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Short communication

Comparative phytochemical analysis of wild and *in vitro*-derived greenhouse-grown tubers, *in vitro* shoots and callus-like basal tissues of *Harpagophytum procumbens*

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Abstract

Comparative phytochemical analysis of wild and *in vitro*-derived greenhouse-grown tubers, *in vitro* shoots and callus-like basal tissues of *Harpagophytum procumbens* was done. Dried samples were ground to fine powders and their total iridoid (colorimetric method), phenolic [Folin–Ciocalteu (Folin C) method] and gallotannin (Rhodanine assay) contents as well as anti-inflammatory activity [cyclooxygenase assays (COX-1 and COX-2)] were determined. The tissue culture-derived tubers had the highest total iridoid content which was significantly higher than that of the tubers collected from the wild and other tissue cultured materials evaluated. This suggests that cultivated plants can be a viable alternative source of the active principle(s). The total phenolic and gallotannin contents of the wild tubers were significantly higher than the tissue culture-derived tubers and other *in vitro*-derived plant materials. The presence of phenolic compounds including gallotannins in the tissue cultured materials is of interest from a pharmacological point of view given the pharmacological role of phenolics. In general, extracts from wild tubers demonstrated better inhibitory activities in both COX-1 and COX-2 assays when compared to the tissue culture-derived tubers. All the petroleum ether (PE) and dichloromethane (DCM) extracts showed moderate (50–70%) to good (>70%) inhibitory activities whereas the ethanol (EtOH) extracts showed poor or no inhibition in both assays. Based on previous reports indicating weak inhibition of COX-2 enzyme by harpagoside, the inhibitory activities of both COX enzymes exhibited by PE and DCM extracts in the current study could be due to the presence of other constituents in the extracts. This points towards the need to identify other active constituents and evaluate their role and mode of action in relation to harpagoside — the main active principle.
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Keywords: Devil's Claw; Gallotannin; Harpagoside; Iridoid; Phenolic compounds; Tissue culture

1. Introduction

The past couple of decades magnified the role of *in vitro* techniques in plant conservation efforts. This is largely due to the rapid decline and threats to the world's biodiversity. Although the protection and sustainable management of wild populations and natural habitats can greatly improve species conservation, the importance of *ex situ* cultivation is becoming a key element of modern day conservation strategies due to increasing urbanization, population growth and industrialization (Pfab and Scholes, 2004). *In vitro* techniques are very useful in ensuring to ensure sustainable, optimized sources of plant-derived natural products. However, *ex situ* cultivation should be preceded by proper evaluation of the plants for their ability to produce the required bioactive constituents before commencing cultivation or introducing the technology to potential growers. The ability of plants to produce certain bioactive substances is largely influenced by the physical and chemical environments in which they grow. Plants also produce certain chemicals to overcome biotic and abiotic stresses (Kuzel et al., 2009). The literature indicates that the majority of pharmacologically important compounds of plant origin are products of defense

Abbreviations: GAE, gallic acid equivalent; HE, harpagoside equivalent; TC-CLT, tissue culture callus-like tissue; TC-S, tissue cultured shoots; TC-T, tissue culture-derived tuber; W-T, wild tuber.

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and secondary metabolism (Andrew et al., 2007; Kuzel et al., 2009; Sudha and Ravishankar, 2002). This ability of plants to respond to physical and/or chemical stimuli can be used for elicitation of pharmacologically active substances by subjecting an intact plant to stress factor(s) (Kuzel et al., 2009). Growing a plant outside its natural environment under ideal conditions may therefore, result in it being unable to produce the desired bioactive substance, hence the need for prior evaluation.

Harpagophytum procumbens, commonly known as Devil's Claw, is one of the most important medicinal plants native to southern Africa. Its storage tubers are harvested, dried and sold by an estimated 10–15 thousand plant gatherers who rely on the plant as a primary source of income (Raimondo and Donaldson, 2002; Strohbach and Cole, 2007). Devil's Claw has been used as part of traditional medicine for centuries. Clinical trials indicated that extracts of the tubers are active in the treatment of degenerative rheumatoid arthritis, osteoarthritis, tendonitis, kidney inflammation and heart disease (Stewart and Cole, 2005). The presence of important bioactive constituents in Devil's Claw contributed to its popularity in the western market. In Germany, Devil's Claw became the third most frequently used medicinal plant in 2001 with sales of ca. 30 million Euro and an overall industry growth of 113% between 1999 and 2000 with an additional 59% in 2000 and 2001 (Strohbach and Cole, 2007). Thereafter, 57 different medicines derived from the plant were produced and licensed in 2003 by 46 different companies for the German pharmaceutical market (Kathe et al., 2003).

Despite these encouraging economic benefits, research on *H. procumbens* largely focused on its pharmacology and regulatory harvesting. Research reports on the biology, mainly propagation and *ex situ* cultivation, of the plant are limited. Low germination rates of seeds and failure of cuttings to produce primary roots result in only a single harvest from a plant; one of the major limitations of conventional propagation (Kathe et al., 2003). There are some reports on the micropropagation of the plant (Bairu et al., 2009; Jain et al., 2009; Levieille and Wilson, 2002). These attempts however, need to be supplemented by growing the plant *ex situ*, evaluating their ability to produce tubers, as well as the occurrence of the bioactive constituents in the tubers and comparing the results with their wild counterparts. Levieille and Wilson (2002) succeeded in growing the plant in the greenhouse and made comparative quantification of iridoids. The pharmacological activity of *H. procumbens* is attributed to the iridoid component of the extract (Levieille and Wilson, 2002).

The aim of this project was to evaluate the initial steps towards domesticating this plant, namely optimizing the growth requirements as well as assessing the biological activity and phytochemical constituents in comparison to tubers collected from the wild.

2. Materials and methods

2.1. Micropropagation and greenhouse tuber production

Micropropagated plantlets, based on the protocol described by Bairu et al. (2009), were acclimatized *ex vitro* (the different developmental stages are presented in Fig. 1A–E). The original plant materials used for the micropropagation were obtained from southern Namibia. *In vitro* acclimatized and rooted plantlets (Fig. 1A) were directly transferred to a greenhouse in a potting mixture containing 1:1 ratio of sand and soil (15 cm pots). Initial stages of acclimatization were achieved by a daily light watering for the first week, watering once every three days during the second week and once weekly for the third and fourth weeks (Fig. 1B). Fully acclimatized plants were then transferred to bigger pots (30 cm) containing the same potting mixture. The plants were left to grow with watering once every ten days until they completed one growth cycle (Fig. 1C). Watering was terminated when the plants started to shed their leaves and stopped producing new shoots to prevent sprouting of tubers; sprouting was observed when lifted intact tubers were left on a tray at room temperature (Fig. 1D). Once the above ground part of the plants died, the tubers (Fig. 1E) were harvested from one-year old greenhouse-grown plants, sliced and air-dried at room temperature (24 ± 2 °C). Tissue culture shoots (TC-S) and tissue culture callus-like tissues (TC-CLT) were taken from maintenance cultures while sub-culturing. Sliced and air-dried wild tubers (W-T) collected from the Tosca-Bray area (north of Vryburg, South Africa) in May 1998, were provided by Professor B-E van Wyk, University of Johannesburg. The dried samples were prepared for phytochemical analysis by grinding to fine powders.

2.2. Preparation of extracts

Wild tubers, tissue culture-derived greenhouse-grown tubers, shoots of *in vitro* plantlets and *in vitro* basal callus-like tissues were air-dried at room temperature (for a period ranging from three weeks to a few months, depending on the sample type) and ground into powder form. These were extracted with 50% methanol (10 ml/g) for 20 min using a sonication bath containing cold water and the extracts were used for the quantification of total phenolic, iridoid and gallotannin content. For the anti-inflammatory assay, the dried, ground materials were extracted with 20 ml/g petroleum ether (PE), dichloromethane (DCM) and 80% ethanol (EtOH) respectively, using a sonication bath containing cold water for 1 h. The use of different solvents of varying polarities helps in extracting metabolites of different chemical nature. The extracts were filtered using Whatman No.1 filter paper and concentrated *in vacuo* at ≤ 40 °C using a Rotary evaporator before drying with a fan at room temperature. The yields (%) of these extracts are presented in Table 1.

2.3. Total iridoid, phenolic and gallotannin quantification

The colorimetric method described by Levieille and Wilson (2002) was used to quantify the total iridoid content of the plant materials. In brief, 1.35 ml of vanillin-sulphuric acid reagent (containing 82 ml methanol, 100 mg vanillin and 8 ml concentrated sulphuric acid) was added to three replicates of 150 µl of each plant extract. For each extract, a blank was prepared by adding 1.35 ml of blank reagent (containing 82 ml methanol

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