both aqueous and ethanol extracts of Eucomis plant parts (leaf, bulb, root).

The domestication and cultivation of medicinal plants from the wild is a complex process and cannot be achieved by simply transferring the plants from their natural habitats to cultivated fields. Environmental conditions in the new, foreign location, in the form of cultivated fields or greenhouses, potentially alter both the suitability of the plant for cultivation and the levels and/or type of the phytochemicals (Máthé 1988). For this reason several studies have been undertaken, using E. autumnalis autumnalis as the test species, to determine the effects of factors such as age, season and growth conditions on the levels of COX-1 inhibitors in the different plant parts (Taylor and Van Staden 2001b). In addition, micropropagated plants were tested for levels of COX-1 inhibitors and homoisoflavonoids (Van Wyk et al. 1997). The aim of this particular study was to ascertain the influence of storing the dormant bulbs under different conditions during the winter months on the anti-inflammatory activity of extracts prepared at the beginning of spring and half way through the growing season.

Materials and Methods

Plant material

Uniform specimens of Eucomis autumnalis autumnalis (Mill.) Chitt. Reyn., grown originally from micropropagated stock, were collected at the end of summer, once the foliage had died back. Eight specimens were stored dry in brown paper bags in the potting shed (18 ± 2°C), eight were stored dry in a coldroom (10 ± 1.5°C) and eight were replaced into the greenhouse (15–24°C). All specimens were dusted with Bexadust® to discourage fungal growth. One harvest was performed at the end of the winter season and one mid-way through the growing season, when the bulbs had developed leaves.

Extraction

Harvested specimens were divided into leaf, bulb and root material, weighed to determine fresh mass, and dried at 50°C for three days. The different plant parts were each ground to a fine powder and 500mg samples were extracted in ethanol (20ml) using a sonication bath (30min). The extracts were filtered through Whatman No 1 filter paper and dried under vacuum at 35°C. Samples were resuspended in ethanol (10mg ml⁻¹) and were tested for inhibitory activity in the COX-1 assay.

Cyclooxygenase (COX) Assay

The COX-1 assay was performed as described by White and Glassman (1974), with minor modifications by Jäger et al. (1996). Ten microlitres of the standardised COX-1 enzyme preparation and 50μl (per sample) of co-factor solution (0.003g L-adrenalin/l-epinephrine and 0.003g reduced glutathione in 10ml; 0.1M Tris buffer, pH 8.2) were preincubated for 15min on ice. This solution (60μl) was added to the test solution (2.5μl of ethanol extract + 17.5μl water) and preincubated for 5min at room temperature. 14C-arachidonic acid (20μl) was added to this enzyme-extract mixture and incubated for exactly 8min in a water bath at 37°C. The reaction was terminated with 10μl 2N HCl.

A sample resuspended at 10mg ml⁻¹ solvent was tested in the assay at a concentration of 250μg ml⁻¹ test solution. In each test four controls were run (2.5μl ethanol + 17.5μl water). Two were backgrounds in which the enzyme was inactivated with HCl before the addition of 14C-arachidonic acid and which were kept on ice, and two were solvent blanks. Indomethacin (5μM) was tested in each assay as a positive control.

Unlabelled prostaglandin carrier solution (0.2mg ml⁻¹) of unlabelled prostaglandins [PGE2:PGF2 in the ratio 1:1] was added to the reaction mixture (4μl per sample), and 14C-prostaglandins synthesised from unmetabolised arachidonic acid by column chromatography using silica columns. Silica gel, (Kieselgel 60, Korngroesse 0.063–0.2mm, 70–230 mesh ASTM) in eluent 1 {hexane:1,4-dioxan:acetic acid [350:150:1 v/v/v]}, was packed to a height of 3cm in Pasteur pipettes stoppered with glass wool. The assay mixture was applied to the column with 1ml eluent 1. This was followed by an additional 4ml eluent 1 to elute the unreacted arachidonic acid, which was then discarded. The prostaglandins were then eluted into scintillation vials using 3ml eluent 2 (ethyl acetate:methanol [85:15v/v]). Scintillation fluid (4ml) was added and the radioactivity was counted after 1h in the dark, using a Beckman LS3801 scintillation counter.

Calculation of inhibition

The percentage inhibition of the extracts was obtained by measuring the amount of radioactivity in the solutions relative to that of the solvent blank. Inhibition refers to the reduction of prostaglandin formation with reference to an untreated sample (solvent blank). To calculate the % inhibition, the background DPM value was subtracted from the DPM measurements of the sample and the solvent blank. The ratio of the resultant DPM values for the sample to the solvent blank was converted to a percentage. All samples were tested in triplicate.

Statistical analyses were based on the results obtained from 3 different samples. One-way ANOVA and Tukey HSD tests were performed using Minitab Xtra version 10.52. The results of the statistical analyses are presented on the graphs.
Results and Discussion

Growth data

Bulbs kept in cold storage maintained their fresh mass at a higher level than did both the dry storage, and leaving the bulbs in soil, during winter (Figure 1A). Those bulbs kept in the soil had a significantly lower dry mass than those stored dry or cold. No significant differences were observed in the root dry mass, but the fresh mass of roots harvested from plants that remained in the soil was significantly higher than that of plants stored dry or cold (Figure 1B). The moisture in the soil prevented the roots from drying out to the same extent as those of the specimens stored dry or cold. The ratio of fresh to dry mass was highest for the bulbs and roots of those specimens kept in soil (Figure 2). This also indicates the higher water content of these specimens. There was no difference in this ratio between the specimens stored dry or cold.

A second group of plants was harvested mid-way through the summer season to determine the regenerative ability of the bulbs after storage. The bulbs kept in cold storage were slower to develop leaves, with both fresh and dry leaf mass accumulation significantly lower than those of plants stored dry or in the soil (Figure 3A). Specimens from the latter two categories did not differ significantly from each other in this respect. Bulb fresh mass showed no significant differences across treatments, while the dry mass of the bulbs stored cold was significantly lower compared to the bulbs stored in the soil, but not compared to those stored dry (Figure 3B). Root fresh and dry mass accumulation was slower for bulbs that had been stored cold (Figure 3C).

The ratio of fresh to dry mass did not differ significantly for the leaves (Figure 4), indicating that the delay in leaf emergence was the probable cause of the lower fresh and dry mass of the leaves in the bulbs stored cold, rather than a change in growth parameters. With respect to the bulbs stored in cold conditions, the fresh to dry mass ratios for the bulbs and roots were significantly higher than that for the plants stored in the soil or dry, which could correspond to the observed delay in leaf emergence and consequently higher reserves still in the bulbs (Figure 4).

Anti-inflammatory activity

Prostaglandins are the primary mediators of the body’s response to pain and inflammation, and are formed from essential fatty acids found in cell membranes (Campbell 1990). This reaction is catalysed by cyclooxygenase, a membrane-associated enzyme (Smith 1990, Goetzl et al. 1995). Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting the activity of cyclooxygenase (COX), the rate-limiting step in the formation of prostaglandins from arachidonic acid (Vane and Botting 1995). The pharmacological studies reported here were based on the cyclooxygenase-1 assay, a mechanism-based assay that tests for the presence of NSAIDs by measuring the degree of inhibition of the COX-1 enzyme, which is active in prostaglandin synthesis.
Inhibition can be caused by denaturing (or destroying) the enzyme, or by acting on the prosthetic group, thus inactivating the enzyme (Tunón et al. 1995).

The COX-1 inhibitory activity determined at the end of winter was significantly lower from bulbs stored in the soil, with little difference exhibited between extracts from bulbs stored dry or cold (Figure 5). The activity of the root extracts did not differ significantly between treatments.

Extracts prepared from the second harvest of plants (midway through summer) showed high levels of COX-1 inhibitory activity in the leaf extracts from plants that had undergone cold storage (Figure 6). Part of this increased activity could be due to the younger physiological status of the leaves. Previous investigations (Taylor and Van Staden 2001b) showed higher levels of COX-1 inhibitory activity in leaf extracts prepared from younger plant specimens, and from micropropagated plants that had been acclimatised. As the plant aged, no significant differences were observed in the levels of COX-1 inhibitory activity in the different plant parts (Taylor and Van Staden 2001b). A common feature to all these examples is a higher water content in the plant material. No significant difference was detected in the activity of the various bulb or root extracts (Figure 6).

Storing the bulbs under cold conditions, at 10°C (in a coldroom) during winter significantly increased the levels of anti-inflammatory activity shown by the bulb extracts (but not the root extracts) at the end of the winter season (Figure 5). After a period of growth during summer, this activity dropped to a level comparable to extracts from plants stored in the soil and those stored dry. At this stage, however, higher levels of activity were observed in the leaf extracts from these plants (Figure 6). These differences could be associated with changes in the water content of the plant parts.

Small-scale farming of indigenous medicinal plants has been proposed as a venture for providing an alternative source of these plants thus alleviating pressure on rapidly shrinking natural populations (Van Staden 1999). The over-exploitation of traditional medicinal plants is a major factor in their current scarcity in the wild. In addition, such ventures can provide valuable income and employment for impover-
ished rural populations. To optimise both rapid growth, and the levels of pharmacologically active compounds, in the crop species, studies into environmental factors affecting these parameters need to be established. Studies such as the one presented here, and in previous reports (Taylor and Van Staden, 2001b), provide the first step in this process and should be followed by field trials. These results can have further implications for the practitioners of traditional medicine. Collection and storage of medicinal plant material is a growing business, increasingly practiced by those untrained in the traditional ways. With the potency of the plant material affected by the growth conditions and storage conditions, it is important to educate the gatherers and sellers of the plants in the best way to standardise and preserve the plant samples on sale.

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