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# Croton gratissimus - essential oil composition and chemometric analysis of an ethnomedicinally important tree from South Africa



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## ABSTRACT

*Croton gratissimus* Burch. is widely used in traditional medicine to treat a range of conditions including malaria, hypertension, diabetes, arthritis, urinary tract infections, gonorrhoea and impotence. The young branches and leaves are pleasantly aromatic, and it is recorded that various ethnic groups in South Africa used the leaves as a perfume. The chemistry of the non-volatile constituents has been widely investigated. However, little scientific data on the volatile constituents are available. Essential oils (EOs) were obtained by hydrodistillation from the leaves and twigs ( $n = 62$ ) collected at different locations in South Africa. The hydro-distilled oils were analysed with gas chromatography coupled simultaneously to a mass spectrometer (MS) and a flame ionisation detector (FID). GC–MS/FID of the EOs showed the presence of mono- and sesquiterpenes as the major class of compounds. The most abundant compounds were  $\beta$ -pinene (nd–13.7%), sabinene (0.2–16.7%),  $\alpha$ -phellandrene (0.4–44.3%),  $Z$ - $\beta$ -ocimene (0.2–12.5%), limonene (0.2–38.3%),  $p$ -cymene (0.1–25.2%), phytol (nd–24.9%) and oxidohimachalene (nd–54.6%). Untargeted analysis of the GC–MS data was performed using SIMCA- $P + 14.0$ , and different methods were used to explore possible chemical variation in the data set. After principal component analysis (PCA), the hierarchical cluster analysis (HCA) revealed three major chemotypes. Loadings and contribution plots identified oxidohimachalene as the main chemical marker for chemotype 1. Chemotype 2 was dominated by  $\alpha$ -phellandrene,  $\alpha$ -pinene and  $p$ -cymene, while chemotype 3 was characterised by low yields of  $\alpha$ -phellandrene and oxidohimachalene. This study revealed mostly quantitative rather than qualitative variation in the EOs constituents of *C. gratissimus*.

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## 1. Introduction

*Croton* is a large genus of ca. 1300 species with the majority of species located in the New World (the Americas). Of the 1300 species, 26 are found in Africa, while 10 species are well known in South Africa (Palmer and Pitman, 1972). Amongst the indigenous African species, *Croton gratissimus* Burch. has been the most used and studied. *Croton gratissimus* (= *C. zambesicus*) is commonly known as “lavender *Croton*, bergboegoe, boog, Kalahari-boegoe, laveltelbos, masquassieboom, rekstokbos (Afrikaans); umahlabekufeni, ihubeshane-elikhula (Zulu); umhuluka (Siswati); moologa (Tswana)” (Palmer and Pitman, 1972). It is a tree found in many areas of South Africa including the Western Cape, North West, Free State, Gauteng, Limpopo and KwaZulu-Natal provinces. The plant grows well on rocky outcrops and usually occurs as a shrub or tree with a rough grey bark. Leaves are alternate with a striking silvery upper surface without hairs, dotted with cinnamon coloured glandular scales and the apex tapering broadly. It has small, cream to golden-yellow

flowers in spikes up to 10 cm long and a 10 mm yellow 3-lobed capsule fruit (Palgrave, 1983).

*Croton gratissimus* is used traditionally in southern Africa for the treatment of bleeding gums, colds, red eyes, rheumatism, management of candidiasis, irritant and painful breathing (Watt and Breyer-Brandwijk, 1962; Palgrave, 1983; Hedberg and Staugard, 1989; Hargreaves, 1991; Masevhe et al., 2015). In the northern part of Africa, the plant is used to treat a range of diseases including hypertension, urinary tract infections, diabetes, malaria, cancer, fever, gonorrhoea, arthritis, diarrhoea, impotence, tetanus, menstrual pain, constipation and syphilis (Iwu, 1993; Ngadjui et al., 2002; Okokon and Nwafor, 2009a; Cheikhoussef et al., 2011; Chinsenu, 2016).

Pharmacological activities reported for the extracts and isolated compounds from *C. gratissimus* include anti-epileptic, antipyretic, anticancer, antiplasmodial, analgesic, anti-inflammatory (Boyom et al., 2009; Okokon and Nwafor, 2009b, 2010; Okokon et al., 2013), immunomodulatory, antileishmanial activity (Okokon et al., 2013), antimicrobial (Van Vuuren and Naidoo, 2010; Van Vuuren and Viljoen, 2008), antidiabetic activity (Baccelli et al., 2005; Robert et al., 2010) and pro-fertility property (Dada and Adeparusi, 2012).

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Due to the reported biological activities, several investigations into the chemistry of *C. gratissimus* have been carried out, and interest in this plant is gaining attention. Some studies on the essential oil composition of *C. gratissimus* have previously been published (Van Vuuren, 2007; Mekki, 1985; El-Kamal et al., 2012; Owolabi et al., 2013; Ogundajo et al., 2014; Lawal et al., 2017). However, in the majority of the studies, usually, a single plant collection was analysed, and the essential oil composition of a plant cannot be solely based on a single collection or single analysis.

In this study, the analysis of essential oils (EOs) of 62 samples was carried out using GC–MS/FID. Chemometric analysis was performed on GC–MS data obtained from geographically distinct populations in South Africa in order to establish any possible chemotypic variation that may exist.

## 2. Materials and methods

### 2.1. Plant collection

The aerial parts of *C. gratissimus* ( $n = 62$ ) were collected in 2014 in the Limpopo, Mpumalanga and Gauteng provinces of South Africa. The plants were identified at the Herbarium of the University of Limpopo and dried at room temperature. Voucher specimens were deposited at the Tshwane University of Technology, South Africa (supplementary documents S1).

### 2.2. Isolation of the essential oils

The essential oil was isolated by hydrodistillation using a Clevenger-type apparatus as per the British Pharmacopoeia Commission (1980). Approximately 300 g of fresh sample was placed in the all-glass Clevenger-type apparatus, and 1 L water was added before plant material was heated for 3 h at 100 °C. The oils were then collected and stored in an amber vial at 4 °C until analysis.

### 2.3. Gas chromatography coupled to mass spectrometry/flame ionisation detector

The samples were prepared by diluting 20  $\mu\text{L}$  of essential oils with 80  $\mu\text{L}$  of hexane. The oil samples were analysed, using gas chromatography coupled to a mass spectrometry/flame ionisation detector (Agilent 6890 N GC system coupled directly to a 5973 MS, Hanova, USA) operating under the following conditions: injection volume of 1  $\mu\text{L}$ , split ratio 200:1 and inlet temperature 250 °C. HP-Innowax polyethylene glycol column (60 m  $\times$  250  $\mu\text{m}$  i.d.  $\times$  0.25  $\mu\text{m}$  film thickness), oven temperature at 60 °C for 10 min, then increasing at a rate of 4 °C/min to 220 °C, held for 10 min and then increasing again at 1 °C/min to 240 °C with helium as carrier gas with a flow rate of 1.2 mL/min. Electron impact mass spectra was at 70 eV, and scan from 35 to 550  $m/z$  was recorded (Kamatou et al., 2010). The composition of the oil was calculated from electronic integration measurements, using flame ionisation detection (FID, 250 °C). The data were processed using ChemStation version 11 software, and the identification of the compounds was carried out using different mass spectrum libraries such as NIST<sup>®</sup>, Mass Finder<sup>®</sup>, flavor<sup>®</sup> and also some authentic standards obtained from Sigma. *n*-Alkanes ( $\text{C}_6$ – $\text{C}_{24}$ ) were used as references points in the calculation of the retention index (RI) (Kamatou et al., 2010).

### 2.4. Chemometrics analyses

#### 2.4.1. Pre-processing of GC–MS data

The GC–MS data were analysed, using an untargeted approach, where the HP Agilent files of all the samples injected were converted to MassLynx (raw) files. The GC–MS data was pre-processed, enabling a comprehensive analysis of the whole chromatogram,

including minor peaks that are usually omitted for targeted analyses with the use of manual integration method. During pre-processing, baseline correction and spectral alignment of all peaks were carried out across all samples, hence identifying differences and similarities within the whole chromatogram throughout the entire data set. The MS Excel file of the aligned data from MarkerLynx<sup>®</sup> was exported to SIMCA-P + 14.0 (Umetrics AB, Malmo, Sweden) for chemometric analysis.

#### 2.6.2. Principal component analysis of GC–MS data using SIMCA-P + 14.0

The GC–MS data were subjected to SIMCA-P + 14.0 for chemometric analysis. Principal component analysis, a primary method was used to investigate trends, patterns and groupings within the data set. Different types of scaling (univariate, pareto and centre scaling) methods were used to determine the scaling that produced the model with the highest statistical parameters (variation and prediction capability). The model performance was evaluated by considering the number of principal components (PCs), cumulative variation within X ( $R^2X_{cum}$ ) and the predictive ability of the model ( $Q^2_{cum}$ ). The resulting model, following preliminary investigations, was subjected to hierarchical cluster analysis to investigate potential chemotypes. In addition, the plot obtained was coloured according to localities to explore possible grouping based on geographical variation (Tankeu et al., 2013).

#### 2.6.3. Hierarchical cluster analysis

The data set was further analysed by hierarchical cluster analysis (HCA) to observe relationships amongst different samples. A dendrogram, together with an interactive score scatter plot, were constructed to allow a pictorial overview of potential relationships between samples. Geographical variation was investigated, and the dendrogram was labelled, according to localities (Tankeu et al., 2013).

#### 2.6.4. Identification of chemical markers

A loadings plot was constructed for the identification of chemical markers involved in the relationships observed using the hierarchical clustering analysis. The use of a loadings plot made it possible to explore the correlation between chemical markers and specify chemical markers for the relationships detected from the dendrogram. In addition to the loadings plot, contribution plots were constructed for each cluster identified from the dendrogram to investigate differences amongst groups. Thus, groups were composed, and relevant chemical makers were allocated to each group, based on the observation from the loadings plot. The identified biomarkers were assigned using various MS libraries in conjunction with mass/retention time pair values.

## 3. Results and discussion

### 3.1. Essential oil composition of *Croton gratissimus* using GC–MS/FID

Hydrodistillation of 62 samples yielded a light yellow oil with percentage oil yield ranging from 0.004 to 0.020 (w/w). More than 33 compounds contributing to 90% of the total oil composition were detected, using GC–MS/FID. A typical total ion chromatogram of a selected sample is shown in Fig. 1. The main chemical classes of compounds identified in the oil were monoterpenes ( $\approx 47\%$ ) and sesquiterpenes ( $\approx 23\%$ ). Mostly quantitative rather than qualitative differences were observed in the essential oil of *C. gratissimus*. The major compounds identified in the oil include  $\beta$ -pinene (nd–13.7%), sabinene (0.2–16.7%),  $\alpha$ -phellandrene (0.4–44.3%), limonene (0.2–38.3%), 1,8-cineole (0.1–18.3%), *p*-cymene (0.1–25.2%), terpinolene (nd–15.1%), camphor (nd–13.1%), linalyl acetate (0.1–18.8%), oxidohimachalene (nd–54.6%) and phytol (nd–24.9%) (Table 1).

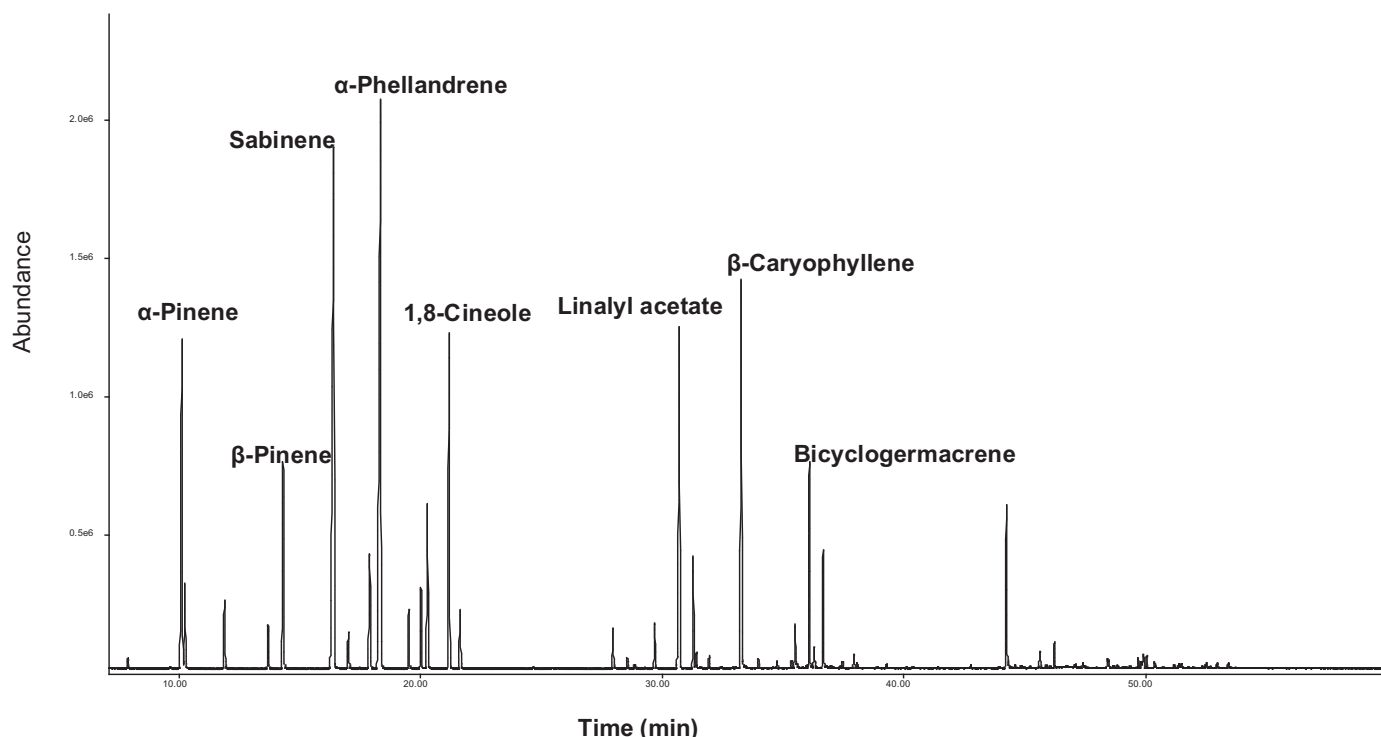


Fig. 1. Typical total ion chromatogram of essential oil of *C. gratissimus* indicating the major constituents.

α-Phellandrene was the dominant compound in the majority of the oils with an average percentage area of  $18.5 \pm 11.2$ .

Some studies on *C. gratissimus* volatiles (Van Vuuren, 2007; Mek-kawi, 1985; Block et al., 2006; Usman et al., 2009; El-Kamal et al., 2012; Owolabi et al., 2013; Ogundajo et al., 2014; Yagi et al., 2016; Lawal et al., 2017) and other *Croton* species (Sacchetti et al., 2005; Doria et al., 2010; Andrade et al., 2013; Turiel et al., 2016) have previously been published. However, in the majority of the studies, a single plant collection was often analysed, and the essential oil

composition cannot be solely based on a single sample. The oil profiles obtained in this study were comparable with other results on *C. gratissimus* essential oil previously investigated in southern Africa although a single sample was used. For example, the results of Van Vuuren (2007) and recently by Lawal et al. (2017) have shown that the essential oil of *C. gratissimus* is characterised by high concentrations of the cyclic monoterpene α-phellandrene, which has been reported to be a skin irritant, and resulting in vomiting and diarrhoea if ingested (Van Vuuren, 2007). The slight difference in the

- Group 1
- Group 2
- Group 3

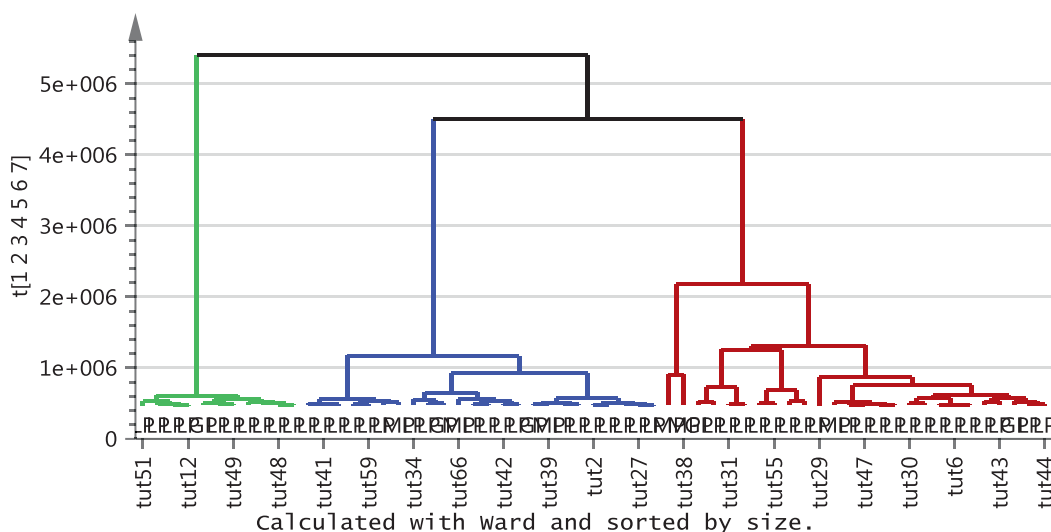


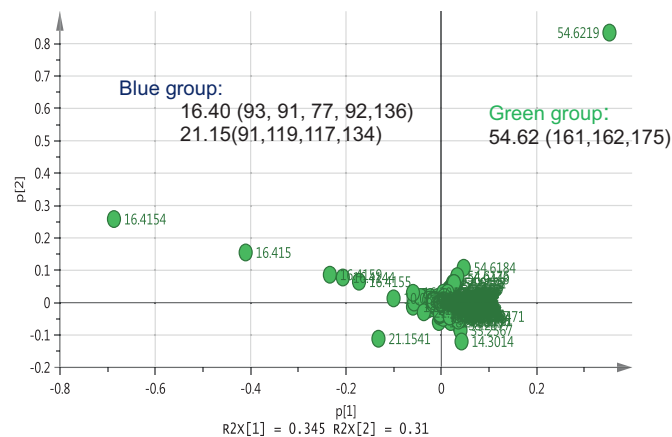
Fig. 2. Dendrogram obtained from the hierarchical clustering analysis using essential oil GC–MS data.

**Table 1**  
Percentage area (min and max, mean ± SD, n = 62) of major constituents of *C. gratissimus* oil as identified by GC–MS/FID.

Compound name	RRI <sub>c</sub>	RRI <sub>p</sub>	Min	Max	Mean	SD	Mean±SD
α-Pinene	1132	1017	0.1	6.3	3.1	1.7	3.1 ± 1.7
α-Thujene	1333	1019	0.1	3.1	1.1	0.6	1.1 ± 0.6
β-Pinene	1201	1104	nd	13.7	1.0	1.8	1.0 ± 1.8
Sabinene	1222	1117	0.2	16.7	2.0	3.3	2.0 ± 3.3
α-Phellandrene	1245	1162	0.4	44.3	18.5	11.2	18.5 ± 11.2
α-Terpinene	1243	1174	0.1	2.0	0.6	0.4	0.6 ± 0.4
Limonene	1267	1193	0.2	38.3	3.6	6.6	3.6 ± 6.6
1,8-Cineole	1229	1202	0.1	18.3	4.2	4.5	4.2 ± 4.5
Z-β-Ocimene	1292	1246	0.2	12.5	1.7	1.7	1.7 ± 1.7
λ-Terpinene	1297	1266	nd	3.8	1.6	0.8	1.6 ± 0.8
E-β-Ocimene	1308	1250	nd	9.2	3.2	2.0	3.2 ± 2.0
p-Cymene	1325	1270	0.1	25.2	4.0	3.8	4.0 ± 3.8
Terpinolene	1338	1281	nd	15.1	1.6	2.3	1.6 ± 2.3
α-Copaene	1495	1493	0.1	8.6	1.3	1.4	1.3 ± 1.4
Camphor	1572	1521	nd	13.1	3.9	3.1	3.9 ± 3.1
Linalyl acetate	1573	1563	0.1	18.8	3.0	3.5	3.0 ± 3.5
β-Caryophyllene	1683	1596	nd	12.2	3.5	3.3	3.5 ± 3.3
α-Humulene	1757	1687	nd	5.9	2.1	1.5	2.1 ± 1.5
α-Cubenene	1772	1736	nd	7.2	0.7	1.5	0.7 ± 1.5
Terpinyl acetate	1778	1708	nd	12.5	2.9	2.7	2.9 ± 2.7
Germacrene D	1790	1760	nd	9.6	1.7	2.1	1.7 ± 2.1
Isoborneol	1799	1702	nd	5.5	1.1	1.4	1.1 ± 1.4
Geranial	1813	1740	nd	9.0	0.6	1.3	0.6 ± 1.3
δ-Selinene	1997	2011	nd	5.9	0.7	0.9	0.7 ± 0.9
Caryophyllene oxide	2013	2010	nd	5.8	0.6	0.9	0.6 ± 0.9
Costol	2058	2045	nd	7.8	0.8	1.4	0.8 ± 1.4
Undecanoic acid	2070	–	nd	8.1	0.5	1.1	0.5 ± 1.1
Carbamic acid	2130	2188	nd	2.9	0.83	0.81	0.8 ± 0.81
Eugenol	2240	–	nd	3.8	0.7	0.8	0.7 ± 0.8
Oxidohimachalene	2358	–	nd	54.6	8.5	12.2	8.5 ± 12.2
Phytol	2398	–	nd	24.9	3.2	4.5	3.2 ± 4.5

nd: not detected, RRI<sub>c</sub> = relative retention index calculated, RRI<sub>p</sub>: relative retention index previously reported (Kamatou et al., 2010).

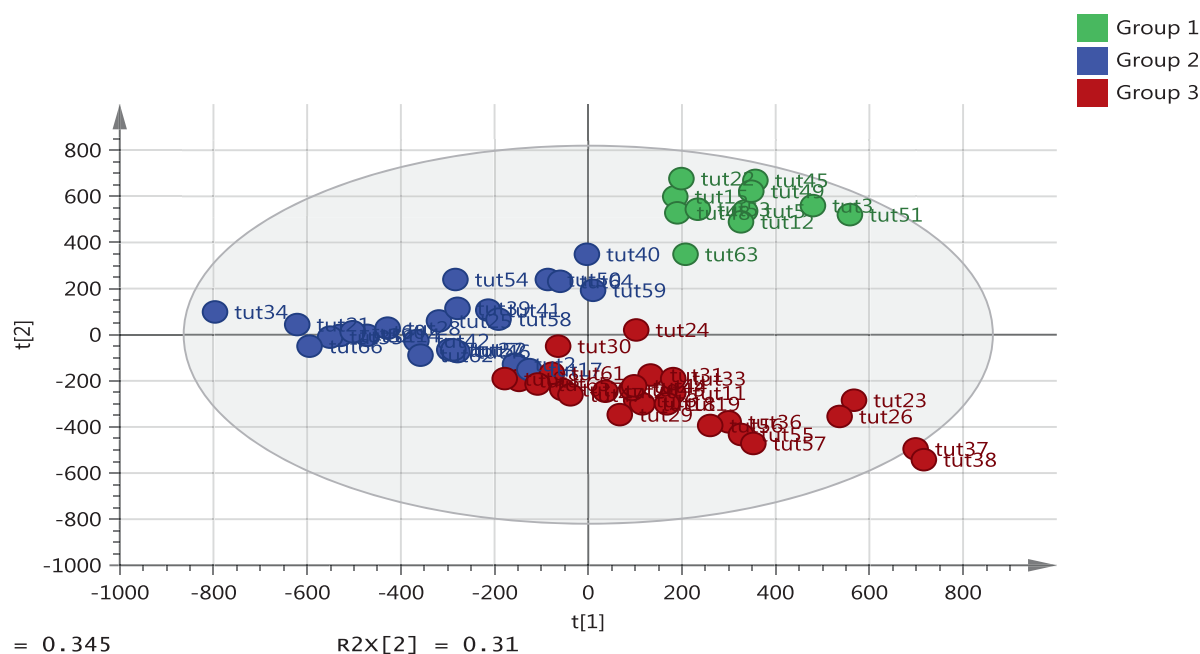
percentage composition between the present findings and those of Van Vuuren (2007) [α-phellandrene (20.7%), germacrene D (8.6%), α-pinene (6.7%)], and Lawal et al. (2017) [(sabinene (14.6%), α-phellandrene (12.3%), β-phellandrene (10.7%), α-pinene (6.1%),



**Fig. 4.** Loading scatter plot obtained from the PCA analysis of essential oil GC–MS data, coloured according to the dendrogram. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

germacrene D (5.9%)] may be due to the geographical location of the plants, vegetative stage of the plant and the plant part from which the oil was isolated. The previous authors (Van Vuuren, 2007; Lawal et al., 2017) investigated the leaf essential oil exclusively, while the aerial parts (leaf and twigs) were used in the current investigation.

The study conducted on various *Croton* species including *C. gratissimus* from North and Central Africa showed that α-phellandrene is usually present in lower quantities (Block et al., 2006; Owolabi et al., 2013; Ogundajo et al., 2014). Several other papers indicated high variability in the chemical composition of the essential oil of *C. gratissimus* collected from different plant parts (leaves, stem barks, roots and twigs) for the same plant and from different locations (Menut et al., 1995; Boyom et al., 2002; Ogundajo et al., 2014) and from various *Croton* species (Radolović et al., 2006; Delima et al., 2010; Rodríguez-Castillo et al., 2012). In the present study, the essential oils of *C. gratissimus* are dominated by monoterpenes (α-phellandrene, 1,8-cineole) and sesquiterpenes (germacrene D,



**Fig. 3.** Score scatter plot obtained from the PCA analysis of essential oil GC–MS data, coloured according to the dendrogram. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



oxidohimachalene), which agrees with the findings by Van Vuuren (2007) and Lawal et al. (2017).

### 3.2. Multivariate analysis of GC–MS chromatography data

#### 3.2.1. Untargeted GC–MS/FID analysis

Principal component analysis produced a ten-component model with Pareto scaling, explaining about 84% variation between the samples with the first two principal components (PCs) accumulating 65% of the variation (**data not shown**). All the essential oil samples of *C. gratissimus* were found within the 95% confidence limit of the model. This observation showed that there were no outliers related to GC–MS values. The score scatter plot obtained was further coloured according to the three provinces (Gauteng, Limpopo and Mpumalanga), where the samples originated, and this revealed that the distribution of the samples within the model was not specific to provinces (**data not shown**). Cumulative variation within X ( $R^2X_{cum}$ ) and the cumulative predictive ability of the model ( $Q^2_{cum}$ ) values were both  $\geq 0.5$ , which indicate good model prediction.

#### 3.2.2. Hierarchical clustering analysis for GC–MS/FID

Since there was no clear pattern observed between the 62 samples, the untargeted approach, hierarchical clustering analysis (HCA) was a good method to investigate possible clusters/relationships within the data set. Therefore, a dendrogram constructed using the GC–MS data set revealed three main branches representing three chemotypes (Fig. 2). The score scatter plot of the PCA was later coloured, based on the three chemotypes distributed on different quadrants of the scatter plot (Fig. 3). Group 1 was on the positive PC1, while group 2 was on the negative PC1 and group 3 was mainly on the positive PC1. Furthermore, groups 1 and 3 could be separated along PC2, with group 1 being distributed on the positive PC2 and group 3 on the negative PC2.

#### 3.2.3. Identification of chemical markers for each chemotype obtained using GC–MS data

To investigate variables that could be correlated to the chemotypes identified in Fig. 3, a loadings scatter plot was constructed (Fig. 4). The plot illustrates that most of the variables (retention times) are concentrated on the X and Y-intercept, suggesting that the compounds represented are present in almost equal amounts in all the samples. However, variables located far from the centre are specific to some of the samples and therefore, to the chemotype distributed on the same quadrant as the variables. In the quadrant that corresponds to positive PC1 and positive PC2, one retention time (54.62 min) is prominent, which was tentatively identified as the sesquiterpene oxidohimachalene. Therefore, this compound represents chemotype 1 (group 1/green group). This means that samples present in this quadrant or belonging to chemotype 1 contain > oxidohimachalene compared to the rest of the samples. On the other hand, the quadrant corresponding to negative PC1 and positive PC2, three retention times can be considered for three compounds that represent the second chemotype (group 2/blue group). These retention times are; 10.08, 16.40, and 21.15 min and correspond to  $\alpha$ -pinene,  $\alpha$ -phellandrene and *p*-cymene, respectively (Fig. 4). These three compounds are dominant in samples from chemotype 2 compared to the rest of the samples. Finally, considering the loadings plot and comparing the quadrant in which chemotype 3 is distributed from the score scatter plot with the quadrants on the loadings plot, no specific retention times are clearly associated that could be allocated to chemotype 3. Therefore, this necessitated further investigation to understand what differentiated chemotype 3 from chemotype 1 and 2.

To confirm previous observations, different contribution plots were constructed for each chemotype to firstly endorse the chemical markers obtained from the loadings plot for chemotype 1 and 2 and

secondly investigate the particularity of chemotype 3 which had no specific chemotype from the loadings plot. Considering that three chemotypes were defined, the use of an S-plot was not possible, and the contribution plots for each chemotype in addition to the loadings plot were further explored. The contribution plot for chemotype 1 (Fig. 5a) clearly specified the retention time 54.62 min (oxidohimachalene) as indicated on the loadings plot. Similarly, the contribution plot for chemotype 2 (Fig. 5b) demonstrated that the retention time 16.41 min ( $\alpha$ -phellandrene) was the main chemical marker for chemotype 2. Finally, the retention times 16.41 min ( $\alpha$ -phellandrene) and 54.62 min (oxidohimachalene), were recognised as chemical markers for chemotypes 2 and 1, respectively were the most relevant chemical markers on the contribution plot obtained for chemotype 3 (Red group) (Fig. 5c). As can be seen on Fig. 5c, these two compounds ( $\alpha$ -phellandrene and oxidohimachalene) with negative score contributions were particularly very low in chemotype 3, and this indicates that chemotype 3 (red group) cannot be defined by specific markers except the observation that  $\alpha$ -phellandrene and oxidohimachalene which are both chemical markers for chemotype 2 and 1, respectively were very low in chemotype 3.

Owolabi et al. (2013) conducted a numeral cluster analysis of 16 *C. gratissimus* oils (from the leaves) from Nigeria, in which they identified two chemotypes: the monoterpene-rich chemotype (I) dominated by  $\beta$ -pinene and limonene, and the sesquiterpenes-rich chemotype (II) dominated by  $\beta$ -caryophyllene and caryophyllene oxide. Rodríguez-Castillo et al. (2012) from the results obtained from an analysis of leaf essential oils of a large number of *Croton* species in Venezuela found that  $\alpha$ -pinene was the major constituent of the essential oil. In this study, three chemotypes were identified and these chemotypes were different from the chemotypes previously reported for *C. gratissimus* oils obtained in Nigeria, which clearly indicates chemogeographical variation in essential oil composition of *C. gratissimus*.

## Conclusions

The essential oil of *C. gratissimus* collected from different localities in South Africa is composed mainly of monoterpenes and sesquiterpenes hydrocarbons and the variation is more quantitative than qualitative. The major compounds identified in the oil included  $\beta$ -pinene, sabinene,  $\alpha$ -phellandrene, limonene, 1,8-cineole, *p*-cymene, terpinolene, camphor, linalyl acetate, oxidohimachalene and phytol. Three chemotypes were identified and the main chemical markers that contribute to the separation were  $\alpha$ -pinene,  $\alpha$ -phellandrene and oxidohimachalene. The composition of the essential oils of *C. gratissimus* were not unique to southern Africa as most of the identified compounds were comparable with those reported from other parts of the continent. The study also highlighted the importance of using several samples when reporting on the essential composition of aromatic plants.

## Declaration of Competing Interest

No conflict of interest

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2020.12.015.

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