UPLC-MS PROFILING, IDENTIFICATION OF MAJOR PEAKS AND COMPARISON OF *HARPAGOPHYTUM PROCUMBENS* EXTRACTS FROM DIFFERENT LOCATIONS

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ABSTRACT:

Organic extracts (methanol: acetonitrile) of the dried and grounded secondary tubers of Devil's Claw, *Harpagophytum procumbens*, produced extracts rich in Iridoid glucosides. An UPLC-MS profiling method was developed that could separate the major compounds found in the extracts and allowed for high resolution mass spectral evaluation of the compounds detected. By using reference standards, the presence of six compounds could be confirmed namely harpagide, verbascoside, isoverbascoside, harpagoside and 6-acetylacteoside. The high mass accuracy of the TOF data also allowed for the tentative identification of three compounds, namely dihydrichinatrienone, totaratrienediol and a possible isomer of 6-acetylacteoside. By using the extracted mass chromatogram of the known compounds of *Harpagophytum procumbens*, a rapid assessment of the identity of the plant extract can be made. A statistical evaluation of *Harpagophytum procumbens* plant material obtained from two different sources using Scores Plot indicated similarity as well as differences between the CSIR and PlantLIBRA samples. A further evaluation of the data via a Hotellings T2 Range comparison indicated that the two samples were >95% similar, while the Scores Plot data revealed that the indicated differences were caused by concentration variations between the CSIR and PlantLIBRA sample. The developed analytical method was applied to the comparison of 12 PlantLIBRA samples and a CSIR sample. The method was found suitable for profiling *Harpagophytum procumbens* extracts and can also be used to manually or statistically evaluate samples for similarities/differences. It would thus be suitable to screen for batch to batch reproducibility of crude plant material as well as processed samples.

KEYWORDS:

Harpagophytum procumbens; UPLC-MS; extract; profiling; quality control; adulteration.

ABBREVIATIONS:

Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS); Time-of-flight (TOF); Principal Component Analysis (PCA).

1. INTRODUCTION

Harpagophytum procumbens is a weedy perennial tuberous herb and is also known as Devil's Claw, The fruits of the plant are covered in small claw-like protrusions, hence the name, Devil's Claw (Grant et al, 2007). The plant is native to the eastern and south eastern parts of Namibia, Southern Botswana and the Kalahari region of the Northern Cape (Raimondo and Donaldson, 2012).

The plant has a primary tuber with a taproot which can be up to 2 meters deep. Secondary tubers develop on the primary tuber and they are up to 25 cm long and 6 cm thick. It is from these secondary tubers that the active constituents are extracted (Schneider, 1997; Wegener, 2000) for potential medicinal uses. Some of the valuable compounds present in the secondary tubers of the plant are iridoid glycosides, particularly harpagoside (trans cinnamoyl harpagide), as well as small amounts of trans-coumaroyl harpagide, procumbide and plant sterols which are thought to have anti-inflammatory effects (Wichtl, 2004). Other medicinal effects of the plant extract as recognised by the British Herbal Pharmacopoeia and the European Pharmacopoeia include being analgesic, sedative and having diuretic properties, while the Khoisan people in the Kalahari Desert use the plant in the form of an ointment to treat skin disorders by (Wegener, 2002). Devil's claw for medicinal use is normally prepared as a herbal extract which is standardised between 1 and 4% harpagoside (Bone, 2001). The specific mode of action of the herb in the body is still unknown, but it has been proven to be effective and safe in clinical investigations (Wegener, 2000, Chrubasik et al., 2003, Gagnier et al., 2004). The standardised preparation of the herbal extract is called Doloteffin and has been used in a number of small scale studies in Germany to compare its efficiency to that of Vioxx. The extract was said to be comparable to Vioxx for the treatment of chronic lower back pain as well as in the treatment of hip and knee pain caused by arthritis (Chrubasik et al., 2003). According to literature, a daily dose of 50 to 100 mg of the extract of the plant has similar effects than 12.5 mg of Vioxx and was well tolerated after more than four years of treatment (Chrubasik et al., 2005; Gagnier et al., 2006). Harpagoside has further been shown to inhibit the leukotriene synthesis pathway in human white blood cells (Tippler et al., 1996) and may also play a role in the inhibition of the lipoxygenase pathway (Loew et al., 2001).

The general method to extract the herb is by first macerating it and then using ethanol-water (6:4 w/w) and 2.5% harpagoside is normally obtained (Günther and Schmidt, 2004). By using super and subcritical carbon dioxide in the presence of 25% (w/w) ethanol as modifier, the harpagoside content of the extract was increased by 20 – 30%, but also resulted in a high level of other lipophilic substances which interfere with analytical assays (Günther et al., 2006). To overcome this, most of the lipophilic substances can be pre-extracted using CO_2/n-propanol (5% w/w) and then the main extraction is done with CO_2/ethanol (25% w/w) (Günther et al., 2006).

A patent by Stumpf et al. (2001), described a method for harpagoside-enriched extract from *Harpagophytum procumbens*. The preferred method according to the patent is to extract the ground herb with 20% ethanol/water and then the extract is concentrated. This concentrate is then stirred with 96% ethanol at room temperature. The soluble and insoluble fractions are separated and the soluble fraction is dried and the extract contains at least 5% harpagoside, while conventional extraction methods result in 1 to 3% harpagoside. Babili et al. (2012) prepared the extract by adding water, 8 to 10 times the weight of the powder, to the dried powder of the secondary tubers and extracting at 50°C for 2 hours. Rolland and Duval (2010) described in a patent, a novel method to prepare concentrated extracts of *Harpagophytum procumbens* by using liquid-liquid extraction with an organic solvent

selected from the esters as using super or subcritical CO_2 is not practical on industrial scale. Through this technology they are able to get a titre between 30 and 55% harpagoside in comparison to the normal 3% when only using water or ethanol/water.

The aim of the paper was to develop an extraction method and then analyse this extract on UPLC-MS to give a representative profile of the more hydrophilic marker compounds in the plant. This profile can then be used in the screening for batch to batch reproducibility of crude plant material as well as authentication of processed samples said to contain extracts of the plant.

2. MATERIALS AND METHODS

All chemicals including reference standards for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchase from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated from a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France). Plant material was sourced from the ECD unit of the CSIR (Dr. Marthinus Horak) locally while the PlantLIBRA plant material was supplied by Dr. Franz Chlodwig (PlantLIBRA).

2.1 Instrumentation and Analysis

A Waters Acquity Classic binary UPLC system, coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer, was used to visualise separated compounds. Two analytical procedures were used to analyse the samples. Three column chemistries were evaluated, namely the Waters CSH C18, HSS T3 and HSS C18 UPLC columns. All the columns were 150 mm x 2.1 mm x 1.7/8 µm in dimension and column temperature controlled at 60 °C. The HSS C18 column produced the best peak shape and was used to develop the chromatographic separation method. Chromatographic separation of the pooled extraction samples was done utilising a Waters HSS C18 column (150 mm x 2.1 mm, 1.8 µm) and the column temperature controlled at 60 °C. A binary solvent mixture was used consisting of water containing 10 mM formic acid (Eluent A) (no pH adjustments; natural pH of 2.3) and acetonitrile containing 10 mM formic acid (Eluent B). The initial conditions were 95% A at a flow rate of 0.4 mL/min and were maintained for 1 minute, followed by 90% A at 3.5 minutes, 80% A at 5 minutes, 75% A at 16 minutes and 10% A at 24 minutes. The conditions were kept constant for 3 minutes and then changed to the initial conditions. The runtime was 30 minutes and the injection volume was 3 µL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 40 spectra per second.

The SYNAPT G1 mass spectrometer was operated in electrospray mode and utilising the V-optics configuration. Leucine enkephalin solution (50 pg/mL in (1:1) water: acetonitrile) was used as reference calibrant to obtain typical mass accuracies between 3 and 5 mDalton (mDa). The mass spectrometer was operated in negative mode with a capillary voltage of 2.0 kV, the sampling cone at 30 V and the extraction cone at 4 V. The scan time was 0.1 seconds covering the 100 to 1000 Dalton mass range. The source temperature was 120 °C and the desolvation temperature was set at 450 °C. Nitrogen gas was used as the nebulisation gas at a flow rate of 800 L/h. The software used to control the hyphenated system and to do all data manipulation was MassLynx 4.1 (SCN 704).

Waters MarkerLynx XS (SCN 678) software was used to evaluate similarities/differences between plant extracts and to visualise the similarities/differences in the chemical profile of plant material obtained from different sources.

2.2 Extraction of the plant material

Different solvents and ratios of solvents were initially tested for extraction of distinctive compounds from *Harpagophytum procumbens* and it was found that a 1:1 (v/v) mixture of methanol: acetonitrile (MeOH:ACN) produced a useful extract from dried plant material. For all subsequent extractions, 1g of plant material obtained from the CSIR Enterprise Creation for Development (ECD) unit was extracted with 10 mL of the solvent mixture in an ultrasonic bath. The samples were extracted four (4) times. Efficiency of extraction was determined through analysis of each re-extraction. The chromatographic method was developed using the combined supernatants.

3. RESULTS AND DISCUSSION

The general method to extract the herb is by first macerating it and then using ethanol-water (6:4 w/w) for extraction (Günther and Schmidt, 2004). Other methods used for extraction of the more hydrophilic compounds for analysis on HPLC include super and subcritical carbon dioxide in the presence of 25% (w/w) ethanol as modifier (Günther et al., 2006), initial extraction with 20% ethanol and following concentration of the extract, stirring it with 96% ethanol at room temperature (Stumpf et al., 2001), extraction of dried powder with water at 50°C for 2 hours (Babili et al., 2012) and liquid-liquid extraction with an organic solvent selected from the esters (Roland and Duval, 2010).

For the work conducted in this paper, preliminary extractions were done using different alcohol to water ratios and a mixture of 1:1 methanol: acetonitrile yielded an extract containing most of the major compounds identified by other researchers on HPLC or HPTLC either linked with DAD or MS (Günther and Schmidt, 2004; Babili et al., 2012; Abdelouahab and Heard, 2008; Karioti et al., 2011; Seger et al., 2005). Six reference standards were analysed with the optimized method to determine the elution patterns of the most important active compounds normally reported in *Harpagophytum procumbens* and is given in Figure 1.

Evaluation of the CSIR ECD sample raw data confirmed a significant number of the compounds listed in the Dictionary of Natural Products (DNP Ver.18.2) was present in the extracted sample. Figure 2 depicts a typical extracted mass chromatogram (XIC) obtained from the analysis of the methanol/ACN extract using the optimized method. The compounds that were positively identified were labelled. Table 1 is a summary of the compounds detected and identified.

Another application of the method could be to determine is plant samples from different locations are similar and if the UPLC-MS can distinguish differences between these samples. Plant samples were received as a gift from Dr. Franz Chlodwig (PlantLIBRA) and compared to the CSIR sample for this study. Eight replicate analysis of each plant extract were done and evaluated with MarkerLynx, a software package designed to mine mass spectral data for biomarkers and that can be used to evaluate the similarity or differences between samples – in this case *Harpagophytum procumbens* samples sourced from different suppliers.

The evaluation of the chemical profiles of the CSIR ECD and PlantLIBRA plant material appeared significantly similar when comparing the BPI mass spectral chromatograms (not shown), but the statistical PCA evaluation using the MarkerLynx software indicated differences between the two samples (Figure 3). The evaluation indicated that the samples (observations) were primarily similar and therefore grouped within the ellipse. The CSIR ECD and PlantLIBRA samples however clustered as unique groups indicating distinct differences between the CSIR ECD and PlantLIBRA samples and therefore placed in opposite quadrants of the Scores Plot. All data was Pareto scaled and no observations were excluded.

The developed method was applied to the analysis and comparison of ten (10) samples obtained from PlantLibra (Dr. Franz Chlodwig), two (2) commercially available samples as well as the CSIR ECD sample obtained from the ECD Unit of the CSIR (Dr. Marthinus Horak). All the samples were supplied as derived from *Harpagophytum procumbens* and the sample names and format supplied in summarised in Table 2. All the samples were prepared as described above (Extraction of plant material) and analysed using the optimised UPLC-MS method. A PCA Scores Plot analysis (Figure 4) of the samples clearly highlighted similarities and differences between the samples. Most of the samples clustered within the ellipse indicating similarities between the samples. The two commercial samples, COM1 and COM2 clustered outside the ellipse indicating substantial differences between the commercial samples and the "raw material" samples. This could be due to sample preparation applied by the manufacturer prior to formulation or packaging.

The effect of sample preparation was evaluated but no clear pattern could be observed as fine and coarse samples scattered within the ellipse and even merged with the resin samples and some of the capsule samples. It can therefore be concluded that the distribution pattern observed in Figure 4 is more related to chemical composition and concentration than physical sample appearance.

4. CONCLUSION

Consumers are more aware of the side-effects of many pharmaceuticals and therefore there is an increase in the use of more natural products to address health problems. The medicinal potential of *Harpagophytum procumbens* has been realised and more products may come onto the market claiming to contain extracts of this plant. A fast and reliable method using the profile of the plant would therefore be very valuable.

An UPLC method was successfully developed using an extract from dried and grounded secondary tubers of *Harpagophytum procumbens* obtained through extracting the plant material with methanol: acetonitrile 1: 1 (v/v). Reference standards confirmed the presence of harpagide, verbascoside, isoverbascoside, 6-acetylacteoside and harpagoside. The use of LockSpray^{TM} and TOF technology rendered high mass accuracy data could be used for tentative identification of three compounds, namely dihydrichinatrienone, totaratrienediol and a possible isomer of 6-acetylacteoside. A comparison was done between *Harpagophytum procumbens* plant material sourced locally and material obtained from PlantLIBRA (PL Ref). The comparison using BPI chromatographic profiles indicated very strong overlap in composition, while the MarkerLynx software indicated differences between the two samples. Statistical evaluation of the data via a Hotellings T2 Range process predicted that the various injections (8 x CSIR; 8 x PlantLIBRA) had a high probability of similarity.

The developed method was tested on thirteen samples, ten samples received from PlantLibra, two commercially available samples in South Africa and one sample obtained from the CSIR ECD unit. The method performed

well and could determine that all the samples were derived from *Harpagophytum procumbens.* The two commercial samples however grouped separately in the PCA Scores Plot analysis indicating that they differed significantly from the unprocessed samples.

The method will be very valuable in screening plant material to unequivocally assess whether the material is indeed originating from *Harpagophytum procumbens.*

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6. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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