Phytochemical Profiling and Chemical Marker Compounds Identification in *Helichrysum caespititium*: A Chemometrics and 2D Gas Chromatography Time of Flight Mass Spectrometry (GCxGC-TOF-MS) Perspective

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**ABSTRACT**

**Introduction:** *Helichrysum caespititium* is a medicinal plant indigenous to South Africa. Hitherto, only 2 compounds—caespitin and 2-methyl-4-[2’,4’,6’-trihydroxy-3’-[2-methylpropanoyl]-phenyl]but-2-enc acetate have been reported from this species. Phytochemical profiling of the plant and identification of chemical markers are limited. **Objectives:** Determining phytochemical profile of *H. caespititium* and identifying the major marker compounds in its extracts. **Methods:** A two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOF-MS) was used to analyze *H. caespititium* extracts (acetone, methanol, and dichloromethane). The marker compounds from the generated phytochemical fingerprints were identified using Column plots and chemometrics orthogonal partial least squares discriminant analysis (OPLS-DA). Polar acetone, methanol, and non-polar dichloromethane extracts were analyzed separately. **Results and Discussions:** A total of 135 (12 from acetone, 13 from methanol, and 110 from dichloromethane extracts) compounds were identified in *H. caespititium*. An OPLSDA score plot with $R^2 = 0.81$ grouped the polar compounds into 2 clusters as phenolic and non-phenolic compounds, while a contribution plot from the score plot then nominated benzene[(methoxymethoxy)methyl], 4-methyl-2,4-bis(p-hydroxyphenyl)penet-1-ene, isoeugenol, and 3,4-dihydroxymandelic acid as marker compounds in the polar extracts. In a second plot with $R^2 = 0.67$, the corresponding contribution plot accentuated 2-methyl-5-(fur-3-yl)pent-3-en-2-ol, 3,5-dimethyl-4-heptanone, 1,2-benzenedicarboxylic acid, dihexyl-1-(4-methylphenyl)-5(2-dimethyl aminothienyl)-1H-tetrazole, and 3,5-dimethyl-4-heptanone as the marker compounds in the dichloromethane extract. **Conclusion:** This study recommends the use of the marker compounds as quality standard of raw materials and commercial products containing extracts or other forms of the South African *H. caespititium*.

**Key words:** Helichrysum caespititium, Phytochemical profiling, Chemometrics, Chemical markers, GCxGC-TOF-MS.

**INTRODUCTION**

*Helichrysum* (Asteraceae) consists of 600 species in Africa, and about 40% these species are endemic to South Africa. The traditional use of ~102 South African *Helichrysum* species as well as the phytochemicals responsible for such uses are very well documented. Noteworthy phytochemicals include flavonoids, terpenoids, pinoembrin chalcone, pinoembrin from *H. acutatum* DC, and acetic acid 1-acetoxymethyl-dodeca-2,4,6,8,10-pentaynyl ester acetylene derivative from *H. adenocarpum*. Some of these, such as terpenoids from *H. appendiculatum* (L.F.) work against respiratory, bacterial, and antifungal infections. Flavanones, phloroglucinols, purones, diterpenes, terpenes, and monocyclic sesquiterpene from *H. callicomum* Harv are used as incense. Some pure compounds, including galangin from *H. aurantiens* Sch. Bip possess antibacterial and antifungal properties. Helichromanochalcone, heliulumone, 5-hydroxy-8-methoxy-7-prenyloxyflavanone, 8a-hydroxy-a-gurjunene, 8a-acetoxy-a-gurjunene, 14-gnaphaladien-8-ol, 1-methyl-4-[(1,5,9-trimethy-4,8-dicadienyl)-1,3-cyclohexadiene, helinudichromene quinone, helindifolin, helindiquinone-6-methyl ether, helindiquinone, and isocomen-5-one has been reported. Three new and six known acylphloroglucinols alongside a known dialcohol triterpene with excellent total antioxidant capacities, as demonstrated by helinivines A and B, were described by Popoola et al. Most studies reported no more than two compounds derived from *H. caespititium*.

Peak overlap and compound co-elution is a major problem encountered during classical chromatography. GCxGC-MS, which analyses samples in two dimensions to circumvent this problem, has helped separation scientists worldwide. However, analyte peaks generated by GCxGC-MS can be numerous and be identified from a relevant library. However, the analysis of GCxGC-MS data...
using classical statistical methods remains a challenge. Furthermore, beyond the identification of compound peaks from the library, the WHO recommends that quality control of medicinal plants be conducted via chemical profiling, which means “using known marker compounds of a natural product as standards for quality evaluation of its raw material and commercial products.” This deploys a holistic perspective and not a targeted approach, which requires standard(s) that are either not commercially available or difficult to isolate.

This study explored the potential of using GCxGC-TOF-MS fingerprint in combination with chemometrics to identify the chemical marker compounds in the South African *H. caespititium*’s polar and non-polar extracts.

**MATERIAL AND METHODS**

**Plant collection**

*H. caespititium* (DC) Harv. matured plant material was collected in January 2017 from Ga-Mamabolo (23.8346°S, 29.8844°E) Masealama, Mankweng district, Limpopo province, South Africa. The plant was botanically identified by indigenous knowledge practitioners and the South African National Biodiversity Institute (SANBI) under specimen number DTH 9006000 in the Pretoria herbarium, Gauteng Province, South Africa. The plant material (350 g) was air-dried in the laboratory, ground to a fine powder using a mill (Kinematica AG, Lauzern, Switzerland), and stored until use.

**Plant extraction**

A total of 333.33 g of the powdered plant material was transferred to a 1000 ml beaker, and 500 mL of dichloromethane was added to it. The mixture was sonicated for 30 min at 25 °C and subsequently filtered using Whatman No. 1 filter paper. The process was repeated twice more with the same plant material. The filtrate was pooled together and evaporated using a rotary Stuart evaporator (Cole Parmer Ltd., UK) connected to a Vacuubrand MZ 2C NT pump (Vacuubrand GmbIH + Co Kg, Wertheim, Germany) until 5.0 mL was left in the flask. The remaining solvent was left to air dry, yielding 5.0 g of the dichloromethane extract. The same extraction protocol was used to obtain the acetone (2.10 g) and methanol (1.45 g) dry extracts.

**Instrumentation and sample preparation**

The *H. caespititium* acetone, methanol, and dichloromethane extracts were individually analyzed without derivatization using a LECO Pegasus 4D Time of Flight mass spectrometer (LECO Corporation, St Joseph, MI, USA) equipped with a modified Agilent 7890A Gas Chromatograph (Agilent Technologies, Inc., Wilmington, DE, USA), a LECO GC×GC modulator, a secondary oven (LECO Corporation, St Joseph, MI, USA), and a split/splitless inlet. Rxi-5 SILMS (29.5 m × 0.25 mm × 0.25 μm) was the primary column and Rxi 17 Sil MS (0.95 m × 0.25 mm × 0.25 μm) the secondary column (Restek, Bellefonte, PA, USA). Helium was used as the carrier gas at a constant flow rate of 1 mL/min at an inlet temperature of 250 °C. An initial oven temperature of 40 °C was held for 0.5 min and then slowly increased at 10 °C/min to 250 °C and then held for 0.5 min at 250 °C. The modulator and secondary oven were run at an offset temperature of 5 °C above the primary oven. The mass spectrometer was set up under the following conditions: transfer line temperature at 250 °C, electron ionization at -70 eV, source temperature at 250 °C; stored mass range: 45–600 μ; acquisition rate: 10 spectra/s for GC×GC-TOF-MS; detector offset voltage was set at 300 V. Retention time alignment, matched filtration, peak detection, and peak matching were performed using ChromaTOF software (LECO, USA). Subsequent identification was performed by comparison with mass spectral databases (NIST, Adams, and EO libraries). Semi-quantification of each compound was calculated based on in percentage. A stock solution of 10 mg/mL of each extract except water was prepared by dissolving 10 mg of the dry extract in a suitable solvent and sonicating for 30 s. Aliquots from the stock solution were further diluted to 1 mg/mL and filtered with 0.25 μL nylon syringe filter attached to a 5 mL syringe prior to injection into the GCxGC-TOF-MS. Pre-injection filtering of each extract (acetone, dichloromethane, and methanol) was performed using a 50/30 μm SPME fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/ CAR/ PDMS) (Supelco, Inc., Bellefonte, PA) before being immediately injected into the GC-MS system for analysis.

**Chemometrics analysis**

The GCxGC-TOF-MS data were exported to Excel 2016 and made compatible with chemometrics analysis. All the artifacts and contaminants, such as polymeric materials and stationary phase silicate complexes were excluded from the data in Excel before the data were made SIMCA-15 compatible prior to chemometrics analysis. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using SIMCA 15 software (Umea, Sweden). From the positively correlated observations of the score plot, a column plot was constructed and analyzed, and the contribution of each compound was determined by comparing its weight against that of all the other compounds. A contribution plot for the selected compound displayed the area and the weight t1 (weight = p1p2) on the Y-axis of the plot. Compounds with the most significant weight (t1) were nominated as marker compounds by the model.

**Statistical analysis**

The average of the replicated data obtained from the GC×GC-TOF-MS analysis was computed, and the results are presented as mean ± standard deviation.

**RESULTS AND DISCUSSION**

From a thorough search of NIST, Adams, and EO mass data libraries, subsequent identification of peaks obtained from the analysis of the acetone and methanol extracts were carried out. The acetone extracts revealed 13 peaks, the methanol extract contained 12 compounds with 1 compound extracted by both solvents to account for 24 compounds present in the analyzed *H. caespititium* polar extracts. The co-occurrence of these phytochemicals in *H. caespititium* could support its traditional use by rural folk as medicine if the biological activities of these compounds are proven.

A total of 110 compounds were annotated and identified from the dichloromethane extract of the plant extract. However, the health benefits of these compounds are not discussed here because dichloromethane is carcinogenic13, unpopular in the preparation of commercial extract and traditional medicine14.

**Chemometric identification of chemical marker compounds from polar (acetone, methanol) extracts of *H. caespititium***

The compound peaks generated from the GCxGC-TOF-MS analysis of the acetone and methanol extracts and identified using NIST, Adams, and EO libraries were exported to Excel 2016. The data were arranged before SIMCA-15 analysis by setting the peak numbers as the primary ID and compound names (synonyms) as the alternative primary ID. The retention time (RT) and the area were set as the secondary observation IDs. These data were then converted to SIMCA-15 and made compatible with chemometrics analysis. A principal component analysis (PCA) model with R2 = 0.807 ability to explain the variation or similarity in the dataset in the first component (t1) and a predictive
power or Q2 of 0.801 in the second component (t2) was constructed and used for the analysis. The information from the scores plot is completely and usually independent of each other.

Score t1 is the first component – horizontal direction in a score scatter plot usually explains the largest variation of the X space, followed by t2, the vertical direction for intra-observation differences. Hence, the scatter plot of t1 vs. t2 is a window in the X space displaying how the X observations are situated with respect to each other. This plot shows the possible presence of outliers, groups, similarities, and other patterns in the data. The score scatter plot (Figure 1) depicted a set of outlier compounds in green out of the 95% confidence level of the analysis. The outliers identified as 1-propanol, 2-mino from the methanol extract, and dl-alanine from the acetone extract were negatively correlated and should have been placed in the blue cluster. However, both compounds (outliers) are amines and are different from the other phenolic compounds and their derivatives that characterize this cluster. The negatively correlated compounds in the blue circle and the positively correlated compounds in the red circle of the polar extract were also observed.

The compounds in the blue and red clusters in the first component t1 were grouped within the 95% confidence level of the model, while the outlier compounds (in green) were placed outside the confidence level. The compounds in each of the clusters were from both the acetone and methanol extracts highlighted that grouping was not the function of the solvent used for extraction, but it occurred due to similarity in the compounds placed in each cluster. Besides bicinnamaldehyde, a phenolic compound that probably, with an improved chemometric model, is positively correlated because it has the least t1 value of -0.39, all the other compounds physically identified, including heamatoporphyrin, methylphosphonyl dichloride, carbon tetrachloride 5-(3-methoxymethoxy-10,13-dimethyl-2,3,4,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthrene-17-yl)-hex-1-ynyl-trime, 17-(15-Dimethylhexyl)-10,13-dimethyl-3-styrylhexadecahydropenta(a) phenanthrene-2-one and 8,9-Dicyanoacenaphthol(1,2-β) pyrazine are non-phenolic compounds and clustered together by the chemometric model used for the analysis.

A similar observation was recorded for most compounds in the positive (red) cluster (Figure 1). Apart from 1,4-Dihydro-2-isopropyl-6-phenlynicotinonitrile and lycopene, most compounds, 3,4-Dihydroxymandelic acid, 2,4,6,8,10,12,14,16-Heptadecaoctanedioic acid, 17-(4-methoxyphenyl)-2-decyl-3-methoxy-5-pentylphenyl ester, isoeugenol, 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-l-ene, 1,2-Benzenedicarboxylic acid, 3,4,5,6-tetrafluoro-dimethyl ester, 3-(4-Bromobenzoylxy)-5,7,8-trimethyl-2-(2-propenyl) phenantherone-1,4-dione, 4-Methyl-2,4-bis(p-hydroxyphenyl) pent-l-ene, 1,4-benzenedi, 2-(4-aminophenoxy)-3,5-dichloro-6-pentadecyl, benzene, [(methoxymethoxy)methyl] in this cluster are phenolic compounds or their derivative carboxylic acids or ketones.

As indicated in the column plot (Figure 2), the green bars represent variation of the compounds in the first component, t1 (horizontally). Upward bars from the origin are positively (significantly) correlated compounds, while the downward bars represent negative variations within the compounds. Peaks 1 and 13 were identified as compounds 1-propanol, 2-mino from the methanol extract, and dl-alanine from the acetone extract clustered as outliers because both compounds are amines ad negatively related and different from the other compounds (phenolic and their derivatives). Conversely, peaks 4 and 5 were identified as 1,4-benzenedi,2-(4-aminophenoxy)-3,5-dichloro-6-pentadecyl and 1,4-Dihydro-2-isopropyl-6-phenylnicotinonitrile were positively correlated in both t1 and t2 as possible chemical marker compounds in the plant’s polar extract. All the other compounds per cluster indicated inter- and intra-correlations with and among each other.

The acid functionality in methylmalonic acid and 9-Desoxo-9-acetoxy-3,8,12-tri-O-acetylingol as nonphenolic acids tends to support clustering, while hydrazine, tungsten, and magnesium, bis(2,4-pentanedionato-O,O’)- (T-4) are considered artifacts picked up during the GCxGC-MS analysis. Their presence in this cluster would be insignificant, considering these are not necessarily plant-
based compounds. The positively correlated compound in the first component, with the largest t1 values, should determine the predominant marker compounds in the polar extract of *H. caespititium*. Observations 9, 10, 23, 24, and 25 with t1 values of 1.25, 1.17, 1.13, 1.18, and 1.21 were identified from the column plot (Figure 3) as representing these compounds. Interpolating these observations underscored benzene[methoxymethoxy]methyl, 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene, isoeugenol, and 3,4-dihydroxymandelic acid (Figure 3) as the marker compounds. However, since 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene was indicated for both methanol and acetone extracts with combined t1 values of 2.36, it was considered one of the major marker compounds alongside 3,4-dihydroxymandelic acid, which is possibly a precursor to caespititin 15 and 2-methyl-4-[2',4',6'-trihydroxy-3'-(2-methylpropanoyl)-phenyl] but-2-enyl acetate, as previously reported. 16

The observation that benzene methoxymethoxy methyl, 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene, isoeugenol, 3,4-dihydroxy mandelic acid defines the polar extract from the column plot was further endorsed using a contribution plot (not shown). A contribution plot for the selected compound was displayed, and the weight of that compound (weight = p1p2) on the Y-axis was recorded (Table 1). Table 1 shows that 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene has a combined weight from acetone and methanol of 1.69, thus having the highest contribution compared to all the other compounds. This was followed by benzene methoxymethoxy methyl (weight = 0.84), 3,4-dihydroxy mandelic acid (weight = 0.82), and isoeugenol (weight = 0.77) as the other major marker compounds.

The dichloromethane extract of *H. caespititium* that was analyzed by GCxGC-MS yielded N = 110 possible compounds matched from the NIST, Adams, and EO libraries. These 110 compounds were placed in an Excel 2016® and subsequently transferred to Simca 15® for analysis to identify which of these compounds were markers that should be used for the identification of the South African *H. caespititium* non-polar extract. A different model was constructed for this analysis. The statistics for the model used for this analysis indicated that it could explain 67.7% of the variation in the data set in the first component (R2 = 0.67) with a predictive ability (Q2) of approximately 50%. The PCA-X score scatter plot (Figure 4) of the unsupervised data revealed about 14 compounds as outliers out of the 95% confidence level of the analysis. The outlier compounds were removed, and a new model was constructed for the analysis. The analysis statistics for this new model did not improve from the previous one as the R2 and Q2 remained the same. This plot did not indicate any distinct cluster, as was the case with the combined acetone-methanol compounds analyzed. This could

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**Figure 2:** Column plot indicating the correlation weights of the compounds in the acetone-methanol extract for each cluster.

**Figure 3:** Structures of the major marker compounds from the aceton-methanol extract of *H. caespititium*. 
imply that the compounds in the dichloromethane extract of the South African *H. caespititium* do not contain compounds that are closely similar or completely different.

However, there was marginal separation from the origin of the score plot of the compounds that are negatively correlated in the blue circle from those that are positively correlated in the red rectangle. Standing out as the major marker compounds from the positively correlated compounds (in red) are compounds with positive correlation value in the first component (t1) of greater than 1 as interpolated from the column plot (Figure 5). The main marker compounds identified were 2-methyl-5-([fur-3-yl]pent-3-en-2-ol, propanedioic acid, diazo-, dimethyl ester, 3,5-dimethyl-4-heptanone, 1,2-Benzenedicarboxylic acid, dihexyl ester, imidazole [4,5-δ],imidazole, 1,4-dithiin,2,5-diphenyl, 5-Methyl-2-(2-methyl-2-tetrahydrofuranyl)tetrahydrofuran, butane diamide, 2-methylene, 1-Aminocyclopentanecarboxylic acid, -n-propargyloxycarbonyl-pentadecyl ester, and 1-(4-methylphenyl)-5(2-dimethyl aminothenyl)-1H-tetrazole. However, the contribution plot weights displayed in Table 2 designated 1-(4-methylphenyl)-

![Figure 4: Score scatter plot of the compounds in the dichloromethane extract of *H. caespititium* indicating 2 clusters of phenolic and allylic compounds.](image)
Figure 5: Column plot indicating the correlation weights of the compounds in the dichloromethane extract for each cluster.

Figure 6: Structures of the marker compounds present in the dichloromethane extract of *H. caespititum*. 
5(2-dimethyl aminothenyl)-1H-tetrazole, 3,5-dimethyl-4-heptanone (Figure 6) as the top three marker compounds because of their weight (percentage area) contribution.

CONCLUSION

Benzene[(methoxymethoxy)methyl],4-methyl-2,4-bis[p-(hydroxyphenyl)penet-1-ene, isoeugenol, and 3,4-dihydroxymandelic acid from the polar extract and 2-methyl-5-((fur-3-yl)pent-3-en-2-ol, propanedioic acid, diazo-, dimethyl ester, 3,5-dimethyl-4-heptanone, 1,2-benzenedicarboxylic acid, dihexyl ester, imidazole[4,5-δ],imidazole, 5-methyl-2-(2-methyl-2tetrahydrofuryl)tetrahydrofuran, butanediamide, 2-methylene, 1-Aminocyclopentane-carboxylic acid, -n-propargyloxycarbonyl-pentadecyl ester, and 1-(4-methylphenyl)-5(2-dimethyl aminothenyl)-1H-tetrazole from the non-polar extract are the marker compounds in the South African

H. caespititium. These marker compounds are recommended for use in the identification, standardization, and quality control of raw materials and commercial products that contain extracts or other forms of the South African H. caespititium.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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